Neuregulin reduces glycaemia by targeting liver metabolism

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LA NEUREGULINA REDUEIX LA GLUCÈMIA ACTUANT SOBRE EL METABOLISME DEL FETGE

Memòria presentada per:

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Per optar al grau de

Doctora per la Universitat de Barcelona.

Aquesta tesi ha estat realitzada sota la direcció de la Dra. Anna María Gumà García al Departament de Bioquímica i Biologia Molecular, Facultat de Biologia, de la Universitat de Barcelona en el Programa de Doctorat en Biomedicina

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Barcelona, 2013

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INTRODUCTION

1. Diabetes and regulation of glucose homeostasis

The prevalence of diabetes is growing in tremendous rates worldwide. According to International Diabetes Federation (http://www.idf.org) 366 million people had diabetes in 2011 and it is expected to rise to 552 million by 2030. Type 2 Diabetes (T2D; also known as non-insulin dependent diabetes or adult-onset diabetes) accounts for at least 90% of all cases of diabetes and is often believed to be associated with growing obesity, sedentary lifestyle, and global population aging (Smyth and Heron, 2006). Traditionally considered a disease of adults, T2D is increasingly diagnosed in children parallel with rising obesity rates. Obesity leads to insulin resistance, the first detectable effect of T2D, in peripheral tissues due to altered expression or secretion of adipokines and infiltration of macrophages and subsequently causes type 2 diabetes, hyperlipidemia and hypertension and finally atherosclerosis (Despres et al., 2006). The pathogenesis of this multi-factor disease involves abnormalities in both insulin action and secretion (Saltiel, 2001)

1.1. Regulation of glucose metabolism by insulin

Glucose homeostasis is a finely tuned process comprised of glucose production and uptake by several organs. Under physiological conditions blood glucose levels are maintained within a narrow range, partly by the action of hormones, which stimulate or inhibit glucose production (Cherrington, 1999) and regulate glucose uptake by peripheral tissues (Ferrannini et al., 1965; DeFronzo et al., 1985). Elevation of blood glucose levels is rapidly sensed by pancreatic β-cells. These archetypical metabolic sensors respond the elevation by secreting insulin or by increasing insulin production through increased proliferation (Kousteni, 2012). β-cells take up the glucose and quickly metabolize it, resulting in the increase of intracellular ATP/ADP ratio (Gross et al., 2008). This rise in ATP results in closing of potassium ATP channels, depolarization of the cell and subsequent opening of voltage-gated calcium channels (Gross et al., 2008), which in turn leads to the secretion of insulin-containing granules. (Newgard and McGarry, 1995; Straub and Sharp, 2002).

Skeletal muscle regulates energy metabolism by contributing to more than 30% of resting metabolic rate and 80% of whole body glucose uptake (de Lange, 2007), while adipocytes adjust energy homeostasis either through secretion of cytokines controlling appetite and insulin sensitivity or by storing excess amount of energy intake as triglycerides and mobilizing them to be oxidized during energy deprivation (Kousteni, 2012). In addition to glucose uptake in muscle and adipocytes, insulin regulates many other pathways including the stimulation of protein

synthesis in muscle and liver, lipid synthesis and storage in liver and adipose tissue, glycolysis and glucose storage in muscle, liver and adipocytes, and the inhibition of ketogenesis and gluconeogenesis in liver and kidney (Farese et al., 2005; Shaham et al., 2008).

Insulin binds to insulin receptor (IR), which in turn autophosphorylates at specific tyrosine residues. That leads to a creation of specific docking sites where insulin receptor substrate (IRS) 1 and IRS2 can be recruited. IRSs binding to IR increases IRS1 and IRS2 tyrosine phosphorylation (White, 2002), which leads to recruitment of adaptor molecules, such as the p85 regulatory subunit of class 1 PI3K (Fruman et al., 1998; Backer et al., 1992). Class 1 PI3K is a lipid kinase and bringing it close to the membrane leads to the production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3). Increases in the membrane content of PIP_3 allow to recruit Akt, where it is phosphorylated by phosphoinositide-dependent protein kinase 1 (PDK1) (Alessi et al., 1997b; Stokoe et al. 1997) and mTOR complex 2 (mTORC2, previously known as PDK2) (Sarbassov et al., 2005). Once activated, Akt phosphorylates and modulates the function of a number of important regulatory proteins, resulting in inhibition of apoptosis, promotion of cell division and stimulation of glucose uptake and storage (Mora et al., 2004).

Liver maintains blood glucose levels through two processes, a combination of what makes up the hepatic glucose production (HGP): (1) gluconeogenesis – a generation of glucose from non-carbohydrate carbon substrates (glycerol, lactate, alanine), and (2) glycogenolysis – a degradation of glycogen. Initially, the glucose that is produced results from the breakdown of liver glycogen stores (glycogenolysis), whereas with prolonged fasting, gluconeogenesis is the primary source of glucose (Gross et al., 2008). These processes become inhibited by transcriptional suppression of the key gluconeogenetic and glycogenolytic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) when insulin levels in blood are rising. Although it is generally accepted that HGP is high in patients with diabetes, the physiology of this abnormality remains disputed.

1.1.1 Glucose transporters

Glucose is a hydrophilic molecule, which cannot diffuse through the lipid bilayer. It needs membrane transporters to enter into the cell. Most mammalian cells import glucose by a process of facilitative diffusion mediated by members of the GLUT (Glucose transporters) family of membrane transport proteins. Fourteen different isoforms of GLUT are expressed in human organism (Scheepers et al., 2004), including also transporters for substrates other than glucose, such as fructose, myoinositol and urate. Most GLUTs catalyze the facilitative (energy-independent) bidirectional transfer of their substrates across membranes, and they may exhibit either symmetric or asymmetric transport kinetics. The well-established glucose transporter isoforms, GLUTs 1-4, are known to have distinct regulatory and/or kinetic properties that reflect their specific roles in cellular and whole body glucose homeostasis.

GLUT1 is ubiquitously expressed and is responsible for basal glucose supply (Mueckler et al., 1995). GLUT1 is highly expressed during foetal development, in erythrocytes and endothelial cells. It is also frequently up-regulated during oncogenesis in many tissues (Ganapathy et al., 2009).

GLUT2 has a uniquely high Km for glucose (~17mM), and it is expressed at very high level in pancreatic β-cells, and in the basolateral membranes of intestinal and kidney epithelial cells and of hepatocytes (Uldry and Thorens, 2004). In all these tissues, glucose transport is not dependent on the number and activity of glucose transporters but on the blood glucose concentration. The rate of glucose metabolism in these cells is controlled at the glucose phosphorylation step.

GLUT3 has a high affinity for glucose and has the highest calculated glucose turnover of the GLUT isoforms (Simpson et al., 2008). Therefore it is predominantly expressed in tissues with high glucose requirement, such as neurons and placenta (Kayano et al., 1988).

GLUT4 is a major glucose transporter expressed in most insulin sensitive tissues, such as skeletal muscle, heart and white adipose tissue (Fukumoto et al., 1989). Insulin stimulates GLUT4 translocation from intracellular membrane compartments to the cell surface, resulting in immediate increase in glucose transport (Bryant et al., 2002).

1.1.2 Insulin signaling cascade regulating glucose metabolism

1.1.2.1. Insulin receptor (IR)

Insulin receptor is expressed in insulin sensitive tissues and is localized on the plasma membrane. The receptor is a heterotetrameric bifunctional complex, which consists of two extracellular insulin-binding α-subunits and two transmembrane β-subunits containing intracellular tyrosine kinase domain. As mentioned above, upon insulin binding, IR undergoes autophosphorylation at different tyrosine residues. These residues are docking sites for several downstream signaling molecules among them insulin receptor substrate (IRS) 1 and 2.

1.1.2.2. Insulin receptor substrate (IRS)

Four IRS proteins, namely IRS1-4, are expressed in rodents, but only three in humans where absence of functional IRS3 gene has been reported (Bjornholm et al., 2002). While IRS4 is largely restricted to hypothalamus and thymus (Numan and Russell, 1999), IRS1 and IRS2 are expressed in brain, muscle heart, adipose tissue, liver kidney, ovary and mammary gland where they contribute to a broad array of physiologic functions including cell growth and metabolic regulation (Dearth et al., 2007). Work with transgenic mice has revealed that most if not all insulin and IGF responses associated with somatic growth, carbohydrate and lipid metabolism are initiated through IRS1 and IRS2 (White, 2003).

IRS proteins have PhosphoTyrosine Binding (PTB) domain and Pleckstrin Homology (PH) domain in their N-terminus. While PTB domain is needed to bind IRS proteins to IR, then through structurally similar but functionally distinct PH domain it binds to several other proteins (Dhe-Paganon et al., 1999). Deletion of that domain changes the signaling potential of IRS1 and IRS2 (Yenush et al., 1998).

The C termini of IRS1 and IRS2, which are poorly conserved, contain several recognizable tyrosine phosphorylation motifs in similar positions that can be phosphorylated by IR and thereafter serve as docking sites signaling molecules containing Src-homology-2 (SH2) domains.

Many distinct pathways are developed in the cells to down-regulate IRS1 and IRS2 by inhibitory serine phosphorylation. For instance, prolonged exposure to insulin itself is able to inhibit IRS proteins binding to IR (Smith et al., 1995, Rui et al., 2001). Activation of mTOR-S6K branch downstream of the PI3K-Akt pathway appears to be a critical event rendering IRS1 and IRS2 unresponsive to insulin and IGF-I and is known as a negative feedback loop mediated through serine/threonine kinase mTOR-S6K (Haruta et al., 2000; Greene et al., 2003; Carlson et al., 2004; Ueno et al., 2005). Activation of stress-sensitive kinases such as Protein Kinase C (PKC), IκB Kinase (IKK) and c-Jun N-terminal Kinase (JNK) are also considered responsible for phosphorylating and inactivating IRS1 and IRS2 (Lowell and Shulman, 2005). As proven in mice, adipose derived molecules, such as tumor necrosis factor α (TNFα) and free fatty acids (FFA), stimulate an inhibitory phosphorylation of IRS1 by JNK (Hotamisligil et al., 1996; Aguirre et al., 2000; Hirosumi et al., 2002).

Several experiments with IRS knockout mice support the concept of the tissue specificity of insulin action, as well as the distinct compensatory mechanisms in the different insulin responsive tissues. For instance, IRS1 deficient mice led researches to suggest that IRS2 could be major player in hepatic insulin action and that IRS2 can compensate IRS1 deficiency more effectively in liver and β-cells than in muscle and adipose tissue (Yamauchi et al., 1996; Higaki et al., 1999; Kahn et al., 2000). Contrary to IRS1 knockout mice, where the binding of PI3K to IRS2 is increased 2.8-fold (Sajan et al., 2004), lack of IRS2 in hepatocytes does not result in enhanced IRS1-associated PI3K activity and phosphorylation of several downstream kinases including Akt, GSK3, FoxO1 and aPKC (Valverde et al., 2003). At the same time, knockout of IRS2 does not inhibit insulin effects on glucose transport in muscle (Higaki et al., 1999).

1.1.2.3. Phosphatidylinositol 3-kinase (PI3K)

The type 1A PI3K is a lipid kinase that associates with IRS1 and IRS2 upon insulin stimulation and phosphorylates the plasma membrane inositol phospholipids at 3'-OH position of the inositol. The most common substrate products are phosphatidylinositol (PI), PI 4-phophate and PI 4,5-bisphosphate, which become PI 3-phosphate, PI 3,4-bisphosphate and PI 3,4,5-triphosphate (PIP_3) respectively. PIP_3 produced by PI3K recruits several Ser/Thr kinases that contain PH domains to plasma membrane, among them PDK1 and Akt (17).

The heterodimer is composed of 110 kDa catalytic subunit – either p110α, p110β or p110δ - and one of the five 85 kDa regulatory subunit isoforms encoded by three different genes – *Pik3r1*, *Pik3r2* and *Pik3r3* (Manning and Cantley, 2007; Ueki et al., 2003; Ueki et al., 2000). PI3K becomes activated when the SH2-domains of its regulatory subunit (p85) are occupying tyrosine-phosphorylated sites of IRS proteins (Backer et al., 1992). The regulatory protein in PI3K not only promotes the stabilization and localization of catalytic subunit activity, but also binds directly to and enhances the lipid phosphatase PTEN activity maintaining in that way the balance of PI3K/PTEN signaling (Chagpar et al., 2010). Partial loss of the PI3K regulatory subunits increases insulin sensitivity (Geering et al., 2007; Terauchi et al, 1999), while knockout mice for catalytic subunit are insulin resistant and glucose intolerant (Brachmann, 2005). Analysis of human subjects and animal models indicates the existence of a correlation between impaired activation of PI3K in insulin target tissues and insulin resistance *in vivo* (Kim et al., 1999; Heydrick et al., 1993; Tsuchida et al., 2001).

1.1.2.4. 3-Phosphoinositide Dependent Protein Kinase (PDK1)

PDK1 is a 63kDa serine/threonine enzyme with a kinase domain at its N-terminus and a PH domain at its C-terminus. The latter interacts with high affinity with PIP_3 and one of its immediate breakdown products PIP_2 (Stephens et al., 1998; Alessi et al., 1997a; Currie et al., 1999).

PDK1 activity is not directly altered by agonist stimulation as suggested by the results showing the same high catalytic activity was obtained immunoprecipitating PDK1 from either unstimulated or growth factor/insulin stimulated cells (Alessi et al., 1997a). Moreover, the binging of PI3K products to PDK1 *in vitro* does not affect its catalytic activity either (Stephens et al., 1998; Alessi et al., 1997a; Currie et al., 1999). PDK1 belongs to an AGC family of protein kinases (named after protein A, G and C families, which share a conserved catalytic domain) that, in order to be activated, require phosphorylation at its T-loop residue (Ser241), also known as activation loop (Casamayor et al., 1999). PDK1 has the ability to phosphorylate its own T-loop residue, mediated by an intermolecular (trans) reaction, rather than intramolecular (cis) reaction (Alessi et al., 1997b; Stokoe et al., 1997; Wick et al., 2003). Activation loop phosphorylation is then followed by secondary phosphorylations among other modifications. That may explain why PDK1 is constitutively active in mammalian cells.

PDK1 also catalyzes efficiently the phosphorylation of threonine residues in the activation loop of Akt (Alessi et al., 1997b; Stokoe et al., 1997), ribosomal S6 Kinase (S6K) (Pullen et al., 1998; Alessi et al., 1998), Serum- and Gluckocorticoid induced protein Kinase (SGK) (Kobayashi and Cohen, 1999; Kobayashi et al., 1999; Park et al., 1999), and atypical Protein Kinase C (aPKC) (LeGood et al., 1998; Chou et al., 1998). Experiments establish that there is a difference in the mechanism how PDK1 recognizes Akt and S6K/SGK/aPKC. Mutual ability of Akt and PDK1 to interact with PIP_3 and PIP_2 via their PH domain, plays an important role in co-localizing these

enzymes at the plasma membrane and enabling PDK1 to phosphorylate, and hence activate Akt (Alessi et al., 1997b; Stokoe et al., 1997; Andjelkovic et al., 1997). The other kinases do not contain a PH domain and, therefore, interact with PDK1 through a hydrophobic motif on PDK1 (PIF – PDK Interacting Fragment) that functions as a substrate-docking site (Collins et al., 2003; Biondi et al., 2001).

PDK1 is important in regulation of cell size. For instance, the PDK1-deficient hearts are smaller due to a reduction in cardiomyocyte size rather than number (Mora et al., 2003). PDK1 is also required for normal embryo development. Mice embryos lacking PDK1 die at day E9.5, displaying multiple abnormalities, including lack of somites, forebrain and neural crest derived tissues (Lawlor et al., 2002).

1.1.2.5. Akt (PKB)

Akt, also known as Protein kinase B (PKB), is a serine/threonine kinase that has three different isoforms: Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ). As mentioned previously, the PH domain in the Akt N-terminus has high affinity to PI3K products, which assist its activation. However, binding to PIP_3 itself does not activate Akt (Alessi et al., 1997b; James et al., 1996), but induces a conformational change that greatly enhances the rate at which it can be phosphorylated at Thr308 by PDK1 (Milburn et al., 2003; Thomas et al., 2002). In addition Akt needs phosphorylation at Ser473 residue, conducted by mTORC2, to be fully active (Hresko and Mueckler, 2005; Sabrassov et al., 2005).

Activated Akt can phosphorylate wide range of substrates relevant for several actions, including GSK3α/β (Glycogen Synthase Kinase 3α/β, inhibiting this kinase and allowing an increase in glycogen synthase activity), AS160 (promotes GLUT4 translocation), the BAD-BCL2 heterodimer (inhibits apoptosis), the FoxO transcription factors (regulate gene expression in liver, β cells and hypothalamus), p21CIP1 and p27KIP1 (blocks cell cycle inhibition), eNOS (stimulates NO synthesis and vasodilation), PDE3b (hydrolyzes cAMP), and mTORC1 (reviewed in Cheng et al., 2010) How and what signaling events Akt regulates depends also on different Akt isoforms and that brings out Akt2 as the isoform contributing to metabolic regulation (Bae et al., 2003; Cho et al., 2001; George et al., 2004; Semple et al., 2009).

1.1.2.6. Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is serine/threonine kinase with highly conserved homologues that are found in all eukaryotes (Schmelze and Hall, 2000). The protein is found in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which represent an important component of signaling network responsive to insulin and growth factor signals, energy status, oxygen availability and amino acid concentrations (Laplante and Sabatini, 2009b).

The primary pathway by which most growth factors and cytokines, including insulin, activate mTOR signaling depends on activation of PI3K pathway (Fingar and Blenis, 2004).

In mTORC1, raptor (Kim et al., 2002; Hara et al., 2002; Loewith et al., 2002) and G protein Beta-subunit-Like protein (GβL) (Kim et al., 2003) are associated with mTOR, forming a complex. Insulin-sensitive signaling proteins downstream of PI3K and upstream of mTORC1 include the Akt, the Tuberous Sclerosis heterodimer Complex TSC1/TSC2 the and small G protein Rheb. TSC1/TSC2 complex functions as a GTPase that drives Rheb (Yamagata et al., 1994) into the inactive GDP-bound state (Garami et al., 2003; Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003) and thereby interrupts the direct Rheb-mTORC1 interaction, contributing to the inhibition of mTORC1. Insulin inhibits the TSC1/TSC2 activity by Akt mediated phosphorylation of TSC2 (Montagne et al., 2001; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). This phosphorylation disrupts TSC2 association with TSC1 (Inoki et al., 2002, Potter et al., 2002; Manning et al., 2002) and targets TSC2 for degradation (Plas and Thompson, 2003) or binding to 14-3-3 protein, thus keeping TSC2 in the cytosol and away from membrane-bound TSC1 (Li et al., 2002b). The latter leads to the activation of mTORC1. However, in response to a decrease in cellular ATP levels, AMP-activated protein Kinase (AMPK) overrides the Akt signal and suppresses Rheb-mTORC1 signaling by activating TSC2 (Corradetti et al., 2004; Inoki et al., 2003; Shaw et al., 2004). That ensures that mTORC1 is activated only under conditions of energy sufficiency.

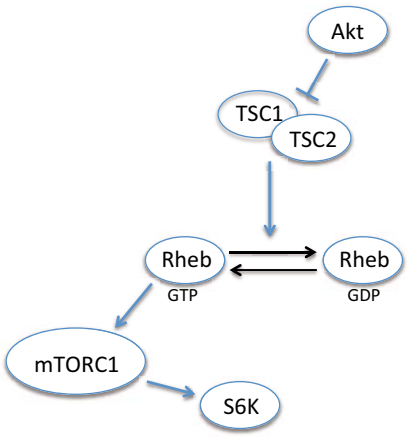


Figure 1. Akt mediated activation of mTORC1/S6K pathway

In mammals, the two well-characterized downstream targets of mTORC1 are ribosomal S6 Kinases (S6K1 and S6K2) and the eukaryotic Initiation Factor E4 (eIF4E)-Binding Protein 1 (4E-BP1) (Long et al., 2005). The complex interacts with its downstream substrates through raptor, which recognizes substrates through their TOR Signaling (TOS) motifs (Schalm et al., 2003) and leads to S6K1/2 phosphorylation and activation and to E4-BP1 phosphorylation and release from the cap-dependent translation factor eIF4E (Gingras et al., 2001). These two events lead to an increase in ribosomal biogenesis and the selective translation of specific mRNA populations. mTORC1 also plays an important role in lipid biosynthesis by promoting the cleavage and

activation of SREBP1, which is the key regulator of lipogenic genes (Laplante and Sabatini, 2009a), as previously mentioned.

The other complex in what mTOR also exists, mTORC2, includes rictor, GβL and mSin1 (mammalian Stress-activated protein kinase interacting protein 1) (Jacinto et al., 2004; Frias et al., 2006; Sarbassov et al., 2005). As described above, mTORC2 is the major kinase involved in regulating the phosphorylation of Ser473 residue on Akt and is mainly regulated by nutrients (Laplante and Sabatini, 2009b).

1.1.2.7. 70kDa ribosomal protein S6 kinase (p70 S6K)

The 70kDa ribosomal protein S6 kinases, S6K1 and S6K2 are two highly homologous serine/threonine kinases. The S6K1 gene encodes two proteins, p70S6K and p85S6K. The p70S6K is the predominant form and is expressed ubiquitously and localized largely but not exclusively in the cytoplasm. Upon growth factor stimulation p70S6K may also shuttle from cytoplasm to the nucleus (Valovka et al., 2003). The other, p85S6K, contains a nuclear localization sequence in its N-terminus and targets, therefore, the nucleus. The two isoforms of S6K2, p56S6K and p54S6K, both locate predominantly in the nucleus of quiescent cells (Koh et al., 1999). S6 kinases are activated in response to growth factors, cytokines, nutrients, and intracellular calcium (Jones and Kazlaukas, 2001; Jones et al., 1999b; Pinzani et al., 1991).

p70S6K1 is rendered active following phosphorylation of a critical Thr229 residue in the activation loop by the PDK1 (Mora et al., 2004; Pullen et al., 1998; Alessi et al., 1998). The phosphorylation of that T-loop residue is only possible once series of serine and threonine residues in the C-terminal regulatory domain have been phosphorylated. For full activation of p70S6K the protein needs mTORC1 to phosphorylate its Thr389, which lies in the kinase extension domain in the conserved sequence known as the hydrophobic motif (Jacinto et al., 2004; Kim et al., 2003; Inoki et al., 2002). The activation typically peaks within 1 hour following the stimulation with growth factors and slowly declines over time. Dephosphorylation of S6K1 is mediated by PP2A (Petritsch et al., 2000). In addition to acute changes in the localization and kinase activity of S6K, alterations in the overall level of S6K can also have an important effect on cellular function.

Growth factor stimulation of cells induces not only phosphorylation of multiple serine/threonine residues of S6K, but also phosphorylation at several tyrosine sites (Rebholz et al., 2006) and acetylation of Lys516 close to the C-terminal PDZ (post-synaptic density protein) binding motif (Fenton et al., 2010a). These modifications do not appear to affect S6K activity and may be important recruiting it into specific complexes and/or subcellular compartments. Also ubiquitination and proteasomal degradation of S6Ks, that has been recently observed, may play a role in S6K-mediated signaling events (Gwalter et al., 2009; Wang et al., 2008).

The S6 kinases have been linked to diverse cellular processes, including protein synthesis, mRNA processing, glucose homeostasis, cell growth and survival (Raught et al., 2004; Wang et al., 2001;

Richardson et al., 2004; Ruvinsky et al., 2005; Dorrello et al., 2006; Harada et al., 2001). Studies with S6K1-/- mice have revealed that the kinase also has a role in suppressing catabolic events, such as lipolysis in adipose tissue and fatty acid oxidation in muscle (Um et al., 2004), but do not regulate SREBP-1c expression, which is also mediated through mTORC1 (Li et al., 2010).

Beside increased lipolysis and β-oxidation, mice lacking S6K1 also display hypoinsulinemia and glucose intolerance, a possible result of significantly smaller pancreatic β-cells and therefore impaired capacity for insulin production (Pende et al., 2000). Despite displaying defects in insulin secretion, these mice show increased sensitivity to insulin (Um et al., 2004). Studies, both *in vitro* and *in vivo*, have shown S6K directly phosphorylating IRS1 on serine residues, which is known to inhibit signaling to PI3K (Harrington et al., 2004; Um et al., 2004; Werner et al., 2004). However, in S6K1-/- mice, the serine sites on IRS1 are not phosphorylated, and Akt remains responsive to insulin (Um et al., 2004), indicating that other kinases have an overlapping action on IRS1 phosphorylation. S6K not only deactivates insulin signaling by serine phosphorylation of IRS1, but also induces a down-regulation of IRS1 protein levels (Harrington et al., 2004) and inhibition of Akt activation by phosphorylating the mTORC2 component, Rictor, at Thr1135 (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2009).

1.1.2.8. FoxO1

FoxO1 is one of the four FoxO isoforms of Forkhead Box O transcription factors in mammals that belong to the class O of the forkhead family that is characterized by an evolutionally conserved DNA binding domain termed the "forkhead box". FoxO proteins consist of four regions: a N-terminal highly conserved DNA-binding domain, a Nuclear Localization Sequence domain (NLS), a Nuclear Export Signal (NES) and a C-terminal transactivation domain. FoxO transcription factors are regulated by wide range of external stimuli, including insulin, IGF-I, nutrients, cytokines, and oxidative stress and therefore play an important role in tumor suppression, energy metabolism, and lifespan extension, apoptosis, energy metabolism, and oxidative stress resistance (Salih and Brunet, 2008; Daitoku and Fukamizu, 2007). Some of the functions of FoxO1 and the other three members of the family, namely FoxO3, FoxO4 and FoxO6, are overlapping (Paik et al., 2007), but each of them also appears to have some unique functions (Arden, 2008).

FoxO1 is highly expressed in insulin-responsive tissues, including pancreas, liver, skeletal muscle, hypothalamus, white and brown adipose tissue. FoxO1 fulfills its functions in all these different tissues as a transcriptional modulator of insulin sensing genes as well as genes that are involved in lipid oxidation and metabolism, mitochondrial activity and energy uptake (Kousteni, 2012). Insulin inhibits FoxO1 transcriptional activity via phosphorylation at Ser256 and subsequent nuclear exclusion (van der Horst and Burgering, 2007). FoxO activity can be altered also by an intricate combination of other post-translational modifications besides phosphorylation, such as acetylation, ubiquitination, and methylation, which in turn regulate subcellular localization, protein levels, DNA-binding properties, and transcriptional activity

(Accili and Arden, 2004; Jing et al., 2007; Calnan and Brunet, 2008; Lai et al., 1993; Matsuzaki et al., 2005; Matsuzaki et al., 2003; Huang et al., 2005; Kitamura et al., 2005). It also has been established that physical interactions between FoxO proteins and their various binding partners have significant consequences on transcriptional activity and other FoxO functions (van der Vos and Coffer, 2008).

In the pancreas, FoxO1 regulates β-cell formation and function by a balanced dual mode of action that suppresses β-cell proliferation (Kitamura et al., 2002; Nakae et al., 2002; Buteau et al., 2006) but promotes survival by means of protecting β-cell function from increases in oxidative stress levels (Kitamura et al., 2006). In liver, Foxo1 promotes HGP by driving the expression of gluconeogenic enzymes (Altomonte et al., 2003; Puigserver et al., 2003; Samuel et al., 2006; Matsumoto et al., 2007) and regulates lipid metabolism (Altomonte et al., 2004). In skeletal muscle FoxO1 maintains energy homeostasis during fasting and plays a role in myocyte/myotube formation as well as skeletal muscle size (Bois and Grosveld, 2003; Hribal et al., 2003; Kamei et al., 2004). In a dual function, FoxO1 regulates energy and nutrient homeostasis through energy storage in with adipose tissue, but promotes energy expenditure in brown adipose tissue (Kousteni, 2012).

Chronic expression of an active FoxO1 mutant in the liver leads to increased expression of genes involved in gluconeogenesis, resulting in elevated plasma glucose and insulin levels, which are not able to maintain normal glycaemia (Zhang et al., 2006a). Moreover, hepatic expression of dominant negative FoxO1 in mice reduces gluconeogenic gene expression and rescues the diabetic phenotype of insulin resistance (Altomonte et al., 2003). However, FoxO1-null mice die in embryogenesis due to developed defects in arterial and venous development (Hosaka et al., 2004).

1.1.2.8.1. Gluconeogenesis regulation by FoxO1

FoxO1 functions as a metabolic switch that shifts metabolic responses with the purpose of re-establishing energy homeostasis. In liver, FoxO1 mediates the expression of genes involved in both glucose and lipid metabolism. Under fasting conditions FoxO1 is activated and locates in the nucleus, where it drives the expression of gluconeogenic enzymes (Altomonte et al., 2003; Puigserver et al., 2003; Samuel et al., 2006; Matsumoto et al., 2007). In the fed state hepatic FoxO1 is inhibited by insulin and excluded from the nucleus (Nakae et al., 1999). This function in the liver shifts glucose metabolism to acetyl CoA for oxidation or conversion to fatty acids (Dong et al., 2008).

In hepatocytes, FoxO1 directly interacts with the peroxisome proliferator-activated receptor γ (PPARγ) coactivator PGC1α to promote the expression of *Pepck* and *G6pc* and thereby stimulating gluconeogenesis (Yoon et al., 2001; Puigserver et al., 2003). At the same time Foxo1 also regulates positively PGC1α expression at the transcriptional level by binding to three insulin

response elements in its promoter (Daitoku et al., 2003). Both FoxO1 activity and PGC1α expression are inhibited by insulin via Akt (Daitoku et al., 2003).

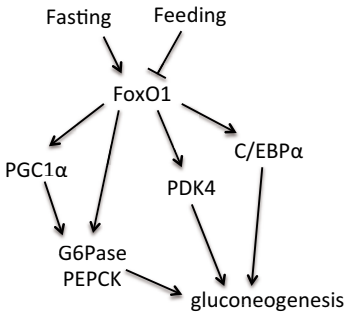


Figure 2. FoxO1-mediated regulation of gluconeogenesis regulation

Another mechanism that promotes gluconeogenesis involves inhibition of the pyruvate dehydroxygenase complex (PDH complex). In hepatocytes, FoxO1 induces the transcription of PDK4 (Kwon et al., 2004), which phosphorylates the E2 subunit of the PDH, thereby inhibiting the conversion of pyruvate to acetyl-CoA, resulting in a shift of pyruvate from the tricarboxylic acid cycle or fatty acid synthesis toward gluconeogenesis (Randle, 1986). In this case, pyruvate will be converted by pyruvate carboxylase to oxaloacetate, which is a substrate for PEPCK. In addition to FoxO1 this process also needs presence of PPARα (Huang et al., 2002).

FoxO1 expression sharply increases before birth. It appears that during late gestation FoxO1 binds to C/EBPα, a critical regulator of perinatal gluconeogenesis as well as glycogen synthesis, (Wang et al., 1995), which leads to binding to PEPCK promoter and an increase of its transcription (Sekine et al., 2007).

While promoting gluconeogenesis, FoxO1 also plays a role in down-regulating the expression of genes involved in glycolysis (glucokinase and pyruvate kinase) and fatty acid synthesis (Sterol Regulatory Element-Binding Protein 1c (SREBP1c), Fatty Acid Synthase (FAS), ATP citrate lyase) (Zhang et al., 2006a).

1.1.2.9. PEPCK

In the post-absorptive state, the liver is the main source of free glucose generated via glycogenolysis and gluconeogenesis. Although the kidney cortex is clearly gluconeogenic *in vitro* (Nishiitsuji-Uwo et al., 1967; Weideman and Krebs, 1969), the contribution of the kidneys to whole-body glucose production has been debated for number of years. The key rate-limiting enzyme responsible for gluconeogenesis is PEPCK.

PEPCKs are ubiquitous and highly conserved throughout the phyla. In vertebrates, there are two isozymes of PEPCK, a mitochondrial (PEPCK-M) and a cytosolic (PEPCK-C), which are encoded by

separate nuclear genes. Despite of that, these two enzymes have very similar catalytic properties and catalyze precisely the same reaction:

Oxalacetate + GTP ↔ Phosphoenolpyruvate + CO_2 + GDP

Most mammals have similar expression of PEPCK-C and PEPCK-M in their livers, but in rats and mice 95% of the PEPCK enzymatic activity comes from PEPCK-C. Contrary to PEPCK-M, which is constitutively expressed in rodents and humans, PEPCK-C is tightly regulated at the level of transcription. The activity of PEPCK-C in the liver can be rapidly induced by altering the concentration of the RNA. The half-life of the mRNA is 30 min (Beale et al., 1981; Beale et al., 1982; Sasaki et al., 1984; Chrapkiewicz et al., 1982) and the half-life of the protein varies from 6 to 8 hours (Hopgood et al., 1973).

The promoter of PEPCK is robust and integrates with numerous signals including cAMP, glucocorticoids, insulin, thyroid hormone, fatty acids and glitazones to regulate transcription of PEPCK-C gene. While cAMP and glycocorticoids enhance PEPCK-C gene transcription in liver (Lamers et al., 1982), insulin dominantly represses it via inhibition of FoxO1 (Puigserver et al., 2003; Hall and Granner, 1999; Hall et al., 2000; Nakae et al., 2001; Yeagley et al., 2001). It is also suggested that insulin inhibits PEPCK-C expression by increasing the concentration of C/EBPβ-C1 (also known as LIP – Liver-enriched Inhibitory Protein), which is able to complete away the activating counterpart of PEPCK-C gene transcription C/EBPβ-B1 (also known as LAP – Liver-enriched activator Protein) (Duong et al., 2002). PEPCK-C gene promoter also contains an SREBP-1 binding site. Known to be the major regulator of lipid metabolism, SREBP-1 is also involved in down-regulating genes in carbohydrate metabolism, such as G6Pase and PEPCK (Chakravarty et al., 2004; Takashi et al., 2004).

Besides being involved in gluconeogenesis in liver and kidney, PEPCK-C has several other proposed metabolic roles, such as glyceroneogenesis in liver and adipose tissue (Ballard and Hanson, 1967) and cataplerosis.

Tissue-specific knockouts as well as tissue-specific over-expression of PEPCK-C have resulted in type 2 diabetes and several other phenotypes including obesity, lipodystrophy, fatty liver, and death (Hanson and Reshef, 1997; She et al., 2000; Olswang et al., 2002). It has been reported that PEPCK overexpression in the liver results in a selective down-regulation of IRS2 expression and failure of insulin to suppress HGP (Sun et al., 2002).

1.1.2.10. G6Pase

In liver, G6Pase (Glucose-6-Phosphatase catalytic subunit) catalyzes the final step in the gluconeogenic and glycogenolytic pathways, the hydrolysis of Glucose-6-Phosphate (G6P) to glucose and inorganic phosphate (van Schaftingen and Gerin, 2002). Within a multi-component enzyme system G6Pase is anchored to the endoplasmic reticulum (ER) by nine transmembrane

helixes whit its N-terminal catalytic site directed towards the lumen of the ER and C-terminus into cytoplasm. The G6P transporters facilitate the delivery of substrate from the cytosol to the active site of the G6Pase and thereafter, transporters of inorganic phosphate and glucose return the reaction products back to the cytosol (van Schaftingen and Gerin, 2002).
G6Pase gene transcription is regulated by multiple factors. The transcription of G6Pase is stimulated by glucose, glucocorticoids, glucagon and fatty acids and repressed by insulin, Tumor necrosis Factor α (TNFα), Epidermal Growth Factor (EGF) and phorbol esters.

1.1.2.11. GSK3β

GSK3 is a serine/threonine kinase composed of two isoforms, GSK3α and GSK3β. GSK3α has slightly higher molecular weight than GSK3β due to an extended N-terminal glycine rich domain. The two isoforms are very similar to each other with a high sequence homology (86% overall and 97% in kinase domains) and biochemical characteristics (Woodgett and Cohen, 1984; Woodgett, 1990; Woodgett, 1991). Both kinases have an inhibitory serine phosphorylation site at their N-termini (Ser21 for α and Ser9 for β) and a facilitative Tyr site in their catalytic loop (Tyr279 for α and Tyr216 for β). Although these isoforms have a lot in common, they do not completely share their physiological roles.
GSK3β is a well-known regulator of glycogen metabolism, gene expression, and cell cycle progression (Kockeritz et al., 2006; Eldar-Finkleman and Krebs, 1997). In basal state it is constitutively activated, but phosphorylation of serine site by Akt inhibits the kinase activity of the enzyme (Stambolic et al., 1994; Cross et al., 1995;Grimes and Jope, 2001).
Glycogen is the major form of glucose storage in liver and its levels in cells are tightly regulated by glycogen synthase (GS) (synthesis) and glycogen phosphorylase (lysis) (Ferrer et al., 2003). During fasting state, GSK3 inhibits glycogen synthesis by suppressing GS through inhibitory phosphorylation. Upon insulin stimulation, however, activation of PI3K-Akt signaling pathway leads to the inhibitory phosphorylation of GSK3 at the regulatory serine residue. This activates GS and thereby glycogen synthesis (Woodgett, 2001; Eldar-Finkelman, 2002; Summers et al., 1999).
It has been shown that GSK3β can attenuate the insulin-stimulated phosphorylation of tyrosine residues in IR and IRS1 by directly phosphorylating these proteins at serine residues (Eldar-Finkleman and Krebs, 1997; Greene and Garofalo, 2002; Liberman and Eldar-Finkelman, 2005). That suggests that GSK3β may be a potential kinase that also induces insulin resistance.

1.1.2.12. AMPK

AMPK is a heterotrimer that functions as a serine/threonine protein kinase (Towler and Hardie, 2007). The complex consists of one catalytic subunit (α1/2) and two regulatory subunits (β1/2

and γ1/2/3) and is activated upon phosphorylation at Thr172 residue in the catalytic domain of α subunit (Lage et al., 2008). The upstream kinases that can regulate AMPK activity include Liver Kinase B1 (LKB1) (Woods et al., 2003), Ca^{2+}/calModulin-dependent protein Kinase Kinase (CaMKK) β (Hawley et al., 2005), and TGFβ-activated kinase 1 (Momcilovic et al., 2006). The first two, LKB1 and CaMKK, are able to increase AMPK activity through direct phosphorylation of Thr172. AMPK itself interacts with insulin signaling pathway through activating TSC2 and thus repressing signaling through mTORC1 (Baum et al., 2009).

Physiologically, AMPK is and an enzyme that responds to the changes in the AMP:ATP ratio, and thus serves as an intracellular sensor for energy homeostasis (Lage et al., 2008). In addition to inducing ATP production by switching off anabolic processes in tissues, the activation of AMPK affects whole body fuel utilization by up-regulating fatty acid oxidation and glucose uptake and inhibiting lipogenesis and adipocyte differentiation (Towler et al., 2007; Lage et al., 2008). In liver, AMPK's role is to inhibit gluconeogenesis and synthesis of glycogen, fatty acids and cholesterol.

In response to diverse stress conditions, such as energy depletion, ER stress, hypoxia, AMPK activation induces autophagy, which is an evolutionally conserved pathway for self-digesting of cytoplasmic components and organelles by lysosomal degradation (Mizushima, 2007; He et al., 2009).

1.2. Defects in regulation of liver glucose metabolism in insulin resistant states

Liver plays a central role in fuel metabolism and regulates dynamic catabolic and anabolic processes to maintain energy homeostasis of organisms. It controls glucose homeostasis due to its ability to consume and produce glucose, and insulin is the principal hormone that controls these processes. In liver, insulin suppresses gluconeogenesis and promotes glucose storage and utilization by inducing glycogen and triglyceride synthesis (DeFronzo, 2004). Failure of the insulin signaling system is associated with many metabolic disorders, including dyslipidemia, hypertension, female infertility, and glucose intolerance that may progress to T2D (Reaven, 1988).

Insulin resistance has been strongly associated with obesity (Kahn and Flier, 2000) and a variety of pathological stress conditions, including inflammatory diseases, hemorrhage, thermal injury, sepsis, and cancer cachexia (Del Aguila et al., 2000; Ikezu et al., 1997; Ma et al., 2004; Houstis et al., 2006). Stress stimuli, such as oxidative conditions (Rudich et al., 1998; Maddux et al., 2001), osmotic shock, increased inflammatory response (Wellen and Hotamisligil, 2005; Shoelson et al., 2006), and the translation inhibitor anisomycin (Aguirre et al., 2000; Hemi at al., 2002) are found to promote Ser phosphorylation of IRS and, therefore, impair metabolic responses to acute insulin stimulation due to decreased IR-IRS interaction (Kanety et al., 1995; Hotamisligil et al.,

1996; Paz et al., 1997). It is known that JNK1 is one of the proteins activated by various stress signals such as cytokines or oxidative stress, and the activity of JNK1 increases under prediabetic or diabetic conditions. As mentioned before, this important kinase is also implicated in the phosphorylation of IRS1 and IRS2 (Hirosumi et al., 2002; Singh et al., 2009; Tuncman et al., 2006). In addition, cellular stressors that activate ErbB receptors induce Ser phosphorylation of IRS proteins through PI3K pathway and promote insulin resistance in hepatocytes (Hemi et al., 2002).

Abnormal fat accumulation in liver is often a prerequisite metabolic event for further pathogenesis (Marra et al., 2008), as it can lead to the generation of oxidative stress, inflammation, and ultimately cause apoptosis (Mantena et al., 2008). Non-alcoholic fatty liver disease (NAFLD) is considered as a main hepatic component of metabolic syndrome (Cornier et al., 2008). Obese people mostly with insulin resistance show excessive fat deposition in the liver, which is closely associated with pathogenic processes of the syndrome (Kotronen and Yki-Järvinen, 2008; Hamaguchi et al., 2005).

Breakdown products of carbohydrate and lipid are common energy sources which are converted to ATP in mitochondria. During the process of catabolism, the mitochondrion serves as the main source of energy for the cell, which converts nutrients into energy via cellular respiration using most of the oxygen derived to the cells (Wallance, 2005). In energy excessive state in the cell, mitochondrial energy production is inhibited and glucose and free fatty acids will be stored as glycogen and fat through anabolic processes. Mitochondrial dysfunction is frequently observed in metabolic syndrome (Petersen et al., 2003). In insulin resistant situations mitochondrial oxidative phosphorylation and ATP production is reduced, and, therefore, the expression of genes encoding for oxidative metabolism are down-regulated (Mootha et al., 2003; Patti et al., 2003; Petersen et al., 2003). In this situation, it is difficult to maintain redox-homeostasis and following changes in mitochondrial membrane permeability cause the release of proapoptotic mediators that can damage DNA and lead to apoptosis (Kantrow and Piantadosi, 1997; Marchetti et al., 1996).

IRS proteins are key mediators of insulin signaling. Although IRS1 and IRS2 share similar expression patterns, the signaling in growth and metabolism of these proteins is mediated tissue-specifically (Araki et al., 1994; Withers et al., 1998; Withers et al., 1999; Kubota et al., 2000). While IRS1-deficient mice are smaller in body size, display insulin resistance in skeletal muscle and have β-cell hyperplasia (Araki et al., 1994; Tamemoto et al., 1994; Yamauchi et al., 1996), mice lacking IRS2 possess defective hepatic insulin action and failure to suppress HGP (Withers et al., 1998; Withers et al., 1999; Kubota et al., 2000, Previs et al., 2000; Kido et al., 2000). Hepatic IRS2 expression is often reduced in mouse models of T2D, which reflects in part the inhibitory effects of hyperinsulinemia on expression of the IRS2 gene (Brady, 2004; White, 2003). For instance, in db/db diabetic mice hepatic IRS2 protein levels are dramatically lower compared to control mice, whereas no change has been observed in levels of IRS1 between the two groups

(Canettieri et al., 2005). Reduction in IRS2 has been associated with increases in gluconeogenic gene expression, including PEPCK, G6Pase and PGC1α (Canettieri et al., 2005).

In type 2 diabetic animals hepatic aPKC activation appears to be conserved (Standaert et al., 2004). Because aPKCs mediate, at least in part, insulin effects on hepatic lipid synthesis, this conservation of hepatic aPKC activation may explain the overproduction of lipids (i.e., VLDL triglycerides) by the liver in obesity, metabolic syndrome, and diabetes (Farese et al., 2005). Moreover, coupled with the compromised activation of Akt in the liver when obesity progresses to diabetes, the conservation of aPKC activation could explain how the diabetic liver generates excessive amounts of both glucose and VLDL lipids, which is a particularly lethal combination for contributing to the development of macrovascular disease (Farese et al., 2005).

Insulin resistance induced by abnormal conditions such as hyperinsulinemia, obesity and excess nutrient availability is accompanied by an increase in S6K1 activity (Um et al., 2004; Tremblay et al., 2005). As described above p70S6K directly increases Ser phosphorylation of IRS1 and IRS2 (Shah et al., 2004: Harrington et al., 2004; Um et al., 2004; Werner et al., 2004), which leads to its interrupted binding with IR and reduction in insulin sensitivity. Moreover, the kinase is also associated with down-regulation of IRS1 protein levels (Harrington et al., 2004) and deactivation of mTORC2 (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2009), which gives an extra layer for attenuation of insulin signaling.

p70S6K represses also the expression of cytochrome P450 (CYP), which among other roles forms part of multi-component electron transfer chain in mitochondria. It does that by phosphorylating and inhibiting pregnane X receptor (PXR) that plays a central role in activating the expression of CYPs in human liver (Pundugula et al., 2009; Lin et al., 2008).

In the fasted state liver is primarily responsible for maintaining glucose levels, with FoxO1 playing a key role in promoting the expression of gluconeogenic enzymes (Gross et al., 2008). However, in type 2 diabetic patients hepatic glucose production is a key physiological process that becomes altered (Bogardus et al., 1984). As described above the HGP is raised due to increased expression of gluconeogenic enzymes PEPCK and G6Pase (Barzilai and Rossetti, 1993; Trinh et al., 1998) and impaired insulin action on inhibiting hyperactivated FoxO1 despite the presence of hyperinsulinemia (Valera et al., 1994; Trinh et al., 1998). Besides decreased insulin receptor signaling in hepatocytes, other factors contribute to elevated gluconeogenesis in diabetes, including increased supply of gluconeogenic precursors to liver (glycerol, amino acids, free fatty acids), increased liver lipid content, altered glucagon/insulin ratios, cytokines and adipokines (Lin and Accili, 2011).

Hyperactivated FoxO1 in hepatic insulin resistant state increases, among the other genes, the expression of HeMe OXygenase 1 (HMOX1), the enzyme that breaks down heme into biliverdin (BV), Fe(III) and CO_2 (Cheng et al., 2009; Cheng and White, 2010). That causes defects in mitochondrial electron transport chain by depleting heme, which is the essential cofactor that facilitates electron transport and ensures the expression, stability and function of electron transport chain components (Converso et al., 2006). Consequently, NADH oxidation impairs,

causing NAD^+/NADH ratio decreases, and subsequently inhibits the SIRT-PGC1α pathway of mitochondrial biogenesis (Cheng et al., 2009; Cheng and White, 2010; Lagouge et al., 2006; Feige et al., 2008; Milne et al., 2007). The result is impaired fatty acid oxidation and increased dyslipidemia in liver.

2. Neuregulin and its receptors

2.1. Neuregulin

Neuregulin (NRG) conform a complex family of proteins that structurally belongs to the EGF family of growth factors. The isoforms are formed by alternative splicing of four genes, namely *nrg1-4* (Buonanno and Fischbach, 2001; Yarden and Sliwkowski, 2001). All the bioactive isoforms have a characteristic EGF-like domain that distinguishes them from the rest of the EGF family.

Neuregulin is predominantly expressed in parenchymal organs and in the embryonic central and peripheral nervous systems (Peles and Yarden, 1993; Carraway and Burden, 1995). They induce growth, differentiation, survival and migration of cells in epithelium (Wen et al., 1992), nerve (Meyer and Birchmeier, 1995), cardiac (Zhao et al., 1998) and skeletal muscle (Florini et al., 1996).

In the early 1990s, a number of groups isolated several neuregulin isoforms naming them Neu Diffention Factor (NDF) (Wen et al., 1992), Heregulin (NRG) (Holmes et al., 1992), Glial Growth Factor (GFF) (Marchionni et al., 1993), AcetylCholine Receptor (AChR)-Inducing Activity (ARIA) (Falls et al., 1993), and Sensory and Motor nerve-Derived Factor (SMDF) (Ho et al., 1995). All of these isoforms are encoded by the same *nrg1* gene.

Nrg1 gene is ≈1.4 megabases long, but less than 0.3% encodes protein. It generates at least 15 different NRG immature isoforms (≈70kDa) due to multiple tissue-specific promoters and to alternative splicing (Buonanno and Fischbach, 2001), as mentioned above. Most of the isoforms contain a transmembrane domain and they differ from each other regarding the type of EGF-like domain (α, β or γ), the N-terminal sequence (I-VI), and the yuxtamembrane extracellular region (a, b or c).

The EGF-like domain of neuregulin is 45-55 amino acids long and includes six cysteine residues, which interact covalently to form three loops and six beta-sheets. Neuregulin is the ligand for ErbB3 and ErbB4 receptors. The EGF-like domain of neuregulin is sufficient for binding and receptor activation. However, although NRG α and NRG β are identical in the EGF-like domain sequence up to fifth cysteine and both directly bind ErbB3 and ErbB4, their binding affinities are quite different (Jones et al., 1999a). In most assays, NRG1β isoforms are 10-100 times more potent than α isoforms. In addition, the splice variants of NRG in ligand-receptor complexes also differ in their ability to recruit a partner ErbB receptor (Tzahar et al., 1997; Landgraf and Eisenberg, 2000; Ferguson et al., 2000), which affects their potency and kinetics of signaling. Partner receptor selectivity in the case of NRG appears to be ErbB2>ErbB3/4>ErbB1. Studies

have shown that the partner selectivity also depends on the EGF-like domain variants of neuregulin. For instance, α- and β-isoforms share the ability to form heterodimers of ErbB3 with ErbB2, but only β-isoforms are capable of stabilizing an ErbB3/ErbB1 heterodimer (Pinkas-Kramarski, 1996a).

