MODULATION OF MUSCLE ENERGY METABOLISM BY BIOACTIVE COMPOUNDS

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Modulation of muscle energy metabolism by bioactive compounds

Doctoral Thesis

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Departament de Bioquímica i Biotecnologia

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FAIG CONSTAR que aquest treball, titulat "Modulation of muscle energy metabolism by bioactive compounds", que presenta Ester Casanova Vallvé per a la obtenció de títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que acompleix els requeriments per poder optar Menció Europea.

Tarragona, 22 de Juliol de 2013

La directora de la tesi doctoral — El co-director de la tesi doctoral

Maria Josepa Salvadó Rovira — Lluís Arola Ferrer

"La duda es el principio de la sabiduría"

Aristóteles

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Abbreviation list

ACC	Acetyl-CoA carboxylase
ACS	Acyl-CoA synthetase
Adipo R1,R2	Adiponectin receptor 1,2
AHA	American Heart Association
AKT	Protein kinase
ALA	alpha linoleic acid
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocase
BAT	Brown adipose tissue
BMCP1	Brain mitochondrial carrier protein-1
BMI	Body mass index
BMPS	Bone morphogenetic proteins
C/EBP	ccaat-enhancer-binding protein
CD36	Cluster of Differentation 36
CHD	Cardiovascular heart failure
COX	Citochorme c oxidase
COX-2	Cyclooxygenase-2
CPT-1	Carnitine palmitoyltransferase I
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
DM	Diabetes Mellitus
Drp1	Dynamin-Related protein1
ECC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EPA	Eicosapentaenoic acid

ERRs	Estrogen-related receptors
ETC	Electron transport chain
FABPpm	Plasma membrane fatty acid binding protein
FAs	Fatty acid synthase
FAT/CD36	Fatty acid translocase
FATPs	Fatty acid transport proteins
FDA	Food and Drug Administration
FFAs	Free fatty acids
Fis1	Fission 1
GLUT 4	Glucose transporter 4
GSH	Glutathione
GSPE	Grape seed proanthocyanidin extract
GTPases	Guanosine triphosphatases
HDL	High density lipoproteins
LA	Linoleic acid
LCFA	Long chain fatty acids
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LXRs	Liver x receptors
MCAD	Medium-chain-acyl-CoA-dehydrogenase
Mff	Mitochondria fission factor
Mnf1	Mitofusin 1
Mfn2	Mitofusin 2
MnSOD	Manganes superoxide dismutase
mtDNA	mitochondrial DNA
NEFAs	None esterified free fatty acid
NRF	Nuclear receptor factor
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
P38MAPK	Phosphor38 mitogen activated protein kinase

PGC1aplha	Peroxisome proliferator-activated receptor gamma coactivator-1-α
PPARs	Peroxisome proliferator-activated receptors
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SCD1	Stearoyl-CoA desaturase-1
SIRT	Sirtuin
SOD	Superoxide dismutase
SREBP1c	Sterol regulatory element binding protein
TCA	Tricarboxylic acid cycle
TG	Triglycerides
TNF-α	Tumor necrosis factor alpha
UCP1,2,3,4,5	Uncoupling proteins 1,2,3,4,5
VLDL	Very-low density lipoprotein
WAT	White adipose tissue

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Summary

Overweight and obesity are markedly increasing, threatening population health in a growing number of countries. In order to develop a new therapeutic strategy against the obesity progression and related diseases, it have been studied the use of natural bioactive compounds to correct metabolic dysfunction associated with obesity. With this pretext, we want to see if the consumption of functional foods with a slight or no change in eating habits, avoiding behavioural problems associated with meal replacements, with the objective to be beneficial beyond obesity and their comorbidities such as heart disease, hypertension, insulin resistance and dyslipidemia.

The aim of this thesis is to evaluate how bioactive compounds present in healthy diets, such as flavonoids, proanthocyanidins and omega-3 fatty acids, such as docosahexaenoic acid (DHA) and the combination of both, could improve or prevent the adverse effects of obesity and related diseases. Studies have focused on, skeletal muscle and adipose tissue, two important organs of body weight control and mitochondrial function, known as the key regulators of energy homeostasis.

To observe the beneficial effects, we evaluated the ability of the compounds to modulate mitochondrial activity in these tissues. First, we quantify the ability of epigallocatechin gallate (EGCG) and/or DHA modulate mitochondrial function in rat muscle cells; for further study in healthy rats, the effects of acute doses of grape seed proanthochyanidin extract (GSPE) and/or DHA-rich oil, combined with a lipid overload. Results indicate that both compounds improve metabolic flexibility and postprandial situation. Moreover, it has also been evaluated the chronic treatment of GSPE and/or DHA-rich oil in obese rats. In both cases there was an improvement of the altered parameters related to the high fat diet intake, improving both, insulin resistance and lipid plasma profile. In addition, there was an increase of fatty acid oxidation concomitant with an increase of mitochondrial oxidative capacity focused in skeletal muscle, and simultaneously with the overexpression of target genes related to β - oxidation, thus improving metabolic flexibility.

Overall, the results of this thesis, achieve the hypothesis, the main objectives, and provide a new opportunity for therapeutic strategy against obesity, with a small change in dietary habits trend and the lifestyle of modern society.

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1. INTRODUCTION

1.1 Obesity

Obesity is defined as a phenotypic manifestation of the abnormal or excessive accumulation of adipose tissue, which alters health and increases mortality [1]. The morbidity and mortality associated with being overweight or obese have been recognised by the medical profession for more than 2000 years. Hippocrates was the first to note a relationship between obesity and sudden death [2]. Recently, data from the life insurance industry and epidemiological studies have proven that obesity plays a major role in the development of various conditions, such as cardiovascular disease (CVD), and thus increases the risk of death [3]. The current obesity epidemic (Figure 1) results from the complex interactions between genetic, behavioural and environmental factors that correspond with chronic and social stays and lifestyles [1].

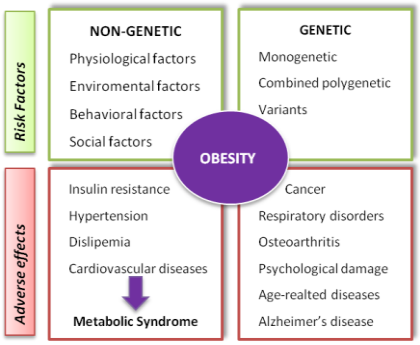


Figure 1. Risk factors and adverse effects associated to obesity based on[1]

A reduction in physical activity due to lifestyle changes, and/or an increase in energy intake due to the increased consumption of energy-dense foods, which result in a reduced satiety index, have led to body weight gain and obesity [4]. Experts predict that the health detriments imposed on children by excess body fat may result in today's youth being the first generation in more than 2 centuries to experience a reduction in life expectancy [5].

Weight gain is primarily caused by a disruption in an individual's energy balance. Energy balance is defined as equilibrium between energy intake and energy expenditure. Excess energy intake and reduced energy expenditure result in abnormal and excessive growth of adipose tissue, which can lead to the development of obesity[6]. The total energy intake is calculated as the sum of energy consumed in food, fluids and dietary supplements. By contrast, energy expenditure is the total energy utilised for basal metabolism, adaptive thermogenesis, food digestion, and activity [7]. In summary, energy balance should be considered as a complex bio-behavioural phenomenon that is influenced by genetics, physiology, early-life, and the environment (Figure 2).

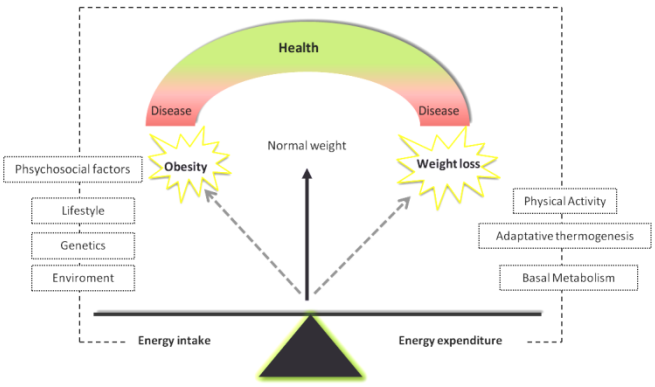


Figure 2. Energy balance

As total body fat content is difficult to measure directly, and because it correlates with the total body mass divided by height squared (Body Mass Index = BMI), another important component in defining obesity is the pattern of fat distribution, which, concomitant with body weight, has been used as an indirect measure of the degree of adiposity [3].

Several studies over the last two decades have aimed to metabolically characterise and quantify visceral adipose tissue, as opposed to subcutaneous adipose tissue. Studies show that in both men and women, the quantity of visceral adipose tissue is directly correlated with altered metabolic risk profiles, which precede metabolic disorders, such as diabetes and hypertension, as well as chronic diseases, such as stroke and sleep [8]. The increased risk of developing such diseases can be largely attributed to obesity, specifically the effect of excess fat on cardiometabolic risk factors stemming from inflammatory processes [5]. The link between obesity and inflammation was first proposed over a decade ago with the finding that the pro-inflammatory cytokine tumour necrosis factor–alpha (TNF-α) was overexpressed in obese subjects. Subsequent studies have demonstrated elevated expression of other inflammatory factors as well. Adipose tissue is able to express many pro-inflammatory molecules in white adipose tissue (WAT), demonstrated and proposed as a potential source of production [9].

Furthermore, obesity is strongly associated with metabolic syndrome, which is characterised by the presence of insulin resistance, hypertension, dyslipidemia and CVD, as mentioned above. A clustering of such factors has been referred to as metabolic syndrome, which is widely recognised as a major public health problem[6]. This syndrome has been found to increase the risk of cardiovascular morbidity and mortality by two- to three- fold [5]. The molecular and cellular mechanisms underlying the relationship between obesity, metabolic syndrome and its associations (Figure 1) with increased cardiovascular risk are not yet entirely understood and have led to focused research on the function of adipose tissue [9].

Preventing and treating excess weight gain is essential to promoting health, combating a rise in the prevalence of obesity and averting devastating CVD outcomes. Global strategies have focused on modifying dietary habits and lifestyle. The fundamental basis for any weight loss intervention, as well as the obvious first defensive action, is lifestyle modification aimed at increasing calories expended during physical activity and decreasing calories consumed from food. The history of success in weight loss interventions targeting diet and exercise has been modest and usually temporary. Lifestyle interventions, including exercise training and at least mild weight reduction via caloric restriction, have led to a nearly 60% reduction in the risk of developing diabetes mellitus [10]. By contrast, pharmacological treatment of obesity has thus far yielded less-than-encouraging results.

Recently, the ability of natural products to counteract obesity has been studied. The anti-obesity effects of these compounds are mediated by the regulation of various pathways, including lipid absorption, intake and expenditure of energy, increasing lipolysis and decreasing lipogenesis[1]. Nutritional genomics methods have the ability to determine which specific nutrients cause phenotypic changes that influence the obesity risk, as well as to establish which interactions are the most important.

There are many different dietary patterns, including some that promote health and others that increase the risk of chronic disease. Despite cultural differences, some characteristics are common to most dietary patterns. Most notably, all diets feature fruits and vegetables, legumes, whole grains, and fish. All are high in fibre, relatively high in omega-3 fatty acids, and low in saturated fat, trans fat and dietary cholesterol. There is appreciable epidemiological evidence that demonstrates a protective role of diets high in fruits and vegetables, legumes, whole grains, and fish against different cancers and cardiovascular diseases [11-14].

1.2 Polyphenols

Polyphenolic compounds constitute one of the most numerous and ubiquitous groups of plant metabolites and take an integral part of both human and animal diets which differ between varieties of the same plants species[15, 16]. Over 8000 phenolic compounds have been isolated from different natural products; they can range from simple molecules, such as phenolic acids, to highly polymerized compounds such as tannins[14, 16]. Polyphenols considered bioactive compounds, are receiving increasing interest from consumers and food manufacturers. Several epidemiological studies, that have examined the relationship between the extent of polyphenol-rich food consumption chronic disease support a protective effect of the compounds upon CVD and several diseases[15, 17]. Moreover in some observational studies, have also been shown an inverse association between the consumption of some classes of polyphenols and overall mortality [18]. According to the recent studies the mechanism by which polyphenols express their beneficial properties appear to involve their interaction with molecular signaling pathways and related machinery that regulate cellular processes such as inflammation [19] insulin signaling pathway [20] and lipogenesis [21] that depend of the polyphenols chemical structure.

Normally polyphenols are present naturally in small amounts in plant products. To understand their impact on human health, it is essential to know the nature of the main polyphenols ingested, their dietary origin, the amounts consumed in different diets, their bioavailability and the factors controlling their bioavailability [15] as it will we described below.

1.2.1 Classification

Polyphenols compounds possess one or more aromatic rings with one or more hydroxyl groups and generally are categorized as phenolic acids, flavonoids, stilbenes, ligands, and tannins [14, 22] (Figure 3).

Flavonoids, also known as flavan-3-ols, represent the most common and widely distributed group of plant phenolics, such as monomers and oligomeric procyanidins [16]. Their basic chemical structure consists of three phenolic rings referred to A, B and C rings. By the other hand flavanoids are further divided into subclasses based on the connection of the B ring to the C ring, as well as the oxidation state and functional groups of the C ring: flavones, flavonols, isoflavones, anthocyanins, flavanols, proanthocyanidins and flavanones (Figure 3) [13, 14, 23, 24].

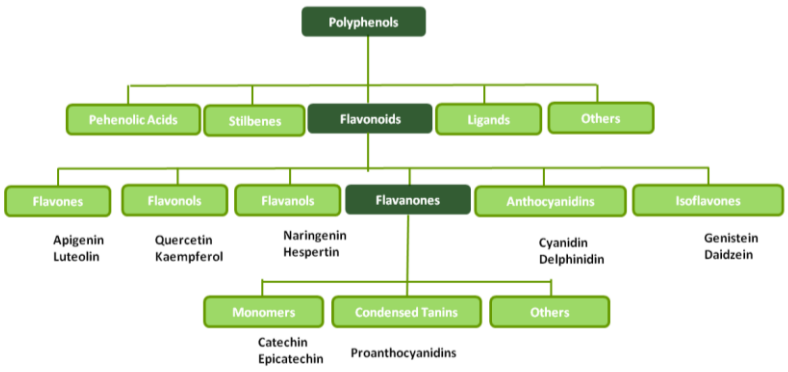


Figure 3. Polyphenols classification base on [14, 25,]

1.2.2 Chemical structure

Chemical structure of different polyphenols in function of the hydroxyl groups on aromatic rings (Figure 4).

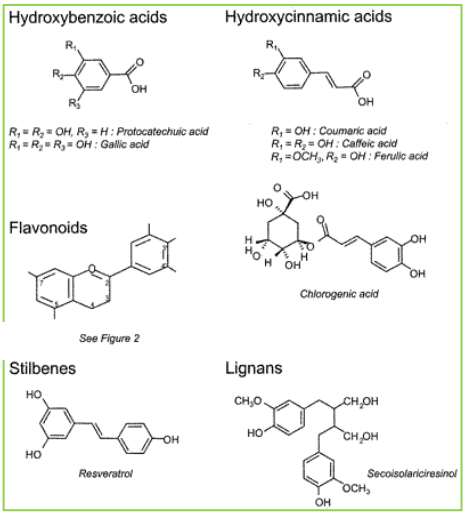


Figure 4. Polyphenols chemical structure base on [26]

1.2.3 Metabolism

Bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways. Bioavailability of polyphenols differs depending of the structure. Before the absorption, these compounds must be hydrolyzed by intestinal enzymes or by colonic microflora. During the course of absorption, polyphenols undergo extensive modification (Figure 5). In fact they are conjugated in intestinal cells and later in liver; as consequence the form reaching the blood and tissues differs from the dose present in food [27]. For the majority of polyphenols, the maximum concentration in the plasma is apparent 1-2h after ingestion[23,28] .

1.2.3.1 Absorption

Polyphenols metabolism occurs via common pathway [15]. During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver. Once ingested, polyphenols/flavonols have several possible fates, including absorption in the small intestine or colon digested into smaller phenolic structures by gut microflora, and/or excretion in the feces or urine. Polyphenols that are not absorbed in the small intestine can be metabolized by colonic microflora into aglycones and phenolic acids, in the ileum and cecum. The site and rate of absorption depend on the chemical structure, degree of glycosylation/acylation, conjugation of other phenolics, size and degree of polymerization [28]. Deglycosilation of flavonoid glycosides has been proposed as the first stage of metabolism [29].

In the small intestine, polyphenols/flavonols can enter the mucosa through passive diffusion; on attachment to a glycoside moiety, the hydrophilicity of the flavonoid molecule is increased, which reduces the possibility of passive transport [29].

1.2.3.2 Distribution & Metabolism

After absorption, circulating flavonoids are bound to albumin and transported to the liver via the portal vein, where are extensively metabolized [30]. Intestine and liver are the major implicated in the flavonoid metabolism. Flavonoids and their derivatives may undergo reactions such as hydroxylation, methylations and reductions. Conjugation reactions with glucuronic acid and/or sulfate seem to be the most common type of metabolic pathways [28, 29]. In recent years have been studied that flavanols and their metabolites also could be accumulate in several organs. Previous experiments have demonstrated the tissue distribution of flavanol metabolites that has been detected at nanomolar levels in tissues such as heart, lung, liver, adipose tissue and muscle [31] .

1.2.3.3 Excretion

The last one, processes is a metabolic detoxication process common to many xenobiotics that restricts their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity [32].

Flavonoid glucoronides and sulfates are polar and water-soluble, excreted by mammals in the urine and bile. When are excreted in bile, are passed into the duodenum and metabolized by intestinal bacteria, which results in the production of fragmentation products and/ or hydrolysis of glucuronide-or sulfoconjugates. The resulting metabolites that are released may be reabsorbed and enter to the hepatic cycle. Flavonoids also are eliminated by renal excretion after conjugation in the liver [29]. The proportion amount of excretion after a dietary intake can vary from 0.8-1.4% [13, 29, 33] .

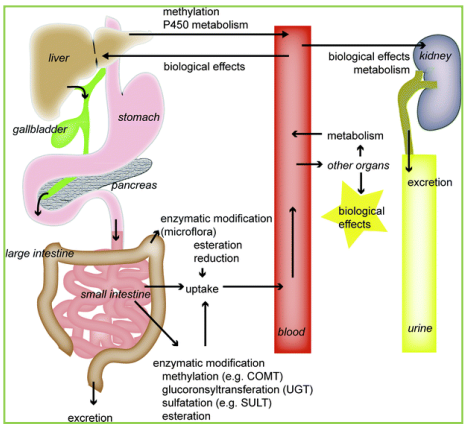


Figure 5. Polyphenols metabolism based on [34]

1.2.4 Food source, bioavailability and dietary intake

Structural diversity of plant flavonoids, their wide distribution in foods, and variations in their content in a given food, are some of the factors that contribute to difficult estimation of dietary consumption.

Polyphenols	Source	Quantity of polyphenol ingested (mg)	Maximum concentration in plasma	Urinary excretion (%of intake)
Anthocyanins				
Cyanidine -3-glucoside	Orange juice (1L)	71 mg Cy-3-glc	0.002	-
Malvidin 3-glucoside	Red wine (500mL)	68 mg Mal-3-glc	0.001	0.016 6h
Flavanols				
Epigallocatechin gallate	Green tea infusion (5gr)	105 mg	0.13-0.31	
Catechin	Red wine (120mL)	34 mg	0.072	
Epicatechin	Chocolate (80 g)	137 mg	0.26	
Catechin	Pure compound	0.36 mg/kg bw	0.14-0.49	1.2-3
Epigallocatechin Gallate	Pure compound	50-1600 mg	0.28-7.4	
Epigallocatechin	Green tea extract	110-328 mg	0.26-7.4	

gallate				
Procyanidin B1	Grape seed extract	18 mg	0.011	
Flavanones				
Hesperidin	Orange juice	61 mg	0.48	4.1
Naringenin	Grape fruit juice	199 mg	5.99	30.2
Hesperetin	Pure compound	135 mg	2.7	3.8
Flavonols				
Quercetin	Apples	107 mg	0.3	3.5
Quercetin 4'glucoside	Pure compound	100 mg	7.0	4.5
Quercetin	Buckwheat tea	200 mg	2.1	1.0
Isoflavones				
Daidzein	Soy milk	108 mg	0.47	37.3
Genistein	Soy milk	102 mg	0.41	20.2
Daidzein	Pure compound	50 mg	0.76	
Glycitein	Pure compound	25 mg	0.72	

Table 1. Polyphenols bioavailability and food source based on [26]

Table 1 presents the estimation of daily dietary intake of individual classes of polyphenols and their maximum concentration in plasma and the % of urinary excretion. It appears that polyphenols intake depends on a large extent dietary habits and preferences. Most of data polyphenol content in food originates from scattered sources. The intake of flavonols, flavones and isoflavones is relatively low compared with that of phenolic acids and other flavonoids, such as proanthocyanidins, anothocyanins and oxidized polyphenols.

Be should know that one of the richest food sources of flavonols are onions, curly kale, leeks, broccoli, apples, and blueberries [14],moreover red wine and tea can also contain a significant amount of flavonols [33, 35]. The consumption of compounds such as quercetin and genistein does not exceed 2-4% of the total polyphenol dietary intake in Western diets [15]. By the other hand an average of 58 mg proanthocyanidin/day [36] is consumed by American population.

1.2.5 Cardioprotective and anti obesity effects of dietary polyphenols

In general terms, polyphenols are common constituents of the human diet, present in most foods and beverages of plaint origin. The assumption that polyhenols, has cardiovascular and anti obesity effects became originally from *in vitro* studies, showing the antioxidant properties, and posteriori with a number of epidemiological studies that related the beneficial effects of polyphenol consumption or polyphenol-rich foods with the risk of cardiovascular diseases and that a moderate wine consumption has consistently association with a reduced risk of cardiovascular diseases[26, 30, 37].

Although, the direct scavenging of free radicals by polyphenols, has often been suggested, that it is not the key mechanism explaining their effects on oxidative stress biomarkers and cardiovascular risk factors [30]. In animal models, enrichment of food with polyphenols is effective against preadipocyte proliferation and adipocyte hypertrophy [38]. The health benefits of consuming polyphenols have been mainly studied in anthocyanidins/anthocyanidines, flavanols and stilbenes (resveratrol) as a part of French Paradox [39]. It could be a positive effect of these molecules to attenuate complications of obesity-induced oxidative stress such as chronic inflammation and insulin resistance, and thus to preserve public health in a therapeutic perspective [40], that interfere with a large number of biochemical signaling pathways and therefore with physiological and pathological processes [30]. Flavonoids exert beneficial effects including anti inflammatory, antioxidant, and antiproliferative

effects. There is an evidence that daily intake of some fruits such as three apples or three pears, or grapefruit (as fresh grapefruit or grapefruit juice or grapefruit capsule), can significantly reduce body weight by 10 or 12 weeks in overweight and obese people independently of the fruit's fiber amount. Moreover other evidence suggest that a long-term (12 weeks) administration of tea catechins in a dose of 400-700 mg/day to Asian subjects reduced body fat and body fat parameters [41]. Matsuyama et al. demonstrated that the daily consumption of 340 ml tea containing 576 mg catechins for 24 weeks reduced body fat ratio and waist circumference compared to control group, who had a daily consumption of 340 ml tea with 75 mg catechins [41]. In our studies, concretely be will focus on epigallocatechin-3-gallate (EGCG) and grape seed proanthocyanidins extract (GSPE); their cardio protective effect will be detailed below.

1.2.5.1 EGCG

EGCG belongs to flavanols and flavonoid subclass. Flavonoids which exist in plants primarily in glucoside forms, as we mentioned above, flavanols are usually present in the aglycone form as monomers, oligomers or esterified with gallic acid to form epigallocatechin (EGC) epicatechin gallate (ECG), and EGCG **[33]**. Catechin and epicatechin are present in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, grapes and more importantly, in tea [32].

Worldwide, tea is a widely consumed beverage which three principal types: green tea, oolong tea and black tea. Green tea contains high levels of polyphenols, which may have a number of positive health effects in the prevention of lifestyle-related diseases [6]. Over the past 10 years, there have been a number of mostly small trials in humans showing favorable effects of green tea catechins (270 to 1200 mg/day) on body weight [42]. Adipose tissue, skeletal muscle, intestine are target organs of green tea, median anti obesity effects [43]. Habitual consumption of green tea extract has been reported to reduce body weight and body fat; this may occur via increased lipolysis in adipose tissue[44, 45] and decreasing lipid blood levels [43, 46]. Also, it was reported that green tea has physiologic effects *in vivo*, decreasing risk for obesity [47, 48]. EGCG may control obesity-associated adipose tissue through mitochondrial remodeling [49], diabetes [50], hypetension [51], dyslipemia[52] and CVD in several epidemiological in selected clinical trials [53]. Green tea supplementation has been shown that significantly improves features of metabolic syndrome such as decreased abdominal adiposity [54], inhibition of inflammation, regulation of nitric oxide, stimulation of specific signal transduction pathways [55] and modulation of other cellular processes such as apoptosis [56]. The antioxidant activities of EGCG are due to the presence of phenolic groups that are sensitive to oxidation and can generate quinine [57]. The antioxidative capacity is further increased by the presence of the trihydroxyl structure in the D ring of EGC [56]. By the other hand EGCG is a powerful radical scavangers, protects from the oxidative damage induced by a commonly used pro-oxidant such as tert-butylhydroperoxide [58]. It had been reported that EGCG can reduced the cytotoxcity evoked by H_2O_2 and increase the levels of enzymes related to the oxidative stress [59].

1.2.5.2 Proanthocyanidins

Grapes and their sub products are consumed worldwide. GSPE is a combination of biologically active polyphenolic flavonoids including oligomeric proanthocyanidins. It is very difficult to value the proanthocyanidins content of foods because proanthocyanidins have a wide range of structure and molecular weights: for example in cider apples, the degree of polymerization ranges from 4 to 11. Proanthocyanidins are responsible for the astringent character of fruit (grape, apples, berries, etc) and beverages (wine, cider, tea, beer, etc) and for the bitterness of chocolate. It is important to note that this astringency changes over the course of maturation and often disappears when the fruit reaches ripeness [32].

It has been demonstrated to exert novel spectrum of biological, pharmacological, therapeutic, and chemoprotective properties against oxygen free radicals and oxidative stress [60, 61]. Apart from that, It have been reported that promote health and decrease CVD risk referred at the French Paradox with red wine is consumed [28, 38]. Moderate red-wine consumption partially prevents body weight gain in rats fed hyperlipidic diet. By the other

hand there are many studies that reported also beneficial effects in anti-inflammatory diseases, insulin resistance [20], Alzheimer [28, 62] and lipid metabolism [60, 63]. A diet rich in polymeric grape tannins proved to lower plasma total cholesterol, triacylglicerides (TGs), low density lipoprotein (LDL) cholesterol concentrations and tends to increase plasma high density lipoprotein (HDL) cholesterol levels in rats more than a diet rich in monomeric catechins cita [64].

1.3 Polyunsaturated fatty acids (PUFAs)

Omega-3 PUFAs are dietary compounds that have demonstrated benefit in health and cardiovascular system. Among populations in which total fat intake is considerably >30% of total energy consumed, where the primary soruce of fat consumed is fish and plant oil, mortality from CVD is low [13, 65-67]. The interest of these fatty acids became when in the 1970s researched discover that the Greenland Eskimos consume diet high in fat and they had a low rate of coronary heart disease. Their research provided the impetus for numerous other studies that have resulted in major recommendations from some organizations to increase the intake of fish oil [68, 69]. Since that it has been identified populations residing in Mediterranean regions by a longitudinal cohort and cross-sectional ecologic studies, that significant reductions in mortality due to myocardial infarction, ischemic heart disease, stroke, sudden cardiac death, and total CVD can be attributed to consumption of fish and other dietary sources omega-3 PUFAs [65]. Epidemiologic studies have shown an inverse relation between the incidence of cardiovascular disease and the consumption of fish oil [70]. Moreover omega-3 PUFAS exhibit anti-inflammatory, antithrombotic, antyarrhythmic and vasodilatory properties, and some of these effects are being modulated through prostaglandins and leukotriene metabolism [71]. So, Interest in dietary intake of marine PUFAs, is due to, especially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

1.3.1 Classification

Numerous fatty acids are synthesized by the human body, and these are known as nonessential fatty acids. Omega-3 PUFAs and omega-6 PUFAs must be obtained from diet because some of them cannot be synthesized *de novo*, called as essential fatty acids [68, 72, 73].

We should know that fatty acids have a carboxylic acid with a long aliphatic tail (the chain) could be either saturated or unsaturated. They could be classified depending on the double bond and the length of the chain fatty acids (Figure 6). Most naturally occurring fatty acids have a chain of an even number of carbon atoms, from 4 to 28. The numbers refers to the location of the first double bond in these unsaturated fatty acids, counting from the carbon on the methyl end of the compound [68,74]. As we can see in the Figure 6 saturated fatty acids derived from animal sources and monounsaturated fatty acids from vegetal oil sources as olive oil and canola oil.

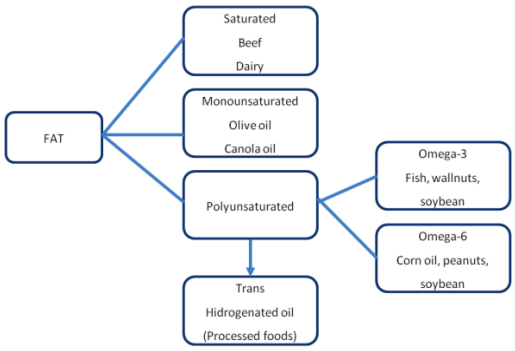


Figure 6. Classification of fat based on [74]

1.3.2 Chemical structure

In the next table 2, we can see the chemical structure of the omega fatty acids and their precursors with their main biologic effects.

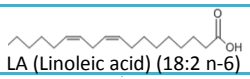
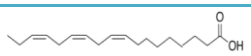
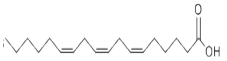
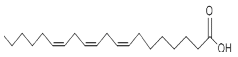
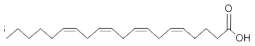
	Omega-6 Fatty acids	Omega-3 Fatty acids
Biologic Effects		
Lowers cholesterol	 LA (Linoleic acid) (18:2 n-6)	 ALA (α-Linolenic acid) (18:3 n-3)
Stearidonic acid (18:4) n-3	 Y-Linoleic acid (18:3 n-6)	 Stearidonic acid (18:4 n-3)
1-Series prostaglandins (anti-inflammatory)	 Dihomo- Y- Linolenic Acid (20:3 n-6)	 20:4 n-3
2-Series prostaglandins 4-series leukotrienes (platelet aggregation, vasoconstriction, proinflammatory)	  AA (Arachidonic acid) (20:4 n-6)	 EPA (Eicosapentaenoic acid) (20:5 n-3)  DHA (Docosahexaenoic acid)

Table 2. Chemical structures, precursors and biological effects of omega fatty acids based on [65]

The long chain 20- and 22-C fatty acids between each class, arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are biologically more active than the 18-C fatty acids, as linoleic acid (LA) and α-linolenic acid (ALA), which serve primarily as substrates for synthesis of the longer-chain, with more highly unsaturated counterparts.

1.3.3 Metabolism

Animals and human subjects cannot synthesize PUFAs which contains double bonds at C-6 and C-3 from the methyl end of the molecule. The metabolic utilization of omega-3 fatty acids differs from omega-6 fatty acid metabolism. Both types of fatty acids are substrates for lipid mediators and are incorporated as structural components, to form the lipid bilayers of cell membranes; however, the structural differences between these classes of fatty acids that determine the specific properties of the lipid mediators and cell membranes are associated with each [65]. The primary precursors of omega-3 essential fatty acids are ALA; in the case of omega-6 essential fatty acid is the LA [68] .

Figure 7 shows the most significant steps processing LA and ALA to their higher unsaturated derivates (AA, EPA, DHA) by the consecutives desaturation and elongation reactions. The conversion of ALA to EPA and DHA occurs primarily in the liver. LA and ALA compete for the enzyme Δ6 desaturase, which is required for further metabolism.

Eicosanoids, are a class of bioactive molecules derived from omega-3 and omega-6 that include leukotrienes, prostanglandines and thromboxanes. Eicosanoids derived from omega-6 are generally proinflamatory and proaggretory and can also increase blood preasure, heart rate and immune response, whereas those derived from

omega-3 are predominantly anti-inflammatory, inhibit platelet aggregation and have benefic effects, opposite to omega-6 [74,65,68] (Figure 7).

It is important a balance between omega-3 and omega-6 fatty acids intakes because compete for the same enzyme systems involved in elongation and desaturation to synthesize the long-chain, to unsaturated and more biologically active fatty acids. They also compete for cyclooxygenase, lipoxygenases involved in the production of prostaglandins and lekotrienes that mediate a range of cell functions important to cardiovascular function [68].

