Regulation of Skeletal Muscle Glucose and Fat Metabolism by DHA and EPA

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Abstract

The objective of this study was to investigate whether docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids can directly regulate glucose and fat metabolism in skeletal muscle, besides exerting anti-inflammatory properties. To accomplish this, L6 skeletal muscle cells were treated with 50μM of either DHA or EPA for 1, 3, and 5 days. Subsequently, we assessed glucose uptake, glycogen synthesis, lactate production, glucose and palmitate oxidation, as well as the phosphorylation and contents of proteins involved in insulin signalling and regulation of cellular oxidative capacity. mRNA expression of several genes involved in inflammation was also measured after DHA or EPA treatment. We found that basal rates of glucose uptake and glycogen synthesis as well as protein kinase B (AKT) and glycogen synthase kinase 3 (GSK3) phosphorylation remained unaffected by DHA or EPA. In addition, similar findings were observed under insulin-stimulated conditions. However, glucose and palmitate oxidation rates were consistently increased (day 1 to 5) by DHA treatment, whereas EPA only increased this variable transiently (day 1). Similarly, only DHA caused significant and sustained increases in AMP-activated protein kinase (AMPK) phosphorylation and contents of carnitine-palmitoyl transferase 1b (CPT1b) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) in skeletal muscle cells. DHA also had a more potent anti-inflammatory effect than EPA in these cells. In conclusion, besides exerting anti-inflammatory effects, DHA and EPA directly regulated glucose and fat metabolism in skeletal muscle cells, although DHA was more potent in doing so than EPA. Thus, by directly enhancing glucose and fat oxidation, DHA may increase glucose disposal and protect skeletal muscle cells against lipotoxicity.

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Table of Contents

List of Acronyms

ACC	Acetyl-CoA carboxylase
ACP	Acyl-transfer protein
ACS	Acyl-CoA synthetase
AKT	Protein kinase B
ALA	Alpha-linolenic acid
AMPK	AMP-activated protein kinase
CACT	Carnitine–acyl carnitine translocase
CCK	Cholecystokinin
CD36	Cluster of differentiation 36
CD40	Cluster of differentiation 40
COX	Cyclooxygenase
CPT1b	Carnitine palmitoyltransferase I
DAG	Diacylglycerol
DGAT	Diacylglycerol Acyltransferase
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ELOVL	Elongation of very long-chain fatty acid
FADH2	Flavin adenine dinucleotide
GLUT 4	Glucose transporter type 4
GPR120	G-protein coupled receptor 120
GS	Glycogen Synthase
GSK3	Glycogen synthase kinase 3
GYS1	Glycogen synthase 1
HDL	High-density lipoprotein
HF	High fat
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IL-6	Interleukin 6
IP3	Inositol triphosphate
IRS-1	Insulin receptor substrate 1
LDL	Low-density lipoprotein
LOX	Lipoxygenases
LPCAT	Lysophosphatidylcholine acyltransferase
MGAT	Monoacylglycerol acyltransferase
MTP	Mitochondrial trifunctional protein
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCTV	Prechylomicron transport vesicle
PKC	Protein Kinase C
PPARα	Peroxisome proliferator-activated receptor alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
T2D	Type-2 diabetes
TAG	Triacylglycerol

TAK1	Transforming growth factor beta-activated kinase 1
TLR 4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
VLCAD	Very-long-chain acyl-CoA dehydrogenase
VLDL	Very low-density lipoprotein

List of Figures

Literature review

Manuscript

Introduction

Omega-3 fatty acids are well known for their beneficial health effects. There are a variety of ω-3 fatty acids; however, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) represent those with the greatest therapeutic potential[1]. Ω-3 fatty acids have therapeutic value for conditions that are characterized by chronic inflammation such as ulcerative colitis[2], rheumatoid arthritis[3], and atherosclerosis[4] among many others. With the drastic rise in the global prevalence of obesity and type-2 diabetes (T2D), there is a greater focus on the potential role that ω-3 fatty acids can play in ameliorating these inflammation-induced metabolic disorders. Although human studies have generally been equivocal, there are several studies that provide support for the role that ω-3 fatty acids have in improving glucose tolerance in healthy humans, as well as in those with metabolic syndrome[5–7]. It was first noted that among Alaskan natives, higher consumption of ω-3-rich fish was associated with a lower incidence of glucose intolerance and T2D[8]. To gain a better understanding of the physiological importance of ω-3 fatty acids, numerous animal and cell culture studies were conducted. To identify the specific effects of ω-3 fatty acids, a portion of mice consuming a high-fat (HF) diet, had 7.5% of their calories replaced with fish oil. The deleterious effects of a HF diet on energy metabolism were reversed in fish oil-fed mice in part by facilitating the upregulation of proteins involved in lipolysis and lipid oxidation in skeletal muscle[9]. Besides having a positive effect on fat metabolism, there is evidence to suggest that insulin signalling can be improved as well. Rats who were fed a HF diet supplemented with 3.4% of calories coming from EPA and DHA had lower blood glucose and higher skeletal muscle expressions of GLUT4, GYS1, and IRS1 than rats fed a pure HF diet[10]. Evidently, ω-3 fatty acids are implicated in glucose and fat metabolism and it appears that their method of action involves the manipulation of skeletal muscle. Importantly, this tissue accounts for ~40% of body weight in men and ~30% in women

and it is responsible for ~80% of post-prandial glucose disposal[11]. Skeletal muscles are also highly adaptable to environmental factors which include alterations to physical activity and diet[12]. For these reasons, the impact of ω-3 fatty acids on skeletal muscle energy metabolism can potentially be of therapeutic value for the treatment and prevention of metabolic disorders. With regard to insulin resistance, the emerging etiological theory is based on excessive intramuscular triglyceride and lipid intermediate accumulation, leading to chronic inflammation that disrupts muscle energy metabolism. In fact, the lipid intermediate, diacylglycerol, can stimulate protein kinase C, leading to the phosphorylation of serine residues on insulin receptor substrate 1 (IRS-1) and impairment of insulin signalling within skeletal muscle tissue[13,14]. Indeed, rodent studies have shown that a HF diet can induce glucose intolerance in as little as 3 days[15]. Thus, an agent that decreases the accumulation of harmful lipid intermediates through the enhancement of fat metabolism may act to prevent insulin resistance. Previous studies support the notion that EPA and DHA can mitigate the detrimental effects of a HF diet on skeletal muscle tissue. Fish oil supplementation was associated with a reduced accumulation of lipid mediators such as ceramides and long chain acyl-CoAs in skeletal muscle tissue of mice fed a HF diet[10]. However, the literature does not provide a detailed evaluation of the molecular mechanisms that underlie these changes.

Ω-3 fatty acids are, however, most well known as powerful anti-inflammatory agents that can reduce whole-body inflammation. Oh et al. demonstrated that ω-3 fatty acids can serve as ligands for GPR120, which is a receptor that is mainly expressed in adipocytes, hepatocytes, and macrophages. Through this process, ω-3 fatty acids can inhibit tumor necrosis factor alpha (TNF-α) and toll-like receptor (TLR) inflammatory pathways as well as reduce the expression of interleukin-6 (IL-6) and other inflammatory genes. Moreover, mice that had a GPR120 knockout demonstrated glucose intolerance and skeletal muscle insulin resistance[16]. In the context of muscle

metabolism, EPA can serve as a ligand that binds to and activate peroxisome proliferator-activated receptor gamma (PPARγ) thereby suppressing the activity of nuclear factor kappa light chain enhancer of activated B cells (NF-κB). Treatment of cultured C2C12 myoblasts, with 50 μM EPA for 24 h reduced IL-6 secretion and mitigated the damaging effects of TNF-α induced inflammation on differentiation[17]. Clearly, EPA has anti-inflammatory capabilities and, through this process, can potentially serve as a mechanism that facilitates muscle energy metabolism[18]. However, in many studies, EPA and DHA are either grouped together[9,10,19,20] or only one of the two is presented[17,21] which prevents a detailed comparison of their independent effects. There is evidence to support the idea that the various ω-3 fatty acids can have different metabolic functions. For instance, one study suggests that EPA has a stronger effect than DHA on skeletal muscle protein metabolism in C2C12 myotubes[22]. However, another study reported that EPA was actually less efficient than DHA in inhibiting protein degradation in C2C12 myotubes[23]. Thus, EPA and DHA can behave differently and the literature is limited in exploring their relative effects on skeletal muscle inflammation and energy metabolism.

For these reasons, the overall aim of this study was to address the underlying molecular mechanisms that are involved in EPA and DHA's independent regulation of skeletal muscle inflammatory genes as well as lipid and glucose metabolism. This was done through a 5-day time-course treatment of cultured L6 myoblasts, which assessed EPA and DHA's relative effects on skeletal muscle function.

Literature review

Digestion of fats

Around 95% of the lipids consumed by humans are triacylglycerols. This macromolecule consists of a glycerol backbone that is esterified to three fatty acids. In order to be properly absorbed and utilized by the body, fats need further digestion and hydrolysis[24]. Teeth begin the physical breakdown of food and saliva serves as the liquid medium that facilitates chewing and swallowing[25]. The majority of saliva is produced from the submandibular, sublingual, and parotid salivary glands. The presence of salivary lipase as a lipolytic enzyme has been disputed in the literature with older work arguing that it is non-existant[26]. However, more recent and comprehensive analysis suggests that there are indeed various lipases that are released and mixed with the saliva to begin partial breakdown of triglycerides[27–30]. Since oral lipases are present in such small quantities, they are not significant contributors to fat digestion[31]. This process usually takes between one and five seconds and is enough time to be able to taste the fat present in foods[32,33]. Therefore, from an evolutionary perspective, the function of oral lipases is likely to facilitate the identification of energy rich foods that are high in fat[32,34]. Once the food forms into a bolus and is swallowed, the esophageal smooth muscle constricts in a wave-like manner to propel the food towards the stomach. The bolus then enters through the cardiac sphincter into the stomach.