The region between transmembrane domain and EGF-like domain contains proteolytic cleavage-sensitive domains that allows the shedding and release of the EGF-like domain by metalloproteases ADAM17/TACE and ADAM19/meltrin-β (Montero et al., 2000; Shirakabe et al., 2001). After the release, the EGF-like domain binds to its receptors in an autocrine, paracrine or juxtacrine manner (Goodearl et al., 1995; Loeb et al., 1998).

The N-terminal sequence consists of a variety of domains, according to what NRG1 isoforms are classified (type I-VI), as mentioned before (Mei and Xiong, 2008). Some of those isoforms contain an immunoglobulin (Ig)-like domain (Ig-NRG), which interacts with heparansulphate groups of proteoglycans of the extracellular matrix and thereby allowing the clustering of NRG in the extracellular space. Cleaved Ig-NRGs are soluble ligands suitable for autocrine and paracrine signaling. Some other isoforms contain a Cysteine-Rich Domain (CRD), which binds to the plasma membrane, thus rendering a second anchoring point for the growth factor. Processing of CRD-NRG precursors generates membrane-bound ligands suitable for juxtacrine signaling (Falls, 2003). Studies with mice have demonstrated that isoforms differing in their N-terminal sequence or in their EGF-like domain have distinct *in vivo* functions (Falls, 2003).

NRG1 knockout mice have a severe reduction in several neural crest-derived cell populations including Schwann cells (the glia of the peripheral nervous system, which form the myelin sheaths of peripheral nerves), neural crest-derived cranial sensory neurons, and sympathetic neurons (Britsch et al., 1998; Meyer et al., 1997). Similarly to knockout of Erb4 or ErbB2, mice defective NRG1 die at embryonic day 10.5 due to defects in cardiogenesis. (Burden and Yarden, 1997; Meyer and Birchmeier, 1995) In adulthood, neuregulin is also implicated in a wide range of processes in the heart such as myocyte proliferation and growth, myofilament structure and organization, survival, myocyte-matrix coupling, glucose uptake, and angiogenesis (Zhao et al., 1998, Russell et al., 1999, Sawyer et al., 2002, Cote et al., 2005, Kuramochi et al., 2006, Bersell et al., 2009, Pentassuglia et al., 2009; Xiong et al., 2001; Yen et al., 2002). In addition to the essential role in cardiac morphogenesis (Meyer et al., 1995), Ig-NRG1 knockout mice expose defects also in cranial sensory neuron, and symphathetic development (Kramer et al., 1996; Britsch et al., 1998). However, mice lacking CRD-NRGs do not have defects in heart development (Wolpowitz et al., 2000) and they survive until birth, when they die because of inability to breath caused by non-functional neuromuscular synapses. Instead, they have a marked reduction in Schwann cell precursors, degeneration of peripheral and cranial nerves and a ≈50% reduction in the number of spinal and motor sensory neurons characteristic to CDR-NRG knockouts. Mice with a targeted mutation that inactivates all NRG isoforms with an α-type EGF-like domain have not been reported to have abnormalities in nervous system or cardiac development, but have marked defects in breast development (Li et al., 2002a). It is proposed that NRGα2 isoform is highly

expressed during late at pregnancy (Fendly et al., 1990), inducing formation of lobuloalveolar structures and increasing formation of milk-producing cells (Yang et al., 1995).
Dysregulation of NRG signaling has been reported in several pathologies including breast cancer (Tsai et al., 2003; Atlas et al., 2003; Li et al., 2004), melanoma (Stove et al., 2005), multiple sclerosis (Frohman et al., 2006), Alzheimer disease (Go et al., 2005; Willem et al., 2006), and schizophrenia (Harrison and Law, 2006).

2.2. Neuregulin receptors – The Epidermal Growth Factor Receptor Family

The ErbB (also named HER for human isoforms) receptor family is a sub-group of the growth factor receptor tyrosine kinase super-family. It consists of four receptors: ErbB1 (also known as Epidermal Growth Factor Receptor (EGFR)), ErbB2 (also known as HER2 or Neu), ErbB3 and ErbB4.
Each of the receptors is a type I tyrosine kinase transmembrane protein. The large extracellular ligand-binding domain consists of four subdomains (I-IV). The sub-domains of the ErbB proteins are relatively conserved among members of the family, despite the fact that the receptors bind different ligands. The intracellular part of the protein consists of a small intracellular juxtamembrane segment, a bilobular tyrosine kinase domain and a carboxyl-terminal tail containing tyrosine phosphorylation sites. Among the four members of ErbB receptor family, ErbB2 and ErbB3 are exceptional, as the first has no known ligand (Klapper et al., 1999; Karunagaran et al., 1996) and the second is kinase-dead (Guy et al., 1994; Pinkas-Kramarski et al., 1996b).
The activation of ErbBs can occur through formation of ligand-bound receptor homo- and heterodimers (Schreiber et al., 1983; Ushiro and Cohen, 1980) and subsequent transphosphorylation of intracellular tyrosine residues.
It is well established that dimerization is provoked by ligand binding and both events are essential for kinase activation (Schlessinger, 2000). In the absence of ligand binding, the majority of ErbBs exist in tethered, autoinhibited form in which the dimerization loop of subdomain II is covered by an intermolecular interaction with subdomain IV. Ligand binding bivalently to the leucine-rich repeats of subdomain I and III traps the receptor in an unthethered conformation, which, thereafter, is able to dimerize with other receptor through the exposed dimerization arm in a 2:2 receptor/ligand stoichiometry (Burgess et al., 2003). The ligandless ErbB2 remains constitutively in the active conformation, with an exposed dimerization loop ready to interact with dimerization partners (Garrett et al., 2003).
The conformational transition of extracellular domains is carried forward to the intracellular receptor portions. It leads to removal of the inhibitory C-terminal tail from the ATP binding cleft, juxtaposition of the C-lobe of the one partner's kinase domain with the N-lobe of the other dimerization partner, and consecutive induction of transphosphorylation of terminal tyrosine residues. (Zhang et al., 2006b) It is believed that the juxtamembrane segment, situated between

the transmembrane domain and the N-lobe, maintains a certain degree of flexibility so that the receptors can switch positions dynamically to activate each other (Zhang et al., 2006b). The activation of ErbB3, being a kinase-dead receptor, is dependent upon heterodimerization with other members of the family.

The phosphorylated tyrosine residues on the C-terminal tail of ErbB receptors serve as docking sites for intracellular signaling molecules or adaptor proteins containing either SH2 or PTB domains. Besides that, ErbB receptors also contain non-tyrosine phosphorylation sites and unusually high number of basic residues in the 35-residue juxtamembrane region for possible association with PKC, acidic phospholipids, calmodulin, which may influence on dimerization (McLaughlin and Murray, 2005; Aifa et al., 2005; Aifa et al., 2006; McLaughlin et al., 2005).

The ligands of ErbB receptors are polypeptides that include an EGF-like consensus sequence consisting of three disulfide-bonded intramolecular loops. Ligands are generated upon the regulated cleavage of glycosylated transmembrane precursor molecules by ADAM (a disintegrin and metalloprotease), which are GPCR (G-protein-coupled receptor) activated and zinc-dependent. Different ligands possess different receptor specificity and binding affinity. Epidermal growth factor (EGF), transforming growth factor-α (TGFα), β-cellulin (BTC), and amphiregulin (AR) are specific ligands only for ErbB1, whereas heparin-binding EGF (HB-EGF) and Epiregulin (EPR) can bind either ErbB1 or ErbB4. The neuregulin (NRG) 1 and 2 can interact either with ErbB3 or ErbB4, but the other two, NRG3 and NRG4, activate only ErbB4. Epigen (EPG) binds to ErbB1, ErbB3 or ErbB4 in the presence of ErbB2 (Kochupurakkal et al., 2005).

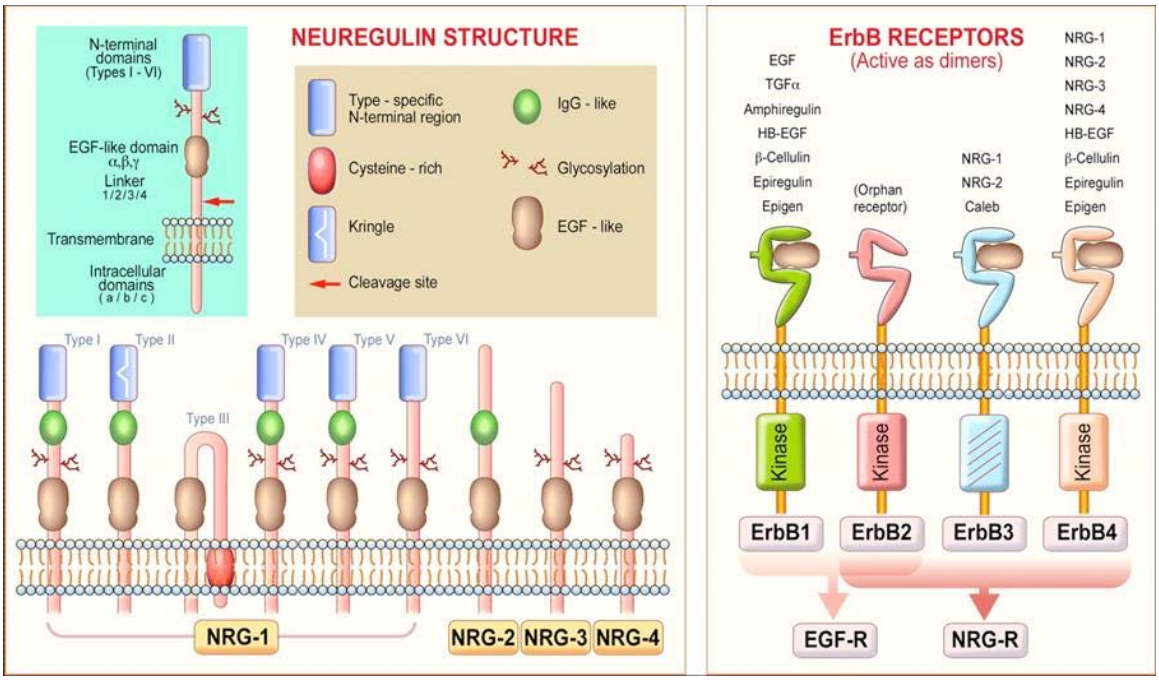


Figure 3. Neuregulin structure and its receptors. (Guma et al., 2010)

Beside ligand-mediated activation of the ErbB network, there are also a ligand-independent mechanisms regulating ErbB signaling. A metalloprotease-mediated cleavage of the extracellular

receptor domain can lead to either truncated or constitutively activated receptors (Codony-Servat et al., 1999), or the receptor nuclear translocation, where it functions as transcription or cotranscription factor (Lin et al., 2001; Ni et al., 2001; Giri et al., 2005). Regarding the intracellular trafficking of ErbB receptors, each of the isoforms has been detected to be present in the nucleus. Moreover, the relocalization of ErbB1 and ErbB4 is ligand dependent and influences gene expression (Carpenter, 2003; Amit et al., 2007). In addition, cleavage and receptor trafficking, unphysiological stimuli, such as oxidative and mechanical stress, UV-light and gamma-radiation, and activation of chemokine receptors can induce a shift in the basal activation and deactivation equilibrium of ErbB receptors (Ficher et al., 2003; Zwang and Yarden, 2006).

Members of ErbB family differ with respect to their tyrosine phosphorylation sites and the combinatorial interactions of ligands and receptors dictate which tyrosine sites will be phosphorylated (Fischer et al., 2003; Olayioye et al., 1998). Therefore, the recruitment of signaling proteins and activation of certain pathways depends critically on the specific ligand and receptor complex composition. The phosphorylation pattern of cytoplasmic receptor tyrosine residues created after receptor activation leads to interaction with specific signaling molecules containing SH2 and PTB domain, taking in account also the amino acids surrounding the tyrosine phosphorylation site.

All ErbB ligands and receptors are able to activate the RAS-MAPK pathway through adaptor proteins such as a Growth factor-Receptor Bound-2 (GRB2) and GRB2-bound exchange factor Son Of Sevenless (SOS). Activation of another important pathway, PI3K, however, is differentially induced. In that situation activation of ErbB3 and ErbB4 plays an important role as they contain respectively six and one direct binding site for the SH2 domain of PI3K p85 regulatory subunit. (Soltoff and Cantley, 1996) ErbB1 and ErbB2 can activate the PI3K pathway, but they do it indirectly through adaptor proteins such as GRB-Associated-Binding protein 1 (GAB1) and c-CBL (E3 ubiquitin-protein ligase, named after Casitas B-lineage Lymphoma). (Soltoff and Cantley, 1996) Other signaling effectors are recruited only to some ErbB family members: PhosphoLipase C-γ (PLC-γ) contains a binding site in ErbB1 and ErbB2; c-SRC, Focal Adhesion Kinase (FAK) and Protein Tyrosine Kinase 2 (PYK2) are activated upon stimulation of ErbB1 with EGF.

The specificity of ErbB-mediated signaling is regulated at multiple levels. The expression profiles of ErbB receptors and ligands differ not only between organs, but are also distinct for specific developmental stages. This allows regulation of biological responses throughout development and adulthood by influencing ErbB homo- or heterodimer formation and the identity of phosphorylation sites within individual ErbBs (Olayioye et al., 1998). Beside that, ligand affinity with its receptor influences the strength and duration of the signal (Kochupurakkal et al., 2005) and the pH stability of this interaction receptor trafficking. For instance, the pH-resistant interaction of ErbB1 with EGF targets the receptor to the lysosome, whereas TGFα and NRG1 dissociate from their receptors in early endosomes, thus favoring receptor recycling and signal potentiation (Ebner and Derynck, 1991).

Dimerization of ErbBs adds an additional level of diversity to the signal. Heterodimer combinations compared to homodimers are more potent and cannot be functionally substituted by homodimers. The ligandless ErbB2 is the preferred heterodimer partner for other ErbBs (Tzahar et al., 1996; Graus Porta et al., 1997), which forms the most potent complexes, due to an increased affinity of ligand binding, relaxed ligand specificity, decreased ligand dissociation, decreased rate of endocytosis, and increased receptor recycling, as well as highly promiscuous PTB and SH2 binding sites (Yarden and Sliwkowski, 2001; Jones et al., 2006).

The ErbB receptor signal attenuation is initiated by both transcription-independent and transcription-based negative feedback initiated by both preexisting and transcriptionally induced negative regulators, which define the signal specificity, duration, and amplitude. The transcription-independent signal attenuation is achieved by either receptor endocytosis (Waterman et al., 2001) or dephosphorylation by tyrosine phosphatases such as Density Enhanced Phosphatase-1 (DEP1) and Protein Tyrosine Phosphatase-1B (PTP1B).

With its widespread expression and highly regulated signaling, ErbB molecules are important in a variety of physiological processes, such as survival, cell growth and development. Dysregulation of its signaling has been detected in pathological states, such as respiratory disease, gastric ulcer disease, cardiomyopathy, inflammatory hyperproliferative diseases and most importantly in cancer (Tapinos et al., 2006; Slamon et al., 1989).

2.2.1. ErbB1

ErbB1 is a prototype member of type 1 receptor tyrosine kinases. After ligand binding, ErbB1 molecules cluster over clathrin-coated regions of the plasma membrane, which invaginate to form endocytic vesicles and mature to early and late endosomes. Gradually decreasing internal pH and accumulation of hydrolytic enzymes in endosomes leads to ErbB1 degradation. Unlike ErbB1, the three other ErbB proteins are endocytosis impaired and are more often recycled back to surface (Pinkas-Kramarski et al., 1996b; Baulida et al., 1996).

Studies with transgenic mice have shown that ErbB1 is important in promoting proliferation and differentiation of the epithelial component of skin, kidney, lung, pancreas and the gastrointestinal tract (Threadgill et al., 1995; Mittinen et al., 1995; Sibilia and Wagner, 1995). In addition, ErbB1 knockout mice show massive apoptosis in cortical and thalamic brain regions (Sibilia et al., 1998).

During pregnancy expression of ErbB1 increases in parallel to up-regulated DNA synthesis in the mammary gland and declines immediately before the onset of lactation (Ederly et al., 1985). Studies have revealed that EGF role in mammary glands is to induce ductal and alveolar epithelial differentiation and suppress the accumulation of milk fat droplets in the alveoli during mid- and late pregnancy, while overexpression of TGFα in mammary glands results in earlier alveolar development (Voonderhaar, 1987; Halter et al., 1992; Spitzer et al., 1995).

Expression of EGF-like ligands often accompanies ErbB1 overexpression in primary tumors. TGFα is considered relevant in human cancers and its co-expression with ErbB1 has been seen in several types of carcinomas (Modjtahedi and Dean, 1994). Particularly in lung, ovary and colon tumors co-expression of ErbB1 and TGFα correlates with poor prognosis (Salomon et al., 1995). Activation of ErbB1 has also been reported as a significant factor in vasoconstriction and hypertension (Hao et al., 2004).

2.2.2. ErbB2

As mentioned above, ErbB2 is a ligandless isoform, which is in constitutively activated state and, therefore, is a preferred heterodimerization partner for other ErbB receptors by decreasing the rate of endocytosis and increasing recycling of its partners (Worthylake and Wiley, 1997; Lenferink et al., 1998). Several factors can regulate ErbB2 intrinsic catalytic activity. While cytokine interleukine-6 promotes ErbB2 tyrosine phosphorylation (Qui et al., 1998), then certain growth factors and hormones, such as Platelet-Derived Growth Factor (PDGF), LipoProtein A (LPA) and EGF by itself, increase phosphorylation on threonine and serine residues and thereby decreasing tyrosine phosphorylation and ligand binding affinity through mechanism involving accelerated recycling of internalized receptors.

Overexpression of ErbB2 has been observed in several types of cancers, including lung adenocarcinomas and breast, gastric and cervical carcinomas (Ross and Fletcher, 1998; Stancovski et al., 1994; Slamon et al., 1989; Slamon et al., 1987). The amplification pushes the normal equilibrium towards formation of ErbB2 homo- and heterodimers, out of which ErbB2/ErbB3 heterodimer is believed to be one of the most biologically active and tumorigenic (Holbro et al., 2003; Hudelist et al., 2003). Excessive ErbB2 level correlates with tumor size, spread of the tumor to lymph nodes, high percentage of S-phase cells, aneuploidy and lack of steroid hormone receptors, implying that ErbB2 confers a strong proliferative advantage in tumor cells (Ross and Fletcher, 1998; Paik and Liu, 2000).

2.2.3. ErbB3

ErbB3 lacks the kinase activity because of substitution in its catalytic domain (Plowman et al., 1990; Kraus et al., 1989) but is capable of activating numerous signaling pathways when transactivated by other receptors. ErbB3 signaling is associated with biologic activities, such as mammary epithelial (Marte et al., 1995; Ram et al., 1995) and breast tumor cell (Holmes et al., 1992; Pietras et al., 1995) growth, and muscle cell differentiation (Bacus et al., 1992; Bacus et al., 1993; Peles et al., 1992; Wen et al., 1992; Falls et al., 1993), Schwann cell precursor proliferation, maturation and survival (Marchionni et al., 1993; Levi et al., 1995; Dong et al., 1995), and proliferation of keratinocytes *in vitro* (Marikovsky et al., 1995). Mice lacking ErbB3 die on

embryonic day 13.5 due to defective cardiac formation (Britsch et al., 1998; Erickson et al., 1997). In addition, they show partial lack of Schwann cells along peripheral and sensory neurons (Erickson et al., 1997; Riethmacher et al., 1997).

ErbB3 signaling participates in several cancers. For instance, in breast cancer ErbB3 heterodimer with ErbB2 contributes to metastasis by enhancing tumor cell invasion and intravasation (Xue et al., 2006), while ErbB1/ErbB3 activation has a role in tumor growth and metastasis *in vivo*. ErbB3 expression in cancer is also reported to be closely associated with relapse-free and overall survival and it is associated with a high risk of metastasis among patients with NSCLC (non-small-cell lung carcinoma) (Chen et al., 2007).

2.2.4. ErbB4

ErbB4 expression pattern is relatively limited. It has several isoforms that differ in their juxtamembrane and carboxyl termini and thus results in differences in the recruitment of PI3K, which activates cell-survival pathways (Elenius et al., 1999).

ErbB4 has been shown to regulate neural development (Jones et al., 2003), including its role in astrogenesis (Sardi et al., 2006) and control of axon navigation (Lopez-Bendito et al., 2006) in the developing mouse brain. Moreover, ErbB4 deficient mice display severe defects in the development of the cranial sensory ganglia following migration from the neural crest. Studies have shown that ErbB4 with its ligand NRG1 are involved in the pathogenesis of schizophrenia and other neurological pathologies such as bipolar disorder, depression and dementia (Corfas et al., 2004; Law et al., 2006; Hahn et al., 2006). In addition, ErbB4 has an important function in mammary differentiation (Jones et al., 2003) and cardiac development (Gassmann et al., 1995).

2.3. Neuregulin signaling in regulation of metabolism

Neuregulin role as a regulator of metabolism has been established in the early 2000s. The studies indicated that in muscle NRG, in addition to its well-known roles as a myogenic and neurotrophic factor, also stimulates glucose uptake, both in an acute and chronic manner (Suárez et al., 2001).

NRG induces glucose uptake in skeletal muscle by translocation of GLUT4 to cell surface membranes (Cantó et al., 2006). The effect is additive to insulin stimulation and thereby suggests that there are independent pathways regulating the action of these two factors (reviewed in Gumà et al., 2010). Studies with different inhibitors reveal that NRG requires PI3K/PDK1/PKCζ pathway to induce glucose transporter translocation in muscle cells and tissue (Cantó et al., 2004; Cantó et al., 2006). Although this cascade is shared partially with insulin action (Bandyopadhyay et al., 1997), both effectors show additive effects on glucose transport, as mentioned, but surprisingly no additive activation could be observed on these signaling elements. Muscle contraction shows additivity with the insulin action on glucose transport.

(Douen et al., 1990; Gao et al., 1994) and has been observed that NRG is involved contraction events (Cantó et al., 2006; Lebrasseur et al., 2003). Muscle contraction induces glucose transport through the second messenger Ca2+ and the energetic state sensitizer 5'-AMP, which activate Calcium/calModulin-dependent protein Kinase II (CaMKII) and AMPK, respectively (Wright et al., 2004). During contraction NRG is cleaved in a calcium/metalloprotease-dependent manner, thereby inducing the NRG EGF-like domain release and ErbB4/ErbB2 receptors activation. Blocking ErbB4 receptor with specific monoclonal antibodies impairs highly the contraction effect on glucose uptake (Cantó et al., 2006). Although CaMKII is not directly activated by NRG, its inhibition impairs glucose uptake induced by neuregulin, so establishing a relationship among the direct PKCζ activation by ErbBs and the calcium-dependent activation of CaMKII under contraction (Cantó et al., 2006).

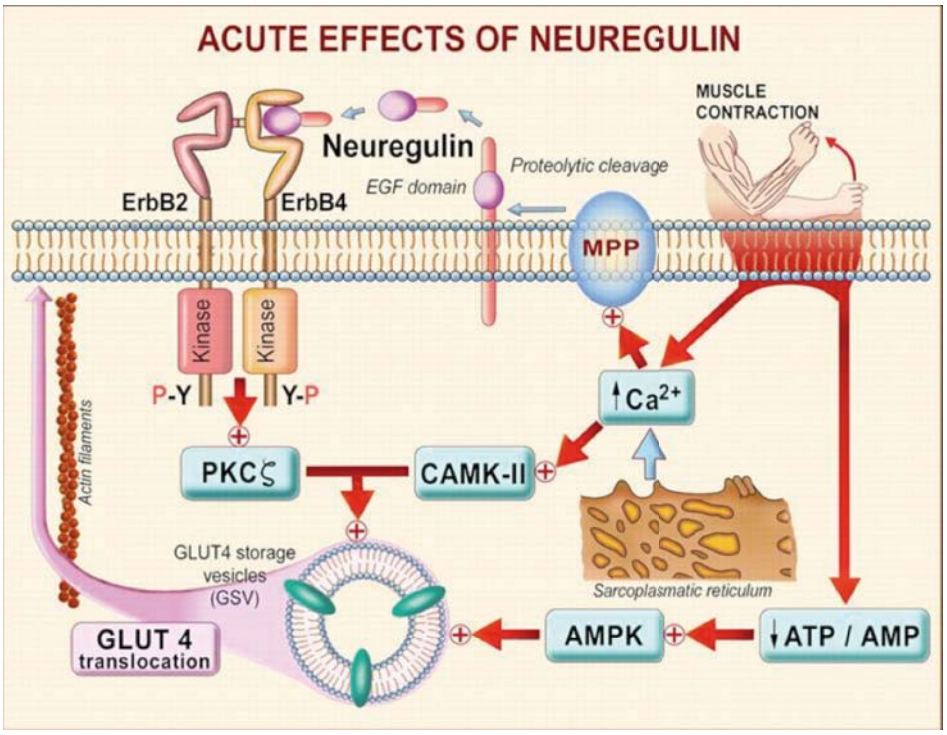


Figure 4. Acute effects of neuregulin in skeletal muscle (Guma et al., 2010)

Chronic treatment with NRG, at picomolar non-myogenic concentrations, induces oxidative capacity by increasing mitochondrial biogenesis and the GLUT4 content in muscle cells (Cantó et al., 2007). Mitochondrial biogenesis is achieved by increases in the expression of PGC1α and PPARδ (Cantó et al., 2007), both of which are also up-regulated after adaption of skeletal muscle to exercise (Baar et al., 2002; Goto et al., 2000; Luquet et al., 2003; Norrbom et al., 2004). Thus, chronic effects of NRG resemble those of muscle training inducing a switch toward oxidative metabolism similar to that of type I oxidative fibers and increasing insulin sensitivity.

Adult skeletal muscle expresses ErbB2 and ErbB4 (Cantó et al., 2006), while in cultured myotubes the predominant isoforms are ErbB2 and ErbB3 and rarely ErbB4 (Moscoso et al., 1995; Zhu et al., 1995). The ErbBs in muscle are accumulated in neuromuscular junction (NMJ),

ErbB2 and ErbB4 at the muscular postsynaptic site and ErbB3 at the presynaptic terminal Schwann cells (Trinidad et al., 2000). In addition, the receptors are also abundant in the transverse tubular (T-tubules) system (Ozcelik et al., 2002; Ueda et al., 2005). Studies in mice with muscle-specific double knockout of ErbB2 and ErbB4 (Martínez-Redondo, Doctoral Thesis, 2011) show smaller size, which corresponds to decreased visceral adiposity compared to wild type (WT). They also show reduced basal glycaemia and triglyceridemia, and contrary to expected, better sensibility to insulin. The characteristic observations of double knockout appear due to lack of ErbB2, because the phenotype of ErbB2 knockout resembles that of double knockout. Contrary, knockout of ErbB4 shows expose lower insulin sensitivity, indicating that muscle ErbB4 is relevant to maintain whole body insulin sensitivity, whereas ErbB2 may be involved in a variety of non-dependent neuregulin effects.

In vivo treatment of rats with an acute dose (25 ng/g body weight) of recombinant NRG show a significant reduction in glycaemia and insulinema during glucose tolerance tests (López-Soldado, manuscript in process). However, analyses of skeletal muscle signaling have shown only minor improvement in phosphorylation of PKCζ and AS160, but no effect on Akt activation. Instead, further studies unfolded a great NRG response on Akt activation in liver (López-Soldado, manuscript in process).

ErbB receptors expression in the liver has a developmentally and circadian regulated pattern, which provide a basis for differential combinatorial ErbB signaling during development and at different times of day (Carver et al., 2002). Adult liver expresses high amount of ErbB1 and ErbB3 receptors, but only trace amount of ErbB4 is detected by some authors (Carver et al., 1997; Carver et al., 1996; Camprecios et al., 2010). The almost ubiquitously expressed ErbB2 is present only in fetal and neonatal livers and persists until the end of the second postnatal week, but after what it declines dramatically. In adult liver ErbB2 is not expressed. At the same time, ErbB3 is not detected until embryonic day 19 from what its expression gradually increases, reaching adult levels during the second and third postnatal week. No major changes have been detected in expression of ErbB1 protein levels. The developmental profile of ErbB proteins in liver predicts different signaling capabilities of these receptors at different developmental stages. While in the adult liver only ErbB1 and ErbB3 are available for heterodimeric pairings, pairings between ErbB1, ErbB2, and ErbB3 are possible in fetus.

Adult animals also have circadian differences in the expression of ErbB1 and ErbB3 (Carver et al., 2002). The expression of ErbB3 and to lesser extent ErbB1 is increased, 6- and 2-fold respectively, late in the light phase and early in the dark phase, which is the end of the resting period and, when insulin and hepatic glycogen levels are lowest (Carver et al., 2002). Studies show that ErbB3 is down-regulated by insulin both *in vitro* (Carver et al., 1996) and *in vivo*. The down-regulation by insulin is mediated through PI3K, whereas p70S6K (Carver et al., 1997), an enzyme downstream of PI3K, cAMP levels (Heyworth and Houslay, 1983; Marchmont and Houslay, 1980) and PKC (Cooper et al., 1990) do not have a role in it. Besides lowering ErbB3 mRNA and protein levels, insulin also inhibits NRG binding to the receptor (Carver et al., 1996).

Moreover, higher expression of ErbB3 observed in two models of insulin deficiency *in vivo* (streptozotocin (STZ)-induced type I diabetes and fasting) suggest that the metabolic state of the animal may control ErbB3 synthesis (Carver et al., 2002; Carver et al., 1997). In normal liver of animals, there are roughly 230000 binding sites for EGF versus 20000 for heregulin during the early light period (Carver et al., 1996). Thus the increase in ErbB3 levels relative to ErbB1 during the late period of the normal day or following prolonged fast, could facilitate the formation of ErbB3/ErbB1 heterodimers compared with ErbB1 homodimers (Carver et al., 2002).

Because of inactive tyrosine kinase domain, ErbB3 is unlikely to initiate intracellular signaling on its own. However, in conjugation with ErbB1 or ErbB2, neither of which binds NRG, ErbB3 has been shown to elicit both differentiation and growth responses (Carver et al., 1997). NRGβ1 induces phosphorylation of ErbB3 and decreases ErbB3 protein levels, suggesting that NRGβ1 activates signaling through the ErbB3 receptor and influences receptor down-regulation.

OBJECTIVES

Previous studies in our laboratory revealed that neuregulin, besides regulating cell growth, differentiation and survival, has a role also in metabolic processes, such as the induction of muscle glucose uptake by an insulin-independent and contraction-related pathway. Moreover, recently we observed that neuregulin had *in vivo* effects lowering whole body glycaemia in control and diabetic ZDF rats. Preliminary studies analyzing signaling cascades involved in the stimulation of glucose uptake through GLUT4 translocation discarded skeletal muscle as the main neuregulin target-tissue in the *in vivo* action in resting (non-exercising) situation. Then, the neuregulin target-tissue(s), which were responsible for the hypoglycemic effect, and the involved mechanisms remained unkown.

Therefore, the objectives for this thesis were:

1. To characterize *in vivo* neuregulin effects on glycaemia in a mice model, in order to assess studies affordable with the amount of recombinant neuregulin necessary and available, developing studies in different metabolic situations: fasted, fed and insulin-deficient mice;

2. To characterize neuregulin effects in another insulin target-tissue, the liver, that has an essential role regulating glycaemia. Analysis of the neuregulin-induced signaling cascade in the liver and its possible interaction with the insulin signaling pathway;

3. To identify neuregulin action on proteins that regulate or are involved in liver glucose metabolism contributing to the neuregulin hypoglycemic effect;

4. To evaluate *in vivo* neuregulin effect on glycaemia in an insulin resistant state, the db/db mice model, that develops type 2 diabetes. Analysis of the neuregulin effects in liver;

5. Analysis of the "neuregulinemia" in mice plasma at different metabolic situations: circadian rhythm, fasted/fed, type I and type II diabetes models: searching for a physiological role of neuregulin as a regulator of glycaemia.

RESULTS

1. *IN VIVO* NEUREGULIN EFFECTS IN CONTROL MICE

All of the studies carried on in this thesis are performed *in vivo* mouse model, mostly with C57BLKS/J strain. In this section, neuregulin effects on glycaemia and insulinemia are characterized on C57BLKS/J-db/+ (control) animals. We used this strain as a control group because they are respective controls to C57BLKS/J-db/db (db/db) type 2 diabetic mice that are described later in section 2.

Mice age during the different studies was between 7-15 weeks. Taking in account the circadian rhythm of hepatic ErbB receptors (Carver et al., 2002) all the necessary experiments, either fasted or fed state, were performed at the same timeframe during the morning, while animals have started their resting period. Knowing that EGF domain from neuregulin alone is able to activate its receptors, ErbB3 and ErbB4, a recombinant NRG 1β1 EGF domain (NRG) dilution in saline was used.

1.1 Effects on plasma metabolites (glucose, lactate and triglycerides) and insulin levels

To understand more clearly the roles of neuregulin on regulating whole-body metabolism, it is utterly important to describe how and by what extent neuregulin changes plasma metabolites (glucose, lactate and triglycerides) and insulinemia in response to different or no substrates on an *in vivo* model, which represents the closest situation to reality. For that purpose mice were treated with neuregulin or saline 15 min prior to glucose or pyruvate injection during glucose or pyruvate tolerance tests, respectively, and blood samples were collected at certain time-points as shown on Figure 1. For measuring neuregulin effect to basal glycaemia, saline was administered instead of substrate and samples were taken on respective time-points to other experiments.

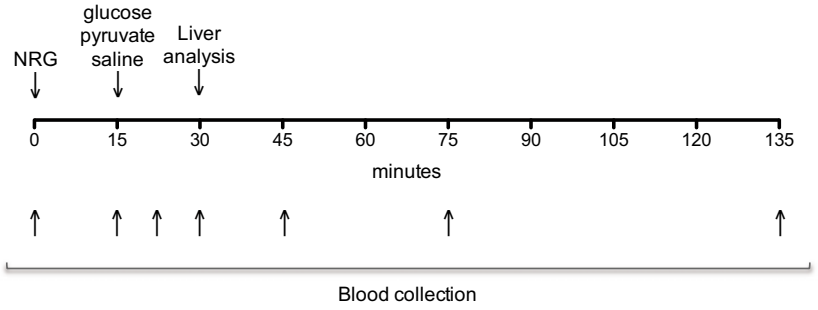


Figure 1. Design of experiments

1.1.1. Neuregulin action on glycaemia and insulinemia under basal conditions in fasted mice

The initial characterization tests consisted in testing the neuregulin effect on glycaemia and insulinemia in fasted mice. Although previous studies with rats, performed in our lab, have shown that NRG is able to reduce glycaemia during GTT, there was no knowledge on a possible action regulating basal glycaemia and insulinemia. In order to test this aspect, we measured glucose concentration in the blood after a short-term action of NRG, administrated intraperitoneally (i.p.). While saline-treated fasted animals during first minutes showed a slight increase in glycaemia, probably due to experiment-caused stress, NRG treatment significantly kept the glycaemia lower almost in every measured time-point (Fig. 2, A). By the end of the test mice that got a dose of NRG often experienced hypoglycaemia, which in few cases reached lower than 30 mg/dL of blood. That refers that the consumption of glucose during the test was higher than glucose production by the body. Despite that severely low blood glucose level, mice did not show any sign of hypoglycemic shock. A finding that neuregulin lowers basal glycaemia comes also out from counting AUC (Fig. 2, B) where NRG treatment gives a significant 35% reduction.

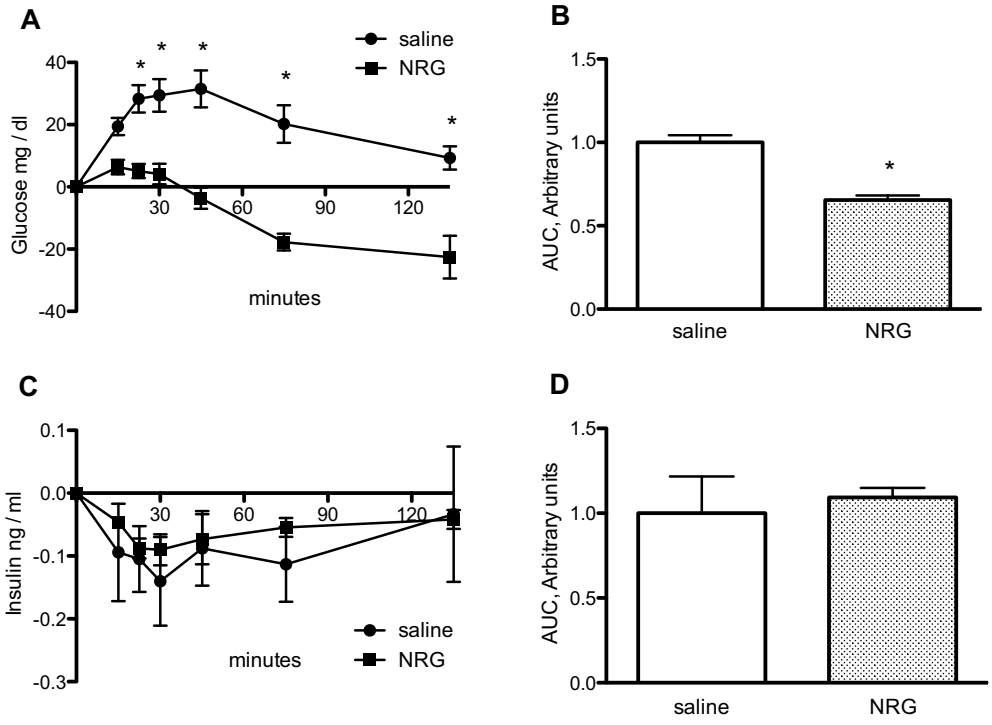


Figure 2. **Neuregulin action on glycaemia and insulinemia under basal conditions in fasted state**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=8-10; (**B**) AUC of glycaemia, n=8-10; (**C**) change in insulin level relative to basal insulinemia, saline vs NRG, n=3; (**D**) AUC of insulinemia, n=3. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**, **C**) or Unpaired Student's t test (**B**, **D**).

NRG did not have any effect at any point measured on basal insulin levels (Fig. 2, C), which was lowering during time independently of the treatment. That can easily be observed also when quantifying the area under the curve (AUC) (Fig. 2, D). Insulin has a short plasma half-life (4-6 min), as would be expected from a necessity to respond rapidly to changes in blood glucose (Duckworth et al., 1998). A non-changing insulin level would, therefore, suggest that NRG by itself does not regulate insulin production by β-cells, but the levels increase according to the need dictated by blood glucose levels.

1.1.2. Neuregulin action on glycaemia and insulinemia under glucose tolerance test (GTT) in fasted mice

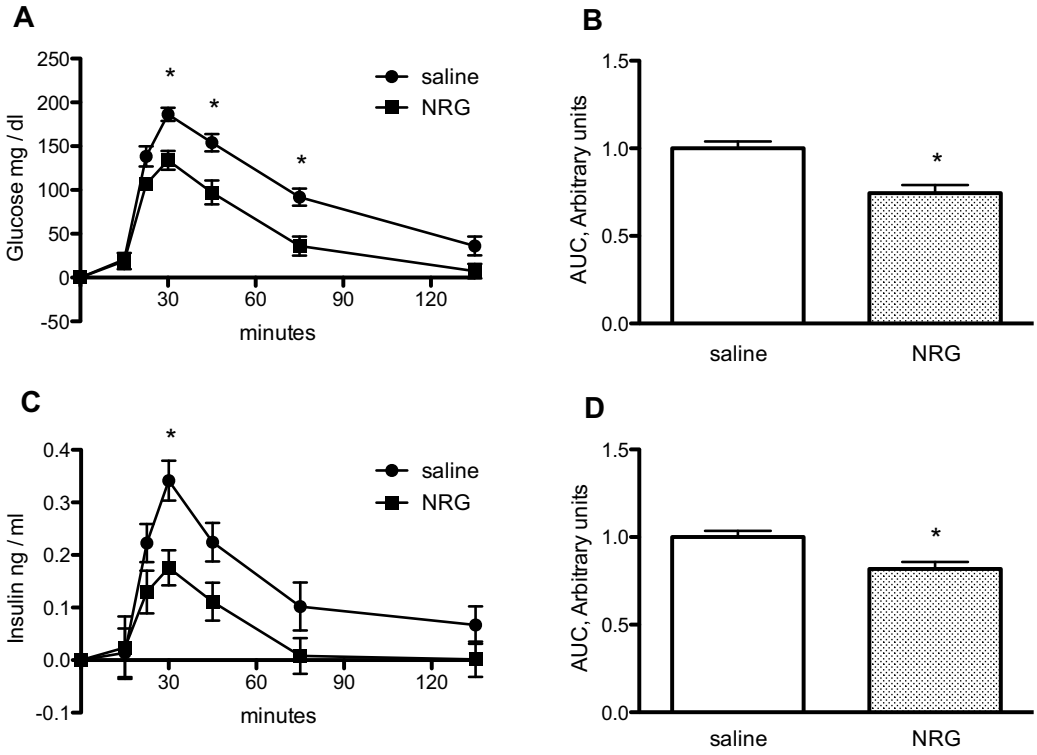


Figure 3. **Neuregulin action on glycaemia and insulinemia under glucose tolerance test in fasted state**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=6-9; (**B**) AUC of glycaemia, n=6-9; (**C**) change in insulin level relative to basal insulinemia, saline vs NRG, n=4-5; (**D**) AUC of insulinemia, n=4-5. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**, **C**) or Unpaired Student's t test (**B**, **D**).

Here we tested the neuregulin effect on glycaemia and insulinemia during intraperitoneal glucose tolerance test (GTT). Although previous studies with rats, performed in our lab, have shown that NRG is able to reduce glycaemia during GTT, it have not been shown on mouse model, which is a different specie with its slight differences in its metabolism and also economically wiser choice for affording *in vivo* studies. One of the differences between rat and mouse model is

the dose of NRG need to be used. While in rats 25ng NRG per gram of body weight gives a reduction in glycaemia, for the same effect in mice a double dose is needed. As shown in Figure 3 A, during GTT in overnight fasted animals glycaemia decreases significantly after acute NRG treatment, confirming the result seen in rats The AUC is supporting this result (Fig. 3 B). AUC of mice that received neuregulin treatment is reduced approximately 25%, which shows clearly that these animals became more glucose tolerant.

Analyzing insulinemia in the same samples indicates that, with decreased glycaemia during GTT, also insulin levels are significantly lowered in NRG-treated mice (Fig. 3, C). A reduction in insulin production in comparable level with lowering of glycaemia shows clearly that NRG lowers the need of insulin for handling the same amount of glucose (Fig. 3, D).

1.1.3. Neuregulin action on glycaemia under pyruvate tolerance test (PTT) in fasted mice

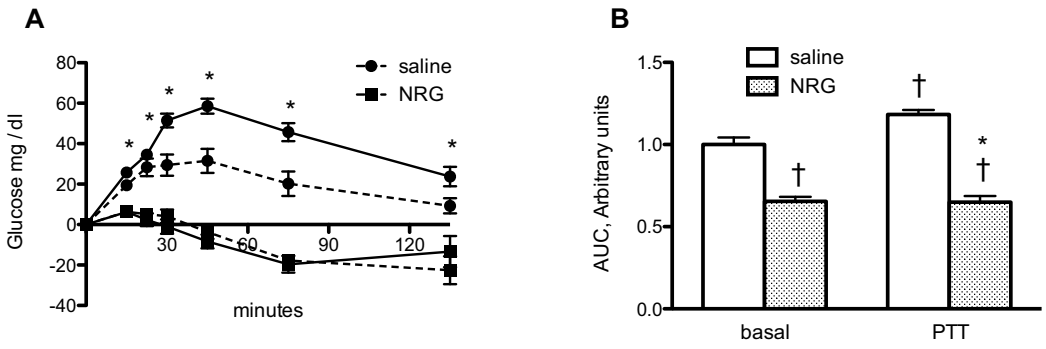


Figure 4. **Neuregulin action on glycaemia under pyruvate tolerance test in comparison with its action on basal glucose levels in fasted state**. (**A**) change in glucose level relative to basal glycaemia during PPT (continuous lines) and basal condition (dashed lines), saline vs NRG (50 ng/g of body weight), n=7-10; (**B**) AUC of glycaemia, n=7-10. * indicates significant difference vs. PTT saline group and † difference vs. basal saline group at $P<0.05$ determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

Taking in account the results from the experiments described above we wanted to measure whether neuregulin have an impact on the input of glucose to blood. For that we performed a pyruvate tolerance test (PTT). Pyruvate is one of the gluconeogenic precursors that will be converted to glucose in liver in states of energy deprivation, such as fasting, and released to bloodstream. An intraperitoneal injection of pyruvate without previous NRG treatment proved us that gluconeogenesis, indeed, is happening and we can observe an increase in blood glucose levels, which is higher than detected changes in basal glycaemia levels reaching upto 60-70 mg/dL over the basal fasted state glycaemia (Fig. 4, A). Administering NRG in such condition clearly inhibits the production of glucose from pyruvate. Moreover, the glycaemia in NRG-treated rodents during PTT follows exactly the detected glycaemia pattern that was measured during neuregulin effect on basal fasted glycaemia, reaching similarly severe hypoglycaemia. Taking in

account the latter it is possible to presume that neuregulin completely blocks HGP. This result is supported by AUC data, which was reduced by half and yields the same value than neuregulin action on basal glycaemia in fasting conditions (Fig. 4, B).

1.1.4. Neuregulin action on lactacidemia under basal / GTT / PTT conditions in fasted mice.

Above described experiments lead to an understanding that a possible role of NRG *in vivo* is to inhibit HGP. That raises questions about where and at which form the consumed glucose will be stored and where ends up the gluconeogenic precursor pyruvate instead of being released to bloodstream as glucose. One of the possibilities is to check blood lactate levels. Lactate is one of the main precursors for gluconeogenesis that will be taken up by liver and converted to pyruvate. The enzyme responsible for that conversion is lactate dehydrogenase (LDH), catalyzing also the reversible reaction converting pyruvate to lactate. The latter happens while pyruvate, exogenous or produced by glycolysis, cannot enter into gluconeogenesis (up-regulated during fasting) or for oxidation in Krebs cycle. Therefore, measuring circulating lactate levels is one of the potential ways to indicate whether neuregulin prevents gluconeogenesis and/or, indirectly, enhances glycolysis.

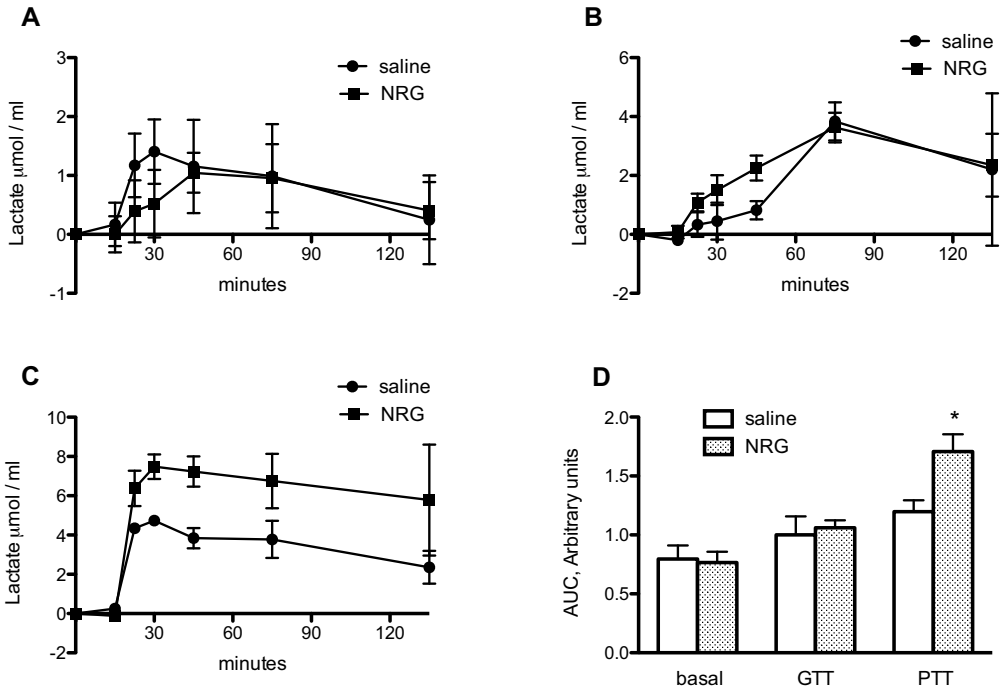


Figure 5. **Neuregulin action on lactacidemia under basal/GTT/PTT conditions in fasted mice**. Change in lactate level relative to basal lactacidemia under (**A**) basal, (**B**) GTT and (**C**) PTT condition, saline vs NRG (50 ng/g of body weight), n=3-4; (**D**) AUC of lactacidemia, n=3-4. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**, B, **C**) or Unpaired Student's t test (**D**).

According to lactate levels during GTT, as well as during solely treating fasted animals with NRG, we are unable to see up-regulated glycolysis. The curves and also AUC show basically no difference between treatments (Fig. 5, A, B, D). The unaffected profiles of lactate by NRG in these two conditions, while significant reduction in glycaemia was detected, can again refer to inhibited HGP or elevated glucose conversion to some other form, such as glycogen or triglycerides, or both.

However, a bigger difference between lactate levels can be observed during PTT (Fig. 5, C). After pyruvate administration during first 15 minutes, lactate levels rise by 4 μmol/ml over the basal level, reaching almost a double effect with previous NRG treatment and following a slow comparable-rate decrease in both groups. While the curves themselves are not statistically significant, we can observe significant difference comparing AUC-s (Fig. 5, D). That supports the finding that a pyruvate conversion to glucose was inhibited due to neuregulin caused down-regulation of gluconeogenesis and, thus, a higher amount of pyruvate could be converted to lactate. However, a different destination of pyruvate can also be considered.