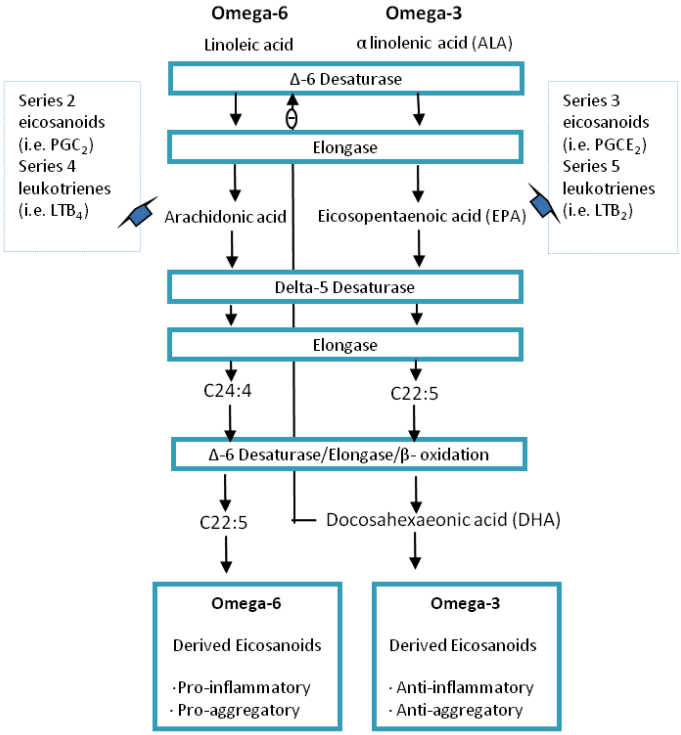


Figure 7. Metabolic pathway of omega-6 and omega-3 fatty acids based on [74]

Excessive intake of LA or ALA increase production of AA slows down the formation of EPA and DHA. Compensation for this imbalance can be achieved in part by increasing consumption of EPA and DHA, but this does not completely satisfy the biologic requirements for omega-3 fatty acids, indicating that ALA has important biologic effects that are independent, being a precursor for EPA and DHA. Each of these fatty acids have different metabolic role, with DHA preferentially, taken up by cell membranes and EPA utilized as a substrate for eicosanoid synthesis.

So, it is very important to establish an appropriate quantity and ratio intake of omega-3 to omega-6 PUFAs in diet, because are determinant of human health. In modern Western diets, the ratio of omega-6/omega-3PUFA increase to 15:1 to 25:1, which may have contributed to the prevalence of the chronic diseases. Traditional diets beings involved on PUFA-rich diet in which the ratio of omega-6/omega-3PUFA is about 1-2:1 to promote brain evolution[72].

1.3.4 Food source, bioavailability and dietary intake

The most prominent omega-6 fatty acids in the human diet are arachidonic AA (animal and meat) and LA (found in vegetable oils, seeds, and nuts) as we can see in table 3, which can be converted into AA acid by a desaturase

enzyme. Major dietary sources of omega-3 are fish containing EPA and DHA. By the other hand nuts, seeds, and vegetable oils have ALA, which can be converted to EPA, and then DHA by the same desaturase enzyme that converts LA acid to ARA acid [74,68]. EPA and DHA can be produced by single celled marine organisms that fish and shellfish regularly consume in their diets. Also, some fish and shellfish, such as herring, mackerel, salmon, sardines or tuna content it. DHA sources, also, are being produced by some algal and fungal sources, that have been cultivated and commercialized.

Product	LA	ALA	AA	EPA + DHA
Omega-6 fatty acid rich food				
Corn oil	50000	900		
Soybean oil	53400	7600		
Sunflower oil	60200	500		
Margarine	17600	1900		
Lard	8600	1000	1070	
Bacon	6080	250	250	
Ham	2480	160	130	
Soya vean	8650	1000		
Peanut	13900	530		
Wallnut	34100	6800	590	
Omega-3 fatty acid rich food				
Canola oil	19100	8600		
Linseed oil	13400	55300		
Herring	150	61.66	36.66	1700
Salmon	440	550	300	1200
Trout	74		30	500
Tuna	260	270	280	400
Cod	4	2	3	300
Data reported as mg/100g Content of omeg-6 and omega-3 fatty acids may slightly vary according to species, sources and analytical methods.				

Tabla 3. Dietary sources of PUFAs selected food base on [75]

Dietary sources of omega-6 PUFAs are abundantly present in liquid vegetable oils, including soybean, corn, sunflower, safflower oil, cotton seed oils, while linseed and canola oils are rich in omega-3 PUFAs (Table 3).
Human's studies recommended doses of 500 mg/day for individuals without underlying over CVD and at least 800-1000 mg/day for individuals with known coronary heart disease (CHD) and heart failure [67] which is traduced with two oily fish meals per week. Recently, The Food and Drug Administration (FDA) approved omega-3 PUFAs at dose of 4 g/day to treat very high triglycerides (TGs) levels (≥500mg/dl) in aim to reduce 30% to 40% of blood levels [76].

Different recommendations by American Heart Association (AHA) have been postulated dependent if population has documented CHD (Table 4).

AHA suggest that suggests that patients with CHD a supplementation in humans of 3 g/day of PUFAs fish oil may be appropriate at least with respect to its ability to modify the lipid profile and to incorporate these fatty acids into plasma phospholipids. Furthermore, patients without documented CHD, consumption of fatty fish at least twice a week would be optima (Figure 7).

Population	Recommendation
Patients without documented CHD	Eat a variety of (preferably fatty) fish at least twice a week. Include food and oils rich in ALA in your diet
Patients with documented CHD	Consume approximately 1 g of EPA + DHA (3g of fish oil) every day, preferably from fatty fish. EPA + DHA (fish oil) supplements could be considered in consultation with a physician.

Table 4. Summary of American Heart Association recommendation for omega-3 fatty acid intake [74]

It is important to consider, that high levels of fish, or oily fish could contain contaminants, methyl mercury. FDA advised children and pregnant avoid fish with a potentially high content of mercury such as swordfish, tile fish, king mackerel, and shark. Salmon, sardines, trout, oysters and herring, are quite low in mercury. Although there are some pregnant studies with an excess of fish intake recommendation; by offspring have better cognitive and behavioral development that others who consumed less fish [67]. The reason is because mercury is water soluble and protein bound, so mercury is present in the muscle of the fish but not in the oil. So, fish oil supplements should contain negligible amounts of mercury [77].

1.3.5 Cardioprotective and anti obesity effect of dietary PUFAs

Western diet has a greater risk for develop obesity and their associate's diseases. Fat are essential nutrient, but also the type and amount of fat ingested can have dramatic effect on health, being important to control these terms. As we mentioned before, in most of the populations examined, reduction of CVD mortality have been associated with fish consumption. High intakes of omega-3 fatty acids are characteristic of fish-consuming populations, attention has largely focused on the benefits of EPA and DHA to explain the protective effects on CVD risk improving dyslipemia, insulin resistance, impaired glucose homeostasis, diabetes and obesity observed in several meta-analysis's and interventional studies [66-68, 71]. The regular consumption of variety of omega-3 fatty acid sources- including fish oils, nuts and soybean oil, that provide concentrated amounts of purified EPA and DHA has been demonstrated to be inversely associated with CVD mortality or promoted significant reductions 30% to 60% in CVD mortality [65]. Focus in omega-3 tends to reduce fasting and postprandial triglyceride levels by 20% to 35% by suppressing hepatic very low density lipoprotein-TG (VLDL-TG) production and accelerated clearance of chylomicron TGs. Also there is a slightly increase in HDL [68].Types of fat in the diet also, determine the relative composition of biomembranes, and PUFAs are indeed substrates for free radical reactions leading to lipidperoxidation [78]. Lipidperoxidation is an important event for reactive oxidative species (ROS), oxidative metabolism and alterations in food constituents or fuel energy generation, may be related to oxidative stress. Therefore, oxidative stress not only may be associated with unsaturated fatty acid content in biomembranes, also may be dependent on metabolic pathways shifting for energy production [70]. Although fatty acids are classically observed as an energy substrate, they are also endogenous ligand for peroxisoe proliferator activator receptors (PPARs) and regulate the expression of genes encoding key proteins controlling fatty acid uptake and metabolism [67].

The mechanism for the mentioned lipid lowering effects seems to involve activation of gene transcriptions via nuclear receptor such as PPARs, liver X receptors (LXRs) and sterol regulatory element binding protein (SREBP1c), controlling the expression of the genes involved in hepatic and skeletal muscle fatty acid oxidation, glucose metabolism and adipogenesis [71] such as carnitine palmitoyl trasnferase-1 (CPT1) acyl-CoA oxidase[7 9], lipoprotein lipase (LPL), fatty acid syntase (FAS), acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase-1 (SCD1) [80] .Metabolic effects of PUFAs are mediated by stimulation of 5'-activated protein kinase (AMPK), a metabolic sensor controlling intracellular metabolic fluxes. Thus, omega-3 PUFAs modulate the functions of all major tissues involved in the development of metabolic syndrome in liver, adipose tissue, and skeletal muscle by multiple mechanism of action. In adipose tissue the incorporation of omega-3 PUFAs indicated an induction of mitochondrial biogenesis adipocytes, adiponectin secretion an amelioration of adipose tissue inflammation [81].

The intake of omega-3 PUFAs apart from lower the risk to CVD and mortality, improve colorectal cancer and breast cancers, rheumatoid arthritis [82], neurosensory organs and depression [83]. In addition, it has been proposed that omega-3 PUFAs must be involved in hormone (eicosanoid) production [84], and they are essential for normal growth and development[68,71]. In omega-6 PUFAs they are some concern over excess consumption of omega-6 PUFAs for their proinflammatory and proaggregatory effects [79], so for that reason has we mentioned before, it important establish optimal ratio of omega-3: omega-6 to promote healthy diet.

1.3.5.1 DHA

As we are going to focus our studies concretely in oil rich in DHA, a briefly description will be made. As we mentioned above, DHA is an essential fatty acid of omega-3 series, the chemical structure is made of 22 atoms of carbon, with 6 double bonds spread along the structure [85]. It is the longest chain and most unsaturated fatty acid commonly found in biological systems. In mammalian species, a part from be available in reasonable quantities in fish, it could be available in ever greatened percentages in oils derived from these animals, and some micro-algae eaten by fish as we mentioned previously. Also, it can be found in small quantities in meat especially when the animals were fed with fish meal or linenseeds [11].

It has been reported a variety of positive effects in some human diseases: cancer [86], heart disease [87], brain development [88], reduce plasma TGs content, obesity [89], reduce risk of CVD [76]. DHA has been shown to be rapidly incorporated into a variety of cells, primarily into phospholipids of the plasma membrane and mitochondria [90] and guaranties the permeability and functionality of cellular membranes [85] .

1.4 Mitochondria

Mitochondria are small, vesicular organelles involved in many metabolic processes. The main function of mitochondria is to transform the chemical energy derived from food and body stores into ATP by a process called oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC) (Figure 8). Mitochondria are unique organelles with a two-layer membrane structure that separates the organelle into four compartments: the outer membrane, intermembrane space, inner membrane, and matrix. The internal aqueous compartment is called the matrix, which contains approximately two-thirds of the total protein in the mitochondria and is also the compartment in which the mitochondrial genome is contained. The matrix contains several enzymes involved in the tricarboxylic acid (TCA) cycle and is the site at which the majority of biochemical reactions occur[89]. The matrix is enclosed by a highly folded, insulating membrane called the inner membrane, which contains the enzymatic machinery of OXPHOS. The inner membrane is separated from the cytosol by a more permeable outer membrane, which is highly permeable to most molecules and contains proteins that perform OXPHOS, protein import and substance exchange. The aqueous compartment between the inner and outer membrane is called the intermembrane space[91] which contains proteins that play major roles in mitochondrial energetic and apoptosis, most notably cytochrome c. Mitochondria possess their own genome, called mitochondrial DNA (mtDNA),that encodes the 37 genes necessary for the assembly of the OXPHOS machinery [92]. Most of the approximately 1500 other mitochondrial proteins are encoded by nuclear genes, translated in the cytoplasm and then imported into

the mitochondria. Mitochondrial function depends on the assembly, maintenance and dynamics of the organelle. Emerging evidence suggests that cellular events that occur outside of the mitochondria are tightly coupled to gene expression patterns within the organelle [93]. The organelles move around the cell by means of the cytoskeleton, and they also continually undergo fission and fusion, which are intimately linked to apoptosis and the removal of dysfunctional mitochondria [94].

Mitochondrial biogenesis and turnover are regulated and coordinated by multiple transcription factors and transcriptional coactivators, notably peroxisome proliferator-activated receptor-ϒ coactivator-1α (PGC-1α)[95], which enable mitochondria to respond to long-term alterations in metabolic demands.

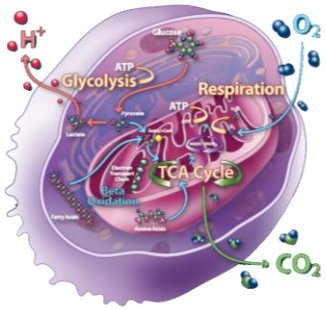


Figure 8. Mitochondria

The main reactions (Figure 9), included in the matrix are TCA cycle, and enzymes involved in fatty acid breakdown (β-oxidation). The TCA cycle oxidizes acetyl-coA to CO_2, generating GTP, NADH and $FADH_2$. Besides providing electron donors for OXPHOS, the TCA cycle is critical for several metabolic functions, where its intermediates are used as substrates for *de novo* synthesis of biomolecules: Succinyl-CoA for synthesis, 2-oxoglutarate for the amino acids glutamate, glutamine, proline, and arginine, oxalacetate for the amino acids aspartate and asparagines and citrate for fatty acid synthesis. Also, TCA cycle serves a role in catabolism where amino acids are degraded to TCA cycle intermediates [96, 97].

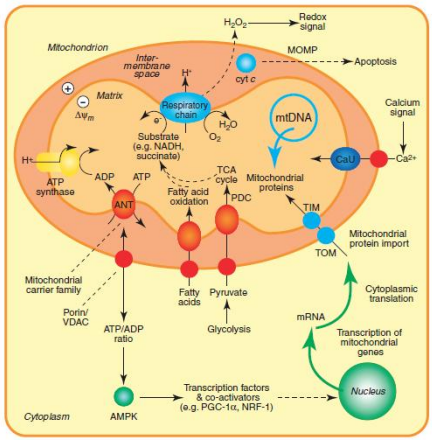


Figure 9. Main mitochondria reactions based on [98]

1.4.1 Electron transport chain and Oxidative phosphorylation

OXPHOS, is a key functional unit in mitochondria, and combine electron transport with cell respiration and ATP synthesis. It not only produces the base majority of cellular energy, but is also involved in radical production and apoptosis [99]. In order to produce energy to the organism, mitochondria carried out to produce ATP through the oxidation of carbohydrates, fats and amino acids by the OXPHOS system. As we can see in the Figure 10, OXPHOS occurs via ETC, located within the mitochondria inner membrane and it is composed with 4 different complexes as we can see below; complex V that produce ATP it is not takes part of ETC, although is a component of OXPHOS system [100].

- Complex I (NADH ubiquinone oxidoreductase)
- Complex II (Succinate ubiquinone oxidoreducate)
- Complex III (Ubiquinone cytochorme c oxidoreductase)
- Complex IV (cytochrome c oxidase) (COX)
- Complex V (F_1F_0-ATP synthase)

The electrons passed through the mitochondrial respiratory chain to drive ATP synthesis by oxidative phosphorylation or coupled respiration. The electron moves down energy gradient from NADH/FADH to oxygen, redox energy is conserved by pumping protons across the inner membrane to build up an electrochemical gradient [89, 100]. OXPHOS activity is limited by the availability of the substrates for ATP synthesis, ADP and phosphate. Thus, OXPHOS activity increases when ATP utilization generates ADP and phosphate [99].

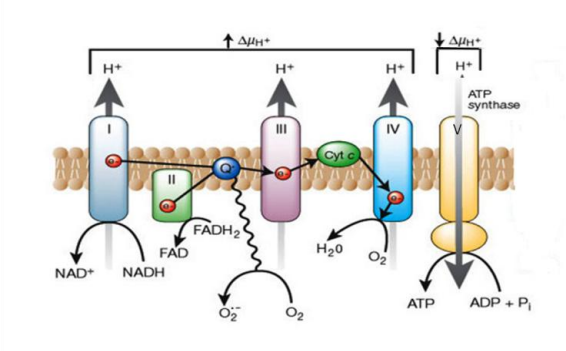


Figure 10. OXPHOS system based on [101]

1.4.2 Efficiency

Control of mitochondrial respiration and OXPHOS is fundamental for the maintenance of cellular homeostasis. At cellular levels, mitochondria are the major determinant of energy transduction efficiency. The main determinant of OXPHOS efficiency is represented by the degree of coupling between oxygen consumption and ATP synthesis [102].

TCA cycle and OXPHOS are critical for cellular metabolism, so different anabolic and catabolic functions of mitochondria must be tightly regulated in response to nutrients, such as carbohydrates, amino acids and fatty acids [96] to obtain energetic sources. Several requirements are essential to allow efficient OXPHOS; impermeability of the mitochondrial inner membrane to protons is a key condition to insure the coupling of the respiration to ATP synthesis. Nonetheless, it is known [103] that the inner membrane is at least partly permeable to protons, especially at high membrane potential. Free permeation of protons (proton leak) across the inner membrane decreases the proton availability for the ATP synthase, and thereby, affects the yield of ATP synthesis per oxygen consumed.

So, the efficiency of the mitochondrial machinery depends on the presence of basal proton leak pathway and futile cycling protons across the inner mitochondrial membrane, which has been reported to account for about 20% of

resting metabolic rate in rats [104]. Mitochondrial energy efficiency regulated in skeletal muscle plays a direct thermogenic role, based on fatty acid-induced mild uncoupling of mitochondrial oxidative phosphorylation[105]. Specific proteins present in the mitochondrial membrane such as uncoupling protein 3 (UCP3) that may facilitate back-leak-age of protons and thus decrease the efficiency of oxidative phosphorylation. UCP3 expression is increased during high fatty acids plasma levels conditions), such as during starvation or after high-fat diet [106].

One way to reduce mitochondrial oxidation of energy substrates is to increase the efficiency of oxidative phosphorylation. A higher efficiency implies that less substrate is needed to burn and obtain ATP [102]. The number, the structures and functions of mitochondria differ in animal cells and tissues, in relation to the energetic needs, and they can vary during development and differentiation in response to physiological or environmental alterations. Mitochondrial proliferation occurs in response to electrical muscle stimulation, following training exercise and during thermogenesis adaptation in rodent brown fat [97]. The mitochondrial malfunctioning is related to aging and to the onset of many diseases, including cancer.

Metabolic imbalance of nutrient signal input, energy production and/or oxidative respiration results in "mitochondrial dysfunction", where WAT play the major role in regulating energy intake, energy expenditure, and insulin resistance [107]. For example excessive energy substrates, typically occurring in situations of obesity and metabolic syndrome, may lead to mitochondrial dysfunction with consequential effects on lipid and glucose metabolism. Moreover abnormal mitochondria function through increased ROS production in adipocytes results in lipid accumulation and insulin resistance [108].

1.4.3 Metabolic regulation via nuclear receptors

Transcriptional regulation

Several regulatory circuits might exist in response to different physiological stimuli or by the activation of different regulatory pathways for the expression of different groups of genes [97].

The expression of many proteins of the OXHPOS complexes, like COX (the terminal component of mitochondrial respiratory chain), is regulated at transcriptional level through specific nucleus-encoded factors. The target genes of nuclear receptor factors (NRFs) encode subunits of the OXPHOS complexes or proteins involved in the expression, assembly, and function of the complexes. Moreover, NRF-1 seems to be related to the expression of mitochondrial cytosolic enzymes of the heme biosynthetic pathway, and with components of the protein import and assembly machinery, suggesting that it plays a role in nuclear-mitochondrial interactions. By the other hand NRF-2 regulates the transcription of mitochondrial encoded COX subunits [109]. The PGC1α family plays a critical role in the control of tissue specific biological processes and in the regulation of mitochondrial oxidative metabolism. PGC1α co-activators are highly versatile, interacting with different transcription factors that directly regulate the expression of certain nuclear genes for mitochondrial products, these include NRF-1 and NRF-2, whose genes, are themselves targets of PGC1α. By the other hand PGC1α and β stimulate biogenesis of mitochondria with different metabolic characteristics, by modulating the relative activity of these two coactivators, cells may achieve fine-tuning of mitochondrial function in response to specific metabolic needs [97]. So, either transcriptional factors or the nuclear co activators goes head the programs of expression of both genomes, essential to cellular energetic, therefore they can be considered main players of the communication between nucleus and mitochondria.

Post-transcriptional regulation

Increasing evidences, demonstrate the importance of mRNA localization, stability, and translocation regulation in the control of gene expression. In both, developmental and differentiated cells, such regulation mostly relies upon the activity of RNA-binding proteins [97].

Different post-transcriptional mechanism operates in the regulation of mitochondrial biogenesis and activity. Future investigation efforts should be devoted to the understanding the relationship between the components of these regulation systems.

1.4.3.1 PGC1α

PGC1α is one of the three members of a gene family of transcriptional co-activators that modulate mitochondrial function by regulating cellular energy metabolism including mitochondrial biogenesis, thermogenesis [110], fatty acid oxidation [111], and also has been shown to exert profound effects on glucose metabolism, by upregulating mitochondrial biogenesis and muscle glucose transport [112]. It has been reported that PGC1α is expressed in many tissues as liver, heart, kidney, white adipose tissue, and skeletal muscle [113], where mitochondria are abundant and oxidative metabolism is active. It is highly likely that PGC1α is involved in disorders such as obesity, diabetes and cardiomyopathy, in concretely in lipid metabolism [114].

Although there are three members of PGC1 family (PGC1α, PGC1β, and PGC1 related coactivator) [110], there a special interest in PGC1α because there is strong evidence that is a potent regulator of energy metabolism under health and disease conditions [115]. By the other hand PGC1α has two putative nuclear localization signals and located in the cell nucleus [116].

Focusing in the adapatative thermogenesis, PGC1α was originally discovered as a cold-inducible transcription coactivator. Brown adipose tissue (BAT) and skeletal muscle are the two major organs involved in this process. The adapatative thermogenic program in both, involves the stimulation of mitochondria biogenesis, increase FA oxidation, and uncoupling of OXPHOS. By the other hand, an increase of PGC1α, induces the transcription of NRF-1 and NRF-2 leading to the increased expression of mitochondrial transcription factor (mtTFA) [113]. In BAT, PGC1α also interacts with other nuclear hormone receptors such as PPARα, retinoic acid receptor, and thyroid receptor to enhance the expression of brown fat specific UCP1 increasing heat production and rate of energy metabolism [110].

1.4.3.2 PPARs

PPARs are members of the nuclear receptor superfamily that function a master regulator of mitochondrial β-oxidation [111] and fatty acid-activated transcription factors. There are three related PPAR family members: PPARα, PPARy and PPARδ with different ligand specificities, and tissue distributions. Evidence supports a link between PPARs, diabetes, obesity, dyslipemia and inflammation [117].

PPARα was the first PPAR to be identified, is highly expressed in liver, BAT, kidney and skeletal muscle, where controls peroxisomal and mitochondrial fatty acid catabolism as a result of upregulation of genes involved in lipid transport, fatty acid β-oxidation, and ketogenesis [117]. For example it has been shown that obese mice treated with PPARα agonist also improved insulin resistance, and decreased the fasting blood glucose level [118]. Furthermore PPARα could be activated by endogenous long-chain saturated and unsaturated fatty acids ligands. In particular DHA and EPA from fish oil, whose beneficial effects had been well studied, including enhancement of fatty acid oxidation throughout a PPARα stimulated process [119], and suppression of hepatic lipogenesis via suppression of nuclear abundance, and expression of SREBP-1c [120]. Moreover, preclinical data suggest a role for PPARα in body weight control, supporting the use of PPARα against obesity treatment [117].

PPARy is abundant in adipose tissues, as a key transcriptional factor for adipogenesis, although it is also expressed in skeletal muscle, liver, pancreatic β-cells, heart, colon, retina, placenta and immune systems [119]. PPARy, also plays an indispensable role in the regulation of adipocyte differentiation, lipid storage, glucose metabolism, and the transcriptional regulation of a number of genes involved in the metabolic processes [121]. Moreover, PPARy agonist attenuates hyperlipidemia-induced elevation due to circulating free fatty acids (FFA), the lipotoxic accumulation of lipid in peripheral tissues and insulin resistance. Promoting increased FFA for storage in WAT, reduce ectopic lipid accumulation particularly in liver and skeletal muscle, and regulate the expression of the

adipokines and inflammatory cytokines that impact hepatic and skeletal muscular glucose metabolism and whole-body insulin sensitivity [122].

There are two isoforms of PPARϒ1, PPARϒ2 present in colon, retina, spleen, hematopoietic cells, liver and skeletal muscle, and PPARϒ2 present also in adipose tissue. Both of them can respond to the same signals: LPL, acyl-CoA synthetase (ACS) and cluster of differentiation (CD36). In muscle the loss of PPARϒ had 80% reduction insulin-stimulated glucose disposal rates, that did not improved with thiazolidinedione treatment[119].

PPARδ is expressed in many tissues including liver, kidneys, cardiac and skeletal muscle, colon and BAT [123]. In rodents treatments with potent agonist, attenuated body weight gain, insulin resistance, and intracellular accumulation of lipid TG in skeletal muscle, liver and adipose tissue increasing expression of skeletal muscle genes involved in lipid catabolism, mitochondrial uncoupling and fatty acid β-oxidation [124] .

1.4.3.3 Nuclear respiratory factors (NRFs)

NRF-1 was first identified as a factor binding of the cytochorme c promoter, and NRF-1 binding sites are found in the promoters of multiple genes encoding mitochondrial proteins, including most subunits of the respiratory chain complexes [96]. Little is known whether NRF-1 functions as a link between nutrient availability and mitochondria [96, 111]. In mammals, NRF-1 and NRF-2 are not the only factors that are targeted by PGC1α for their effect on mitochondria. The nuclear receptor ERRα (estrogen related receptor alpha) plays an additional important role to control mitochondria, in response to external stimuli.ERRα is co-activated by PGC1α through direct binding. Moreover, ERRα and NRF-2 were shown to function in a double positive-feedback loop: Both factors were induced by PGC1α expression, and ERRα stimulated NRF-2 expression and vice versa. Strikingly, it had been demonstrated that ERRα inhibitor could counteract the PGC1α effect on mitochondria, demonstrating a functional link between the two proteins [96, 111].

1.4.4 AMP-activated protein kinase (AMPK)

AMPK is present in all tissues as a heterotrimeric complex consisting with a catalytic α subunit, regulatory β and γ subunits. Both β and γ are required for optimal activity of α-catalytic subunit [125-127]. Depending on the muscle fiber type composition, multiple mammalian isoforms of the three subunits exist; all subunits are differentially expressed in rodent and human skeletal muscle [126]. In the case of human skeletal muscle, the majority AMPK complexes contain both α2 and β2 subunits [128].

Reversible phosphorylation at Thyrosine (Thr172) within the activation loop of the α-subunit is the most potent activator of AMPK [125, 128] .

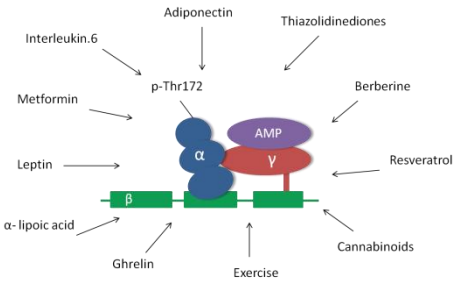


Figure 11. AMPK activators based on [129]

A number of hormones and natural compounds have been reported to activate AMPK by an indirect manner (Figure 11). Changes in mitochondrial uncoupling and cellular energy state could account for mechanism leading to cellular AMPK activation [129].

Signaling cascade is sensitive to many stimuli, such as cell stress, oxidative damage, hypoxia, glucose deprivation, AMP/ATP ratio increased [128], adipokines, exercise and pharmacological substances. Once activated, AMPK induces an up-regulation of ATP-generating systems (fatty acid oxidation, glycolisis and mitochondrial biogenesis) and stimulate mitochondria biosynthesis, while simultaneously down-regulate process that consumes energy (fatty acid synthesis and gluconeogenesis). AMPK signaling pathway exerts beneficial effects against diseases, such as diabetes, obesity, cardiac hypertrophy and cancer [130]. Previous research had been shown that AMPK regulates numerous proteins including Sirtuin (SIRT), Ciclooxigenase-2 (COX-2), SREBP, PPAR, Protein kinase B (Akt), UCPs [126] associated with diseases.

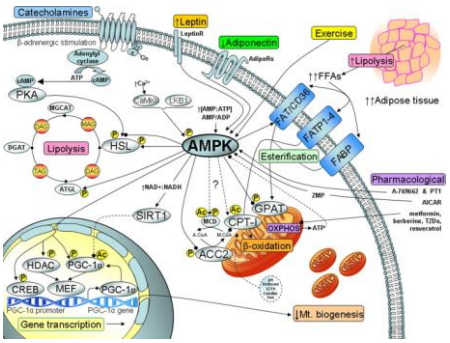


Figure 12. Schematic diagram of lipid metabolism and regulation of AMPK pathway [129]

In general terms AMPK, carries out its function as a pleiotropic regulator of cell metabolism by regulating a large number of processes (Figure 12):

- AMPK is expressed and functions as an intracellular fuel sensor activated by depletion of high energy phosphorylation [127]. Phosphorylation plays a key role in regulating the activity of AMPK regulating cellular energy levels [131]. Activation is induced in response to diverse stimuli that disturb the energy balance of the cell either by inhibiting ATP generation or accelerating ATP expenditure, resulting in an increase in the AMP/ATP ratio [126].

- AMPK activation increases cellular NAD^+ levels and enhances SIRT 1 activity, resulting in the deacetylation and activation of PGC1α [129]. Concomitant with an increase of mitochondrial genes in skeletal muscle, in turn stimulate the expression of genes that contribute to ATP generation, such as those that function in fatty acid oxidation, glicolysis and mitochondrial biogenesis [126].

- AMPK is also modulated by many adipose-derived factors (Adipokines) that regulate whole-body energy metabolism, such as leptin, adiponectin and resistin. Leptin and adiponectin induce muscle glucose uptake and fatty acid oxidation by AMPK activation [129], whereas resistin which inhibits AMPK, appears to have opposite effects [125, 131, 132] .

- AMPK phosphorylation, also increase phosphorylation of ACC [129, 133]. The AMPK-dependent phosphorylation of ACC reduces the conversion of acetyl-coA into malonyl- CoA as we can see in Figure 12. Malonyl-CoA an important precursor for lipid synthesis is reduced concomitant with a decrease fatty acid synthesis, and also inhibits fatty acid oxidation, with an increase of mitochondrial ATP formation. By the other hand, fatty acid are mainly transported and stored as TGs.

- Activated AMPK and increased PGC1α have been suggested to mediate the regulatory effects of adiponectin on mitochondrial biogenesis and function. These results demonstrate that adiponectin plays an indispensible role in regulating mitochondrial biogenesis and function [129, 133].

- AMPK stimulates the translocation of the glucose transporter (GLUT4) to plasma membrane, where it facilitates the entry of glucose in skeletal muscle and heart cells.

There are some natural products that have effects in AMPK activation. EGCG, resveratrol, quercetin, catechin, berberine had been shown that accelerates significantly AMPK phosphoryaltion and is successfully employed in the prevention and treatment of variety diseases [125, 129]. Concretely EGCG, a main catechin of green tea, suppressed hepatic gluconeogenesis by AMPK activation mediated by CaMKK. By the other hand, berberine caused inhibition of mitochondrial function which increases AMP/ATP ratio, followed by the activation of the AMPK pathway [129]. Interestingly, like physical activity, some of the antidiabetic drugs used are known to activate AMPK, which may promote beneficial effects, including glucose uptake into skeletal muscle and promoting insulin sensitivity [126].

1.5 Mitochondrial dynamics

The overall processes of mitochondrial fusion and fission have not yet been thoroughly defined; however, the relevant molecular mechanisms mediated by genes controlling mitochondrial dynamics that promote mitochondrial fusion and fission, in addition to the posttranscriptional regulation of these genes, have been elucidated [134].

Changes in mitochondrial morphology may be relevant with regard to obesity and related cardiovascular diseases. Mitochondrial dynamics are controlled by the activity of a group of guanosine triphosphatases (GTPases) related to a dynamin family (Figure 13). Remodelling of the mitochondria and their ultrastructures and crests can be observed in transitions between respiratory states or during apoptosis [135].

It has been well established that mitochondria are highly dynamic organelles; their distribution and activity can be altered by fusion and fission events, which are essential in mammals and are implicated in multiple functions, including the maintenance of mitochondrial morphology, mtDNA stability, respiratory capacity, apoptosis, and the response to cellular stress. Thus, mitochondria take on a variety of shapes, ranging from long, interconnected networks to individual, small spheres [137, 138]. All of these characteristics are involved in mitochondrial dynamics and are thus very important for maintaining an equilibrium that is appropriate for the mitochondrial quality. It is also important that fusion and fission events remain in balance for the maintenance of healthy mitochondria [139, 139].

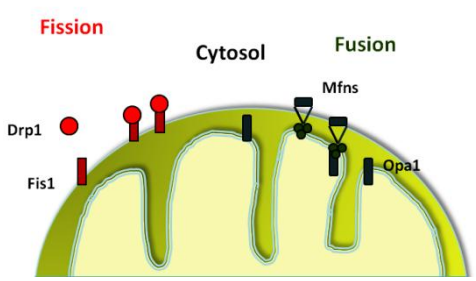


Figure 13. Mitochondrial dynamics and Mfn, OPA1, Drp1 and Fis1 localitzation. Mfns and Fis1 anchored in the outer membrane. Drp1 is a soluble cytosolic protein that binds to Fis1[136]. Mitofusin (Mfn); optic atrophy 1 (OPA1); Dynamin-Related Protein 1(Drp1), Fission 1 (Fis1)

1.5.1 Fusion

1.5.1.1 Mitofusins

In mammals there are 2 mitofusins homologues, Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), located in the mitochondrial outer membrane, where they can initiate the interaction of different mitochondrias [134, 137]. Both molecules are necessary otnormal levels of mitochondrial fusion and have similar biochemical activities. So, they may interact with each other to coordinate fusion of the external mitochondrial membrane of opposing mitochondria [135]. When mitofusins are overexpressed, cause aberrations in mitochondrial morphology. When mitochondrial fusion is reduced, mitochondrial population is fragmented into short tubules or small spheres [139, 140].

Mfn2 has relevance in the activity in distinct key cellular functions such as oxidative metabolism, cell cycle, cell death, and mitochondrial axonal transport involved in some pathologies [134]. So, the action in these proteins could help to reverse some pathology. It has been shown that the overexpression of Mfn2 directly increases the activity of respiratory complexes, mitochondrial oxidation, and glucose utilization [135].