Here, the bolus is exposed to an extremely hostile chemical environment. Gastric acid is secreted by parietal cells of the stomach to facilitate digestion. Gastric acid is mainly composed of water, enzymes, electrolytes, and hydrochloric acid. The latter component is responsible for the low pH of the stomach interior. The maintenance of this acidic environment is critical for many digestive processes, one of which is protein denaturation[35]. This allows for lipid molecules to dissociate from proteins which improves the access that lipases have to them. However, the water-soluble nature of gastric and pancreatic lipases presents a barrier to their interactions with lipids.

The stomach forcefully breaks down its contents through the constricting and churning action of the gastric musculature. This causes an oil in water emulsification which helps lipases to overcome aqueous barrier and engage in lipid hydrolysis[36,37]. The products of gastric lipid digestion are unesterified fatty acids, diacylglycerols and monoacylglycerols among many other nutrients that combine together in an acidic mixture termed chyme.

The stomach's contribution to fat digestion and the human's overall capacity to digest fat is greatly influenced by physiological maturation and health status. Once emerging from the womb and beginning to feed on breast milk, infants must deal with the demanding process of fat digestion. As an infant, with minimal production of bile salts from the liver and lipase from the pancreas, digestion of fats presents a significant challenge. Nonetheless, the properties of breast milk optimize its own digestion due to the presence of bile-salt stimulated lipase. This lipase maximizes the efficiency of the small amount of bile-salts that are produced by infants. Like adults, infants also secrete gastric lipase; however, unlike adults, the infant's accessory organs are not fully developed and therefore rely heavily on gastric lipase to break down lipids[38]. Moreover, health conditions that compromise the function of the pancreas, such as pancreatitis and cystic fibrosis, lead to an increased reliance on the stomach for fat digestion[39]. However, in an otherwise healthy individual, once consumption of solid foods begins, the primary location of fat digestion shifts to the duodenum. In fact, among healthy adults, only 10-30% of fat digestion occurs in the stomach[40,41].

Thus, as the solution exits the stomach through the pyloric sphincter, it enters the duodenum. The presence of free fatty acids within the chyme is sensed by duodenal receptors, triggering the release of cholecystokinin (CCK) which leads to a series of responses. CCK causes the pylorus to contract and the body and the fundus of the stomach to relax which ultimately delays stomach

emptying and prolongs gastric digestion[42]. CCK also binds to CCK1 receptors that induce satiety. In addition, pancreatic lipase is released which is a major contributor towards lipolysis. CCK also promotes gall bladder contraction which leads to the release of concentrated bile. Bile is a substance that is composed of 95% water, however, it also contains bile salts, cholesterol, bilirubin phospholipids, vitamins, and minerals[43]. The alkaline bile helps to neutralize the acidic chyme within the duodenum. In the context of fat metabolism, the amphipathic nature of bile salts allows for further emulsification of dietary fat which facilitates the access of the primary lipolytic enzyme, pancreatic lipase, to its substrate. However, the bile salt itself inhibits pancreatic lipase. For this reason, the pancreas also secretes colipase which enables the interaction between pancreatic lipase and the bile salt-lipid complex[44,45]. As a consequence, pancreatic lipase is able to engage in lipid hydrolysis and produce monoglycerols, free fatty acids, and glycerol molecules. Knowledge of this physiological machinery allows for its manipulation in order to manage physiological dysfunction. For instance, Orlistat is a drug that has been proposed as a method to manage obesity through its ability to bond to serine residues within the active sites of pancreatic and gastric lipases, thereby inhibiting their function[46,47]. This ultimately lowers lipolysis, decreases the absorption of fat, and increases fat excretion[48]. However, this also means that the free fatty acid product would be produced in lesser quantities which can have a detrimental downstream effect.

Nonetheless, the products of TAG breakdown exit the intestinal lumen and enter intestinal epithelial cells called enterocytes through a variety of pathways. Predominantly, this occurs through passive diffusion into the enterocyte, however, this process is highly dependant on the saturation of the phospholipid membrane. Lysophosphatidylcholine acyltransferase (LPCAT) is an enzyme that is responsible for the reacylation and addition of polyunsaturated fatty acids to the membrane. Mouse models that have a LPCAT gene knockout lead to diminished presence of

unsaturated fatty acids in the enterocyte membrane. Dietary deficiency in essential fatty acids also leads to less incorporation of unsaturated fatty acids into the lipid bilayer. In both cases, fat malabsorption stems from insufficient unsaturation of the enterocyte phospholipid membrane[49]. Aside from passive diffusion, there are numerous proteins that are also implicated in intestinal lipid absorption. The most prevalent is CD36, which is a protein that is highly expressed in the small intestine, muscle tissue, and adipose tissue. These peripheral tissues rely heavily on CD36 for adequate fatty acid uptake, whereas passive diffusion is more common for intestinal fat absorption[49]. In fact, mice with CD36 knockouts still had normal levels of circulating TAG of intestinal origin. Despite the intestine's apparent independence, CD36 has still shown to be particularly important for the uptake of very long chain fatty acids[49].

Once inside the enterocyte, lipids are then transferred to the endoplasmic reticulum where TAGs are resynthesized. This is done through the help of monoacylglycerol acyltransferase (MGAT) and diacylglycerol transferase (DGAT). Apo B48 and many other proteins are added for structural and recognition purposes. Finally, cholesterol is added and this structure of lipids and proteins is packaged into a prechylomicron transport vesicle (PCTV) to be sent to the Golgi complex. There, it undergoes further modification until it leaves the Golgi as a mature chylomicron, undergoes exocytosis from the basolateral enterocyte membrane, and enters the lymphatic system (figure 1). Chylomicrons travel along lymphatic vessels and enter the circulatory system through the thoracic duct where they deposit TAGs and cholesterol in peripheral tissues. In order for chylomicrons to be utilized by peripheral tissues, they must be hydrolyzed. For this reason, endothelial cells of capillaries that are next to muscle, adipose, and heart tissue contain lipoprotein lipase which isolates the fatty acids from the rest of the chylomicron. For this process to occur, Apo C-II is required as a cofactor and is carried by the chylomicron itself. After lipolysis,

the fatty acids enter the tissue through the use of CD36 and other fatty acid transport proteins. The remaining chylomicron remnant is recycled by the liver and the cholesterol that is present is used for the production of bile acids or very low density lipoprotein (VLDL). Similar to chylomicrons, VLDL is composed of triglycerides and cholesterol, however, this is done in the hepatocyte endoplasmic reticulum and Apo B-100 is required for its synthesis[50]. VLDL particles travel to peripheral tissues where it competes with chylomicrons for access to LPL in order to undergo fatty acid hydrolysis. Once triglycerides are removed from the VLDL particle, VLDL remnants remain which are termed intermediate density lipoproteins (IDL)[50]. Similar to chylomicron remnants, IDL is cleared by the liver, however not as effectively, and approximately 50% of IDL remains in circulation. Through the use of hepatic lipase, the liver lowers the lipid content of the IDL molecules and several apolipoproteins are exchanged which leads to the creation of low density lipoprotein (LDL)[50]. Therefore, the predominant lipid in LDL is cholesterol and it is recognized by many tissues that have the LDL receptor. The liver, however, is the major tissue responsible for the clearance of circulating LDL. The abundance of LDL receptors that a cell has is inversely proportional to the amount of cholesterol found within that cell. Once inside, the lipoprotein is degraded by lysozymes leading to the release of cholesterol in the tissue. This decreases the expression of HMG-CoA reductase, a critical enzyme for cholesterol synthesis, thereby leading to less endogenous cholesterol synthesis. HDL cholesterol is formed from Apo A-I as well as cholesterol and phospholipids that are obtained from enterocytes, hepatocytes, and many other peripheral tissues. ABCA1 allows the transfer of cholesterol to Apo A-I molecules, whereas ABCG1 accomplishes this role for mature HDL molecules. Moreover, HDL can obtain cholesterol from VLDL and chylomicrons when they are being hydrolyzed by LPL. Therefore, HDL and LDL

have opposing functions in the body, with the former extracting cholesterol from tissues, and the latter transporting cholesterol to tissues.