1.1.5. Neuregulin action on triglyceridemia under basal / GTT / PTT conditions in fasted mice

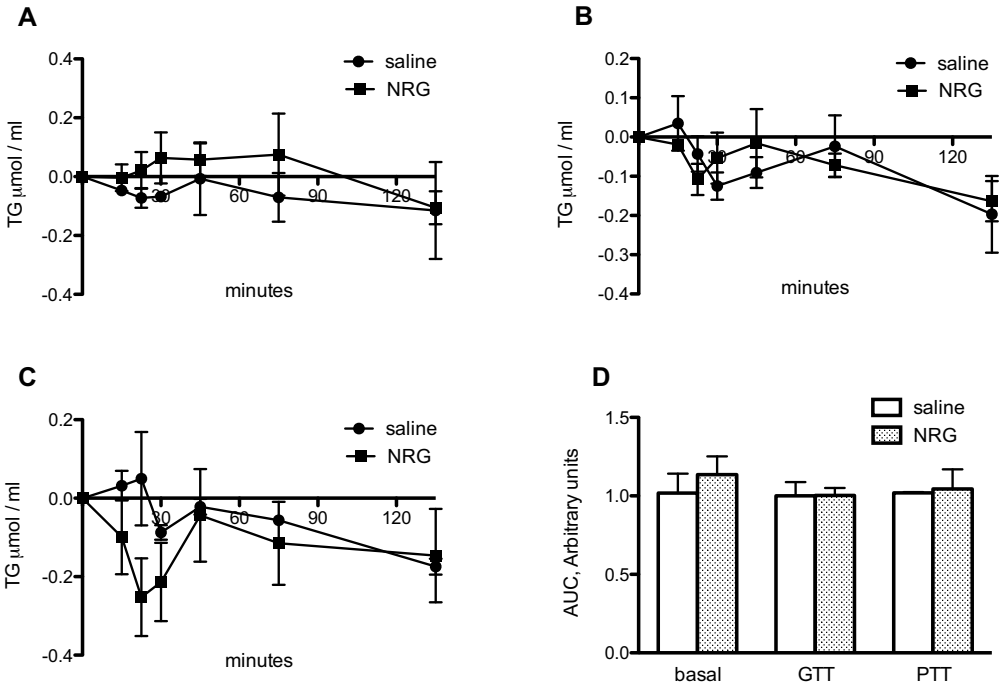


Figure 6. **Neuregulin action on triglyceridemia under basal/GTT/PTT conditions in fasted mice**. Change in triglyceride level relative to basal triglyceridemia under (**A**) basal, (**B**) GTT and (**C**) PTT condition, saline vs NRG (50 ng/g of body weight), n=3-5; (**D**) AUC of triglyceridemia, n=3-4. Statistical test applied: Two-way ANOVA with post-hoc Tukey's test (**A**, B, **C**) or Unpaired Student's t test (**D**).

Besides lactate, we also measured blood triglyceridemia during basal, GTT and PTT condition as one of the potential destination that can explain the differences seen in glycaemia. Comparing saline-treated animals with NRG group, we could not point out any differences between the groups. The curves in all three conditions, likewise calculated AUC-s showed the same profile regardless the mice had got a dose of growth factor or not (Fig. 6, A-D). The experiments give a good assumption that NRG neither up-regulate lipogenesis nor alter consumption of fatty acids in healthy animals.

1.1.6. Neuregulin action on glycaemia under basal conditions in fed mice

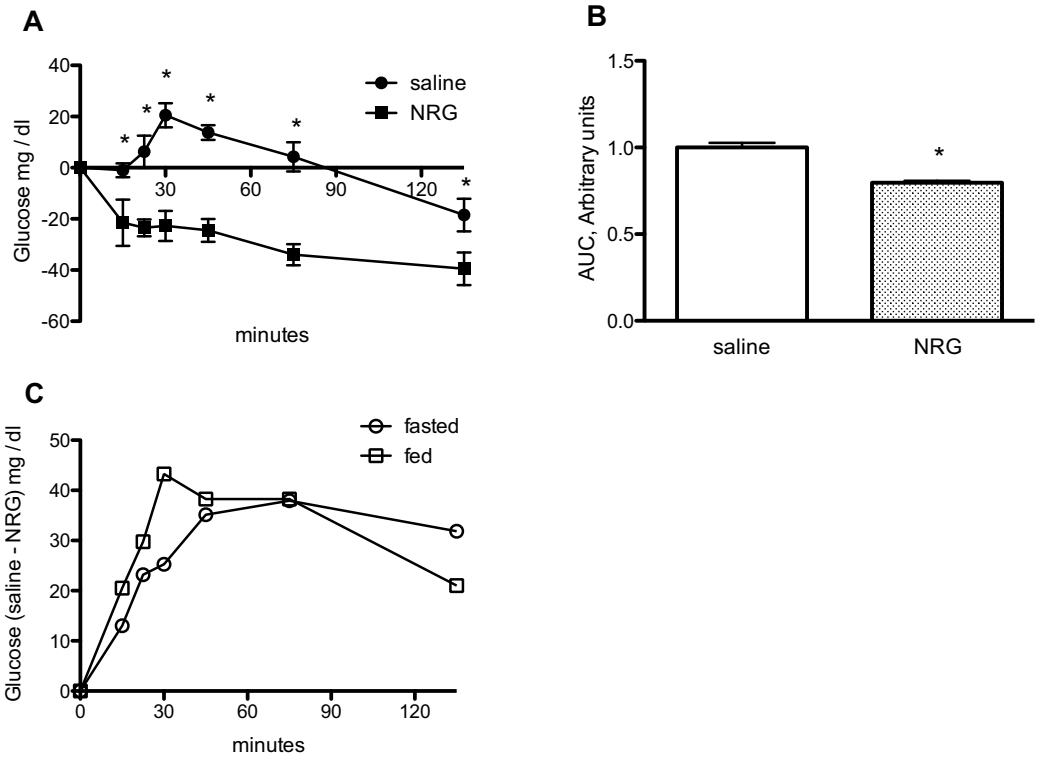


Figure 7. **Neuregulin action on glycaemia under basal conditions in fed state**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=4; (**B**) AUC of glycaemia, n=4; (**C**) the effect of neuregulin on glycaemia relative to saline-treatment – difference of means, fasted vs fed. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

Neuregulin have shown *in vivo* a remarkable effect on glucose metabolism in fasted rodents. Especially clear and important is the consequence seen in PTT, pointing out liver as a possible target tissue of neuregulin action on changing glucose homeostasis. Carver and colleges have demonstrated that a sole neuregulin receptor in liver is ErbB3 (Carver et al., 1997; Carver et al., 1996) and it has a circadian rhythm, which depends on animal metabolic state (Carver et al., 2002). While the ErbB3 is up-regulated at the end of the resting period or when rodents are

fasted, in fed state the expression of the receptor decreases 6-fold (Carver et al., 2002). This information made us question whether we can see the same effects as in fasted state, also in fed mice.

Measuring neuregulin ability to lower basal glycaemia in fed mice, unexpectedly yielded in a significant reduction in glucose levels (Fig. 7, A) also seen in AUC (Fig. 7, B). While no extra glucose was given to these animals, we can speculate that insulinemia during the experiment lowered and therefore, the binding between NRG and its receptors became better, giving a significant effect. A decrease in NRG-treated group, already at 15 min after injecting the growth factor, informs that despite the lower expected level of receptors it still can convert the signal to metabolic changes and inhibit HGP. Moreover, neuregulin inhibits HGP by the same extent in fed animals than fasted ones, reaching upto 40 mg/dL (Fig. 7, C).

1.1.7. Neuregulin action on glycaemia under glucose tolerance test in fed mice

Contrary to fasted mice, that were described above, during GTT neuregulin did not change glycaemia at any measured point in fed state (Fig. 8, A). The cause could be mentioned decrease in levels of receptors and therefore a low enough signal to make an observable change. However, the reason can also be a weakened binding of NRG to its receptor in the presence of higher insulin levels (Carver et al., 1996), which, indeed, by some extent occur in fed state and rise following the intraperitoneal glucose injection. As both groups showed similar changes in glycaemia over 135-minute test, there were also no differences between AUC (Fig. 8, B).

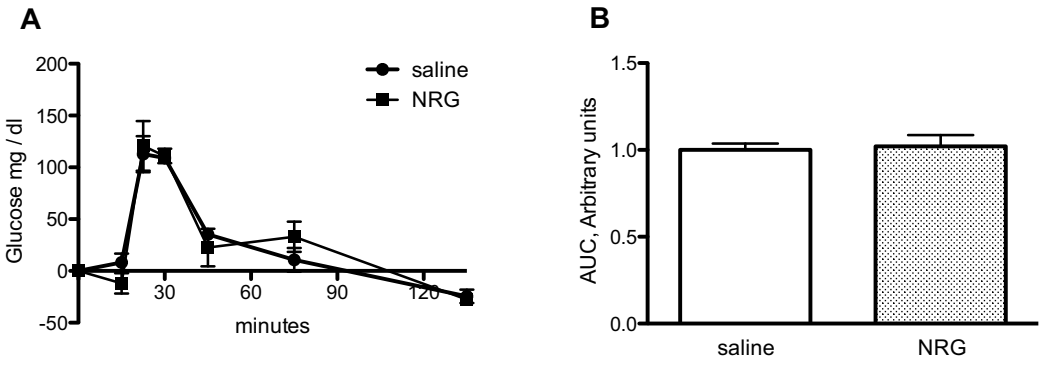


Figure 8. **Neuregulin action on glycaemia under glucose tolerance test in fed state**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=3-4; (**B**) AUC of glycaemia, n=3-4. Statistical test applied: Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

1.1.8. Neuregulin action on glycaemia under pyruvate tolerance test in fed mice

In fed state gluconeogenic pathway is down-regulated and so is the conversion of gluconeogenic precursors to glucose. Relaying on that fact, as expected, the gluconeogenesis is not taking place. Instead, the result is a graph that is similar to basal glycaemia measurements over the time, which proves above-mentioned (Fig. 9, A). Administering NRG to mice in these conditions produces a statistically significant reduction in glycaemia. The extent of reduction is lower than observed in fasted animals, which is expected due inhibited gluconeogenesis (Fig. 9, C). However, the lowering is comparable with neuregulin effect on basal fed state glycaemia. That is reasonable, because the gluconeogenic substrate, pyruvate, cannot be converted to glucose and NRG, therefore, can only inhibit the same component as in basal condition. Measuring AUC supports the latter with showing no difference between the basal glycaemia and pyruvate administration, and displays a decrease by approximately 20% in both cases (Fig. 9, B).

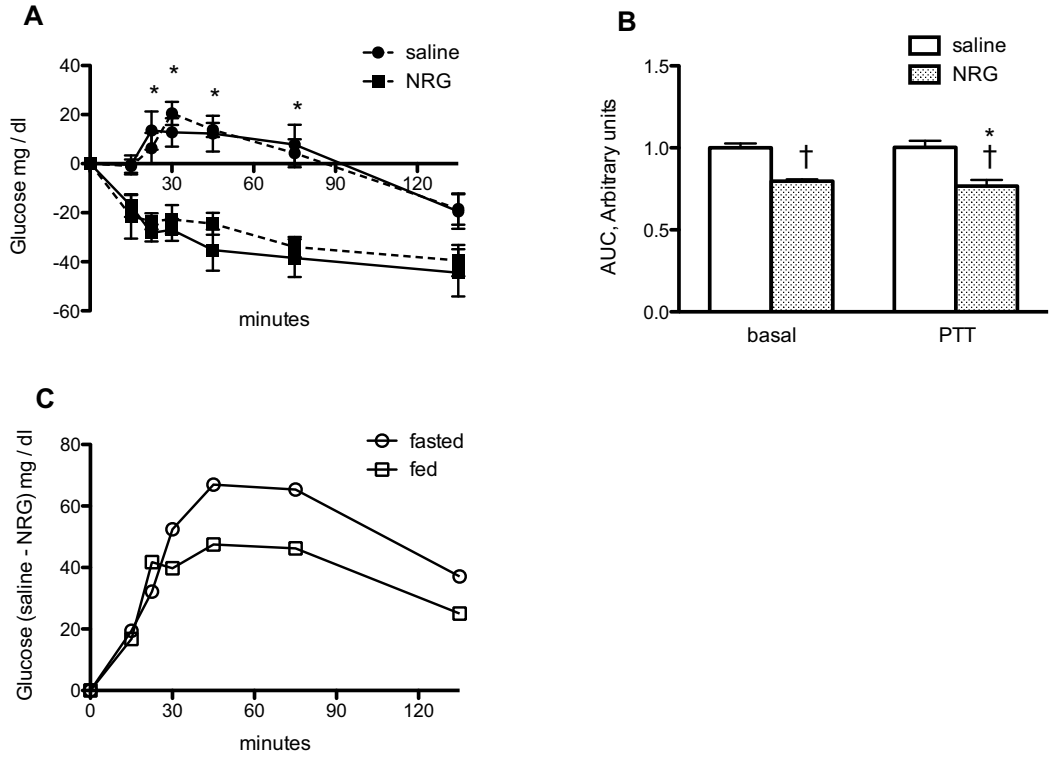


Figure 9. **Neuregulin action on glycaemia under pyruvate tolerance test in comparison with its action on basal glucose levels in fed state**. (**A**) change in glucose level relative to basal glycaemia during PPT (continuous lines) and basal condition (dashed lines), saline vs NRG (50 ng/g of body weight), n=4; (**B**) AUC of glycaemia, n=4; (**C**) the effect of neuregulin on glycaemia relative to saline-treatment in fasted and fed state – difference of means, fasted vs fed. * indicates significant difference vs. PTT saline group and † difference vs. basal saline group at $P<0.05$ determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

1.1.9. Neuregulin action under B / GTT / PTT conditions in insulin-deficient mice.

When comparing fasted and fed mice, we observed relevant differences in the neuregulin action on the regulation of glycaemia, as shown above. Insulin levels are deeply different in fasted compared to fed state. Here, we analyzed the role of insulin on the neuregulin action regulating glycaemia. In order to address this objective, we analyzed neuregulin effects in streptozotocin (STZ)-induced type 1 diabetic mice, a suitable physiological *in vivo* model of insulin deficiency.
STZ is particularly toxic to insulin producing β-cells and is widely used to induce type 1 diabetes in animals. After treatment with STZ the glycaemia of these insulin-deficient animals gradually increases, which is a sign of successful treatment. In our experiments we used STZ-treated C57BL6 strain mice, which developed hyperglycaemia during two weeks after injecting a dose (50 mg/kg of body weight) of STZ in five consecutive days. Mice remained normal weight throughout 2 months and had higher sensitivity to overnight fasting.

1.1.9.1. Effects on basal glycaemia

First, we wanted to measure how neuregulin was affecting basal glycaemia in insulin-deficient mice. During the experiment, basal glucose concentration slightly increased, probably due to stress caused by injections and collecting blood samples (Fig. 10, A). NRG was mildly lowering basal glycaemia in fasted STZ-treated mice, although not reaching significant difference at any measured point. That is consistent with AUC data, where a slight insignificant decrease was shown (Fig. 10, B). This is consistent with the contention in the neuregulin action reducing basal glycaemia, in a fasted state, seen previously in control mice.

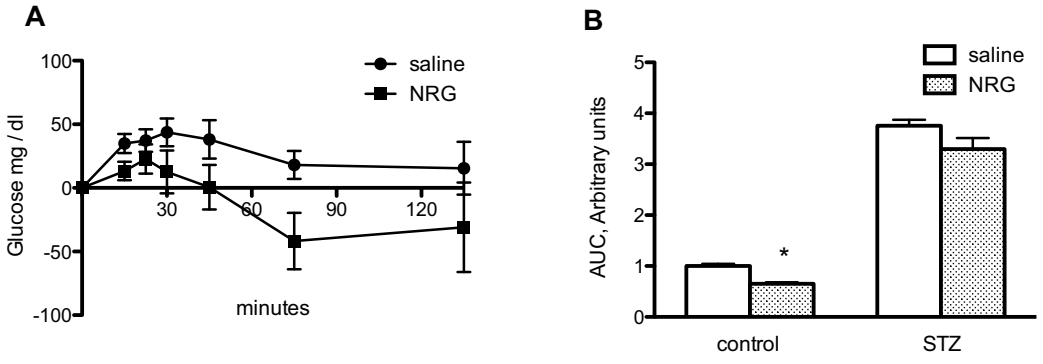


Figure 10. **Neuregulin action on glycaemia under basal conditions in STZ-treated mice in fasted state**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=6; (**B**) AUC of glycaemia in comparison with control mice, n=6. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

1.1.9.2. Effects on glycaemia in GTT assays

Here, we initially analyzed *in vivo* NRG action on glycaemia in GTT assays. A dose of glucose injected intraperitoneally to STZ-treated mice raised glucose levels quickly during first 7.5 minutes, which thereafter started to decline and dropped almost 240 mg/dL below the fasted state glycaemia at the end of the test (Fig. 11, A). However, with administration of NRG 15 minutes prior to glucose injection, the increase in glucose levels continued longer and reached comparable maximum level with saline-treated animals. Surprisingly, the following fall was much slower, keeping the glucose levels significantly higher than in saline group until the end of the experiment. NRG effect on impairing STZ–treated mice glucose tolerance, contrary to control animals, was also observed with AUC data, which increased by 25% (Fig. 11, B).

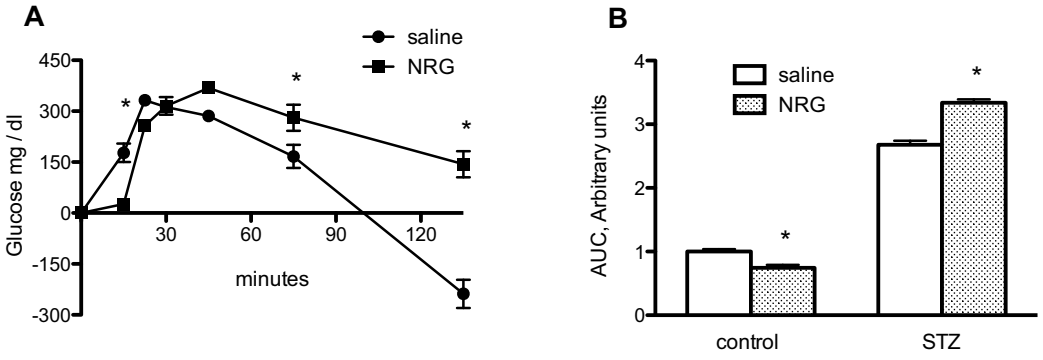


Figure 11. **Neuregulin action on glycaemia under glucose tolerance test in STZ-treated mice in fasted state**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=4; (**B**) AUC of glycaemia in comparison with control mice, n=4. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

1.1.9.3. Effects on glycaemia in PTT assays.

Next, we wanted to analyze how insulin deficiency affects neuregulin action reducing glycaemia in pyruvate tolerance tests under fasting conditions. Glycaemia increased after a dose of pyruvate in saline-treated insulin-deficient mice (Fig. 12, A and B), indicating the gluconeogenesis was properly functioning. Similarly to control mice, NRG inhibited the raising in glycaemia after the pyruvate administration in insulin-deficient mice indicating that neuregulin was significantly inhibiting gluconeogenesis.

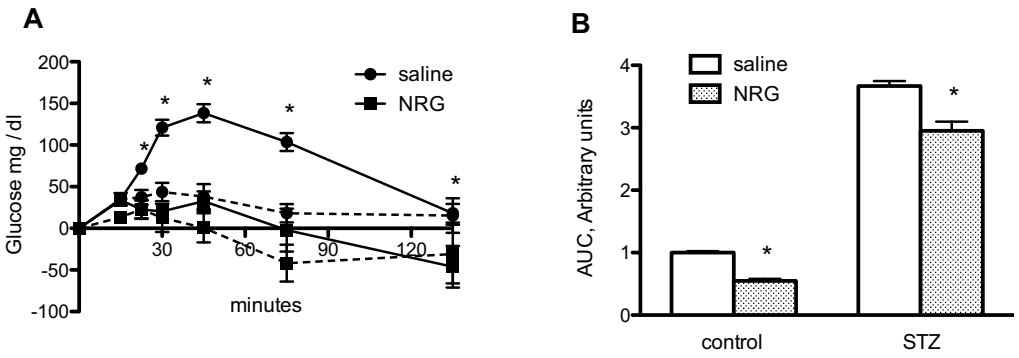


Figure 12. **Neuregulin action on glycaemia under pyruvate tolerance test in STZ-treated mice in comparison with its action on basal glucose levels in fasted state**. (**A**) change in glucose level relative to basal glycaemia during PPT (continuous lines) and basal condition (dashed lines), saline vs NRG (50 ng/g of body weight), n=4-6; (**B**) AUC of glycaemia in comparison with control mice, n=4-6. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

1.1.9.4. Effects on lactacidemia in basal / GTT / PTT conditions.

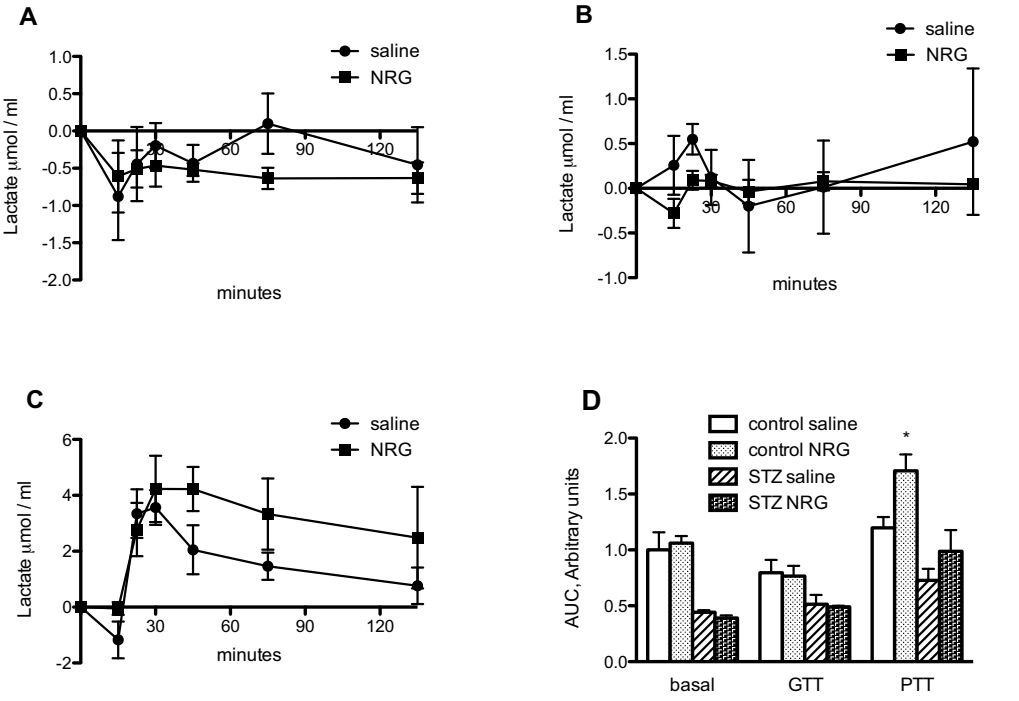


Figure 13. **Neuregulin action on lactacidemia under basal/GTT/PTT conditions in STZ-treated mice**. Change in lactate level relative to basal lactacidemia under (**A**) basal, (**B**) GTT and (**C**) PTT condition, saline vs NRG (50 ng/g of body weight), n=3-4; (**D**) AUC of lactacidemia in comparison with control mice, n=3-4. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**, B, **C**) or Unpaired Student's t test (**D**).

Lactate levels in control animals were increased with NRG treatment during PTT, but not during GTT and when we measured NRG influence to basal fasted glycaemia. That suggested that NRG supported pyruvate conversion to lactate by inhibiting gluconeogenesis, but did not enhance lactate production from glucose. To understand how the situation is in STZ-treated mice, where NRG has also shown an inhibiting effect on gluconeogenesis. Therefore, we measured lactate levels in plasma collected during three previously described experiments. It revealed that situation in insulin-deficient mice is comparable with what we have seen in control animals. In insulin STZ-treated as well in control animals NRG did not change lactate concentration during GTT and investigation of NRG effect on basal glycaemia, which suggests that NRG does not influence lactate production (Fig. 13, A and B). The lactate levels picture was similar also in case of PTT. During saline treatment STZ mice lactate concentration rose approximately by 3.5 μmol/ml 15 minutes after injection of pyruvate after what it started slowly to decrease (Fig. 13, C). In NRG-treated insulin-deficient mice the rise was slightly higher and the rate of decrease parallel to saline-treatment. In overall, the difference between saline and NRG-treated animals was smaller in STZ group compared to control. The trend that NRG favors pyruvate conversion to lactate, either in control or insulin-deficient mice, is well seen also in calculated AUC (Fig. 13, D).

1.1.9.5. Effects on triglyceridemia in basal / GTT / PTT conditions

We also analyzed STZ mice TG levels and whether they are influenced with NRG treatment during the three experiments. During GTT a slight lowering in overall TG levels was observed during first 30 minutes, but it recovered by the end of the experiment (Fig. 14, A). A same pattern with a little delay occurred when animals were treated with NRG, thought that did not change significantly TG concentration in any measured point. During the experiment when we measured neuregulin influence to basal glycaemia we saw almost identical consumption of TG in both saline and NRG-treated group of mice (Fig. 14, B). No fluctuations and also no influence by acute NRG treatment on TG concentration were observed during PTT (Fig. 14, C). All these results together, as presented also as AUC, suggest that NRG in STZ-treated mice, likewise in control animals, does not regulate TG metabolism (Fig. 14, D).

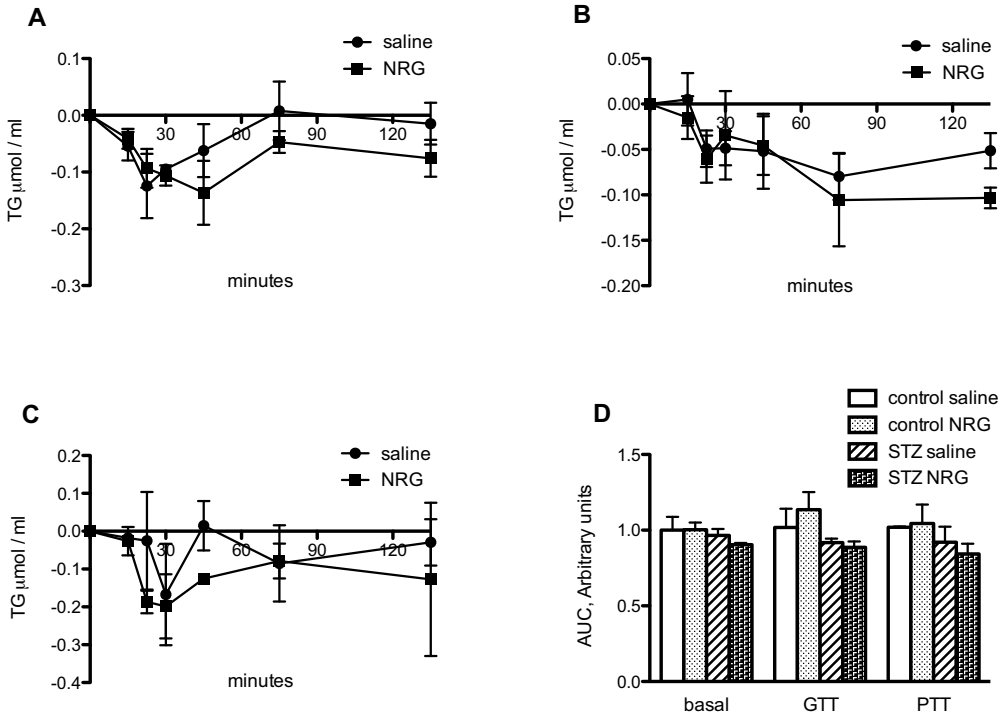


Figure 14. **Neuregulin action on triglyceridemia under basal/GTT/PTT conditions in STZ-treated mice**. Change in triglyceride level relative to basal triglyceridemia under (**A**) basal, (**B**) GTT and (**C**) PTT condition, saline vs NRG (50 ng/g of body weight), n=3-5; (**D**) AUC of triglyceridemia in comparison with control mice, n=3-4. Statistical test applied: Two-way ANOVA with post-hoc Tukey's test (**A**, B, **C**) or Unpaired Student's t test (**D**).

1.2. *In vivo* neuregulin action on the liver of control mice.

Liver is a principal organ in maintaining blood glucose homeostasis through its ability to both take up and release glucose. In fasted state, HGP is increased and the glucose produced by liver derives from combination of activated glycogenolysis and gluconeogenesis. PTTs have clearly shown that neuregulin reduce glucose levels possibly by inhibiting HGP. That led us to investigate neuregulin role in regulating liver metabolism.

All the analysis described in this section to examine different aspects of neuregulin action on signaling cascades were performed on liver that was removed 30 minutes after acute treatment with saline or NRG *in vivo*.

1.2.1. Neuregulin receptors in the liver. Effect of an ErbB3 blocking antibody on the neuregulin action reducing glycaemia.

Adult liver is known to express high levels of ErbB1 and ErbB3, and according to some published data also trace amounts of ErB4 (Carver et al., 1997; Carver et al., 1996; Camprecios et al., 2010). We analyzed ErbB receptor protein levels in liver by Western blot (WB) and, as expected, the organ expressed high amount of ErbB1 and ErbB3, with barely detectable level of ErbB4 (Fig. 15). Erb2 was not detected. Comparison of liver ErbB receptor levels of saline and NRG-treated mice showed no differences between the groups. That ensures that the number of ErbB receptors was not changing after an acute effect of NRG.

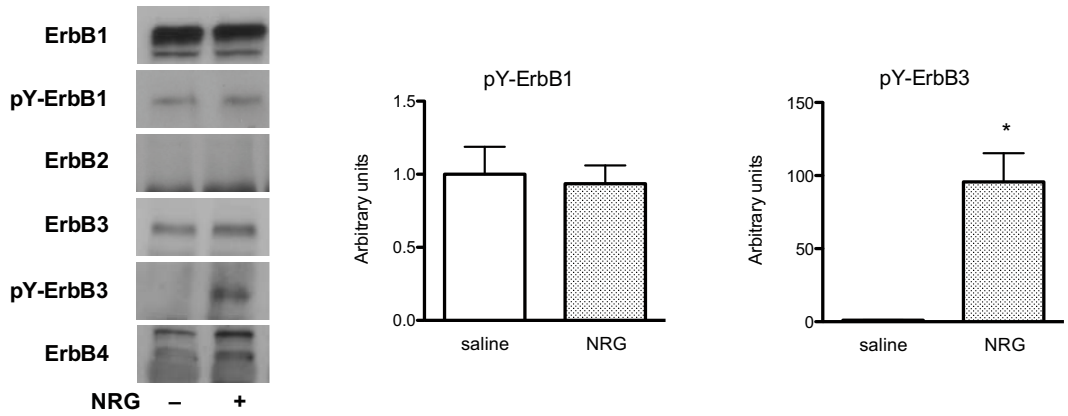


Figure 15. **Expression and tyrosine phosphorylation of ErbB receptors in liver of control mice**. Total lysates obtained from liver were used for IP and WB to detect ErbB receptor expression and tyrosine phosphorylation of ErbB1 and ErbB3. Tyrosine phosphorylation levels were adjusted against total respective ErbB receptor expression level. * indicates significant difference vs. saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Next, we analyzed NRG influence on ErbB1 and ErbB3 phosphorylation and activation of the receptor by immunoprecipitation (IP) studies. An IP with anti-phospho-tyrosine (anti-pY) and subsequent WB with anti-ErbB1 yielded in similar levels pY-ErbB1 in both groups. Contrary to that phosphorylation of neuregulin receptor ErbB3 (IP: anti-ErbB3, WB: anti-pY) was increased almost 100-fold after acute administration of NRG. These results suggested that neuregulin triggered signaling in hepatocytes through activating ErbB3, using ErbB1 as a coreceptor to induce ErbB3 tyrosine phosphorylation. ErbB1 was unable to become phosphorylated since ErbB3 is a tyrosine kinase-dead receptor.

To evaluate the relevance of ErbB3 in the hypoglycemic effects of neuregulin, we decided to inhibit liver ErbB3, by administrating anti-ErbB3 antibodies in three small volume injections to the abdominal area comprising the liver localization, 15 min before the NRG administration (Fig. 16). Control groups (Ig and Ig+NRG) were treated with an equivalent dose of irrelevant immunoglobulins (Ig).

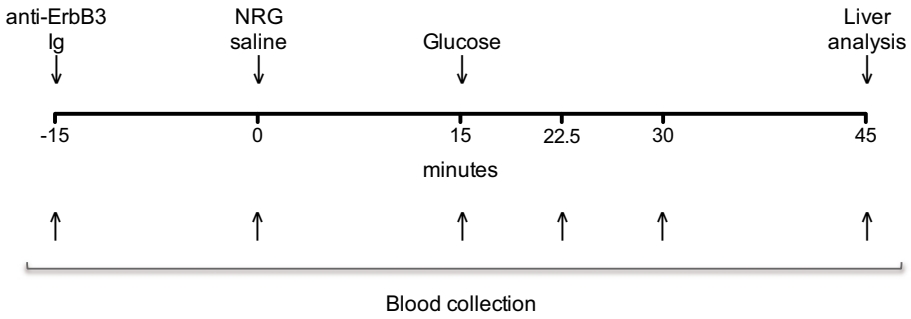


Figure 16. Design of ErbB3 blocking experiment.

The first thing that we examined was tyrosine phosphorylation of ErbB3 because it would indicate how well the blocking antibodies were able to inhibit ErbB3 receptor activation. In control animals we observed a 72-fold increase in phosphorylation of ErbB3 with NRG treatment (Fig. 17). That increase was abolished when anti-erbB3 was injected before the neuregulin treatment. This result proved that anti-ErbB3 antibodies, indeed, blocked ErbB3 receptor in liver. Later, the same experimental groups (Ig / Ig+NRG / anti-ErbB3+NRG) were submitted to a GTT assay. In Ig and Ig+NRG groups it was observed the neuregulin-induced reduction in glycaemia described previously (Fig. 18, A and B), suggesting that the presence of irrelevant Ig was not affecting neuregulin impact on glycaemia. When ErbB3-blocking antibodies were administrated, NRG was unable to reduce glycaemia (Fig. 17, A). That was indicating very clearly that NRG was reducing glycaemia by effects, at least, on liver ErbB3, in control fasted mice. This result was also supported by AUC data (Fig. 17, B).

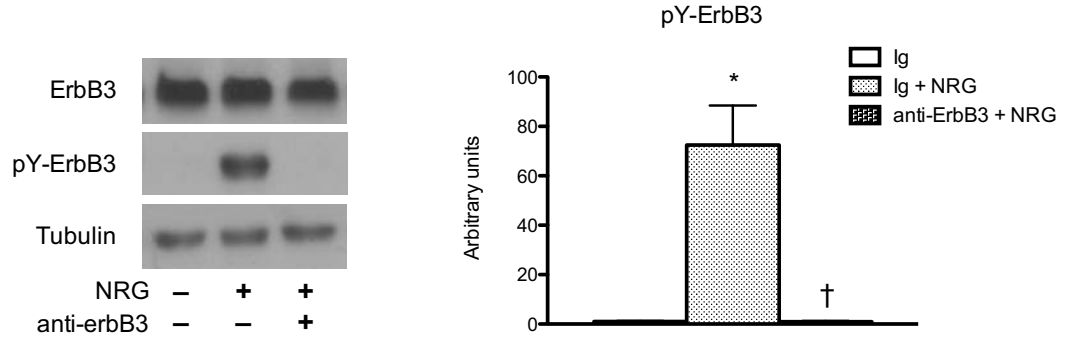


Figure 17. **Anti-ErbB3 prevents ErbB3 phosphorylation**. Ig, Ig+NRG or anti-ErbB3+NRG-treated mice liver total lysates were used for IP and WB assays to measure ErbB3 tyrosine phosphorylation. Tyrosine phosphorylation levels were adjusted against total ErbB3 receptor expression level. * indicates significant difference vs. Ig group and † difference vs. Ig+NRG at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

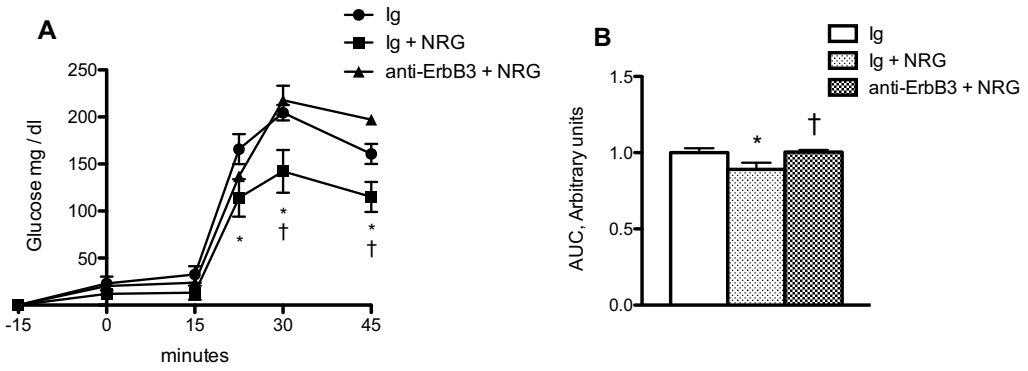


Figure 18. **Anti-ErbB3 abolishes neuregulin effect on glycaemia under glucose tolerance test**. 20μg of anti-ErbB3 or Ig was administered 15 minutes prior treatment with saline of NRG (50 ng/g of body weight) and 30 minute prior glucose (2 g/kg of body weight). (**A**) change in glucose level relative to basal glycaemia in Ig, Ig+NRG and anti-ErbB3 groups, n=4; (**B**) AUC of glycaemia, n=4. * indicates significant difference vs. Ig group and † difference vs. Ig+NRG group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

Next, we analyzed the liver ErbB3 activation under *in vivo* NRG action in a model of insulin deficiency, the STZ-treated mice. For that, livers were dissected from overnight fasted mice, 30 minutes after saline or NRG treatment. After preparing total lysates, we immunoprecipitated ErbB3 and measured by immunoblot assay its total phosphorylation at tyrosine residues. Results showed that *in vivo* NRG treatment caused hepatic ErbB3 phosphorylation at tyrosine residues as much as in control animal livers (Fig. 19), suggesting that insulin, or its downstream signaling cascade, is not participating in the ErbB3 activation.

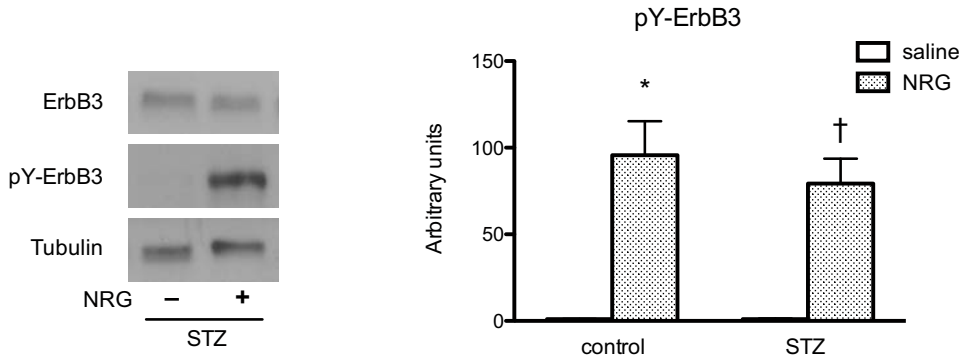


Figure 19. **Neuregulin influence on ErbB3 tyrosine phosphorylation in STZ mice in comparison with control mice**. Total lysates obtained from liver were immunoprecipitated with ErbB3 and tyrosine phosphorylation was detected with anti-pY in WB. Tyrosine phosphorylation levels were adjusted against total ErbB3. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.2. Neuregulin effect on ErbB3 and on insulin receptor substrates (IRS1 and IRS2) tyrosine phosphorylation. Consequences on the ability to recruit PI3K.

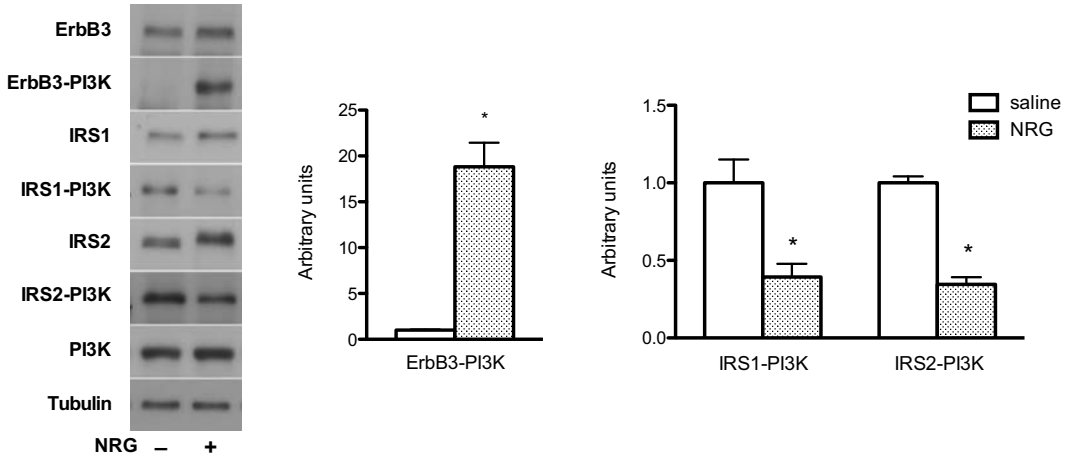


Figure 20. **Neuregulin effect on p85 PI3K interaction with ErbB3 and IRS proteins in control mice**. ErbB3, IRS1 or IRS2 was immunoprecipitated from liver total lysates and bound p85 PI3K was detected with WB. Detected PI3K was adjusted against total ErbB3, IRS1 or IRS2, respectively. Tubulin was used as a loading control. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

On intracellular domain, ErbB3 contains six binding sites for p85 PI3K (Soltoff and Cantley, 1996), which is an important enzyme also on classical insulin signaling pathway regulating energy metabolism. Therefore, measuring interaction between ErbB3 and PI3K became in our

interest. Detection of p85 PI3K by WB on ErbB3 immunoprecipitated samples showed approximately 20-fold increase in mice that had got a dose of NRG compared to saline-treated animals, demonstrating that neuregulin activated a pathway dependent on PI3K (Fig. 20).

While PI3K binding to ErbB3 increased significantly after neuregulin treatment, we wanted to know what happens with interactions between PI3K and IRS proteins that are important for insulin to transmit its signal. We immunoprecipitated IRS1 and IRS2 and found that level of p85 PI3K bound to these proteins was decreased by 60-65% in both cases in NRG group (Fig. 20). The latter suggests that neuregulin had a role in inhibiting insulin signaling.

The use of anti-ErbB3 antibodies previously to neuregulin reverted the preferent binding of PI3K to ErbB3, redistributing it to the IRSs, what indicated the essential role of ErbB3 on the neuregulin action competing for the insulin mediators cascade at initial steps or antagonizing with the insulin signaling (Fig. 21).

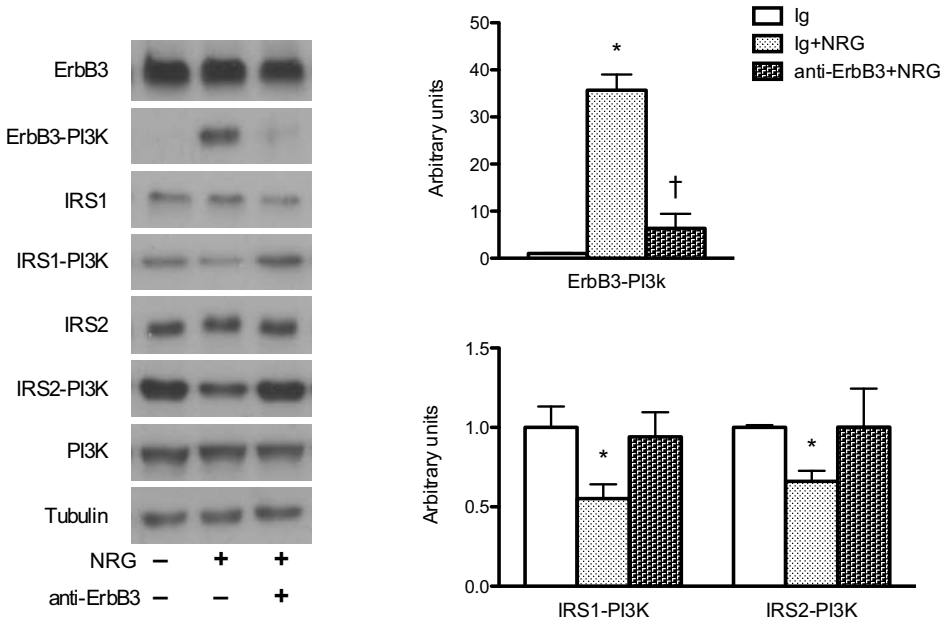


Figure 21. **Neuregulin regulates PI3K interaction with ErbB3 and IRS2 proteins through activating ErbB3**. Liver total lysates from Ig, Ig+NRG and anti-ErbB3+NRG-treated mice were used to immunoprecipitate ErbB3, IRS1 and IRS2 and bound p85 PI3K was detected with WB. Detected p85 PI3K was adjusted against total ErbB3, IRS1 and IRS2, respectively. Tubulin was used as a loading control. * indicates significant difference vs. Ig group and † difference vs. Ig+NRG group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

When analyzing the ErbB3 and the IRS1/IRS2 interaction with PI3K in the insulin-deficient model, there was no statistical difference in the ErbB3-PI3K interaction regarding control mice indicating that the lack of insulin did not have benefits on the capacity of ErbB3 to recruit PI3K, probably indicating that it was maximal either in the presence or absence of insulin. When analyzing neuregulin effect on the binding of PI3K to IRS proteins, it decreased for IRS2 as in

controls but did not have any effect on IRS1, which suggest the poor contribution of the later to the activation of PI3K in this model (Fig. 22).

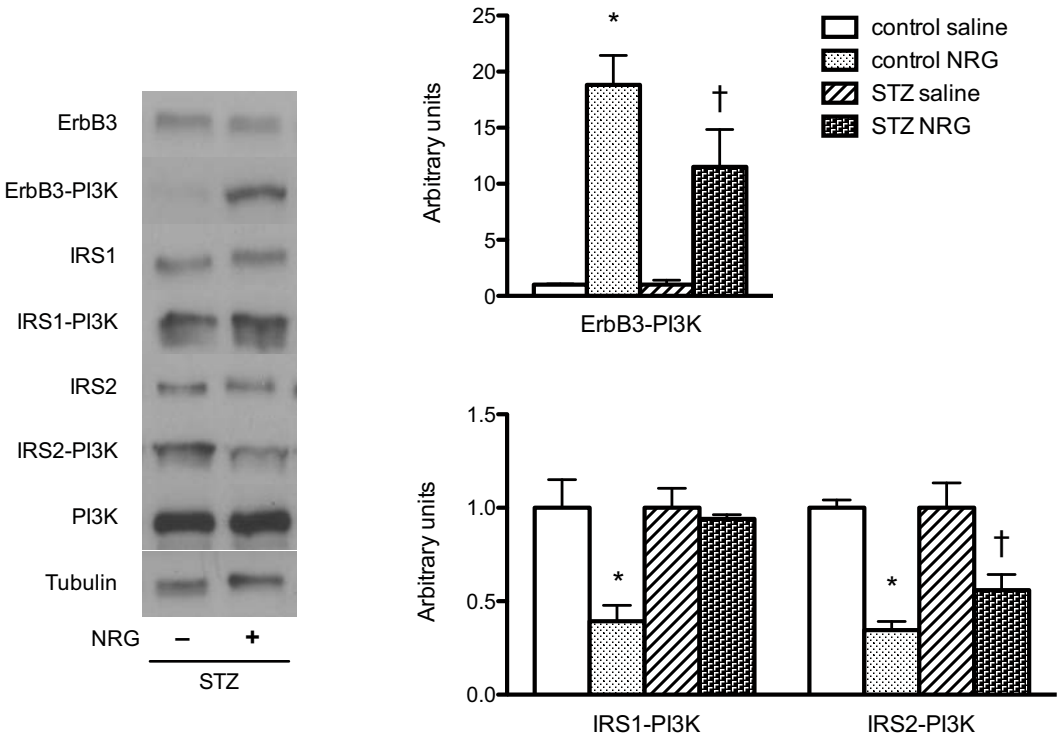


Figure 22. **Neuregulin effect on PI3K interaction with ErbB3 and IRS proteins in control and STZ mice in comparison with control mice**. ErbB3, IRS1 or IRS2 was immunoprecipitated from liver total lysates and bound p85 PI3K was detected with WB. Detected PI3K was adjusted against total ErbB3, IRS1 or IRS2, respectively. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

A decrease in tyrosine phosphorylation and increase in serine phosphorylation of IRS proteins inhibits insulin derived signaling due to diminished interaction between IR and IRS. The situation follows with a weaker binding of PI3K to IRS. An observed decrease in IRS1 and IRS2 interaction with PI3K made us question whether the inhibiting effect of NRG is a result of reduced tyrosine phosphorylation of IRS proteins. Indeed, as WB analysis of IRS1 and IRS2 immunoprecipitated samples showed, NRG significantly reduced tyrosine phosphorylation of both proteins by 74% and 81%, respectively (Fig. 23). Moreover, the change in phosphorylation was also possible to notice from the slower mobility of total IRS1 and IRS2 bands in WB (Fig. 23), which generally refers to increased serine/threonine phosphorylation (Harrington et al., 2004). A reduction in tyrosine phosphorylation and possible increase in serine phosphorylation of IRS1 and IRS2 after NRG treatment indicate that this may be the cause of the weaker interaction with PI3K.