1.5.1.2 Optic atrophy 1

Optic atrophy 1 (OPA1) are dynamin family GTPases, located within the mitochondrial intermembrane space and are associated with the inner membrane [134]. OPA1 participates in the remodeling of mitochondria crests and the approach and fusion of the internal mitochondrial membrane [134, 135]. OPA1 deficiency leads to cellular defects, including reduction and disorganization of cristae membranes, severely reduced respiratory capacity, reduce oxygen consumption and sensitivity to apoptosis [134]. So, the decrease in the concentration of OPA1 protein leads to the development of fragmented mitochondria with a lower oxygen uptake and lower mitochondrial membrane potential [135, 137].

1.5.1.3 Mechanism Fusion

Mitochondrial fusion is necessary for respiration function cells [137]. In the mechanism of fusion 2 distinct membrane fusion events occur. The outer and inner membranes, which delineate a mitochondrion, merge with the corresponding membranes on another mitochondrion [139, 141].

Three GTPases are essential for mitochondria fusion (Figure 14.): Mitofusins (Mfn1 and Mfn2) which are transmembrane GTPases, and OPA1, which is a dynamin related GTPases associated with the mitochondrial inner membrane or intermembrane space [135, 138]. Mitochondrial fusion is necessarily with multistep process, because mitochondria have double membranes. To profusion protein located in the inner mitochondrial membrane (OPA1) and the pro-fusion proteins located in the outer membrane (mitofusins) are detected in the same complex, so 2 steps are co regulated or coordinated. When any of this GTPases are depleted, the mitochondria fusion is reduced [139]. The single disruption of Mfn or OPA1 is sufficient to stop the mitochondrial fusion process [136].

In this event there are a mixing of the membranes, the intermembrane space and matrix. It is assumed that outer and inner membrane fusion is tightly coordinated [137]. With mitochondrial fusion, the contents of the organelles are intermittently homogenized, and therefore the mitochondria could operate as a coherent population. When mitochondrial fusion is disrupted, the loss of content mixing may be responsible for many of the functional defects such as mtDNA instability or reduced respiratory capacity [139].

1.5.2 Fission

1.5.2.1 Dynamin-Related Protein 1

Dynamin-Related Protein 1 (Drp1) is a key component of the mitochondrial fission machinery. Large amount of Drp1 is located in the cytosol, but a subpool is located on mitochondria tubules [137].

This protein is essential for most types of mitochondrial fission. The role of Drp1 in mediating mitochondrial fission is thought to be similar to that of dynamin, in scission of endocytic vesicles [139]. The inhibition of Drp1, leads to increase length and interconnectivity of mitochondrial tubules, secondary to inhibition of fission [137] and results in very elongated mitochondria.

1.5.2.2 Fisssion 1 & Mitochondrial Fission Factor

Fission 1 (Fis1), is a key component of fission events. It is a small protein located in the outer membrane of mitochondria, although most of the protein faces in cytosol [137]. It is not yet well studied his mechanism actions, but possibly Fis1 may play an essential role in mitochondrial fission in only specific cell types. In other cell types other molecules such as mitochondria fission factor (Mff), may plan a dominant role which is the best established receptor for Drp1 [139].

1.5.2.3 Mechanism Fission

Mitochondrial fission process usually occurs in all cells in normal conditions or in conditions of metabolic stress, as well as autophagy, and apoptosis [135]. Components of fission machinery, Drp1 and Fis1, are the central players of the mammalian mitochondrial fission[135, 137]. Although little known about their molecular mechanism of action. Mitochondrial fission likely is coordinated with other cellular processes. Fission generally results in daughter mitochondria that each contains at least one nucleotide, even in cells with highly fragmented mitochondrial. The fission may also be linked to the cell cycle. Therefore, cell may differe their mitochondria to facilitate segregation during cell division [137].

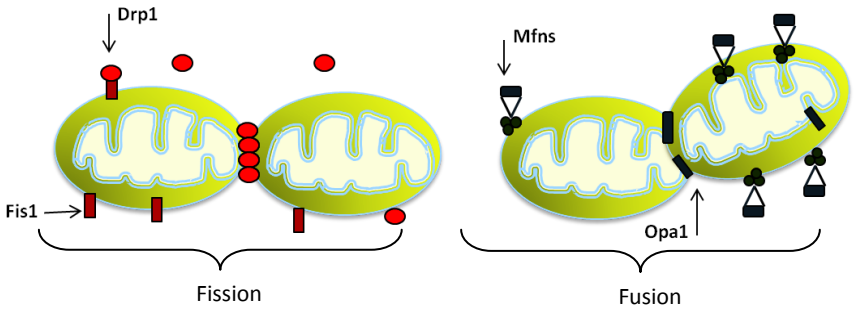


Figure 14. Mitochondrial dynamics: factors that regulate mitochondrial morphology in fusion and fission events [135]

1.5.3 Cell biology of fusion and fission events

Mitochondrial function is involved in certain pathological conditions [139]. In mammals, rats and humans, there are several alterations in mitochondrial dynamics implicated in energy metabolism (Table 5). Mitochondrial size is reduced in the skeletal muscle of obese and type 2 diabetic patients compared to lean subjects. This alteration is correlated with a repression of the mitochondrial fusion protein Mfn2 in skeletal muscle [136].

The morphology of mitochondria depends on the balance between the opposing processes of fusion and fission. Unbalanced fission leads to fragmentation, whereas unbalanced fusion leads to elongation. In a normal physiological state, the control of these processes can alter the shape of mitochondria to suit a particular developmental function[137]. Changes in mitochondrial dynamics can allow cells to adapt to certain types of stress. The control of mitochondrial shape, fusion and fission are important in mitochondrial bioenergetics. Whereas mitochondrial fission often plays a proapoptotic role, mitochondrial fusion tends to protect cells from cell death. Mitochondrial fusion is reduced following the induction of apoptosis, and the overexpression of mitofusins results in poor cell growth[137].

When mitochondrial fusion is reduced, the mitochondria become fragmented due to unbalanced fission; however, mitochondrial tubules can be restored by the simultaneous inhibition of fission [137]. By contrast, if mitochondrial fission is reduced, the mitochondria become elongated and excessively interconnected due to unbalanced fusion. As such, these opposing processes work in concert to maintain the appropriate shape, size, and number of mitochondria[139].

In cells lacking mitofusins or OPA1, mitochondrial function is greatly diminished and manifests as a reduction in glucose oxidation and oxygen consumption, which causes a reduction in membrane potential in the presence of several substrates [142]. Cells with low Mfn2 activity rely on anaerobic glycolysis to generate energy. Alterations in OPA1 expression also affect mitochondrial metabolism, and its depletion causes a reduction in both basal respiration and the capacity to enhance oxygen consumption in the presence of uncoupling proteins, as well as in the absence of Mfn [135, 142]. Mitochondrial fusion can also protect cells from the detrimental effects of mtDNA mutations by allowing functional complementation of mtDNA gene products [137].

In summary, mitochondrial dynamics are important not only for the control of mitochondrial shape but also for mitochondrial function.

Protein	Metabolic effects
↑Mfn2	High mitochondrial membrane potential increased glucose oxidation
↑Mfn2Δ602-757	No fusion activity High mitochondrial membrane potential Increase glucose metabolism
↓Mfn2	Low mitochondrial membrane potential Reduction of oxygen consumption Decrease in glucose and palmitate oxidation Low activity of respiratory complexes
↓OPA	Low mitochondrial membrane potential Reduction of oxygen consumption Low activity of respiratory complexes

Table 5. Mitochondrial fusion proteins in cell metabolism [142] .Gain of function (↑) and/or loss of function (↑) studies have revealed a regulatory role of Mfn2 and OPA1 on cell metabolism.

1.6 Energetic homeostasis in different tissues

1.6.1 Adipose tissue

In mammals, adipose tissue develops in many different sites through the body, and generally occurs in areas of loose connective tissue, such as subcutaneous layers between muscle and dermis [143]. In general terms, there are two types of adipose tissue: WAT and BAT. The adipose tissue functionality is a key for the energy homeostasis of the organism and is considered a metabolically active storage tissue for lipids while BAT is considered a thermogenic adipose tissue with higher oxidative capacity. Experimental evidence suggests a link between adipose tissue malfunctioning, obesity and their related diseases [144].

BAT is well accepted as an important metabolically organ in small mammals (rats, mice) and predominate in interscapular area. From years was thought that in humans BAT was present mainly in the neonatal period for thermoregulation but was scarce in adults. Recent studies, evidenced that significant amount of BAT is also

present in adult humans in neck and around clavicles, extending in some people into the supraclavicular and thoracic region [145, 146] and take importance to be metabolically significant in adult human's tissues. These discover was obtained by positron emission tomography and could be a useful way to avoid obesity and also prevent the side effects of associated diseases [108].

1.6.1.1 White adipose tissue

WAT is a metabolic organ that specializes in energy storage in TGs containing intracellular droplet. It represents around 10% of total body weight in lean adults, but can achieve >50% in obese subjects, disrupting whole-body energy homeostasis inducing changes in WAT mitochondria [108].

Adipose tissue grows by hypertrophy (cell size increase) and hyperplasia (cell number increase). When energy intake exceeds energy expenditure, energy continues with the storage in adipocytes leading to hypertrophy and weight gain. Although adipocyte hyperplasia is not necessary to directly promote adiposity, the adiposity number set during childhood and adolescence is likely to have a dominant role in determining the lipid-storing capacity of adipose tissue and fat mass in adults [147].

Mitochondria in adipose tissue play an important role in lipogenesis by providing key intermediates for TGs synthesis; in adipocytes, mitochondria is also involved in the regulation of lipolysis. Fatty acids resulting from lipolysis can be oxidized by the fatty acids β-oxidation pathway into the mitochondria matrix compartment, with the objective to prevent lipotoxicity that induce insulin resistance in other organs such as liver, muscle or β- cells [107, 108,]. The imbalance between lipid storage and lipid utilization predisposes to adipocyte dysfunction and promote the proinflammatory response and ROS production involved in severe metabolic disorders [148].

WAT besides affecting multiple biological systems also affect the immune system, with the secretion of hormones that regulate energy balance as adipokines (the leptin, adiponectin and resistin) [143, 147, 148]; it is also capable of emitting signals to regulate food intake and energy expenditure and thereby to orchestrate changes in energy balance and whole body nutritional status .It is believed that modulation of cellular and molecular events in adipogenesis could serve as an effective means to control body-weight gain and obesity [147].

1.6.1.2 Brown adipose tissue

Brown fat cells are characterized by a polyglonal shape with multilocular lipid droplets and an increased number of large and spherical mitochondria, which give their brown coloration [108]. In contrast to lipid storage function in WAT; BAT provides heat via dissipation of energy, known as thermogenesis, possessing the ability to transfer directly, energy from food into heat, maintaining the appropriate balance between energy storage and expenditure. The main part of cellular thermogenesis comes from mitochondria where a large part of the mitochondrial respiration energy can be dissipated spontaneously as heat.

There are different thermogenesis mechanisms; cold environment, energy oversupply stimulated by catecholamines that activates thyroid hormones, and also activated by lipase to release FFA from TGs. So, FFA and thyroid hormones are activators of the inner mitochondrial membrane UCP1 that uncouples mitochondrial respiration from ATP production by causing protons to leak across the inner membrane, enabling energy dissipation as heat [149].

UCP1 allows protons to move down their electrochemical gradient, by passing ATP synthase and therefore ATP production (Figure 15). So, uncouples aerobic respiration from substrate oxidation by producing heat instead of ATP. As the results brown adipocytes, oxidizes their own fat stores and circulating substrates at a fast rate, thus releasing heat [150] apart from circulating lipids and glucose.

This observation thus highlights BAT thermogenesis as an attractive therapeutic anti- obesity target in the regulation of body weight [108, 148, 150, 151]. There are studies that demonstrate rodents feed cafeteria diet activates

and expands BAT [152]. This effect could be in response to the hypercaloric diet for defense excess of energy supply by dissipating part of the energy excess and thus reducing body weight gain and also its comorbilities [150].

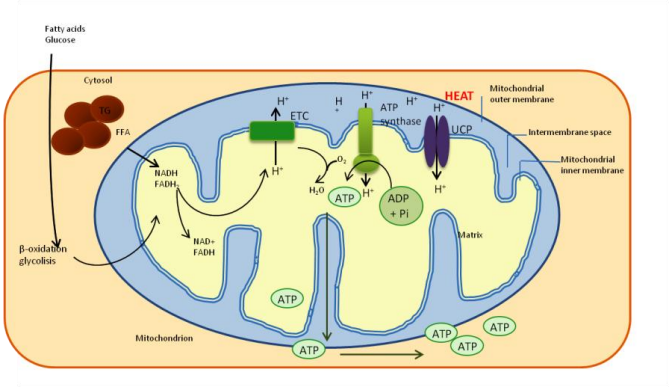


Figure 15. Mitochondrial functionality in brown adipose tissue

1.6.1.3 Regulation of brown adipogenesis

Brown adipogenesis is regulated with grown transcription factors. At trancriptional level, several proteins enhance or inhibit brown fat development, Bone morphogenetic proteins (BMPs) are main regulators, but also PGC1α, PPARγ and UCP1 [108]. PPARγ, and ccaat-enhancer-binding protein (C/EBP) family, that in combination with proteins such as PGC1α have a critical role to determine brown adipogenesis. PGC1α with their expression is a key gene, involved in BAT development that plays a critical role in the mitochondrial biogenesis and oxidation in metabolic pathways [150]. The high oxidative capacity of BAT is due to its high mitochondrial density, expression of fatty acid oxidation enzymes and respiratory chain components, similarly to the muscle.

1.6.2 Skeletal muscle

In healthy humans, skeletal muscle accounts for 40-45% of the total body mass and is a major determinant of resting energy expenditure, accounting for 40-50% of the variability in basal metabolic rate and for up to 75% of total muscle glucose disposal [153]. Additionally, in terms of metabolic health, skeletal muscle physiology plays a role in the body's overall nutrient balance, characterised by the capacity to utilise either lipid or carbohydrate fuels and to effectively transition between these fuels. Thus, skeletal muscle is essential for the maintenance of whole-body energy homeostasis under a wide range of physiological conditions [154]. Skeletal muscle accounts for the majority of insulin-stimulated glucose utilisation and is therefore the major site of insulin resistance in patients with obesity and type 2 diabetes mellitus (DM). Obesity and type 2 DM, in addition to insulin resistance, affect the composition of skeletal muscle by increasing the lipid content within and around muscle fibres, manifesting inflexibility in the transition between lipid and carbohydrate fuels [155] (Figure 16). Increases in intramyocellular lipids in the form of diacylglycerol and ceramides, as well as increases in acylcarnitines (due to incomplete mitochondrial fatty acid oxidation), adipocytokines associated with inflammation, branch chain amino acids and ROS, have all been implicated as potential causes of defects in insulin signalling. There is also evidence to suggest that OXPHOS is decreased. Skeletal muscle rapidly increases its rate of energy consumption in the form of ATP in situations where explosive contractions are required. There are two metabolic pathways, namely, anaerobic and aerobic metabolism [156].

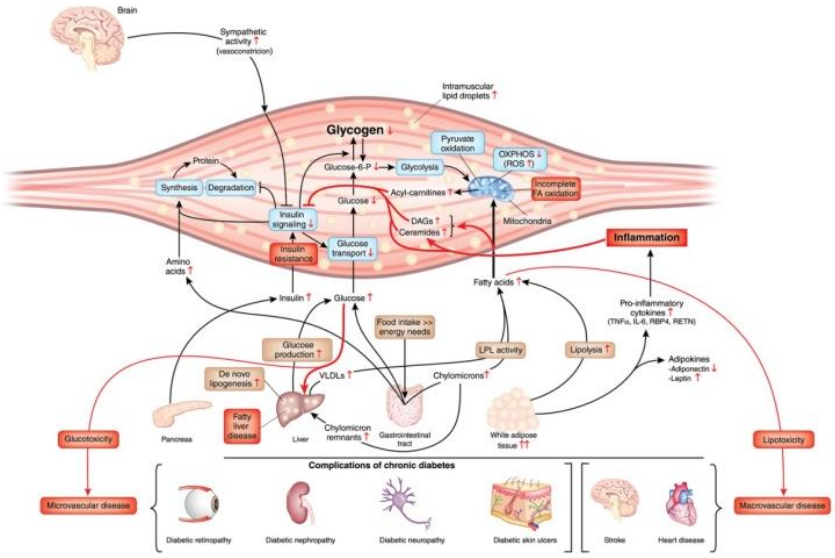


Figure 16. Defects in muscle metabolism by metabolic syndrome based on [157]

1.6.2.1 Skeletal muscle and glucose metabolism

Glucose, which is hydrophilic enters the cytoplasm by a process of facilitates transport. To date, 13 isoforms of GLUTs have been identified. The variety in their maximal rates of glucose transport and sensitivity to hormonal regulations [158] GLUT1 and GLUT4 are the main glucose transporters in skeletal muscle [159]. In addition GLUT1 is predominantly responsible for glucose transports under basal conditions and GLUT4 mediates the effect of insulin on promoting glucose uptake in skeletal muscle, a process that accounts for about 75% of whole-body insulin-stimulated glucose uptake [160].

1.6.2.2 Skeletal muscle and fatty acid oxidation

Skeletal muscle has a limited capacity to store lipids; there are two pools of fatty oxidative metabolism in skelta muscle: circulating TGs and intramyocellular TGs. LPL act as a door keeper in tissue fatty acid metabolism by hydrolyzing blood TGs [133].

Membrane transport of long chain fatty acids (LCFA) is tightly regulated to prevent intramuscular lipid accumulation. Fatty acid transport in skeletal muscle is regulated at the plasma and mitochondrial membranes via both common and distinct mechanism. Uptake of LCFA by the expression of activity of carrier proteins including fatty acid translocase/cluster differentiation 36 (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm) and fatty acid transport proteins (FATPs)[161] .Transport of LCFA is considered as the rate limiting step in fatty acid oxidation. Once fatty acids are inside cytosol, could be esterified and metabolized to lipid or β-oxidized in mitochondria by CPT system. CPT1 located on the outer mitochondrial membrane facilitates the binding of carnitine to long chain acyl-CoA [161].

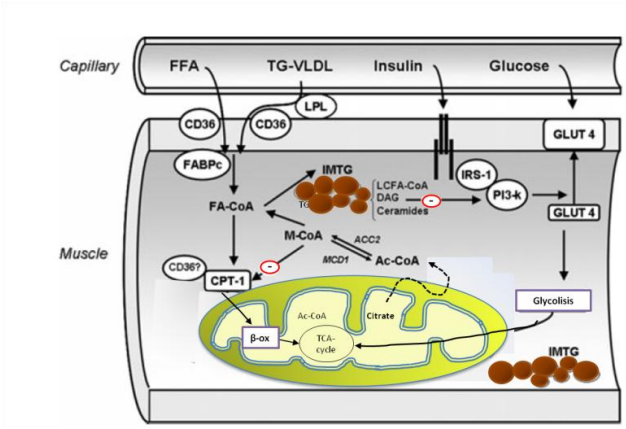


Figure 17 Cell metabolism in mycyte: key factors in pathogenesis of fat-induced muscular insulin resistance[30]

By the other hand malonyl-CoA, first intermediate in lipogenesis, regulated CPT1 system as alloesteric inhibitor. When ACC, that control synthesis of malonyl-CoA, is inhibited by phosphorylation, the reduction in malonyl-CoA synthesis, release the inhibition of CPT1, and transport of LCFA into the mitochondrial increases, being the rate limiting step in mitochondria fatty acids oxidation as we mentioned above So, fatty acid carriers and CPT1 may be important, under conditions in which there is a demand increase of fatty acid oxidation including during exercise or muscle contraction [162] (Figure 17).

1.6.3 Hormonal control of adipose and muscle energy metabolism

Adipose tissue acts as an endocrine organ, and plays an important substantial role in the pathogenesis and complications of obesity and CVD. Adipose tissues and obesity have clued adiponectin, which has antidiabetic and antiarteriosclerotic effects, and leptin, which has regulatory effects on feeding. In addition, adipokines with undesirable properties have also been identified, such as TNF-α which causes diabetes and artheriosclerosis [122].

Specifically, obesity results as a pro-inflammatory state with increased visceral fat deposits and alteration of adipokine secretion (insulin, leptin and adiponectin), concomitant with insulin resistance [9].

1.6.3.1 Insulin

Insulin is a peptide hormone, synthesized in β-cells of the pancreatic islets of Langherhans; it is the pivotal hormone regulating cellular energy supply and macronutrient balance. Insulin stimulates glucose uptake, promotes lipogenesis while suppressing lipolysis, and hence, free fatty acid flux into the bloodstream [163] directing anabolic processes of fed state. Insulin is essential for the intra-cellular transport of glucose into insulin-dependent tissues such as muscle and adipose tissue [164]. Glucose is the principal stimulus for insulin secretion, through other macronutrients, hormones, humoral factors and neural input, which may modifies this response. Insulin together with its principal counter-regulatory hormone glucagon regulates blood glucose concentrations [165].

Insulin in muscle glucose uptake is essential and dependent via GLUT4; muscle accounts for about 60-70% of whole-body insulin mediated uptake. In the fed state insulin promotes glycogen synthesis via activation of glycogen synthase [166]. Insulin therefore promotes glucose entry to enables oxidations, recycling lactate, synthesizes and stores glycogen, and also lipid synthesis in muscle cells, while suppressing lipolysis and gluconeogenesis from muscle amino acid [164]. A decrease in insulin sensitivity to skeletal muscles induces an increase in basal insulin plasma levels.

By the other hand, in adipose tissue intracellular glucose transport into adipocytes in postprandial state is insulin dependent via GLUT4; it is estimated that adipose tissue accounts that whole body glucose uptake is stimulated about 10% by insulin. Ingestion of lipid may also modify the insulin response to glucose by effects on

gastrointestinal hormones and gastric empting [167]. In carbohydrate metabolism facilitated diffusion of glucose into fat and muscle cells via GLUT 4 modulation[168].

In general terms, insulin modifies the supply of nutrients to muscle through a direct vasodilatory effect and reduce arterial metabolites concentrations of glucose, amino acids and NEFAs due to the inhibition of hepatic gluconeogenesis, coupled with inhibition of lipolysisis and stimulation of lipogenesis in adipose tissue [169].

1.6.3.2 Leptin

Leptin appears to be an important adipokine, performing a significant role in the regulation of food intake and energy homeostasis in peripheral tissues such as liver and muscle [147], with body weight regulation particularly related with CVD [10].

The synthesis and secretion of leptin are regulated by numerous factors, including insulin, steroid hormones, β-adrenergic stimulation, growth hormone, glucocorticoids and nutrients such as glucose, leucine and some polyunsaturated fatty acids [170]. Leptin secretion is positively associated with fat mass content. After a meal, leptin is secreted by adipose tissue and goes through specific areas of the hypothalamus to regulate signal satiety. By the other hand it has been reported that leptin stimulates phosphoyrlation and activation of the α2 catalyic subunit of AMPK in skeletal muscle increasing fatty acid oxidation. Moreover in neuron decrease the release of orexigenic neuropetides with regulatory effects on food intake [132].

Acute leptin treatment rapidly activates fatty acid oxidation by increasing the AMP/ATP ratio, which in turn activates AMPK by phosphorylation[171]. Chronic leptin treatment effects on muscle fatty acid oxidation via rapid and longer term mechanisms and were related to increase mRNA levels of medium-chain-acyl-CoA dehydrogenase (MCAD), CPT1 and UCP2 [172].

1.6.3.3 Adiponectin

Adiponectin was initially identified as an adipocyte derived hormone secreted from adipose tissue and served as an anti-inflammatory adipokine that regulates energy homeostasis by increasing insulin sensitivity. Plasma adiponectin levels are negatively correlated to fat mass [172] .Two adiponectin receptors are known: Adiponectin receptor (Adipo R)1 and Adipo R2. Adipo R1 is expressed in skeletal muscle, and Adipo R2 is mostly expressed in liver [133]. Adiponectin is also known as a target of AMPK activity to decrease gluconeogenesis, increase glucose uptake and improve oxidative metabolism of fatty acid oxidation liver and muscle, through its own receptors and downstream signaling, resulting in ameliorating insulin sensitivity [133, 173].

The effect of adiponectin on mitochondrial biogenesis and fatty acid oxidation is dependent of AMPK and phospho38 mitogen activated protein kinase (p38MAPK) signaling pathways (Figure 18). The effect of adiponectin on AMPK and p38MAPK activation in fact is linked. Adiponectin activates AMPK which in turn activates p38MAPK, and then PGC1α and PPARα ultimately, resulting in increased mitochondrial biogenesis and function [172]. The increased of fatty acid oxidation was in part the result of increased β-oxidation enzyme expression and gene expression of genes encoding proteins involved in fatty acid transport as CD36, acyl-CoA oxidase, UCPs and LPL activity which should provide fatty acids as a substrates for oxidation [133], resulting in enhanced fatty combustion and energy expenditure [174]. Replenishment of adiponectin might represent a novel treatment strategy for insulin resistance and type 2 DM. In addition, adiponectin has potential anti-inflammatory properties that might prevent or retard atherogenesis [175].

1.6.4 UCPs homolegs

UCPs are anion carriers across the mitochondrial inner membrane, which bring protons back into the mitochondrial matrix .Mitochondrial uncoupling is mediated by UCPs, among those UCP1 was the first protein to be identified in brown adipose tissue which, dissipates redox energy and provides heat to the animal. UCP1 is followed by its four homologs: UCP2, which is widely expressed in several tissues of the body (e.g brain, pancreas, spleen, macrophages, stomach, skeletal muscle, lung) [176]; UCP3 expressed at high levels in skeletal muscle, heart as well as BAT; UCP4and brain mitochondrial carrier protein 1 (BMCP1) or UCP5 are predominantly expressed in the central nervous system[177-179].

In general terms, these novel uncoupling proteins have several hypothesized functions including thermogenesis in certain tissues, neuroprotection, export of fatty acids, mediation of insulin secretion, protection from ROS and preventing obesity and diabetes [180,181], these mechanism actions are illustred in Figure 18.

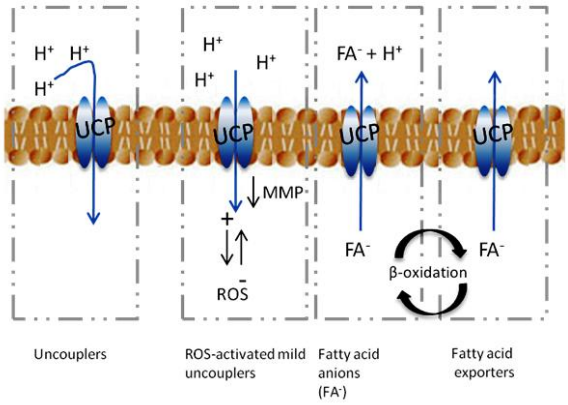


Figure 18. UCps analogs functions

1.6.4.1 UCP2 & UCP3

The amino acid sequence of UCP1 is 59% and 57% identical to the sequence of UCP2 and UCP3, respectively [182].

While UCP1 is responsible of thermogenesis, as already discussed in the section of brown adipose tissue, proteins UCP2 and UCP3 are usually not considered thermogenic proteins, but nonetheless they might be significantly thermogenic when is fully activated by endogenous or exogenous environmental factors [182].

UCP3 is activated by fatty acids when thermogenesis is required, suggesting that the activation of UCP3 by physiological activators might cause significant thermogenesis under certain conditions [183] . By the other hand, it has been reported that lack of UCP3 in mice has a tendency to accumulate fat compared with wild type mice [184]. So the

Furthermore, skeletal muscle plays an important role in energy expenditure by activation of UCPs. It has been suggested that UCP3 expression could be upregulated by diet fish oil and DHA [185] and also with oleic acid [186] promoting benefits in lipid metabolism and prevention of obesity and diabetes [187, 188].

UCP2 is involved in a wide range of functions including satiety signaling, insulin release, and immune cell functions; also has been implicated in physiological and pathological processes related to glucose and lipid metabolism [189]. It has also been proposed that UCP2 may serve as the enigmatic calcium uniporter [190]. Previous data suggest that function as uncoupler when activated by superoxide and other metabolites of lipids and peptides [191, 192] by increasing proton leak, decreasing ROS production and protecting against oxidative stress [193-195] .

The mechanism of fatty acids to induce uncoupling, may involve PUFAs in activating UCP2 and uncoupling activity. The PUFAs, their hydroperoxides, and hydroxyl fatty acids derivatives originated from lipoperoxidation can be cleaved off by phospholyphase A and may activate UCP2 instantly. Such activation may present a downregulating feedback mechanism of ROS formation [192, 195, 196]. Moreover it has been shown that dietary fat is influenced in the expression of UCP2 and UCP3 [176, 197]. The expression of both UCPs are elevated in fasting conditions or other states, including high fat diets, were circulating fatty acids are elevated and there are a shift between carbohydrate and lipid oxidation. Although the expression depends on strain and tissue type, the upregulation by high fat diet of UCP3 also occurs in human skeletal muscle [198].

In vitro studies support the idea that UCP2 and UCP3 can be activated by fatty acids and inhibited by purine nucleotides. UCPs in general have a purine nucleotide binding site located into the intermembrane space. The purine nucleotides ATP, ADP, GTP and GDP are inhibitors of uncoupling activity [199] .

1.6.4.2 UCP4&UCP5

UCP4 and UCP5 have the characteristic structure of other UCPs. UCP4 is mainly expressed in the central nervous system[199] and was the first identified in humans as novel member of the UCP family in 1999 by Mao et al.[177].By the other hand UCP5, is strongly expressed in amygdale, dorsomedial hypothalamic nucleus, mediodorsal thalamic nucleous, and vetromedial hypothalamus in mice [200]. Thus UCP4 and UCP5 perform the essential function of an uncoupler of oxidative phosphorylation accompanied by a reduction of oxidative stress [201] . UCP4 and UCP5 play a role in apoptosis in the brain [202].

A part from the mentioned mechanism of UCPs; it is thought that also they can act as fatty acid anion flipases causing the outward movement of the fatty acid anion head groups across the inner membrane (Figure 18), once on the outer surface, the fatty acids pick up a proton and then flip-flops rapidly will be back into the matrix. Thus, UCPS des not conduct protons *per se*; movement rather it enables fatty acids to behave as cycling protonophores [91].

1.7 Oxidative stress

ROS are used to describe a variety of molecules and free radicals (chemical species with one unpaired electron) derived from the metabolism of molecular oxygen. In living cells, the major site of nonezymatic production of ROS is the mitochondrial respiratory chain [180]. The 0.2% to 2% of oxygen consumed by cells is incomplete metabolized; such that when an electron is accepted from the ETC, superoxide anions ($O2^{-\cdot}$) are generated as obligatory byproducts, with complexes I and III, the major ROS generation in mitochondrial respiratory chain, being the primary source of ROS [107, 180,]. Recent work suggests that other mitochondrial enzymes are also involved in ROS generation, as succinate dehydrogenase (Complex II) [203].

High levels of ROS are associated with significant cell damage and mitochondrial dysfunction in a process known as oxidative stress, usually associated by with a etiology of obesity, insulin resistance and type 2 DM[108]. Oxidative stress occurs in cells as a consequence of an imbalance between the prooxidant/antioxidant systems, which could be produced with the feeding of high/fat/high sucrose diet. This oxidative stress cause damage to cellular macromolecules such has nucleic acids, proteins, structural carbohydrates and lipids, increase mutation in mtDNA and apoptosis [204]. The peroxidation of lipids is particularly negative because the formation of lipid peroxidation products leads to a simple propagation of free radical reactions [107, 108, 205], with fluidity and permeability changes, inhibition of metabolic processes, and alterations of ion transport. The damage on mitochondrial induced by lipid peroxidation can lead to further ROS generation [180] .

Antioxidants could reduce ROS-mediated damage to mitochondrial macromolecules. However, an alternative approach would be modulate mitochondrial function, with the aim of producing less ROS in the first place while

maintaining adequate ATP production [206].The natural antioxidant system could be classified into two main groups: enzymes and low molecular weight antioxidants. The enzymes scavenging ROS includes superoxide dismutase (SOD), catalase, and glutathione peroxidase. The low molecular weight antioxidants include ascorbate, glutathione (GSH), phenolic compounds and tocopherols. In mitochondria, superoxide anions are converted to H_2O_2 by manganese superoxide dismutase (MnSOD) (present in the matrix and intermembrane space), converted subsequent to water by the glutathione peroxidase enzyme. Alternatively, hydrogen peroxide can diffuse from the mitochondria into the cytoplasm [180].

It has been showed that skeletal muscle insulin resistance development in response to a high fat diet could be prevented by specifically inhibiting ROS emission with a mitochondrial-targeted antioxidant [172]. So, targeting natural antioxidants in mitochondria could be a good choice to prevent ROS damage.

1.7.1 Mild uncoupling

Apart from use antioxidant compounds to decrease ROS products, another way for the prevention of ROS exces, bases on the observation that there is a possible physiological role of UCPs to attenuate this damaged caused by mitochondrial ROS [180] (Figure 18). Proton motive force is a key factor influencing free radical production at complexes I and III, the mild mitochondrial uncoupling and dissipation of mitochondrial gradient could be a strategy to scavenge ROS [206].

Adenine nucleotide translocase (ANT) and the UCPs 1 to 3, represent the most well described proteins involved in proton leak, although proton leak mechanism by ANT are not well understood. Proton leak through UCP2 and UCP3 is statistically associated, and activated by ROS or their lipid by products, such as 4-HNE [183] to decrease mitochondria ROS emission [207] .