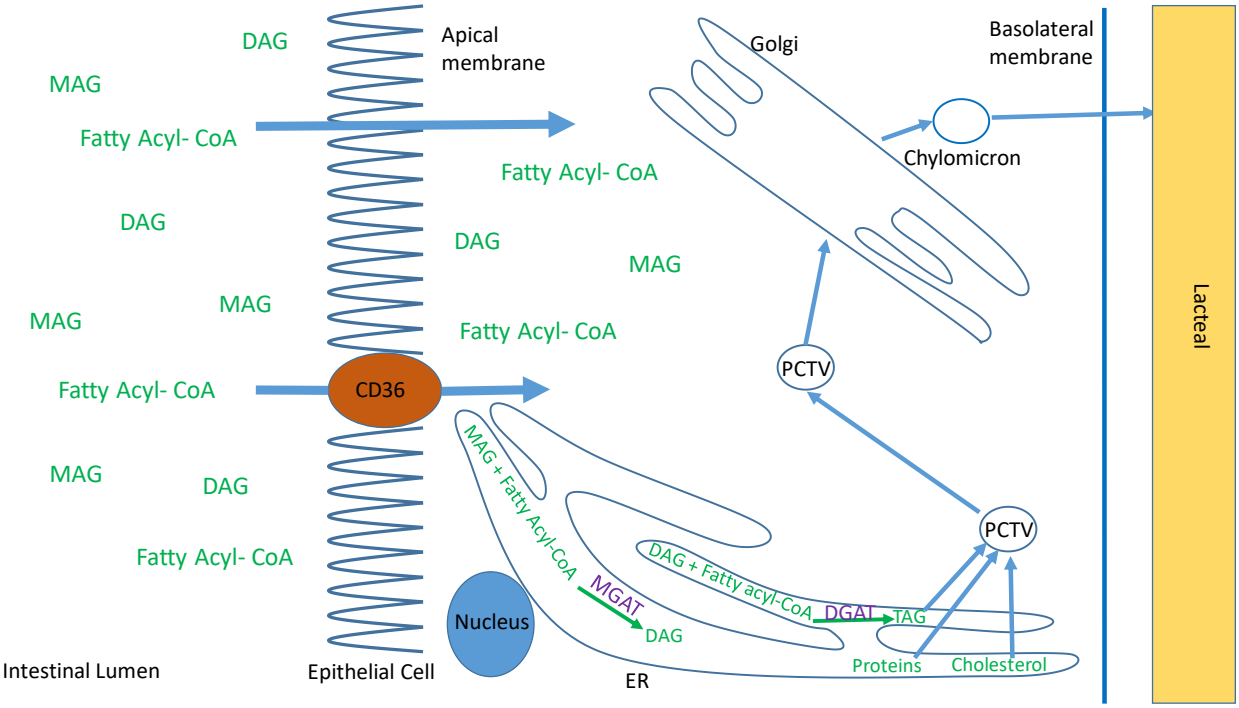

Figure 1: Outline of intestinal lipid absorption. The products of TAG breakdown enter the epithelial cell either through passive diffusion or through fatty acid transport proteins. MGAT catalyzes the reaction that forms DAG from MAG and a free acyl-CoA. DGAT catalyzes the reaction that forms TAG. The PCTV, composed of TAG, proteins, and cholesterol, matures in the Golgi, exits the epithelial cell through the basolateral membrane, and enters the lymphatic system.

Once inside the tissue, fatty acids can either be re-esterified and stored in the form of triglycerides or they can be oxidized. Short and medium chain fatty acids that are eight carbon units in length or smaller are able to diffuse directly through the mitochondrial membrane into the matrix where they are activated by CoA. On the other hand, long-chain acyl-CoA synthetase (ACS) is required to activate long chain fatty acids and they are subsequently transported into the mitochondrion through the use of the carnitine shuttle[51]. Along the outer mitochondrial membrane, carnitine palmitoyltransferase 1 (CPT1) catalyzes the rate limiting reaction in which a fatty acyl CoA is carnitinized to yield a fatty acyl carnitine. Once formed, the fatty acyl carnitine is able to cross the inner mitochondrial membrane through the help of carnitine–acyl carnitine translocase

(CACT)[52]. Inside the matrix, CPT2 catalyzes a reaction in which the acyl chain is transferred from carnitine to CoA in order to commence β-oxidation. Meanwhile, carnitine is free to diffuse back into the intermembrane space. β-oxidation is a cycle that leads to shortening of acyl-CoAs through the repetitive cleavage of 2-carbon acetyl-CoA units. It begins with very long chain acyl-CoA dehydrogenase (VLCAD) striping a hydrogen atom from the second and third carbons thereby creating trans-2-enoyl-CoA and $FADH_2$ in the process. The mitochondrial trifunctional protein (MTP), which has hydratase, dehydrogenase, and thiolase activity, carries out the ensuing three reactions[53]. MTP adds H_20 which breaks the double bond and adds a hydroxyl group to the third carbon, creating 3-hydroxyacyl-CoA. Then, a dehydrogenation reaction occurs in which NADH and β-ketoacyl CoA are formed. Subsequently, an acetyl-CoA unit is cleaved on the β carbon from the carboxyl end through MTP's thiolase activity. The existing acyl chain re-enters the cycle in order for β-oxidation to proceed. Each acetyl-CoA that emerges from this cycle can then enter the citric acid cycle and form ATP through the electron transport chain.

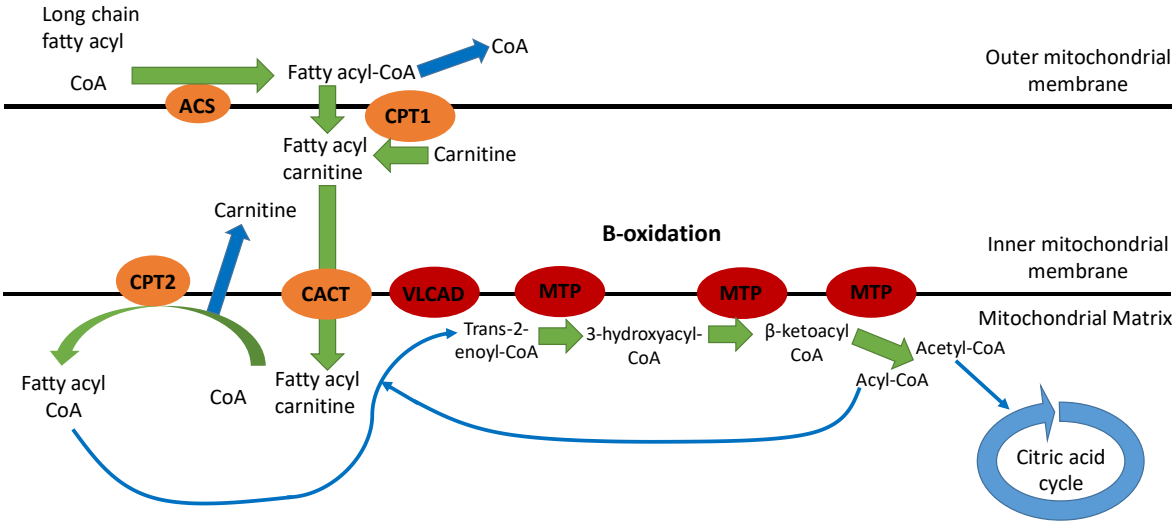

Figure 2: Long chain fatty acid transport into mitochondria and β-oxidation. Long chain fatty acids are activated with CoA prior to entering the mitochondrion. They bind to carnitine in order to enter the matrix. Subsequently, the fatty acids dissociate from the carnitine shuttle and proceed with β-oxidation.

Fatty acid synthesis

Elongation

In order to appreciate the significance of why ω-3 fatty acids are considered to be essential, there needs to be a thorough understanding of fatty acid synthesis. Within mammalian de novo lipogenesis, the carbon source can originate from carbohydrates and proteins. Amino acids can enter the cellular respiration pathway at various stages. However, glucose enters in the first step of glycolysis, which occurs in the cytosol. Pyruvate, the product of glycolysis, enters the mitochondrion, where pyruvate dehydrogenase produces acetyl CoA. These acetyl CoA units must be transferred from the mitochondrion back to the cytosol, where de novo lipogenesis takes place. To accomplish this, acetyl-CoA enters the Krebs cycle and combines with oxaloacetate to produce citrate, a molecule that is able to cross the mitochondrial membrane[54]. The tricarboxylate transport system facilitates the crossing of the membrane. Once in the cytosol, citrate is converted back into acetyl CoA by ATP citrate lyase and is now ready for fatty acid biosynthesis. Meanwhile, oxaloacetate can be converted to malate and then pyruvate which can then re-enter into the mitochondrion. Once acetyl-CoA is in the cytoplasm, acetyl-CoA carboxylase (ACC) adds a carboxyl group to create malonyl CoA. Acetyl CoAs and malonyl CoAs are stripped of their CoA components and are both transferred to acyl-transfer proteins (ACP) through the activity of malonyl-acetyl transferase[54]. Acetyl ACP and malonyl ACP undergo a condensation reaction which creates 3-ketoacetoacyl ACP and releases CO_2 in the process. A reduction reaction follows in which NADPH (from pentose phosphate pathway) is oxidized to NADP and facilitates the creation of 3-hydroxyacyl ACP. This compound is then dehydrated in order to create enoyl ACP which has a double bond between the second and third carbons[54]. Enoyl ACP is reduced once again to create butyryl ACP, a saturated 4-carbon unit. This cycle is then repeated until palmitic acid, a saturated 16-carbon fatty acid, is formed (Figure 3). Although the location of fatty acid

elongation beyond this point is shifted to the endoplasmic reticulum membrane, the process remains similar to the one described above. With the addition of one more malonyl CoA, stearic acid, an 18-carbon saturated fatty acid, is formed. This reaction and all subsequent elongations are catalyzed by elongation of very long chain fatty acids (ELOVL) enzymes. There are seven ELOVL proteins all of which vary in expression depending on saturation level[54].