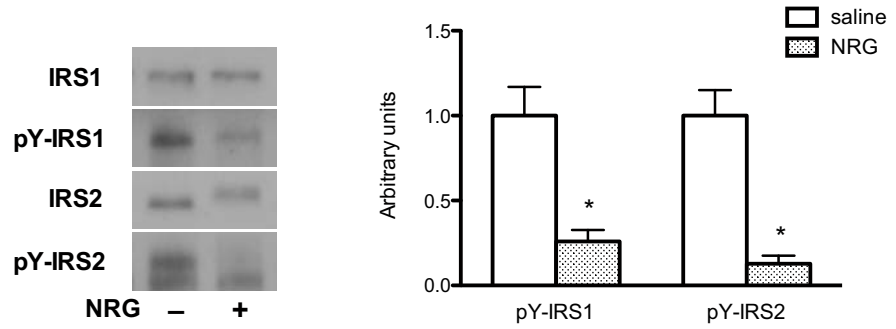


Figure 23. **Neuregulin effect on tyrosine phosphorylation of IRS1 and IRS2 in control mice**. IRS1 or IRS2 was immunoprecipitated from liver total lysates and tyrosine phosphorylation was detected with anti-pY. Detected tyrosine phosphorylation was adjusted against total IRS1 or IRS2, respectively. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.3. Neuregulin effects on Akt and on PKCζ activation.

Next, based on above described results, we focused on determining neuregulin effects on insulin signaling pathway elements downstream PI3K. Akt, among them, plays a central role in activating different aspects of glucose metabolism by phosphorylating wide range of its substrates. Therefore we measured with WB the phosphorylation of Akt at its Thr306 and Ser473 residues, conducted by PDK1 and mTORC2, respectively (Milburn et al., 2003; Thomas et al., 2002; Hresko and Mueckler, 2005; Sabrassov et al., 2005). It appears that neuregulin fully activated Akt increasing 100-fold the phosphorylation of Thr308 and 70-fold the phosphorylation of Ser473 (Fig. 24), suggesting that both, PI3K and mTORC2 mediated pathways are initiated with NRG administration.

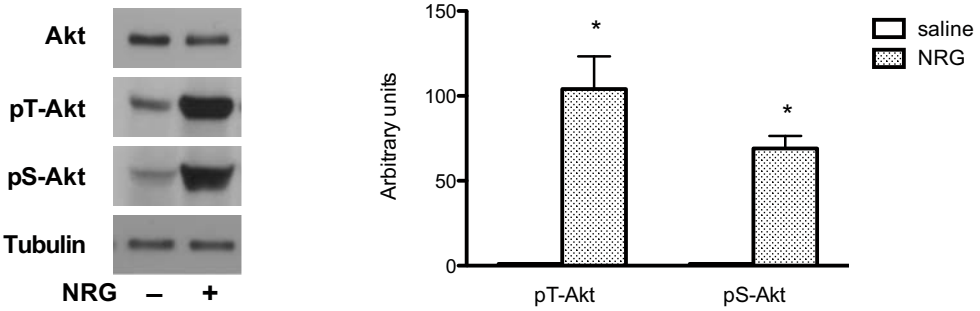


Figure 24. **Neuregulin influence on Akt phosphorylation at Thr308 and Ser473 residues**. Liver total lysates were used to detect Akt and phosphorylation at Thr 308 and Ser473 residues with WB. Detected phosphorylation was adjusted against total Akt. Tubulin was used as a loading control. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

The use of anti-ErbB3 antibodies, prior to NRG administration, prevented Akt phosphorylation, and therefore activation, in the liver of control mice (Fig. 25). That proves that NRG binding to liver ErbB3 receptor triggers Akt phosphorylation at both sites.

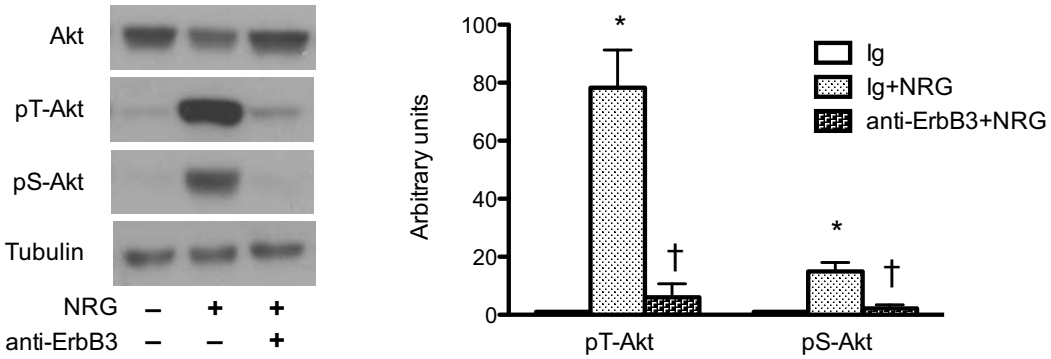


Figure 25. **Neuregulin induces Akt phosphorylation through activating ErbB3**. Liver total lysates from Ig, Ig+NRG and anti-ErbB3+NRG-treated mice were used to detect Akt and its phosphorylation at Thr308 and Ser473 residue with WB. Detected phosphorylation was adjusted against total Akt. Tubulin was used as a loading control. * indicates significant difference vs. Ig group and † difference vs. Ig+NRG group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

When analyzing Akt phosphorylation on both sites after NRG treatment, in liver of STZ-treated mice we observed that NRG caused 22-fold increase on Thr308 residue and 14-fold increase on Ser 473 residue, which in both cases was approximately 5-fold less increased phosphorylation than observed in control mice (Fig. 26).

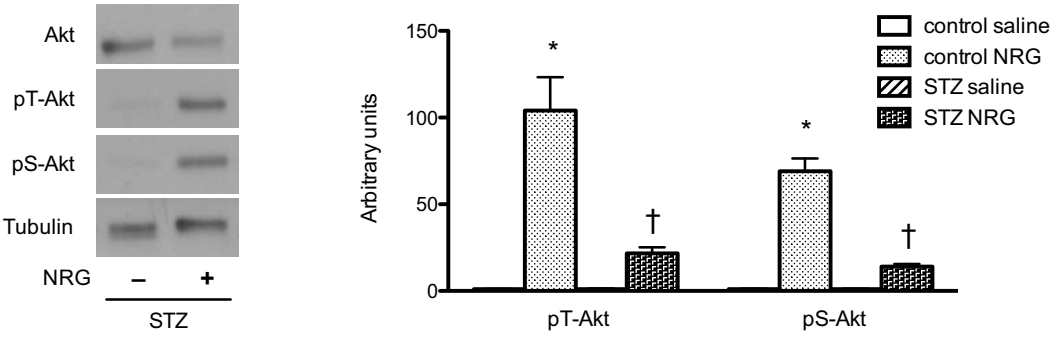


Figure 26. **Neuregulin influence on Akt phosphorylation in control and STZ mice in comparison with control mice**. Liver total lysates were used to detect Akt and phosphorylation at Thr 308 and Ser473 residues with WB. Detected phosphorylation was adjusted against total Akt. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

These results suggested that although neuregulin and insulin compete by PI3K recruitment, somehow the action of both might be improving Akt activation. Alternatively, the sustained lack

of insulin in the STZ-treated model generated alterations in its signaling cascade that might affect the common steps with the neuregulin cascade. To better understand this observation, further studies should be done.

Studies with skeletal muscle cells culture have revealed that neuregulin induces GLUT4 translocation to plasma membrane through a PI3K/PKCζ pathway and therefore up-regulate glucose transport (Cantó et al., 2004; Suárez et al., 2001). However, liver expresses GLUT2 instead of GLUT4. Contrary to skeletal muscle, hepatic glucose transport does not depend on the number and activity of GLUT2 in the surface membranes, but on increases in the concentration of blood glucose, due to the low affinity of GLUT2 to this metabolite. To detect whether PKCζ has a role in regulation of neuregulin effects in hepatocytes, we immunoprecipitated PKCζ and detected the phosphorylation using anti-phospho-PKCζ antibody. Despite previously demonstrated importance for mediating neuregulin signal in skeletal muscle, *in vivo* administration of NRG failed to activate PKCζ in liver (Fig. 27), suggesting that it is not included in signaling pathways causing the observed glycaemia-lowering effect.

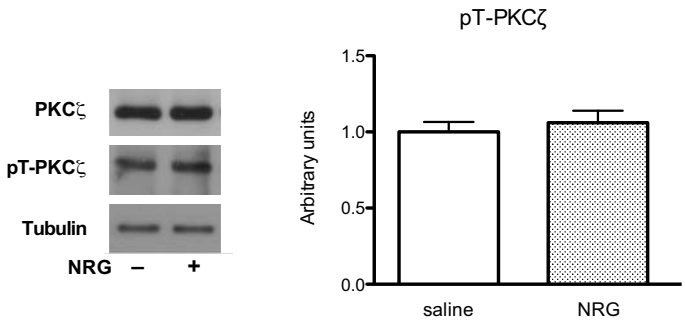


Figure 27. **Neuregulin influence on PKCζ phosphorylation at Thr410 in liver**. PKCζ was immunoprecipitated from liver total lysates and detected with anti-phospho-PKCζ in WB. Detected phosphorylation was adjusted against total PKCζ. Tubulin was used as a loading control. Statistical test applied: Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.4. Neuregulin effects on downstream Akt activation proteins involved in the regulation of glucose metabolism in the liver.

1.2.4.1. Effects on FoxO1: consequences for the neuregulin effect repressing hepatic glucose production.

PTT studies described above have suggested that one of the main roles of neuregulin is an inhibition of gluconeogenesis. The enzymes responsible for glucose production from non-carbohydrate carbon substrates are transcriptionally regulated by an Akt substrate FoxO1 (Altomonte et al., 2003; Puigserver et al., 2003; Samuel et al., 2006; Matsumoto et al., 2007). Therefore, it is important to investigate different aspects regulated by transcription factor FoxO1,

which in fasted state is active and locates in nucleus, but will be excluded and inactivated after insulin signal (Nakae et al., 1999)– a signal that neuregulin seems to mimic.

At first we wanted to know whether NRG, indeed, is able to phosphorylate and inactivate FoxO1. As shown on figure (Fig. 28, A), WB shows a clear 8-fold increase in phosphorylation of the transcription factor after an acute treatment with neuregulin. While phosphorylation of FoxO1 targets the protein to degradation, we also measured NRG effect on transcription of FoxO1 itself and did not observe a significant difference between the groups (Fig. 28, B). The latter is supported by WB data of protein levels that during the 30-minute treatment have remained same. As expected the results suggest that NRG is an inhibitor of FoxO1, but an acute treatment does not have effect on either FoxO1 protein or mRNA levels.

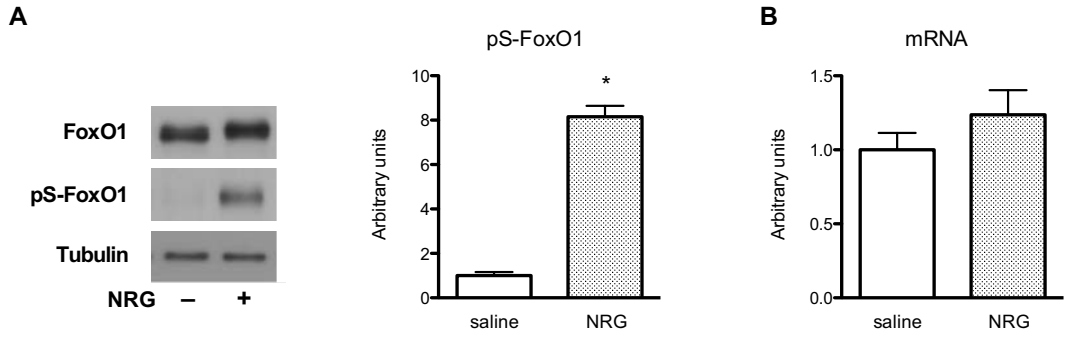


Figure 28. **Neuregulin influence on FoxO1 phosphorylation at Ser256 residue in control mice**. **(A)** Liver total lysates were used to detect FoxO1 and its phosphorylation at Ser256 residue with WB. Detected phosphorylation was adjusted against total FoxO1. Tubulin was used as a loading control; **(B)** FoxO1 mRNA levels were determined by RT-PCR. * indicates significant difference vs. saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

An inhibiting effect of NRG on Foxo1 suggests that the expression of gluconeogenic genes is reduced. To control that argument we measured mRNA levels of *Pepck*, *G6pase* and *Pgc1α*. The latter interacts directly with FoxO1 to promote expression of *Pepck* and *G6pase*, but FoxO1 also regulates its transcription (Yoon et al., 2001; Puigserver et al., 2003). RT-PCR results of *Pgc1α* showed approximately 40% decrease after half an hour *in vivo* treatment with NRG, though that outcome was not significant (Fig. 29, A). A change in same direction, and similarly not significant, was observed in *Pepck* and *G6Pase* mRNA levels, decreasing by 20% and 40%, respectively. As expected from transcription data, no change was observed in protein levels of PEPCK between the two groups (Fig. 29, B). These results suggest that inhibition of FoxO1 by NRG causes a trend of lowering gluconeogenic gene expression, which may not be fully developed because of very short treatment period.

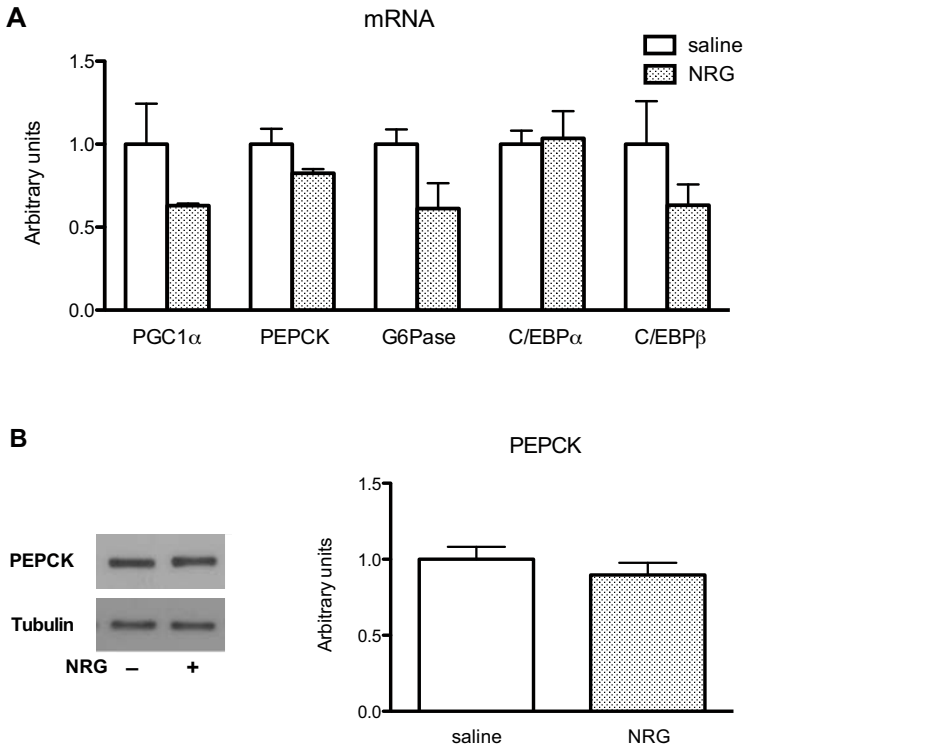


Figure 29. **Neuregulin effect on gluconeogenic gene expression and PEPCK protein in control mice**. **(A)** RNA was extracted from saline and NRG-treated livers and analyzed by RT-PCR; **(B)** Liver total lysates were used to detect PEPCK levels. Tubulin was used as a loading control. Statistical test applied: Unpaired Student's t test. These images are representative of 4 independent experiments.

CCAAT/enhancer-binding proteins (C/EBP) α and β are transcription factors, with high hepatic expression that also regulate gluconeogenesis in liver. C/EBPα is essential for neonatal glycogenesis, where co-operation with FoxO1 promotes Pepck and G6pase transcription (Sekine et al., 2007). C/EBPβ activates gluconeogenesis by binding to several sequences of the PEPCK gene promoter (Park et al., 1990). Its expression, like gluconeogenesis, is stimulated by cAMP (Nizielski et al., 1996) and down-regulated by insulin (Bosch et al., 1995). Therefore, our interest was to check also neuregulin influence to C/EBP transcription factors. RT-PCR did not reveal any influence on transcription of *C/ebpα* mRNA (Fig. 29, A), which could be expected as C/EBPα regulates gluconeogenesis in developing liver. Contrary to that, a similar decrease to *Pgc1α* and *G6pase* was observed in *C/ebpβ* mRNA levels, though again non-statistically important.

The use of anti-ErbB3 prior to NRG administration prevented FoxO1 phosphorylation, and therefore inactivation, indicating that under *in vivo* neuregulin action, liver ErbB3 is responsible for actions on FoxO1 (Fig. 30). PEPCK protein levels did not change in a significant manner, which is probably due to the short time at which we allowed neuregulin action.

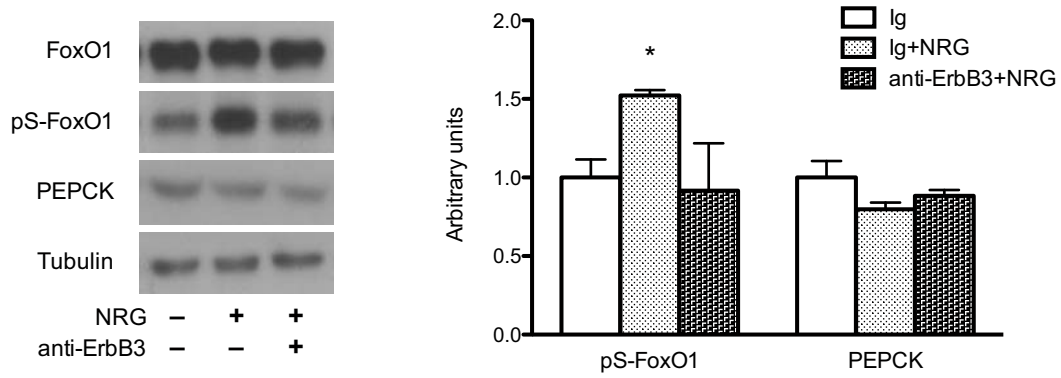


Figure 30. **Neuregulin inhibits FoxO1 through activating ErbB3**. Liver total lysates from Ig, Ig+NRG and anti-ErbB3+NRG-treated mice were used to detect FoxO1, its phosphorylation at Ser256 residue and PEPCK with WB. Detected phosphorylation was adjusted against total FoxO1. Tubulin was used as a loading control. * indicates significant difference vs. Ig group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

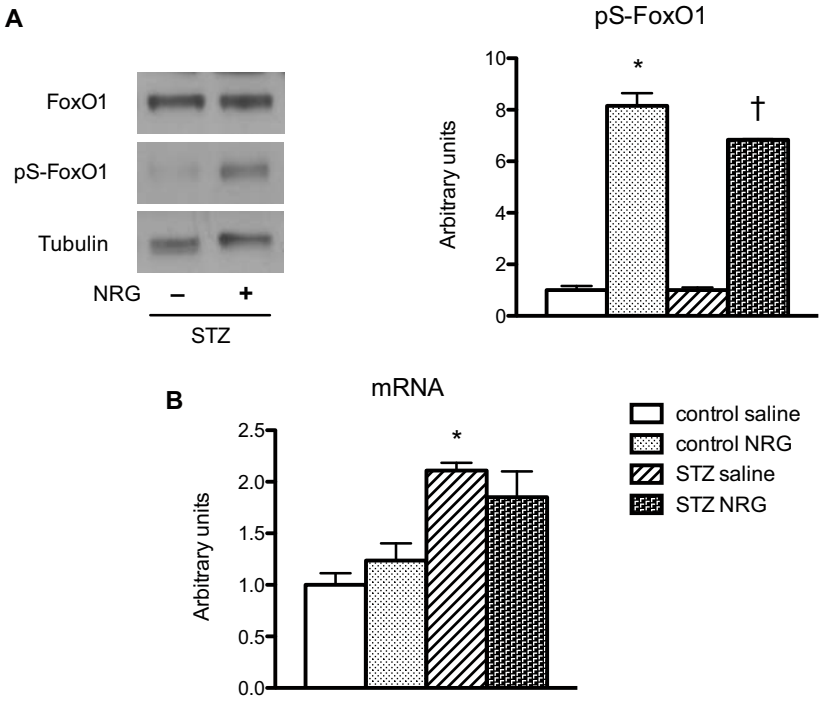


Figure 31. **Neuregulin influence on FoxO1 phosphorylation and transcription in STZ mice in comparison with control mice**. **(A)** Liver total lysates were used to detect FoxO1 and its phosphorylation at Ser256 residue with WB. Detected phosphorylation was adjusted against total FoxO1. Tubulin was used as a loading control; **(B)** FoxO1 hepatic mRNA levels were determined by RT-PCR. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Next, we analyzed deactivation of FoxO1, by immunoblotting of phosphor-FoxO1, under NRG treatment in livers of insulin-deficient mice. Although FoxO1 is a target protein of Akt, which was

lower activated in this model by NRG treatment, results showed almost 7-fold increase in phosphorylation of FoxO1 at Ser256 per protein after NRG treatment (Fig. 31, A). It was comparable to what was observed in control animals. That suggests that Akt activity was not limiting for FoxO1 deactivation levels under NRG treatment in this insulin-deficient model. Therefore, independently of the insulin presence, NRG should be able to block gluconeogenesis which was totally in accordance with the results obtained in the PTT assays on glycaemia in STZ - treated mice.

To measure NRG influence to hepatic FoxO1 transcription, we performed RT-PCR analysis. The experiment indicated that STZ-treated livers contained twice as much FoxO1 mRNA as control animals, but the level was not regulated by NRG treatment (Fig. 31, B).

When analyzing the transcription of several gluconeogenic genes that are tightly regulated by FoxO1 in insulin-deficient mice we observed that at our NRG treatment conditions both PEPCK and G6Pase showed a trend for decrease as expected (Fig. 32, A). That tendency, however, was not detectable in level of PEPCK protein, which suggested that this process needed more time to be effective (Fig. 32, B).

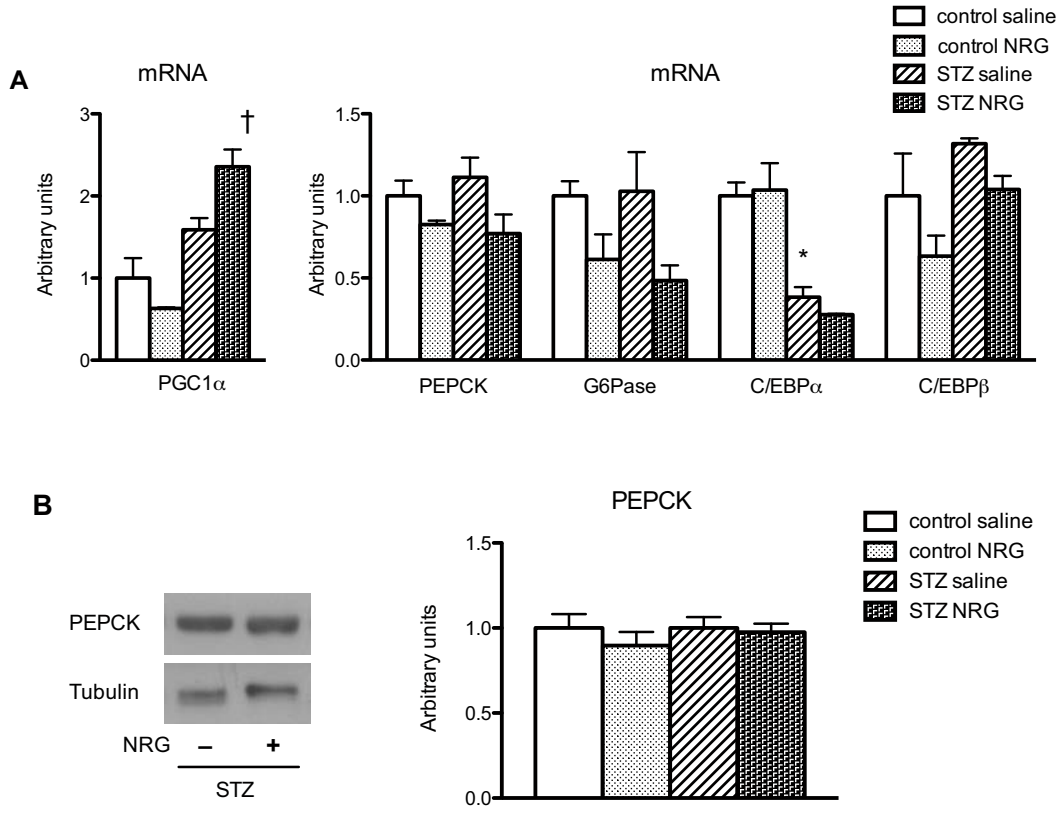


Figure 32. **Neuregulin effect on gluconeogenic gene expression and PEPCK protein STZ mice in comparison with control mice**. **(A)** RNA was extracted from saline and NRG-treated livers and analyzed by RT-PCR; **(B)** Liver total lysates were used to detect PEPCK levels. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Contrarily to expected, PGC1 α transcription was significantly up-regulated (Fig. 32, A) indicating that other signals, involved in its expression and contraries to the neuregulin action, might be participating under long-term insulin deficiency.

Studies have shown that C/EBP β plays a role in full induction of PEPCK and G6Pase genes, increases in gluconeogenesis rate and blood glucose significantly in STZ-treated animals (Arizmendi et al., 1999). Therefore, in situation where NRG activates several elements of insulin signaling pathway, also in STZ-treated mice, it is interesting to see what influence neuregulin have on C/EBP α and C/EBP β which expression is normally blocked by insulin. It is reported that during STZ-induced diabetes, liver C/EBP α mRNA is decreased and C/EBPβ mRNA is increased (Bosch et al., 1995), which was also observable in our case. An acute neuregulin treatment in this situation is giving a slightly lowering trend to transcription of both transcription factors, suggesting that it might be another way how NRG was contributing to inhibition of gluconeogenesis in insulin-deficient mice. Although, further studies will have to be accomplish to obtain statistically significance in the mentioned tendencies of change.

1.2.4.1.1. Effects on FoxO1 targeting PC and L-PK

When analyzing the NRG action on the first enzyme in the gluconeogenic pathway, pyruvate carboxylase (PC), which catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate, the results showed no influence on transcriptional activity of this enzyme (Fig. 33). Despite, we cannot discard the existence of other mechanisms regulating PC activity under NRG action, including allosteric inhibition that should be approached in the future.

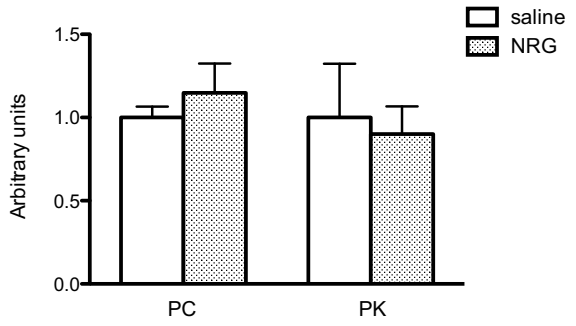


Figure 33. **Neuregulin influence on pyruvate carboxylase (PC) and liver-type pyruvate kinase (PK) transcription**. RNA was extracted from saline and NRG-treated livers and analyzed by RT-PCR. Statistical test applied: Unpaired Student's t test. These images are representative of 4 independent experiments.

Another possible way to reduce pyruvate conversion to glucose is also to activate liver-type pyruvate kinase (PK), which is an enzyme involved in glycolysis. PK catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP), a product of PEPCK, to ADP and yielding a

molecule of pyruvate and ATP. Moreover, Zhang and colleagues have reported that active FoxO1 plays a role in down-regulating the expression PK gene (Zhang et al., 2006a). Since neuregulin phosphorylated and deactivated FoxO1, we expected an increase in mRNA of this glycolytic enzyme. Nonetheless, there was no change in transcriptional activity under NRG action (Fig. 33). These results suggest that neuregulin, at short-term, did not influence gluconeogenesis by changing the activity of PC and PK at the transcriptional level.

I.2.4.1.2. Effects on FoxO1 target involved in hepatic glucose utilization: GK

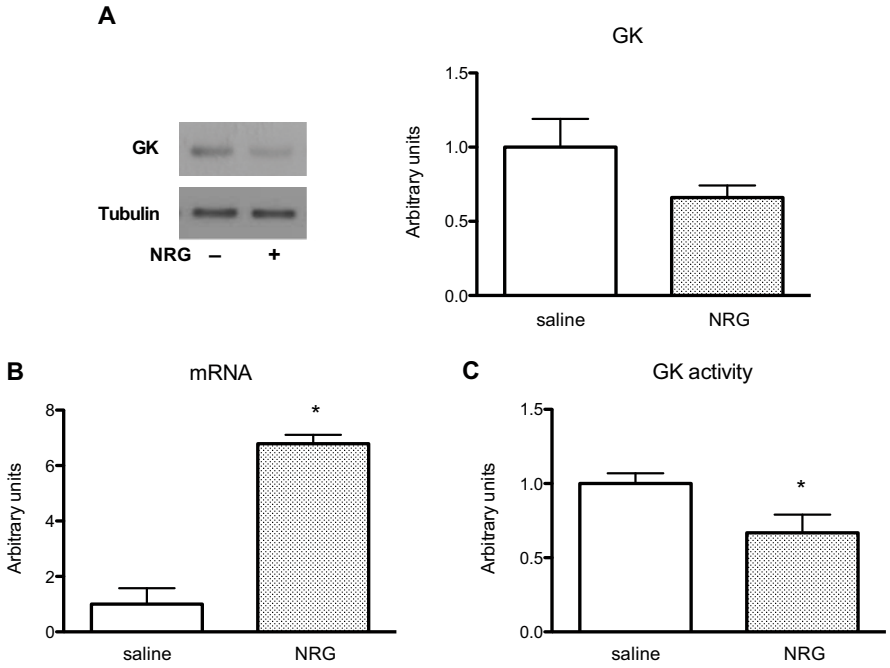


Figure 34. **Neuregulin influence on glucokinase protein levels, protein activity and transcription**. **(A)** Liver total lysates were used to detect GK. Tubulin was used as a loading control; **(B)** GK hepatic mRNA levels were determined by RT-PCR; **(C)** GK activity was measured from a fresh liver extract in a spectrophotometric method. * indicates significant difference vs. saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Another glycolytic enzyme that is proposed to be transcriptionally down-regulated by active FoxO1 is glucokinase (GK) (Zhang et al., 2006), which is an enzyme that facilitates phosphorylation of glucose to glucose-6-phosphate (G6P). However, insulin increases its expression through Akt-mediated activation of SREBP1c transcription factor (Laplante and Sabatini, 2009). At the light of previous reports, NRG should influence GK synthesis positively. Indeed, mRNA increased almost 7-fold under NRG action (Fig. 34, B). Unexpectedly, protein levels of GK showed a large trend of degradation (34%), although not statistically significant (Fig. 34, A), but confident enough since it was confirmed by GK activity by measuring conversion of

glucose to G6P at the same time-point (Fig. 34, C). These results suggested that while neuregulin activated GK transcription, there was also another mechanism operating that induced GK degradation and/or deactivation.

Next, we wondered if all previously described effects were under the control of ErbB3. For this purpose, we used ErbB3 blocking antibodies. In this approach, *in vivo* NRG action remained for 45 minutes, what allowed us to observe a significant decrease in GK levels (Fig. 35). This decrease, however was smaller (10%), maybe due to the up regulation of GK transcriptional activity seen in previous experiments or to the administration of glucose, that raises insulin levels, what was not done while extracting livers from previous experiment. The use of ErbB3 blocking antibodies abrogated NRG effects lowering GK protein levels, suggesting that the unknown mechanism that triggered GK degradation was mediated by Erb3.

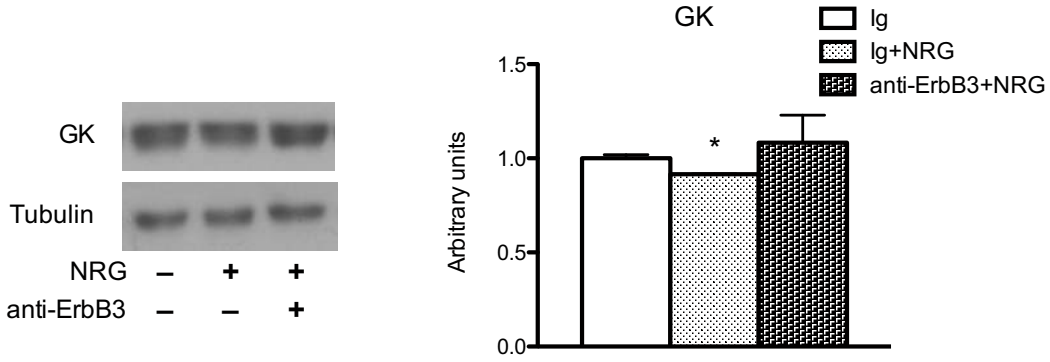


Figure 35. **Neuregulin affects GK through activating ErbB3**. Liver total lysates from Ig, Ig+NRG and anti-ErbB3+NRG-treated mice were used to detect GK with WB. Tubulin was used as a loading control. * indicates significant difference vs. Ig group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

To pursue in the role of neuregulin on GK, we analyzed NRG action in insulin-deficient mice. Immunoblots revealed that liver GK protein levels dropped under *in vivo* neuregulin action. This decrease in STZ-treated mice, however, was much greater, remaining only 26% of the total after 30 minute stimulation with NRG (Fig. 36, A). Therefore, the suggested degradation mechanism induced by NRG on GK appears to be much more effective in STZ-treated mice. To understand whether the higher GK degradation rate, in STZ-treated mice compared to controls, might be a cause of NRG regulating differently mRNA levels, we performed RT-PCR assay. The result clearly showed that basal mRNA levels of those two animal groups were similar, but while in control animals with acute NRG treatment GK transcription increased almost 7-fold, this effect was impaired in STZ-treated mice (Fig. 36, B). Therefore, neuregulin inability to up-regulate transcription of hepatic GK, which seemed to be an insulin-dependent effect, or at least the mechanisms to up-regulate GK transcription were altered in insulin-deficient mice, may be a supporting factor in remarkable and quick degradation of GK protein. When analyzing GK

activity, we also saw a significant reduction (60%) in glucose conversion to G6P under NRG action (Fig. 36, C).

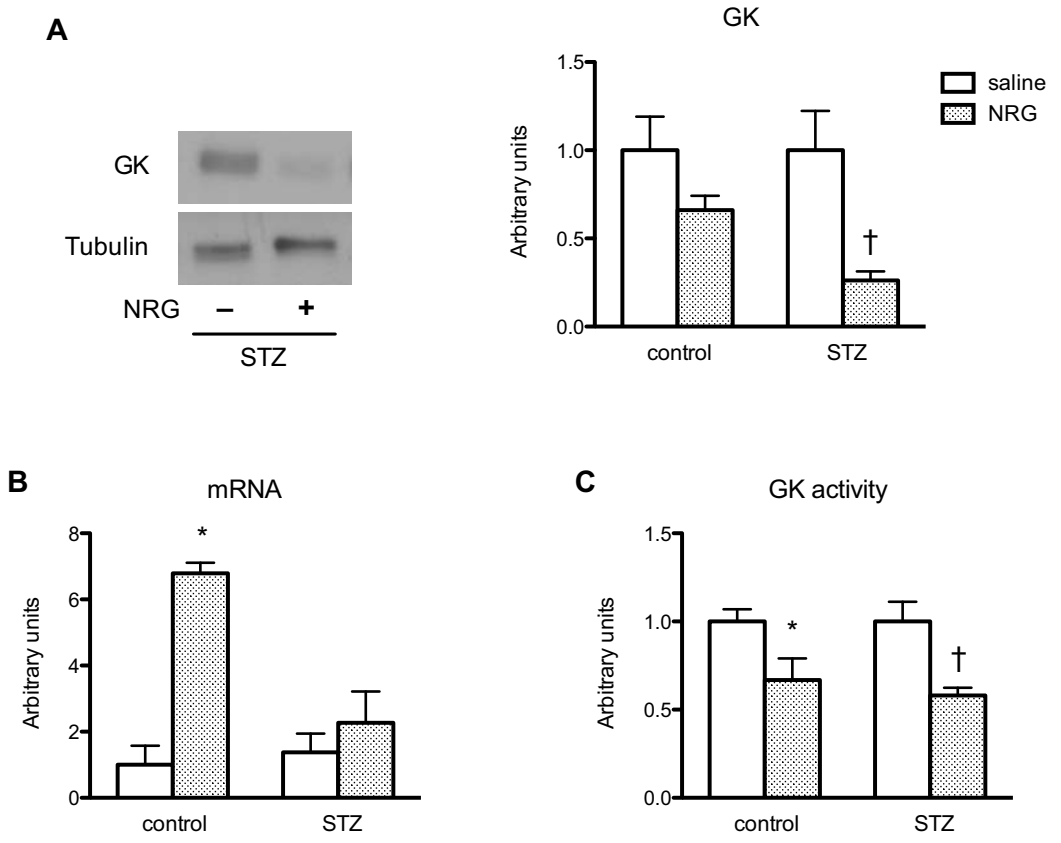


Figure 36. **Neuregulin influence on glucokinase protein levels, protein activity and transcription in STZ mice in comparison with control mice**. **(A)** Liver total lysates were used to detect GK protein. Tubulin was used as a loading control; **(B)** GK hepatic mRNA levels were determined by RT-PCR; **(C)** GK activity was measured from a fresh liver extract in a spectrophotometric method. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.4.1.3. Effects on the FoxO1 target, IRS2

Likewise GK, IRS2 promoter is activated by FoxO proteins through an insulin response element. Moreover, SREBPs also in case of IRS2 directly repress its transcription and inhibit hepatic insulin signaling (Ide et al., 2004). As expected, RT-PCR assays showed a significant reduction in IRS2 mRNA from NRG-treated mice liver (Fig. 37). This observation enforced the concept that neuregulin had a negative impact on insulin signal transduction at IRS proteins.

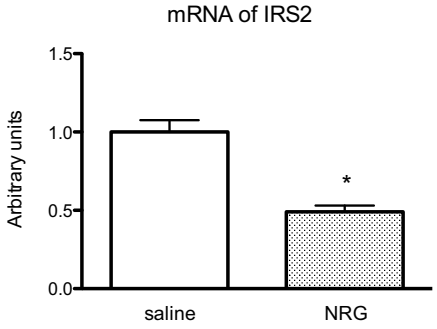


Figure 37. **Neuregulin influence on IRS2 transcription**. IRS2 hepatic mRNA levels were determined by RT-PCR.. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Next, we wanted to see how neuregulin affected IRS2 transcription in insulin-deficient situation. Measurements of IRS2 mRNA levels by RT-PCR showed that neuregulin action was at least partially impaired in STZ-treated mice (Fig. 38).

Figure 38. **Neuregulin influence on IRS2 transcription in STZ mice in comparison with control mice**. IRS2 hepatic mRNA levels were determined by RT-PCR. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.4.2. Effects on GSK3β: consequences for a neuregulin effect inducing glycogen synthesis.

Glycogenesis induces glucose storage as glycogen. Insulin initiates the process through PI3K/Akt pathway by inhibitory phosphorylation of GSK3β at Ser9 and following activation of glycogen synthase. GSK3β is a direct substrate of Akt. WB analysis revealed that NRG increases serine phosphorylation of GSK3β significantly almost 6-fold (Fig. 39) and therefore gave bases to purpose an induction in glucose conversion to glycogen. Therefore, we quantified glycogen amount in the same livers treated *in vivo* during 30 minutes either with saline or NRG. Although on average a slight increase was detected, it was not significantly different. The latter can be a cause of too short time interval to a see a significant change in glucose conversion rate to glycogen, accompanied with a lack and impossibility to measure intracellular glycogen levels

before the treatment. However, recent preliminary data from radioactive glucose conversion to glycogen supports the fact that neuregulin, indeed, induce glycogenesis (data not shown).

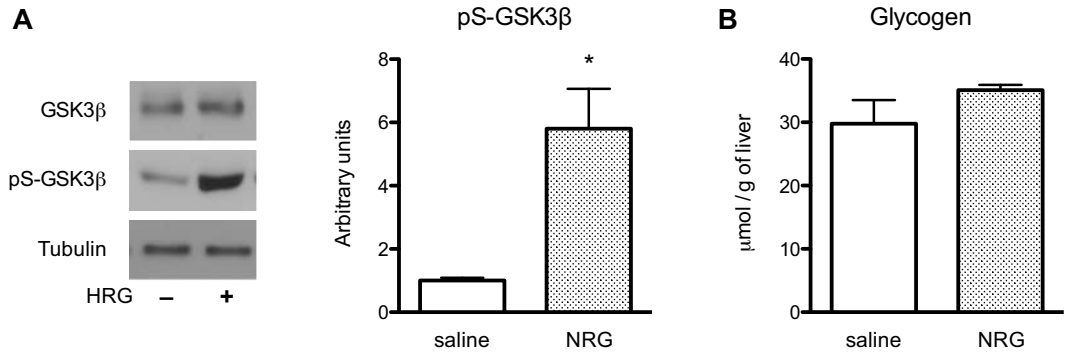


Figure 39. **Neuregulin influence on GSK3β phosphorylation on Ser9 residue and glycogen synthesis in control mice**. **(A)** Liver total lysates were used to detect GSK3β and its phosphorylation at Ser9 residue with WB. Detected phosphorylation was adjusted against total GSK3β. Tubulin was used as a loading control; **(B)** liver glycogen content was determined with a spectrophotometric assay. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

To prove that this neuregulin effect on GSK3 β arises from liver ErbB3 activation, we analyzed the anti-ErbB3 blocking antibodies impact on its Ser9 residue phosphorylation. In this situation, *in vivo* administrated neuregulin was unable to induce GSK3 β phosphorylation indicating the relevance of hepatic ErbB3 for this neuregulin effect (Fig. 40).

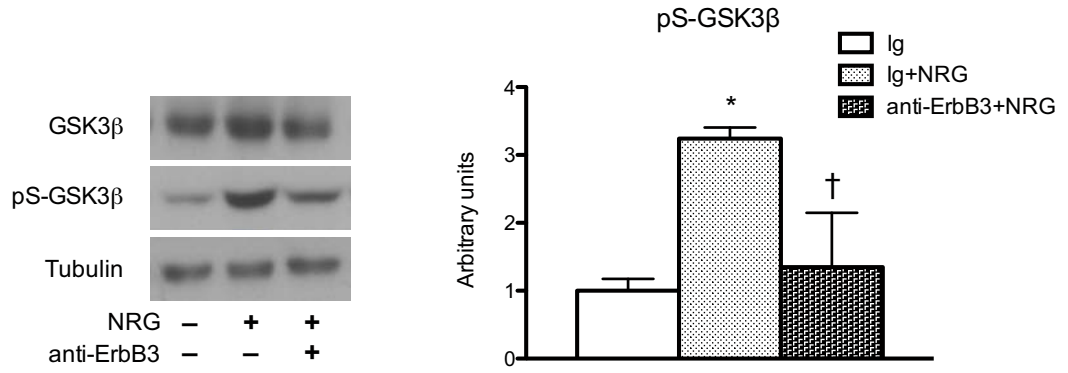


Figure 40. **Neuregulin inhibits GSK3β through activating ErbB3**. Liver total lysates from Ig, Ig+NRG and anti-ErbB3+NRG-treated mice were used to detect GSK3β and its phosphorylation at Ser9 residue with WB. Detected phosphorylation was adjusted against total GSK3β. Tubulin was used as a loading control. * indicates significant difference vs. Ig group and † difference vs. Ig+NRG group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Glycogen levels in STZ-treated mice are remarkably reduced (Jourdan et al., 2009). Since NRG induced signaling that enhances glycogenesis, by inhibiting GSK3β, we wanted to analyze how

NRG may influence the phosphorylation state of this kinase in STZ-treated mice. The results from WB assay showed that phosphorylation at Ser9 residue increased 2.7-fold after acute treatment with neuregulin (Fig. 41). That was almost half of what was the increase in phosphorylation per protein loaded found in control animals, but remained to be significant. The result suggested that even phosphorylation per protein level has increased less than in control mice, NRG still gave a signal to inhibit GSK3β in insulin-deficient mice.

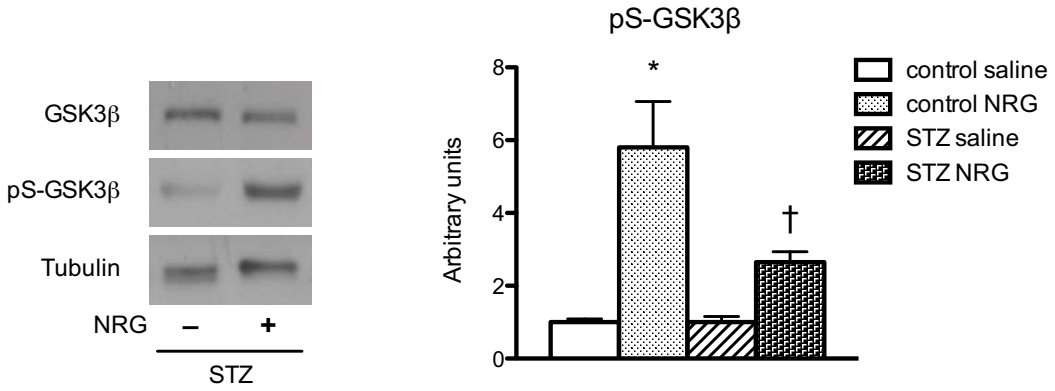


Figure 41. **Neuregulin influence on GSK3β phosphorylation and glycogen synthesis in STZ mice in comparison with control mice**. **(A)** Liver total lysates were used to detect GSK3β and its phosphorylation at Ser9 residue with WB. Detected phosphorylation was adjusted against total GSK3β. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. STZ saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.4.3. Effects on the mTORC1-p70S6K pathway: consequences on IRSs deactivation

Another pathway activated by Akt is the mTORC1-p70S6K pathway, which has a positive influence on protein synthesis and is also connected to increased serine phosphorylation and inhibition of IRS (Harrington et al., 2004; Um et al., 2004; Werner et al., 2004). The latter is known as an insulin negative feedback loop (Haruta et al., 2000; Greene et al., 2003; Carlson et al., 2004). Since neuregulin action induced a decrease in IRS1 and IRS2 interaction with PI3K possibly by increased serine phosphorylation of IRS proteins and observing a clear activation of Akt dependent of ErbB3, an examination of p70S6K seemed as a necessary task to fulfill. An easily detectable increase of phospho-(Thr389)-p70S6K in WB varied slightly between neuregulin-treated animals, but despite the fact, gave a significant result with an average of 60-fold higher phosphorylation (Fig. 42). The outcome strongly supports the assumption that in liver neuregulin might be disrupting insulin action at the IRS proteins level by activating p70S6K, which in turn, mediate serine phosphorylation of IRS proteins.

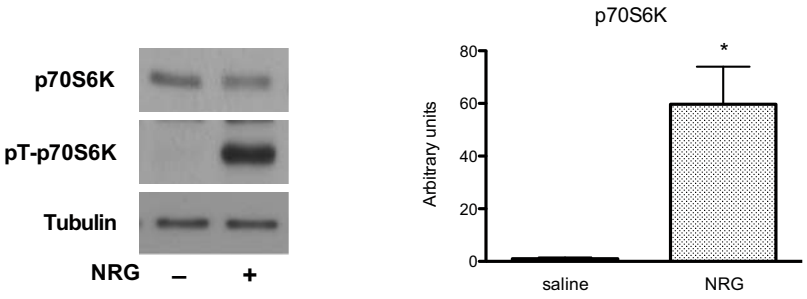


Figure 42. **Neuregulin influence on p70S6K phosphorylation at Thr389 residue in control mice**. Liver total lysates were used to detect p70S6K and its phosphorylation at Thr389 residue with WB. Detected phosphorylation was adjusted against total p70S6K. Tubulin was used as a loading control. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

To ensure the specificity of the neuregulin action, we analyzed the ErbB3 blocking antibodies effect on the neuregulin action inducing p70S6K phosphorylation at the liver. Here, we observed that blocking antibodies completely inhibited activation on p70S6K, indicating that liver ErbB3 is essential for this neuregulin action (Fig. 43).

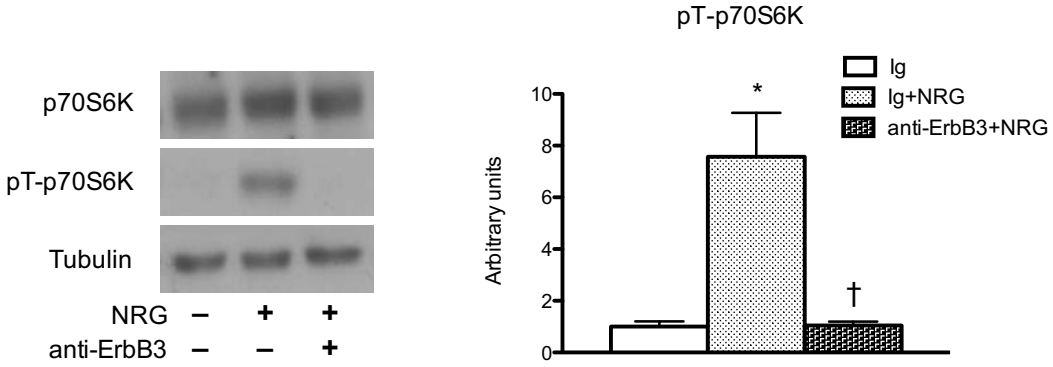


Figure 43. **Neuregulin activates mTORC1-p70S6K pathway through binding to ErbB3**. Liver total lysates from Ig, Ig+NRG and anti-ErbB3+NRG-treated mice were used to detect p70S6K and its phosphorylation at Thr389 residue with WB. Detected phosphorylation was adjusted against total p70S6K. Tubulin was used as a loading control. * indicates significant difference vs. Ig group and † difference vs. Ig+NRG group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

We also analyzed NRG ability to activate p70S6K in livers of STZ-treated mice. Surprisingly, neuregulin failed to activate this kinase in livers of insulin-deficient mice (Fig. 44). As previously observed in this STZ-treated mice model, deficiencies observed in the neuregulin action could be a consequence of an altered pattern of protein expression as a consequence of the long-term absence of insulin expression and action that may drive to a secondary manifestation of insulin resistance, and as shown, of neuregulin resistance.