The interrelationship between cellular fuel oxidation, mitochondrial bioenergetics and ROS signaling has been improved. It has been proposed that one function of both, UCP2 and UCP3 is to mildly uncouple respiration allowing a more rapid electron flux (Figure 19), thus reducing membrane potential resulting in reduced ROS production. Since even mild uncoupling, has a large effect on reducing ROS production, this hypothesis has strong support, and now is generally accepted [149, 181]. The ability of UCP2 and UCP3 to modulate the frequency and duration of ROS emission by mitochondria could have profound physiological effects protecting against ROS production and tissue oxidative damage [207, 208].

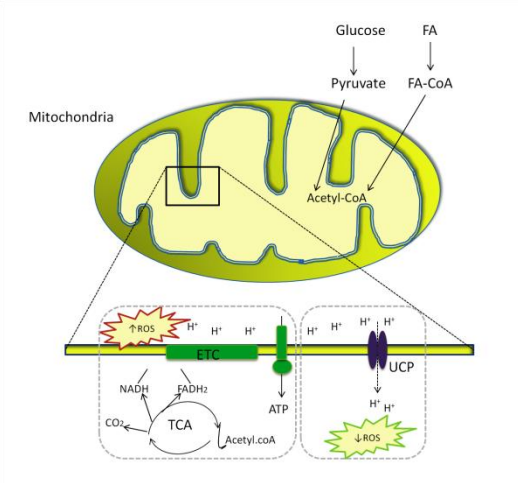


Figure 19. UCPs and ROS accumulation based on [148]

1.8 Metabolic flexibility

The availability of substrates is a major determinant in energy metabolism of skeletal muscle. Fat overload may represent a metabolic challenge for many individuals, even in energy balance conditions, who may indeed fail to appropriately upregulate skeletal muscle lipid oxidation, producing intracellular lipid accumulation and insulin resistance.

So, the ability of a system to adjust fuel oxidation to fuel availability is known as metabolic flexibility [209, 210]. The switch in fuel oxidation will depend on the type of amount of nutrient available for oxidation at the cellular level [154]. In response to fuel oversupply, anabolic pathways are activated. The ability to change substrate oxidation in response nutritional status will depend on the genetically determined balance between cellular oxidation and storage capacities [161]. For example during an overnight fast or in response to a high fat diets switch from carbohydrate to lipid oxidation should also be part of the assessment of metabolic flexibility (Figure 20). A consequence the oxidative capacity for skeletal muscle may be of utmost importance to boost lipid oxidation to the level of lipid supply and therefore modulate insulin sensitivity.

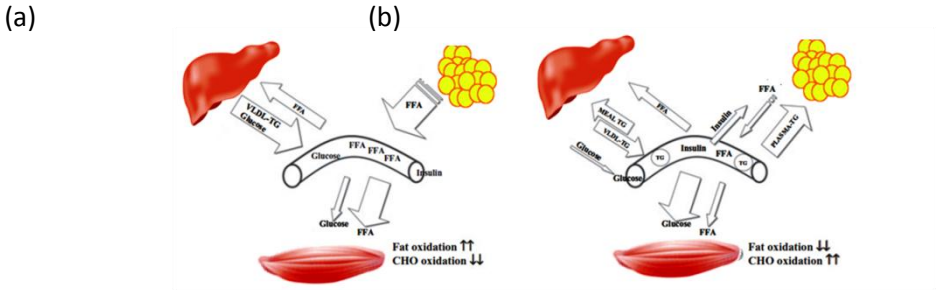


Figure 20. Schematic overview of metabolic fluxes of fatty acids and glucose between organs, in healthy subjects during **(a)** fasting and **(b)** postprandial phase, in the same conditions [30]

It will be important to test whether whole body or skeletal muscle metabolic flexibility are affected by muscle mitochondrial characteristic such as density, morphology and activity because mitochondrial abnormalities may be a primary cause of metabolic inflexibility raised [211], although probably metabolic flexibility also depends on the ability of adipose tissue to handle fatty acids, apart from the rate at which nutrients are available to the cells, as we mentioned above.

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2. GLOBAL GOAL

Obesity is the outcome of a long-term imbalance between energy intake (calories obtained from food) and energy expenditure (calories expended in basal metabolism, growth, development and physical activity) and manifests an increase in adipocyte number and size due to increased TGs storage in white adipose tissue. Obesity is a multifactorial disorder that results from complex and largely unknown interactions between genes, food intake and physical activity, which together determine body weight and fat distribution. Obesity is also a systemic phenomenon involving the interplay of multiple organs, such as white adipose tissue, brown adipose tissue, muscle and liver.

Certain dietary patterns and specific food components have been associated with a lower prevalence of cardiovascular diseases within the population. In this context, the traditional Mediterranean diet, characterised by a high intake of fibre, low-glycaemic index carbohydrates, unsaturated fats (monounsaturated oleic acid from olive oil and omega-3 PUFAs from nuts and sea fish), vitamins and antioxidant polyphenols, has been linked to a lower incidence of cardiovascular diseases, obesity and type 2 DM. Metabolic syndrome patients following a Mediterranean diet exhibit significant reductions in total weight, insulin resistance, and inflammation markers, as well as ameliorated endothelial function.

Proanthocyanidins, which comprise the oligomeric forms of (+)-catechin and (-)-epicatechin, in addition to their glycosylated and gallated derivatives, are found in wine, grapes, berries, apples, nuts, and chocolate. Numerous population and intervention studies have demonstrated that proanthocyanidin-rich diets reduce the risk of cardiovascular diseases. Studies using animal models have demonstrated the benefits of proanthocyanidins in terms of preventing and ameliorating obesity, diabetes, atherosclerosis, and hypertension. Because oxidative stress and subsequent inflammation represent a common backdrop to these diseases, the beneficial effects of proanthocyanidins have been largely attributed to their well-known antioxidant activities. Proanthocyanidins have also been shown to interact with plasma membrane and nuclear receptors, functioning as signalling agents and eventually modulating gene expression and cell metabolism. A similar effect has been described for the green tea polyphenol EGCG. Proanthocyanidins and EGCG repress proinflammatory NFϰβ-dependent gene expression in a wide range of cell types, including macrophages.

Green tee catechins, including EGCG, initially attracted attention as anti-inflammatory and anti-tumour compounds and have recently been recognised as potential therapeutic agents to treat and prevent obesity and diabetes. Intervention studies have shown the potential of green tea catechins to reduce human body weight, blood pressure and plasma low density lipoprotein (LDL)-cholesterol in obese patients. In animals, EGCG supplementation prevents diet-induced obesity, while reducing postprandial plasma levels of glucose, TGs and leptin, as well as lipogenesis in white adipose tissue; furthermore, EGCG is able to reverse established diet-induced obesity.

The diet of the Inuit population in arctic Canada is also correlated with a very low incidence of CVD, a phenomenon that has been ascribed to its high content of unsaturated fatty acids of sea fish origin. Numerous epidemiological studies and clinical trials have shown that unsaturated FAs (both polyunsaturated and monounsaturated) reduce the incidence of CVD and type 2 DM. Two natural omega-3 PUFAs, namely EPA (20:5n-3) and DHA (22:6n-3), which are abundant in sea fish, prevented the development of obesity and insulin resistance in rodents fed a high-fat diet. Studies in obese humans demonstrated a reduction in adiposity following supplementation with omega-3 PUFAs. These FAs act as hypolipidemlipidemics, reduce cardiac events, and decrease the progression of atherosclerosis. The hypolipidemic and anti-obesity effects of omega-3 PUFAs likely depend on the suppression of lipogenesis and an increase in FA oxidation in the liver, inhibiting carbohydrate-responsive element-binding protein (CHREBP) and SREBP-1c expression and activation, as well as on the enhanced mitochondrial biogenesis and β-oxidation of FA in white adipose tissue.

Current obesity treatments based on caloric restriction and physical exercise are not sufficient to combat the obesity epidemic; in addition, the available pharmacological options have shown very limited results and tend to cause undesirable side effects. Therefore, there is an evident societal need for new strategies to combat obesity.

Global goal

Following this rationale, our approach to developing new therapeutic strategies against the development and progression of obesity and its associated diseases has focused on the use of natural bioactive food components, which have the potential to be used as food additives to create functional foods and nutraceuticals. Hence, these components may be consumed by people with minor or no changes in their dietary habits, thereby avoiding the behavioural problems associated with meal replacements**.**

For this study, we have selected food ingredients that are characteristic of healthy diets, have documented beneficial effects on obesity or cardiovascular disease risk-factors in human studies and have no adverse effects on human health; these include the plant polyphenols EGCG and proanthocyanidins, as well as the sea fish omega-3 PUFAs and DHA. These compounds act through a variety of mechanisms, and therefore, we expect them to display additive and/or synergistic effects when used in appropriate combinations, thereby reducing the quantity of each individual compound needed to attain beneficial effects *in vivo*.

Thus, the **global goal** of the present thesis is the development of a new preventative and therapeutic strategy that is simultaneously effective against obesity and its major associated diseases, specifically insulin resistance. Societal trends continue to favour the intake of calorie-dense foods, as well as a sedentary lifestyle, and behavioural interventions have shown little success in combating the obesity epidemic; thus, we are pursuing a new anti-obesity strategy that requires only minor modifications of dietary habits.

Our work focused on the mitochondrial function of skeletal muscle, a major player in the body's overall nutrient balance, and adipose tissue, which is the key to energy homeostasis in the organism affected by obesity and related diseases, with the purpose to study the follow hypothesis: **Proanthocyanidins and omega-3 PUFAs oil rich in DHA may modulate metabolic flexibility and ameliorate the obesity dysfunction.**

The validity of this hypothesis was evaluated using the following main objectives:

Objective 1:

To understand the mitochondrial physiology, dynamics and target genes of the oxidative phosphorylation system through *in vitro* experiments in L6 myocytes, using pure polyphenols and polyunsaturated fatty acid molecules, such as EGCG and DHA, respectively.

Objective 2:

To study the postprandial effects of an acute dose of proanthocyanidins from GSPE and omega-3 PUFA oil rich in DHA combined with a fatty acid overload *in vivo* in healthy rats. We are interested in evaluating metabolic flexibility by studying the key interactions in gene expression related to lipid metabolism of adipose tissue and skeletal muscle, concomitant with the study of mitochondrial functionality.

Objective 3:

To characterise both the anti-obesity effects of dietary proanthocyanidins from GSPE and omega-3 PUFAs oil rich in DHA, concomitant with a high-fat diet, and the capacity to improve metabolic flexibility *in vivo* in obese rats. We focused primarily on determining the role of skeletal muscle in energy metabolism, particularly in lipid catabolism.

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3. RESULTS & DISCUSSION

3.1 Manuscript I

" Epigallocatechin gallate counteracts oxidative stress in docosahexaenoic acid- treated myocytes"

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Abstract

Skeletal muscle is a key organ of mammalian energy metabolism, and its mitochondria are multifunction organelles that are targets of dietary bioactive compounds. The goal of this work was to examine the regulation of mitochondrial dynamics and functionality and cell energy parameters using docosahexaenoic acid (DHA), epigallocatechin gallate (EGCG) and a combination of both in L6 myocytes. Compounds (at 25 μM) were incubated for 4 hours. Cells cultured with DHA displayed higher intracellular reactive oxygen species (ROS) levels, a higher ADP/ATP ratio, and higher intracellular calcium levels when calcium was added to the culture media. The mitochondrial membrane potential was lower; *Ucp2, Ucp3,* and *Mnsod* were upregulated; and *Cox* and *Ant1* were downregulated. Myocytes cultured with DHA consumed less oxygen and had a higher mitochondrial mass and a higher proportion of large and elongated mitochondria, whereas the fission genes *Drp1* and *Fis1* and the fusion gene *Mfn2* were downregulated. In myocytes co-incubated with DHA and EGCG, ROS levels and the ADP/ATP ratio were similar to untreated myocytes, whereas the intracellular calcium level was still higher. The mitochondrial membrane potential and *MnSod*, *Cox* and *Ant1* expression were similar to untreated cells; however, *Ucp2* and *Ucp3* were upregulated similar to the DHA-treated cells. When EGCG was co-cultured with DHA, the myocytes consumed less oxygen, had a higher mitochondrial mass, and had reduced *Drp1, Fis1* and *Mfn2* levels similar to the DHA-treated cells. However, the mitochondrial network was restored. The addition of EGCG to DHA returned the cells to the control conditions in terms of mitochondrial morphology and the energy and redox status, which were unbalanced in the DHA-treated myocytes.

Keywords:
Docosahexaenoic acid, Epigallocatechin gallate, Skeletal muscle, Mitochondria, Reactive oxygen species, Antioxidants

Abbreviations: Ant, adenine nucleotide translocase 1; Cox, cytochrome C oxidase subunit V;Cs, citrate synthase; DCFH-DA, 2',7'-dichlorofluorescin diacetate; DHA, docosahexaenoic acid; Drp1, dynamin-related protein 1;EGCG, Epigallocatechin-3-gallate; ETC, Electron transport chain; ETS, Electorn transport capapcitu ; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; Fis1, mitochondrial fission 1 protein; Mfn2, mitofusin 2; MMP, mitochondrial membrane potential; Mnsod, manganese superoxide dismutase;mtDNA, mitochondrial DNA; nDNA, nuclear DNA; Opa1, optic atrophy 1; PUFAs polyunsaturated fatty acids; Rhodamine 123, Rhd123; ROS, reactive oxygen species; ROX, residual oxygen consumption; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3.

Introduction

Mitochondria are ubiquitous organelles in eukaryotic cells whose primary functions are to generate energy, regulate the cellular redox state, calcium homeostasis, and initiate cellular apoptosis [1]. In addition, mitochondria are the main intracellular source and immediate target of reactive oxygen species (ROS), which are continuously generated as byproducts of aerobic metabolism in mammalian cells. Thus, mitochondria play a pivotal role in the determination of the life and death of the mammalian cell [2]. The size, shape, and abundance of mitochondria vary dramatically in different cell types and may change under different energy demands and physiological environmental conditions [3]. In many cell types, especially muscle fibres, mitochondria form tubular structures or networks [4]. Mitochondria are highly dynamic organelles with constant fusion and fission events mediated by conserved cellular machineries. The frequencies of these fusion and fission events are balanced to maintain the overall morphology of the mitochondrial population [5] and to control mitochondrial energy metabolism, protecting cells from mitochondrial damage [6, 7].

Mitochondria are recognised as major targets of bioactive compounds, such as omega-3 polyunsaturated fatty acids (PUFAs) and flavonoids, which are found in healthy diets. The current data support a role for omega 3 PUFAs supplementation, particularly docosahexaenoic acid (DHA), which is strongly associated with changes in and the remodelling of mitochondrial phospholipid composition, fluidity, plasticity and organisational domains [8-13]. Although DHA is a likely target for oxidation [14-16], its health benefits are largely derived from DHA (22:6n-3)

incorporation in place of other fatty acids into biological membranes and its cell signalling mechanisms. Incorporation of 22:6n-3 influences membrane structure and function [17], increasing membrane permeability [18] and, altering conformational states with their acyl chains, which are extremely flexible [12], and influences the physical properties of biological membranes, thereby altering protein function and fusion [12, 19]. In addition to being susceptible to lipid peroxidation, DHA could decrease mitochondrial function simply as a result of the accumulation of oxidised products [20], altering the lipid bilayer and decreasing bioenergetic activities due to membrane perturbations [18]. In mitochondria, PUFAs play a role in several mitochondrial processes, including mitochondrial calcium homeostasis, gene expression, and respiratory function, and act as protonophores to reduce mitochondrial ROS production through uncoupling protein (UCP)-mediated decreases in mitochondrial membrane potential [16].

Mitochondria can also be regarded as important intracellular targets of agents that protect from the undesirable action of ROS, such as polyphenols, which prevent against many pathological states involving oxidative cell damage [21]. Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol isolated from green tea and is widely studied because it promotes cardiovascular and metabolic health by acting as a potent antioxidant that may have therapeutic applications in the treatment of many disorders. EGCG has antioxidant properties [22-24] with powerful radical scavengers. These antioxidant activities are due to the presence of phenolic groups that are sensitive to oxidation and are increased by the presence of the trihydroxyl structure in the D ring in EGCG [25]. Therefore, the presence of antioxidants jointly with omega 3 PUFAs would prevent possible oxidative deterioration.

Taking into account that skeletal muscle tissue is a major determinant of whole-body energy metabolism, the aim of this study was to examine how EGCG and DHA, alone or in combination, affect cell energy, mitochondrial morphology, oxidative phosphorylation, ROS generation and calcium homeostasis in L6 myocytes.

Materials and Methods

Chemicals

(-) Epigallocatechin-3-gallate (EGCG) from green tea, cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), fatty acid-free BSA, 2',7'-dichlorofluorescin diacetate (DCFH-DA), DMSO, Bradford reagent, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin, rotenone, antimycin A, rhodamine 123 (Rhd123), ethanol, succinate and Fluo-3 acetoxymethyl ester (Fluo-3 AM) were obtained from Sigma-Aldrich (Madrid, Spain), and the MitoTracker FM was obtained from Molecular Probes (Eugene, Oregon, USA). Dulbecco's Modified Eagle's Medium (DMEM), glutamine, fetal bovine serum (FBS), penicillin and streptomycin were obtained from BioWhittaker (Verviers, Belgium).

Cell culture

L6 myocyte cells (kindly supplied from Dr Manuel Portero-Otín) were routinely cultivated in DMEM supplemented with 2 mM glutamine, 10% FBS, 1% penicillin (126.6 U/mL) and 1% streptomycin (0.126 mg/mL) at 37ºC in an atmosphere of 5% CO_2. The cells were grown to approximately 80% confluence and then induced to differentiate into myotubes in DMEM supplemented with 2% FBS. After 7 days, myotube differentiation was complete, and the experimental procedure was initiated. L6 cells were serum-starved for 4 hours before the assay. All experiments were performed in triplicate in 3 independent experiments.

Cell treatment

To study the effects of EGCG, DHA and the combination of both compounds on mitochondrial function, metabolism and morphology, L6 myocyte cells were treated with the vehicle control, 25 µM EGCG, 25 µM DHA, or 25 µM EGCG + 25 µM DHA. Both compounds were dissolved in ethanol and added to the culture media. The medium used during the treatment was serum-free DMEM containing 2% BSA. The experiments were performed

in triplicate and with 3 different passages. The final concentration of ethanol in the media was 0.05%, a nontoxic percentage. After 4 hours, the cells were used for the different analysis.

Cell citotoxicity assay using the lactate dehydrogenase (LDL) method

LDH is released into the culture media following loss of membrane integrity. Therefore, membrane integrity was assessed by estimating the amount of LDH present in the media. The assay was performed according to the manufacturer's instructions for the LDH kit (QCA; Amposta, Spain). Cell cytotoxicity (%) was normalised to the control group.

Measurement of intracellular ROS generation using the DCFH assay

The levels of intracellular ROS were quantified using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) [26]. A stock solution (10 mM) of DCFH was prepared in DMSO. Cells (2×10^6 per well) were incubated in black 24-well plates with clear bottoms. DCFH-DA diffuses into cells and becomes trapped inside the cell after being cleaved by intracellular esterases.

After treatment, the cells were washed twice with warmed PBS, and 10 µM of DCFH diluted in PBS was then loaded into the wells for 30 minutes at 37ºC in the dark. The cells were then gently washed twice in PBS to remove the excess dye and resuspended in 0.5 mL of PBS per well. The fluorescence intensity was recorded over 4 hours as a measure of the degree of cellular oxidative stress. Intracellular ROS production was measured using an FLx800 Multi-Detection Microplate Reader (BioTek, Winooski, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (37ºC). The measured fluorescence values are expressed as a percentage of fluorescence with respect to the control group.

ADP/ATP ratio

The total ADP/ATP ratio in the muscle cells was determined after 4 hours of treatment using the APosensor™ ADP/ATP ratio Assay kit (Biovision, Mountain View, CA, USA) following the manufacturer's instructions.

Oxygen consumption in intact cells

In vivo measurements of oxygen consumption with intact cells were performed using a high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria) to quantify the respiration states. L6 myocytes were treated in 6-well plates and removed from culture dishes through trypsinisation (0.05% trypsin-EDTA). After 5 minutes of centrifugation at 200 g (room temperature), the cells were resuspended in warmed respiration medium (DMEM without fetal bovine serum) and transferred to the corresponding respiration chamber at a concentration of 4-6 × 10^6 cells/mL. Analyses of respiration rates were performed in 2 mL of respiration medium at 37ºC with stirring at 750 rpm. When the oxygen concentration was stabilised, basal respiration was recorded (Routine state) to control the levels of respiration and phosphorylation in a physiologically coupled state, which was supported by exogenous substrates in the culture media. Following stabilisation of the Routine state, ATP synthesis was inhibited with 2 µg/mL oligomycin, and the nonphosphorylating or resting state (Leak state) was recorded. Subsequently, 10-12 µM FCCP was added to stimulate respiration maximally at a level flow, measuring the electron transport capacity (ETS) in the noncoupled state (ETS state). In sum, respiration was blocked with 2.5 µM rotenone and 2.5 µM antimycin, representing the residual oxygen consumption (ROX) state that remains after electron transport chain (ETC) inhibition. The results are expressed as oxygen flow per number of cells (pmol oxygen/10^6 cells*s). All results were corrected using the ROX state capacity. Oxygen consumption was calculated using DataGraph Software from Oroboros Instruments (Innsbruck, Austria).

Mitochondrial membrane potential (MMP)

MMP was monitored using Rhd123 dye fluorescence with an excitation wavelength of 525 nm and an emission wavelength of 485 nm using an FLx800 Multi-Detection Microplate Reader (BioTek; Winooski, USA) at 37ºC. Rhd123 is a membrane-permeant cation that is strongly sequestered in mitochondria due to their negative

membrane potential. If mitochondria become depolarised, Rhd 123 is redistributed from the mitochondria to the cytosol, where it becomes diluted and, as a consequence, the fluorescence increases. Therefore, a decrease in fluorescence corresponds to an increase in MMP. To perform the analysis, cells (2×10^6 per well) were incubated in black 24-well plates with clear bottoms. Rh123 was dissolved in ethanol as a 0.5 mM stock solution. After treatment, the cells were washed 3 times with warm PBS and permeated with a permeating solution (0.5 µg/mL digitonin, 250 mM sucrose, 1 mM EDTA, 50 mM KCl, 2 mM KH_2PO_4, 25 mM Tris-HCl (pH 7.4)). To initiate the assay, 6 mM of succinate and 0.5 µg/ml Rhd 123 were added to the wells. Fluorescence was recorded after the addition of 2 µg/mL oligomycin to inhibit the ATP synthase, and 10 µg/mL of FCCP was then added to visualise the complete depolarisation. The results are expressed as a % compared to the control group.

Intracellular Ca^{2+} levels

Intracellular Ca^{2+} was measured using the fluorescent calcium indicator Fluo-3 AM. This dye is a fat-soluble reagent, is not fluorescent and is membrane permeate. Intracellular esterases break down the Fluo-3 AM ester into acetoxymethyl and Fluo-3, which can then combine with free intracellular calcium ions. The intensity of the fluorescence is dependent on the free calcium concentration. L6 cells were treated in 24-well black plates with clear bottoms. After treatment, the cells were washed three times in standard medium (141 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES (pH 7.4)). The cells were loaded with Fluo-3 AM (5 µM) for 45 minutes (37ºC) in the dark in the standard medium. The cells were then washed again to allow for the cleavage of the acetoxymethyl esters and resuspended in the standard medium or Ca^{2+}-free solution (the same as the standard medium except for the addition of calcium). Fluorescence was measured after 30 minutes using an FLx800 Multi-Detection Microplate Reader (Biotek, Winooski, USA) with an excitation wavelength of 503 and emission wavelength of 526 (37ºC). The Ca^{2+} level is expressed as a percentage of the fluorescence intensity relative to the control group's fluorescence intensity.

RNA extraction and quantitative real time PCR (qRT-PCR) analysis

Total RNA was obtained from L6 cells using a RNeasy Mini kit (Qiagen; Valencia,Spain) according to the manufacturer's protocol. RNA (4 µg) was reverse transcribed to complementary DNA from the total RNA using a reverse transcription reagent kit (Applied Biosystems; Madrid, Spain). Gene expression was analysed by qRT-PCR amplification using TaqMan Universal 2X PCR Master Mix (Applied Biosystems; Madrid, Spain) and a PCR 7300 system (Applied Biosystems; Madrid, Spain) according to the manufacturer's instructions.

The thermal cycling consisted of an initial step at 50°C for 2 minutes, followed by a polymerase activation step at 95°C for 10 min and a cycling step with the following conditions: 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Specific TaqMan assay-on-Demand probes were used to amplify the cDNA:

Cyclophilin peptidylprolyl isomerase A (*Ppia*) (Rn00690933_m1) (used as an endogenous control gene), cytochrome C oxidase subunit V (*Cox5a*) (Rn 00821806_m1), citrate synthase (*Cs*) (Rn00756225_m1), ATP5A1 (*ATPase*) (Rn01527025_m1), adenine nucleotide translocase 1 (*Ant1*) (Rn 00821477_g1), uncoupling protein 3 (*Ucp3*) (Rn00565874_m1), uncoupling protein 2 (*Ucp2*) (Rn00571166_m1), manganese superoxide dismutase (*MnSod*) (Rn00566942_g1), mitochondrial fission 1 protein (*Fis1*) (Rn01480911_m1), dynamin-related protein 1 (*Drp1*) (Rn00586466_m1), mitofusin 2 (Rn00500120_m1 (*Mfn2*), optic atrophy 1 (Rn_00592200_m1) (*Opa1*). The expression levels were normalised to cyclophilin using a comparative ($2^{-\Delta\Delta Ct}$) method.

mtDNA content using quantitative real-time PCR

Total DNA was extracted from cells using a Qiamp DNA mini kit (Qiagen; Valencia, Spain) according to the manufacturer's instructions. The relative mitochondrial DNA (mtDNA) levels were measured by real-time PCR using the PCR 7300 system (Applied Biosystems, Madrid, Spain) and normalised by simultaneous measurement of the nuclear DNA (nDNA), (mtDNA/nDNA ratio). Primers and probes for quantitative PCR (qPCR) were designed using Primer Express (Applied Biosystems, Madrid, Spain): *Nd3* mitochondrial gene (forward: 5'-cttatcttttatcctcatttcaattgca-3', reverse: 5'- gtagtgggtattggttgtttgaatcgctc-3') and *Gadph* as a nuclear gene (forward: 5'-ccagaacatcatccctgcat-3'; reverse: 5'-gttcagctctgggatgacctt-3'). PCR conditions were 95ºC for 15 min, followed by 40 cycles of 95ºC for 15 s, and 60ºC for 60 s. The threshold cycle number (Ct) values for *Nd3* and *Gadph* were

determined. The results were calculated from the difference in threshold cycle (ΔC_t) values for mtDNA and nuclear-specific amplification.

Mitochondrial morphology and mass

To determine the mitochondrial morphology of individual cells, L6 myocytes were grown on coverslips inside 6-well collagen-treated plates filled with the appropriate culture medium. After treatment, the media was removed from the dish, and the cells were washed 3 times with prewarmed PBS. A concentration of 400 nM MitoTracker Green FM was added in PBS. The cells were incubated at 37ºC for 30 minutes. The solution was then replaced with fresh media, and the cells were coverslipped for fluorescence microscopy to examine their morphology. Confocal images were obtained using a confocal laser-scanning microscope (NIKON TE-2000; Tokyo, Japan) with a 60× objective. MitoTracker Green FM preferentially accumulates in mitochondria regardless of the mitochondrial membrane potential and provides an accurate assessment of mitochondrial mass.

Statistical Analysis

The results are expressed as the mean ± SEM of 6 animals. SPSS Statistics version 19 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Significant differences were analysed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A *p-value* ≤ 0.05 was considered statistically significant.

Results

EGCG reverses the increase of intracellular ROS caused by DHA treatment

The selected physiological concentration of 25 µM for EGCG and DHA was non-toxic to
L6 cells. The treatment with 25 µM DHA for 4 hours significantly increased (150%) the intracellular ROS levels (Figure 1) compared to the control and EGCG groups. In contrast, treatment with 25 µM DHA concomitant with 25 µM EGCG attenuated and reversed the increase in ROS levels induced by DHA.

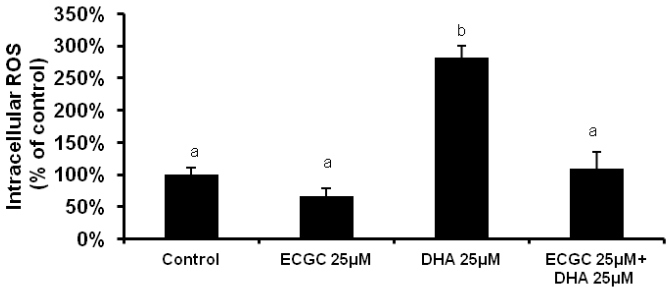

Figure 1. ROS levels in L6 cells treated with EGCG, DHA or EGCG+DHA. Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. ROS production was then measured using the DCFH assay. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences (p≤0.05) among the different groups.

DHA increases the ADP/ATP ratio in addition to lowering oxygen consumption in intact L6 cells even when combined with EGCG

Figure 2 presents the changes in oxygen consumption caused by the different treatments in Routine, Leak and ETS states. The Routine state (Figure 2a) controls physiological respiration through the cellular energy demand and is supported by the substrates in the culture media. Here, in the Routine state, treatment with DHA or DHA+EGCG for 4 hours significantly reduced the oxygen consumption of the L6 cells, whereas there were no significant differences in oxygen consumption between the EGCG and control groups. By measuring the respiration rate in the presence of oligomycin, an ATPase inhibitor that is a direct measure of uncoupled respiration (Leak state) *in situ* (Figure 2b), it has been shown that in the DHA and EGCG + DHA groups, oxygen consumption was significantly

decreased. In the ETS state (Figure 2c), in the presence of FCCP, an uncoupler and a strong indicator of potential mitochondrial dysfunction, a decrease in the DHA and EGCG + DHA groups compared to the control and EGCG groups was evident, though not statistically significant. The reduction in oxygen consumption (approximately 69%) in the Leak state compared to the routine conditions of the EGCG+DHA group differs slightly compared to the remaining groups, with the DHA group having a reduction in oxygen consumption of 59%, similar to the control group, whereas, in the EGCG group, there was a reduction of approximately 50%.

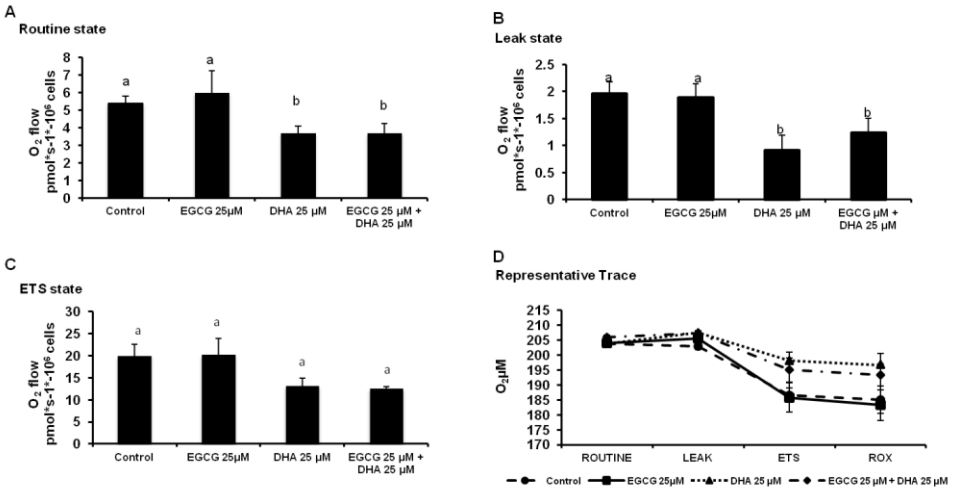

Figure 2. Oxygen consumption of L6 cells under different treatments Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. *In vivo* oxygen consumption was then measured using high-resolution respirometry and different intact cell states: A. Routine state, B. Leak state, C. ETS state. D. Oxygen consumption trace in the chamber. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically ($p \leq 0.05$)

Figure 2d presents a representative trace for the oxygen concentration in the chamber. This chart shows the differences between the groups in terms of the oxygen consumed in the different states and dependent on the treatment used. It clearly indicates that the DHA group tends to consume less oxygen during all recording states, especially in ETS states. Moreover, the ADP/ATP ratio (Figure 3) was significantly higher in the DHA group and significantly lower in the EGCG group, whereas in the EGCG+DHA group, the ADP/ ATP ratio was similar to that of the control group.

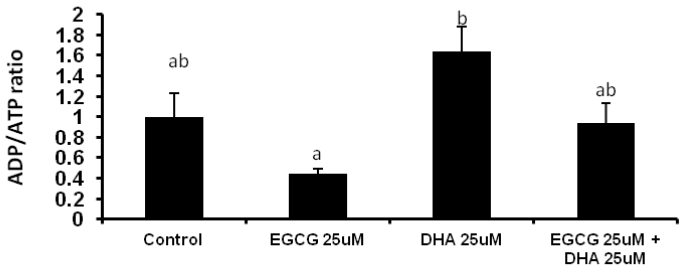

Figure 3. ADP/ATP ratio in L6 cells treated with EGCG, DHA or DHA+EGCG.. Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. The ADP/ATP ratio was then measured using an Aposensor kit. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences ($p \leq 0.05$) among the different groups.

DHA decreases mitochondrial membrane potential simultaneously with an increase of intracellular Ca^{2+} levels

Figure 4 shows the results for the MMP measurements. When oligomycin was added, there was a significant increase in arbitrary fluorescence units (AFU) values for the DHA treatment with respect to the control conditions,

indicating a decrease in the membrane potential. By the other hand intracellular calcium was elevated in DHA treatment in the presence of Ca^{2+} in the medium (Figure 5a). This elevation was not produced when a Ca^{2+}-free solution was used (Figure 5b).