Desaturation

Mammalian cells have Δ5, Δ6, and Δ9-desaturase capabilities. Each number refers to the carbon, counting from the carboxyl end, where the enzyme can form a double bond[55]. Continuing from stearic acid, Δ9-desaturase is able to form oleic acid, a monounsaturated fat that is the major component of olive oil[56]. A Δ12-desaturase is required to create linoleic acid, a polyunsaturated ω-6 fatty acid. Due to the fact that mammals lack the desaturase required to form this fatty acid, it is termed essential. The name, ω-6 comes from the fact that the first double bond counting from the methyl end occurs on the 6th carbon. Furthermore, a Δ15-desaturase is required in order to convert linoleic acid to α-linolenic acid (ALA), an ω-3 fatty acid. This desaturase is only present in plants which makes ALA an essential fatty acid as well. Once present in the body, ALA can undergo Δ6-desaturation, followed by a series of elongations and desaturations resulting in the creation of EPA and then DHA the two ω-3 fatty acids that have the greatest bioavailability and therapeutic potential. In theory, ALA is the only essential ω-3 fatty acid because humans have the desaturases necessary to produce the rest. However, the rate limiting step is Δ6-desaturation of ALA since linoleic acid competes for access to this enzyme (Figure 3). This results in less than 10% of ALA being converted to DHA in females and less than 3% in males. Thus, in practical terms, due to the inefficiency of their conversions, both EPA and DHA are necessary to obtain from the diet[57].

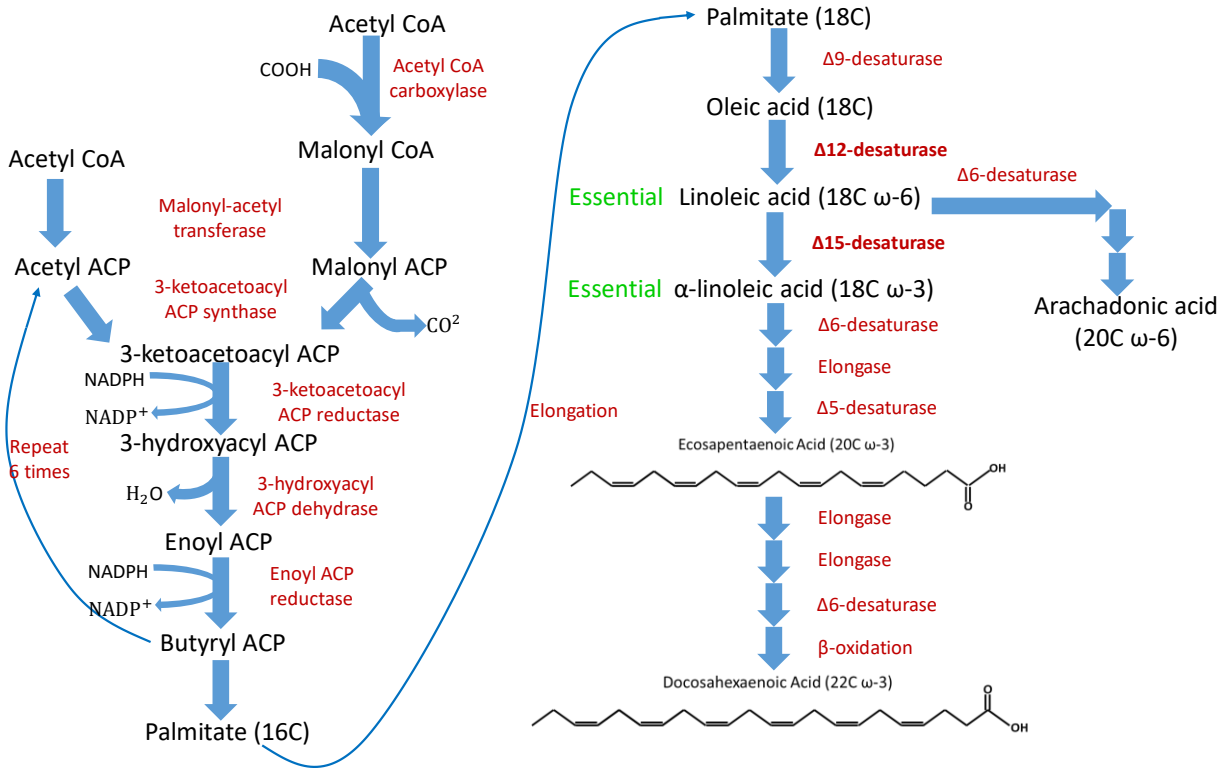

Figure 3: Fatty acid synthesis pathway. Repetitive addition of malonyl CoA yields palmitate, a saturated 16-carbon fatty acid. Absence of Δ12 and Δ15 desaturases in humans means that linoleic and α-linoleic acid are both essential. Competition for Δ6-desaturase leads to low quantities of EPA and DHA.

Sources of omega-3 fatty acids

It is thought that prior to the agricultural revolution, hominid diet consisted largely of seafood which yielded an ω-6: ω-3 ratio of approximately one[58]. Thus, from an evolutionary prospective, it would not have been any more advantageous to be able to synthesize ω-3 fatty acids. However, with a typical present-day western diet, the ratio of ω-6 to ω-3 is around 20:1. In the last 20 years, the ratio of ω-6 to ω-3 has also increased in breast milk which has played a role in the rising rates of childhood obesity[59]. Ω-6 fatty acids promote the production of pro-inflammatory mediators and are contributors to the increased prevalence of conditions that are characterized by chronic inflammation. Thus, it is imperative to have an adequate amount serum and tissue ω-3 fatty acid content to help counteract the negative effects of ω-6 fatty acids. The abundance of ω-3 fatty acids in adipose tissue, the main storage site for fatty acids, is relatively small. ALA is the

major ω-3 fatty acid stored in this tissue and it accounts for approximately 1% of all fatty acids[60]. Thus, EPA and DHA are stored in limited quantities and are therefore required to be consumed consistently through the diet. There are a variety of sources from which one can obtain ω-3 fatty acids such as seeds, vegetables, oils, fish, and supplements. For instance, chia seeds, hemps seeds, and flaxseed oil are all excellent sources of ALA with 17.8g, 8.7g, and 53.4g respectively of ALA per 100g[59]. However, as mentioned earlier, the bioavailability of ALA is relatively limited, and instead, sources of EPA and DHA should be sought. It is also important to note that in nature, polyunsaturated fatty acids do not exist in isolation, but rather a mixture of ω-3, ω-6, monounsaturated, and/or saturated fatty acids and their relative proportions can vary overtime. For example, the average avocado has 13.3g of monounsaturated fat, 2.5g of polyunsaturated fat, and 2.9g of saturated fat. As the avocado ripens, its saturated fat content slightly decreases, while its monounsaturated fat increases slightly[61]. Phytoplankton are the main source of ω-3 fatty acids in the aquatic environment. As a consequence, fish and crustaceans such as krill that consume phytoplankton are rich in ω-3 fatty acids. Fish can be categorized based on their relative fat content as well as the distribution of lipids within their various tissues. Lean fish such as bass, cod, and catfish have relatively little fat and use their liver as the predominant lipid storage area. However, fatty fish such as salmon, herring, and mackerel are lipid rich and have a higher percentage of lipids stored within their muscle and skin tissue[62,63]. Fatty fish as well as the liver of lean fish are excellent sources of essential polyunsaturated fatty acids and are recommended to consume for this reason. Each fish has a different proportion of polyunsaturated fatty acids based on their environmental conditions. Wild sources of fish have higher ω-3 fatty acid content than their farmed counterparts. This is because wild fish feed on phytoplankton as opposed to the cereal-based formula that farmed fish eat. Furthermore, fish that come from cold water as opposed to warm

water environments have a higher proportion of long chain polyunsaturated fatty acids[59]. However, fish can accumulate contaminants such as mercury which has a half life of 70-90 days and heavy consumers of fish can suffer the consequences of toxicity[64]. To minimize the risk of toxicity and still benefit from the ω-3 fatty acids present in fish, the European Food Safety Agency, the American Heart Association, and the World Health Organization all recommend approximately 1-2 servings of fatty fish a week[65]. Furthermore, the commercialized extraction of oil from marine organisms has provided an alternative source of ω-3 for humans. Fish oil supplements reflect the diversity in fatty acid makeup among fish in nature. There are a variety of options to choose from such as krill oil, cod liver oil, and algal oil. Krill oil is considered to have a higher bioavailability than other fish oils because it has 35% of its DHA in the form of phospholipids which are easier to integrate as opposed to triglycerides[66]. Overconsumption of fish oil supplements can also increase the risk of lipid peroxidation which can damage muscle, nerves, and membranes. To mitigate this risk, it is necessary to consume adequate levels of vitamin E and regulate the frequency of fish oil supplement consumption[64].

Mechanism of action

On a cellular level, fatty acids are central components of the phospholipid bilayer that forms the foundation of all cell membranes. Phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and phosphatidylcholine are the four most common phospholipids found in animal membranes[67]. The phospholipids in the inner leaflet are arranged with their polar heads directed towards the cytosol, while those in the outer leaflet have their polar heads pointed towards the extracellular matrix. The two layers of phospholipids have their non-polar fatty acid chains facing each other which creates a hydrophobic interior. Incorporation of ω-3 fatty acids into a cell membrane can increase the fluidity of the cell membrane due to the kinks that are created by the double bonds present in the structure of the fatty acid[68]. Membrane fluidity can enhance the

permeability to molecules of smaller size thereby altering the plasma membrane environment[69]. This increased fluidity can also alter the function of channels, receptors and membrane processes as a whole[70,71]. Proteins that require mobility within the membrane are especially dependent on membrane fluidity and are thus indirectly affected by the presence of polyunsaturated fatty acids. Ω-3 fatty acids can also have a direct effect on the activity of receptors and integral membrane proteins by restricting conformational changes and associating with hydrophobic regions of proteins[72]. Moreover, higher quantities of polyunsaturated fatty acids in membranes have been associated with greater proportion of insulin receptors[73,74]. This provides further evidence for the role that ω-3 fatty acids have in altering the plasma membrane lipid environment and altering cell function.