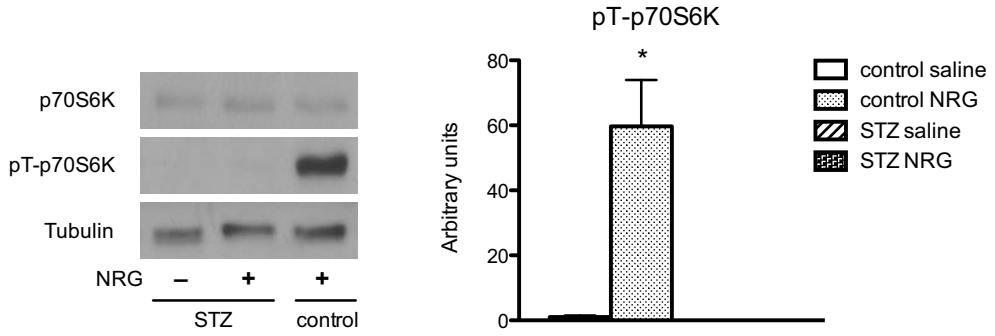


Figure 44. **Neuregulin influence on p70S6K phosphorylation in STZ mice in comparison with control mice**. Liver total lysates were used to detect p70S6K and its phosphorylation at Thr389 residue with WB. Detected phosphorylation was adjusted against total p70S6K. Tubulin was used as a loading control. * indicates significant difference vs. control saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

1.2.4.4. Effects on AMPK

AMPK is an intracellular sensor of energy homeostasis (Lage et al., 2008) that interacts with insulin signaling pathway through activating TSC2 and thus repressing signaling through mTORC1 (Baum et al., 2009). In liver, an active phosphorylated AMPK is also responsible for inhibiting gluconeogenesis and glycogenesis among other ATP consuming processes. Being an important regulator of glucose metabolism and playing an important role in areas where we can see NRG impact, we questioned whether NRG might have an influence on AMPK activity. The outcome of WB of AMPK catalytic subunit phosphorylation at Thr172 did not unfold any differences between saline and neuregulin-treated livers (Fig. 45). It suggests that NRG caused acute effect in mice during 30 minutes does not include participation of AMPK.

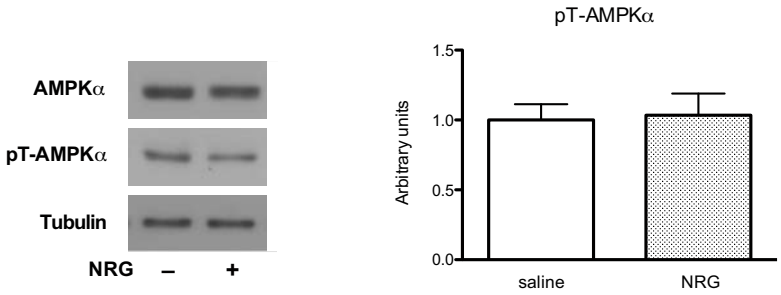


Figure 45. **Neuregulin influence on AMPKα phosphorylation on Thr172 residue in control mice**. **(A)** Liver total lysates were used to detect AMPKα and its phosphorylation at Thr172 residue with WB. Detected phosphorylation was adjusted against total AMPKα. Tubulin was used as a loading control. Statistical test applied: Unpaired Student's t test. These images are representative of 4 independent experiments.

2. *In vivo* neuregulin effects in type 2 diabetic mice model (db/db)

In insulin resistant state insulin sensing tissues are ineffective clearing the glucose from the blood and the physiological levels, therefore, remain much higher than in healthy organisms. In addition, gluconeogenesis, which is normally down-regulated by insulin after a meal, often remains active in T2D contributing as well to higher glycaemia. In control mice, neuregulin have shown to stimulate glycogenesis and inhibit HGP by activating a pathway that is under classical insulin signaling, but without a contribution of IR/IRS, which in insulin resistance is often impaired. Therefore, it is important to investigate whether neuregulin signaling in T2D is intact and the growth factor can be used as a therapeutic tool to relieve the pathological situation.

The most widely used and one of the first mouse models for type 2 diabetes is leptin receptor deficient C57BLKS/J-db/db (db/db) (Hummel et al., 1966). The mice are remarkably obese (Fig. 46, A) and develop severe hyperglycaemia and hyperinsulinemia. According to supplier information the strain do not expose any insulin response to glucose, which shows a strong defect in β-cell function.

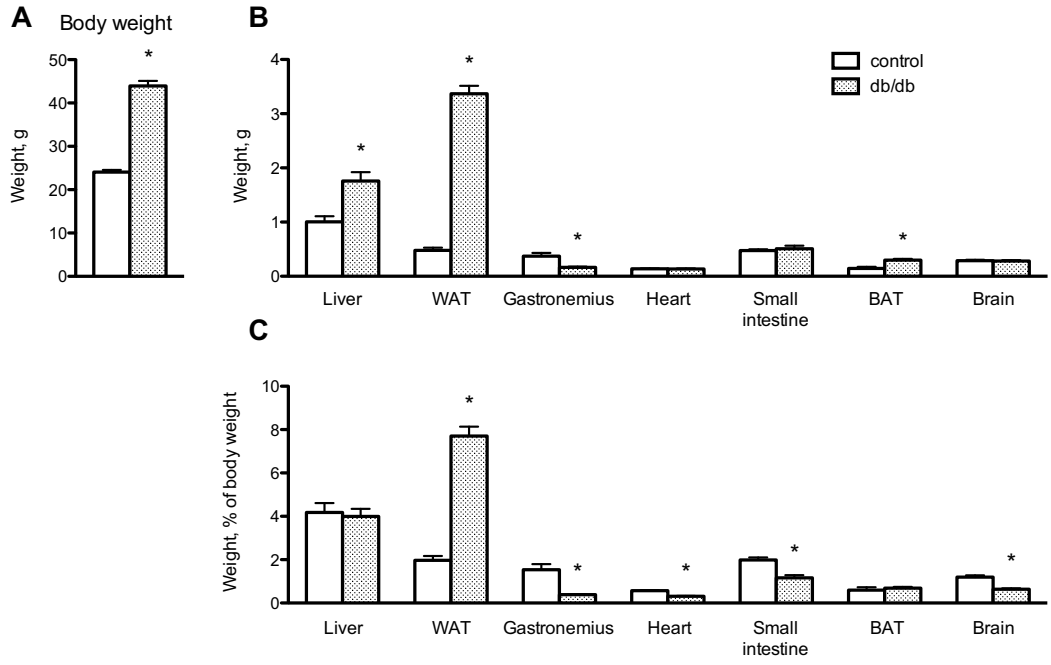


Figure 46. **Characterization of db/db mice body composition. (A)** Body weight; **(B)** weight of tissues in grams and **(C)** as ratio to body weight. * indicates significant difference vs. control saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 6 independent experiments.

In addition to hyperglycaemia and hyperinsulinemia, db/db mice show changes also in body composition (Fig. 46, B and C) and appearance of tissues. The biggest change is in mice adiposity. The amount of visceral adipose tissue solely has increased 7-fold (3.5-fold compared to body weight). Liver, which shows signs of steatosis, and brown adipose tissue (BAT) have increased in comparable extent with body weight. However, there is a visible decrease in weight of gastrocnemius muscle that becomes even more apparent comparing it with body weight. The total mass of other tissues, such as heart, small intestine and brain, has not changed.

2.1. *In vivo* neuregulin effect on plasma metabolites (glucose, lactate and triglycerides) and insulin levels.

As in control animals, we started with detection of neuregulin effects on blood glycaemia and insulinemias of db/db mice in different conditions to see the effects that the growth factor can produce. The physiological measurements help us to understand whether neuregulin signaling in diabetic state is working properly and gives comparable results with control animals. It has to be noted that in these experiments mice in NRG group got a same neuregulin dose (50ng/g body weight) as control group.

2.1.1. Neuregulin effects on basal glycaemia and insulinemia in fasted mice

Here, we wanted to see what happens with basal fasted blood glucose levels in hyperglycemic db/db mice after an injection of NRG. As in control animals then also diabetic mice with a dose of saline an increase in basal glycaemia appeared, which most probably is a sign of stress caused by the experiment. However, the glucose measurements were significantly lower in NRG group compared to saline group at 30, 45 and 75 minutes after administration of the growth factor (Fig. 47, A) and when measuring AUC (Fig. 47, B). While in db/db mice lowering of glycaemia happens in greater extent than in control animals it may be due to big differences between basal fasted glycaemia in these groups. That is supported by observation that the higher glycaemia an animal reaches the bigger is NRG generated effect. The mechanism that may be operating to refine neuregulin effects according to departing glycaemia levels are unknown and look to be more precise than the ones that operate in response to insulin.

In control animals no influence on insulinemia was observed when animals were treated with NRG. At the same time, db/db mice, although being in fasted conditions, still have higher levels of insulin than healthy animals. Therefore there is a need to know if and how neuregulin treats this situation. Measurements of insulin in an ELISA assay showed that NRG reduced basal insulinemia, reaching significance at 75 minutes after NRG injection (Fig. 47, C), although the overall AUC did not significant difference despite 32% reduction (Fig. 47, D).

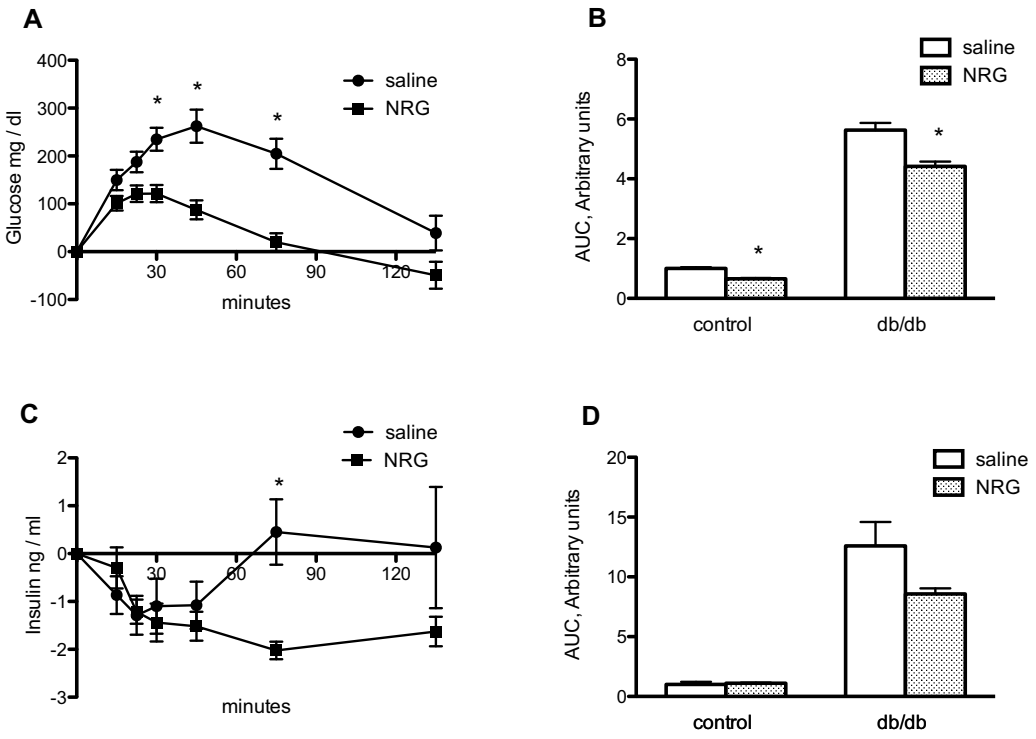


Figure 47. **Neuregulin action on glycaemia and insulinemia under basal conditions in fasted db/db mice in comparison with control mice**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=7; (**B**) AUC of glycaemia, n=7; (**C**) change in insulin level relative to basal insulinemia, saline vs NRG, n=4; (**D**) AUC of insulinemia, n=4. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**, **C**) or Unpaired Student's t test (**B**, **D**).

2.1.2. Neuregulin effects on glycaemia and insulinemia under glucose tolerance test in fasted mice

Here, we performed a GTT with db/db mice. A dose of glucose (2g/kg of body weight), administered 15 minutes after treatment with saline or NRG, created a huge increase in blood glucose levels, which in its highest point, 60 min after administration of glucose, reached more than 700 mg/dL over the basal fasted state glycaemia (Fig. 48, A). The glycaemia stayed high throughout the 120 minutes, which is characteristic to insulin resistant state. Administration of neuregulin greatly reduced glucose levels in blood letting it to raise upto 400 mg/dL over the basal glycaemia and making a significant difference between the groups already 30 minutes after the treatment. The fact that short-term neuregulin impairs glucose tolerance is well seen in calculations of AUC, which is 28% smaller than we can observe in the saline-treated diabetic mice (Fig. 48, B). While AUC of diabetic animals is remarkably higher, which is characteristic to diabetic situation, reduction seen in control animals is, in absolute glucose values, smaller but percentage-wise comparable (25%).

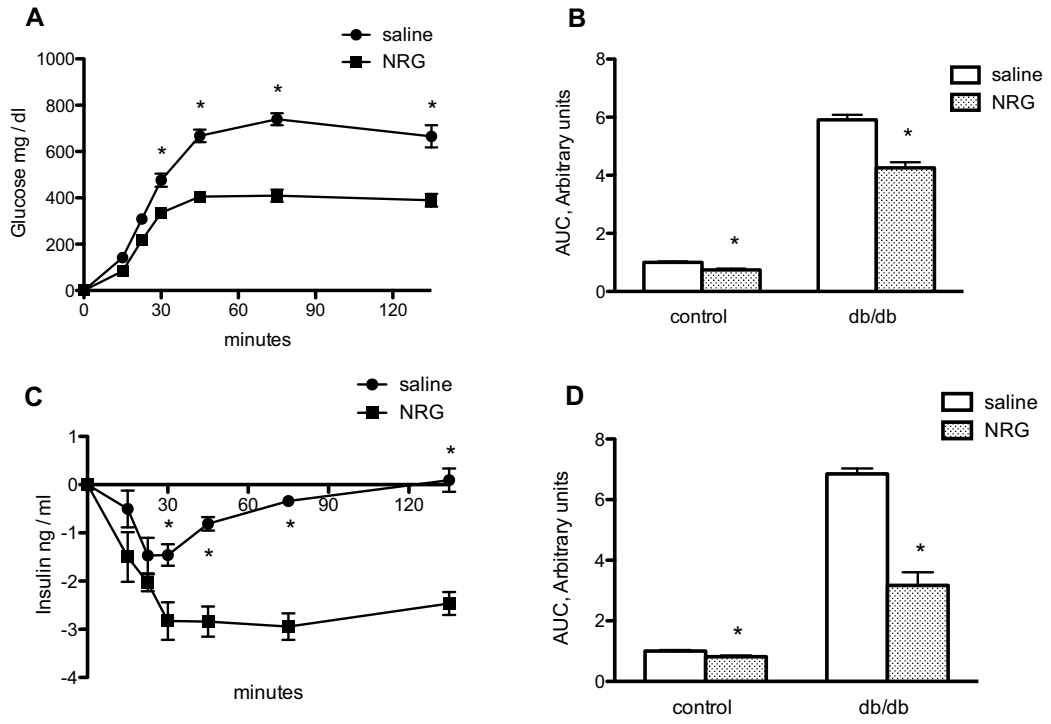


Figure 48. **Neuregulin action on glycaemia and insulinemia under glucose tolerance test in fasted db/db mice in comparison with control mice**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=15; (**B**) AUC of glycaemia, n=15; (**C**) change in insulin level relative to basal insulinemia, saline vs NRG, n=4-5; (**D**) AUC of insulinemia, n=4-5. * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**, **C**) or Unpaired Student's t test (**B**, **D**).

NRG ability to lower glucose accompanied with decreased need for insulin during GTT in control animals. Db/db mice are hyperinsulinemic, but as the supplier of animals claims that this strain does not expose any insulin response to glucose (Hellman et al., 1974). Therefore it is important to check what happens with insulin in neuregulin administration. In saline group during the first 30 minutes of the experiment we could observe a lowering in insulinemia, which it is supposed to be caused by stress from repeated injections and taking the blood samples. By the end of the test, insulinemia arrived back to basal fasted state levels (Fig. 48, C). However, neuregulin administration causes extensive decrease in insulin levels, measuring almost 3 ng/ml less than in starting point 30 minutes after neuregulin administration. That significantly different level is kept until 75 minutes have past from beginning GTT and thereafter slowly starts to rise. The effect on insulinemia is clearly seen also in AUC, where data from NRG group gives 54% lower bar (Fig. 48, D). The reduction seen in db/db mice is greater than in control animals (18% lower), but also the conditions of these animals are different.

2.1.3. Neuregulin effects on glycaemia under pyruvate tolerance test in fasted db/db mice

In T2D, HGP is up-regulated due to increased expression of gluconeogenic enzymes PEPCK and G6Pase (Barzilai and Rossetti, 1993; Trinh et al., 1998). Studies in control mice revealed that short-term treatment with neuregulin before administration of pyruvate was able to completely block gluconeogenesis. However, in db/db mice neuregulin only could impair partially the hepatic glucose production based on pyruvate tolerance test (Fig. 49, A). Treatment with neuregulin decreased blood glucose levels significantly at 30, 45 and 75 minutes from the start of the test, which is also seen in AUC data (Fig. 49, B). The blockage of gluconeogenesis observed was comparable to that seen when analyzing neuregulin action on basal glycaemia in fasted db/db mice (Fig. 49, C). This result indicated that in insulin resistant situation neuregulin affects HGP only partially and fails to completely inhibit it.

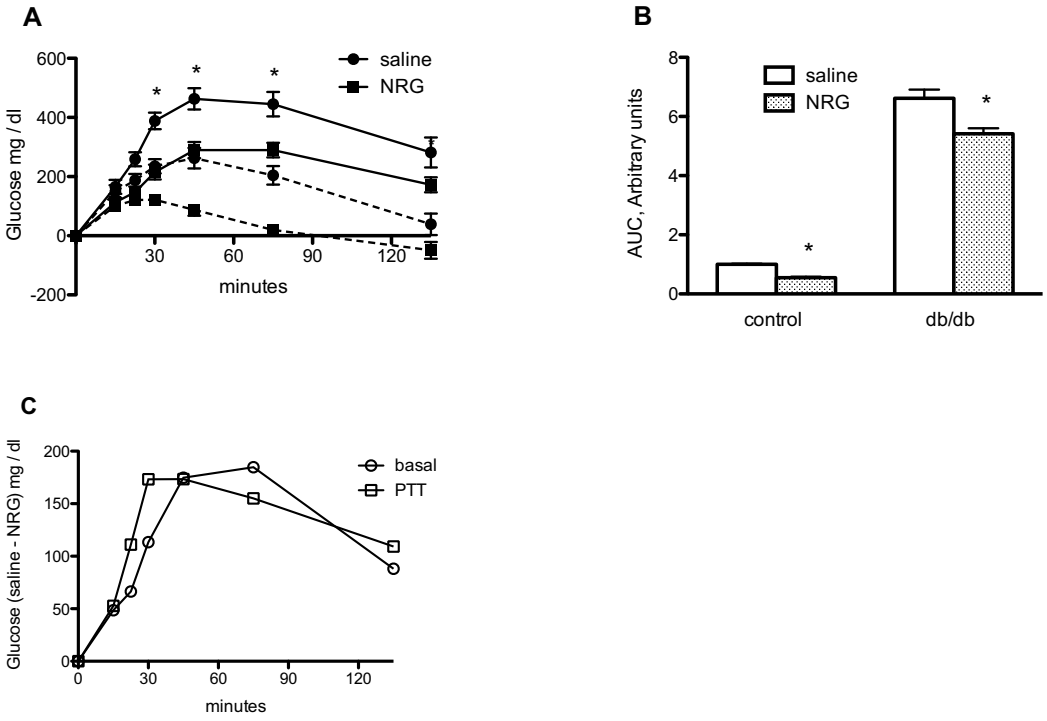


Figure 49. **Neuregulin action on glycaemia under pyruvate tolerance test in fasted db/db mice in comparison with control mice**. (**A**) change in glucose level relative to basal glycaemia during PPT (continuous lines) and basal condition (dashed lines), saline vs NRG (50 ng/g of body weight), n=7; (**B**) AUC of glycaemia, n=7; (**C**) the effect of neuregulin on glycaemia relative to saline-treatment – difference of means, fasted vs fed. * indicates significant difference vs. PTT saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

2.1.4. Effects of neuregulin on lactacidemia and trigliceridemia under Basal / GTT / PTT conditions in fasted db/db mice

Here, we analyzed neuregulin effect on basal lactacidemia in fasted db/db mice (Figure 50, A). The result is comparable with control animals, with a non-statistically significant neuregulin effect on lactacidemia. The same conclusion can be read out from calculations of AUC (Fig. 50, D). In samples from GTT assays, a rise in lactate production soon after the glucose administration in an independent manner of the neuregulin action, indicating that it was consequence of the glucose administration (Fig. 50, B). Contrarily to control mice, the increase in lactacidemia was continuous in diabetic animals. Despite of that, AUC data shows no significant differences in overall lactate production between healthy and diabetic mice (Fig. 50, D).

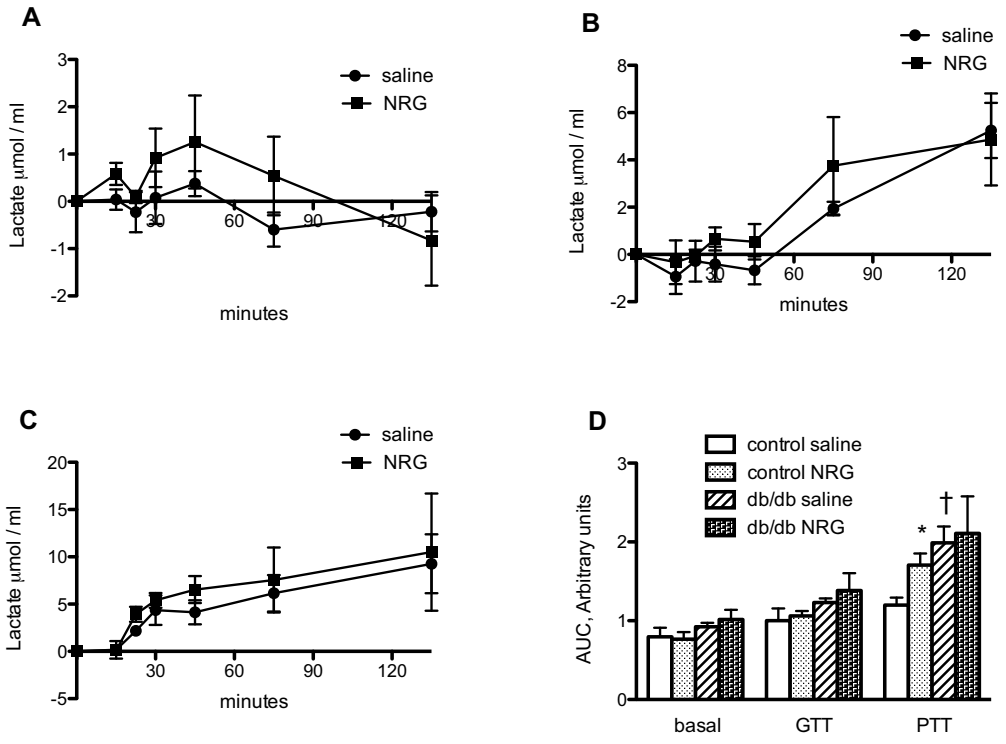


Figure 50. **Neuregulin action on lactacidemia under basal/GTT/PTT conditions in db/db mice in comparison with control mice**. Change in lactate level relative to basal lactacidemia under (**A**) basal, (**B**) GTT and (**C**) PTT condition, saline vs NRG (50 ng/g of body weight), n=3-4; (**D**) AUC of lactacidemia, n=3-4. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A, B, C**) or Unpaired Student's t test (**D**).

PTT assays also gave a different result than observed in healthy mice. In db/db group like in control group, lactate concentration in plasma increased sharply after pyruvate injection, but later on, instead of starting to lower, as observed in control animals, it kept increasing (Fig. 50, C). Another difference came from neuregulin impact on lactate accumulation in blood because no extra increase as in control animals was observed after growth factor treatment of diabetic mice.

Both contradictions, increased overall accumulation of pyruvate and neuregulin inability to contribute that process was also visible on Fig 50, D. That may suggest that neuregulin failed to prevent pyruvate entering to gluconeogenesis and therefore it was converted to lactate with the same rate despite animals were treated with NRG or not.

It is known that lipids, including TG, are overproduced in diabetic organisms. Therefore it is important to see if and how neuregulin influences lipid synthesis in different situation. A good way to observe possible changes is to measure TG levels in plasma of collected samples on basal fasted conditions or during GTT or PTT in order to analyze the NRG action.

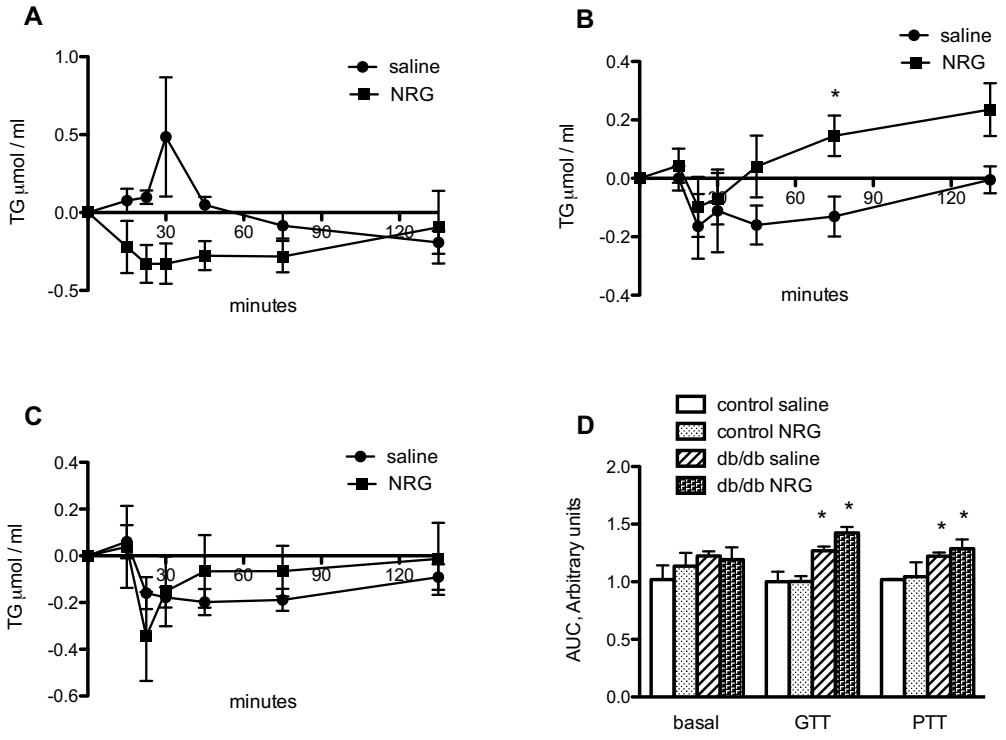


Figure 51. **Neuregulin action on triglyceridemia under basal/GTT/PTT conditions in db/db mice in comparison with control mice**. Change in triglyceride level relative to basal triglyceridemia under (**A**) basal, (**B**) GTT and (**C**) PTT condition, saline vs NRG (50 ng/g of body weight), n=3-5; (**D**) AUC of triglyceridemia, n=3-4. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A, B, C**) or Unpaired Student's t test (**D**).

Triglyceridemia was not affected by neuregulin action, either on basal situation or under a PTT in fasted db/db diabetic mice (Fig. 51, A and C). Contrarily, during a GTT, after the administration of glucose to db/db mice, 15 minutes later started a continuing increase in plasma triglycerides content, at first in NRG-treated group and later in saline treated group (Fig. 51, B). The increase in synthesis in similar rate, though with a shift towards higher concentrations in NRG group, was seen from 45 minutes to the end of the test, with being significantly different at 75-minute measuring point. Neuregulin ability to direct glucose to TG synthesis may be as sign of regulating lipid synthesis. It suggests that the increased glucose amount taken up by tissues during GTT was

used up to produce TG. However, when looking at AUC data, the detected increase was not relevant. Significant differences exist only between db/db and control animals (GTT and PTT), which is possibly due to existing hyperglycaemia (Fig. 51, D).

2.1.5. Neuregulin effects on glycaemia under basal / GTT / PTT conditions in fed db/db mice.

First, we measured neuregulin effect on basal glycaemia in fed state. In that condition glycaemia decreases during the time and neuregulin just lowers glucose levels a bit more at most by 50 mg/dL, but insignificantly (Fig. 52, A). As presented with AUC, also that data fails to show any difference (Fig. 52, B). That is different from fasted state where the neuregulin influence was times bigger and also significant.

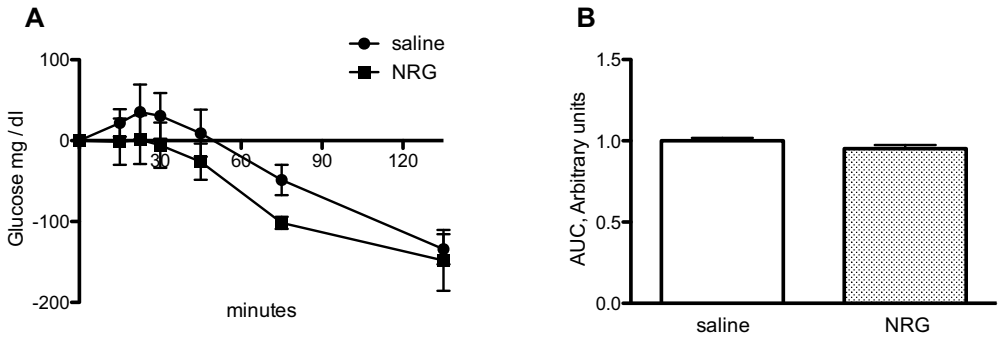


Figure 52. **Neuregulin action on glycaemia under basal condition in fed db/db mice**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=4-6; (**B**) AUC of glycaemia, n=4-6. Statistical test applied: Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

Next, we wanted to investigate whether that remarkable decrease in glycaemia that we observed during GTT in fasted db/db mice was also seen in fed state, while glycaemia and insulinemia are even higher than in fasted state. An intraperitoneal injection of glucose caused an increase in glycaemia up to 400 mg/dL over the basal fed state glucose level, reaching the same range as in fasted state (Fig. 53, A). Despite that glycaemia levels were similar, regardless the state of animal, a neuregulin reducing effect was much smaller in feeding conditions. In the last measuring point, the only time-point where it was significantly different, NRG was able to lower glycaemia only by 140 mg/dL compared to 300 mg/dL what was observed in fasted state. That suggest that higher hyperglycaemia can play a role in it, supported by published data that states that insulin weakens neuregulin binding to its receptor (Carver et al., 1996). However, similarly to fed control animals, NRG did not get significant differences on glycaemia when data is expressed as AUC (Fig. 53, B).

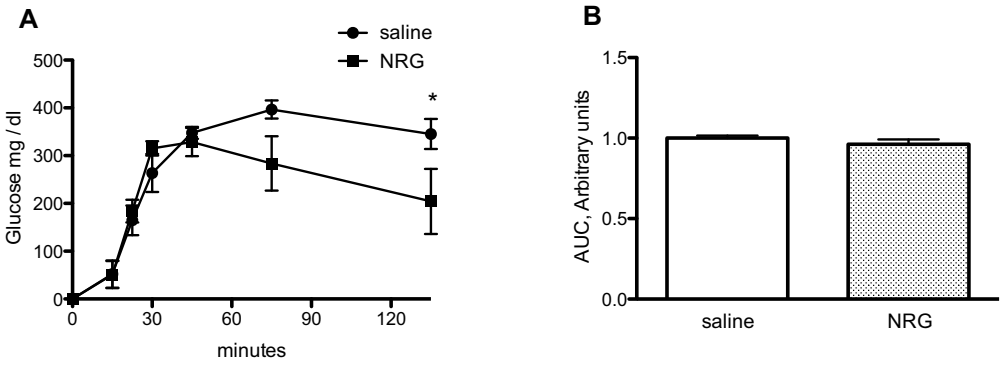


Figure 53. **Neuregulin action on glycaemia under glucose tolerance test in fed db/db mice**. (**A**) change in glucose level relative to basal glycaemia, saline vs NRG (50 ng/g of body weight), n=4; (**B**) AUC of glycaemia, n=4; * indicates significant difference vs. saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

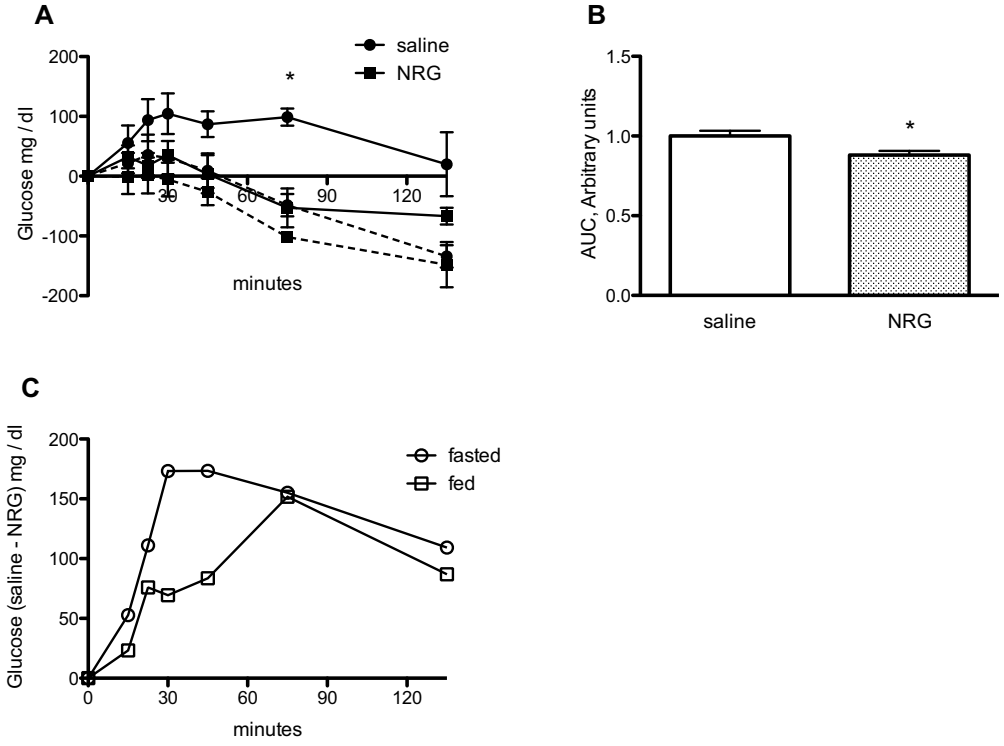


Figure 54. **Neuregulin action on glycaemia under pyruvate tolerance test in fed db/db mice**. (**A**) change in glucose level relative to basal glycaemia during PPT (continuous lines) and basal condition (dashed lines), saline vs NRG (50 ng/g of body weight), n=3-6; (**B**) AUC of glycaemia, n=3-6; (**C**) the effect of neuregulin on glycaemia relative to saline-treatment – difference of means, fasted vs fed. * indicates significant difference vs. PTT saline group at P<0.05 determined by Two-way ANOVA with post-hoc Tukey's test (**A**) or Unpaired Student's t test (**B**).

Next we wanted to know what happens with gluconeogenesis in fed diabetic animals when treated with NRG. Gluconeogenesis itself is up-regulated in these conditions, but with slower rate than in fasted animals (Fig. 54, A), but much higher than observed in fed control animals where it

hardly happened. Despite down-regulated gluconeogenesis, in fed control animals neuregulin inhibited HGP. In db/db mice we could see a reduction in glycaemia, under a pyruvate tolerance test, being significantly different at 75 minutes after NRG injection. The fact that the reduction was significantly important was supported also by AUC data (Fig. 54, B). While in control fed animals neuregulin inhibited HGP by lesser extent than in fasted situation, as expected from differences between gluconeogenic states, in fed diabetic mice the NRG effect reached the same level as in fasted state after 75-minute treatment (Fig. 54, C). The delay in reaching the maximum effect was also seen during GTT of fed db/db mice.

2.2. *In vivo* neuregulin action on the liver of diabetic db/db mice.

From experiments with control animals it revealed clearly that liver plays an important part in NRG-mediated regulation of glucose metabolism. While results from db/db mice showed distinct differences by having a greater effect on basal glycaemia and under GTT conditions, but failing completely to inhibit HGP, it is essential to investigate whether these differences were a consequence of alterations in the biology of neuregulin in diabetic liver.

2.2.1. Neuregulin and its receptors expression in the liver of diabetic mice.

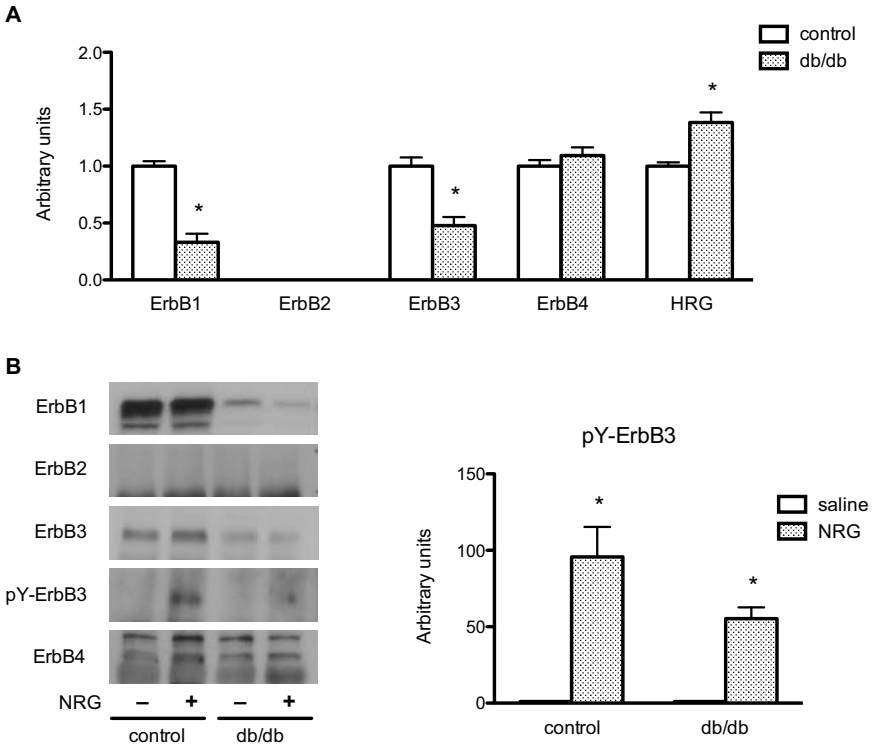


Figure 55. **Expression of ErbB receptors and neuregulin in liver of control and db/db mice. Tyrosine phosphorylation of ErbB3**. Total lysates obtained from liver were used for IP and WB to detect (**A**) expression of ErbB receptors and neuregulin in control and db/db mice and (**B**) tyrosine phosphorylation of ErbB3 upon NRG stimulation. Tyrosine phosphorylation levels were adjusted against total ErbB3 expression level. * indicates significant difference vs. saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

As a first thing we wanted to make clear how is the situation with ErbB receptor expression in db/db mice livers compared healthy animals. We observed that in insulin resistant livers, ErbB1 protein was reduced by 66% and the neuregulin receptor ErbB3 by 52% (Fig 55, A). ErbB2 remained absent and trace amounts of ErbB4 were observed with no significant changes in

expression levels. That reduction in the two main ErbB receptors in liver gives a first picture of serious differences between liver availability to convey neuregulin signal in healthy and diabetic mice.

Neuregulin mainly act as autocrine, paracrine or juxtacrine cytokine (Goodearl et al., 1995; Loeb et al., 1998) and, therefore, it influences in the immediate environment of cells in relatively short distance. It made us question whether neuregulin expression by the liver as well may have changed with diabetes. WB revealed that contrary to down-regulated ErbB receptors, neuregulin expression in liver had risen by 38% with diabetes. This result may suggest that liver was responding in front to fewer number of ErbBs receptors by increasing neuregulin expression to maintain neuregulin signaling.

To measure if and how well the neuregulin receptor ErbB3, in spite of greatly reduced levels, is activated, we immunoprecipitated ErbB3 and detected tyrosine phosphorylation by WB. The detected phosphorylation was increased 55 times over the unstimulated level, which is rather less than observed control mice per amount of protein (Fig, 55, B). That suggests that while in T2D ErbB3 expression is largely reduced, also activation of the number of neuregulin receptors present is weaker in db/db mice.

2.2.2. PI3K recruitment to ErbB3, IRS1 and IRS2

Here, we examined how an insulin resistant state affected neuregulin signal transduction to activate its pathways. The most direct way was to analyze whether there was some change in the p85 PI3K bound to immunoprecipitated ErbB3. PI3K interaction with ErbB3 was, after 30-minute of NRG treatment, significantly increased 13-fold per amount of receptor, which is less than observed in control animals, where the increase was 19-fold (Fig. 56). It again confirmed the fact that besides lesser number of ErbB3 receptors in db/db mice liver, also receptor activation is reduced.

Activation of neuregulin signaling in liver of control animals also disrupted insulin signaling pathway by reducing interactions of IRS proteins with PI3K. To see how neuregulin behaved in diabetic situation, we immunoprecipitated IRS1 and IRS2 followed by WB to measure p85 PI3K amount attached. In case of IRS1 we observed a little but insignificant reduction in interaction with PI3K after neuregulin treatment (Fig. 56) what was different from control animals where NRG caused approximately 60% decrease. However, PI3K interaction with IRS2 declined significantly by 32%, which is still less than in control animals, according to relative data per amount of IRS2 expressed. In that step it has to be noted and it is also well described in literature that IRS2 protein levels are dramatically reduced in livers of db/db mice compared to controls, whereas no change has been observed in levels of IRS1 between the groups (Canettieri et al., 2005). This is in accordance with our results. Altogether suggest that with weaker activation of ErbB3, there was also reduced effect on disrupting insulin signaling.

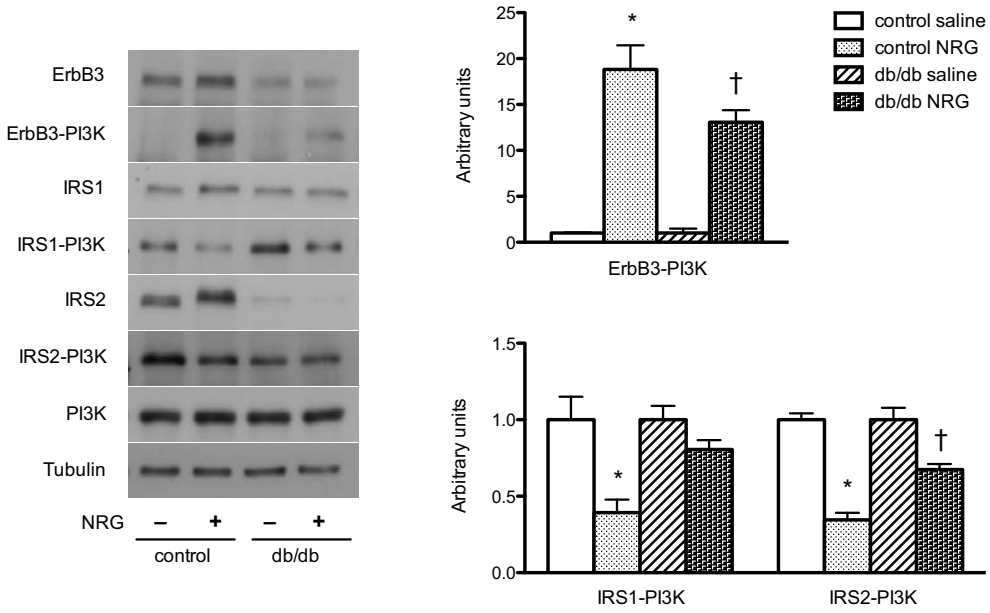


Figure 56. **Neuregulin effect on PI3K interaction with ErbB3 and IRS proteins in db/db mice in comparison with control mice**. ErbB3, IRS1 or IRS2 was immunoprecipitated from liver total lysates and bound p85 PI3K was detected with WB. Detected PI3K was adjusted against total ErbB3, IRS1 or IRS2, respectively. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

2.2.3. Neuregulin effect on Akt activation in db/db mice

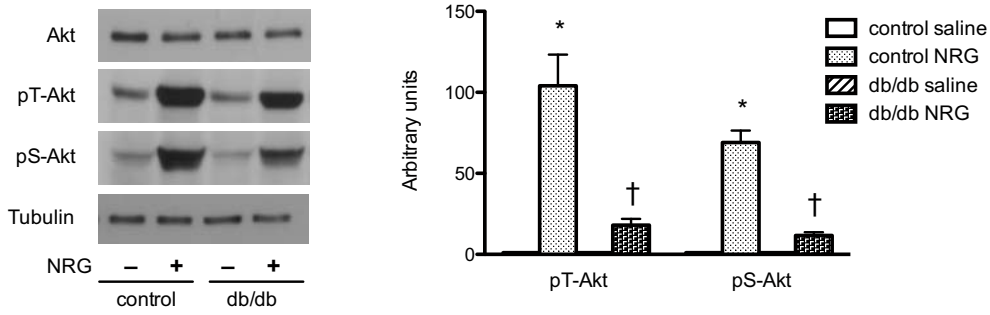


Figure 57. **Neuregulin influence on Akt phosphorylation in db/db mice in comparison with control mice**. Liver total lysates were used to detect Akt and phosphorylation at Thr 308 and Ser473 residues with WB. Detected phosphorylation was adjusted against total Akt. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

A next step on PI3K dependent pathway that neuregulin activates is Akt. Its expression in db/db mice livers did not change. Despite of that, a weaker signal from neuregulin is unable to maintain the same activation level in diabetic liver compared to healthy as detected from WB assay (Fig.

57). In db/db mice NRG induced phosphorylation of Thr308 residue 21-fold and phosphorylation of Ser473 12-fold. In control animals the increases were 104-fold and 69-fold, respectively, which is 5-6 times more than in db/db. That again proves that NRG signal in insulin resistant hepatocytes is largely reduced.

2.2.4. Neuregulin effects on downstream Akt activation proteins involved in the regulation of glucose metabolism in the liver of diabetic db/db mice.

2.2.4.1. Effects on FoxO1 and downstream target enzymes involved in gluconeogenic and glycolytic pathways. Effects on IRS2.

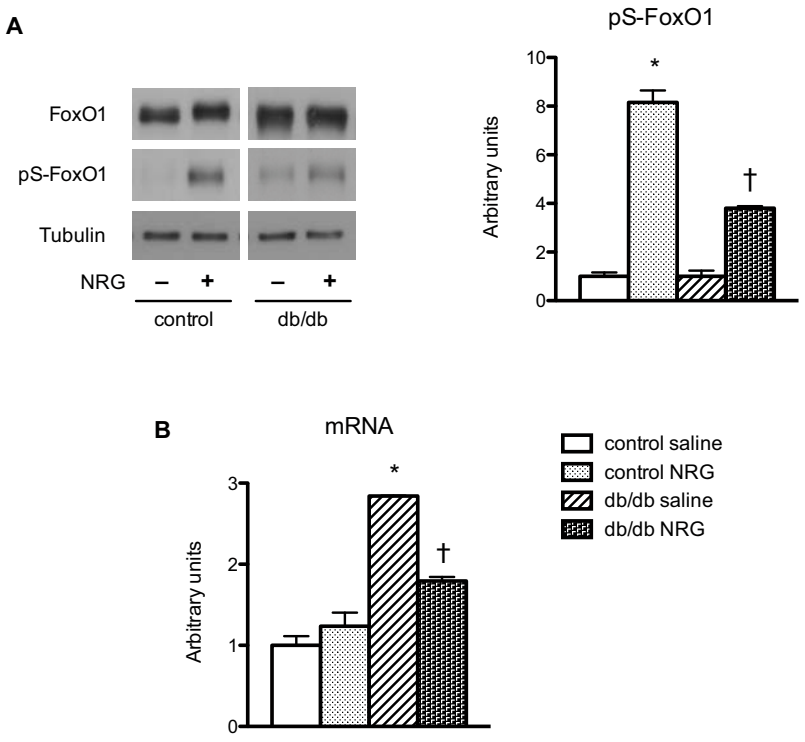


Figure 58. **Neuregulin influence on FoxO1 phosphorylation and transcription in db/db mice in comparison with control mice**. (**A**) Liver total lysates were used to detect FoxO1 and its phosphorylation at Ser256 residue with WB. Detected phosphorylation was adjusted against total FoxO1. Tubulin was used as a loading control; (B) FoxO1 hepatic mRNA levels were determined from extracted RNA by RT-PCR. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

HGP is altered in type 2 diabetic liver due to increased expression of gluconeogenic enzymes PEPCK and G6Pase (Barzilai and Rossetti, 1993; Trinh et al., 1998) and impaired insulin action on inhibiting hyperactivated FoxO1 despite the presence of hyperinsulinemia (Valera et al., 1994; Trinh et al., 1998). An inhibition of gluconeogenesis and one of its main regulators, FoxO1, was

observed after NRG treatment in control animals. However, inhibition of gluconeogenesis by NRG was defective in livers of db/db mice. Therefore, it was necessary to investigate thoroughly signaling mediated by FoxO1. In db/db mice liver, NRG induced FoxO1 phosphorylation at Ser256 residue what inactivates it (Fig 58, A). That effect, however, was half of what was observed in control animals per total FoxO1 protein immnunodetected. That suggested that in diabetic liver, where FoxO1 was hyperactivated and neuregulin signaling to inhibit it was weaker, neuregulin action may fail to produce an effect as consistent as it was on normal healthy state.

While looking at the neuregulin effect on FoxO1 transcription, we surprisingly observed a 37% decrease (Fig. 58, B). However, no reduction was observed in FoxO1 protein content 30 minutes after *in vivo* neuregulin treatment. That suggests that even if weaker neuregulin signal in db/db mice fails to inhibit FoxO1 completely, in longer term it may have an effect FoxO1 regulated machinery through down-regulating the protein levels.