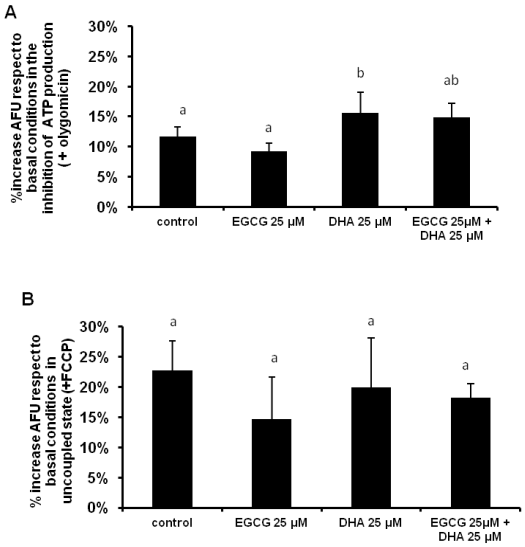

Figure 4. Mitochondrial membrane potential in L6 cells treated with EGCG, DHA or DHA+EGCG. Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. The mitochondrial membrane potential was then measured using: A. oligomycin and B. the FCCP condition. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences (p≤0.05) among the different groups. Arbitrary fluorescence units (AFU).

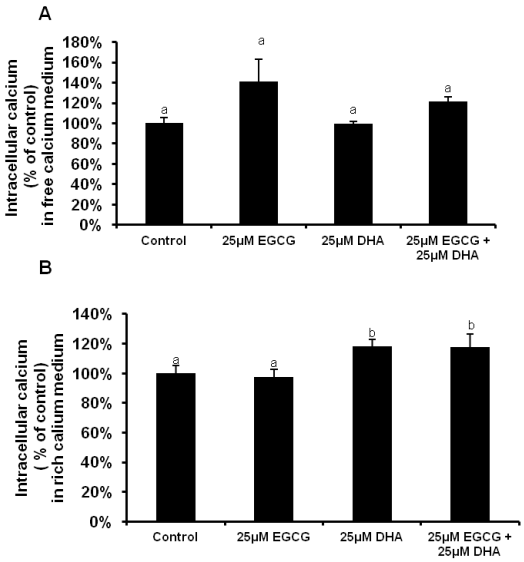

Figure 5. Intracellular calcium levels in L6 cells treated with EGCG, DHA or DHA+EGCG.
Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. Intracellular calcium levels were then measured in different medium conditions: A. Calcium-free medium and B. Calcium-replete medium. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences (p≤0.05) among the different groups.

DHA downregulates genes related to mitochondrial function, upregulates MnSod in parallels with UCPs, which maintained upregulation with EGCG cotreatment

The effects of DHA, EGCG and DHA+EGCG on the mRNA gene expression of marker enzymes of mitochondrial functionality are shown in Table 1. As in the respiration analyses, treatment with 25 µM EGCG did not induce a change in mitochondrial gene expression. In the case of 25 µM DHA treatment, *Cs* and *ATPase* gene expression did not differ between the groups. In contrast, *Cox* and *Ant1* gene expression were downregulated compared to the control group; however, *Cox* and *Ant1* were not downregulated when the cells were treated with DHA concomitant with EGCG. Both uncoupling protein genes (*Ucp2* and *Ucp3*) are expressed in muscle cells and were significantly upregulated in the DHA and EGCG + DHA groups. In examining the antioxidant system of the mitochondria, *MnSod* gene expression was significantly upregulated by DHA treatment compared to the control, but not in the co-treatment group.

Table 1. mRNA expression indicating mitochondrial function in L6 cell treated with DHA, EGCG or DHA+EGCG

	Control	EGCG 25µM	DHA 25µM	EGCG 25µM+ DHA 25µM
Cox	1.00±0.03a	0.99±0.09ab	0.82±0.03b	0.77±0.08ab
Cs	1.00±0.03a	0.92±0.07a	1.02±0.06a	0.92±0.06a
Atpase	1.00±0.02a	1.04±0.03a	1.01±0.05a	0.85±0.08a
Ant1	1.00±0.02a	0.89±0.07ab	0.86±0.03b	0.89±0.04ab
Ucp3	1.23±0.15a	1.32±0.28a	2.51±0.29b	2.03±0.37b
Ucp2	1.05±0.16a	1.22±0.12a	2.48±0.07b	2.11±0.37b
Mnsod	1.00±0.052a	1.21±0.16ab	1.18±0.17b	1.13±0.01ab

Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. Different mRNA levels associated with the OXPHOS system were then analysed using real-time qRT-PCR. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences (p≤0.05) among the different groups.

DHA and EGCG+DHA increase mtDNA and downregulate enzymes involved in mitochondrial dynamics

As shown in Figure 6, the relative amount of mtDNA undergoes a 58% increase in the DHA-treated cells compared to the control group. Moreover, in the EGCG+DHA group, there was also an increase of approximately 61% compared with the control group.

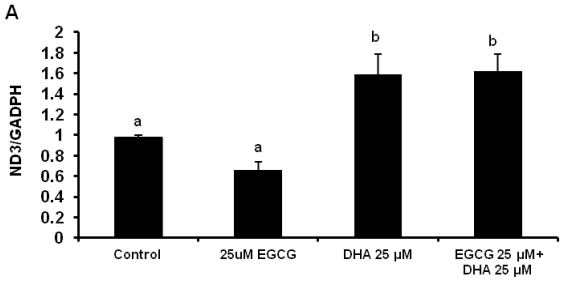

Figure 6. Mitochondrial mass of L6 cells treated with EGCG, DHA or DHA+EGCG.
Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. The mitochondrial mass: mtDNA/nDNA ratio (ND3/GADPH by real time PCR) was then analysed. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences (p≤0.05) among the different groups.

Dynamin-related GTPases mediate the fission and fusion of mitochondrial membranes. In the outer membrane, Mfn1and Mfn2, are involved in the dynamic formation of the mitochondrial network. Similarly, OPA1, which is in the inner membrane, controls mitochondrial membrane fusion, whereas Drp1 triggers fission events [27].

Table 2. mRNA expression for mitochondrial dynamics in L6 cells treated with DHA, EGCG or DHA+EGCG

	Control	EGCG 25µM	DHA 25µM	EGCG 25µM+ DHA 25µM
Fiss1	1.00 ± 0.01a	0.96 ± 0.04a	0.67 ± 0.04b	0.71 ± 0.04b
Drp1	1.00 ± 0.03a	0.90 ± 0.05ab	0.77 ± 0.0b	0.72 ± 0.02b
Mfn2	1.00 ± 0.03a	0.92 ± 0.0a	0.76 ± 0.01b	0.85 ± 0.05ab
Opa1	1.01 ± 0.05a	1.05 ± 0.05a	0.93 ± 0.08a	0.90 ± 0.04a

Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours to analyse different mRNA levels associated with the mitochondrial dynamics system, which were analysed using real-time qRT-PCR. The results are expressed as the mean ± SEM of triplicate measurements and are representative of three independent experiments. Different letters indicate statistically significant differences (p≤0.05) among the different groups.

The results of how EGCG and/ or DHA compounds modulated the expression of genes implicated in mitochondrial dynamics are shown in Table 2. The results revealed that fission genes were downregulated, specifically *Fis1* (located in the outer membrane) and *Drp1*, in the DHA treatment compared to the control and EGCG groups. Likewise, *Mfn2* was significantly downregulated with the DHA treatment compared to the control group; however, in terms of *Opa1* expression, there were no significant differences between the groups.

Mitochonarıaı morphology and mass after the DHA and EGCG treatments

To investigate whether EGCG and DHA directly alter mitochondrial dynamics, a morphological analysis was performed using fluorescence microscopy with the mitochondrial mass marker MitoTracker Green, which provided information about the mitochondrial organisation and dynamics in L6 myocytes. Figure 6a shows that the L6 control cells exhibited tubular networks with round and stubby mitochondria. After EGCG treatment (Figure 7b), the mitochondrial morphology was similar to the control groups, with round spheres and large shapes. In contrast, the mitochondrial morphology after DHA treatment (Figure 7c) was shifted toward a fragmented and tubular discontinuous network with a higher proportion of large and elongated mitochondria. Co-treatment with EGCG (Figure 7d) reversed the mitochondrial fragmentation, restoring the mitochondrial network with elongated and interconnected myotubes.

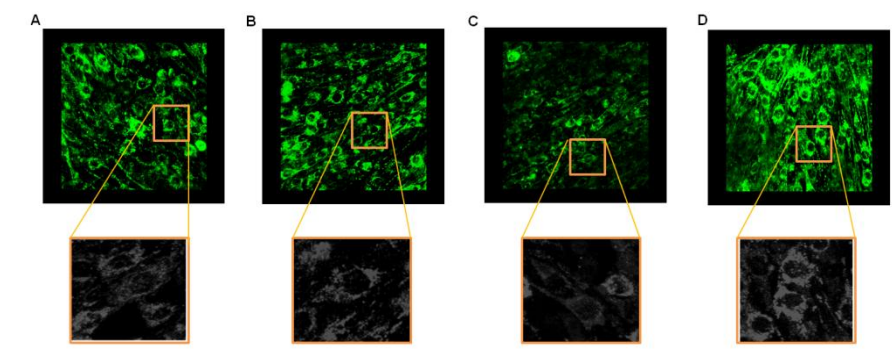

Figure 7. Mitochondrial morphology of L6 cells treated with EGCG, DHA or DHA+EGCG. Cells were incubated with 25 µM EGCG, DHA or EGCG + DHA for 4 hours. The visual mitochondrial network and morphology were then visualised using MitoTracker Green FM staining and a confocal microscope. A. Control, C. 25 µM EGCG, C. 25 µM DHA and D. EGCG + DHA (25 µM each).

Discussion

In aerobic organisms, approximately 85–90% of cellular oxygen is consumed by mitochondria to produce energy in the form of ATP molecules, concomitant with the formation of ROS. ROS induce chemical modifications in other molecules, generating oxidative damage, regulating signal transduction components, and acting as second messengers for various physiological and pathological stimuli [28]. The incubation of L6 myocytes with 25 μM DHA for 4 hours led to an increase (150%) in intracellular ROS production without causing lost of cell viability. This result is in agreement with the interaction or incorporation of added DHA into the cell membrane phospholipid composition [29, 30] and the susceptibility of most polyunsaturated n-3 fatty acids to produce oxidative damage in cells [31]. In addition, many lipid peroxidation products are themselves very potent ROS producers that can induce considerable damage to other biological molecules [32]. The increased ROS levels in the DHA group led to MnSod overexpression, which is one of the primary antioxidant responses to elevated ROS production [33, 34].

Mitochondrial respiration was also deregulated by the DHA treatment. The results from the *in vivo* oxygen consumption assay indicate that substrate oxidation was altered, decreased in the Routine and Leak state, by the DHA and EGCG+DHA treatments. In addition, DHA-treated cells clearly consumed less oxygen during the entire recording protocol. A few studies have used dietary fish oil to investigate the influence of omega 3 PUFAs on mitochondrial respiration and have reported either no change or decreases during some measurements of various substrates [18, 32]. Here, with the decrease in mitochondrial respiration, mitochondrial functionality was also affected. DHA may have contributed as a potent deregulator of O_2 consumption and oxidative phosphorylation with the decreasing bioenergetic activities being, in part, due to the result of membrane perturbations caused by DHA [18]. Thus, DHA treatment lowered mitochondrial functionality through ETC and OXPHOS system disruptions, concomitant with *Cox* downregulation, the increased ADP/ATP ratio and *Ant1* downregulation, which blocked the exchange of ADP and ATP across the mitochondrial inner membrane. The impairment in mitochondrial functionality was reversed in the EGCG+DHA group, whereas the ROS levels remained at the same levels as the control cells, which is consistent with the role of EGCG as a potent antioxidant [35] and as an uncoupler-like [36] compound, decreasing ROS production. The polyphenol structure of EGCG facilitates its capacity to penetrate membranes, resulting in ROS scavenging between the mitochondrial membrane and matrix [37]. Moreover, *Cox* and *Ant1* expression were unaltered, and the ADP/ATP ratio was similar to the control cell value. Furthermore, *Ucp3* was overexpressed with DHA treatment. The role of *Ucp3* overexpression during DHA treatment is to protect myocytes from ROS; as already described, *Ucp3* overexpression neutralises oxidative stress in mouse myotubes [38]. It is important to emphasise that the overexpression of *Ucp2* and *Ucp3* was maintained in the DHA+EGCG group because, despite the antioxidant effects of EGCG, DHA was still present in the culture media.

Furthermore, as mitochondria participate in intracellular Ca^{2+} homeostasis via several Ca^{2+} uptake and release pathways, ROS production could affect Ca^{2+} homeostasis due to the deterioration of membranes that contain the intracellular Ca^{2+} stores [39, 40]. The results from the present study reveal that the DHA group displayed higher intracellular Ca^{2+} levels when Ca^{2+} was added to the culture media. However, if calcium was not supplemented, this increase was not observed. Accordingly, it appears that the high levels of ROS produced by DHA incorporation or through alterations of the cell membrane phospholipid composition induced a calcium influx of external calcium that was not from mitochondria or other calcium stores.

It appears that the respiratory activity of ETC is linked to mitochondrial morphology because metabolic changes could be produced by variations in membrane fatty acid composition [32], as described above. The downregulation of fission (*Fis1*) and fusion (*Mfn2*) gene expression during DHA treatment is a mechanism to compensate for mitochondrial function and to restore mitochondria tubules [7], in accordance with the low respiratory capacity, low oxidative phosphorylation, decreased MMP and the incapacity to increase respiration upon the addition of FCCP shown in the DHA group, as other authors have reported [41-43]. Another compensatory mechanism is the higher mtDNA/nDNA ratio of the DHA-treated cells, suggesting that endogenous and exogenous oxidative stress are factors involved in the increase of the mitochondrial abundance and mtDNA copy number in human and animal cells [3], resulting from a feedback response that compensates for defective mitochondria, hallmarks of an impaired respiratory chain or mutated mtDNA [44]. The *Fis1* and *Mfn2* gene were also downregulated in the EGCG+DHA group, in which endogenous respiration in the Routine state was also low

and concomitant with altered mitochondrial morphology and cellular bioenergetic dynamics [45-47], as in the DHA group, due to the PUFA-containing mitochondrial membranes [43] that led to the oxidative damage. In addition, *Drp1* was also downregulated in the L6 cells treated with DHA and after the EGCG+DHA treatment. It has previously been demonstrated that *Drp1* downregulation in HeLa cells leads to slower cell growth, ETC uncoupling, decreased cellular respiration and increased ROS levels [45], as occurred here in L6 myocytes after DHA treatment. The imbalance in mitochondrial architecture by the decreased fusion and fission gene expression levels revealed that the majority of mitochondria were shorter, more elongated and tubular, and sometimes fragmented, as described in 3T3-L1 adipocyte studies [48, 49]. In addition, this deregulated mitochondria morphology might lead to dysfunctions in oxidative phosphorylation [7, 50] in cells treated with DHA, as mentioned above. After EGCG+DHA treatment, the mitochondria were more rounded with a reticulum network [4, 51], developing functional machineries and restoring mitochondrial tubules [52]; however, *Fis1* and *Mfn2* were still down regulated. In addition to possessing the antioxidant and uncoupling abilities mentioned above, polyphenols, specifically EGCG, also influence mitochondrial morphology [36]. Thus, ROS generation produces a downregulation in gene expression levels implicated in ETC complexes due to DHA membrane incorporation with a rapid alteration in mitochondrial fusion/fission gene expression levels, fragmented and elongated mitochondrial tubule networks, an unbalanced mitochondrial morphology, and increased mtDNA level to compensate for mitochondrial dysfunction.

Despite the few changes observed in the L6 myocytes when treated only with EGCG, co-administration with DHA reduced DHA intracellular ROS overproduction, altering the ADP/ATP ratio and expression of *Cox* and *Ant1* to control levels, restoring mitochondrial morphology. In conclusion, the combination of DHA+EGCG could be a good choice for avoiding and correcting the possible deleterious effects of DHA.

Acknowledgements

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3.2 Manuscript II

"Omega-3 polyunsaturated fatty acids and proanthocyanidins improve postprandial metabolic flexibility in rat"

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Omega-3 polyunsaturated fatty acids and proanthocyanidins improve postprandial metabolic flexibility in rat

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Abstract

Postprandial lipemia influences the development of atherosclerosis, which itself constitutes a risk factor for the development of cardiovascular diseases. The introduction of bioactive compounds may prevent these deleterious effects. Proanthocyanidins are potent antioxidants that have hypolipidemic properties, while omega-3 polyunsaturated fatty acids (ω3 PUFAs) stimulate fatty acid oxidation and gene expression programs, controlling mitochondrial functions. In this study, we investigated the effects of acute treatment of ω3 PUFAs and proanthocyanidins on the metabolic flexibility and lipid handling profiles in the skeletal muscle and adipose tissue of rats that were raised on diets, high in saturated fatty acids. For this, oil rich in docosahexaenoic (DHA-OR), grape seed proanthocyanidins extract (GSPE), or both substances (GSPE + DHA-OR) were administered with an overload of lard oil to healthy Wistar rats. Our results indicate that the addition of DHA-OR to lard oil increases insulin sensitivity and redirects fatty acids toward skeletal muscle, thereby activating fatty acid oxidation. We also observed an improvement in adipose mitochondrial functionality and uncoupling. In contrast, GSPE lowers lipidemia, prevents muscle reactive oxygen species (ROS) production and damage, furthermore, activates mitochondrial biogenesis and lipogenesis in adipose tissue. The addition of GSPE+DHA-OR to lard resulted in nearly all the effects of DHA-OR and GSPE administered individually, but the combined administration resulted in a more attenuated profile. © 2013 BioFactors, 00(00):000–000, 2013

Keywords: *docosahexaenoic acid; proanthocyanidins; metabolic flexibility; skeletal muscle; adipose tissue*

1. Introduction

Animal physiologies require many adaptations to guard against major discontinuities in both the supply of and demand for energy. Such capacities characterize the healthy state and have been collectively termed "metabolic flexibility." The ability of subjects to respond to nutritional challenges may reflect the flexibility of their biological systems; as a result, the study of postprandial events in disease development has attracted significant interest. Epidemiological studies have demonstrated that postprandial hypertriglyceridemia possess an independent risk for coronary atherosclerosis [1,2]. Furthermore, the efficiency with which the body manages incoming dietary lipids can modulate the risk of chronic disease, such as obesity [3], type 2 diabetes [4,5], and non-alcoholic fatty liver disease (NAFLD) [6]. The physiological and biochemical responses to dietary perturbations are complex; these responses primarily affect the mechanisms of energy storage, which are mostly controlled by insulin and involve metabolic switches in several key organs [7]. Skeletal muscle is a major player in maintaining energy balance, as it accounts for >20% of the body's total energy expenditure, in addition is the major tissue involved in insulin-stimulated glucose uptake and glucose storage (possessing >4 times the liver's glycogen capacity) and strongly influences metabolism by modulating lipid flux (both circulating and stored lipids) [8]. Skeletal muscle is also the most important organ in balancing the uptake and oxidation of fatty

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acids (FA) and is a major consumer of O_2 because it accounts for a large share of the total body mass [9]. Fat overload may represent a metabolic challenge for many apparently healthy individuals, who may be unable to appropriately upregulate skeletal muscle lipid oxidation, resulting in intracellular lipid accumulation and the development of insulin resistance [9,10]. As a result, skeletal muscle studies are particularly relevant to the characterization of whole-body metabolic flexibility [8]. Adipose tissue, which stores energy in the form of triglycerides (TG) also, plays a major role in maintaining metabolic flexibility. Specifically, adipose tissue buffers the daily influx of dietary fat entering to the circulation and prevents lipotoxicity due to excessive exposure of other tissues to non-esterified fatty acids (NEFAs) [11].

Herein, we investigated the postprandial state following the oral administration of lard. We hypothesized that the variation in fat types caused by adding oils rich in omega-3 polyunsaturated fatty acids (ω3 PUFAs) could cause changes in the activity of muscle and white adipose tissue and potentially improve metabolic flexibility. The ω3 PUFAs consisted primarily of docosahexaenoic acid (DHA) and were administered in small quantities compared with the total amount of lard. Many studies have demonstrated that ω3 PUFAs are involved in a variety of mitochondrial processes, including homeostasis, gene expression, and apoptosis [12]. It has also been shown that ω3 PUFAs stimulate the expression of genes related to mitochondrial functionality, fatty acid oxidation, and the inhibition of fatty acid synthesis [13,14]. Moreover, ω3 PUFAs have also been shown to improve insulin sensitivity [15] and are more potent inducers of ROS generation [16] than either saturated or mono-unsaturated fatty acids. An increase in ROS may be associated with the inhibition of the respiratory chain complex because ω3 PUFAs may change the fluidity of the mitochondrial membrane as a result of their incorporation into the membrane bilayer [12,17]. Furthermore, ω3 PUFAs are believed to aid in the prevention of various chronic diseases [18], including coronary heart disease [18], strokes [19], and certain types of cancer.

We also hypothesized that the addition of proanthocyanidins, which constitute the most abundant polyphenols in the human diet, could further improve postprandial metabolic flexibility because these substances are potent antioxidants [20] with hypolipidemic effects [21]. Likewise, proanthocyanidins are anti-inflammatory [22], cardioprotective [20], and decrease risk factors associated with cardiovascular diseases (CVD), such as insulin resistance [23]. Animal studies have demonstrated that proanthocyanidins reduce the plasma levels of atherogenic apolipoprotein B (ApoB) rich in TG and low density lipoprotein (LDL)-cholesterol while simultaneously increasing the levels of antiatherogenic high density lipoprotein (HDL)-cholesterol; the reduction of plasma TGs constitutes their strongest effect [21]. Furthermore, polyphenols may modulate muscle lipid metabolism [24]. In fact, several studies have demonstrated that polyphenols possess muscle lipid-lowering properties [25] and also downregulate the expression of cardiac fatty acid translocase/cluster of differentiation 36 (*Cd36*) [26].

The goal of this study was to compare the postprandial responses following a challenge with high levels of saturated fat, supplemented with ω3 PUFAs or proanthocyanidins. Moreover, the synergistic capacity of both compounds improving metabolic flexibility has been also evaluated. The results were evaluated in skeletal muscle and white adipose tissue, which are both key organs in the maintenance of metabolic flexibility.

2. Experimental Procedures

2.1. Reagents

Grape seed proanthocyanidin extract (GSPE) was provided by Les Dérives Résiniques et Terpéniques (Dax, France). GSPE contains 21.3% monomeric, 17.4% dimeric, 16.3% trimeric, 13.3% tetrameric, 5–13 units of oligomeric, and 31.7% proanthocyanidins.

The ω3 PUFAs oil was provided by Market DHA^{TM}-S (Columbia, MD). The nutritional oil is derived from the marine algae *Schizochytrium* sp., which is a rich source of docosahexaenoic acid (DHA) and is supplemented with sunflower lecithin and rosemary extract (flavoring). The fatty acid profile of oil rich in DHA (DHA-OR) was 5.6% 14:0, 16.1% 16:0, 0.9% 18:0, 15% 18:1n-9, 1.4% 18:2n-6, 0.5% 20:4n-6 (ARA), 1.1% 20:5n-3 (EPA), 16.2% 22:5n-6 (DPA), 38.8% 22:6n-3 (DHA), and 4.4% other species. Tocopherols and ascorbyl palmitate are added as antioxidants to provide stability.

2.2. Animals and Treatments

Male Wistar rats weighing 200 ± 50 g were supplied by Charles River (Barcelona, Spain). The animals were housed in a 12 h light/dark cycle at 21–23 °C, fed with a standard chow diet (Panlab 04, Barcelona, Spain) *ad libitum* and provided access to tap water during the adaptation week.

After 1 week of adaptation, the animals were randomly divided into four equal groups (six rats/group) and deprived of food for 14 h before the experiment. To induce a metabolic challenge, the rats were orally gavaged with 2.5 mL/kg body weight (bw) of lard oil. Concomitant with the lard oil, three groups were also treated with 250 mg GSPE/kg bw (GSPE group), 250 mg ω3 PUFAs/kg bw (DHA-OR group) or 250 mg GSPE/kg bw plus 250 mg ω3 PUFAs/kg bw (GSPE + DHA-OR group). The group fed with lard oil alone, was named the lard oil group. Five hours after treatment, the animals were anesthetized using ketamine (70 g/kg bw, Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg/kg bw, Bayer, Barcelona, Spain) and sacrificed by exsanguination from the abdominal aorta. Blood was collected using heparin (Delta Laboratory, Barcelona, Spain) as anticoagulant. Plasma was obtained by centrifugation (1,500*g*, 15 min, 4 °C) and stored at −80 °C until the subsequent plasmatic parameter analysis. The gastrocnemius skeletal muscle and visceral adipose tissue were excised, weighed, and immediately frozen in liquid N_2 and stored at −80 °C until further analysis. Portions of the gastrocnemius skeletal muscle and visceral adipose tissue were also

placed on ice and used for mitochondrial fraction isolations to perform the enzymatic assays. The remaining gastrocnemius muscle was also rapidly used for mitochondrial isolation to study mitochondrial functionality. All procedures were approved by the Experimental Animal Ethics Committee of the University Rovira i Virgili.

2.3. Plasmatic Parameter Analysis

Plasma total cholesterol, TGs, glucose, and creatine kinase (CK) were measured using an enzymatic colorimetric kit (QCA, Barcelona, Spain). NEFAs were measured using a kit (WACO Chemicals (Neuss, Germany). Plasma insulin levels were determined by rat insulin ELISA (Mercodia, Uppsala, Sweden).

2.4. Measurement of Intracellular ROS

To monitor the intracellular ROS production, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Madrid, Spain) was used. Briefly, frozen gastrocnemius skeletal muscle was placed on ice cold buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, 50 mM EDTA, pH 7.4.) and homogenized at 4 °C in a Teflon/glass homogenizer. Once the tissue was homogenized, 1 mM DCFH-DA was added to the sample and protected from light for 30 min. The intracellular ROS was analyzed by spectrofluorometry, using 507 nm as the excitation wavelength and 530 nm as the emission wavelength using FLx800 Multi Detection Microplate Reader (Biotek, Anaheim, California). Cellular ROS levels were normalized to total protein content and expressed as relative units of intensity of fluorescence in %.

2.5. Tissue Lipid Content Measurement

The lipid content of the gastrocnemius muscle was estimated using ethanolic KOH tissue solubilization. Briefly, about 50 mg of the tissue was weighed and digested in 0.15 mL of 3 M alcoholic KOH for 2 h at 70 °C. The resulting homogenates supernatants were diluted 10-fold with deionized water (dH_2O) and the TGs levels were measured using a colorimetric triglycerides kit (QCA, Barcelona, Spain).

2.6. Mitochondrial Respiration by High-Resolution Tracking

A piece of gastrocnemius muscle was rapidly excised, weighed and placed in ice-cold homogenization medium buffer (100 mM sucrose, 50 mM KCl, 20 mM K^+-TES, 1 mM EDTA, and 0.2% (w/w) BSA) for mitochondrial respiratory assays. Fresh skeletal muscle mitochondria were isolated by differential centrifugation as described previously by Hoeks et al. [27] and was used for subsequent respiratory assays. All procedures were performed on ice. The tissue was kept in isolation medium (100 mM sucrose, 50 mM KCl, 20 mM K^+-TES, 1 mM EDTA, and 0.2% (w/w) bovine serum albumin (BSA)), cooled on ice and minced with scissors and a mechanical Potter homogenizer in a presence of proteinase (Subtilisin®, 1 mg/g of tissue (Sigma-Aldrich, Madrid, Spain)). Volumes were adjusted to 35 mL with isolation medium. The homogenates were then centrifuged at 8,500*g* for 10 min at 4 °C, and the resulting pellet was resuspended in isolation medium and centrifuged again at 800*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 850*g* for 10 min at 4 °C. The final pellet was gently resuspended by hand-homogenization in a small glass homogenizer with a Teflon pestle in a small volume of isolation medium.

The protein contents of the mitochondrial preparations were determined using fluorescamine Fluram® (Sigma-Aldrich, Madrid, Spain) and BSA was used to generate a standard curve [28].

Mitochondrial function was measured *ex vivo* and recorded at 37 °C using a polarographic oxygen sensor in a two-chamber Oxygraph (OROBOROS® Instruments, Innsbruck, Austria). The oxygen flux was expressed in nmol O_2 per mg mitochondrial protein per minute.

To view the bioenergetics in freshly prepared mitochondria, we modulated the oxidative phosphorylation (OXPHOS) function in several ways: the basal state (state 2), the OXPHOS coupling effect (state 3) stimulated with adenosine diphosphate (ADP), non-phosphorylating respiration (state 4) with the inhibition of adenosine triphosphate (ATP) synthesis by the addition of oligomycin and OXPHOS non-coupling effect (state U) with the addition of cyanide trifluoromthoxyphenylhydrazone (FCCP). The measurements of the OXPHOS system were performed with three different sets of substrates: 5 mM pyruvate (glycolytic substrate) to show the functionality of the pyruvate dehydrogenase enzyme and tricarboxylic acid (TCA) cycle functionality; 10 mM glutamate (complex I substrate) + 10 mM succinate (to activate complex I and complex II with the maximum coupled oxidative capacity); and 2 mM L-carnitine + 50 μM palmitoyl-CoA (a fatty acid substrate to induce *β*-oxidation). Briefly, 0.2 mg (pyruvate/glutamate + succinate sets) or 0.5 mg (carnitine + palmitoyl-CoA set) of freshly isolated mitochondria isolated from the gastrocnemius skeletal muscle was incubated in 2 mL of respiration buffer, which consisted of 100 mM sucrose, 20 mM K^+-TES (pH 7.2), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 4 mM KH_2PO_4, 3 mM malate, and 0.1% BSA.

In each protocol, we used the same sequence of measurements to determine the respiratory states with different titrations: an initial state 2 or basal (presence of substrates), state 3 (+ 450 μM ADP), state 4 with 1 μg/mL of oligomycin to block respiration and finally, state U (+ 0.5 μM FCCP) to obtain the maximum respiratory capacity.

2.7. Mitochondrial Enzymatic Activity

Pieces of gastrocnemius muscle and visceral adipose tissue were weighed and placed on ice for subsequent mitochondrial fraction isolation and for further enzymatic assays. Pieces were isolated according to Fuster et al. [29]. Briefly, the tissues were homogenized at 4 °C at ratio of 1:3 (w:w) in a buffer (250 mM sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4) with a Teflon/glass homogenizer. The homogenate was centrifuged at 700*g* for 10 min at 4 °C and the supernatant were centrifuged at 12,000*g* for 10 min at 4 °C. The resulting pellet, which contained the purified mitochondria, was resuspended in 100 μL of a buffer containing 70 mM sucrose, 220 mM mannitol, 2 mM

TABLE 1 *Taqman probes*

Gene symbol	*Protein name*	*Reference*
Cpt1b	Carnitine palmitoyltransferase 1b, muscle	Rn00564242_m1
Srebpc	Sterol regulatory element binding transcription factor 1	Rn01495769_m1
Pgc1α	Peroxisome proliferative activated receptor, gamma, coactivator 1	Rn00580241_m1
Pparα	Peroxisome proliferator activated receptor alpha	Rn00566193_m1
Ucp3	Uncoupling protein 3	Rn00565874_m1
Ucp2	Uncoupling protein 2	Rn00571166_m1
Cox5a	Cytochrome c oxidase subunit Va	Rn00821806_m1
Cs	Citrate synthase	Rn00756225_m1
Atp5a1	ATP synthase	Rn01527025_m1
Ant1	Adenine nucleotide translocase 1	Rn 00821477_g1
Mnsod	Superoxide dismutase 2, mitochondrial	Rn00566942_g1
Pdhb	Pyruvate dehydrogenase beta	Rn01537771_g1
Lpl	Lipoprotein Lipase	Rn00561482_m1
Cd36	Fatty acid translocator	Rn00580728_m1
Glut4	Glucose transporter type 4	Rn01752377_m1

List of specific Taqman assay-on-demand probes used to analyze gene expression by qRT-PCR.

HEPES, and 1 mM EDTA, pH 7.4 to analyze cytochrome *c* oxidase (COX) and citrate synthase (CS) activities.

COX activity was determined spectrophotometrically, as described by Fuster et al. [29]. The activity was measured at 550 nm and 37 °C in the presence of the reduced substrate, 50 M cytochrome *c* in 10 mM phosphate buffer. The reduced substrate was obtained by dialysis of 0.8 mM oxidized ferrocytochrome c (Sigma-Aldrich, Madrid, Spain) in 1 mM phosphate buffer, pH 7, with 50 mM ascorbic acid (Sigma-Aldrich, Madrid, Spain). Finally, 10 μL of the diluted mitochondrial fractions were added to initiate the reaction.

CS activity was measured by the Srere method [30], which measures the reduction of 5.5-dithiobis(2-nitrobenzoic acid) (DTNB) in a coupled reaction with acetyl-CoA and oxalacetate at 412 nm. A reaction mixture containing 0.2 M Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, 0.1 mM DTNB and 10–15 μL of the mitochondrial fraction was measured following the addition of 0.5 mM oxaloacetate to initiate the reaction. The absorbance was monitored for 5 min.