Within the phospholipid bilayer, ω-3 fatty acids can integrate into lipid rafts and alter their composition and function. Lipid rafts are components of the cell membrane that are generally composed of glycosphingolipids and cholesterol[75]. Various regulatory membrane proteins routinely assimilate into lipid rafts, which presents these rafts with cell signalling properties[76]. Stulnig et al. demonstrated that human T-cells treated with polyunsaturated fatty acids had a significantly greater proportion of unsaturated acyl chains within the lipid raft than untreated cells, which altered the fate of critical signalling proteins[77]. Therefore, ω-3 fatty acids can influence cell signalling indirectly by integrating into lipid rafts and modulating their lipid composition.

The various phospholipids that form the basis of the plasma membrane can be used as substrates by phospholipases for the production of second messengers that are involved in various signalling pathways. For instance, the second messengers diacylglycerol (DAG) and inositol triphosphate (IP3) are produced from the phospholipids, phosphatidylinositol bisphosphate, and phosphatidyl-choline. In addition, protein kinase C (PKC), an enzyme that is involved in cell

signalling, requires phosphatidylserine for its activation and certain isoforms of PKC are also activated by DAG[78]. Kishimoto et al. have shown that DAGs that contain unsaturated fatty acid moieties, such as diarachidonoylglycerol and dioleoylglycerol, are better able to activate PKC than DAGs containing saturated fatty acids[79]. Therefore, the activity of certain enzymes and their products are highly dependent on the type of phospholipid and its constituent fatty acid[78]. Membrane phospholipids can also be cleaved by phospholipases for alternative fates. Ω-3 fatty acids can be used by cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to synthesize eicosanoids such as prostaglandins, thromboxanes, lipoxins, and leukotrienes[80]. Eicosanoids facilitate the inflammatory response, however the fatty acid precursor that is used as the substrate in eicosanoid production dictates the intensity and the physiological role of the eicosanoid. Ω-3 fatty acids in the phospholipid bilayer would compete with and lower the amount of arachidonic acid, an ω-6 fatty acid, incorporated into membrane phospholipids. Consequently, there is less arachidonic acid substrate available for eicosanoid synthesis. The eicosanoids that are produced from ω-3 fatty acids have different structures and functions from those produced by arachidonic acid, with the former having a bioactivity that is less efficient than the latter. The leukotriene originating from ω-3 fatty acids is up to 100 times less potent of an attractant to neutrophils than leukotrienes originating from ω-6 fatty acids[81]. Wada et al. also demonstrated that ω-3-derived prostaglandins were 50-80% less potent than their ω-6 counterparts towards the eicosanoid receptors[82]. Therefore, ω-3 fatty acids can not only act as second messengers that regulate signalling pathways, but can also dampen the inflammatory response by mediating eicosanoid synthesis.

Within the cell membrane, there are four types of transmembrane G-protein coupled receptors that bind fatty acids, with GPR40 and GPR120 binding long chain fatty acids[83,84]. These

receptors trigger intracellular signalling pathways that can produce anti-inflammatory and metabolic responses. Once EPA and DHA bind to and activate GPR120, the B-arrestin 2 protein is recruited to the plasma membrane where it forms a complex with GPR120. This complex then penetrates the cytoplasm and binds to the transforming growth factor-β-activated kinase 1 (TAK1)-binding protein. As a result, the TAK1-binding protein's ability to interact with TAK1 is hindered which reduces the activation of TAK1 and consequently the NF-κB signalling pathway[81]. Through the decreased phosphorylation of the inhibitory subunit, IkB, the NF-κB complex is no longer able to translocate to the nucleus to transcribe inflammatory cytokines[85]. The TNF receptor and the TLR4 inflammatory signalling pathway are dependent on the binding of TAK1 to TAK1-binding protein as well. This means that ω-3 fatty acids can suppress the expression of pro-inflammatory genes like TNFα and IL-6, but also interrupts the signalling of TLR4 and NF-κB signalling pathways[81]. Through the activation of GPR120, DHA has been shown to be able to stimulate GLUT4 translocation to the surface of adipocyte cells and consequently promote glucose uptake. In fact, 30 min of exposure to DHA (100 μM) increased basal glucose uptake by ~2-fold, which was equivalent to 30-50% of the insulin-stimulated glucose uptake response in 3T3L1 adipocytes[16]. Therefore, by binding to G-protein coupled receptors, ω-3 fatty acids can exert anti-inflammatory effects and alter uptake and metabolism of glucose in cells.

Ω-3 fatty acids along with their eicosanoid products are able to act as ligands for peroxisome proliferator-activated receptors (PPAR), which have various isoforms (α, δ and γ). Upon binding to their ligands, PPARs translocate to the nucleus where they associate with the retinoid X receptor and trigger the transcription of genes[86]. PPARα is highly expressed in skeletal muscle and is responsible for regulating β-oxidation and lipid homeostasis. For instance, activation of PPARα in skeletal muscle has been shown to enhance lipid oxidation and increase the

expression of carnitine palmitoyltransferase I (CPT-1), a rate limiting enzyme of β-oxidation of long-chain fatty acids[87]. At a tissue-specific level, muscle can display impairment in insulin-stimulated glucose uptake due to impaired ability to oxidize fat and lipid accumulation[88,89]. Therefore, the ability of ω-3 fatty acids to enhance fat oxidation in muscle tissue through PPARα activation can reduce insulin resistance and improve glucose utilization[90]. With regard to the other PPAR isoforms, it has been demonstrated that there is a functional overlap between PPARδ and PPARα[91]. Indeed, an increase in fatty acid uptake and oxidation was found when L6 skeletal muscle cells were treated with PPARδ agonists[92]. PPARδ is also implicated in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) upregulation and mitochondrial biogenesis, which is consistent with improved cellular oxidative capacity[93–96]. The activation of PPARγ, despite having an expression in muscle that is 5-10% of its expression in adipose tissue, has a significant role in muscle insulin sensitivity[97,98]. In fact, pioglitazone, a PPARγ agonist has been shown to ameliorate insulin sensitivity of an *in vivo* type II diabetic model[99]. Deletion of PPARγ in skeletal muscle has led to extreme glucose intolerance and insulin resistance in mice[100]. In addition, when PPARγ is activated, it can physically interfere with the translocation of NF-κB to the nucleus, which provides an alternative mechanism of shutting down the NF-κB inflammatory pathway[80]. Thus, ω-3 fatty acids can potentially influence skeletal muscle energy metabolism through multiple mechanisms.

In the context of cellular energetics, EPA and DHA can serve as agonists of AMP-activated protein kinase (AMPK). AMPK is an important protein that regulates mitochondrial biogenesis and oxidative capacity through the activation of PGC1-α and nuclear respiratory factors[101]. AMPK also acutely influences fatty acid oxidation through the phosphorylation and inactivation of acetyl CoA carboxylase (ACC), which limits the production of the CPT-1 inhibitor malonyl CoA[102].

Activation of AMPK can facilitate the translocation of GLUT4 to the plasma membrane and increase skeletal muscle glucose uptake[103,104]. Furthermore, AMPK can upregulate the expression of PPARγ in skeletal muscle, which demonstrates the diverse functions of this protein[105]. Aside from AMPK activation, rats fed a diet high in polyunsaturated fatty acids have been shown to increase the number of insulin receptors as well as the expression of GLUT4 and IRS1 receptors in skeletal muscle[106,107]. Therefore, ω-3 fatty acids can potentially play an insulin sensitizing role and promote glucose uptake and its metabolism. In this context, the AKT protein, which is central to the insulin signalling pathway, is of particular interest. The ability of ω-3 fatty acids to modulate this pathway has been demonstrated in neuroblastoma cells in mice, epithelial cells of human lung carcinoma, and human pancreatic epithelial cells[108–110]. The phosphorylation of AKT would lead to a cascade of intracellular events that promote GLUT4 translocation, phosphorylation/inhibition of glycogen synthase kinase 3 (GSK3), and enhanced activity of glycogen synthase (GS).