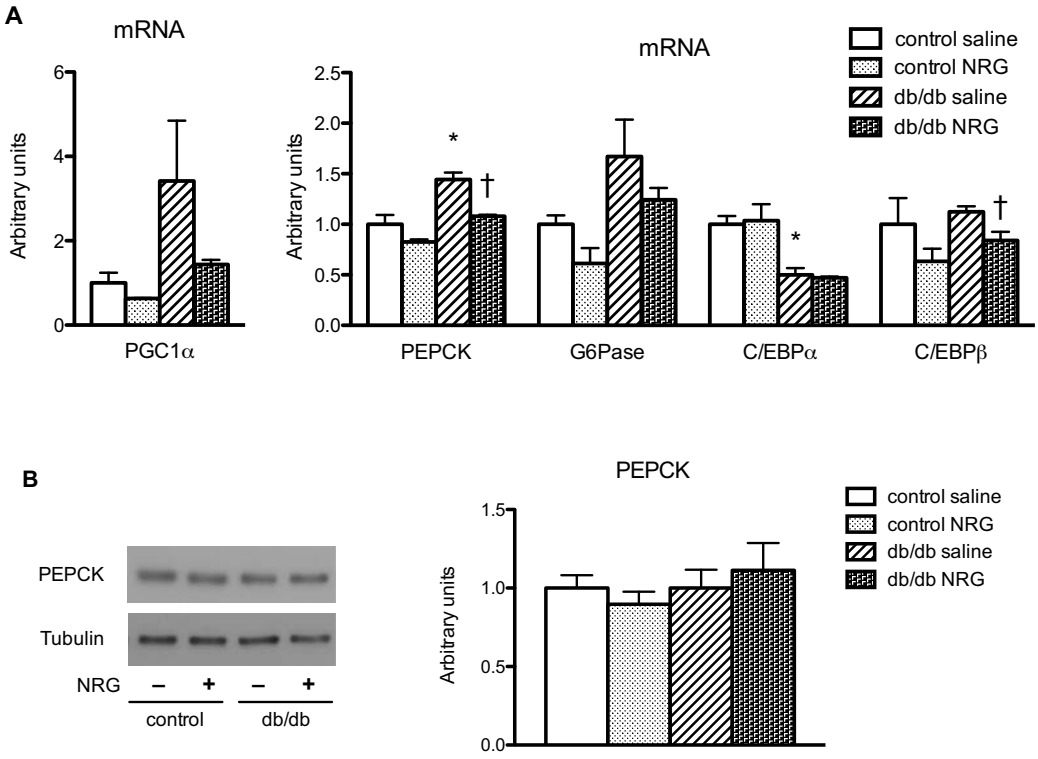


Figure 59. **Neuregulin effect on gluconeogenic gene expression and PEPCK protein level in db/db mice in comparison with control mice**. **(A)** RNA was extracted from saline and NRG-treated livers and analyzed by RT-PCR; **(B)** Liver total lysates were used to detect PEPCK levels by WB. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Next, we focused on transcription of genes, *Pgc1α, Pepck* and *G6pase*, which are essential for gluconeogenesis and at the same time are regulated by FoxO1. Data obtained from RT-PCR showed a discrete increase in mRNA levels of all three genes when comparing control and db/db

saline-treated groups, but the increase was significant only in case of the *Pepck* transcript (Fig. 59, A). *Pepck* transcription diminished under NRG action, being the unique significantly altered gene expression of the ones analyzed. However, no change during 30 minutes was observed in PEPCK protein levels (Fig. 59, B).

The other transcription factors that participate in gluconeogenic gene expression and which expression is regulated by insulin are C/EBPα and C/EBPβ. NRG, which uses multiple elements of classical insulin signaling pathway, significantly down-regulated the latter and had no influence on the former, which already had lower expression in db/db compared to control group. This data suggested that, despite the weaker signaling we observed throughout the pathway, NRG is still able to negatively alter transcription activity of important regulators of gluconeogenesis.

Pyruvate Carboxylase (PC) is the enzyme that provides oxaloacetate for PEPCK in order to drive carbons to the gluconeogenesis. In db/db mice we observed a small but significant elevation in overall mRNA level of PC compared to controls (Fig. 60, A). Similarly to non-diabetic mice, PC mRNA level was not altered with 30-minute NRG treatment.

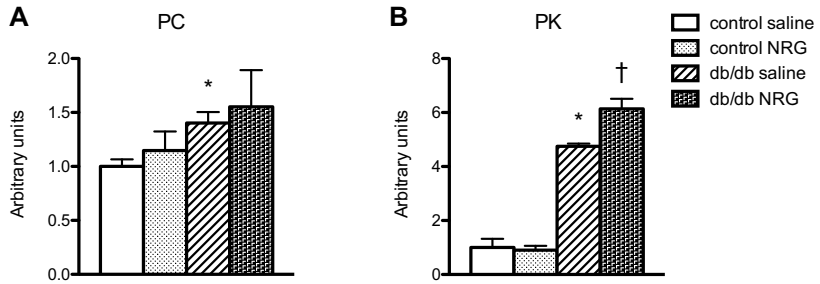


Figure 60. **Neuregulin influence on pyruvate carboxylase or liver-type pyruvate kinase transcription in db/db mice in comparison with control mice**. RNA was extracted from saline and NRG-treated livers and analyzed by RT-PCR to detect mRNA of (**A**) PC and (**B**) PK. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

Pyruvate kinase (PK) an enzyme that converts PEPCK product PEP to pyruvate, is reported to be elevated in livers of db/db mice (Chan et al., 1975). Being one of the proteins, which FoxO1 transcriptionally regulates and having in mind that NRG could be enhancing glucose utilization by improving the glycolytic pathway, it seemed important to measure PK mRNA level. As expected PK transcriptional activity was increased (4.7-fold) compared to control animals (Fig. 60, B). Contrarily to the lack of NRG influence on PK in control animals, levels of liver PK mRNA achieved an extra 30% elevation over the saline-treated level. This suggests that neuregulin enhanced even more the up-regulated glycolysis and therefore can explain partly the huge effect on reducing glycaemia during GTT in db/db mice.

Glucokinase, GK, is another of the glucose metabolism enzymes, which activity is up-regulated in db/db strain (Chan et al., 1975) and, likewise PK, its transcription is reduced with active FoxO1. Studies in control animals, showed that although mRNA of GK was increased with NRG 7-fold,

probably partly due to inducing inhibitory phosphorylation of FoxO1, the protein levels, surprisingly, showed strong trend of degradation. However, analysis of GK protein levels in db/db mice did not reveal any sign of possible degradation between saline- and NRG-treated groups (Fig. 61, A). Diabetic mice showed 5–fold higher levels of GK mRNA compared to control animals (Fig. 61, B). Acute neuregulin treatment raised already elevated level of GK mRNA by more than 70%. While in the control mice obtained results suggest that it might be operating a mechanism that degrades GK protein, this mechanism seemed to be impaired in livers of db/db mice.

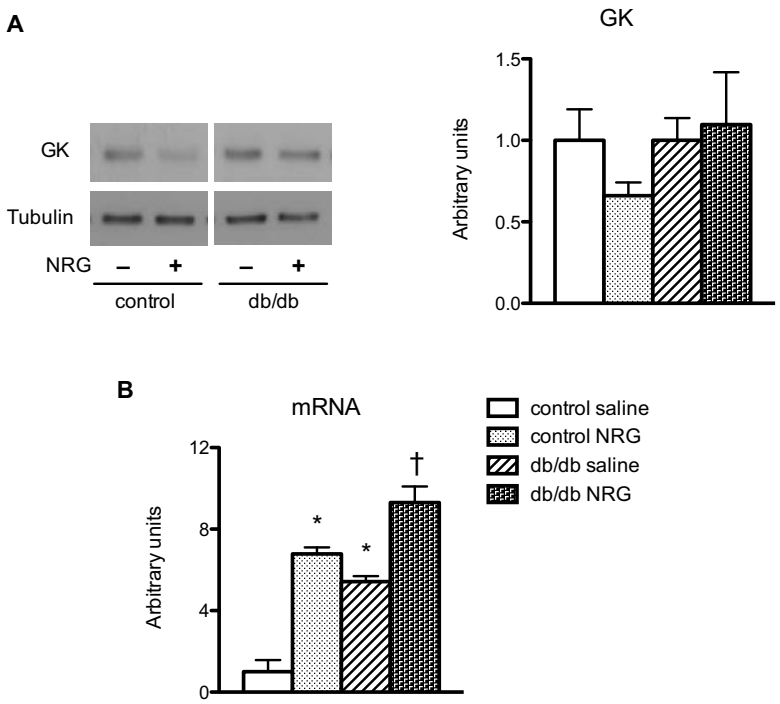


Figure 61. **Neuregulin influence on glucokinase protein level and transcription in db/db mice in comparison with control mice**. **(A)** Liver total lysates were used to detect GK protein. Tubulin was used as a loading control; **(B)** GK hepatic mRNA levels were determined from extracted RNA by RT-PCR. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

As mentioned above, IRS2 protein levels in db/db mice livers are remarkably reduced (Canettieri et al., 2005). While in livers of control animals we observed a 2-fold decrease in FoxO1 regulated transcription of IRS2 30 minutes after NRG injection, we questioned how it could affect it in animals where it is already greatly decreased. Our RT-PCR data showed that in db/db mice IRS2 mRNA is reduced by 65% compared to controls. NRG, however, was not able to reduce it further (Fig. 62). It suggested that *in vivo* NRG treatment was unable to enforce the reduction of the IRS2 transcription.

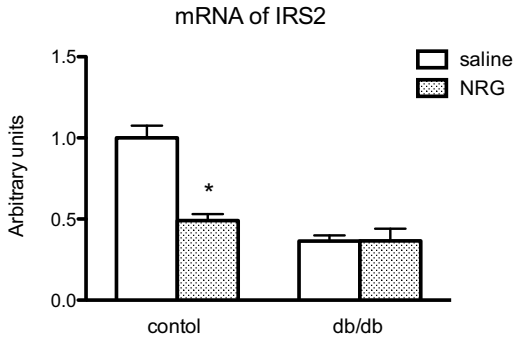


Figure 62. **Neuregulin influence on IRS2 transcription in db/db mice in comparison with control mice**. IRS2 hepatic mRNA levels were determined by RT-PCR. * indicates significant difference vs. saline group at P<0.05 determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

2.2.4.2. Effects on GSK3β and glycogen content.

Despite insulin resistance in db/db mice, glycogen synthase (GS) levels as well as hepatic glycogen synthesis is markedly increased (Chan et al., 1975). Non-phosphorylated and active GSK3β is a protein that suppresses GS activity by inhibitory phosphorylation, as previously mentioned. However, in db/db mice GSK3β levels were greatly reduced (Fig. 63, A), which suggested that there was less functional protein to inhibit increased levels of GS and therefore the hyperglycaemia established might be driving glucose to the liver for increasing storage as glycogen, meanwhile the pools are expandable. In this situation it is important to see how NRG impacted the situation. WB analysis reveals that an acute NRG treatment on db/db mice increased phosphorylation of GSK3β at Ser9 residue in a minor manner (2-fold) than in control mice (6-fold). Since GSK3β is a substrate for Akt, the reduction in Akt phosphorylation, and therefore in activity, in db/db mice is in accordance with the lower GSK3 phosphorylation. Interestingly, GSK3β showed a higher basal phosphorylation level in diabetic than in control mice, which was unrelated to the phosphorylated Akt level observed, therefore, other mechanisms, independent of Akt activity, might also be involved in the activation of GSK3β in diabetic mice.

To investigate whether neuregulin promoted glycogen synthesis, we measured glycogen content in the livers of saline- and NRG-treated db/db mice. In saline-treated diabetic animals, liver contained 6 times more glycogen than in control ones with the same treatment, which is expected (Fig. 63, B). Although neuregulin showed a tendency to increase glycogen content in db/db mice, at the time of treatment assayed (30 min after NRG administration) the differences did not reach significance. A reason for that may also lay on individual variability, what could not be solved due to the impossibility to measure glycogen content in liver in each mice before NRG administration. Nonetheless, this data supported the view that neuregulin contributed to enhance glycogen synthesis in db/db mice.

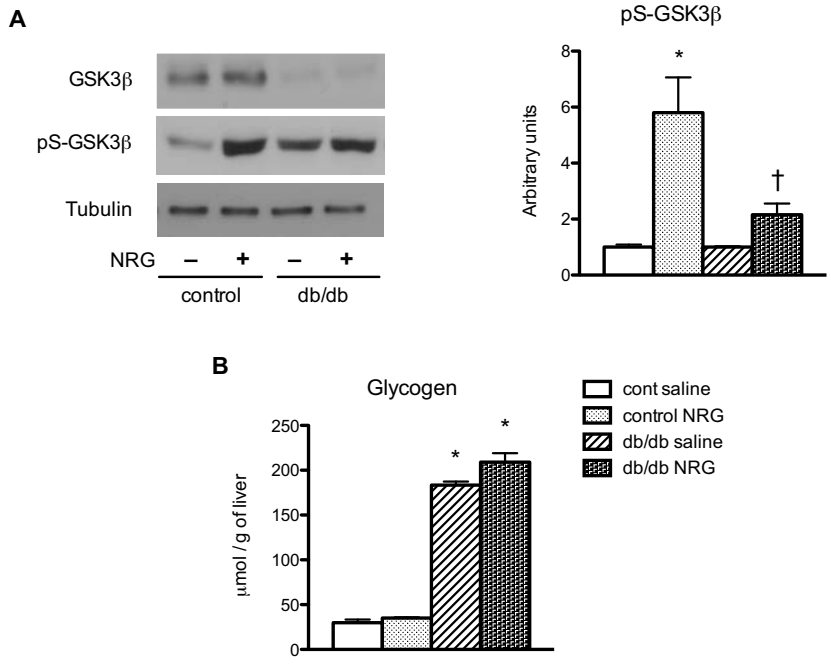


Figure 63. **Neuregulin influence on GSK3β phosphorylation and glycogen synthesis in db/db mice in comparison with control mice**. **(A)** Liver total lysates were used to detect GSK3β and its phosphorylation at Ser9 residue with WB. Detected phosphorylation was adjusted against total GSK3β. Tubulin was used as a loading control; **(B)** liver glycogen content was determined with a spectrophotometric assay. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

2.2.4.3. Effects on the mTORC1-p70S6K pathway: consequences on IRSs deactivation and AMPK phosphorylation.

Another kinase under Akt signaling that neuregulin showed to regulate in control animals is p70S6K, which is associated with serine phosphorylation of IRS proteins. Since neuregulin influence on weakening IRS1 as well as IRS2 interaction with PI3K was reduced in diabetic animals, it is possible to suspect that it might be caused by reduced activation of p70S6K. Therefore, we determined by WB assay the phosphorylation of p70S6K at Thr389 residue. In liver of db/db mice, there was a 41-folds increase in p70S6K phosphorylation by NRG treatment (Fig. 64). That was approximately two thirds of the NRG-induced phosphorylation seen in control mice what, again, it was in accordance with the reduced Akt phosphorylation observed in diabetic mice.

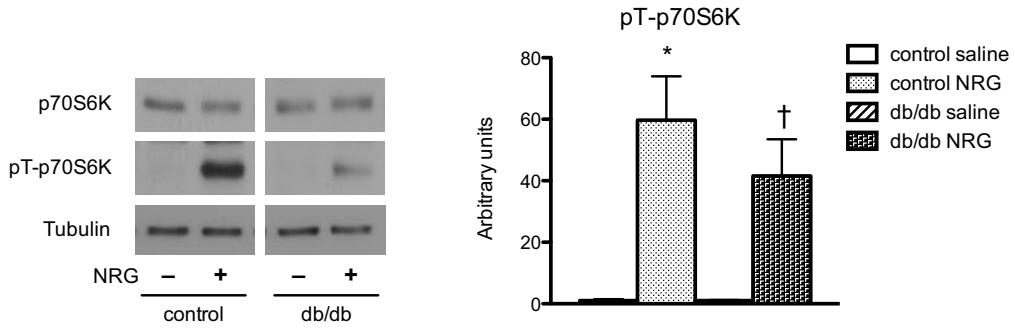


Figure 64. **Neuregulin influence on p70S6K phosphorylation in db/db mice in comparison with control mice**. Liver total lysates were used to detect p70S6K and its phosphorylation at Thr389 residue with WB. Detected phosphorylation was adjusted against total p70S6K. Tubulin was used as a loading control. * indicates significant difference vs. control saline group and † difference vs. db/db saline group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4 independent experiments.

3. Characterization of endogenous neuregulin-1 circadian rhythm in control, db/db and STZ-treated mouse models

Hepatic ErbB3 and by lesser extent ErbB1 have a circadian rhythm in their expression. In rats these receptors are up-regulated at the end of the resting or non-feeding period, before the transition from light to dark when animals become active and fed (Carver et al., 2002). Moreover, studies have also shown that insulin plays an important role in down-regulating ErbB3 (Carver et al., 1996, Carver et al., 1997), which is the hepatic neuregulin receptor. Taking this in account, we wandered whether also circulating neuregulin has an endogenous circadian rhythm and whether it is depends on insulinemia or glycaemia.

3.1. Characterization of blood metabolites during overnight monitoring

For understanding whether neuregulin has a circadian rhythm and if it depends on insulinemia, glycaemia or other metabolites in blood circulation we executed a 24-hour study. We collected blood samples from control, db/db and STZ-treated mice, three situations characterized by showing normoinsulinema, hyperinsulinemia and absence of circulating insulin, respectively. Samples were obtained from the tail after every three hours, starting from 9:00 am, when animals were fed and were entering to resting-fasting period. After first collection of blood samples the food was removed and kept away until obtaining the blood samples at 21:00 pm in order to make sure that all the animals went over the same rhythm in food intake and fasting period.

3.1.1. Circadian rhythm of circulating neuregulin

At first we analyzed neuregulin levels in plasma samples at different times throughout the day with a commercially available ELISA test to detect mice NRG1.
In control animals the starting NRG1 concentration ranged from 400-1100 pg/ml of plasma (Fig. 65, A). During the daily fasting period the levels slowly decreased and some animals had a slight increase in NRG release to bloodstream at 3:00 am, which is 6 hours after being re-fed.
NRG1 levels in db/db mice at 9:00 am in fed state were similar as observed in control animals ranging from 350-1100 pg/ml of plasma (Fig. 65, B). During the daytime fasting period NRG1 concentration in blood remained unaltered until 21:00 pm, when half of the animals showed an

increase in NRG1 levels. Returning to re-fed state lowered the concentration again, but turned into a new slow raise at 3:00 and 6:00 am.

Differently from control and db/db mice, changes in NRG1 concentration have clearer outlines in STZ-treated mice (Fig. 65, C). At 9:00 am, in fed state, neuregulin levels of insulin-deficient mice were in average remarkably higher than in the other two groups, ranging from 750 to 5300 pg/ml of plasma. After removal of food, the concentration sharply dropped comparable levels with other animal groups and remained there for 6 hours almost in all animals. At the last point before re-feeding at 21:00, the NRG1 concentration had increased remarkably to a range between 2300 and 5800 pg/ml of plasma. Re-feeding lowered the concentration by approximately same extent in next three hours, from where it slowly started to increase again. In STZ-treated mice, the overall neuregulin amount found in plasma was also remarkably higher, which is well observed when results are shown as AUC (Fig. 65, D). Obtained results suggested that this insulin-deficient model had greater control over body than in normo- or hyperinsulinemia and it also depended on animals feeding state.

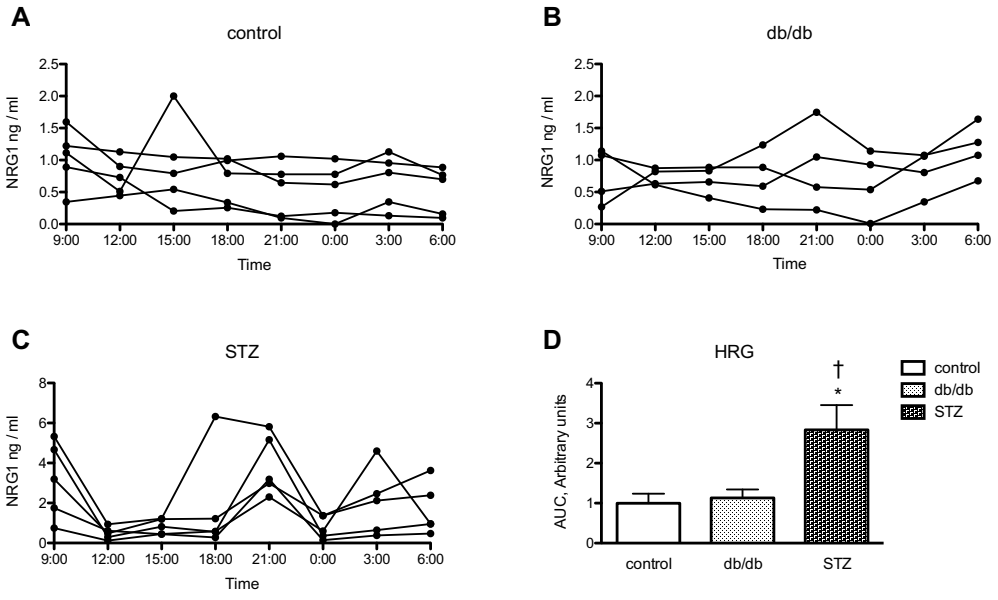


Figure 65. **Endogenous NRG1 levels during the 24-hour study**. Blood samples were collected from the tail from (**A**) control, (**B**) db/db and (**C**) STZ-treated mice in every 3 hours starting from 9:00 am. The samples were analyzed with commercially available ELISA. (**D**) AUC of neuregulin levels. * indicates significant difference of AUC vs. control group and † difference vs. db/db group at $P<0.05$ determined by Unpaired Student's t test. These images are representative of 4-5 independent experiments.

3.1.2. Insulinema during overnight monitoring

Carver *et al.* pointed out an insulin role in lowering ErbB3 expression in liver, but to date it has not been investigated whether insulin also regulates blood neuregulin concentration. To study

this question we measured mice insulin concentrations in control and db/db mice with another commercially available ELISA test for mice insulin to compare directly insulin and neuregulin1 level changes along the day. We skipped STZ-treated mice from this test due to the lack of insulin characteristic of this model.

As expected, insulin levels in control mice were remarkably lower than in db/db mice and both groups followed an expected pattern throughout the study (Fig. 66, A and B). In both groups, insulin decreased after taking away the food and increased again with re-feeding. The changes were more outstanding in diabetic animals. A light detected increase in db/db neuregulin concentration, which matches with a lowest measured insulinemia at 21:00 pm, suggests that lowering insulinemia up-regulates NRG production by the body. However, that kind of change is not observable in control animals, which suggest that it either happens only in insulin resistant situations or a bigger decrease in insulinemia is needed to up-regulate neuregulin concentration in plasma.

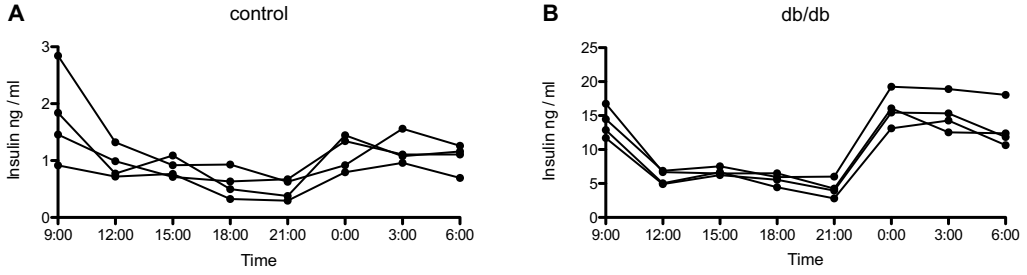


Figure 66. **Insulinemia during the 24-hour study**. Blood samples were collected from the tail from (**A**) control and (**B**) db/db mice in every 3 hours starting from 9:00 am. The samples were analyzed with commercially available ELISA. n=4.

3.1.3. Glycaemia during overnight monitoring

To investigate whether glycaemia affected neuregulin concentration in plasma we investigated the possible correlations between glucose and NRG1 levels during the overnight study. To do that, we initially we measured glycaemias in all collected samples. In control animals glucose levels stayed stable the first 6 hours (from 9:00 am to 15:00 pm) and reached its minimum just before re-feeding at 21:00 pm (Fig. 67, A). Thereafter, it raised and stayed higher until the end of experiment. In db/db mice, it was observed a reduction in glycaemia from noon to 21:00 pm and it increased 3 hours after re-feeding, changing to a slow decreasing trend later on (Fig. 67, B). In STZ-treated mice, however, a rapid decrease in glycaemia was observable already from the beginning of the study at 9:00 am and reached its lowest point by 21:00 pm (Fig. 67, C). Food availability increased blood glucose concentration of insulin-deficient mice, observed at midnight (00:00), by approximately 400-500 mg/dL, and later on it slowly decreased until the end of experiment. Comparing these patterns to the corresponding NRG1 curves we could see that the level of plasma neuregulin in db/db and STZ-treated mice was higher when glucose was at the

lowest level or when glucose started to lower after reaching its maximum at midnight. Moreover, a decrease in neuregulin occurred when glycaemia reached the highest level after re-feeding, but also kept low levels during first hours of fasting. However, it was difficult to observe a clear correlation between glucose and NRG1 levels in control animals. The results suggested that neuregulin concentrations in bloodstream may have a relationship with the animal metabolic situation and up-regulation could be a retarded response to a demand of energy supply. Alternatively, it may also reflect that neuregulin was primarily acting locally, and its release to the circulating blood could be a consequence of the lower requirement by these tissues, after their metabolic demands were fulfilled.

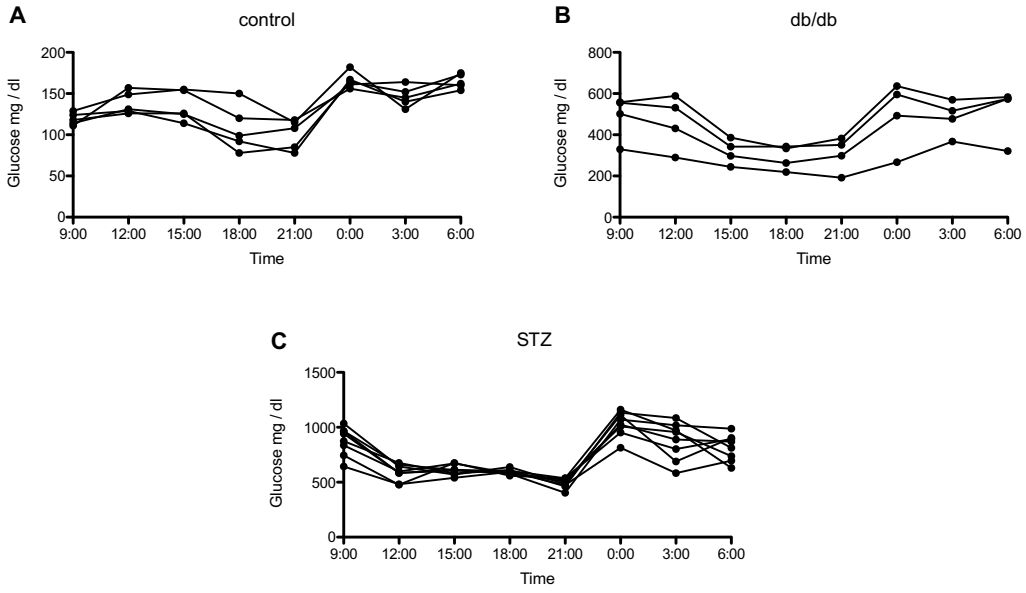


Figure 67. **Glycaemia during the 24-hour study**. Blood samples were collected from the tail from (**A**) control, (**B**) db/db and (**C**) STZ-treated mice in every 3 hours starting from 9:00 am. The samples were analyzed with commercially available Glucose Assay Reagent. n=4-8.

DISCUSSION

The prevalence of type II diabetes and other metabolic disorders, which already is taken an epidemic form, has predicted to grow in progressive rate in next 20 years, becoming one of the most serious risk factors to the health. This is well supported by the fact that only 1-2% of the cases of T2D are due to genetic mutations, while 98% of the incidences are related to nowadays poor lifestyle, which among other things stands out with increased caloric intake and decreased physical activity. A very characteristic and early sign of T2D is insulin resistance and, therefore, increased fasting blood glucose levels. Thus, it is necessary to understand the physiological mechanisms that cause the pathological situation and regulate energy metabolism to find therapeutical tools ameliorate what is known as *Metabolic Syndrome*.

Liver is one of the organs where metabolic functions become altered with T2D. Liver uses glucose as a fuel, has the ability to store it as glycogen and synthesize it from non-carbohydrate precursors. Gluconeogenesis, which normally becomes inhibited in postprandial condition, is still hyperactivated in insulin resistant liver, contributing in that way to elevated glucose level in fed as well as in fasted state. Besides that, patients with T2D often, but not always, have abnormal accumulation of glycogen and fatty acids in the liver, rendering hepatic steatosis, which contributes to the metabolic pathology. Despite accumulation of glycogen, glycogenolysis has not been shown to be increased in the liver of patients with type 2 diabetes (Consoli, et al., 1989).

Results obtained in the studies of this thesis project revealed that neuregulin had hypoglycemic effects in mice, as previously described in rats, although requiring double dose in mice for unknown reasons. Liver appeared as a strong neuregulin target tissue, which responded rapidly by inhibiting hepatic glucose production. FoxO1, a relevant transcription factor inducing gluconeogenic enzymes expression, was deactivated by neuregulin in an Akt-induced phosphorylation mechanism. Neuregulin and insulin signaling pathways used common steps, initiated with competition for PI3K by ErbB3 and IRS proteins, respectively. Moreover, insulin and neuregulin appeared to silentiate each other's signal. Previous reports indicated that insulin reduces binding of neuregulin to ErbB3 in cultured hepatocytes, whereas our results pointed out a mechanism by which neuregulin induced the mTORC1-p70S6K pathway, which, it is well known to impair insulin action through IRS serine phosphorylation. Somehow, liver appeared to manage discerning between insulin and neuregulin actions avoiding simultanious effects. When analyzing levels of neuregulin in plasma under different metabolic situations we could not observe a clear relationship between "neuregulinemia" in front to acute changes in glucose or insulin. A sustained hyperinsulinemia situation showed a lower level of neuregulin in plasma and the opposite situation was observed in the insulin-deficient model used. Many questions remained to be solved considering that neuregulin is a local growth factor and each tissue could be regulating both its own neuregulin and ErbBs receptors expression under different metabolic states.

1. **Neuregulin action on glycaemia and liver metabolism in control mice**

1.1. The relevance of working in basal conditions in the neuregulin action reducing glycaemia.

Starting from a piece of information that neuregulin lowers glycaemia during GTT (López-Soldado, manuscript in process), we soon realized that it also produced an effect in basal state without administrating glucose, and therefore, without inducing insulin release from pancreas. The fact that this effect in basal state was significant revealed that something else might lay under *in vivo* NRG action than just making the organism more glucose/insulin tolerant. Therefore one of the first challenges during this thesis was to understand what conditions were the best for investigating the neuregulin action *in vivo* – either it should be done in basal or absorptive state.
In absolute values, the effect what we saw on glycaemia in basal state was slightly smaller than in absorptive state. In absorptive state, body is forced to uptake and normalize the glucose levels, while in basal, especially fasted state, where glycaemia is already relatively low, liver, through increased HGP, manages to keep glucose concentration normal. In this situation, where in one state glucose mainly moves from blood to cells (absorptive) and in the other state the "in and out" of cell movements are rather balanced (basal), we can speculate that part of the glucose in basal fasted state is compensated at first by normally up-regulated HGP and, therefore, giving a less-extensive result under neuregulin action, what is accordance with our results. Despite of that, insulin is reported to be a factor that negatively affects NRG binding to ErbB receptor (Carver et al., 1996), as previously mentioned. Therefore, it suggested that the NRG signal through ErbB receptor should be weakened when insulin levels are elevated. In one way this information makes more difficult to understand the bigger effect observed during GTT, but we have to take into account that data about insulin disrupting NRG binding to its receptor originates from an *in vitro* cultured hepatocytes' assay, and it was never been tested *in vivo* preceding our results. We also have to take in consideration that other tissues may contribute as well. Skeletal muscle, for example, responds to neuregulin inducing glucose uptake in an insulin independent and additive manner according to *in vitro* muscle incubations (Suárez et al., 2001; Cantó et al. 2004), although, unpublised studies in our laboratory (López-Soldado, manuscript in process) indicated that *in vivo* NRG had minor, if any, effect on the activation of the Akt/AS160, which is one of the signaling pathways that drives to GLUT4 translocation to surface membranes and is needed to induce a rapid glucose uptake by the muscle. Considering the available data, a basal fasted state seems to be a good condition to investigate NRG effects, unaffected by the influence of the insulin presence, on the regulation of glycaemia and glucose metabolism.

Another reason to add the basal condition all along our studies was due to the proposed role of NRG as an inhibitor of HGP, which clearly came out from our results while administering pyruvate. Insulin has the same function in body and, therefore, can disrupt the clear understanding of how much of the effects were produced by NRG and how much by insulin.

1.2. Comparing fed and fasting situation.

Carver and colleagues have clearly shown that the two metabolically important hepatic ErbB receptors, ErbB1 and ErbB3, have circadian rhythms (Carver et al., 2002) and their expression is higher in fasted than in fed animals (Carver et al., 1997). Then, it is logical to expect that in fed state the neuregulin effect on glycaemia would be smaller than in fasted animals. With the GTT assays we could nicely prove it. The effect on glycaemia obtained in fasted state, was completely abolished in fed state. To this lack of effect in fed conditions could be contributing the higher concentration of insulin. Surprisingly, the effect did not change between fed and fasted state while we analyzed neuregulin influence on basal glycaemia. In studies on basal glycaemia, the insulin levels corresponding to a fed state should be lower than the ones generated as a consequence of a GTT assay, considering that the insulin itself has short half-life and during the 2-hour assay the mice remained without available food, although coming from a fed state. Besides, in fed state, insulinemia is already higher than in fasted state and due to the bolus of glucose it also stayed higher, probably difficulting NRG efficiency during the GTT assay.

While in fasted state NRG completely inhibited HGP, then in fed state, we saw an identical picture to NRG effect on basal fed state glycaemia, suggesting that no pyruvate was converted to glucose. It is expectable because in healthy organism gluconeogenesis is down-regulated with feeding. The result is also in correspondence with the fact that NRG lowers basal fed state glycaemia, showing that in both cases it affects glucose levels through the same mechanism.

From these results we can conclude that the departing state of the animal plays an important role on whether we were able to see a higher or lower neuregulin effect on glycaemia. As mentioned, this is not easily explainable only with the lowered number of receptors, but also may depend on other factors, such as availability of insulin in bloodstream. Comparing circadian concentration of neuregulin, insulin and glucose in plasma, it revealed that NRG in some conditions, such as insulin deficiency, developed a circadian pattern. It showed that neuregulin was released to the bloodstream at the end of the daily resting and fasting period when glycaemia is lowest and was abolished right after mice become feed. In STZ-treated mice it is known that ErbB3 expression in liver is up-regulated, but there is no information about if they follow similar circadian expression of ErbB3 receptors as in controls. Although, we may question it due to the lack of insulin, it should be proved with a study. However, we were not able to see this pattern of neuregulin release in normoinsulinemic control mice. The results rather suggested that neuregulin might become important when insulinemia and glycaemia were at the lowest levels, which at the same time, matches with higher expression of ErbBs in the liver. It raises questions, whether in insulin

deficiency neuregulin has a more important role in regulating glycaemia, while in control mice it depends on several factors. That all needs further investigation to clarify neuregulin physiological role in it.

1.3. Neuregulin and insulin - help each other or counteract?

Insulin is one of the factors that is proposed to negatively affect neuregulin signaling – it targets ErbB3 receptor for degradation and interrupts NRG binding to it. To better understand the interaction of insulin with the neuregulin signaling pathway, we may compare fasted state control animals with STZ-treated ones because both have high expression of ErbB3, but differ in insulin availability. Besides, in fasted state there is a provisional low insulinemia, whereas in insulin-deficient mice there is a permanent lack of insulin, which probably induces mice to be adapted to this condition. When analyzing in these two models signaling events taking place in liver, results revealed differences. For instance, in insulin-deficient mice neuregulin did not activate p70S6K and had smaller effects on Akt. It looked like insulin was enhancing neuregulin signaling, but the situation was not that simple. When neuregulin was binding to ErbB3, the activated receptor created an interaction with the PI3K regulatory subunit, p85. At the same time interactions between PI3K and IRS proteins were weakened significantly, which indicated that NRG inhibited insulin signal transmission through IRS1 and IRS2. We also have to take into account that ErbB3 has 6 binding sites for PI3K and therefore, constitutes one of the most potent recruiter of the PI3K while competing with IRS proteins. A possible pathway how neuregulin might affect the interaction is through activation of p70S6K and subsequent serine phosphorylation of IRS proteins (Ueno et al., 2005). In western blot assays we were able to observe a change in IRSs band mobility under neuregulin action. It has been reported that a slight reduction in IRSs mobility is observed when IRSs serine/threonine phosphorylation has increased (Harrington et al., 2004). While in the insulin action it is a manner to down-regulate its own signaling returning to a basal situation, then in the neuregulin action, it possibly is a manner to give priority to neuregulin action. In the insulin-deficient mice p70S6K was not activated, but despite of that, interaction between IRS2 and PI3K was still interrupted. The results suggest that there may be also another mechanism for regulating IRS2 interaction with PI3K. However, further studies with inhibitors of mTORC1/p70S6K pathway would be needed to light on this topic.

Another question rises about how p70S6K becomes activated. While in insulin deficiency neuregulin does not activate p70S6K, then in a system, where neuregulin counteracts to insulin signal transmission, somehow insulin presence under NRG action creates a situation where p70S6K is active. In this situation it seems like insulin enhances neuregulin signaling, or contrary, neuregulin enhances insulin signaling. The explanation can also lay in the developed metabolical differences in mice after STZ treatment. However, this situation as well needs more clarification.

Taken together, these results suggest that low levels of insulin do not represent a negative influence on neuregulin signaling in liver. In control animals the effect is rather more comprehensive than in insulin-deficient situation. Despite of that, we do not know how could the situation change with higher insulinemias.

1.4. Liver ErbB3 role in neuregulin effect on glycaemia

After *in vivo* neuregulin treatment liver ErbB3 became phosphorylated at intracellular tyrosine residues and therefore it initiated neuregulin signaling pathway. Because it is a kinase-dead receptor, it needs, in order to be activated, to dimerize with another ErbB receptor. ErbB3 heterodimerizes preferently with the coreceptor ErbB2, an orphan receptor with a potent tyrosine kinase activity (Tzahar et al., 1996; Graus Porta et al., 1997). In liver, ErbB2 expression decreases during differentiation reaching undetectable levels in adults (Carver et al., 2002). ErbB1, the EGF receptor, which also displays a ligand-inducible tyrosine kinase activity, is largely expressed in adult liver. Here we reported that ErbB1 may act as a coreceptor for ligand-bound ErbB3 receptors. In the presence of *in vivo* neuregulin, ErbB1 remained unphosphorylated; therefore, neuregulin acts exclusively through ErbB3 but the presence of ErbB1 is necessary for ErbB3 tyrosine phosphorylation. It is interesting to note that neuregulin action on metabolism appears to be signaling by ErbB3 or ErbB4 activation, whereas ErbB1 or ErbB2 act as coreceptors in order to phosphorylate the formers. For instance, neuregulin involvement in the muscle contraction-induced glucose uptake requires ErbB4 activation, which in the context of skeletal muscle, dimerizes with ErbB2 coreceptors (Cantó 2006). Moreover, ErbB3 and ErbB4 are mutually exclusionary in the skeletal muscle, being more abundant of ErbB3 at the undifferentiated state, whereas ErbB4 expression increases with differentiation and muscle contraction, at the time that ErbB2, although decreasing, remains expressed during the whole process (Cantó and Gumà, unpublished results). So, neuregulin targets tissues through different receptors, and therefore its actions could be also tissue-specifics rendering a much more complex view on the regulation of the metabolism by a single ligand, which, otherwise, acts locally, probably in response to signals generated by the own tissue.

The fact that neuregulin acted through ErbB3 is favored also by studies with ErbB3 blocking antibody, which showed that all the effects what we saw with neuregulin treatment during GTT were abolished whenever neuregulin could not bind to ErbB3. Moreover, the studies strongly supported that ErbB3 is the neuregulin receptor that mediates NRG control over glycaemia. These results also suggest that skeletal muscle hardly has any role in neuregulin-regulated control of whole-body glycaemia. The neuregulin binding receptor in skeletal muscle is ErbB4. While blocking ErbB3 already completely abolishes the effect on glycaemia, it is hard to see that ErbB4 mediated activation of pathways in muscle contributes to lowering of glucose levels. Even if *in vitro* experiments with muscle strips and in myocyte cell culture it has been shown that NRG induces glucose uptake (Cantó et al., 2004; Cantó et al., 2006), analyses of *in vivo* treated rat

skeletal muscle barely show differences in signaling cascade (López-Soldado, manuscript in process) and support the view that muscle contribution is minimal. Moreover, recent results of our laboratory obtained in muscle-specific conditional ErbB4 knockout mice showed a significant effect of neuregulin lowering glycaemia under GTT assay (Gumà, A. unpublished results) indicating that *in vivo* neuregulin had an scarce effect on muscle glucose utilization in resting fasting conditions, enforcing the view that the physiological role of neuregulin regulating muscle glucose uptake falls in the context of muscle contraction. At the time, a new view on a selective and tissue-specific neuregulin action on metabolism emerges from our results.

1.5. Neuregulin activates liver Akt, but not PKCζ

Acute neuregulin treatment triggered the activation of Akt in liver, which is an expected step after seeing remarkably increased binding of PI3K regulatory subunit to activated ErbB3 receptor. In L6E9 muscle cells, which express ErbB3 and ErbB2 neuregulin receptors, but not ErbB4, Akt is also phosphorylated under neuregulin action but only at the Thr308 residue, what it is targeted by PDK1. The Akt Ser473 residue, targeted by mTORC2, is not phosphorylated under neuregulin action and dominant negative models for Akt activation, overexpressing Akt with mutated phosphorylatable residues does not impact on the neuregulin action inducing glucose uptake in muscle cells (Cantó et al., 2004). Therefore, Akt is only partially activated in muscle cells and does not participate in the neuregulin action on glucose uptake. Contrarily, liver Akt is phosphorylated in both sites, in an ErbB3-dependent manner, under *in vivo* neuregulin action.

Previous studies with a muscle cell line and *in vitro* incubated rat muscle tissue revealed that neuregulin induced GLUT4 translocation and glucose uptake through a PI3K/PDK1/PKCζ pathway (Cantó et al., 2004; Cantó et al., 2006). When analyzing *in vivo* neuregulin effects on PKCζ we observed the previously described activation in skeletal muscle (López-Soldado, manuscript in process), but not in liver. These results again suggested that neuregulin action in skeletal muscle and liver was different, but, as mentioned previously, also the receptors expressed for neuregulin are different in these tissues and therefore could easily activate different signaling cascades.

1.6. Neuregulin as a hepatic glucose output inhibitor.

PTTs indicated that neuregulin was inhibiting HGP, which resulted in a severe hypoglycemic effect reaching the low glucose levels that were obtained by neuregulin action in fasted basal conditions (30 mg/dL or even lower). It was a sign that liver was unable to supply enough glucose to compensate the utilization in these fasting conditions, but also hepatic glucose

utilization was reduced according to the fall in glucokinase protein. This could be a compensatory mechanism to preserve glucose for other more glucose-dependent tissues and cells.

We also got a similar outcome from STZ-treated mice. Gluconeogenesis in these mice is highly up-regulated, which we could also observe in how much and quickly glycaemia rose under PTT. This could also be a consequence of the insulin lack, which should induce a higher glucose production. Despite of the higher conversion rate, neuregulin completely inhibited the HGP, proving that insulin presence did not have a role in this neuregulin action.

While looking the signaling cascade in liver 30 minutes after the neuregulin treatment, we could see that the main inducer of gluconeogenic genes transcription, FoxO1, was rapidly inhibited by phosphorylation, which is targeted by Akt that is highly activated in liver by neuregulin, as mentioned. When analyzing mRNA levels of gluconeogenic enzymes PEPCK and G6Pase but also the ones of the relevant co-activator PGC1α there was, in all the cases, a trend to decrease under neuregulin action. Probably allowing a longer time of neuregulin treatment the differences would be significant. But this raised another question: what is the fast mechanism induced by neuregulin to reduce the hepatic glucose production, since there is not enough time to change gluconeogenic enzymes expression? It may be that the answer is in the activity of these proteins, but it also can be that neuregulin induced an oxidative destination for pyruvate. The last was not the case, since during the PTT assay, lactate levels were increasing, indicating that pyruvate was just accumulating intracellularly and metabolized by lactate deshydrogenase rendering lactate to the circulation. Since the main consumer of pyruvate in fasting conditions is the liver, we assumed that neuregulin was indeed blocking gluconeogenic pathway, without altering the oxidative rates of pyruvate consumption. Therefore, an unknown short-term mechanism down-regulating gluconeogenesis must be induced by neuregulin in the liver.

1.7. Neuregulin as an inhibitor of hepatic glucose utilization

One of the neuregulin roles came to light when we were working with insulin-deficient mice. During the GTT assays, we observed a relevant difference between control and insulin-deficient mice, in how neuregulin behaved with whole-body glycaemia. Our results suggested that in STZ-treated mice neuregulin did not reduce glycaemia, as we observed in control mice, but prevented glucose to be absorbed remaining the levels higher until the end of the experiment. A rate-limiting step in hepatic glucose utilization is phosphorylation of glucose by glucokinase (Hornichter and Brown, 1969). While looking our data obtained from analysis of STZ-treated mice liver extracts we noticed that glucokinase protein was remarkably reduced after acute neuregulin treatment. Moreover, also glucokinase activity in the extract was reduced. While looking the protein and activity data together, the latter rather confirms that the extract contained less protein than the protein itself was less active. This could be due to the fact that glucokinase activity was referred to total protein content and not specifically to glucokinase

protein content, which as mentioned, decayed under neuregulin action. Glucokinase degradation was rapid and clearly observed in 30 minutes after neuregulin administration. Although neuregulin has similar, but not that extensive, influence on glucokinase also in healthy mice, we still see a decrease in glycaemia after glucose administration, which may be due to insulin presence. Insulin increases glucose uptake mainly in peripheral tissues, such as skeletal and cardiac muscle as well as white and brown adipose tissue, and up-regulates glucokinase in liver allowing a higher glucose utilization in this tissue in absorptive conditions. This situation raises a question what is the mechanism by what neuregulin causes the quick degradation of the protein and why the final influence on whole-body glycaemia is different in STZ-treated and control mice. Contrary to the glucokinase protein levels, neuregulin was inducing glucokinase mRNA in control mice but not in STZ-treated mice. Glucokinase transcription is known to be activated by insulin, which STZ mice simply lack. In wide view, NRG has shown to mimic insulin action in several steps of the signaling pathway, including the inhibition of FoxO1, which in active state is an inhibitor of glucokinase transcription. NRG action, however, has not proven to be enough to initiate glucokinase transcription in insulin-deficient mice despite the deactivation of FoxO1 by same extent as in controls. One possibility is that NRG may not control the activity of another transcription factor, SREBP-1c, that is involved in the insulin action on glucokinase transcription.
While one factor of greater decrease in glucokinase protein levels, in STZ-treated mice, could be that neuregulin was unable to up-regulate transcription, the other may be related to differences in activation of mTORC1/p70S6K pathway. Among other targets, activated mTORC1/p70S6K pathway leads to increased protein synthesis. Phosphorylation of p70S6K was triggered by neuregulin binding to ErbB3, since we impaired activation of p70S6K by use of blocking ErbB3 antibody. Our results showed that, differently from control mice, neuregulin was unable to activate this pathway in the insulin-deficient model. It suggested that also translation process may be affected in insulin-deficient situation and, therefore, resulting in greater glucokinase protein decrease. Nonetheless, the described results, however, raise questions whether insulin presence promotes transmission of neuregulin signal to some signaling elements and helps through that to keep glucokinase from falling drastically.
Above described results do not completely explain why do we see a trend for decrease in protein level even when there is more mRNA synthesized and p70S6K activated after NRG treatment in control mice. According to published data, glucokinase half-life is greater than 30 hours (Iynedjian et al., 1988; Niemeyer et al., 1974), which supports the fact that there has to be a mechanism that targets glucokinase for degradation under neuregulin action. To determine, what exactly is the mechanism and what is the physiological importance of degrading glucokinase at the same time with activated glycogenesis and inhibited gluconeogenesis needs a further investigation. However, based on our circadian data on plasma neuregulin, we can speculate that neuregulin may be involved in down-regulating metabolical processes after fasting avoiding liver energy depletion and, at the same time, keeping the glycaemia from falling too low.

At the light of these events, it would be also interesting to know how glucokinase is regulated with neuregulin action in β-cells, where it serves as a principal control for the secretion of insulin in response to rising levels of blood glucose. Our data, though, rejects that pancreas may be responding to *in vivo* neuregulin treatment since insulinemias remained unaltered during the 135 minutes of neuregulin action.

1.8. Neuregulin as activator of glycogenesis

Acute neuregulin treatment inactivated GSK3β by inducing its phosphorylation. This kinase is metabolically important to activate glycogen synthase and subsequently, glycogenesis. The fact that GSK3β was a target of ErbB3-mediated neuregulin signaling, was also proved by using ErbB3 blocking antibodies. Despite of that, liver glycogen content in NRG-treated mice livers did not increase significantly compared to saline-treated mice livers, probably due to the short term neuregulin action. 30 minutes is very short period to measure neuregulin-caused difference in total liver glycogen even when the animals were previously fasted and their liver glycogen content was decreased. Besides short time-interval, while glucose incorporation to glycogen could happen, a reason for not detecting a difference can also be that we were measuring a relative small change compared to the total glycogen amount. We do not know what was the liver glycogen content at the moment when neuregulin was administered. Differences in starting point could easily mask the effect and make it difficult to understand how much of it changed. Therefore, a radioactive method is better option, because it makes possible to measure *de novo* synthesis and gives an actual picture. Preliminary results, obtained when ending these studies, with radioactive glucose revealed that neuregulin induced glucose incorporation into glycogen (data not shown).