2.8. RNA Extraction and Real-Time Quantitative PCR (qRT-PCR) Analysis

Total RNA was isolated from the gastrocnemius skeletal muscle and visceral adipose tissue using TRIzol (Invitrogen, Barcelona, Spain) and the RNeasy Kit (Qiagen, Valencia, CA), according to the manufacturers' protocols. RNA was reverse-transcribed from the total RNA to complementary DNA using the reverse transcription reagent kit (Applied Biosystems, Madrid, Spain). Gene expression was analyzed by qRT-PCR amplification using the TaqMan Universal 2X PCR Master Mix (Applied Biosystems, Madrid, Spain) and the PCR 7300 system (Applied Biosystems, Madrid, Spain), according to the manufacturer's instructions. The thermal cycling comprised an initial step at 50 °C for 2 min, followed by a polymerase activation step at 95 °C for 10 min and a cycling sequence with the following conditions: 40 cycles of denaturation at 95 °C for 15 s, followed by annealing at 60 °C for 1 min. Specific Taqman Assay-on-Demand Probes were used as described in Table 1. Cyclophilin peptidylprolyl isomerase A (ppia) was used as an endogenous control gene (Rn00690933_m1). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.9. Statistical Analysis

The results are expressed as the mean ± SEM of six animals. The software package SPSS Statistics version 19 (SPSS, Chicago, IL) was used for statistical analysis. Significant differences were analyzed by one-way analysis of variance (ANOVA)

TABLE 2 *mRNA Expression in Skeletal Muscle and Adipose Tissue*

	Lard oil	*GSPE*	*DHA-OR*	*GSPE + DHA-OR*
Adipose tissue				
Pparα	1.03 ± 0.12a	1.39 ± 0.13ab	2.02 ± 0.30b	0.35 ± 0.20b
Pgc1α	1.03 ± 0.13a	1.65 ± 0.35b	0.88 ± 0.20a	0.72 ± 0.08a
Srebpc	1.03 ± 0.10a	1.71 ± 0.25b	0.64 ± 0.15a	0.66 ± 0.21a
Ucp2	1.00 ± 0.01a	1.2 ± 0.11b	3.19 ± 1.22b	2.38 ± 0.45b
Cd36	1.08 ± 0.04a	1.08 ± 0.03a	1.04 ± 0.08a	1.01 ± 0.09a
Lpl	1.01 ± 0.08a	1.00 ± 0.09ab	0.9 ± 0.02ab	0.77 ± 0.04b
Skeletal muscle				
Pparα	1.04 ± 0.14a	0.45 ± 0.10b	1.31 ± 0.40ab	0.59 ± 0.07b
Pgc1α	1.1 ± 0.25a	0.36 ± 0.05b	0.69 ± 0.15ab	0.58 ± 0.02b
Ant1	1.05 ± 0.15ab	0.94 ± 0.06a	1.43 ± 0.18b	1.06 ± 0.07ab
Cox5a	1.08 ± 0.19a	1.43 ± 0.49a	1.59 ± 0.36a	0.9 ± 0.04a
Cs	1.00 ± 0.03a	0.82 ± 0.07a	1.33 ± 0.20a	0.97 ± 0.11a
Atp5a1	1.10 ± 0.16a	0.89 ± 0.13a	1.75 ± 0.18b	1.13 ± 0.16a
Ucp3	0.82 ± 0.11a	0.71 ± 0.06a	1.68 ± 0.30b	1.17 ± 0.16b
Srebpc	1.07 ± 0.20a	1.46 ± 0.31a	1.75 ± 0.25a	2.15 ± 0.42a
Cpt1b	1.01 ± 0.09a	1.05 ± 0.27ab	1.34 ± 0.08b	1.51 ± 0.07b
Cd36	0.88 ± 0.15a	0.75 ± 0.18a	1.64 ± 0.27b	1.04 ± 0.12ab
Lpl	0.98 ± 0.30ab	1.06 ± 0.08a	2.04 ± 0.70b	1.20 ± 0.35ab
Glut4	1.1 ± 0.17ab	0.75 ± 0.06a	1.73 ± 0.22c	1.20 ± 0.09b
Pdhb	1.07 ± 0.24a	0.99 ± 0.16a	0.97 ± 0.15a	1.18 ± 0.23a
Mnsod	1.02 ± 0.11a	0.43 ± 0.07b	0.75 ± 0.14ab	0.56 ± 0.01b

Effects of GSPE and/or DHA-OR on gene expression in skeletal muscle and adipose tissue. Rats were fasted for 14 h and orally gavaged with lard oil (2.5 mL/kg) with or without supplementation with proanthocyanidins (GSPE; 250 mg/kg bw) or oil rich in docosahexaenoic acid (DHA-OR; 250 mg/kg bw) or both compounds (GSPE + DHA-OR) after five hours. Each value is the mean ± SEM of six animals per group. The letters a, b, and c indicate significant differences ($P < 0.05$) between groups by ANOVA analysis.

followed by the Tukey *post hoc* test. A P-value ≤ 0.05 was considered statistically significant.

3. Results

We analyzed the interactions and amelioration of metabolic flexibility caused by the addition of bioactive compounds, including proanthocyanidins (GSPE) and ω3 PUFAs oil rich in DHA (DHA-OR). The compounds were administered in response to a postprandial challenge of lipid intake in healthy Wistar rats. To observe metabolic changes, we focused our studies on gastrocnemius skeletal muscle and adipose tissue.

3.1. DHA-OR Administration Counteracts the Homeostatic Distortion Caused by an Oral Overload of Saturated Fat

The supplementation of lard oil with DHA-OR resulted in a significant overexpression of muscle *Lpl* and fatty acid translocase *Cd36* (Table 2). *Cpt1b* was significantly overexpressed (Table 2), and the intramuscular lipid content was increased (Fig. 1). Moreover, the mRNAs of the mitochondrial membrane

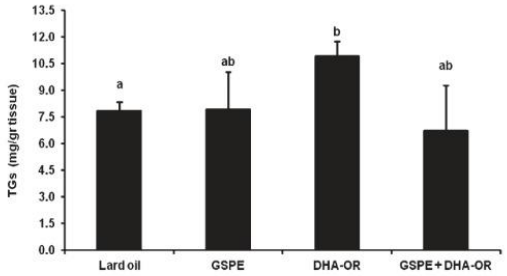

FIG 1 Skeletal muscle lipid content. Total levels of TGs were measured by the digestion of skeletal muscle in alcoholic 3 M KOH. Experimental details as in Table 2. Each value is the mean ± SEM of six animals per group. The letters a, b indicate significant differences ($P < 0.05$) between groups by ANOVA analysis.

proteins *Atp5a1*, *Ant1*, and *Ucp3* were also significantly overexpressed (Table 2). The mitochondrial respiration studies showed a significant decrease in oxygen consumption during basal state 2 and the uncoupled state U (Table 3) when DHA-OR was administered in the glycolytic substrates protocol. Moreover, no significant differences were found regarding CS and COX activity in the DHA-OR group (Table 4). Likewise, the activity of CS of the adipose tissue of the animals in the DHA-OR group was higher than in the lard group (Table 4) and the mRNAs of the uncoupling protein *Ucp2* were higher as well (Table 2). No significant differences were observed in plasma glucose levels after oral DHA-OR, GSPE, and GSPE + DHA-OR administration (Table 5). With respect to the concentrations of plasma TGs, NEFAs, and cholesterol, there were no significant differences in rats gavaged with DHA-OR compared with rats in the lard oil group. In contrast, the plasma insulin levels of the DHA-OR group were significantly decreased compared

TABLE 3 ***Oxygen consumption of skeletal muscle mitochondria in different substrates conditions***

	Lard oil	*GSPE*	*DHA-OR*	*GSPE + DHA-OR*
Oxygen consumption (nmols O_2/ mg protein/min)				
Pyruvate + Malate				
State 2	31.45 ± 4.34ab	39.14 ± 3.08a	24.15 ± 4.33b	30.21 ± 5.24ab
State 3 (+ADP)	524.05 ± 60.26a	568.35 ± 33.63a	438.65 ± 75.54a	431.12 ± 113.71a
State 4 (+oligomycin)	33.49 ± 7.19a	40.03 ± 3.40a	34.14 ± 6.36a	32.75 ± 4.90a
State U (+FCCP)	687.69 ± 25.53a	701.50 ± 45.18a	599.03 ± 92.53a	659.08 ± 135.44a
Palmityl-CoA+Carnitine				
State 2	17.50 ± 0.91a	25.38 ± 0.47b	13.18 ± 1.56a	20.70 ± 3.60ab
State 3(+ ADP)	106.72 ± 3.49a	120.84 ± 8.46a	107.95 ± 21.55a	92.92 ± 15.75a
State 4 (+oligomycin)	31.02 ± 1.76a	37.88 ± 1.84a	29.96 ± 7.16a	30.57 ± 3.50a
State U (+FCCP)	169.27 ± 29.99a	194.14 ± 9.11a	188.32 ± 30.78a	202.43 ± 39.67a
Glutamate-Malate-Succinate				
State 2	31.53 ± 1.92a	30.09 ± 1.51a	19.87 ± 4.79b	30.00 ± 3.34b
State 3 (+ADP)	470.83 ± 20.18a	421.62 ± 27.53a	360.89 ± 53.50a	345.81 ± 63.30a
State 3 (+Succiante)	423.54 ± 53.13a	488.86 ± 7.67a	453.82 ± 102.20a	263.59 ± 35.67a
State 3 (+ADP)	615.47 ± 73.25a	603.67 ± 10.38a	514.68 ± 80.82a	252.64 ± 109.56b
State 4 (+oligomycin)	79.10 ± 9.62a	73.51 ± 5.34a	56.92 ± 7.51a	64.41 ± 8.35a
State U (+FCCP)	479.91 ± 26.30a	482.29 ± 27.87a	284.27 ± 30.52b	365.59 ± 43.20c

Each value is the mean ± SEM of 6 animals per group. The letters a, b, c indicate significant differences ($P < 0.05$) between groups by ANOVA analysis.

TABLE 4 *Enzymatic activities of COX and CS in skeletal muscle and adipose tissue mitochondrial fractions*

	Lard oil	*GSPE*	*DHA-OR*	*GSPE + DHA-OR*
Mitochondrial enzymatic activities (μmols/ mg protein/min)				
Adipose tissue				
Citrate synthase (CS)	27.17 ± 5.96a	37.36 ± 7.04ab	51.59 ± 6.15b	51.59 ± 12.01ab
Cytochrome c oxidase (COX)	33.96 ± 1.22a	58.40 ± 10.58b	40.92 ± 5.17a	72.53 ± 15.97b
Skeletal muscle				
Citrate synthase (CS)	110.70 ± 6.98ab	93.05 ± 20.47ab	124.85 ± 11.12a	91.25 ± 6.42b
Cytochrome c oxidase (COX)	209.69 ± 23.82a	179.87 ± 45.74a	196.34 ± 15.48a	178.13 ± 62.38a

Each value is the mean ± SEM of six animals per group. The letters a, b indicate significant differences ($P < 0.05$) between groups by ANOVA analysis.

with rats of the lard oil group. *Glut4* was also overexpressed in skeletal muscle (Table 2).

3.2. The GSPE Supply Lowers Circulating Lipid Levels and Protects Muscle from the Saturated Fatty Acid Overload

There is a significant lowering of plasma CK concentrations and intracellular ROS production (Fig. 2); moreover, the expression levels of *Pgc1α*, *Pparα*, *Ucp3*, and *Mnsod* were downregulated (Table 2). In contrast, *ex vivo* mitochondrial respiration studies showed a significant increase in oxygen consumption in basal state 2 with palmitoyl-CoA as a substrate (Table 3). There were no changes in the activities of CS and COX (Table 4) in GSPE group respect to the lard oil group. In adipose tissue, GSPE supplementation was found to increase COX activity and upregulate the expressions of *Pgc1α*, *Srebpc*,

TABLE 5 *Plasma parameters*

	Lard oil	*GSPE*	*DHA-OR*	*GSPE + DHA-OR*
Plasma parameters				
Lipid metabolites				
TGs (mg/dL)	89.68 ± 11.07a	59.76 ± 5.52b	94.528 ± 6.57a	82.00 ± 9.62ab
NEFAs (mg/dL)	29.09 ± 2.17a	24.51 ± 1.41b	25.48 ± 0.17ab	21.64 ± 1.78b
Cholesterol (mg/dL)	66.53 ± 3.98a	53.92 ± 3.40b	58.95 ± 4.55ab	59.25 ± 2.25ab
Glucose homeostasis				
Glucose (mg/dL)	98.33 ± 6.30a	108.60 ± 11.16a	113.28 ± 12.97a	137.24 ± 25.75a
Insulin (ρmol/L)	64.13 ± 4.44a	62.30 ± 0.57ab	57.86 ± 0.54b	62.07 ± 5.86ab

Each value is the mean ± SEM of six animals per group. The letters a, b indicate significant differences ($P < 0.05$) between groups by ANOVA analysis.

and *Ucp2* (Table 2). With respect to plasma TGs, NEFAs and cholesterol concentrations (Table 5) were more significantly lower in the GSPE group than in the lard oil group.

3.3. GSPE Plus DHA-OR Modulates Metabolic Flexibility in Adipose and Muscular Tissues

In muscle, the GSPE+DHA-OR supplementation resulted in significant overexpression of *Cpt1b*, *Ucp3,* and *Glut4* (Table 2) in a manner similar to that of the DHA-OR group. However, *Pparα* and *Mnsod* were downregulated, as observed in the GSPE group (Table 2). Likewise, in the GSPE+DHA-OR group, the total mitochondria oxygen consumption in state U and state 3 was significantly lower in the glutamate-malate-succinate protocol (Table 3) result which correlates with a lower CS activity (Table 4). Concerning the effect of GSPE+DHA-OR in adipose tissue, *Ucp2* was upregulated as in the DHA-OR group, while *Lpl* was downregulated (Table 2). The mitochondrial fraction showed a significant increase in COX activity compared with the DHA-OR and lard oil groups (Table 4). The plasma levels of NEFAs were significantly lower compared with the lard oil group (Table 5), as in the presence of GSPE supplementation.

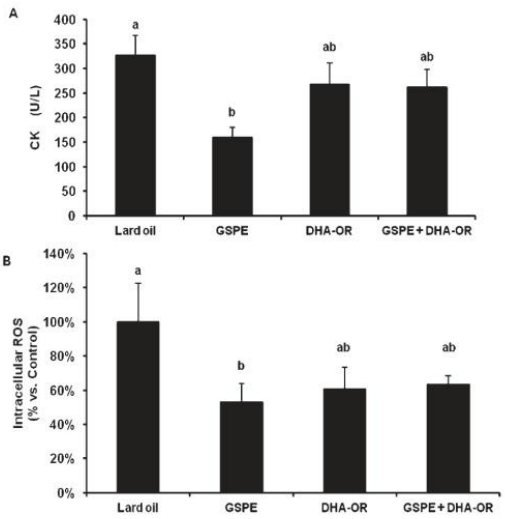

FIG 2 Creatine kinase plasma values and ROS production in skeletal muscle. A: Creatine kinase values were measured in plasma. B: ROS production was measured with cell-permeant DCFH-DA in a homogenized preparation of skeletal muscle. Experimental details as in Table 2. Each value is the mean ± SEM of six animals per group. The letters a, b indicate significant differences ($P < 0.05$) between groups by ANOVA analysis.

4. Discussion

Changes in lifestyle, dietary intake, and environmental factors lead to an increase in energetic density, with emerging metabolic dysfunctions and cardiovascular diseases determined to be concomitant with oxidative and metabolic stress, homeostatic perturbation, and inflammatory diseases [31]. In this context, the introduction of bioactive compounds may prevent these deleterious effects and offer opportunities to improve metabolic flexibility. The primary goal of this investigation was to examine the influence of DHA-OR and GSPE and their synergism on postprandial metabolic flexibility after an oral lard oil challenge. To this end, we have used a single non-toxic dose of both GSPE [32] and DHA-OR [33], and analyzed metabolic flexibility 5 h after treatments. Studies have shown that these bioactive compounds possess beneficial effects in treating metabolic syndrome and cardiovascular disease [34–36]. Herein, we explore the therapeutic potential of diet changes in improving metabolic flexibility and lipid handling in skeletal muscle and adipose tissues. Fatty acid metabolism in these tissues depends on many factors, including fatty acid availability, uptake, storage, and oxidation. In this study, we analyzed the gene expression patterns of key proteins, plasma parameters, intracellular ROS levels, and intramyocellular lipid accumulation. The activities of CS and COX and *ex vivo* mitochondrial function were also determined.

LPL is a major determinant of fatty acid delivery to muscle, where may be considered a metabolic gatekeeper and preferentially hydrolyzes TG rich chylomicrons (CMs) to release FA for tissue uptake and its regulation is tissue-specific. The supply of DHA-OR upregulates *Lpl* and *Cd36* in skeletal muscle (Table 2). CD36 is a membrane protein that functions in facilitating and regulating the entry and uptake of long chain fatty acids (LCFA) into the cell [37] apart from being insulin-sensitive. This recycling of CD36 seems to be pivotal for the proper regulation of cellular substrate uptake. For instance, this mechanism may be invoked to clear TG-derived FAs from the plasma, such as during the late phase following a meal [38]. Furthermore, it has been suggested that CD36 may act as a LCFA acceptor in the CPT1 system for the mitochondrial uptake of LCFAs [39]. In our observations, DHA-OR upregulated *Cpt1b* (Table 2), indicating an increased flux for FA oxidation in mitochondria. In addition, the muscles were found to exhibit a high lipid content (Fig. 1), which is normal in the existing postprandial situation [40] and may imply storage usage for subsequent fatty acid oxidation [41,42]. Concomitant with the upregulation of *Cpt1b*, there was significant overexpression of *Atp5a1, Ucp3, and Ant1* mRNA with respect to the lard oil group (Table 2). The increase of expression of *Ucp3* and *Ant1* tends to compensate for increasing demand of mitochondrial ATP production and is involved in the control of both mitochondrial energy conversion and ATP/ADP transport across the inner mitochondrial membrane [43].

Moreover, there was no significant difference in the activities of CS and COX (Table 4), which are markers of the TCA and the inner membrane integrity, respectively [44]. The lower oxygen consumption shown in state 2 with malate-glutamate

as substrates and also with pyruvate-malate, in the absence of adenylates, reflected a lower respiration rate that was specific to complex I (Table 3). Likewise, the overexpression of *Ucp3* caused by DHA-OR (Table 2) implied a mild uncoupling, because ATP continued to be generated and oxygen consumption in state U was lower than in the lard oil group (Table 3). UCP 3 may also reduce the production of mitochondrial ROS and thereby protect against mitochondrial dysfunction in skeletal muscle and free fatty acids (FFA) peroxide overload [45,46]. UCP 3 has been suggested to specifically fulfill this role under high-fat conditions. DHA-OR also leads to an increase in TG storage (Fig. 1); if this condition is chronic, it may also be associated with impairments in insulin signaling [41]. Our experiments indicate that this is not the case because DHA-OR improves insulin sensitivity (Table 5). This finding was described by Suresh and Das [15], who noted decreasing levels of plasma insulin without changes in plasma glucose, as well as overexpression of the *Glut4* gene, which is essential for insulin-stimulated glucose uptake in muscle cells [47]. Likewise, it has been reported that DHA-OR exerts a tissue-specific influence in which the expression of *Glut4* (Table 2) may be mediated by altering the membrane phospholipid [36]. Despite the modifications caused by DHA-OR in insulinemia and in FA uptake, storage, and oxidation in muscles, lipidemia did not change significantly with respect to the lard oil group (Table 5). In contrast, adipose tissue did not overexpress *Lpl* or *Cd36* (Table 2). When TGs on CMs are hydrolyzed by LPL, not all the fatty acids are taken up; some fatty acids spill over into the systemic plasma [48–50]. In the early postprandial period (0–2 h), most FFAs are trapped (100%–80%), but in the late postprandial period (4–6 h), additional FFAs escape entrapment (50%–80%) [38,51]. Thus, a considerable portion of the circulatory postprandial FFAs (30%–40%) originates from TG hydrolysis in the vasculature of adipose tissue [51]. Contrary to muscle LPL, which is considered to be a metabolic gatekeeper, Frayn et al. [52] proposed that the adipose LPL does not function as gatekeeper and is the hormone-sensitive lipase (HSL) and FA esterification which govern fatty acid mobilization and deposition in adipose tissue. It is important to emphasize that, despite the lack of changes in lipidemia, the higher CS activity (Table 5) and the overexpression of *Ucp2* mRNA (Table 2) indicate that the functionality, oxidative capacity, and uncoupling occur in the adipose tissue mitochondria of the DHA-OR group [53]. In general, all the changes caused by unsaturated fatty acids added to lard oil tend to redirect nutrient partitioning toward skeletal muscles, thereby improving the tissue's capacity to regulate fatty acid oxidation and thus improve metabolic flexibility.

When GSPE is administered with lard oil, other parameters are being modified differently from those DHA-OR. Indeed, the oral intake of GSPE improved lipidemia; the increase of plasma TG induced by lard oil was blocked (Table 5), as observed in previous studies [23,54]. The plasma levels of NEFAs (Table 5) may result in decreased FA availability for TG synthesis in the liver following GSPE administration with reduced very low-density lipoprotein (VLDL)-TG secretion [24]. Amin et al. demonstrated that a high-fat diet produced an increase in serum CK activity in rats [55,56], which suggested that feeding a high-lipid diet induced oxidative stress and had the possibility of injuring skeletal muscle [57]. Herein, the GSPE supply lowered the plasma CK levels and muscle ROS species in lard-fed rats (Fig. 2), which improved the condition of the skeletal muscle state. This reduction in ROS agrees with the notion that fatty acid oxidation decreases due to the down-regulation of *Pgc1α* (Table 2) and its target genes, as well as a direct scavenging effect of GSPE [58,59]. As a result, the down-regulation of *Mnsod* mRNA observed in the GSPE group (Table 2) should be due in part to the lowered ROS content inside the mitochondria because these antioxidant enzymes are substrate-inducible [60]. The maintenance of mitochondria functionality in muscle is supported because no changes were detected in CS and COX activities (Table 4), although the increase in oxygen consumption in state 2 with palmitoyl-CoA as a substrate (Table 3) indicates that there is a proton leak causing a slight uncoupling of mitochondria, as well as a decrease in ROS (Fig. 2b), which is in agreement with the results of Pajuelo et al. [25]. Apart from the GSPE effects mentioned previously, there was a significant overexpression of *Srebpc* and *Pgc1α* (Table 3), as well as an increase of COX activity (Table 4), in adipose tissue. The mitochondrial biogenesis and COX activation in adipose tissue agree with promoted fatty acid β-oxidation, which would protect the organism against fatty acid leakage from adipocytes. This effect, together with our previous results, showing GSPE repression of hepatic lipogenesis and VLDL assembly in the liver, would account for the improved lipidemia in the GSPE group.

In general, all these changes indicate that, contrary to ω3 PUFAS, proanthocyanidins added to lard oil will not redirect fat toward skeletal muscle but, rather, will improve the capacity to regulate lipogenesis and the oxidation of fatty acids in adipose tissue and thus improve the overall metabolic flexibility. The combination of GSPE plus DHA-OR maintained the beneficial effects of GSPE, improving lipidemia tending to decrease levels of TGs, NEFAs (Table 5), and TGs accumulation in skeletal muscle compared with DHA-OR alone (Fig. 1). Moreover, *Glut4* was overexpressed in a manner similar to the DHA-OR group (Table 2). Interestingly, the oxidative capacity and uncoupled effects in the mitochondria of adipose tissue are preserved in the combined group, as shown by the *Ucp2* overexpression (Table 2) and the higher COX activity (Table 4). In skeletal muscle, the significant upregulation of *Cpt1b* (Table 2) a key enzyme of the β-oxidation pathway was observed to direct the flux of fatty acids to the mitochondrial oxidation.

In addition, GSPE plus DHA-OR caused an uncoupling effect due to the overexpression of *Ucp3* (Table 3) and the decrease of oxygen consumption in state 3 (Table 4), in addition to lowering CS activity (Table 5). However, the mild uncoupling was produced because the oxygen consumption in state U (glutamate-malate-succinate protocol) (Table 3) was decreased in the isolated mitochondria [61]. In general, the

combination of both bioactive compounds tend to decrease lipidemia and prevented ROS production and muscle damage in a manner similar to GSPE but with a smaller impact (Fig. 2). Like DHA-OR, the combination mildly uncoupled oxidative phosphorylation, but the effect was greater than with DHA-OR alone. This finding correlates with the reduced oxygen consumption in state 3; therefore, ATP synthesis is less efficient in the muscles of the GSPE+DHA-OR group. The addition of GSPE+DHA-OR to lard oil maintains nearly all the beneficial effects that have been previously observed using each compound individually through the modulation and coordination of fatty acid metabolism between skeletal muscle tissue and adipose tissue.

In summary, DHA-OR and GSPE improve metabolic flexibility with different ways. The addition of DHA-OR to lard oil increases insulin sensitivity, redirects fatty acids toward skeletal muscle, and activates the capacity to regulate fatty acid oxidation. This treatment, also improves adipose mitochondrial functionality and uncoupling by *Ucp2* overexpression. GSPE activates lipid accumulation and their oxidation in adipose tissue, thereby decreasing lipidemia and prevents muscle damage and ROS production; furthermore, GSPE activates mitochondria biogenesis and lipogenesis in adipose tissue. The addition of GSPE+DHA-OR to lard oil maintained almost all the effects of DHA-OR and GSPE when each factor is supplied individually but resulted in changes not so deep.

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3.3 Manuscript III

"Chronic intake of proanthocyanidins and oil rich in docosahexaenoic acid improve skeletal muscle oxidative capacity in diet-obese rats"

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Abstract

Background: Obesity has become a worldwide epidemic. The cafeteria diet (CD) induces obesity and oxidative stress-associated insulin resistance. Polyunsaturated fatty acids and polyphenols are dietary compounds that are intensively studied as products that can reduce the health complications related to obesity by counteracting metabolic inflexibility.

Objective: To evaluate the effects of 21 days of supplementation with grape seed proanthocyanidins extract (GSPE), docosahexaenoic-rich oil (DHA-OR) or both compounds (GSPE+DHA-OR) on skeletal muscle metabolism in diet-obese rats.

Methods: Wistar rats were allowed free access to a standard diet (STD) or a cafeteria diet, which was composed of STD plus highly palatable, energy-dense foods, for 10 weeks.

Results: The supplementation with different treatments for 21 days did not reduce body weight. All groups used more fat as fuel, particularly when both products were co-administered; muscle β-oxidation was activated and the mitochondrial oxidative capacity was higher. In addition to these outcomes shared by all treatments, GSPE reduced insulin resistance, reduced food intake, corrected oxidative stress and improved muscle status. DHA-OR upregulated manganese superoxide dismutase (*MnSod)* gene expression to neutralize increased reactive oxygen species (ROS) production. GSPE+DHA-OR counteracted ROS production and improved muscle status. All of these treatments increased 5'-AMP-activated protein kinase (AMPK) phosphorylation. AMPK activation by GSPE was consistent with higher plasma adiponectin levels. AMPK activation by DHA-OR correlated with an upregulation of peroxisome proliferator-activated receptor alpha (*Pparα)* gene expression, and with an upregulation of uncoupling protein 2 (*Ucp2)* gene expression by GSPE+DHA-OR.

Conclusion: Although the body weight of CD-fed rats did not change, GSPE and DHA increased fat burning and activate mitochondrial functionality and fatty acid FA oxidation in skeletal muscle. These effects are mediated, at least in part, through the AMPK signaling pathway. Thus, modifications at the cellular and molecular levels improved muscle status and could counterbalance the deleterious effects of obesity.

Keywords: obesity, docosahexaenoic acid, proanthocyanidins, skeletal muscle, mitochondria, β-oxidation

Introduction

Obesity is a complex, multifactorial disease characterized by increased body weight or, more specifically, an increase in adipose tissue. It is prevalent in both developed and developing countries and affects children as well as adults (1). Obesity produces adverse health consequences, such as dyslipidemia, insulin resistance, hypertension, type 2 diabetes and cardiovascular disease. Traditional anti-obesity strategies focus on reducing food intake and increasing physical activity. However, recent results suggest that enhancing cellular energy expenditure may be an attractive alternative therapy (2). Skeletal muscle has an important role in the overall utilization and oxidation of fatty acids (FAs). Specifically, skeletal manifests reduced reliance upon FA oxidation in obesity compared to skeletal muscle of lean individuals. Biochemical marker levels are consistent with these findings, Carnitine palmitoyl translocase (CPT) 1 expression is diminished in obesity, with reduced capacity for FA oxidation (3). Obesity may also interfere with mitochondrial bioenergetics by affecting cellular respiratory functions and oxidative pathways. In addition, there is a chronic elevation of circulating FA levels, a reduction in the expression of genes involved in mitochondrial biogenesis, an increased production of reactive oxygen species (ROS), and impaired mitochondrial biogenesis and function in skeletal muscle (4, 5). The decreased capacity for fuel usage, the impaired capacity to increase fat oxidation upon increased FA availability, and with reduced mitochondrial oxidation and phosphorylation activity is a fundamental characteristic of metabolic inflexibility in metabolic syndromes (1). The cafeteria diet (CD) is a robust model of human metabolic syndrome compared to

traditional lard-based high-fat diets (6). In this model, animals are fed with a standard diet (STD) and are concurrently offered highly palatable, energy-dense foods *ad libitum*. This diet promotes voluntary hyperphagia that results in rapid weight gain and increases fat pad mass and prediabetic parameters, such as glucose and insulin intolerance and dyslipidemia (6).

The use of dietary compounds that reduce health complications related to these pathologies is appearing as a new approach. Previous reports have raised the possibility that polyphenols and omega-3 polyunsaturated fatty acids (PUFAs) are good dietary compounds for reducing obesity-induced metabolic syndrome (7-10). Treatment of rodents with different polyphenols, such as resveratrol, genistein and epigallocatechin gallate, results in the upregulation of genes related to oxidative phosphorylation, the electron transport chain (ETC) and ATP synthesis (11). Furthermore, mitochondrial FA oxidation genes are upregulated (12), whereas FA synthesis genes are downregulated (13). Likewise, omega-3 PUFAs are involved in a variety of mitochondrial processes. Particularly, docosahexaenoic acid (DHA) consumption stimulated genes related to mitochondrial functionality, FA oxidation and inhibited genes involved in FA synthesis (14). Moreover, they are natural ligands of peroxisome proliferator-activated receptors (PPARs); such as PPARα that is activated by several PUFAs (15, 16), with eicosapentaenoic acid (EPA) and DHA as the predominantly active biological components (17, 18).

Some studies show a synergistic effect between polyphenols and omega-3 PUFAs (19). In the present study, we considered the possibility that intake of grape seed proanthocyanidins extract (GSPE) and/or DHA-rich oil (DHA-OR) concomitant with a CD could influence muscle, fat and carbohydrate oxidation rates, while improving the metabolic inflexibility associated with obesity-induced metabolic syndrome. Our hypothesis was that the consumption of GSPE and DHA-OR could increase mitochondrial oxidative capacity in skeletal muscle and that this could be a strategy to combat the adverse effects of obesity. Thus, we evaluated the effect of individual or simultaneous administration of GSPE and DHA-OR by analyzing the respiratory quotient (RQ) ratio (using indirect calorimetry (IC)), mitochondrial bioenergetics (by measuring oxygen consumption of mitochondria isolated from skeletal muscle), expression of key mitochondrial genes, and enzymatic activities.

Material and Methods

Grape seed proanthocyanidins extract

GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France) and its composition was described previously by Casanova et al.(20)

Oil rich in docosahexaenoic acid

The DHA-OR was provided by Market DHA^{Tm}-S (Columbia, MD, USA). The nutritional composition of the oil derived from marine alga, *Schizochytrium sp*., a rich source of DHA (38.8 %), was described previously by Casanova et al. (20)

Animals and diets

Male Wistar rats weighing 200±50 g were supplied by Charles River Laboratories (Barcelona, Spain). The animals were housed in a 12 h light-dark-cycle at 21-23ºC. Following adaptation week, animals were divided into five equal groups (7 rats/group) and housed individually to control the food intake. One group was the STD group, fed with standard chow diet (Panlab 04, Barcelona, Spain) *ad libitum*, and the remaining 4 groups were fed with STD plus CD as a hypercaloric diet, composed of 34.50 % carbohydrate, 37 % fat and 11.50 % protein. Animals were allowed free access to standard chow and water in addition to CD *ad libitum*. The CD consists of cookies, cheese portions, bacon, foie-gras, sugary milk, cupcakes and carrots. During the day, food was removed. Food consumption and weight gain were monitored weekly for 10 weeks until animals were 20 % overweight compared with the STD group. Subsequently, different treatments were administered jointly with the CD for 21 days. One group (CD+GSPE) was supplemented with 25 mg /kg body weight GPSE dissolved in 5 % Arabic gum (Sigma-Aldrich, Madrid, Spain). The second group was supplemented with 500 mg oil-rich DHA (38.8 %/) / kg body weight dissolved in 5 % of gum Arabic (CD+DHA-OR). The third group received GSPE (25 mg/kg body weight) plus 500 mg

oil-rich DHA (38.8 %)//kg body weight dissolved in 5 % of gum arabic (CD+GSPE+DHA-OR)). All groups received the same volume of gum arabic. The rats were treated in the afternoon, and then allowed free access to fresh portions of chow and/or CD during the night. On day 21 of treatment, all rats were fasted for 3 hours before being anesthetized using 50 mg/kg body weight of sodium pentobarbital (Fagron Iberica, Terrasa, Spain) and sacrificed by abdominal aorta exsanguination. Blood was collected using heparin (Deltalab, Barcelona, Spain). Plasma was obtained by centrifugation (1500 x *g,* 15 min, 4ºC*)* and stored at -80ºC until the subsequent plasmatic parameter analysis. Gastrocnemius skeletal muscles were excised, weighed, immediately frozen in liquid N_2 and stored at -80ºC until further analysis. Portions of the gastrocnemius skeletal muscle were also placed on ice and used to isolate mitochondrial fractions for enzymatic assays. The remaining gastrocnemius muscle was rapidly utilized for mitochondrial isolation to study mitochondrial functionality. All procedures were approved by the Animal Ethics Committee of our university.

Plasmatic Parameters

Creatine Kinase (CK) was measured using an enzymatic colorimetric kit (QCA, Barcelona, Spain). Adiponectin and Leptin levels were quantified using specific EIAs according to the manufacturer's instructions (Biosource International, Inc, USA).

Indirect calorimetry

Assessment of respiratory metabolism and dates of analysis were performed in a ventilated hood system (Panlab Harvard Apparatus, Barcelona, Spain). RQ values (VO_2 / VCO_2) were recorded by IC measurements at 10 and 20 days of treatment during 6 hours of the postprandial period (9.00 h – 15.00 h).