Rodent and human studies exploring the effects of ω-3 fatty acids

In the 1950s and 1960s it became apparent that Alaskan natives have much lower rates of insulin resistance, type-2 diabetes, and metabolic syndrome than the general population[111]. Here, the authors of the study ruled out any influence of diet on these findings. However, subsequent studies analyzed data collected from the Alaska Siberia Project in order to specifically determine whether there was a link between plasma polyunsaturated fatty acid concentrations and the prevalence of metabolic syndrome. Three hundred and fifteen Eskimos between the age of 35 and 74 who lived in 4 different villages in Northern Alaska were used for this study. Their results show that ω-3 fatty acids are related to higher levels of plasma HDL concentration and lower levels of fasting insulin, triglyceride concentrations and 2-hour blood glucose[112]. Several other studies have documented that populations with high fish consumption have a lower incidence of type-2 diabetes. In this context, Helland et al. investigated the whether the type of fish is a factor. To

conduct the study, 76 overweight or obese adults ranging from 18 to 69 years old were recruited to either consume 750g per week of cod (lean fish) or salmon (fatty fish) for eight weeks. A third group ate no fish and served as the control. Their results show that the fatty fish group, but not the lean fish group, had lowered their postprandial glucose and insulin concentrations which are indicative of improved insulin sensitivity and glucose tolerance. They also measured leukocyte membrane fatty acid composition and determined that fatty fish consumption leads to lower ω-6 and higher ω-3 fatty acid membrane composition. This means that ω-6-derived eicosanoids would decrease while augmenting the production of resolvins and protectins, and ω-3-derived anti-inflammatory eicosanoids. In a study by Lalia et al., 7 female and 5 male adults between the ages of 65 and 85 were evaluated at baseline and after 16 weeks of daily ω-3 fatty acid supplementation of 3.9g. Although mitochondrial respiration levels remained the same, there was a 25% decrease in reactive oxygen specie production. This study demonstrates the potential for ω-3 fatty acids to limit the release of oxidants and therefore ameliorate mitochondrial function[113]. However, many human studies provide findings that are contrary to this. For instance, among 4941 participants from the National Heart, Lung, and Blood Institute Family Heart Study, of which 21% had metabolic syndrome, neither ω-3 fatty acids nor fish consumption was associated with metabolic syndrome[114]. Another study showed that in 8 patients with impaired glucose tolerance, 3.8g of EPA and 2.5g of DHA added to their regular diet daily for 2 weeks led to no changes in fasting glucose and insulin concentrations[115]. Similarly, as compared to those receiving placebo, insulin-resistant adults who received a 3.9g EPA + DHA mixture for 6 months showed no measurable improvement in peripheral glucose disposal or skeletal muscle mitochondrial function[116]. Thus, human studies have been generally equivocal; however, rodent studies have been more conclusive in demonstrating the therapeutic value of ω-3 fatty acids in insulin resistant states. Indeed, fat-1

transgenic mice, capable of synthesizing ω-3 fatty acids from ω-6 fatty acids, demonstrate improved whole-body glucose tolerance when compared to wild-type mice[117]. In another study, Bidu et al. wanted to test whether the transplantation of a microbiome that was altered due to elevated ω-3 fatty acid concentrations could mitigate the deleterious effects of a high sucrose high fat diet. For this study, they used fat-1 transgenic mice which effectively balanced the ratio of ω-6 to ω-3. The fat-1 mice exhibited increased cecal microbiome diversity, reduced endotoxemia, and preserved intestinal integrity that is usually broken down with a high fat diet. In addition, transplantation led to the reversal of metabolic dysfunction brought on by a high sucrose high fat diet in wild type mice[118]. Here, it is evident that ω-3 fatty acids have broad and potent effects on multiple tissues throughout the body. However, in these transgenic mice, it is challenging to identify the specific effects of ω-3 fatty acids, due to the simultaneous fluctuation in ω-6 fatty acid concentration. For this reason, Lamping KG et al. induced obesity in mice through a 12 week HF diet and subsequently replaced half of the calories with monounsaturated, ω-3, or ω-6 fatty acids. The mice that were fed ω-3 fats were better able, as compared to mice who were fed monounsaturated or ω-6 fatty acids, to ameliorate glucose tolerance and AKT phosphorylation in skeletal muscle tissue[119]. Similarly, rats fed a high fat fish oil-based diet as opposed to a standard HF diet, showed greater insulin sensitivity and lower systemic inflammation and adipocyte diameter[106]. In cultured adipocytes, EPA was actually able to stimulate AMPK phosphorylation and could potentially improve insulin insensitivity through this mechanism[120]. In a separate study, obese rats who consumed a HF diet supplemented with 10% v/w ω-3 fish oil, led to an increase in AMPK-α1 gene expression in the soleus muscle. This was complemented with a reduction in serum free fatty acids, triglycerides, total cholesterol, and glucose levels. Evidently, there is a

plethora of studies that have provided support for the therapeutic value that ω-3 fatty acids can have for insulin resistance.

Objectives and hypothesis

Ω-3 fatty acids are known for their anti-inflammatory properties and as a result have been associated with beneficial outcomes for conditions that are characterized by chronic inflammation such as type-2 diabetes, insulin resistance, and metabolic syndrome. Due to the fact that skeletal muscle tissue accounts for ~40% of body weight in men and ~30% in women and is responsible for ~80% of post-prandial glucose disposal, it likely plays an important role in this response. However, it was unclear whether ω-3 fatty acids exert their effects on skeletal muscle tissue through a reduction in inflammation or through the direct regulation of energy metabolism. Thus, the objective of this study was to investigate whether docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids can directly regulate glucose and fat metabolism in L6 rat skeletal muscle cells, independent of their anti-inflammatory properties. In this context, we hypothesized that besides exerting an anti-inflammatory effect, ω-3 fatty acids could indeed improve glucose and fat metabolism in skeletal muscle cells through their direct regulation of proteins that underlie these pathways.

Also, because EPA and DHA segregate differently between raft and non-raft cell membrane domains[121] and seem to distinctly regulate skeletal muscle mitochondrial oxidative capacity[122], we tested these fatty acids individually. We exposed L6 skeletal muscle cells to either EPA or DHA for 1, 3, and 5 days and then assessed several parameters of glucose and fatty acid metabolism along with the phosphorylation and content of various proteins that underlie these processes. Lastly, mRNA was extracted to assess the expression of various genes implicated in inflammation.

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DHA and EPA differentially regulate glucose and fatty acid metabolism in L6 rat skeletal muscle cells independent of their anti-inflammatory effects

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Abstract

The objective of this study was to investigate whether docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids can directly regulate glucose and fat metabolism in skeletal muscle, besides exerting anti-inflammatory properties. To accomplish this, L6 skeletal muscle cells were treated with 50μM of either DHA or EPA for 1, 3, and 5 days. Subsequently, we assessed glucose uptake, glycogen synthesis, lactate production, glucose and palmitate oxidation, as well as the phosphorylation and contents of proteins involved in insulin signalling and regulation of cellular oxidative capacity. mRNA expression of several genes involved in inflammation was also measured after DHA or EPA treatment. We found that basal rates of glucose uptake and glycogen synthesis as well as protein kinase B (AKT) and glycogen synthase kinase 3 (GSK3) phosphorylation remained unaffected by DHA or EPA. In addition, similar findings were observed under insulin-stimulated conditions. However, glucose and palmitate oxidation rates were consistently increased (day 1 to 5) by DHA treatment, whereas EPA only increased this variable transiently (day 1). Similarly, only DHA caused significant and sustained increases in AMP-activated protein kinase (AMPK) phosphorylation and contents of carnitine-palmitoyl transferase 1b (CPT1b) and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) in skeletal muscle cells. DHA also had a more potent anti-inflammatory effect than EPA in these cells. In conclusion, besides exerting anti-inflammatory effects, DHA and EPA directly regulated glucose and fat metabolism in skeletal muscle cells, although DHA was more potent in doing so than EPA. Thus, by directly enhancing glucose and fat oxidation, DHA may increase glucose disposal and protect skeletal muscle cells against lipotoxicity.

Introduction

Ω-3 fatty acids are well known for their anti-inflammatory properties and therapeutic value for conditions that are characterized by chronic inflammation[2,4]. With the rise in the global prevalence of obesity and its related metabolic disorders, there has been great focus on the potential role that ω-3 fatty acids can play in ameliorating the inflammation-induced metabolic disorders that often accompany obesity. Indeed, there are reports that intake of ω-3 fatty acids improves glucose tolerance in healthy humans and in those with metabolic syndrome[5–8]. To identify the specific effects of ω-3 fatty acids, mice consuming a high-fat (HF) diet had 7.5% of their calories replaced with fish oil. The dysfunctional metabolic alterations in skeletal muscle caused by a HF diet were reversed in fish oil-fed mice, in part by facilitating the upregulation of proteins involved in lipolysis and lipid oxidation in this tissue[9]. Also, rats fed a HF diet with 3.4% of calories coming from eicosapentaenoic and docosahexaenoic acids (EPA and DHA, respectively) had lower blood glucose and higher skeletal muscle expressions of GLUT4, GYS1, and IRS1 than rats fed a regular HF diet[10]. Based on animal studies, it is evident that ω-3 fatty acids can beneficially affect whole-body glycemic control, and they appear to do so, at least in part, through their regulation of glucose and fat metabolism in skeletal muscle cells. However, human studies have not consistently recapitulated rodent findings, so the validity of supplementing ω-3 fatty acids for improvement in glycemic control in humans remains a contentious topic[123].

With respect to the etiology of insulin resistance, it has been proposed that the excessive intramyocellular accumulation of triglycerides (TAG) and diacylglycerols (DAG) leads to the disruption of insulin signaling in skeletal muscle cells. This is based on the observation that DAG can activate protein kinase C (PKC), which, in turn, phosphorylates serine residues on IRS-1 and impairs the ability of insulin to induce phosphorylation of tyrosine residues on this protein[13,124].