Why neuregulin was enhancing glycogen synthesis at the time that it was down-regulating glucokinase protein content and what would be the overall physiological consequences for liver metabolism remains to be determined. Although, we can say that most, but not all, described neuregulin effects in liver metabolism mimicked insulin action. It is well known that insulin, besides its own induced effects, also counteract the glucagon action. In this thesis we could not approach the interaction between glucagon and neuregulin action, which may be relevant to fully understand neuregulin effects in liver.

2. Neuregulin action on glycaemia and liver metabolism in type 2 diabetes.

2.1. Reduced ErbB receptors and increased endogenous neuregulin expression in db/db mice.

Carver and colleagues have shown that insulin is a factor that causes down-regulation of liver ErbB3 receptor expression in cultured primary hepatocytes as well as in healthy and STZ-treated insulin-deficient mice (Carver et al., 1996; Carver et al., 2002). They have also reported that the pathway is sensitive to PI3K inhibitors (Carver et al., 1997). However, no information is published about hepatic ErbB receptor expression in insulin resistant animal models. Our results showed clearly that both of the main receptors, ErbB1 and ErbB3, were remarkably reduced in type 2 diabetic db/db mice, by 66% and 52%, respectively. Moreover, in our laboratory, a similar result was also observed in type 2 diabetic ZDF rats (López-Soldado, manuscript in process). However, ErbB4 receptor, which we were able to detect in trace amounts, did not show changes at the expression level.

Endogenous hepatic neuregulin expression was up-regulated by one third. While neuregulin preferentially acts by an autocrine, paracrine or juxtacrine manner (Goodearl et al., 1995; Loeb et al., 1998), this situation suggested that liver was compensating decreases in ErbBs by increasing neuregulin expression in order to maintain its effects. Despite of that, while we are comparing NRG1 levels in blood circulation throughout the day, we do not see any significant differences between control and db/db mice. Therefore hepatic neuregulin expression did not impact on the overall neuregulin circulating amount. These results suggested that the same hepatic neuregulin amount has cleaved and released to bloodstream, independently of the expression level, or alternatively, hepatic neuregulin has relevance only locally. The importance of neuregulin in its local activity is a concept emerging from several of our exposed results.

2.2. Extensive effect on glycaemia

In db/db mice, neuregulin caused a more extensive effect reducing the hyperglycaemia, which is characteristic of this model, in both, basal and absorptive conditions. It is difficult to understand this neuregulin action in animals, which are hyperinsulinemic and have a decrease in hepatic ErbB3 protein levels – two conditions that would prevent neuregulin signal transmition in the

liver and subsequent effect on whole-body glycaemia. However, in db/db mice the activity of glycolytic enzymes, such as glucokinase and pyruvate kinase, is elevated (Chan et al., 1975) and at transcriptional level, neuregulin shows to up-regulate both of the enzymes in this mouse model. Whereas in control and STZ-treated mice, neuregulin-caused degradation of glucokinase protein, in db/db mice we were not able to see the degradation, which suggests that the unknown mechanism that causes it, is non-functional in diabetic mice. The unchanged glucokinase level, therefore, sets basis for improved glucose utilization when comparing control and db/db mice under neuregulin action. However, glucokinase activity would be also needed to support the data. These results may explain partly the greater effect on glycaemia, but not why the effect exists.

Neuregulin inhibited transcription factor FoxO1, which is one of the factors that hinders the expression of GK and PK, but this effect on FoxO1 is attenuated in db/db mice. The higher transcription levels of glycolytic limiting enzymes, in db/db mice, could induce higher glucose utilization by liver, when comparing with the control mice.

Up-regulated HGP and failure to suppress it in absorptive condition are commonly observed defects in insulin resistant state. Inhibiting it by neuregulin, as seen in control mice, may therefore account for part of the effect the growth factor had on basal glycaemia. However, the effect in absorptive conditions is more extensive than in basal condition, which means that at least the glucose that was utilized more than in basal condition has to be stored in some form or converted to another metabolite.

As in control mice, GSK3β was also inhibited after acute neuregulin treatment in db/db mice, although in a lower degree, as expected for the decay in the neuregulin signaling action, particularly in the Akt phosphorylation. Glycogen synthesis activity is already high in liver of db/db mice (Chan et al., 1975), which we could detect in our studies as a higher basal phosphorylation levels of GSK3β. Indeed, db/db mice showed a relevant increase in total liver glycogen content, and although neuregulin action showed a trend to increase total content, this was not significant. As argued before, studies of radioactive glucose incorporation to glycogen would be needed to ensure this neuregulin glycogenic effects.

In an absorptive state, lactacidemia increases independently of the neuregulin action and with no significant effects, although levels are slightly over the saline-treated group during a period of time (approx. 1 hour) of the GTT assay. At the same time there was an increase in triglyceridemia in NRG-treated group of db/db mice during the GTT, reaching significance after 1 hour of the glucose administration, suggesting that it is one of the destinations of the glucose that was utilized more than in basal state where no changes were detected. Although it has been described that db/db mice have increased fatty acid synthesis (Memon et al., 1994), it needs further investigation which is the neuregulin-stimulated mechanism that induces lipid synthesis in db/db mice – if that is by providing more substrate or by other regulatory effects on lipid metabolism. The lack of significant effects of neuregulin on triglyceridemia in control mice could also be reflecting the poor glucose utilization that the liver of db/db mice have.

Taken together, there can be several reasons for that more extensive lowering rate of glucose concentrations with acute neuregulin treatment. During next studies we should not forget that there might be also contribution from the other tissues, although at the light of results in control animals it is difficult to see it.

2.3. Neuregulin effects in fed versus fasted diabetic mice

As we observed differences between neuregulin action in fasted and fed state in control animals, we saw it also in db/db mice. While we have information about the connection of the hepatic ErbB3 expression circadian rhythm and insulin levels, there is no such information about hyperinsulinemic db/db mice, where ErbB3 was much less expressed. However, insulinemias have different values in fasted and fed state.

The effect between fasted and fed db/db mice was in some ways different of what we saw in healthy mice, but a lot of it can be due to the complications of an insulin resistant organism. One of the major differences what is widely known about T2D and what we can see also in our results is the inability of db/db mice to completely block gluconeogenesis in fed state. Neuregulin after administration of pyruvate inhibited the raise of glycaemia at least by the part that concerns to pyruvate conversion to glucose. We were unable to say it about the total fed state HGP, because of not knowing the basal state rate of gluconeogenesis or the part that comes from glycogenolysis. From the point of view that basal glycaemia lowers during the experiment regardless the treatment, supports the fact that HGP is rather inhibited while mice are fed, but could be up-regulated when to increase the level of substrates for gluconeogenesis. A presence of lowered HGP in fed state favors also the point that there practically is no extra decrease in basal glycaemia after neuregulin treatment. That, however, is different from fasted state, where the decreasing effect of neuregulin on glycaemia is tremendous. Moreover, also when comparing GTT in fasted and fed state, the remarkable decrease in fasted mice was diminished to much smaller one in fed diabetic mice. In that situation we have to take in account that in fasted insulin resistant organism HGP is up-regulated, and the result may originate from neuregulin-initiated inhibition of it.

These results suggested that neuregulin influences on the magnitude of whole-body glycaemia depending also in insulin resistant mice on the state of the animal, being diminished in fed state when insulinemia is higher. However, the effect in fed state db/db mice seemed to increase by the end of the experiment, which may be a caused by some lowering at the insulinemia during the test. Beside the fact that insulin decreases rapidly when food is taken away (approximately by 2/3 in 3 hours), the experiments in fasted state, basal and absorptive conditions, both showed that neuregulin kept insulinemia significantly lower and from rising. Therefore, after 2 hours from the beginning of the test the fed animals' condition may be closer to fasted ones and be more affected by neuregulin.

In hyperinsulinemia (insulin content around 12-17 ng/ml), such as the situation that characterizes the fed type 2 diabetic mice, neuregulin failed to complete its action. It suggested that neuregulin ability *in vivo* to bind to its receptor and activate the pathways impairs already below the insulin concentration of 50nM (290 ng/ml) that was used in an experiment that showed that insulin disrupts NRG binding to its receptor (Carver et al., 1996). However, insulin concentration at 15-17ng/ml constitutes already that high level, which a normal healthy organism does not reach. It suggested that with normal daily insulin physiological levels, including postprandial state, neuregulin should not have a difficulty to activate signaling pathways. Despite of that, we do not see an effect of neuregulin lowering the glycaemia in fed state after administration of glucose as we see in fasted state. It may be a combination of several things, among them: I) there is less neuregulin receptor expressed in liver; II) already higher and raising insulin activates signaling pathways and neuregulin is not able to either enhance or block it due presumable weaker signal; III) HGP, which neuregulin has shown to block, is already inhibited with feeding. All these sides need a further investigation to clearly understand why this situation the result is different.

2.4. Complications in inhibiting hepatic glucose output in db/db mice

Previous chapters have revealed that inhibition of HGP is one of the major processes through what neuregulin *in vivo* regulates glycaemia. However, in db/db mice there are alterations that disable this neuregulin task. Interestingly, we see a neuregulin-induced lowering in glucose levels after pyruvate injection by exactly the same extent as in basal state, suggesting that in both conditions neuregulin has an influence through the same mechanism of HGP. The fact that the pyruvate is incorporated into metabolic processes leading to glucose production in similar rate in saline and neuregulin-treated mice is supported by comparable plasma lactate concentrations throughout the experiment. No extensive increase as in control mice has been seen. Therefore, the data suggested that in insulin resistant situation neuregulin failed to inhibit gluconeogenesis, but it may have an inhibiting effect on the other component of HGP, which is glycogenolysis. Glycogenolysis in db/db mice is up-regulated (Chan et al., 1975), but we have seen, as described before, that neuregulin induced GSK3β phosphorylation and therefore deactivation, enhancing in that way glycogen synthesis. The basal rate of glycogen synthesis/degradation in the liver, which would be different in each of the metabolic situations studied, may contribute to the neuregulin effect that finally lead to reduction in glycaemia in db/db mice, probably by improving glucose utilization and metabolism, as mentioned above, either by storing or consuming this substrate. Further studies will be needed to complete the view, at the same time considering the presence of a clear neuregulin resistance in db/db mice.

Gluconeogenesis in db/db mice functions with no interruption despite neuregulin-caused deactivation of FoxO1. A sign of inhibited FoxO1 was the observation of significant decrease in *Pepck* mRNA levels. In addition, also transcription of other FoxO1-induced genes, *Pgc1α* and

G6pase, had a trend to reduce. However, no changes were detectable in PEPCK protein level. The importance of FoxO1 signal in causing the acute effects on gluconeogenesis is questionable, because the lack of effects on gluconeogenic enzymes was also seen in control mice, but contrary to db/db mice, gluconeogenesis in control mice was inhibited. Therefore, investigation of other short-term events/modulators is needed.

2.5. Decreased activation in the neuregulin signaling pathway

Despite the bigger influence on blood glucose levels that we see in basal and absorptive conditions compared to control animals, the neuregulin effect on signaling cascade elements is reduced almost in every measured step. It started with approximately 2-fold lower activation of its own receptor, ErbB3, which already is expressed in lower level. That enforced the concept that insulin may be a factor that disrupts NRG binding to ErbB3 even in situations (db/db mice) where there is more neuregulin per receptor molecules, when comparing with the control mice. The cascade follows understandably with lower binding to PI3K and almost 5-6-fold decrease in Akt phosphorylation per protein amount in the sample. This weaker induction in the neuregulin signaling pathway rendered a lower action on pathway effectors and in some cases, such as on glucokinase and gluconeogenesis, neuregulin failed to regulate it. This raises question whether the hyperinsulinemia or the adaption to an insulin resistance situation is the cause to the observed failure in neuregulin action in db/db mice? Although the neuregulin signaling in diabetic liver is disrupted, neuregulin administration in fasted state to db/db mice causes a strong lowering of insulinemia both in basal and absorptive conditions, suggesting that neuregulin actually gives a push to make the organism more insulin sensitive, probably by contributing to lower glycaemia. Moreover, if we look at the interactions between PI3K and IRS proteins, we see that in the case of IRS1, the binding did not decreased with neuregulin signaling. The latter, however, can be caused by reduced activation of signaling cascade, including p70S6K, which in turn has weaker influence on IRS1 serine phosphorylation and its interaction with PI3K.

3. Opening a new view in the consideration of neuregulin as a regulator of whole-body glycaemia.

3.1. Where do the carbons go?

Throughout the development of this thesis we have seen that neuregulin lowers glycaemia levels, but also that it blocks HGP and, interestingly, even that it does not let the glucose to be consumed that quickly. It suggests that there are several processes that function in parallels at the same time and what are affected by neuregulin. To describe the situation clearer we should know what is the destination of all these carbons originating from glucose and pyruvate.

While we are talking about glucose that was consumed quicker, then it can be stored as glycogen because neuregulin activates a signal for that through GSK3β. Moreover, as previously mentioned, the preliminary results with radiolabelled glucose also proved it. However, the excess glucose also can enter to glycolytic pathway and metabolized. This in light of our current results is difficult to see, because at least in plasma lactate and triglyceride levels of control animals no changes are observable. For unfold the actual situation it is relevant to perform experiments with glucose-C14 in order to check liver selected metabolic products, including CO_2-C14 for controlling oxidation, lactate-C14 for controlling glycolysis and FFA-C14 for controlling fatty acid synthesis.

In case of pyruvate, we know that neuregulin prevents it from converting to glucose and, therefore, at least part of it will be converted to lactate and released to bloodstream. However, the step in gluconeogenesis pathway that neuregulin inhibits is not that clear regarding our results. We do see inhibition of FoxO1, which regulates transcription of gluconeogenic genes, but a rapid effect in PTT suggests that there is something else than stopping protein synthesis, especially when in 30 minutes we do not see a significant down-regulation in the gluconeogenesis rate-limiting enzyme, PEPCK. To understand how neuregulin actually influences glucose production, it would be good to know at which step of gluconeogenesis pyruvate is rejected. It can be that it is simply not entering to mitochondria or it enters, but will be directed to some other pathway in one of the following steps. Methods, such as metabolomic profiling or measurements of enzyme activities, would benefit in this occasion.

3.2. Long-term effects

30 minutes after *in vivo* administration of neuregulin we see activation of signaling cascades, which also lead to changes in transcriptional level. However, these changes are rather small and would not cause the quick changes in glucose levels as we see with acute neuregulin treatment. Despite of that, it is possible that it affects metabolism in longer term. Based on mRNA data, there are signs that enzymes that are important for gluconeogenesis, such as PGC1α, PEPCK, G6Pase and C/EBPβ, as well as IRS2 will be down-regulated with longer exposure to neuregulin in healthy control mice. In case of diabetic mice, a similar trend was seen regarding the transcription of gluconeogenic genes, which suggests that even if we cannot inhibit gluconeogenesis with acute treatment, there may be help from chronic exposure to neuregulin. That, though, has to be tested, because as seen in case of glucokinase, the effect on protein might not be that directly connected to the influence on mRNA. To perform long-term studies on *in vivo* model, however, may become complicated due to neuregulin circulating half-life, which is reported to be rather short - 30 minutes to few hours (Chang et al., 2013). Cell cultures, at the same time, may give valuable results, but do not represent completely a physiology of a whole body.

Whether there will be other shifts in metabolic state of liver after a chronic neuregulin treatment, such as increased mitochondrial biogenesis and oxidative capacity seen in muscle cell culture (Cantó et al., 2007), needs a further investigation.

3.3. Neuregulin as a therapeutical tool

It is interesting to note that in our studies, although fasting the animals, neuregulin treatment did not induce an hypoglycemic shock that puts in risk the survival of the mice. In this condition, insulin is stronger and much more dangerous in the down-regulation of glycaemia, driving to death. In fact, this is one of the most problematic matter when insulin is required for therapeutical needs either in type I or in advanced type II diabetes. On the light of these findings, neuregulin could be a much more advisable for the hyperglycaemia treatment. But, we observed neuregulin-resistance in the type 2 diabetes model db/db, what let us to question whether the pathological situation of the diabetes is a consequence of a unique fail in the insulin action or it also requires failing in other regulatory factors as neuregulin. But again, why there is no hypoglycemic shock in fasting animals under neuregulin action? One hypothesis could be that *in vivo* neuregulin treatment may target not only the classical insulin responsive tissues, but also others such as the central nervous system. Neurons display ErbB4 and ErbB2 receptors. Somehow, administrated neuregulin may improve glucose utilization by neurons in a manner that at glycaemias of 20-30 mg/dL the animal can survive. Neuregulin plays multiple roles in central neuron system and defects in neuregulin are linked to the development of several

neuropathies, such as schizophrenia, bipolar disorders, depression and dementia (Corfas et al., 2004; Law et al., 2006; Hahn et al., 2006). Interestingly, an insulin resistance syndrome has been associated in some of these pathologies, such as schizophrenia. As a whole, we cannot discard an active role of neuregulin on the neuron, and probably glia, improving glucose uptake and utilization. In the future, studies will have to be addressed to analyze these possible neuregulin effects on energetic metabolism in central nervous system and its possible involvement, as a metabolic regulator, in the development of the neuropathy.

3.4. Physiological importance of neuregulin action regulating glycaemia

Neuregulin is a growth factor that is expressed by a variety of tissues and very few information has been reported regarding to its effects on metabolism. It is clear that ErbB3 expression depends on the metabolic state of animal and it can be easily changed with food intake. Therefore, ErbB3 receptor expression could be tightly connected to the control of glucose homeostasis in the tissues involved in this process.

While looking at the circadian data about endogenous neuregulin in circulation, it is difficult to observe major changes throughout the day in control and db/db mice, but very clearly it comes to a peak in insulin-deficient model. Neuregulin content, when no insulin is present, is highest before transition to active and feeding period of the day. It is the moment when blood glucose levels are the lowest and, in case of insulin presence, also insulinemia would be the lowest. Neuregulin disappears right after re-feeding. A little of similar effect is observable also in few of type 2 diabetic mice. It suggests that in moments when body senses hours of energy depletion, it activates neuregulin signaling and by sending a signal to degrade glucokinase, it blocks further liver glucose uptake to prevent from severe hypoglycaemia. It also supports the process by inhibiting transmition of insulin signal through IRS proteins. At the same time it inhibits HGP, which may indicate that pyruvate, instead of being converted to glucose, will be used up quicker in tricarboxylic acid cycle to produce ATP and supply the tissue with energy. A reason of why we do not see that clear fluctuations of endogenous neuregulin in control and db/db mice may be that in these metabolic conditions the organism is able to supply enough energy for longer period and there is no need for neuregulin to be up-regulated that fast. We also have to take in account that neuregulin usually acts in an autocrine, paracrine or juxtacrine manner, which may not be detected in analyzing plasma.

As a whole, the growth factor neuregulin had hypoglycemic effects in absorptive conditions by targeting liver metabolism reducing hepatic glucose production in an ErbB3-dependent manner. Neuregulin was competing with the insulin action by the use of common upstream steps. Although, in downstream events, somehow insulin and neuregulin may be cooperating, for clearer understanding, more studies will have to focus on this objective. A type 2 diabetic model showed liver neuregulin resistance due to lowering ErbB3 receptors and a weakness in downstream signaling cascade. Despite of this, neuregulin induced a profound decrease in the

hyperglycaemia. It raised the question whether a deficient insulin action allowed neuregulin to act in a more efficient manner, even having deficiencies in its own neuregulin signaling pathway, specially considering that liver could improve glucose uptake in this situation, instead of reducing glucose production. Alternatively, other tissues targeted by neuregulin may be contributing to down-regulate glycaemia by inducing glucose utilization, which should not necessarily be insulin-responsive tissue, since neuregulin receptors are expressed in a larger variety of them. The view of the physiological relevance of these neuregulin effects will have to be fulfilled in future studies, but also current investigation allows to hypothesize that neuregulin may be linked and sensing the energetic state of the tissue and acting in consequence regulating glucose disposal.

CONCLUSIONS

1. Neuregulin regulates glucose homeostasis in an acute manner. Within 30 minutes it activated signaling cascades in liver and affected whole-body glycaemia.

2. *In vivo* neuregulin treatment increased ErbB3 tyrosine phosphorylation in liver. It lead to increased ErbB3 interaction with PI3K regulatory subunit and activation of downstream signaling pathways, including Akt, FoxO1, GSK3β and p70S6K. At the same time neuregulin reduced PI3K interaction with IRS1 and IRS2 probably by increasing IRS1 and IRS2 serine phosphorylation

3. Acute neuregulin treatment caused reduction in hepatic glucokinase activity in control and insulin-deficient mice, probably due to targeting glucokinase for degradation.

4. Neuregulin inhibited hepatic glucose output. It not only completely blocked pyruvate conversion to glucose, but lowered glycaemia even more reaching levels that were comparable to what was seen in its effect on basal glycaemia, leading mice to severe hypoglycaemia.

5. Blocking ErbB3 receptor completely abolished neuregulin effect under glucose tolerance test. Upon neuregulin stimulation it prevented ErbB3 tyrosine phosphorylation and abrogated neuregulin effect on downstream signaling cascade, including Akt, FoxO1, GSK3β and p70S6K.

6. Hepatic ErbB1 and ErbB3 expression in type 2 diabetic db/db mice was remarkably reduced. Contrary to that, endogenous hepatic neuregulin protein expression was up-regulated.

7. Neuregulin caused weaker activation of signaling cascade in db/db mice compared to controls, but had greater effect on lowering whole-body glycaemia.

8. In db/db mice neuregulin failed to inhibit pyruvate conversion to glucose. Under pyruvate tolerance test neuregulin lowered glycaemia by same extent as under basal fasted condition.

9. Endogenous neuregulin concentration in plasma of insulin-deficient mice increased sharply by the end of daily resting and fasting period and decreased sharply after re-feeding. It coincided with a moment when animals usually were experiencing lowest glycaemia and insulinemias.

MATERIALS AND METHODS

1. Animal models

An animal model that was chosen for this thesis, is a house mouse, *Mus musculus*, strain C57BLKS/J. It is the most commonly used mouse strain in studies of T2D. Therefore our control group consisted of C57BLKS/J male mice with heterozygous $Lepr^{db}$ mutation (C57BLKS/J-db/+), which are respective controls to type 2 diabetic homozygous $Lepr^{db}$ mutation (C57BLKS/J-db/db). For insulin deficiency studies we chose male mice with C57BL/6J strain, which is estimated to have more than 70% of the genetic background of C57BLKS/J. All the mice were purchased from Harlan Ibérica S.A. The mice were maintained in the animal facility of Faculty of Biology in an environmentally controlled room with 12-hour light/dark cycle. The animals were supplied *ad libitum* with Teklad Global 18% Protein Rodent Diet (Harlan Laboratories) and water.

2. Manipulations with animals

2.1. Glucose tolerance test (GTT)

GTT was performed both in fasted and fed state at 9 o'clock in the morning taking in account circadian rhythm of hepatic ErbB3, which depends on time and feeding state of animal. For overnight fasting (14h), mice were placed in a special cage with ad libitum access to water. In the morning of experiment a 20% glucose solution in saline (0.9% NaCl) was prepared. Recombinant heregulin (445 μg/ml), which was diluted in saline, was prepared in the morning of experiment or kept at +4°C maximum for 2 days. The concentrations of both substances were prepared so that the injected volume would not cause pain for the animals and do not cause difficulties in diffusion. At time-point 0, NRG (50 ng/g of body weight) or respective volume of saline was administrated intraperitoneally, followed by intraperitoneal glucose injection (2 g/kg of body weight) 15 minutes later. Blood samples were collected from tail at different times (0, 15, 22.5, 30, 45, 75 and 135) for glucose, insulin, lactate and triglyceride measurements and placed to ice. Between sample collections mice were placed back to their cage. After taking the last sample mice were permitted an access to food. The samples were centrifuged as soon as possible with 3000g for 20 minutes at +4°C, plasma was collected and frozen at -20°C.

In GTT, where we wanted to measure ErbB3 importance in NRG caused effect, 20μg of ErbB3 blocking antibody (Thermo Scientific) in 100ul of saline was injected 15 before NRG to zone of liver. This test was terminated 45 minutes after NRG and 30 minutes after glucose injection with anesthesia to take liver samples for following analyses

2.2. NRG effect on basal glycaemia

Similarly to GTT, NRG effect on basal glycaemia was measured in overnight fasted (14h) or fed state at 9am. In the morning mice were administer intraperitoneally a 50 ng/g of body weight dose of NRG solution (445 μg/ml of saline) or respective volume of saline. Blood samples were collected at 0, 15, 22.5, 30, 45, 75 and 135 minutes after NRG treatment. The tubes were placed in the ice until centrifugation with 3000g for 20 minutes at +4°C. Plasma was removed and frozen at -20°C for later measurements of blood metabolites. Mice between sample collections were placed back to their cage and after the experiment were allowed *ad libitum* consumption of food.

2.3. Pyruvate tolerance test (PTT)

Pyruvate is one of the substrates for gluconeogenesis, which is mainly happening in liver. It is normally up-regulated in fasting state and down-regulated in fed state, which does not happen in case of diabetes. PTT is an experiment with what it easy to measure gluconeogenesis activity. PTT in our studies was performed either in overnight fasted (14h) or fed state at 9:00am. A 10% sodium pyruvate solution was prepared in saline prior the experiment. At the beginning of the experiment (time-point 0), mice were injected intraperitoneally 50 ng/g of body weight of NRG solution in saline (445 μg/ml), followed by intraperitoneal administration of sodium pyruvate (1 g/kg of body weight) 15 minutes later. Blood samples were collected from tail at different time-points (0, 15, 22.5, 30, 45, 75 and 135 minutes) and placed in ice until centrifugation with 3000g for 20 minutes at +4°C as soon as possible. Collected plasma was preserved at -20°C for later experiments. Mice stayed in their cages between sample collections and were allowed to an access to food.

2.4. *In vivo* treatment with NRG

In vivo model is the most physiological model and characterizes the best the real metabolic situation and signaling events in different tissues. To obtain this sort of information about signaling pathways an *in vivo* treatment is needed prior dissection of the tissue where the investigation will be performed. In our study, mice were fasted overnight for 14h and *in vivo* treatment was started at 9:00am to represent as much as possible the situation where other *in vivo* experiments were executed. Then, 50 ng/g of body weight NRG solution in saline (445 μg/ml) or respective volume of saline was injected intraperitoneally and the mice were placed back into their cage. 30 minutes later the animals were anesthetized with isofluorane.

2.5. Anesthesia and dissection of tissues

Previous to any chirurgic intervention, mice must be anesthetized to prevent any suffering or pain caused by manipulation Isofluorane is an inhalable anesthetic that works practically instantly and as long as animal is exposed to it. For anesthetization mice were placed at first in a chamber that isofluorane concentration is regulated by a specific system. When unconscious, the chamber was replaced with the mouthpiece of isofluorine system to continuously receive anesthetic and allow an access for chirurgic manipulations. Chirurgic procedures were started after it was made sure that all the animal reflexes are completely absent by pressing the feet. At first was removed liver for signaling studies followed by gastrocnemius, with adipose tissue, small intestine, heart, brown adipose tissue and brain for body composition characterization. All the tissues were instantly frozen in liquid nitrogen containing containers and preserved for following analyses at -80°C.

2.6. Streptozotocin (STZ) treatment

Streptozotocin (STZ) is chemical that is particularly toxic to pancreatic β-cells, which produce the insulin. Therefore, it is widely used in medical research to produce type 1 diabetic animal models. At maximum 20 minutes prior administration STZ was weighed and dissolved in 10mM citrate buffer, pH 4.5. Diabetes in 7-week old mice was induced by a daily intraperitoneal injection of 50 mg/kg of body weight of STZ for five consecutive days. During the next 2 weeks non-fasting glycaemia was monitored to secure the treatment efficiency. With developed hyperglycaemia mice were ready for other examinations.

2.7. Overnight monitoring

An overnight study was performed on groups of animals to see possible correlations between changes endogenous blood metabolites. The study was started at 9:00am with fed mice. In every three hours from 9am to 6am in the next morning, blood was collected from the tail of all the mice and placed into ice until centrifugation with 3000g for 20 minutes at +4°C. Separated plasma was preserved at -20°C until later metabolite analyses. After collecting the first blood sample, the food was removed for next 12 hours to have clearer and more synchronized results from a period when mice normally rest and fast. Food was returned at 9 o'clock in the evening after taking a blood sample. During all the times mice had *ad libitum* access to water.

3. Measurement of metabolites

3.1. Measurement of glycaemia

Glycaemia in this thesis was measured with two different methods depending on the animal model. In control animals, blood glucose levels were measured with automatic glucometer (Accu-Chek, Roche) directly from the tail blood before collecting a sample. In the other two models, db/db and STZ-treated mice, it was impossible to measure all the experiment points with the glucometer due to being over the range of the machine. Therefore, for these animal groups' samples a Glucose Assay Reagent (Sigma) was used. For determination 2μl of plasma sample was pipetted in duplicate into 96-well plate and 200 μl of glucose assay reagent was added. After 15-minute incubation on room temperature the absorption was measured with spectrophotometer at wavelength 340nm and concentration was calculated from the standard curve.

3.2. Measurement of insulinemias

In studies of glucose homeostasis it is important to know also circulating insulin concentration. In this thesis insulin levels were determined using Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem #90080). Supplier instructions were followed for determination of insulin concentration in 5 μl of preserved plasma

3.3. Measurement of blood lactate concentrations

Lactate levels were determined from previously separated and preserved plasma samples. For that a commercial kit from HORIBA ABX was used. 5 μl of plasma sample or lactate standard and 200μl of prepared reagent from the kit was plated in duplicates on 96-well plate and the covered plate was incubated for 5 minutes at 37°C. Therefore, absorbance was measured at two wavelengths – 546 nm for determining the signal and 660 nm for determining the baseline. For calculations the measurement at 660 nm was subtracted from the measurement at 546 nm and concentrations were calculated using the standard curve.

3.4. Measurement of blood triglycerides concentrations

To determine triglyceride in collected plasma a triglyceride measuring kit (BioSystems) was used. 5μl of sample or a standard from the kit was pipetted in duplicates to 96-well plate. 200 μl of reagent was added and the plate was left room temperature for 15 minutes. The absorbance of

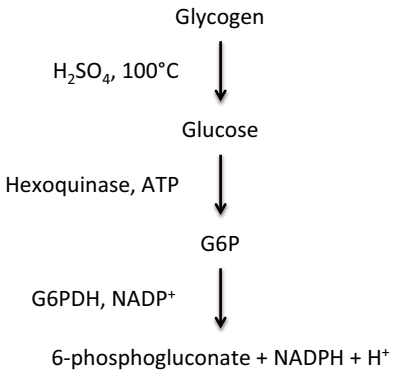
the formed product was measured with spectrophotometer at wavelength 500 nm. The concentration of each sample was calculated by using the standard curve of the same plate.

3.5. Measurement of endogenous NRG1 in blood

Neuregulin is relatively fragile protein that from gained experience is sensitive to temperature, storage and mixing. For mouse NRG1 detection it is recommended to have a fresh sample without long time storage to keep a change of protein degradation or denaturalization low because otherwise it may lead to wrong results. It is also suggested to avoid repeated freeze-thaw cycles. Endogenous NRG1 concentrations from plasma were determined with a commercial Mouse neuregulin-1 ELISA Kit (Cusabio) by following manufacturer-provided instructions. The used sample size was 30-40 μl to where Milli-Q water was added to have a total sample volume of 100 μl.

3.6. Glycogen determination in liver

Glycogen is one of the forms of energy storage in liver and in skeletal muscle. To understand completely animal metabolic profile it is necessary to measure also the amount of glycogen. In our studies hepatocytes seem to play a central role in glucose metabolism and therefore it was measured in previously obtained frozen liver samples with saline or NRG treatment. For that we used the classical method based on the spectrophotometry detection of NADPH + H^+:



Before performing a glycogen extraction and purification protocol the sample weight was marked down. For glycogen determination the sample size should be approximately 200 mg for control mice and 20 mg for db/db mice. The sample was placed into a glass tube and added 1ml of 30% KOH. The tube was placed into hot water bathe at 100°C until the tissue was digested (approximately 10min). Once the tubes were cooled down, 2 ml of ice-cold ethanol (99.5%) was added. The tube was covered with parafilm and left at -20°C for 24 hours. The next day the tubes

were centrifuged 15 minutes at 2000g (+4°C). The supernatant was removed and 1 ml of distilled water was added to dissolve pellet. After adding 2 ml of ice-cold ethanol the tube was centrifuged again at the same conditions and supernatant was removed. Once purified, 1 ml of 5N H_2SO_4 was added and the tube was placed into hot water bath at 100°C for 2 hours. After cooling to room temperature, few drops of pH indicator phenolphthalein was added and neutralized with NaOH solution (until pink color is barely noticeable). Once neutralized 250 μl of 0.2M KH2PO4/K2HPO4, pH 7.4, was added and the total volume was risen to 3 ml using distilled water. In that stage the sample is safe to store at -20°C until quantification, but can also be continued with quantification.

For quantification it is necessary to prepare a 1mM glucose stock. It will be for preparing a standard with concentrations between 0-50 nM. As a next step a glycogen measurement mix needs to be prepared.

GLYCOGEN MEASUREMENT MIX		
Tris-HCl 0.5M, pH 7.4		41.4ml
ATP 100mM	1mM	0.45ml
$NADP^+$ 1%	0.05%	2.25ml
$MgCl_2$ 0.5M	0.5mM	0.45ml
G6PDH 5mg/ml, dil 1:20		0.45ml

10 μl of standard or sample and 190 μl of glycogen measurement mix was plated in duplicates on 96-well plate. The absorption was measured by spectrophotometer at 340 nm with previous mixing of the plate. This first lecture represents a basal concentration of G6P in the sample. Next, 2 μl of hexokinase, which is an enzyme that converts glucose to G6P, was added, the plate was agitated and left at room temperature. 60 minutes and 90 minutes after adding hexokinase the absorption was measured again with spectrophotometer at 340 nm. The basal absorption value was subtracted from the maximum lecture obtained at 340nm, glycogen concentration was calculated using the standard curve and corrugated with the taken tissue weight.

4. General techniques of protein manipulation

4.1. Obtention of total lysates (normal+ripa)

A detergent supplemented buffer helps of to permeabilize cell membranes and solubilize the whole cellular protein content, including also membrane protein complexes. While detergents can also compromise protein-protein interactions or structural features that can affect posterior

detection it is important to choose a suitable detergent for lysis buffer. In this thesis 2 different lysis buffers were used – a regular lysis buffer for most of the proteins and a RIPA buffer for proteins, which need a more aggressive approach, such as the ErbB receptors. Regardless the type of lysis buffer, the protocol for obtention of total lysates was in both cases the same.

LYSIS BUFFER	
Stored at 4°C, pH 7.4	
Tris-HCl	50mM
NaCl	150mM
EDTA	1mM
$Na_4P_2O_7$	5mM
Na_3VO_4	1mM
NaF	50mM
NP-40	1% (v/v)
Add fresly to 100ml	
Protease inhibitors:	
Pepstatin A	2µM
Leupeptin	2µM
PMSF	1mM

RIPA BUFFER	
Stored at 4°C, pH 7.4	
Tris-HCl	50mM
NaCl	150mM
Na_3VO_4	1mM
NaF	1mM
Triton-X	1% (w/v)
SDS	0.1%(w/v)
DOC	0.5% (w/v)
Add fresly to 100ml	
Protease inhibitors:	
Pepstatin A	2µM
Leupeptin	2µM
PMSF	1mM

For obtaining total lysates from liver, an approximately 30mg piece of frozen tissue was placed in a tube with 1ml of lysis buffer. It was immediately homogenized using a Polytron for 2 times of 15 seconds. All these processes were performed using ice bath to maintain the lysis buffer and sample as cold as possible to avoid any possible degradation. Next, the sample was incubated for 1 hour at +4°C on a rotating wheel. After incubation the samples were centrifuged at 13000g for 15 minutes at +4°C and the protein-containing supernatant was transferred into ne tube so that the upper layer of fatty acids was avoided. When needed, centrifugation and collection of supernatant was repeated. The obtained sample was preserved at -20°C or used for needed analysis

4.2. Measurement of protein content

For following analyses of proteins we need to know the concentration in obtained total lysates. In this thesis protein quantification was performed using a commercial kit (Pierce) for bicynconytic acid (BCA) method. This method is based on the reduction of Cu^{2+} to Cu^{+}, which takes place when proteins react with Cu2+. Produced Cu+ together with BCA reagent will form a violet compound, which can be measured at 562 nm. For quantification 2 µl of total lysates or γ-globulin standard

was pipetted in duplicates to 96-well plate and 200 μl of BCA reagent was added. The covered plate was incubated at 37°C for 40 minutes absorption was measured in spectrophotometer. Protein concentration was calculated using the standard curve.

4.3. Immunoprecipitation

Immunoprecipitation is widely used procedure to isolate a specific protein or proteins groups together with interacting proteins from a heterogeneous sample. This technique is useful for example for studying interactions or concentrating the sample. As detergent type used in lysis buffer plays a critical role in how efficient immunoprecipitation will be, in this thesis a total lysate sample for this procedure was prepared with regular lysis buffer.

In immunoprecipitation, an antibody that recognizes a protein that we are interested in is non-covalently bound to protein-G, which in turn is attached to sepharose beads. That makes possible to separate antibody-linked proteins from the remaining homogenate.

For immunoprecipitation, 10-15 μl of G-protein linked sepharose beads (Sigma) were pipetted to an eppendorf tube and washed twice with 1 ml of ice-cold Tris-buffered saline (TBS). The tube was centrifuged at 3000g for 1 minute at +4°C after both washes and the upper phase was aspirated. After washing, 2-5 μl of antibody was added and the tubes were placed to rotating wheel to incubate for 45 minutes at +4°C. During that period the specific antibody attaches to protein-G. Next, the beads were washed twice with 1 ml of ice-cold lysis buffer, centrifuged at 3000g for 1 minute at +4°C after both washes and the upper phase was aspirated. This procedure is needed to remove the non-bound antibody, which otherwise would interact with the protein we are interested and washed out later together with the protein. After washing, a sample containing 0.5-2 mg of protein was added to the beads and the tube was returned to rotating wheel at +4°C, where it rotated overnight. During that period, the attached antibody creates interactions with the protein we are interested in. In the next morning the samples were centrifuged at 3000g for 1 minute at +4°C and the supernatant was removed. The beads were washed 3 times with 1 ml of regular lysis buffer and once with lysis buffer that did not contain detergent. The samples were centrifuged at the same conditions after each wash and the upper phase was aspirated. After last aspiration, 10-20 μl of 4 times concentrated loading buffer (4xLSB) with β-mercaptoethanol (950μl of 4xLSB with 50μl of β-mercaptoethanol) was added to the beads and the samples were placed to thermo-block for 5 minutes at 99°C. That causes denaturation and separation of the proteins from the beads. After heating the samples we let to cool followed by centrifugation at 13000g for 2 minutes. In that moment the liquid part of the sample is ready to load to acrylamide gel.

4.4. Western blot (WB)

Western blot is a quantitative analytical technique used to detect specific proteins or their post-translational modifications in a given sample. It consists of gel separation of the heterogeneous sample by length of the polypeptide (SDS-PAGE, sodium dodecylsulfate-polyacrylamid gel electrophoresis), immobilization to PVDF or nitrocellulose membrane and immunodetection with specific antibodies.

4.4.1. Sample preparation

A protein samples must be mixed with Laemmli Sample Buffer (LSB, loading buffer) to load into SDS-PAGE gel. It contains SDS, a detergent that gives a negative charge to the protein proportionally to their molecular weight. LSB is prepared as a 4-fold concentrated solution (4xLSB), but has to be added to the sample so that in final volume it is 1xLSB. LSB can be prepared including β-mercaptoethanol, which is used to denaturize the protein. In this thesis 50 μl of β-mercaptoethanol was added to 950 μl 4xLSB before adding the needed volume to sample. After adding LSB, the samples were placed to thermo-block at 99°C for 5 minutes to allow the protein to denaturize. Next, the samples were spinned down and loaded to polyacrylamide gel or stored at -20°C.

4x LAEMMLI SAMPLE BUFFER (4xLSB)	
H_2O	2,36ml
Tris-HCl 2M, pH 6.8	1ml
Glycerol	3,2ml
SDS	0,64g
Bromophenyl blue 0.05%	0,8ml

4.4.2. SDS-PAGE electrophoresis

SDS-PAGE is a technique that allows separation of proteins in a previously prepared sample in polyacrylamide gel based on the molecular weight while using electric field. The sampled proteins are covered with negatively charged SDS and move to positively charged electrode through pores in the gel.

A SDS-PAGE gel consists of two types of gels, which differ in their pH and percentage of acrylamide: the running gel and the stacking gel. In stacking gel, which has very low percentage of acrylamide (3%), proteins migrate fast and become concentrated before entering to running gel. In running gel, where the percentage of acrylamide is higher (7.5-12%), proteins will be

separated by their molecular weight. With varying the percentage of acrylamide in running gel it is possible to change the resolution of proteins, depending on what size of proteins are looked for. The higher the percentage of acrylamide the better the lower molecular weight proteins are separated, and contrary. To identify molecular weight of detected proteins and also to monitor electrophoresis progress a pre-stained molecular weight marker is used in one of the wells.
During this thesis, we used the Mini-Protean electrophoresis system (BioRad), which allows to work with relatively low sample quantities (10-80 µl) and easily manipulate the gels during the process.

RUNNING GEL 7.5% (10ml/gel)	
Distilled water	4.9ml
Tris-HCl 1.5M, pH 8.8	2.5ml
Acrylamide solution	2.5ml
SDS 10%	100µl
APS 10%	100µl
TEMED	10µl

RUNNING GEL 10% (10ml/gel)	
Distilled water	4ml
Tris-HCl 1.5M, pH 8.8	2.5ml
Acrylamide solution	3.35ml
SDS 10%	100µl
APS 10%	100µl
TEMED	10µl

RUNNING GEL 12% (10ml/gel)	
Distilled water	3.35ml
Tris-HCl 1.5M, pH 8.8	2.5ml
Acrylamide solution	4ml
SDS 10%	100µl
APS 10%	100µl
TEMED	10µl

STACKING GEL 3% (5ml/gel)	
Distilled water	3.1ml
Tris-HCl 1.5M, pH 8.8	1.25ml
Acrylamide solution	0.55ml
SDS 10%	50µl
APS 10%	50µl
TEMED	5µl

For making a gel, at first, glass-plate sandwiches are prepared and locked to the casting stand. It is followed by making the separating gel with desired percentage of acrylamide. For that, all the components in the recipe, besides TEMED and ammonium persulfate (APS), are mixed together. TEMED and APS are the gelling agents that trigger the crosslinking of acrylamide and, therefore, have to be added quickly before used for polymerization. After adding all the components and mixing to ensure homogenization, the running gel solution is poured into the glass-plate sandwich until it reaches 75% of the height of the small glass. A layer of water is added carefully on the top of the gel with a pipette to provide a barrier to oxygen that inhibits polymerization and to allow a formation of flat interface during polymerization. After the gel is polymerized, the water layer is removed and a teflon comb, which forms the wells, is inserted into the sandwich. Next, a stacking gel is prepared with again adding the TEMED and APS as last components. The

mixed-trough stacking gel solution is applied into the sandwich with a pipette so that no air bubbles would be trapped under the comb.

ELECTROPHORESIS BUFFER 10X (1L)	
Tris-Base	30g
Glycine	142g
SDS	10g

Once the stacking gel is polymerized the gel sandwich is attached to electrophoresis system. The inner chamber of the electrophoresis system is filled with electrophoresis buffer (1x) and monitored for possible leaks. If none is detected, the outer chamber is filled upto few centimeters over the lower edge of the gel sandwiches. The teflon combs were carefully removed. In that moment the system is ready for loading the prepared samples and standard with a 10μl pipette. After loading the samples, the electrophoresis chamber is closed and connected to power supply. During this thesis, the voltage was regulated to 170V and let run approximately 40-50 minutes until the frontline reached at the end of the gel or when desired molecular weight was satisfactorily separated. Then the electrophoresis is stopped and the glass sandwiches are removed from the system. The glass sandwiches are carefully opened, the gels are removed from glasses and the stacking gel is separated and discarded. In this point the gel can be used for transferring the proteins to a membrane or various staining methods.

4.4.3. Tranference

The next step of WB consist of transferring the proteins that were separated by SDS-PAGE from within the gel onto a synthetic PVDF or nitrocellulose membrane where in later step they can be visualized. In the development of this thesis, the proteins were transferred to PVDF membranes (Immobilon-P Transfer Millipore)

Before preparing separating gel from electrophoresis for transfer, it is necessary to complete few procedures. Transfer buffer should be prepared and one piece of PVDF membrane and two pieces of Whatman paper per each gel should be cut in a size of the gel. PVDF membrane, differently from nitrocellulose, needs to be "activated". For that it is soaked in methanol for 1 minute and then submerged into transfer buffer for hydration for another minute. Whatman paper is also submerged into transfer buffer before proceeding to preparing the sandwich for tranferene.

TRANSFERENCE BUFFER 10X (1L)	
Tris-Base	30.3g
Glycine	144g

TRANSFERENCE BUFFER 1X (1L)	
Transference buffer	100ml
Methanol	200ml
Distilled water	700ml

Building a sandwich for transfer is performed in a tray filled with transfer buffer to avoid air-bubbles to be trapped between the sandwich layers. Bubbles can cause defects in transfer and later in visualization of proteins. For building a sandwich an opened gel-holder with black side down is submerged in the transfer buffer in the tray. Next, the layers of sandwich are placed over the black side of the holder in the following order: one sponge, one Whatman paper, the gel, the membrane, one Whatman paper and another sponge. After placing the second Whatman paper, the sandwich is carefully rolled over with a plastic tube to eliminate that trapped bubbles. After finishing with layer the holder is closed and placed into the transfer cassette (Mini-Protean TransBlot Cell, BioRad) in the tank. The tank is filled with transfer buffer and a block of ice in plastic container is added into the tank to cool the buffer during the process. The tank is closed and placed in an ice bath for extra cooling from outside. Next, the system is connected to power supply and transfer is performed. In this thesis, the transfer process lasted 100 minutes at constant 250mA. These parameters can be modified because proteins with higher molecular weight are more difficult to transfer and it depends also on the quantity of protein. When the transfer process is finished the membranes are removed from the sandwiches. Before continuing with immunodetection the transfer efficiency and quality can be assessed with Ponceau S dye (Sigma). For that the membrane is submerged into Ponceau dye for few seconds and washed with TBS to remove the excess and visualize the proteins.

4.4.4. Immunodetection

Once the transferred proteins are transferred to the membrane surface, it is possible to detect the proteins under interest with a specific antibodies and measure their the quantity and size. The procedure consists of exposing the membrane at first to primary antibody, which specifically binds to protein, followed secondary antibody that recognizes the used primary antibody. The secondary antibody in turn is conjugated with an enzyme, horseradish peroxidase (HRP), which after reaction with ECL emits chemoluminicent and can be recorded.

Tris Buffered Saline (TBS) 10x (1L)		
pH 7.4		
Tris-HCl	500mM	78.8g
NaCl	1.5M	87.66g

TBS-T (1L)		
Distilled water		899ml
TBS 10x	1x	100ml
Tween-20	0.1%	1ml

BLOCKING BUFFER (100ml)/ ANTIBODY DILUTION BUFFER		
TBS-Tween		100ml
Skimmed milk or BSA	5%	5g

PHOSPHO-Y BLOCKING BUFFER	
Tris-HCl 0.2M, pH6	5ml
NaCl 1.5M	5ml
Tween-20	25µl
Brij	50µl
Ovoalbumin	0.5g
BSA	0.5g

Prior exposing the membrane to antibodies, all non-specific binding sites should be blocked with non-reactive proteins. For that a blocking buffer containing milk or BSA is used. The usage of BSA is needed when we want to detect phosphorylated proteins, because milk proteins in that case can non-specifically bind the antibody and, therefore, give a higher background in visualization process. In case the sample, in this thesis, was from immunoprecipitation with phospho-tyrosine, a special phospho-tyrosine blocking buffer was used. For blocking, the membrane is soaked in blocking buffer and placed on rocking platform for 1 hour at room temperature.

Next, the membrane is removed from blocking solution and placed in a falcon tube that contains dilution of the primary antibody, which is specific to each antibody. The primary antibodies used during this thesis and their dilutions are specified in Annex. For incubation the falcon tube with membrane and antibody is place overnight on rolling board at +4°C. Next, the membrane is removed from the tube and washed three times of 5 minutes with TBS-Tween to remove excess and non-specifically bound primary antibody. For that the, the membrane is submerged to a plastic pox containing TBS-Tween and placed on rocking board at room temperature. After washing the membrane is placed into another falcon tube containing dilution of secondary antibody. In this thesis, the used secondary antibodies at dilution 1:25000 were from Jackson: HRP-conjugated donkey anti-rabbit IgG and HRP-conjugated donkey anti-mouse IgG. Incubation with secondary antibody is performed on rolling board at room temperature for 1 hour. It is followed by three 10-minute washes with TBS-Tween as described above to remove excess secondary antibody. In this step the membrane is incubated with all the antibodies and ready for visualization with ECL reaction.