Mitochondrial respiration by high-resolution tracking

A piece of gastrocnemius muscle was rapidly excised, weighed and placed in ice-cold homogenization medium buffer (100 mM sucrose, 50 mM KCl, 20 mM K^+-TES, 1 mM EDTA and 0.2 % (w/w) FA free-BSA (Sigma-Aldrich, Madrid, Spain) for mitochondrial respiratory assays. Fresh skeletal muscle mitochondria used for respiratory assays were isolated by differential centrifugations as described by Hoeks, J. (21).

Mitochondrial functionality was measured *ex vivo* and recorded at 37ºC by a polarographic oxygen sensor in a two-chamber Oxygraph (OROBOROS ® Instruments, Innsbruck, Austria). The oxygen flux was expressed in nmol O_2 per mg mitochondrial protein per minute.

To view the bioenergetics in freshly prepared mitochondria, we modulated oxidative phosphorylation (OXPHOS) function in the following ways: basal state (State2), the OXPHOS coupling effect (State3) stimulated with + 450 µM ADP (Sigma-Aldrich, Madrid, Spain), non-phosphorylating respiration (State4) with the inhibition of ATP synthesis by the addition of 1 µg/ml oligomycin, and OXPHOS non-coupling effect (StateU) with + 0.5 µM trifluoromthoxyphenylhydrazone (FCCP) (Sigma-Aldrich, Madrid, Spain) addition to obtain the maximal respiratory capacity. The measurement of the OXPHOS system and the operation of the tricarboxylic acid cycle (TCA) were carried out using 3 different sets of substrates: 5 mM pyruvate + 2.5 mM malate (glycolytic substrates) to show the functionality of the pyruvate dehydrogenase enzyme and tricarboxylic acid cycle (TCA) functionality; 10 mM glutamate (complex I substrate) + 10 mM succinate (complex II substrate) were used in combination of 0.5 µM of rotenone to inhibit complex I in order to study complex II capacity alone; 2 mM L-carnitine + 50 µM palmitoyl-CoA (FA substrate to induce β-oxidation). Briefly, 0.2 mg (pyruvate / glutamate + succinate sets) or 0.5 mg (carnitine + palmitoyl-CoA set) of freshly isolated mitochondria were incubated in 2 ml respiration buffer.

Mitochondrial enzymatic activity

For the enzymatic activity assays, pieces of gastrocnemius tissue were weighed and placed on ice for subsequent mitochondrial fraction isolation to analyze cytochrome *c* oxidase (COX), citrate synthase (CS) and ATPase activities

according to the methods of Fuster et al. (22), Srere (23) and Bergmeyer (24), respectively, as we have detailed previously (25).

Determination of mitochondrial ROS produced in isolated mitochondria

To monitor mitochondrial ROS production, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, Madrid. Spain) was used (26, 27). Isolated mitochondria (0.1 mg protein/ml) were obtained as described in the mitochondrial respiration method, and incubated at 30ºC in phosphate-buffered saline (PBS). DCF fluorescence intensity was measured by spectrofluorometry using 507 nm excitation and 530 nm emission wavelengths (FLx800 Multi-Detection Microplate Reader, Biotek, USA) following the addition of 10 µM DCFH-DA to the mitochondrial suspension. Gastrocnemius mitochondrial ROS levels were normalized to total protein content and expressed as relative units of fluorescence intensity (%).

Lipid peroxidation in skeletal muscle-Malondialdehyde (MDA) Assay

Malondialdehyde (MDA), a lipid peroxide product, is an indicator of oxidative stress, and was measured using an assay kit according to the manufacturer's instructions (Oxford Biomed, Barcelona, Spain).

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from the gastrocnemius skeletal muscle tissue as detailed previously by Casanova et al. (20). Specific Taqman Assay-on-Demand Probes were used: Peroxisome proliferator activated receptor alpha (*Pparα*) (Rn00566193_m1), Carnitine palmitoyltransferase 1b (*Cpt1b*) *(Rn005664242_m1), Uncoupling protein 3 (Ucp3) (Rn00565874_m1),* Uncoupling protein 2 (*Ucp2*) (Rn00571166_m1), ATP synthase 5a1 (*Atpase*) (Rn01527025_m1), *Manganese* Superoxide dismutase (*Mnsod*) (Rn00566942_g1), Lipoprotein Lipase (*Lpl*) (Rn00561482_m1), FA translocator CD36 (*Cd36*) (Rn00580728_m1). Cyclophilin peptidylprolyl isomerase A (*Ppia*) was used as an endogenous control gene (Rn00690933_m1). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot (WB) analyses

Protein was extracted from the frozen gastrocnemius skeletal muscle in RIPA lysis buffer (150 mM Tris-HCl, 1 % Triton x-100, SDS 0.1 %, 167 mM NaCl, 0.5 % Na-deoxycholate) with a protease inhibitor cocktail 1/1000 (Sigma – Aldrich St. Louis, MO, USA), PMSF 1/100 (FLUKA-Biochemika, Switzerland) and a phosphatase inhibitor cocktail: cocktail II 1/100, cocktail III 1/100 (Sigma-Aldrich St.Louis, MO, USA). Total protein levels were determined using the Bradford method (28). Proteins were loaded and run on a 10 % SDS-polyacrylamide gel. Samples were transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA) using a transblot apparatus (Bio-Rad, Hercules, CA, USA) and subsequently blocked at room temperature with 5 % (wt/vol) non-fat milk in TBS-T buffer (Tris-Buffered saline, 0.5 % Tween-20). Primary antibodies were incubated overnight with shaking at 4ºC: rabbit AMPKα primary antibody, phospho AMPK α primary antibody (Cell Signaling Technology, Beverly, MA, USA), or rabbit α-tubulin (Sigma- Aldrich St. Louis, MO, USA). Blots were washed in TBST buffer and incubated with peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK) for 1 hour. Immunoreactive proteins were visualized as previously detailed by Castell-Auví et al.(29). The results were expressed as the relative intensity of p-AMPK/AMPK.

Statistical Analysis

The results are expressed as the mean ± SEM of seven animals. SPSS Statistics version 19 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Significant differences were analyzed by one-way ANOVA followed by the Tukey post-hoc test. A *p-value* ≤ 0.05 was considered statistically significant.

Results

GSPE treatment produced a tendency towards decreased body weight and food intake over 21 days

CD significantly increased body weight as compared to STD at 2 weeks. The difference was more pronounced at 10 weeks (STD: 439.14±10.35; CD: 518.50±6.97), concomitant with significantly lower RQ values in CD respect to STD group (STD: 1.00±0.01; CD: 0.88±0.01), which indicated a preference for lipid oxidation.

Figure 1A and Figure 1C show that the CD+GSPE group tended towards a decrease in body weight with respect to CD group beginning at 10 days of treatment and continuing through the end of testing. Figure 1B shows the food intake of each group during the entire treatment. Importantly, at day 5 of treatment, the CD+GSPE+DHA-OR group increased their food intake significantly compared to the control CD group. Although there is a trend through the last 5 days of treatment, after 21 days (Figure 1D), food intake was decreased significantly in the CD+GSPE group compared to CD group.

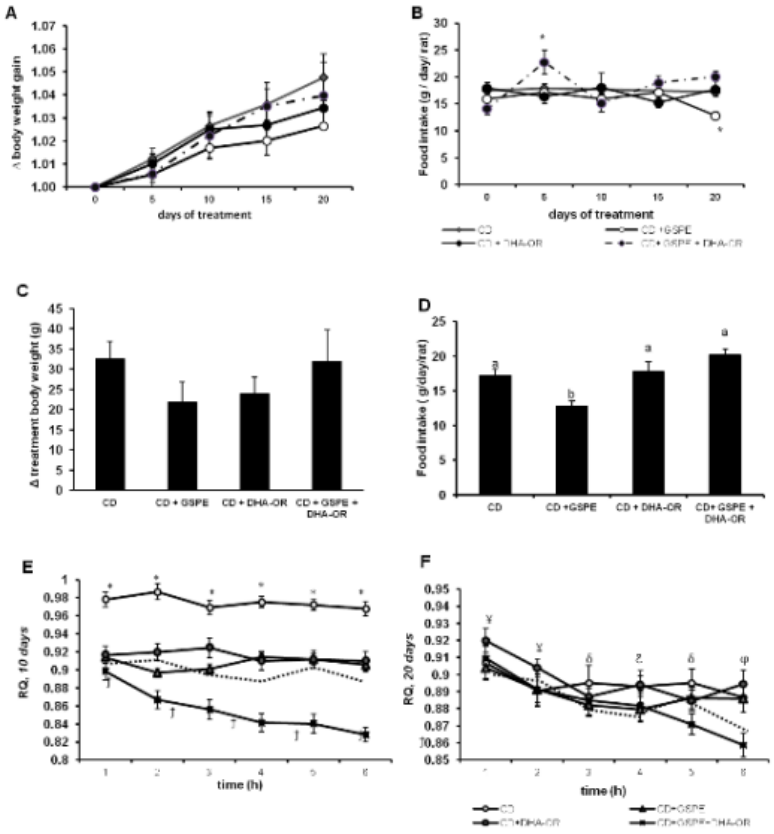

Figure 1. Effect of proanthocyanidins and/or DHA supplementation on energy metabolism in obese rats.A. Increase of body weight during the treatment. **B.** Food intake during the entire treatment. **C.** Increase in body weight at 21 day of treatment. **D.** Food intake at 21 days of treatment. **E.** RQ values at 10 days of treatment. **F.** RQ values at 20 days of treatment. Rats were fed cafeteria diet (CD) or standard diet (STD) for 10 weeks. After 10 weeks, rats were treated orally with vehicle (CD), 25 mg GSPE/kg body weight (CD+GSPE), 500 mg DHA/kg body weight (CD+DHA-OR) or 25 mg GSPE/kg body weight + 500 mg DHA/kg body weight (CD+GSPE+DHA-OR) for 21 days, concomitant with CD. Body weight, indirect calorimetry during 6 hours in postprandial state, and food intake were monitored regularly during the experiment. Data are means ± SEM; seven animals per group. Symbols denote a significant difference between groups by ANOVA analysis ($p< 0.05$). A, B: * difference with respect to all treated groups; C, D : * difference between CD+GSPE group and all groups; E,F: * difference with respect to all treated groups; ϯ difference between CD+GSPE+DHA-OR and all groups; ¥ difference between CD+DHA-OR and all groups; δ difference

between CD and all groups; ƨ difference of CD and CD+DHA-OR between CD+GSPE and CD+GSEP+DHA-OR; φ difference between CD+GSPE and CD+DHA-OR and CD+GSPE+DHA-OR. The dashed line indicates the baseline values on the standard diet.

GSPE+DHA-OR administration with CD decreased RQ values significantly at 10 days of treatment.

At 10 days of treatment (Figure 1E), RQ values from the CD group were significantly higher throughout the postprandial period compared to the treated groups. In contrast, the CD+GSPE+DHA-OR group had significantly decreased RQ values starting at 2 hours of treatment. The RQ values from this treatment group were lower than those of the STD group values, marked as a dashed line. Figure 1F shows that the differences in RQ values between the CD groups are decreased at 20 days of treatment. During the first 2 hours, there were significant differences between the CD+DHA-OR group and the remaining groups. However, the RQ values were significantly lower in the CD+GSPE+DHA-OR group compared to the remaining groups at the end of the measurement (5-6 hours).

Table 1. Effect of proanthocyanidins and/or DHA supplementation on oxygen consumption by skeletal muscle mitochondria in obese rats

	CD	CD+GSPE	CD+DHA-OR	CD+GSPE+DHA-OR
Oxygen consumption flux (nmols O_2 / mg*min)				
protocol pyruvate-malate				
State 2 (+Pyr)	48.7 ± 31.2a	55.3 ± 4.4a	67.4 ± 6.4a	40.9 ± 7.6a
State 3 (+ADP)	72.3 ± 35.9a	103.6 ± 43.7a	604.7 ± 100.0b	173.6 ± 9.3a
State 4 +oligomycin)	18.4 ± 7.6a	33.3 ± 7.4ab	73.2 ± 8.5b	44.5 ± 4.6ab
State U (+FCCP)	47.5 ± 22.8a	154.2 ± 60.0b	623.4 ± 75.3b	291.7 ± 40.3b
protocol GMS(r)				
State 2 (+GM)	28.7 ±3.9a	34.0 ± 9.3ab	77.1 ± 0.9b	32.6 ± 6.4a
State 3 (+ADP)	33.3 ± 20.6a	107.7 ± 12.9a	182.1 ±25.0b	150.6 ± 37.3ab
State 3 (+rot)	17.4 ± 7.2ab	24.9 ± 3.5a	47.5 ± 6.5b	20.9 ± 5.6a
State 3 (+Succ)	64.9 ± 22.7a	102.7 ± 17.4ab	181.8 ± 18.4b	82.0 ± 16.0a
State 4 (+oligomycin)	56.9 ± 20.2a	74.0 ± 10.0a	136.2 ± 11.1b	48.4 ± 8.2a
State U (+FCCP)	43.6 ± 12.9a	63.5 ± 9.1a	167.4 ± 12.2b	54.5 ± 11.4a
protocol Palmitoyl-CoA				
State 2 (+C)	8.7 ± 2.3a	42.4 ± 10.9ab	57.1 ± 9.1b	21.1 ± 8.4ab
State 2 (+CPCoA)	10.0 ± 6.5a	85.8 ± 27.9b	64.1 ± 10.2ab	27.6 ± 6.3a
State 3 (+ ADP)	9.0 ± 6.3a	150.2 ± 45.8ab	231.2 ± 29.8b	39.6 ± 12.5a
State 4 (+oligomycin)	2.7 ± 1.1a	76.9 ± 23.6b	97.8 ± 17.7ab	28.3 ± 6.3a
State U (+FCCP)	2.0 ± 1.1a	103.5 ± 53.5b	243.3 ± 60.7c	60.0 ± 33.3b

Abbreviation: Pyr, pyruvate; GM, Glutamate-Malate; rot, rotenone; succ, succiante; c, carnitine; CPCoA, carnitine + palmitoyl-CoA. Data are means ± SEM of seven animals per group. a and b denote significant differences between groups by ANOVA analysis ($p< 0.05$).

GSPE and DHA-OR administration with CD improved mitochondrial respiration compared to the obese group.

The results of assessing the functionality of the OXPHOS system are described in Table 1Animals fed with CD+DHA-OR had higher rates of respiration in all states compared to the other groups (Table 1) using three different sets of

substrates. The CD+DHA-OR group had higher rates in State3 and the respiratory maximum capacity was present in StateU, which increased ATP production and the maximum respiratory capacity.

Palmitoyl-CoA oxidation was increased by CD+GSPE, CD+DHA-OR and CD+GSPE+DHA-OR treatment. Furthermore, maximum respiratory capacity was increased significantly by GSPE and/or DHA-OR.

AMPK phosphorylation was activated by GSPE or DHA-OR treatments in skeletal muscle.

Figure 2 shows that the administration of either GSPE or DHA-OR activated AMPK phosphorylation and significantly increased the ratio of phosphorylated/unphosphorylated AMPK compared to CD and STD.

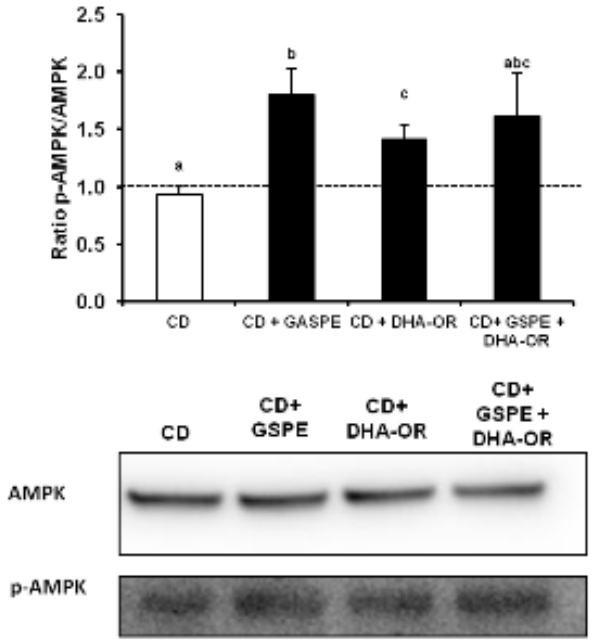

Figure 2. Effect of proanthocyanidins and/or DHA supplementation on AMPK phosphorylation in the skeletal muscle of obese rats. p-AMPK/AMPK protein levels. Experimental details as in figure 1. Data are means ± SEM of seven animals per group. a, b, and c denote a significant difference ($p < 0.05$) between groups by ANOVA analysis. The dashed line indicates the baseline values on the standard diet.

Both GSPE and DHA-OR administration affected the expression of genes related to FA oxidation in skeletal muscle.

Figure 3 shows that CD+DHA-OR treatment significantly increased *Pparα*, *Cd36, Lpl* and *Cpt1b* expression in muscle compared to the CD group. Additionally, CD+GSPE treatment significantly increased *Lpl, Cd36* and *Cpt1b* gene expression. For the CD+GSPE+DHA-OR group, *Ucp2, Lpl* and *Cpt1b* were significantly overexpressed.

It is important to note that *Ucp3* mRNA levels in rats fed with CD were higher than in those on the STD diet, although there were no differences between treated groups. None of the treatments altered *ATPase* or *Mnsod* gene expression.

GSPE administration increased ATPase activity, while GSPE and DHA-OR coadministration increased CS activity.

COX activity showed no difference between groups. However, ATPase activity was higher in the CD+GSPE group compared with the other groups. CS activity in the CD+GSPE+DHA group was significantly elevated compared to the CD group (Table 2).

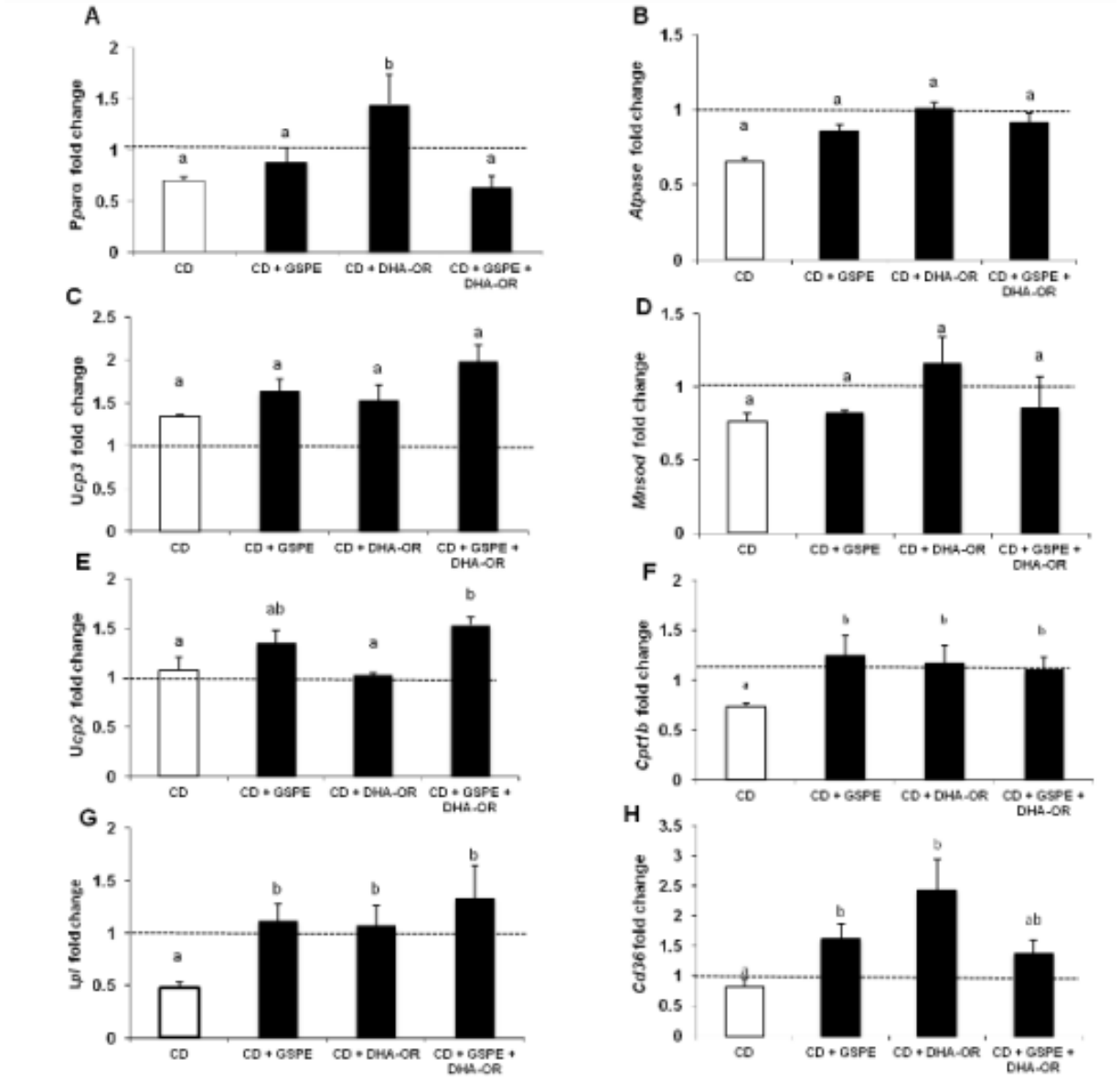

Figure 3. Effect of proanthocyanidins and/or DHA supplementation on gene expression in the skeletal muscle of obese rats.mRNA gene expression of **A.** *Pparα.* **B.** *ATPase*. **C.** *Ucp3*. **D.** *Mnsod.* **E.** *Ucp2*. **F.** *Cpt1b*. **G.** *Lpl*. **H.** *Cd36*. Experimental details as in figure 1. Data are means ± SEM of seven animals per group. a, b, and c denote a significant difference between groups by ANOVA analysis ($p < 0.05$). The dashed line indicates the baseline values on the standard diet.

GSPE administration decreased CK plasma values and inhibited DHA-OR-induced ROS production, whereas DHA-OR increased lipid peroxidation.

CK, an indicator of muscle damage, was significantly decreased by CD+GSPE administration with respect to the CD group, and also when administered concomitantly with DHA-OR. CK levels in both groups appeared to be restored to levels comparable to that in the STD group (Table 2). However, CD+DHA-OR or CD+GSPE+DHA-OR treatment induced lipid peroxidation and significantly increased MDA levels in muscle compared to the CD group (Table 2).

Moreover, ROS produced in mitochondria isolated from muscle were significantly increased in CD+DHA-OR-treated rats compared to the CD group and were decreased when GSPE was administered (Table 2).

Table 2: Effect of proanthocyanidins and/or DHA supplementation on the mitochondrial enzymatic activities and oxidative parameters of the muscle and mitochondria of obese rats

	CD	CD+GSPE	CD+DHA-OR	CD+GSPE+DHAOR
*Mitochondrial enzymatic activities (*µmols/min*mg protein)				
cytochrome c oxidase (COX)	18.7 ± 4.4a	19.5 ± 11.7a	10.1±3.3a	12.6±4.9a
citrate synthase (CS)	65.7 ± 24.0a	122.9 ± 4.8ab	130.6 ± 17.1ab	181.9±40.9b
ATPase	5.6 ± 1.3ab	8.8± 2.7b	4.8 ± 0.5a	5.1±1.1ab
Mitochondrial and muscle oxidative parameters				
ROS (%)	100.5 %±0.1a	128.2 %±0.4ab	182 %±0.4b	110.1 %±0.2a
CK (U/L)	261.5±17.3a	159.4±27.1b	208.0±36.2ab	145.6±22.3b
mM MDA / g muscle	71.5±3.7a	82.9±5.1ab	93.2±5.5b	88.0±3.7b

Measurement of markers of muscle damage and lipid peroxidation in the skeletal muscle of treated obese rats**. A.** CK plasma values. **B.** MDA values corresponding to lipid peroxidation. Experimental details as in figure 1. Data are means ± SEM of seven animals per group. a and b denote a significant difference ($p< 0.05$) between groups by ANOVA analysis.

CD+GSPE administration increased adiponectin levels, whereas CD+GSPE+DHA-OR treatment significantly increased leptin levels.

Adiponectin levels were significantly increased by CD+GSPE administration compared to the CD group, but no significant differences were found in the other treatment groups (Figure 4A). Moreover, leptin levels (Figure 4B) were significantly increased after CD+GSPE+DHA-OR administration compared to the other treatments.

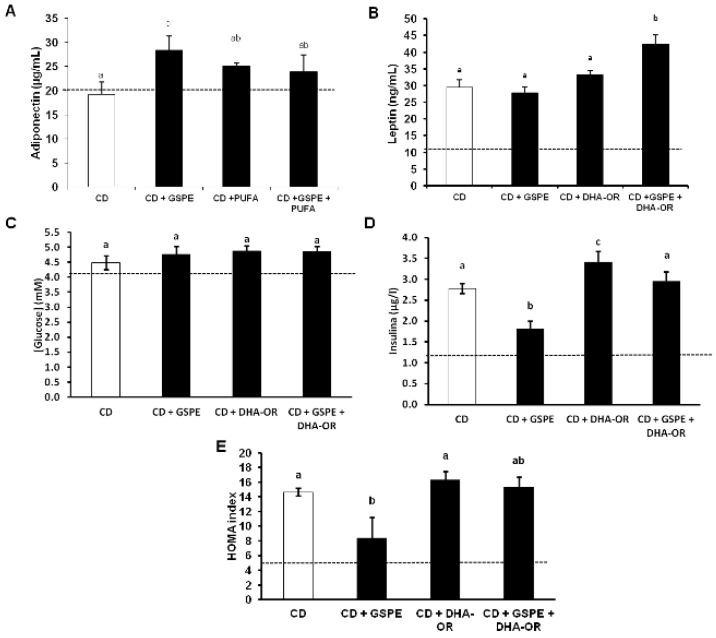

Figure 4. Effect of proanthocyanidins and/or DHA supplementation on adipocytokine and plasma glucose, and insulin profiles in obese rats. A. Adiponectin **B.** Leptin. **C.** Glucose concentration **D.** Insulin **E.** HOMA index Experimental details as in figure 1. Data are means ± SEM of seven animals per group. a and b denote a significant difference between groups by ANOVA analysis ($p < 0.05$). The dashed line indicates the baseline values on the standard diet.

GSPE administration reversed insulin resistance induced by CD

Glucose plasma values did not differ between the CD and treated groups although all of them were increased compared to the values for the STD group (Figure 4C). Plasma insulin was decreased significantly in the CD+GSPE group, approaching the STD group values (Figure 4D). The HOMA index (Figure 4E) was significantly decreased in the CD+GSPE group compared to the CD+DHA-OR group, and was similar to STD group values.

Discussion

Due to its high palatability, CD allows rodents to become hyperphagic by reducing their control over their food intake. As a result of 10 weeks of eating the hypercaloric CD *ad libitum*, Wistar rats become obese, showing a 20 % increase in body weight with higher mesenteric, perirenal and epididymal adipose tissue weights, and an adiposity index approximately twice that of STD-fed rats (data not shown). Furthermore, CD rats were hypertriglyceridemic, hypercholesterolemic, hyperinsulinemic, and hyperleptinemic, with a higher HOMA index than SD rats (30, 31). After 10 weeks of consuming CD, GSPE, DHA-OR or a combination of both were added to the CD for 21 days. None of these treatments significantly changed body weights or the adiposity index of CD rats (data not shown), and food intake was reduced only in the CD+GSPE group at the end of period. Additionally, leptin plasma levels did not change during CD+GSPE treatment, which correlated with the extent of the TG stores. Obesity is associated with leptin production and high concentrations of plasma leptin (32). Occasionally, the rise in leptin was unable to prevent weight gain in obese humans. This apparent “leptin resistance” may result from a decrease in brain transport or attenuation of leptin signaling in the hypothalamus and other central nervous system targets (33). However, the addition of GSPE to the CD lowered insulinemia and the HOMA index, thus improving the insulin resistance shown by CD rats apart from their decreased TGs plasma levels observed in previous studies (20, 30, 34, 35).

The CD also induced oxidative stress (36). Considering that treatment with GSPE and/or DHA-OR increased mitochondrial oxidative capacity and FA oxidation, it was expected that these compounds affected the redox

status of skeletal muscle because excess ROS are produced in mitochondria when FA β-oxidation accounts for the bulk of mitochondrial respiration (4, 37). GSPE in particular, due to its antioxidant capacity, can counteract the production of toxic substances induced by the high fat diet and prevent the deleterious effects of ROS. It has been shown that proteins of the OXPHOS system and DHA in mitochondrial membranes are key targets of ROS (38). The antioxidant properties of GSPE are shown here to reduce the plasma CK values, thus preventing muscle damage. Furthermore, CD+DHA-OR treatment clearly triggered a decrease in ROS production. When GSPE was not present, significantly elevated transcript levels of *Mnsod* were found in CD+DHA-OR-fed rats to counteract the excessive ROS production. Nonetheless, GSPE did not reduce the production of MDA in the same way as ROS. This increase in MDA levels in CD+DHA-OR and CD+GSPE+DHA-OR treated groups could be due to the substantial influence of DHA on membrane structure/function (39). Curiously, muscle status was also improved by CD+GSPE treatment despite the fact that ROS and MDA levels were similar to those associated with the CD. Likewise, although no differences were found in *Ucp3* gene expression between any of the treated groups, *Ucp3* mRNA values were elevated compared to the values for the STD group. Other authors have reported that UCP3 plays an important role in facilitating the transport of FA anions that cannot be oxidized from the mitochondrial matrix, thereby protecting against lipid-induced mitochondrial damage by attenuating the production of free radicals and mitigating the effects of ROS produced by macronutrients of the diet (40).

At 10 days of treatment, while rats fed CD used mainly carbohydrates as fuel throughout the postprandial period (6 h), CD+GSPE and CD+DHA-OR rats used a higher percentage of fat, and CD+GSPE+DHA-OR rats used essentially only FAs as fuel. At 20 days of treatment, the CD rats did not predominantly use glucose; like CD+GSPE and CD+DHA-OR-treated rats, they used more FAs as fuel and had a lower RQ ratio than at 10 days. Meanwhile, CD+GSPE+DHA-OR rats, in spite of an RQ ratio closer to the other groups, fuelled essentially on only FAs at the end of treatment. This enhanced fat combustion is consistent with the significantly elevated transcript levels of *Lpl*, *Cd36* and *Cpt1b* in the skeletal muscle of CD+GSPE, CD+DHA-OR or CD+GSPE+DHA-OR-treated rats.

In the case of CD+GSPE rats, the significantly elevated transcript levels of *Lpl, Cd36, Cpt1b* and higher ATPase activity, concomitant with the reduced TG levels in plasma, was in accord with the role of GSPE in redirecting circulating TGs to skeletal muscle for fat oxidation and ATP synthesis. In a study with a similar obesity model in rats, GSPE improved both lipidemia and the fat content in the liver (9). The increase in FA oxidation following CD+GSPE treatment was substantiated by the high-resolution respirometry outcomes. Higher oxygen consumption in State2 and State4 of the palmitoyl-CoA oxidation pathway rather than in the pyruvate and glutamate/malate/succinate pathways demonstrated the increase of FA oxidation rates in *ex vivo* isolated mitochondria. Moreover, there is a high facility to increase the maximum respiratory capacity, StateU, in extreme conditions by pyruvate/malate and palmitoyl-CoA pathways in CD+GSPE rats. Our results indicate that GSPE modulates mitochondrial function in skeletal muscle, increases the oxidative capacity and has a potential role to adapt the OXPHOS system in special conditions by increasing FA oxidation in CD rats, as Pajuelo et. al. demonstrated in white adipose tissue and brown adipose tissue (25). All of these changes were consistent with the high circulating adiponectin levels in the CD+GSPE group, because adiponectin increases the expression of molecules involved in FA transport (CD36) or combustion (acyl-coenzyme A oxidase) in skeletal muscle (32). In our experiment, we observed that adiponectin may also stimulate ß-oxidation via AMPK (41), which reduces the levels of circulating free FAs and prevents insulin resistance (42, 43).

In a previous study in which healthy rats were given an acute dose of DHA-OR plus a high fat overload (20), the results show that CD+DHA-OR facilitates the entry and uptake of long-chain FAs in skeletal muscle via *Lpl* and *Cd36* overexpression, and increases the flux of FA oxidation to mitochondria by *Cpt1b* overexpression, as found in the present study. This is likely to occur through upregulation of *Pparα*, but may also be due to activated AMPK. It has been shown that the PPARα agonist omega-3 PUFA could efficiently activate AMPK-α1 mRNA expression in skeletal muscle (44). Mitochondrial respiratory capacity was significantly increased in the CD+DHA-OR group using different substrates and protocol sequences. Thus, DHA-OR, apart from increasing the β-oxidation in skeletal

muscle, is also capable of increasing mitochondrial OXPHOS functionality with a maximum functional mitochondrial capacity.

In terms of muscle energy homeostasis and oxidative capacity, the effects of CD+GSPE+DHA-OR were similar to those of each separate compound. Again, gene expression of key markers of β-oxidation, *Lpl* and *Cpt1b* was significantly upregulated, increasing the functionality of mitochondrial capacity. Furthermore, the combination of both compounds caused the highest burning of fat overload. Once again, these effects occur through AMPK activation, which was concomitant with significantly elevated transcript levels of *Ucp2.* UCP2 acts as a sensor of mitochondrial oxidative stress and its function is an important component of local feedback mechanisms controlling the production of mitochondrial ROS (45). Pecqueur et. al. (46) showed that UCP2 promotes mitochondrial FA oxidation while limiting mitochondrial catabolism of pyruvate, suggesting that the metabolic and proliferative alterations triggered by UCP2 are distinct from uncoupling.