Thus, it has been suggested that suppressing the accumulation of harmful lipid intermediates through the enhancement of fatty acid oxidation within skeletal muscle cells may prevent insulin resistance in this tissue. Consistent with this idea are reports that fish oil supplementation is associated with a reduced accumulation of lipid mediators such as ceramides and long chain acyl-CoAs in skeletal muscle tissue of mice fed a HF diet[10]. Whether these effects derive from ω-3 fatty acids directly regulating the ability of skeletal muscle cells to uptake and metabolize fatty acids or indirectly through their anti-inflammatory properties remains undetermined. However, ω-3 fatty acids display unique chemical and physical properties that can affect membrane fluidity and cell function. For instance, DHA is a highly flexible molecule that can rapidly transition between numerous conformers with the ability to enter binding pockets of proteins and also act as ligands for receptor proteins[125]. In addition, ω-3 fatty acids can be incorporated into cell membranes and form lipid rafts, which could then interact with specific proteins and regulate downstream signaling pathways[121,126], as well as modulate the expression of genes related to mitochondrial biogenesis, oxidative capacity, and cellular energy supply[122]. In this context, we hypothesized that besides exerting anti-inflammatory properties, ω-3 fatty acids could directly regulate glucose and fat metabolism in skeletal muscle cells. Also, because EPA and DHA segregate differently between raft and non-raft cell membrane domains[121] and seem to distinctly regulate skeletal muscle mitochondrial oxidative capacity[122], we tested these fatty acids individually. We exposed L6 skeletal muscle cells to either EPA or DHA for 1, 3, and 5 days and then assessed several parameters of glucose and fatty acid metabolism along with the phosphorylation and content of various proteins involved in these processes. Lastly, mRNA was extracted to assess the expression of various genes implicated in inflammation. Here, we show that DHA, and EPA can directly affect glucose and fat oxidation in skeletal muscle cells. However, DHA was the most consistent in doing

so. Furthermore, these effects were accompanied by sustained elevations in AMPK phosphorylation and CPT1b and PGC-1α contents. DHA treatment also consistently reduced the mRNA expression of genes involved in inflammation, whereas EPA only exerted transient effects. Therefore, DHA seems to be more effective than EPA in directly enhancing glucose and fat oxidation in skeletal muscle cells, as well as in simultaneously attenuating the ability of these cells to respond to inflammatory mediators.

Methods

Chemicals and reagents – Antibiotic-antimycotic (Cat # 450-115-EL), α-minimal essential medium (α-MEM) with (Cat # 310-010-CL) and without phenol red (Cat # 310-015-CL), Fetal Bovine Serum (FBS) (Cat # 080-150), trypsin (Cat # 325-040-EL), Phosphate Buffered Saline (PBS) (311-010-CL), were purchased from Wisent Bioproducts (St-Bruno, Quebec, Canada). Fatty acid-free BSA (Cat # A3803) was purchased from Sigma-Aldrich (St. Louis, MO). DHA (Cat# 90310), EPA (Cat # 90110), and α-tocopherol (Cat # 25985) were purchased from Cayman Chemical (Ann Arbour, MI). Protease inhibitor cocktail (Cat # 5892953001) and phosphatase PhosphoStop inhibitor cocktail (Cat # 4906837001) were from Roche Diagnostics (Mannheim, Germany). 2x Laemmli Sample Buffer (Cat # 1610737) was bought from Bio Rad (Mississauga, ON, Canada) and Immobilon Forte Western HRP Substrate (Cat # WBLUF) was purchased from Millipore (Billerica, MA). 2-deoxy-D-[H^3] glucose was purchased from Perkin Elmer (Seer Green, United Kingdom), [1-^{14}C] palmitic acid was from American Radiolabeled Chemicals (St. Louis, MO) and d-[U-^{14}C] glucose was from GE Healthcare Radiochemicals (Quebec City, QC, Canada). Regular human insulin was bought from Eli Lilly (Indianapolis, IN). The lactate colorimetric assay kit (Cat# K607-100) was purchased from BioVision (San Francisco, CA) and the CellTiter-Glo®

Luminescent Cell Viability Assay (Cat # G7570) was from Promega (Madison, WI). TRIzol™ Reagent (Cat # 15596026) was bought from ThermoFisher Scientific (Waltham, MA). EasyScript™ cDNA Synthesis Kit (Cat # G491) and BrightGreen 2X qPCR Mastermix (Cat # Mastermix-S) was obtained from ABM (Richmond, BC, Canada). All antibodies were purchased from Cell Signaling (Danvers, MA), other than PGC-1α which was obtained from Millipore (Billerica, MA) and CPT1b that was bought from Bosterbio (Pleasanton, CA).

Complexation of EPA and DHA with BSA – DHA and EPA stock solutions (10 mM) were prepared after complexation of each fatty acid with fat-free BSA (12.5%, w/v) in α-MEM. Two separate solutions containing fat-free BSA either with EPA or DHA were incubated overnight in a shaking water bath at 50 ^{o}C. Subsequently, each solution was filtered and an aliquot was collected to measure fatty acid concentration using the Wako NEFA kit. α-tocopherol (40 μM) was added to the mixture to prevent lipid peroxidation and the final solution was split into aliquots to be stored at -20 ^{o}C.

Cell culture and treatment – Rat L6 skeletal muscle cells (American Type Culture Collection) were grown in α-MEM containing FBS (10%, v/v) and 1% (v/v) antibiotic-antimycotic solution. Cells were grown in a humidified environment that was maintained at 37°C and 5% CO_2 as previously described[127]. Cell growth was conducted in T75 flasks while maintaining confluence levels of approximately 80%. Cells for all treatment conditions were seeded onto a plate/dish at the same time and placed in the 37°C incubator. Wells that contain the 5-day treatment condition were treated first, then 2 days later the 3-day treatment condition was added, and two days later the 1-day treatment condition was performed. Thus, the cells were treated with either EPA, DHA or control media for 1, 3, or 5 days. The assays were conducted immediately after day 5 of treatment with all cells equally aged. Previous studies have shown that a 50 μM ω-3 fatty acid

concentration would maintain cell viability while still being representative of a serum ω-3 concentration which consequently informed our decision to use this concentration for our study[17,128–131].

Measurement of glucose uptake – Cells were seeded into 24-well plates, treated for 1, 3, and 5 days, and on the day of the experiment, cells were serum starved for 4h. This means that they were exposed to α-MEM containing 1% (v/v) antibiotic-antimycotic, but without FBS. Half of the cells were treated with 100 nM insulin for 30 min after which all the plates were removed from the 37°C incubator and washed with a Hepes-buffered saline (HBS) solution (20 mM Hepes-Na, 1 mM $CaCl_2$, 2.5 mM $MgSO_4$, 140 mM NaCl and 5 mM KCl, pH 7.4). As previously described, a solution containing 10 µM 2-deoxy-D-glucose and 0.5 µCi/ml of radiolabelled 2-deoxy-D-[^{3}H] glucose dissolved in HBS was given to the cells[132]. To account for non-specific background radioactivity, 200 µl of the existing radiolabelled solution containing 10 µl of cytochalasin B (10 µM) was given to one well in each plate. Cells were then incubated at room temperature for 5 min and then immediately placed on ice. The incubation media was quickly aspirated and then the cells were washed with cold 0.9% saline twice. A lysis solution (0.05 M NaOH) was then added to every well and the plate was placed on a shaker for 15 min to lyse the cells. The cell lysates that formed were placed in scintillation vials for radioactivity counting. A 10 µl aliquot of the lysate was used to determine protein concentrations using the Bradford assay.

Measurement of glucose and palmitate oxidation – Cells were seeded into 35x10 mm dishes, treated for 1, 3, and 5 days, and on the day of the experiment they were serum starved for 4h. All wells were then aspirated and given different solutions depending on the assay. To measure glucose oxidation, cells were given α-MEM containing 0.1 µCi/ml radiolabelled D-[^{14}C] glucose as previously described[133]. For palmitate oxidation, cells were given α-MEM containing 20 µM

palmitate as well as 0.15 µCi/ml radiolabelled [1-^{14}C] palmitic acid. For both assays, dishes were air-sealed using parafilm, while a piece of Whatman paper is attached on the inside and placed in the 37°C incubator for 1h. Afterwards, 200 µl of 4 M H_2SO_4 was applied to the cells and 100 µl of a phenylethylamine-methanol (1:1) mixture was applied to the Whatman paper. The dishes were then placed back into the 37°C incubator for 1h to collect $^{14}CO_2$ released by the cells. The Whatman papers were then carefully removed from the dish such that the papers did not touch the media. For both assays, the papers were then placed in scintillation vials for radioactivity counting.

Measurement of glycogen synthesis – Cells were seeded into 12-well plates, treated for 1, 3, and 5 days, and on the day of the experiment they were serum starved for 4 h. The wells were aspirated and given 0.2 µCi/mL of D-[^{14}C] glucose either with or without 100 nM insulin and were incubated (37°C) for 2 h. Subsequently, each plate was placed on ice and the wells were aspirated and washed with cold PBS. After aspirating every well, 450 µl of 1 M KOH was added to each well and the plates were placed on a shaker for 15 min. Afterwards, the contents of the wells were placed into microtubes and heated at 65°C for 30 min. One hundred µl of type-2 glycogen from oyster dissolved in water (25 mg/ml), 80 µl of saturated Na_2SO_4, and 1.2 ml of 100% cold ethanol were then added to each of the tubes containing cell lysates. Tubes were vortexed and stored at -20°C overnight. Tubes were then centrifuged (7000 rpm) for 20 min. The supernatant was discarded and the remaining pellet was resuspended in 500 µl of distilled water. Four hundred µl of this mixture was placed in scintillation vials to count for radioactivity.

Measurement of lactate production – Lactate production was measured using the Lactate Colorimetric Assay Kit according to the manufacturer's instructions. The cells were seeded into 6-well plates and treated for 1, 3, and 5 days. Phenol red media would interfere with the microplate's absorbance reading. For this reason, on the last day of media change, α-MEM media

without phenol red was used instead. On the day of the experiment, 0.5 ml of media extracts were collected into microtubes and stored at -80°C until ready for analysis. Each sample was deproteinized by passing them through 10 kDa filters. To measure lactate, a 96-well clear plate compatible with the spectrophotometer was used.