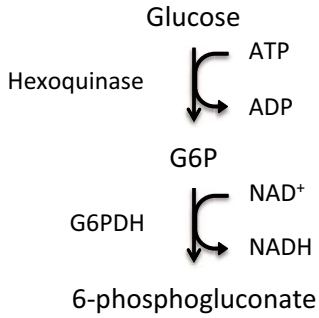
ECL1 (50ml)	
Tris-HCL 1M pH 8.5	5ml
Luminol 5M in DMSO	250µl
p-Coumaric Acid 79.2mM in DMSO	250µl
Upto 50ml with Milli-Q water	

ECL2 (50ml)	
Tris-HCL 1M pH 8.5	5ml
H2O2 8.8M (30% stock)	32µl
Upto 50ml with Milli-Q water	

In this thesis, a non-commercial ECL was used. For performing incubation with ECL the membrane is placed in the plastic box. 1ml of ECL1 and 1 ml of ECL2 are applied on the membrane, followed by slight agitation to mix the two components of ECL and to cover equally the membrane for even reaction. After approximately 1-minute reaction the membranes are gently tried with tissue to get rid of excess ECL and placed between the transparent films in cassette where the exposure to photographic films takes place. In a dark room, the photographic films (Fujifilm) are exposed to membranes for the desired times and processed with automatic revelator (Fujifilm FPM-100A). In this moment the film is ready to use for densitometric quantification. The membrane can be washed and incubate again with another antibody or preserved at +4°C.

4.5. Glucokinase activity assay

A spectrophotometric method is developed by determination of glucokinase or hexokinase activity in cellular extracts. The method detects a capacity of the sample to convert given glucose to G6P. Since G6P cannot be measured directly, it is oxidized further to 6-phosphogluconate with G6PDH that is added to a reaction mix. The latter reaction generates NADH, which is measurable with spectrophotometer at wavelength 340 nm. Knowing the scheme of the reaction and given that the enzymes are working in saturated conditions we can calculate how much G6P product per minute and per mg of protein is produced. By modifying the starting concentration of glucose we can determine the fraction of phosphorylation activity that is coming from glucokinase and the fraction of activity that comes from other hexokinase. This is due to differences in glucokinase and hexokinase affinity to glucose. When a starting concentration of glucose is 0.5mM we can only measure activities of hexokinase I, II and III, which have high affinity to glucose, but when glucose concentration is 100 mM, we measure activities of all hexokinases, including glucokinase. Therefore subtracting the activity obtained with 0.5 mM glucose from an activity obtained with 100mM glucose gives a fraction of activity that is coming from hexokinases that have low affinity to glucose and can be assigned to glucokinase.



For measuring glucokinase activity in tissues it is necessary to work with a fresh sample, which has not been frozen. In current thesis, 100 mg of liver was placed in 1ml of glucokinase buffer and homogenized using Polytron. The sample was transferred to ultracentrifuge tubes and centrifuged at 100000g for 30 min at +4°C. The supernatant was collected and protein concentration was measured using a BCA method.

GLUCOKINASE BUFFER	
Tris-HCl	50mM
KCl	100mM
EDTA	1mM
Saccarose	10%
β-mercaptoethanol	0.1mM

GLUCOKINASE ACTIVITY REACTION MIX			
	0mM glucose	**0.5mM glucose**	**100mM glucose**
Tris-HCl 1M, pH 7.4	1080µl	1080µl	1080µl
ATP/Mg 36/80mM	2160µl	2160µl	2160µl
NAD^+ 30mM	360µl	360µl	360µl
G6PDH 1000UI/ml	60µl	60µl	60µl
Glucose 1M	-	-	1080µl
Glucose 10mM	-	540µl	-
H_2O	5340µl	4800µl	4260µl

At this point the sample is ready for performing the glucokinase activity test with different concentrations of glucose: 0 mM that gives the background measurement; 0.5 mM that gives the activity of hexokinases I, II and III; and 100mM that gives the activity of all hexokinases. For that 15 µl sample, 35 µl distilled water and 250 µl of reaction mix is pipetted in duplicates to 96-well plate. The plate is placed immediately into spectrophotometer, which is set at 37°C. The release of NADH is measured at 340 nm after every 10 seconds for 20 minutes and the activity is calculated from the ascents of the curve. The result was normalized against protein concentration.

5. General techniques of RNA manipulation

Work with RNA requires utilization of sterile material, free of RNAses. RNAses are enzymes that degrade any kind of RNA. For eliminating the potential damage of RNAses all equipment should be autoclaved or left under UV radiation for some time, all the reactives must me RNAse free and

ultra-pure water should be used. It is necessary to work quickly, in cold conditions and always wear clean gloves.

5.1. Extraction and purification of RNA

RNA extraction is a widely used technique in molecular biology. It gives us an opportunity to compare expression levels of a concrete gene in different models or to monitor changes in expression patterns after a manipulation

During this thesis for RNA purification a combined procedure of Trizol (GilbcoBRL) and RNAeasy Kit columns (Invitrogen) was used. 50 mg of frozen liver sample was transferred to a 15 ml tube, containing 1ml of Trizol reagent and homogenized with Polytron. The homogenates were transferred to eppendorf tubes, 200 μl of chloroform was added and the tube was vigorously mixed. After 2-3-minute incubation at room temperature, it was centrifuged at 12000g for 15 minutes at +4°C. The upper aqueous phase was carefully collected avoiding any of the interphase and transferred to new eppendorf. Extraction with chloroform was repeated once more and the upper phase was transferred to new tube. After adding 500 μl of absolute ethanol, it was transferred to RNAeasy column and followed the manufacturers instructions provided with the kit. The samples were preserved in -80°C.

5.2. RNA quantification and quality control

At the end of the extraction and purification of RNA it is necessary to check the purity of the sample and also quantify the concentration of RNA. The both things can be measured from 1 μl of sample by Nanodrop spectrophotometer. From absorbance at 260 nm it calculates the concentration of RNA in ng/μl. For evaluating the quality and purity it gives two relative absorbances. The absorbance ratio between 260 nm and 280 nm (260/280) shows contamination of phenol and proteins and the absorbance ration between 260 nm and 230 nm (260/230) indicates contamination of Trizol, EDTA and other organic solvents. Both of the values should be higher than 2.0 to ensure the sample quality.

5.3. mRNA retrotranscription to cDNA

mRNA transcription levels can be measures with Real Time PCR (RT-PCR). For that it is needed to transform the extracted RNA into complementary DNA (cDNA). The process is known as retrotranscription and can be performed thanks to an enzyme retrotranscriptase polymerase. Before retrotranscriptase PCR reaction it is necessary to clean the sample from any possible

genomic DNA contamination that can happen when extracting RNA. In this thesis, this part was already included in the purification process of extracted RNA sample with the RNAeasy Kit columns.

SSII REACTION MIX	
Buffer 5x	4µl
DTT 0.2M	2µl
RNaseOUT	1µl
dNTP 10mM	1µl

The RNA was converted into cDNA using SuperScript II Reverse Transcriptase (SSII) system. Into each PCR tube was pipetted 2 µg of RNA, 1 µl of Oligo(dt) primer and ultra-pure RNAse-free water upto 11 µl. The tubes were incubated at 65°C for 5 minutes. The tubes were cooled in ice and 8 µl of reaction mix was added. The tubes were incubated at 42°C for 2 minutes in PCR machine. Next, directly in the heating block, 1 µl of SSII was added to each tube and the incubation was continued at 42°C for 50 minutes. After that, the temperature was quickly changed to 70°C for 15 minutes to stop retrotranscription reaction. In that moment all the tubes contain 2 µg of cDNA and be diluted to desired concentration. The samples are stores at -20°C.

5.4. Real-Time PCR

RT-PCR is a technique that allows to trace amplification of specific cDNA through a fluorescent signal produced while new DNA is synthesized. While real-time PCR can approximate an absolute quantification of a certain mRNA in samples, in our experiments we always performed relative quantifications against a housekeeping gene (ARP) whose expression is not altered by the experimental conditions.

In this thesis two methods for RT-PCR, Sybr Green and TaqMan, was used. In Sybr Green method, where the reaction is initiated by previously designed primers, the detectable a fluorescent compound intercalates with double-strand DNA. With the product accumulation the signal intensity increases and is, therefore, quantifiable. However, a fluorescence coming from non-specific PCR products or formation of primer dimers is a problematic point of this method. For TaqMan method the probes are designed by Applied Biosystems. The specific oligonucleotides for detecting desired genes in these probes are coupled to a fluorophore, which is activated with exonuclease activity of Taq DNA polymerase while they are broken down during elongation step of the temperature cycle.

For both of the methods, the RT-PCR mixtures are commercial, containing already the buffer, the nucleotides and Taq polymerase. There is only need to add the primers, the synthesized cDNA

and Milli-Q water to reach desired volume. The reaction mixture is plated on a RT-PCR plate and sealed with a plastic adhesive. The plate is centrifuged to collect the sample on the bottom of the well and placed into a thermocycler.

SybrGreen RT-PCR	
cDNA 2ng/µl	5µl
primer forward 10µM	0.48µl
primer reverse 10µM	0.48µl
SybrGreen Mix	8µl
H_2O Milli-Q	2.04µl

TaqMan RT-PCR	
cDNA 2ng/µl	5µl
TaqMan probe 20x	0.8µl
TaqMan Universal® PCR Master Mix 2x	8µl
H_2O Milli-Q	1.2µl

5.4.1. Real-time PCR primers for Sybr Green method

Abbreviation	Name	Primers
ARP	ribosomal protein, large, P0	AAGCGCGTCCTGGCATTGTCT CCGCAGGGGCAGCAGTGGT
C/EBPα	CCAAT/enhancer binding protein alpha	CGCAAGAGCCGAGATAAAGC CGGTCATTGTCACTGGTCAACT
GK	glucokinase	CCCTGAGTGGCTTACAGTTC ACGGATGTGGAGTGTTGAAGC
Irs2	insulin receptor substrate 2	TCCAGAACGGCCTCAACTAT AGTGATGGGACAGGAAGTCG
PK	liver pyruvate kinase	CTGGAACACCTCTGCCTTCTG CACAATTTCCACCTCCGACTC
PEPCK	phosphoenolpyruvate carboxykinase 1	GTGCTGGAGTGGATGTTCGG CTGGCTGATTCTCTGTTTCAGG
PGC1α	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	GAAAGGGCCAAACAGAGAGA GTAAATCACACGGCGCTCTT

5.4.2. Real time PCR probes for Taqman method

Abbreviation	Name	ID number (Invitrogen)
C/EBPβ	CCAAT/enhancer binding protein, beta	Mm00843434_s1
FoxO1	forkhead box O1	Mm00490671_m1
G6Pase	Glucose-6-phosphatase	Mm00839363_m1

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RESUM EN CATALÀ

Introducció

La diabetis i la regulació de l'homeòstasi de la glucosa

La prevalença de la diabetis està creixent a nivell internacional amb gran rapidesa. Segons la Federació Internacional de Diabetis (http://www.idf.org) 366 milions de persones tenien diabetis en el 2011 i s'espera que augmenti a 552 milions en 2030. La diabetis tipus 2 (T2D, també coneguda com a diabetis no dependent d'insulina o diabetis de l'adult) representa almenys el 90% de tots els casos de diabetis i sovint es creu que està associada amb el creixement de l'obesitat, el sedentarisme i la l'envelliment de la població mundial (Smyth i Heron, 2006). Tradicionalment considerada una malaltia d'adults, la diabetis de tipus 2 es diagnostica cada cop més en els nens en paral·lel amb l'augment de les taxes d'obesitat. L'obesitat condueix a la resistència a la insulina, el primer efecte detectable de la T2D, en els teixits perifèrics, a causa d'una alterada expressió o secreció d'adipoquines i de la infiltració de macròfags que, posteriorment, promou l'aparició de la diabetis tipus 2, hiperlipidèmia i hipertensió i, finalment, l'aterosclerosi (Despres, et al., 2006). La patogènesi d'aquesta malaltia multifactorial inclou anomalies que afecten a l'acció i secreció de la insulina (Saltiel, 2001)

Regulació del metabolisme de la glucosa per la insulina

L'homeòstasi de la glucosa és un procés finament controlat en el que es regula la producció de glucosa i la captació per diversos òrgans. En condicions fisiològiques els nivells de glucosa en sang es mantenen dins d'un rang estret, en part per l'acció de les hormones, que estimulen o inhibeixen la producció de glucosa (Cherrington, 1999) i regulen la captació de glucosa pels teixits perifèrics (Ferrannini et al, 1965;. DeFronzo et al. , 1985). L'increment dels nivells de glucosa a la sang és detectada ràpidament per les cèl·lules β pancreàtiques. Aquests sensors metabòlics arquetípics responen a aquest increment de glucosa mitjançant la secreció d'insulina o augmentant la producció d'insulina a través d'una major taxa proliferativa (Kousteni, 2012).

El múscul esquelètic regula el metabolisme energètic, contribuint amb més del 30% a la taxa metabòlica en repòs i amb el 80% de la captació de glucosa sanguínia en condicions absortives (de Lange, 2007), mentre que els adipòcits ajusten l'homeòstasi energètica ja sigui a través de la secreció de citoquines que controlen la gana i la sensibilitat a la insulina o emmagatzemant els excedents energètics en forma de triglicèrids a fi de mobilitzar-los per a la seva oxidació durant els estats de baixada energètica (Kousteni, 2012).

A més de l'acció de la insulina induint la captació de glucosa a múscul i adipòcits, aquesta hormona regula moltes altres vies que inclouen l'estimulació de la síntesi de proteïnes al múscul i al fetge, la síntesi de lípids i el seu emmagatzemament al fetge i al teixit adipós, la glucòlisi i

l'emmagatzemament de la glucosa al múscul, fetge i adipòcits, i la inhibició de la cetogènesis i de la gluconeogènesis al fetge i al ronyó (Farese et al. 2005; Shaham et al. 2008).

La insulina s'uneix al receptor de la insulina (IR), el qual s'autofosforila a residus específics de tirosina. Aquest fet condueix a la creació de llocs específics de reconeixement per part del substrat del receptor de la insulina (IRS)1 i IRS2. La unió dels IRSs al IR indueix la fosforilació en tirosines d'aquests darrers (White, 2002) que condueix al reclutament de molècules adaptadores, com la subunitat p85 reguladora de les PI3K de classe 1 (Fruman et al. 1998; Backer et al., 1992). La PI3K de classe 1 és una quinasa de lípids que en acostar-se a la membrana genera un segon missatger, el fosfatidilinositol-3,4,5-trifosfat (PIP_3). Increments en el contingut de PIP_3 a la membrana permet el reclutament de la Akt (o Proteïna Quinasa B, PKB), a on serà fosforilada per la proteïna quinasa dependent de fosfoinosítids 1 (PDK1) (Alessi et al., 1997; Stokoe et al., 1997) i per el mTOR complexe 2 (mTORC2, també conegut com a PDK2) (Sarbassov et al. 2005). Un cop activada, la Akt fosforila i modula un número important de proteïnes reguladores, resultant en la inhibició de l'apoptosi, l'estimulació de la divisió cel·lular i l'estimulació de la captació i emmagatzemament de la glucosa (Mora et al., 2004).

El fetge manté els nivells de glucosa a la sang a través de la combinació de dos processos, la producció de glucosa hepàtica (HGP): (1) gluconeogènesi - la generació de la glucosa a partir de substrats no carbohidrats (glicerol, lactat, d'alanina), i (2) glicogenòlisi - la degradació de glicogen. Inicialment, la glucosa que es produeix resulta de la descomposició de les reserves de glicogen hepàtic (glicogenòlisi), mentre que amb el dejuni prolongat, la gluconeogènesi és la font primària de glucosa (Gross et al., 2008). La gluconeogènesi resulta inhibida per la supressió de la transcripció del enzims clau d'aquesta via, fosfoenolpiruvat carboxiquinasa (PEPCK) i la glucosa-6-fosfatasa (G6Pasa) que esdevé quan els nivells d'insulina a la sang augmenten. Tanmateix, en els pacients amb diabetes de tipus 2, la producció hepatica de glucosa és un procés fisiològic clau que es veu alterat (Bogardus et al, 1984) a causa de la hiperactivació del factor transcripcional FoxO1. La resistència a la insulina també s'acompanya d'un augment de l'activitat S6K1 (Um et al, 2004; .. Tremblay et al, 2005), que té una influència inhibidora sobre la senyalització de la insulina a través de la fosforilació en serines de les proteïnes IRS (Um et al., 2004; Tremblay et al., 2007; Bae et al., 2007) i, per tant, redueix les respostes metabòliques a l'estimulació aguda per insulina a causa de la disminució de la interacció IR-IRS (Kanety et al., 1995;. Hotamisligil et al, 1996;. Pau et al, 1997).

La neuregulina i els seus receptors

La neuregulina (NRG) forma part d'una complexa família de proteïnes que estructuralment pertany a la família del factor de creixement epidermal, EGF. Totes les isoformes bioactives tenen un domini *EGF* que els hi és característic i que les distingeix de la resta de la família EGF. La neuregulina s'expressa predominantment en els teixits parenquimals i en el sistema nerviós

central i perifèric (Peles i Yarden, 1993; Carraway i Burden, 1995) i té un paper en la regulació de la proliferació, la diferenciació, la supervivència, el metabolisme i la migració de les cèl·lules.

La neuregulina és el lligand per als receptors ErbB3 i ErbB4. El domini *EGF* de la neuregulina, el qual és suficient per a l'activació del receptor, s'escindeix proteolíticament del domini transmembrana per l'acció de les metal·loproteases ADAM17/TACE i ADAM19/meltrin-β (Montero et al, 2000; Shirakabe et al, 2001) i s'uneix als seus receptors generant accions autocrines, paracrines o yuxtacrines (Goodearl et al, 1995; Loeb et al, 1998).

ErbB3 i ErbB4 pertanyen a la familia ErbB (també anomenada HER per a les isoformes humanes) que és un subgrup de la super-familia de receptors tirosina quinasa i consta de quatre isoformes: ErbB1 -4. Cadascun dels receptors conté un gran domini extracel·lular d'unió al lligand. La part intracel·lular de la proteïna consisteix en un domini tirosina quinasa bilobulat i una cua carboxi-terminal que conté les tirosines fosforilables. Entre els quatre membres de la família de receptors ErbB, ErbB2 i ErbB3 són excepcionals, ja que el primer no té lligant conegut (Klapper et al, 1999; Karunagaran et al, 1996) i el segon no te activa la quinasa (Guy et al., 1994; Pinkas-Kramarski et al., 1996b). L'activació dels receptors ErbB requereix de la formació d'homo- o heterodimers, activada per la unió del lligand al receptor (Schreiber et al, 1983;. Ushiro i Cohen, 1980), i la posterior transfosforilació de residus de tirosina intracel·lulars. L'acció de lligands específics als diversos complexes de receptors, determina quines proteïnes proteïnes de senyalització són reclutades i quines vies son activades. Una d'aquestes proteïnes és la PI3K, que la neuregulina requereix, per exemple, per a induïr la captació de glucosa en cèl·lules i teixit muscular (Cantó et al, 2004; .. Cantó et al, 2006). No obstant això, després d'una dosi aguda (25 ng / g de pes corporal) de NRG recombinant administrada in vivo, el fetge va demostrar tenir una gran resposta a la NRG amb una baixada significativa de la glucèmia i de la insulinemia en assaigs de test de tolerància a la glucosa (López-Soldat, manuscrit en procés).

El fetge expressa grans quantitats de ErbB1 i ErbB3, traces d'ErbB4 i gens ErbB2. L'expressió d'ErbB1 i ErbB3 al fetge adult té un patró regulat circadiari, que proporciona una base per a les diverses combinatòries diferencials de la senyalització dels ErbB en diferents moments del dia (Carver et al., 2002). Els estudis han demostrat que ErbB3 és regulat negativament per la insulina tant *in vitro* (Carver et al., 1996) com *in vivo.* D'altra banda, una major expressió d'ErbB3 s'observa en dos models de disminuïda presencia o absència d'insulina *in vivo*: la diabetes de tipus I induïda per estreptozotocina (STZ) i el dejuni. Els autors proposen que l'estat metabòlic de l'animal pot controlar la síntesi d'ErbB3 (Carver et al, 1997;. Carver et al., 2002).

Objectius

1. Caracteritzar els efectes *in vivo* de la neuregulina sobre la glucèmia en models de ratolins, per tal de fer assequibles aquests estudis amb la quantitat de recombinant de neuregulina

necessaria i disponible, desenvolupant estudis en diferents situacions metabòliques: ratolins dejunats, alimentats i amb deficiència d'insulina.

2. Caracteritzar els efectes neuregulina en un altre teixit diana de la insulina, el fetge, que té un paper essencial regulant la glucèmia. Anàlisi de la cascada de senyalització induïda per la neuregulina en el fetge i la seva possible interacció amb la via de senyalització de la insulina.

3. Identificar l'acció neuregulina sobre proteïnes que regulen o estan involucrats en el metabolisme hepàtic de la glucosa i que contribueixin a l'efecte hipoglucèmic de la neuregulina.

4. Avaluar l'efecte *in vivo* de la neuregulina sobre la glucèmia en un estat de resistència a la insulina, el model de ratolins db/db, que desenvolupen diabetis de tipus 2. Anàlisi dels efectes de la neuregulina en el fetge.

5. L'anàlisi de la "neuregulinemia" en plasma de ratolins sota diferents situacions metabòliques: el ritme circadià, dejuni/alimentació, models de diabetis tipus I i II. Recerca del paper fisiològic de la neuregulina com a regulador de la glucèmia

Resultats i discussió

Acció de la neuregulin sobre la glucèmia i el metabolisme del fetge en els ratolins control

En primer lloc varem provar que l'administració aguda de NRG recombinant (50 ng / g de pes corporal) disminuïa significativament la glucèmia durant el test de tolerància a la glucosa (GTT). A continuació, varem comprovar que aquest factor de creixement tenia un efecte comparable sobre els nivells basals de glucosa, portant als ratolins sovint a una hipoglucèmia severa. La insulinèmia mostrava una reducció significativa durant el GTT, però no en estat basal de ratolí dejunat, mostrant que la neuregulina no te influència sobre la producció d'insulina i l'hormona es secreta a partir de cèl·lules β en funció dels nivells de glucosa. Aquests resultats suggereixen que els canvis en els nivells de glucosa que hem observat poden ser causats ja sigui per l'augment de sensibilitat a la insulina o per la inhibició de la producció hepàtica de glucosa (HGP). El test de tolerància al piruvat ens va mostrar que la neuregulina te un paper important en la inhibició de la HGP. La neuregulina no només va blocar completament la conversió de piruvat a glucosa, sinó que encara va rebaixar més els nivells de glucèmia assolint valors comparables als que s'observa en el seu efecte sobre la glucèmia basal. Aquest fet indicava que en els efectes *in vivo* el fetge juga un

important paper sobre els canvis metabòlics induïts per la neuregulina, recolzat els resultats que s'han vist prèviament en rates (López-Soldat, manuscrit en procés).

Sabent que la insulina, així com l'estat de dejuni/alimentació dels animals, condicionen la senyalització a través ErbB3 (Carver et al., 1997), varem decidir fer assaigs en ratolins alimentats sense un dejuni previ. En aquesta situació, és lògic esperar que en l'estat alimentat l'efecte de la neuregulina sobre la glucèmia sigui menor que en animals en dejú. En estudis de test de tolerància a la glucosa, varem poder demostrar-ho. L'acció hipoglucemiant de la neuregulina observada en l'estat de dejuni, va ser completament abolida en estat alimentat. Això podia ser degut a un menor nombre de receptors de la neuregulina disponibles per a transmetre el senyal en l'estat alimentat, però també a la contribució de les majors concentracions de la insulina. Sorprenentment, l'efecte no era diferent entre ratolins alimentats i dejunats quan s'analitzava la influència de la neuregulina sobre la glucèmia basal. Mentre que la unió de la neuregulina al seu receptor es veu afectada negativament amb concentracions altes d'insulina, però alhora la vida mitja de la insulina és curta (4-6 min) (Duckworth et al., 1998), varem deduir que l'efecte apareixia a conseqüència de la reducció dels nivells d'insulina, la qual cosa no és el cas en assaigs de GTT. En estat alimentat, la insulinèmia de partida ja és més alta que en l'estat de dejuni i a causa de l'administració del bolus de glucosa, es manté elevada, no permetent que la neuregulina pugui treballar eficaçment durant el GTT. Quan es van fer assaigs de PTT en estat alimentat, l'efecte de la neuregulina mostrava un patró idèntic a l'observat sobre la glucèmia basal. Això era d'esperar ja que la gluconeogènesi en estat alimentat s'inhibeix i l'efecte sobre els nivells de glucosa el més probable és que sigui produït pel mateix mecanisme que en la condició basal. A partir d'aquests resultats es pot concloure que l'estat de l'animal juga un paper important en si som capaços de veure un efecte de neuregulina sobre la glucèmia o no.

En veure aquestes diferències rellevants en l'estat alimentat i en dejú, vam decidir investigar quina és la influència de la insulina sobre l'acció neuregulina. Per tal d'abordar aquest objectiu, es van analitzar els efectes de la neuregulina en ratolins amb diabetes de tipus 1 induïda per estreptozotocina (STZ), un model fisiològic adequat de deficiència d'insulina, que alhora, hauria de mantenir alta l'expressió de ErbB3 en el fetge. En aquests ratolins, la neuregulina rebaixava lleugerament la glucèmia basal i tenia un efecte similar, en assaigs de PTT, als observats en ratolins control dejunats, el que suggeria que la presència/absència d'insulina no afectava aquests processos. Sorprenentment, durant els assaigs de GTT, la neuregulina deixava de tenir un efecte hipoglucemiant i fins i tot exercia un efecte contrari als fenòmens de recuperació de la normoglucèmia, provocant un manteniment de l'estat d'hiperglucèmia fins al final de l'assaig.

Tot plegat ens va induir a investigar els efectes que la neuregulina induïa sobre el metabolisme hepatic. Amb aquesta finalitat varem estudiar les accions de la neuregulina en el període comprés dintre dels primers 30 minuts d'acció quan ja es verificaven les accions *in vivo* reguladores de la glucèmia. Tanmateix es pretenia minimitzar les interferències per part de la insulina en els assaigs on es provocava un increment en l'alliberament d'aquesta hormona.

En ratolins, el fetge expressava gran quantitat de receptors ErbB1 i ErbB3, nivells apenes detectables d'ErbB4 i indetectables per a ErbB2. La neuregulina induïa la fosforilació de ErbB3,

però no d'ErbB1, indicant que la senyalització posterior partía d'ErbB3, mentre que ErbB1 actuava merament com a co-receptor dimeritzant amb ErbB3 i fosforilant-lo en tirosines. Aquest resultat era esperable donada la incapacitat d'ErbB3 d'actuar com a tirosina quinasa.

Per avaluar la rellevància del ErbB3 hepàtic en els efectes hipoglucemiants de la neuregulina, vam decidir inhibir ErbB3, mitjançant l'administració d'anticossos específics de blocatge, en tres injeccions de petit volum a la zona abdominal alta que comprèn la localització del fetge, 15 min abans de l'administració de la neuregulina. El grup control va rebre una dosi equivalent d'immunoglobulines irrellevants (Ig). Els anticossos anti-ErbB3 van blocar l'acció activadora de la neuregulina sobre ErbB3 al fetge. Alhora es va abolir totalment l'efecte hipoglucemiant de la neuregulina, descrit prèviament en ratolins controls sotmesos a un test de tolerància a la glucosa. Aquest fet suggeria que la neuregulina estava reduint la glucèmia actuant molt probablement sobre els receptors ErbB3 d'origen hepàtic en ratolins control dejunats. Alhora donava evidència del minim, o inexistent, paper del múscul esquelètic en l'acció hipoglucemiant de la neuregulina, ja que en aquest teixit la principal acció de la neuregulina induint la captació de glucosa es produeix en el context dels efectes de la contracció muscular i cursen principalment a través d'ErbB4, no d'ErbB3 (Cantó et al. 2006). Més encara, estudis recents d'acció *in vivo* de la neuregulina, en ratolins *knockout* condicionals de múscul esquelètic per a ErbB4, mostraven efectes significativament hipoglucemiants en assaigs de GTT (Gumà, A. resultats no publicats).

El domini intracel·lular d'ErbB3 conté sis llocs d'unió a la subunitat p85 de la PI3K (Soltoff i Cantley, 1996). La neuregulina provocava un increment significatiu de la unió de PI3K a ErbB3 al fetge. Donat que la PI3K és un important enzim que també forma part de la via d'acció de la insulina, varem voler esbrinar si la neuregulina interferia en la interacció de la PI3K amb les proteïnes IRSs. Els resultats indicaven que aquesta interacción disminuïa en un 60-65% tant per a IRS1 com per a IRS2. Les diferències en mobilitat que mostraven les IRSs en assaigs d'immunodetecció, sota l'acció de la neuregulina, eren compatibles amb un augment de l'estat de fosforilació en serines, situació que comporta una pèrdua en la capacitat de ser fosforilades en tirosines, generant així una situación de resistència a la insulina. La presència d'anticossos de blocatge d'ErbB3 revertia aquesta situació. Així doncs, la neuregulina, a través de l'activació d'ErbB3, podria estar provocant una disminució en l'acció de la insulina per una reducció en la interacció entre les IRSs i la PI3K, però també podria estar provocant una ràpida situació de resistència a la insulina al provocar la fosforilació en serines dels IRSs.

Posteriorment a l'activació de la PI3K, la neuregulina produia un important increment en el nivell de fosforilació de la Akt, però no de la PKCζ, aquesta última involucrada de manera esencial en les accións de la neuregulina activant la translocació de GLUT4 i el transport de glucosa en el múscul esquelètic (Suárez et al, 2001; Cantó et al, 2004). El blocatge d'ErbB3 amb anticossos específics inhibía la fosforilació d'Akt per la neuregulina al fetge, indicant la relevància de l'acció de la neuregulina sobre l'activació de la via PI3K/PDK1-mTORC2/Akt. A banda, el fetge expressa GLUT2 en lloc de GLUT4 i al contrari del múscul esquelètic, el transport de glucosa hepàtica no depèn del nombre i l'activitat de GLUT2 en les membranes de la superfície, sinó dels augments en la concentració de glucosa a la sang, a causa de la baixa afinitat de GLUT2 a aquest metabòlit.

Degut a l'aparent falta d'efecte de la neuregulina sobre el múscul, els seus efectes hipoglucemiants difícilment podrien explicar-se per una incrementada utilització de la glucosa per part d'aquest teixit. En canvi, sí s'havien observat clars efectes de la neuregulina inhibint la producció hepàtica de glucosa, en base a assaigs de tests de tolerància al piruvat, en ratolins dejunats. Per tant, l'acció de la neuregulina semblava centrada en una inhibició de la gluconeogènesis al fetge, com a eix central per a reduir la glucèmia. Cercant elements de control de les taxes gluconeogèniques, varem investigar les possibles accions de la neuregulina sobre proteïnes regulades per la Akt, amb accions sobre enzims gluconeogènics. FoxO1, un factor transcripcional essencial per a promoure l'expressió de diversos enzims limitants de la via gluconeogènica, induint així l'activitat d'aquesta, és substrat de la Akt i en ser fosforilat esdevé inactiu (Altomonte et al, 2003; Puigserver et al, 2003; Samuel et al, 2006; Matsumoto et al, 2007). Tal i com era d'esperar, la neuregulina indueix la fosforilació, i per tant la inactivació, de FoxO1. Malgrat tot, l'estudi dels gens diana de FoxO1, com el gen de la PEPCK, no mostra caigudes significatives dels nivells de mRNA, tot i mostrar una clara tendència a una disminució, ni tampoc hi ha canvis significatius en els nivells d'aquesta proteïna. Aquesta falta d'efecte bé podria ser conseqüència del curt espai de temps en que deixàvem actuar a la neuregulina (30 min). Aquestes dades indicaven que les ràpides accions hipoglucemiants de la neuregulina, no podien ser mediades per caigudes en els nivells de gens diana de FoxO1, com la PEPCK o la G6Pasa, sinó que probablement inhibien la conversió del piruvat a glucosa a través d'altres mecanismes on no mediaven canvis d'expressió de proteïnes, mecanismes ràpids de regulació. Els experiments amb anticossos de blocatge d'ErbB3 corroboraven aquestes conclusions.

Una altra proteïna que es veia afectat per l'acció aguda de la neuregulina era la glucoquinasa (GK), enzim que facilita la fosforilació de la glucosa a glucosa-6-fosfat (G6P) i és un pas determinant de la velocitat d'utilització de glucosa al fetge (Hornichter i Brown, 1969). Mentre que en ratolins control el tractament amb neuregulina mostrava una clara tendència a la caiguda en els nivells de proteïna GK, alhora potenciava l'expressió d'aquest gen al fetge. Els efectes transcripcionals, similars als de la insulina, semblaven dependre d'aquesta hormona, ja que estudis en ratolins tractats amb estreptozotocina, insulino-deficients, les caigudes induïdes per la neuregulina sobre els nivells de la proteïna GK eren més acusades, efecte que anava en paral·lel a la mesura de l'activitat d'aquest enzim. L'anàlisi dels nivells de proteïna i d'activitat, conjuntament, suggerien que l'extracte de fetge contenia menys proteïna GK i que aquesta era, en si mateixa, menys activa. Tot plegat indicava que alhora que la neuregulina inhibia la gluconeogènesi, també reduïa la utilització hepàtica de glucosa, el que contribuïa a explicar el fet que en ratolins insulino-deficients es veiés un defecte en la recuperació de la normoglucemia en el grup tractat amb neuregulina en resposta a tests de tolerància a la glucosa.

El tractament agut amb neuregulina també afectava la GSK3β, induint la seva fosforilació i desactivació, èssent aquest un mecanisme important per a activar la glicogen sintasa i per tant, la síntesi de glicogen. L'especificitat en l'acció de la neuregulina-ErbB3 es veia confirmada pels estudis amb l'ús d'anticossos de blocatge d'ErbB3. Tot i la desactivació de la GSK3β, no vàrem veure un increment en el contingut de glicogen en el fetge per acció de la neuregulina. 30 minuts

d'acció neuregulina és certament molt poc temps per veure les diferències en els nivells totals de glicogen. No obstant això, resultats preliminars d'incorporació de glucosa radioactiva a glicogen van revelar que la neuregulina potenciava la vía de síntesi de glicogen.

Una altra via sota el control de la Akt, i que s'activa per neuregulina, és la via mTORC1-p70S6K, que té una influència positiva en la síntesi de proteïnes i també està connectada a un augment de la fosforilació en serines de les IRSs i per tant, de desactivació de la via de la insulina (Harrington et al, 2004;. Um et al, 2004;. Werner et al., 2004). Aquest darrer efecte és conegut com un circuit de retroalimentació negativa de la via d'acció de la insulina (Haruta et al., 2000; Greene et al., 2003; Carlson et al., 2004). La neuregulina a través de l'activació, per fosforilació, de la p70s6K, podia induir un major estat de fosforilació en serines de les IRS, i per tant, antagonitzar de manera directa amb la insulina.

Acció de la neuregulina sobre la glucèmia i el metabolisme del fetge en la diabetes de tipus 2

En situació de resistència a la insulina, els teixits diana de la insulina no son efectius en la utilització i reducció dels nivells de glucosa a la sang i per tant, els nivells fisiològics son superiors als dels organismes sans. A més, la gluconeogènesi, que normalment es troba inhibida per la insulina en condicions absortives, es manté relativament activa en la T2D contribuint així a una major glucèmia. En ratolins de control, la neuregulina promou les vies d'activació de la síntesi de glicogen i inhibeix la producció hepàtica de glucosa per una via que clàssicament indueix la insulina, però en la que no intervé el receptor de la insulina ni les IRSs, les quals es troben alterades manifestant així la situació de resistència a la insulina. Per tant, és important investigar si la senyalització de la neuregulina en T2D està intacta i si aquest factor de creixement es pogués utilitzar com una eina terapèutica per a alleujar la situació patològica Per a aquests estudis hem triat el model de ratolí més àmpliament utilitzat per a la diabetes tipus 2: ratolins deficients al receptor de la leptina C57BLKS / J-db/db (db/db).

Una de les grans diferències entre el ratolins control i els hiperinsulinèmics db/db és l'expressió d'ErbBs al fetge. Els nostres resultats van mostrar clarament que els dos principals receptors, ErbB1 i ErbB3, es van reduir notablement en ratolins diabètics db/db, en un 66% i 52%, respectivament. A més, en el nostre laboratori, un resultat similar s'ha observat també en rates amb diabetes de tipus 2, rates ZDF (López-Soldat, manuscrit en procés). Alhora, l'expressió endògena de neuregulina hepàtica augmenta una tercera part. Mentre que la neuregulina actua preferentment en una forma autocrina, paracrina o yuxtacrina (Goodearl et al, 1995; Loeb et al, 1998), aquesta situació suggereix que l'increment de neuregulina expressada pel fetge és una resposta compensatòria a la reducció en els nivells de receptors disponibles.

Mentre que en T2D l'expressió de ErbB3 es redueix en gran mesura, també l'activació dels receptors de la neuregulina presents és més feble en els ratolins db/db. Aquesta situació afectava

la interacció d'ErbB3 amb PI3K, però també la unió de PI3K a les IRS. En reduïr-se la unió d'ErbB3 a la PI3K, els seus efectes inhibint la via de senyalització de la insulina son menys potents que els observats en ratolins control. La disminució en el efecte inhibidor de l'acció de la insulina per part de la neuregulina es pot explicar per la menor activació de la p70S6K-mTORC1 i per tant per una debilitació en el bucle de realimentació negativa de la insulina. Un resultat similar s'observa també en l'activació de l'Akt, on la fosforilació d'ambdós residus, pel total de proteïna Akt expressada, es redueix en unes 5-6-vegades en comparació amb els animals control.

Malgrat el menor nombre de receptors i l'activació més feble de la via PI3K-Akt, els nostres resultats van revelar que en els ratolins db/db la reducció de la glucèmia, després de l'administració neuregulina, succeeix tant en condicions basals com absortives i en major mesura (en valors absoluts) que en els animals control. Aquest fenomen podria ser degut a grans diferències entre la glucèmia basal, en dejú, en aquests grups. La raó també podria estar en la incapacitat de la neuregulina per a degradar ràpidament la GK, hiperactivada en ratolins db/db, la qual cosa és contraria al que hem observat en els ratolins control i deficients d'insulina. L'administració de neuregulina també va causar un extensiu decrement dels nivells de la insulina.

Així com vam observar diferències entre l'acció de la neuregulina en estat de dejuni i d'alimentació en els animals control, també ho varem veure en el cas dels ratolins db/db. En ratolins alimentats db/db es va abolir l'efecte de neuregulina sobre la glucèmia basal i durant els assaigs de GTT va quedar molt disminuïda donant únicament diferències significatives en l'últim punt de mesura. Mentre que els ritmes circadians semblen afectar l'expressió del receptor hepàtic ErbB3, així com els nivells d'insulina també mostren certa connexió, en ratolins control, no hi ha informació sobre l'existència de ritmes circadians en ratolins hiperinsulinèmics db/db.

A partir d'experiments amb ratolins control hem observat que la inhibició de la producció hepàtica de glucosa és un dels majors processos sobre els que actua la neuregulina *in vivo* regulant la glucèmia. Ara bé, els ratolins db/db presenten complicacions a fi de realitzar adequadament la tasca de la mateixa manera que els animals control. Amb tot, varem veure baixades en els nivells de glucosa per acció de neuregulina en assaigs de test de tolerància al piruvat, en extensió similar a la baixada que s'observa quan es treballa sobre la glucèmia basal, suggerint que en ambdues condicions, la neuregulina actua sobre el mateix component de la producció hepàtica de glucosa. Quan s'analitza la senyalització al fetge, la neuregulina va inhibir FoxO1 en menor extensió que en animals control, però l'efecte gluconeogènic modulant l'activitat de la PEPCK, a nivell transcripcional i de proteïna va ser similar. Aquest fet dona un suport encara major al fet que deu haver un altre pas sobre el que actua la neuregulina per a inhibir la gluconeogènesis, diferent a la regulació transcripcional per FoxO1.

A l'igual que per a la resta de elements de la via d'acció de la neuregulina, la fosforilació de l'Akt i de la GSK3β també es veu afeblida. La menor inhibició de la GSK3β va amb paral·lel amb la més moderada a incrementar el contingut de glicogen en els ratolins db/db. Pel temps de tractament amb neuregulina establert (30 min), les diferències no van assolir canvis significatius. La causa

pot ser atribuïble a grans diferències individuals en quant al contingut de glicogen abans d'iniciar-se el tractament amb neuregulina. Amb tot, aquestes dades donen suport a una visió per la que la neuregulina segueix contribuint a estimular la síntesi de glicogen en ratolins db/db.

Noves expectatives en la consideració de la neuregulina com un regulador de la glucèmia.

Durant el desenvolupament d'aquesta tesis hem vist que la neuregulina redueix els nivells de glucosa en sang, que bloca la producció hepàtica de glucosa, i curiosament, no afavoreix un consum ràpid de la glucosa. Aquestes observacions suggereixen que hi ha diversos processos que funcionen en paral·lel, alguns d'ells afectats per la neuregulina. A fi de descriure més nítidament la situació, hauríem d'aclarir quin és el principal destí dels carbonis originaris de la glucosa o del piruvat administrat. Analitzant alguns metabòlits a la sang, com ara el lactat i els triglicèrids, hem vist que cap d'ells es constitueix com a principal destí de la glucosa ni en situació control ni en ratolins insulino-deficients.

En ratolins db/db la síntesi de triglicèrids aumenta lleugerament per acció de la neuregulina en condicions absortives. Durant els assaigs de PTT, el piruvat no es va poder incorporar a la via gluconeogènica i es va convertir ràpidament a lactat, cosa que no va passar en els ratolins db/db,on la caiguda en l'acció de la neuregulina va alleugerir el blocatge en la conversió de piruvat a glucosa. A banda de un possible destí dels carbonis de la glucosa o del piruvat cap a glicogen, que no pot descartar-se, hi ha altres possibles destins metabòlics als que podrien anar adreçats però que no s'han investigat, i tampoc no podem excloure que en situació de resistència a la insulina, altres teixits, no necessàriament diana de la neuregulina poden estar contribuint a la depleció de la glucosa sanguinia.

Si bé no ha estat objectiu d'aquesta tesi, és possible que la neuregulina tingui efectes sobre el metabolisme a llarg termini, consolidant els canvis en l'expressió de múltiples gens amb funcions limitants o reguladores de vies metabòliques, com ja es pot entreveure en aquest estudi d'efectes aguts. Així, prèviament ja s'ha descrit l'existència d'efectes crònics de la neuregulina incrementant el metabolisme oxidatiu, la biogenesis mitocondrial i en conjunt, la sensibilitat a la insulina, en línies establertes musculars (Cantó et al. 2007). Els possibles efectes crònics de la neuregulina sobre el metabolisme hepàtic hauran de ser objecte de futures investigacions.

A banda dels efectes d'una administració exògena de recombinant de neuregulina, cal tenir en compte que la neuregulina s'expressa endogenament i que cal entendre la rellevància fisiològica de la neuregulina en relació als seus efectes reguladors del metabolisme de diversos organs i teixits. Ja queda clar, gràcies a les nostres aportacions i a les d'altres autors, que l'expressió d'ErbB3 es troba regulada per l'estat metabòlic/energètic de l'animal i que la ingesta pot induir canvis d'expressió. Per tant, és fàcil imaginar que aquest receptor, i possiblement els seus

lligands, puguin estar sotmesos i estretament connectats a processos d'homeòstasis de la glucosa i/o a l'estat energètic dels teixits consumidors d'aquesta glucosa.

Les dades dels estudis circadians que ens indiquen les variacions de neuregulina a la circulació durant el dia, en ratolins control i db/db, no mostren grans canvis apreciables, però en el model insulino-deficient, incrementant el contingut de neuregulina quan la glucèmia i la insulinèmia estan en els seus nivells més baixos, just al final del període de llum, en la transició a un estat actiu d'alimentació. La neuregulina circulant desapareix just després de la realimentació. Una certa, però matisada similitud s'observa en alguns ratolins amb diabetes de tipus 2. Tot plegat suggereix que en moments en que es detecten situacions de depleció energètica per part dels teixits, l'increment de neuregulina a la sang podria senyalitzar a nivell hepàtic reduint la disponibilitat de glucoquinasa, i per tant la captació directa de glucosa per part del fetge, a fi de prevenir i evitar estats de severa hipoglucèmia. Alhora, la neuregulina inhibiria l'acció de la insulina a nivell de IRSs. Alhora, l'efecte de la neuregulina inhibint la producció hepàtica de glucosa podria permetre que aquesta glucosa fos completament oxidada en el cicle de l'àcid tricarboxílic a fi de produir ATP i suplementar al teixit amb aport d'energia. El motiu pel que no veuríem clares fluctuacions de neuregulina circulant en ratolins control i db/db podria ser degut al fet que en aquestes condicions metabòliques l'organisme disposa de reservoris per a proveir d'energia per períodes de temps llarg i no caldria una regulació ràpida incrementant l'aport de neuregulina. Entenent que la neuregulina actua de manera local en efectes autocrins, paracrins o juxtacrins, les fluctuacions de menor grau probablement no transcendeixin als valors plasmàtics, i siguin aquests, més moderats, els que autènticament actuïn com a sensors locals de l'estat energètic cel·lular. Aquestes consideracions en les que podria radicar la rellevància fisiològica de la neuregulina resten obertes a fi de ser estudiades en un futur, però la contribució d'aquesta tesi permet obrir noves portes a l'establiment d'aquestes noves hipòtesis de treball que podrien revolucionar el nostre enteniment sobre el control de la glucèmia i de l'estat energètic cel·lular, més enllà de la insulina.

Conclusions

1. La neuregulina regula l'homeostasi de la glucosa de manera aguda. En 30 minuts s'activen cascades de senyalització en el fetge que provoquen canvis sobre la glucemia.
2. El tractament *in vivo* amb neuregulina incrementava la fosforilació en tirosines d'ErbB3 al fetge que conduïa a un augment en la interacció entre ErbB3 i la subunitat reguladora de PI3K i a una activació de la via de senyalització que inclou Akt, FoxO1, GSK3β i p70S6K. Alhora la neuregulina redueix la interacció de la PI3K amb IRS1 i IRS2, probablement per una inducció de la seva fosforilació en serines.

3. El tractament agut amb neuregulina provocava una reducció en l'activitat glucoquinasa hepàtica en ratolins control i insulino-deficients, probablement per una inducció cap a la degradació de la glucoquinasa.
4. La neuregulina inhibia la producció hepàtica de glucosa. No només blocava completament la conversió de piruvat a glucosa, a més reduïa la glucèmia fins a nivells similars als induïts sobre la glucèmia basal, conduint als ratolins a un estat de severa hipoglucèmia.
5. El blocatge del receptor ErbB3 abolia completament l'efecte de la neuregulina sota el test de tolerància a la glucosa. Sota l'estimulació de la neuregulina, prevenia la fosforilació en tirosines d'ErbB3 i anulava l'efecte de la neuregulina sobre la seva cascada de senyalització, incloent Akt, FoxO1, GSK3β i p70S6K.
6. L'expressió hepàtica d'ErbB1 i ErbB3 en ratolins db/db amb diabetes de tipus 2, es va reduïr considerablement. Contrariament, l'expressió hepàtica de neuregulina endògena es va incrementar.
7. La neuregulina va causar una activació dèbil de la cascada de senyalització en ratolins db/db comparat amb els controls, però va tenir un efecte superior en la reducció de la glucèmia.
8. En ratolins db/db, la neuregulina va fallar en el seu efecte inhibidor de la conversió de piruvat a glucosa. En tests de tolerància al piruvat, la neuregulina va baixar la glucèmia fins al punt d'assolir els nivells obtinguts en condicions basals en dejuni.
9. La concentració de neuregulina endògena al plasma dels ratolins insulino-deficients incrementava bruscament al final del període diari de descans i dejuni, i decreixien també bruscament després de la realimentació. Aquest increment de nivells de neuregulina coincidia amb els moments en que els animals experimentaven els nivells més baixos de glucèmia i de insulinemia.

ANNEX: LIST OF ANTIBODIES

Antibody	Supplier	Raised in	Used for	MW (kDa)
Akt	Cell Signaling #9272	Rabbit	WB 1:1000	60
AMPK α-subunits	Cell Signaling #2532	Rabbit	WB 1:1000	62
ErbB1 (EGFR (1005))	Santa Cruz Inc. #sc-03	Rabbit	WB 1:250	175
ErbB2	Santa Cruz Inc. #sc-284	Rabbit	WB 1:250	185
ErbB3 (c-17)	Santa Cruz Inc. #sc-285	Rabbit	WB 1:250 IP 3µl/sample	185
ErbB3 Blocking Antibody	NeoMarkers #MS-303-P	Mouse	ErbB3 blockage	-
ErbB4	Millipore Corp. #06-572	Rabbit	WB 1:1000	180
FoxO1	Cell Signaling #2880	Rabbit	WB 1:1000	82
GK		Rabbit	WB 1:1000	50
GSK3β	Transduction Laboratories #610201	Mouse	WB 1:2000	46
IRS1	Cell Signaling #2382	Rabbit	WB 1:1000 IP 2µl/sample	180
IRS2	Millipore Corp. #MABS15	Mouse	WB 1:1000 IP 3µl/sample	185
NRG	Thermo Scientific #RB-276	Rabbit	WB 1:250	44
p70S6K	Cell Signaling #9202	Rabbit	WB 1:1000	70
PEPCK	Abcam #ab137580	Rabbit	WB 1:1000	67
Phospho-Akt (Ser473)	Cell Signaling #9271	Rabbit	WB 1:1000	60
Phospho-Akt (Thr308)	Cell Signaling #9275	Rabbit	WB 1:1000	60
Phospho-AMPK (Thr172)	Cell Signaling #2531	Rabbit	WB 1:1000	62
Phospho-FoxO1 (Ser256)	Cell Signaling #9461	Rabbit	WB 1:1000	82
Phospho-GSK3β (Ser9)	Cell Signaling #9336	Rabbit	WB 1:1000	46
Phospho-p70S6K (Thr389)	Sigma #S6311	Rabbit	WB 1:1000	70
Phospho-PKCζ/λ (Thr410/403)	Cell Signaling #9378S	Rabbit	IP 3µl	75
Phospho-Tyrosine	Transduction Laboratories #610000	Mouse	WB 1:1000 IP 5µl/sample	-
PI3K (p85 subunit)	Millipore Corp. #06-195	Rabbit	WB 1:1000	85
PKCζ (c-20)	Santa Cruz Inc. #sc-216	Rabbit	WB 1:1000	75
α-tubulin		Mouse	WB 1:500	52