In conclusion, although no changes in body and adipose weights were observed, GSPE, DHA-OR and the combination of the two improved the metabolic inflexibility shown by CD-fed rats, implying that AMPK regulation was present in all of the treatment groups.

Acknowledgements

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Conflict of interest

The authors declare that no conflict of interest exists.

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4. GLOBAL DISCUSSION

Overweight and obesity are growing threats to the overall health of populations in an increasing number of countries. The fundamental cause of the epidemic obesity and its increased prevalence is an energetic imbalance due to a sedentary lifestyle and high-fat, energy-dense diet, which is the result of societal trends. The problem appears to have increased rapidly in both children and adults; however, the actual, long-term health consequences will only be fully apparent in the future. Despite the substantial progression of the obesity epidemic in recent years, now we have begun to identify the neurohumoural mechanisms underlying obesity, whereas nonsurgical obesity treatments have improved little over the years. If obesity involves the biological defence of an elevated level of body fat, as current evidence suggests, advice to simply "eat less, move more" cannot be expected to resolve the problem. Interventions that reduce body fat stores without simultaneously decreasing the level of fat mass, elicit compensatory responses that promote the recovery of lost fat and are difficult to consciously override.

Our approach to developing new therapeutic strategies that combat the development and progress of obesity and related diseases has focused on the utilisation of functional foods with natural bioactive compounds able to correct the metabolic dysfunction associated with obesity. People may consume functional foods while making only minor or no changes to their dietary habits, thus avoiding the behavioural problems associated with meal replacements. The consumption of these bioactive compounds may also have beneficial effects beyond counteracting obesity and its comorbidities, which include coronary heart disease, hypertension, insulin resistance, and dyslipidemia.

The natural bioactive compounds selected for this study –EGCG, proanthocyanidins and omega-3 PUFAs oil rich in DHA – are characteristic of healthy diets and have well-documented beneficial effects with regard to obesity and CVD. Previous studies have suggested that proanthocyanidins act on mitochondria through skeletal muscle, brown adipose tissue and the liver by partially correcting mitochondrial dysfunction linked to obesity[1-3] positively modulating oxidative stress[4,5] and correcting dyslipidemic parameters in different situations[6,7]. EGCG has antioxidative properties[8] and has been shown to increase fat oxidation in skeletal muscle [9]. Oil rich in DHA has the capacity to influence mitochondrial physiology via the upregulation of genes that promote fatty acid oxidation by PPARs activation [10] and the repression of lipid synthesis[11].

While it has been demonstrated that these compounds participate in a variety of mechanisms that can partially correct the metabolic dysfunction associated with obesity, our hypothesis suggests that these compounds could potentially display additive or synergistic effects when used in appropriate combinations, thus increasing their potential to completely correct the dysfunction.

This doctoral thesis focuses on skeletal muscle and adipose tissue, both key organs in the control of body weight, as well as on the study of mitochondrial function, known as a key regulator of energy homeostasis.

We studied how proanthocyanidins, EGCG and omega-3 PUFAs oil rich in DHA could modulate metabolic flexibility and ameliorate the metabolic dysfunction associated with obesity in three different experimental models:

i) *In vitro* studies using L6 myocytes to assess the response of mitochondrial physiology and dynamics to a short treatment with EGCG and DHA.

ii) *In vivo* studies on healthy rats fed a diet overloaded with saturated fatty acid, concomitant with an acute oral intake of proanthocyanidins and omega-3 PUFAs oil rich in DHA, to evaluate the short-term effects on metabolic flexibility in skeletal muscle and adipose tissue.

iii) *In vivo* experiments involving obese rats overfed a high-fat diet, concomitant with 21 days of treatment with proanthocyanidins and/or omega-3 PUFAs oil rich in DHA, to assess the improvement of altered metabolic responses related to energetic metabolism via skeletal muscle studies.

First, an *in vitro* experiment was conducted **(manuscript I)** using EGCG and/or DHA at physiological concentrations of 25 µM, over a short time period of 4 hours. In this experiment, the DHA treatment considerably increased the intracellular ROS production, as expected based on their structural composition and their ability to be oxidised. The mitochondrial antioxidant defence gene, *Mnsod*, is thus overexpressed to avoid this oxidative stress. In addition, the concomitant administration of EGCG, a potential antioxidant polyphenol, reversed this effect.

In analysing the ETC marker gene, we found that DHA reduced the expression of the *Cox* and *Ant1* genes. Importantly, neither EGCG nor DHA were influenced by markers of TCA functionality, as no changes in *Cs* mRNA expression were shown. Perhaps the increase in intracellular ROS production caused by DHA interferes with mitochondrial functionality by way of the ETC and OXPHOS systems; such interference is likely produced by variation in the membrane fatty acid composition, which facilitates ROS production followed by a decrease in mitochondrial function, although cell viability is maintained.

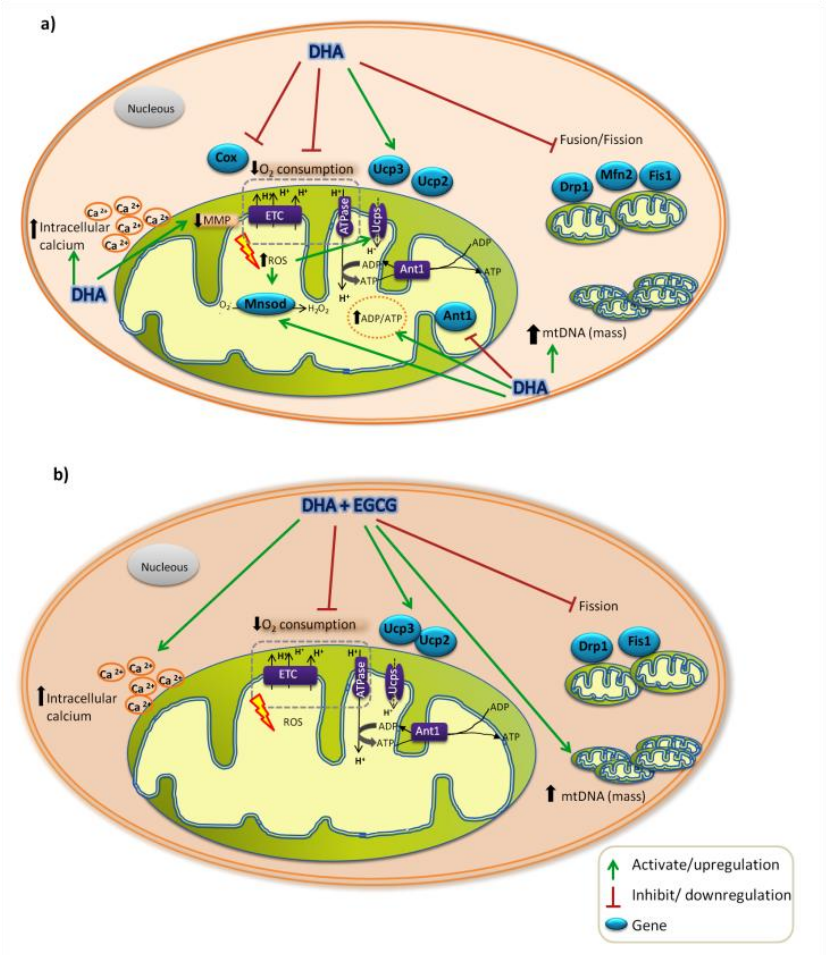

Figure 21. Main effects of a) DHA and b) DHA + EGCG combination on L6 myocytes in 4 hours of treatment.

In vivo mitochondrial respiration in intact L6 myocytes indicates that substrate oxidation is altered, decreasing the routine and LEAK states following DHA treatment, even in combination with EGCG. This decrease in oxygen

consumption due to DHA treatment is a result of the decrease in *Cox* gene expression and the increase in the ADP/ATP ratio, as DHA is a potent deregulator of O2 consumption and oxidative phosphorylation. Moreover, the decrease in oxygen consumption is accompanied by the overexpression of the uncoupled proteins *Ucp2* and *Ucp3*, as well as a decrease in the mitochondrial membrane potential. Overexpression may be due to the production of ROS, which could stimulate mitochondrial uncoupling, also leading to a dissipation of the proton gradient generated by the respiratory chain and reduced oxygen consumption. To compensate for this decline in respiratory function, mtDNA is increased in response to the oxidative stress produced by DHA treatment.

The disruption of mitochondrial membranes by DHA also alters mitochondrial dynamics via the deregulation of the genes involved in the fusion and fission process, indicating that despite the high mitochondrial mass due to treatment with DHA, the mitochondria remain inactive. The decrease in fusion and fission genes simultaneously balance the function of the mitochondria. Furthermore, the mitochondria are restored when EGCG and DHA are added simultaneously. Therefore, the reduction in endogenous respiration by DHA could be attributed to the downregulation of fission and fusion events, due to the influence of mitochondrial morphology and dynamics through OXPHOS and cellular bioenergetics.

Although intracellular ROS decreased following treatment with the combined products, the membrane fatty acid composition and the oxidised products were not sufficiently reduced to maintain the mitochondrial dynamism and functionality in EGCG+DHA-treated cells. It is likely that 4 hours of treatment is not sufficient to restore mitochondrial functionality, even though the levels of mitochondrial mass are increased and mitochondrial morphology is typically restored by the combined treatment with EGCG+DHA.

Second, we studied the *in vivo* postprandial (5 hours) effects of an acute dose of proanthocyanidins from GSPE (250 mg/Kg bw) and omega-3 PUFAs oil rich in DHA (250 mg/Kg bw), combined with a challenge fatty acid overload test (2.5 mL lard oil/Kg bw), in healthy rats **(manuscript II)**. The objective was to determine if these ingredients could modulate metabolic flexibility – via the interactions of expressed genes related to lipid metabolism and mitochondrial functionality – simultaneously in skeletal muscle and adipose tissue, thus counteracting the metabolic dysfunction associated with obesity.

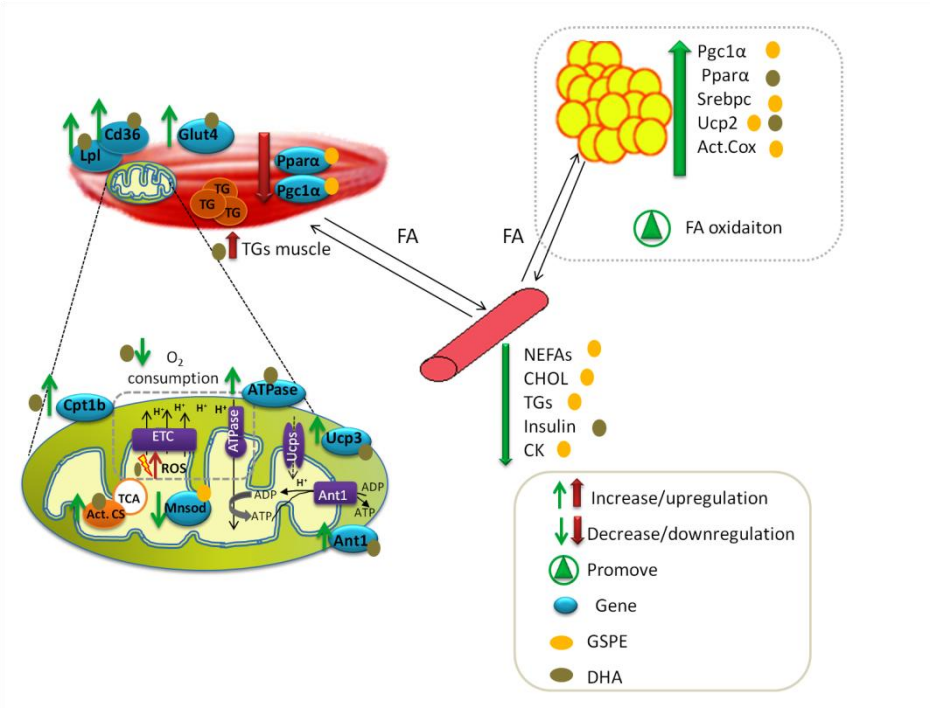

Figure 22.Main effects of 250 mg GSPE/kg*bw or 250 mg DHA-OR/kg*bw in skeletal mucle, adipose tissue and plasma (Acute dose-Manuscript II)

In this study, we showed that the administration of oil rich in DHA upregulates gene expression of *Lpl* and *Cd36* in skeletal muscle, aiding the entrance of fatty acid into the cell. A simultaneous upregulation of *Cpt1b* expression increases the flux of FA oxidation through mitochondria. Although the activities of TCA and ETC are neither altered nor increased, there is a clear increase in ETC functionality mediated by *Atpase* and *Ant1* overexpression, indicating a higher exchange of ADP and ATP. Furthermore, *Ucp3* is also upregulated, which correlates with increased ETC functionality and which also involves a mild uncoupling that could reduce ROS production in skeletal muscle following treatment with oil rich in DHA.

Moreover, oil rich in DHA appears to improve insulin sensitivity, as we noted a decrease in plasma insulin levels with no change in plasma glucose levels, indicating that the expression of *Glut4* could be a result of the altered membrane phospholipids. By contrast, in adipose tissue, although it appears that neither fatty acid oxidation nor fatty acid uptake increase, the tissue remains functional with regard to oxidative capacity and uncoupling due to increases in CS activity and *Pparα* and *Ucp2* gene expression, respectively.

Chiefly, these results indicate that treatment with GSPE and oil rich in DHA, concomitant with a fatty acid overload, tends to improve metabolic flexibility in various ways. The addition of oil rich in DHA increases insulin sensitivity and redirects fatty acid oxidation from the diet towards skeletal muscle by *Lpl*, *Cd36* and *Cpt1b* overexpression. The addition of GSPE improves adipose tissue mitochondrial functionality by activating mitochondrial biogenesis and uncoupling by *Ucp2* overexpression. GSPE causes lipid accumulation and oxidation in adipose tissue, thereby decreasing lipidemia and preventing muscle damage and ROS production. When GSPE + oil rich in DHA is combined with a fatty acid overload, nearly all of the aforementioned effects are maintained, though attenuated.

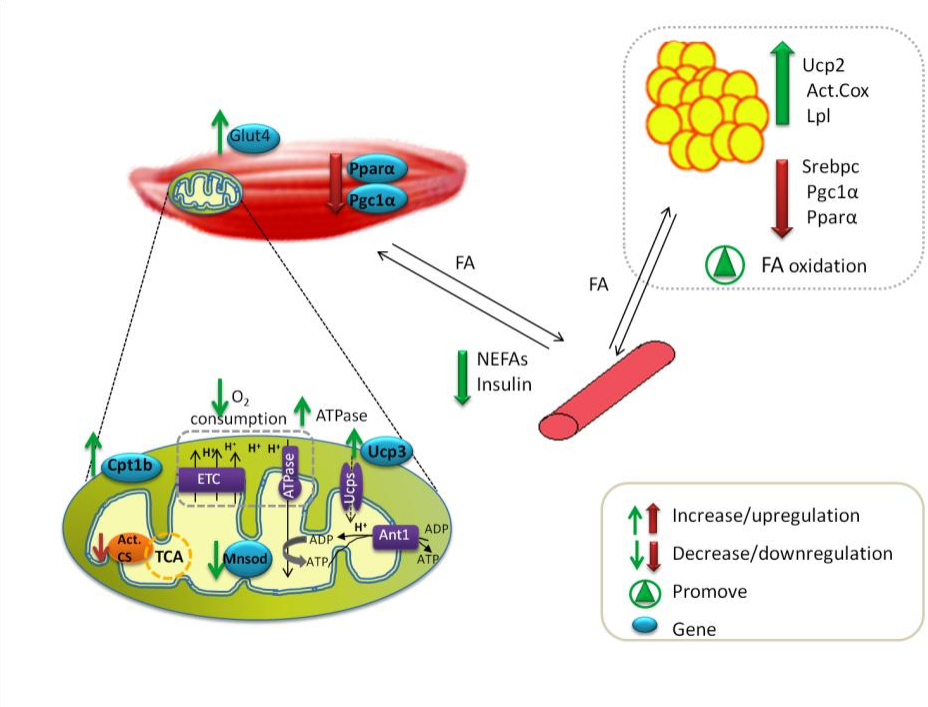

Figure 23.Main effects of 250 mg GSPE/kg*bw and 250 mg DHA-OR/kg*bw in skeletal muscle, adipose tissue and plasma (Acute dose-Manuscript II)

Although GSPE and oil rich in DHA activate different pathways, when combined with a lipid overload, both improve metabolic flexibility. In an effort to determine whether these effects are maintained in an obesity profile, healthy Wistar rats were overfed a high-fat caloric diet, namely a cafeteria diet **(Manuscript III)**. Our aim was to achieve a robust obesity model to test our bioactive compounds and their potential to improve obesity-related diseases.

For 21 days, obese rats were given proanthocyanidins (25 mg GSPE/ kg bw) and/or omega3-PUFAs rich in DHA (500 mg DHA-OR/ Kg bw), concomitant with a cafeteria diet. The metabolic effects were studied in the skeletal muscle, which has a major role in the overall utilisation of mitochondrial energy and fatty acid oxidation.

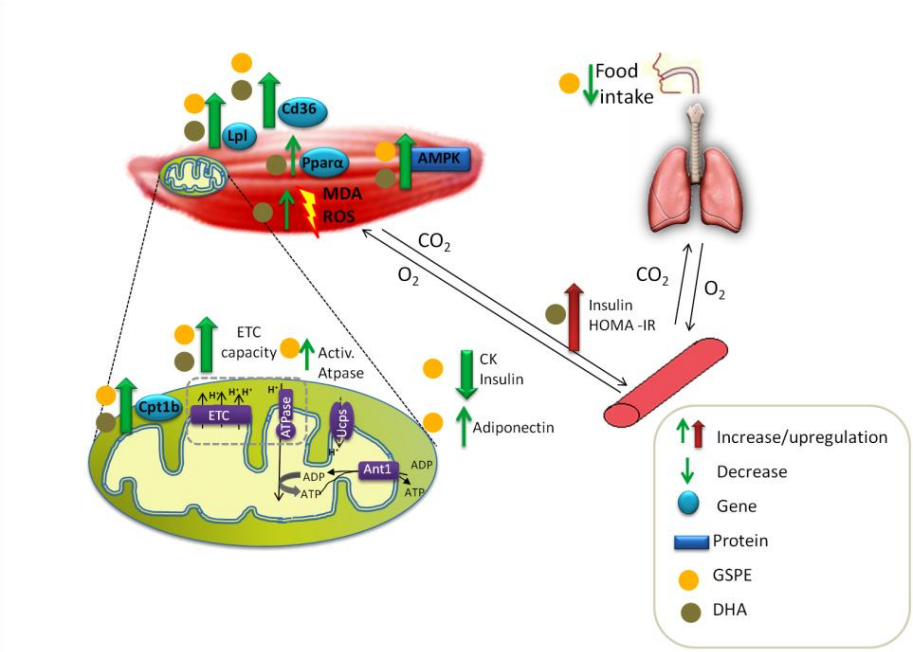

Figure 24. Main effects of 25 mg GSPE/kw*bw or 500 mg DHA-OR/kg*bw in skeletal muscle, plasma and energy balance control (Chronic dose-Manuscript III)

Both GSPE and oil rich in DHA cause significant overexpression of *Lpl, Cd36* and *Cpt1b*, in addition to enhanced fat combustion. In the case of GSPE administration, *Lpl* mediates TG clearance from the circulatory system. Additionally, *Cd36* overexpression increases the rate of fatty acid transport to the muscle and, concomitant with *Cpt1b* overexpression, the mitochondrial fatty acid oxidation in the muscle is increased. Interestingly, AMPK phosphorylation is also increased in both groups, indicating an increase in lipid metabolism.

In the DHA group, *Pparα*, which promotes fatty acid catabolism and the upregulation of genes involved in fatty acid metabolism and mitochondrial β-oxidation, is overexpressed. These results regarding lipid metabolism were reaffirmed when elevated respiration was studied in isolated mitochondria. Both compounds increase the maximal respiratory capacity in obese rats; this result is more prominent, however, in the group administered oil rich in DHA.

The two compounds were tested together to determine whether the observed metabolic changes are reproducible. Our results showed that although AMPK phosphorylation does not change, there is a tendency to maintain high levels of AMPK phosphorylation. Such a tendency is accompanied by an upregulation of the β-oxidation marker *Cpt1b*, concomitant with an upregulation of *Lpl* and *Ucp2*, which increase fatty acid uptake, thus promoting a role in lipid metabolism transport. Additionally, an increase in the TCA cycle, via elevated citrate synthase activity, and in mitochondrial respiration was determined by *ex vivo* measurements.

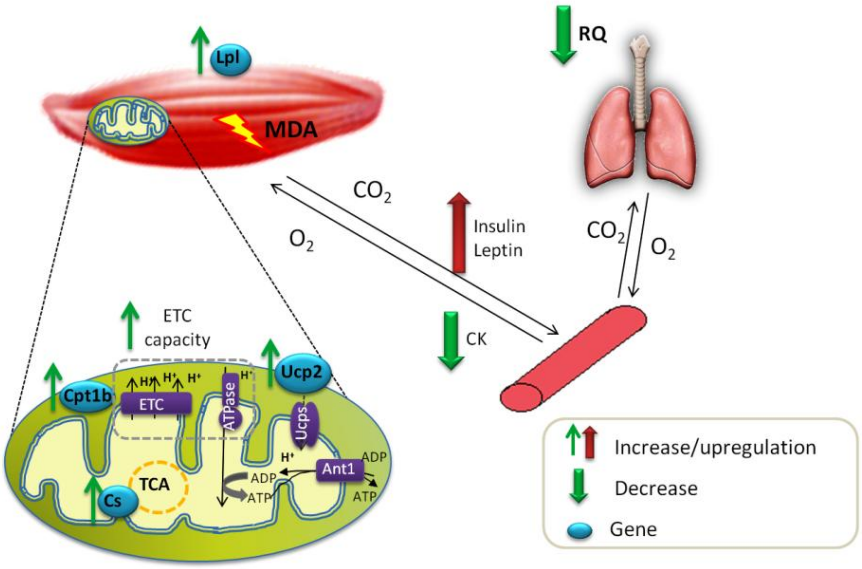

Figure 25. Main effects of 25 mg GSPE/kw*bw and 500 mg DHA-OR/kg*bw in skeletal muscle, plasma and energy balance control (Chronic dose-Manuscript III)

Considering that GSPE and oil rich in DHA increase the oxidative capacity and fatty acid oxidation by the different markers mentioned above, the antioxidant capacity of GSPE was studied. Our results showed a reduction in ROS produced by DHA in the group treated with both compounds. In a similar manner, the CK values were also reduced by GSPE when administered in the combination treatment, thus reversing the muscle damage induced by the high-fat diet.

No significant differences with regard to body weight and food intake were found between the groups receiving treatment with oil rich in DHA and treatment with GSPE. GSPE, however, has a tendency to cause reductions in body weight and food intake at the end of the course of treatment. Interestingly, in the group where the two compounds were tested together, the respiratory quotient ratios were significantly decreased on the 10th day of treatment and maintained until the end of the treatment regime, with a more pronounced profile between 5 and 6 h of postprandial measure. This result indicates a preference for fatty acid catabolism, although this preference is not reflected in the organisms' body weight. The complementary effects of these compounds enable them to increase the maximal oxidation capacity of mitochondria and to increase lipid catabolism, thus improving the lipidemia profile and metabolic flexibility, with the objective of reducing excessive fatty acid in the diet.

Overall, the study of energetic metabolism in the skeletal muscle showed that the interactions of GSPE in different situations tend to decrease ROS production in the muscle tissue, thus protecting against oxidative stress produced by the dietary intake of fatty acids, including saturated and polyunsaturated fatty acids, concomitant with muscle damage protection. Moreover, lipidemia profiles improve as plasma TGs decrease due to increased uptake, associated with *LpL* overexpression, in muscle tissue. Additionally, fatty acid oxidation in the mitochondria increases due to the increase in AMPK phosphorylation. *Cpt1b* and *Cd36* overexpression leads to increased mitochondrial maximal respiration capacity following chronic treatment with a high-fat diet profile; furthermore, increased biogenesis and respiratory chain uncoupling are observed following acute treatment, concomitant with a high-fat overload, due to lipogenesis in adipose tissue, which avoids lipotoxicity in other tissues. Pure DHA and oil rich in DHA increase mitochondrial fatty acid oxidation by increasing the expression of *Lpl, Cpt1b* and *Cd36*; this increase is reflected in the increase in maximal respiratory function in isolated mitochondria of skeletal muscle, concomitant with increased expression of the natural ligand *Pparα* in both chronic and acute treatment.

Additionally, an increase in the expression of uncoupling proteins and biogenesis genes in adipose tissue is observed following acute treatment. These metabolic changes affect mitochondrial morphology over a short period of time by disrupting the mitochondrial membrane in *in vitro* studies using myocytes.

Based on our results, we can conclude that this thesis achieved the main hypothesis and objectives, thus presenting a new therapeutic strategy to combat obesity, which involves only minor changes in societal trends with regard to dietary habits and lifestyle.

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5. CONCLUSIONS

1. Myocytes treated with 25 µM EGCG concomitant with 25 µM DHA exhibit decreased ROS production. Additionally, the myocytes treated with 25 µM DHA uncouples mitochondrial respiration via *Ucp2* and *Ucp3* overexpression, thus increasing the ADP/ATP ratio and decreasing oxygen consumption and membrane potential, concomitant with a reduction in ETC functionality by *Cox* and *Ant1* downregulation.

2. Myocytes treated with 25 µM DHA display an increase of mitochondrial mass with an increased ROS production and *Mnsod* overexpression, concomitant with a decrease in the occurrence of fission and fusion events, to preserve minimum mitochondrial functionality. Additionally, the treatment with 25 µM DHA, also causes disruption in mitochondria morphology which is reversed when 25 µM DHA + 25 µM EGCG were combined in the same treatment maintaining the *Ucp2* and *Ucp3* overexpression.

3. Acute treatment with 250 mg oil rich DHA/Kg bw or 250 mg GSPE/kg bw simultaneously with saturated fatty acid overload, improves metabolic flexibility in the postprandial period. Oil rich in DHA increases insulin sensitivity, redirects fatty acid towards the skeletal muscle and increases the capacity to regulate fatty acid oxidation. This treatment improves adipose mitochondrial functionality and uncoupling by *Ucp2* overexpression.

4. Acute treatment with 250 mg GSPE/kg bw, in tandem with saturated fatty acid overload, activates lipid accumulation and oxidation in adipose tissue, activating mitochondrial biogenesis and lipogenesis and thereby decreasing lipidemia and preventing muscle damage and ROS production.

5. Obese rats fed a cafeteria diet and simultaneously treated with 25 mg GSPE/kg bw for 21 days show an improvement of energy homeostasis, lipid metabolism and insulin resistance induced by the cafeteria diet, which increases adiponectin levels, AMPK phosphorylation, mitochondrial β-oxidation and ATPase activity in skeletal muscle.

6. Obese rats fed a cafeteria diet and simultaneously treated with 500 mg oil rich in DHA/kg bw for 21 days display increased AMPK phosphorylation, concomitant with *Pparα* overexpression, increased mitochondrial β-oxidation and maximal respiratory capacity in the skeletal muscle.

7. Obese rats fed a cafeteria diet and simultaneously treated with 25 mg GSPE/kg bw plus 500 mg oil rich in DHA/kg bw for 21 days have decreased respiratory quotient ratio in postprandial period, concomitant with an increase of CS activity and fatty acid oxidation in skeletal muscle.

8. Treatment with chronic (25 mg GSPE/kg bw for 21 days) or acute doses of GSPE (250 mg GSPE/kg bw for 5 hours) decreases CK plasma levels while avoiding muscle damage and ROS accumulation.

.

6. **ANNEX I**

Annex I

Resum

El sobrepès i l'obesitat estan incrementant de forma desmesurada, amenaçant la salut de la població en un nombre cada cop més gran de països. Per tal de desenvolupar una nova estratègia terapèutica en contra del desenvolupament i la progressió de l'obesitat i les malalties relaciones, s'ha estudiat la utilització de components bioactius naturals per corregir la disfunció metabòlica associada a l'obesitat. Amb aquest pretext, doncs, es vol veure si consumint aliments funcionals amb un lleu o sense cap canvi en els hàbits alimentaris, evitant així problemes de comportament associats als substituts d'àpats, hi ha un efecte beneficiós més enllà de l'obesitat i les seves comorbiditats com la malaltia coronaria, la hipertensió, la resistència a la insulina i la dislipemia.

L'objectiu d'aquesta tesis és avaluar com compostos bioactius presents en dietes saludables, i la combinació d'ambdós, com els flavonoids, tal com les proantocyanidines i els àcids grassos omega-3, tal com l'àcid docosahexaenoic (DHA), poden prevenir o millorar els efectes adversos de l'obesitat i les malalties relacionades. Els estudis s'han centrat tant en múscul esquelètic com en teixit adipós, dos òrgans importants en el control del pes corporal i en l'estudi de la funció mitocondrial, coneguda com a reguladora clau en l`homeòstasis energètica.

Per tal de veure els efectes beneficiosos, es va avaluar la capacitat que tenen els compostos en modular l'activitat mitocondrial en els teixits esmentats. En primer lloc, vam quantificar si epigallocatechin gallate (EGCG) i DHA, tan sols com combinats, tenen la capacitat de modular la funcionalitat mitocondrial en cèl·lules musculars de rata, per posteriorment estudiar en rates sanes com una sobrecàrrega lipídica i dosis agudes tan de proanthochyanidines provinents d'extracte de llavor de raïm (GSPE) com oli ric en DHA, i la seva combinació, afecta a la flexibilitat metabòlica i millora la situació postprandial. Per altra banda, també s'ha avaluat com el tractament crònic de rates obeses amb GSPE i oli ric en DHA podria millorar els efectes adversos de la obesitat. En ambdós casos s'ha observat una millora dels paràmetres que es veuen alterats degut a la ingesta d'una dieta alta en greixos, millorant tant la resistència la insulina com el perfil lipídic en plasma quant els animals han estat tractats amb GSPE. A més a més, s'observa un augment de l' oxidació d'àcids grassos en múscul, en incrementar-se la capacitat oxidativa de la mitocòndria, simultàniament amb la sobreexpressió de gens diana relacionats amb la β-oxidació, millorant així la flexibilitat metabòlica quant aquestes rates han estat tractades tant amb GSPE com amb oli ric en DHA i la seva combinació.

De forma global els resultats d'aquesta tesi, assoleixen la hipòtesi i els objectius principals i donen una nova oportunitat en l'estratègia terapèutica contra l'obesitat, amb un petit canvi en la tendència de la societat actual amb els hàbits alimentaris i l'estil de vida.

Conclusions

1. En miòcits tractats amb 25 µM EGCG conjuntament amb 25 µM DHA disminueix la producció de ROS. Addicionalment els tractats amb 25 µM, DHA desacoblen la respiració mitocondrial mitjançà la sobreexpressió de *Ucp2* i *Ucp3*, augmentant el ràtio ADP/ATP i disminuint el consum d'oxigen i el potencial de membrana conjuntament amb la reducció de la funcionalitat de la cadena de transport d'electrons en relació amb la repressió dels gens *Cox i Ant1.*

2. Miòcits tractats amb 25 µM DHA sofreixen un increment de la massa mitocondrial amb un augment de la producció de ROS, conjuntament amb la disminució dels esdeveniments de fusió i fissió de la mitocòndria per tal de preservar la mínima funcionalitat mitocondrial. A més a més 25 µM DHA causa una disrupció de la morfologia mitocòndria que és reverteix quan els miòcits són tractats amb la combinació de 25 µM DHA+25 µM EGC mantenint la sobreexpressió dels gens *UCp2* i *Ucp3*.

3. El tractament agut amb 250 mg per kg de pes corporal d'oli ric amb DHA o 250 mg per kg de pes corporal de GSPE, simultàniament amb una sobrecarrega d'àcids grassos saturats, millora la flexibilitat metabòlica en període postprandial. L'oli ric en DHA augmenta la sensibilitat a la insulina i redirigeix els àcids grassos cap a múscul esquelètic i n'activa la capacitat de regular l'oxidació d'àcids grassos. Aquest tractament millora la funcionalitat mitocondrial de teixit adipós i en desacobla la respiració amb la sobreexpressió de la *Ucp2.*

4. El tractament agut amb 250 mg per kg de pes corporal d'oli ric amb DHA o 250 mg per kg de pes corporal de GSPE, simultàniament amb una sobrecarrega d'àcids grassos saturats, activa l'acumulació lipídica i la seva oxidació en teixit adipós activant la biogènesis mitocondrial i lipogènesis,
disminuint així la lipidèmia, prevenint el dany muscular i la producció de ROS a múscul.

5. En rates obeses alimentades amb dieta de cafeteria i tractades durant 21 dies amb 25 mg GSPE per kg de pes corporal millora la homeòstasi energètica i el metabolisme lipidic, així com la millora de la resistència a l'insulina alterat per la dieta de cafeteria. Conjuntament amb un increment de nivells de adiponectina en plasma, augmentant la fosforilació d'AMPK, β-oxidació i l'activitat ATPase mitocondrial en múscul esquelètic.

6. En rates obeses alimentades amb dieta de cafeteria i tractades durant 21 dies amb 500 mg DHA per kg de pes corporal, la fosforilació d'AMPK es augmentada, conjuntament amb la sobreexpressió de *PPARα*, augmentant la β-oxidació i la capacitat respiratòria màxima en múscul esquelètic.

7. En rates obeses alimentades amb dieta de cafeteria i tractades durant 21 dies amb 25 mg GSPE més 500 mg oli ric en DHA per kg de pes corporal disminueix el ràtio de coeficient respiratori en període postprandial conjuntament amb un augment de l'oxidació d'àcids grassos en múscul esquelètic.

8. El tractament crònic 25 mg GSPE per kg de pes corporal durant 21 dies o dosis agudes 250 mg GSPE per kg de pes corporal durant 5 hores disminueix nivells plasmàtics de CK evitant el dany muscular i l'excés de ROS.