Assessment of cell viability – The CellTiter-Glo® Luminescent Cell Viability Assay was used to conduct the experiment. Cells were seeded into 96-well clear plates that were compatible with the spectrophotometer and were treated for 1, 3, and 5 days. On the last day of media change, 50 µl of α-MEM media without phenol red was used in each well instead. ATP quantity in media was determined according to the manufacturer's instructions. ATP media content is directly proportional to the number of metabolically active cells present.

Western Blotting Analysis – Cells were seeded into 6-well plates and treated for 1, 3, and 5 days. On the day of lysis, wells were aspirated and washed with PBS. A lysis buffer that contains 25 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 25 mM Tris-HCl, 10% glycerol, and 1% Triton was made. Protease and phosphatase inhibitors were added to the buffer and 100 µl of this buffer was added to each well. Cells were then further mechanically broken down using a 23-gauge needle and a 1 ml syringe. Samples were then diluted 1:1 (v/v) with 2x Laemmli sample buffer. Samples were subsequently loaded and run through a gel, transferred onto a PVDF membrane, probed against the primary (1:1000 dilution) and secondary (1:2000 dilution) antibody of choice, and then developed in a dark room using Immobilon HRP substrate. The primary antibodies used were as follows: P-AKT (60 kDa, Cat # 9271); AKT (60 kDa, Cat # 9272); P-GS (85-90 kDa, Cat # 3891); GS (84 kDa, Cat # 3886); P-GSK3α (51 kDa, Cat # 9327); GSK3α/β (51 and 46 kDa, Cat # 5676); PGC-1α (100 kDa, Cat # AB3242); P-AMPK (62 kDa, Cat # 2535); AMPK (62 kDa, Cat # 2532);

CPT1b (87 kDa, Cat # PB9491); β-actin (45 kDa, Cat # 4967) served as a loading control. The Scion Image program was used to perform densitometry analyses on all developed blots.

RNA isolation and quantitative PCR – Cells were seeded into 6-well plates and treated for 1, 3, and 5 days. The following day, the wells were aspirated and washed with PBS. The TRIzol™ protocol for cells was followed to extract RNA. cDNA was synthesized with 2 µg of RNA using the EasyScript™ cDNA Synthesis Kit. cDNA synthesis was completed at 50°C for 50 min and then the reaction was stopped by heating the samples at 85°C for 5 min. Using a 96-well plate, 4 µl of cDNA, 0.4 µl of the primer of choice, 10 µl of EvaGreen qPCR Mastermix, and 5.6 µl of RNase-free water was placed into each well. The plate was then centrifuged at 2500 rpm for 5 min at 4°C and amplified according to the following conditions: 95°C (10 min); 40 cycles of 95°C (15 s), 60°C (60 s). All genes were normalized to TBP. The ΔΔCt method was used to determine the difference in gene expression between groups[134]. The primers used were as follows: TBP forward (TAC AGG TGG CAG CAT GAA GTG ACA) and reverse (AAC CAA CAA TCA CCA GCA GCA GTG); FAS forward (AAG GCA TCA CCA TAG CTA CAG CCT) and reverse (TAT GCT TCT CAC AGT GGC CAC ACA); CD40 forward (AGA TTA TCC CGG TCA CAA CAC) and reverse (CTG AGA TGC GAC TCT CTT TAC C); IL6r forward (TGG CAA CCT TAG TGC TCA TT) and reverse (TGT CTG CTC CAG CTT GTT AC); NF-κB forward (TCC AGC TGC TAT TGG ATT ACA C) and reverse (GGG ACT GCG ATA CCT TAA TGA C); TLR4 forward (ACC TAA GGA GAG GAG GCT AAG) and reverse (GGT AAC TGC AGC ACA CTA CA) and TNFR2 forward (CCA TGC TCA CAG ATT CCA CA) and reverse (GAC CTA ACA AGT CTG TCC CAA G).

Statistical analyses – The GraphPad Prism statistical software was used for statistical analyses. One- and two-way ANOVA with Bonferroni post-hoc tests were used to compare differences between conditions. Statistical significance was set at $p < 0.05$.

Results

Effects of DHA and EPA on glucose uptake, glycogen synthesis, glucose oxidation, and lactate production – Under basal conditions with DHA treatment, glucose uptake remained unchanged (Figure 1A), unlike EPA treatment which led to a 39% increase in glucose uptake exclusively after 5 days (Figure 1B). With insulin stimulation, DHA resulted in an 18% reduction in glucose uptake only after 3 days, whereas EPA treatment led to no change for all time points. DHA and EPA treatment produced no change in basal glycogen synthesis (Figure 1 C, D respectively). Insulin-stimulated glycogen synthesis increased by 19% and 27% only after 1 day of DHA and EPA treatment respectively. The effect of insulin on glucose uptake and glycogen synthesis was maintained for all time points for DHA and EPA. DHA increased glucose oxidation by 70%, 70%, and 54% after 1, 3, and 5 days of treatment, respectively (Figure 1E). On the other hand, EPA's influence was blunted with a 58% increase in glucose oxidation that was only maintained for 1 day of treatment (Figure 1F). To assess alternative fates of glucose, lactate production in L6 myoblasts was measured. With DHA treatment, there was no change in lactate production (Figure 1G). On the other hand, EPA has shown to increase lactate production across all time points with significance being reached only after three and five days of treatment with 18% and 26% increases respectively (Figure 1H).

Effects of DHA and EPA on insulin signaling – In order to evaluate the impact of ω-3 fatty acids on the insulin signalling pathway, western blotting was conducted to assess phosphorylated and total AKT, GSK3α, and GS proteins. DHA treatment significantly increased insulin-stimulated phosphorylation of AKT after 5 days by 51% and remained unaffected under basal conditions (Figure 2A). EPA exposure led to no change in either basal or insulin-stimulated AKT phosphorylation (Figure 2D). After 1 day of DHA treatment, insulin-stimulated GSK3α phosphorylation was elevated by 92%, whereas no change was reported under basal conditions (Figure 2B). EPA caused no alteration in GSK3α phosphorylation with and without insulin (Figure 2E). DHA and EPA had no effect on GS phosphorylation under basal and insulin-stimulated conditions (Figure 2 C, F respectively). Lastly, the effect of insulin was maintained for AKT and GSK3α phosphorylation, but not for GS.

Effects of DHA and EPA on palmitate oxidation, AMPK phosphorylation and PGC-1α and CPT1b content – DHA increased palmitate oxidation by 81%, 55%, and 64% after 1, 3, and 5 days of treatment respectively (Figure 3A). In contrast to DHA, EPA led to a 69% increase in palmitate oxidation that lasted for only 1 day (Figure 3B). To unravel the molecular mechanisms that are implicated in these functional changes, western blotting was done with L6 skeletal muscle cells. DHA exposure led to a uniform 6-fold, 7.8-fold, and 5.2-fold increases in the phosphorylation of AMPK with 1, 3, and 5 days of treatment respectively (Figure 3C). The content of CPT1b increased by 2.1-fold after both 1 and 5 days of DHA treatment (Figure 3D). On the other hand, EPA treatment showed no effect on AMPK phosphorylation (Figure 3F) and CPT1b content (Figure 3G). The content of PGC-1α was augmented by 5.9-fold, 5.3-fold, and 5.2-fold after 1, 3, and 5 days of DHA treatment respectively (Figure 3E). Following 1 day of EPA treatment, PGC-1α content increased by 2.3-fold (Figure 3H).

Effects of DHA and EPA on the mRNA expressions of IL-6R, TNFαR, CD40, TLR4, FAS, and NF-κB – To assess the anti-inflammatory role that ω-3 fatty acids can have directly on L6 skeletal muscle cells, PCR was done on several inflammatory genes. There was no effect of DHA on the mRNA expression of IL-6R (Figure 4A), whereas EPA led to a 39% reduction that lasted for 1 day (Figure 4B). TNFαR mRNA expression was decreased by 50%, 50%, and 55% after 1, 3, and 5 days of DHA treatment respectively (Figure 4C). However, EPA resulted in a 53% reduction in TNFαR mRNA expression that was maintained for 1 day (Figure 4D). CD40 mRNA expression was decreased by 42%, 59%, and 49% after 1, 3, and 5 days of DHA treatment respectively (Figure 4E). On the contrary, CD40 was decreased by 56% when L6 cells were treated with EPA exclusively with 1 day of treatment. (Figure 4F). TLR4 mRNA expression decreased only after 1 and 5 days of DHA treatment by 21% and 33% respectively (Figure 5A). Conversely, EPA treatment reduced TLR4 mRNA expression after 1, 3, and 5 days by 45%, 39%, and 43% respectively (Figure 5B). FAS mRNA expression decreased after three and five days of DHA treatment by 53% and 51% respectively (Figure 5C). On the other hand, EPA produced a 60% decrease in the mRNA expression of FAS, lasting for only 1 day (figure 5D). DHA treatment decreased the mRNA expression of NF-κB by 57%, 42%, and 47% after 1, 3, and 5 days respectively (Figure 5E), as opposed to EPA that led to no change (Figure 5F).

Effects of DHA and EPA on cell viability – The concentration of ATP in media was measured to assess the cell viability of a time-course ω-3 fatty acid treatment. Exposure of DHA (Figure 6A) and EPA (Figure 6B) to L6 cells have led to no change in ATP production.

Discussion

Herein, we provide evidence for the differential effects of EPA and DHA on glucose and fat metabolism, as well as their ability to reduce the expression of inflammatory genes in skeletal muscle cells. It has previously been shown that human rhabdomyosarcoma cells treated with ω-3 fatty acids for 24 hours and 48 hours can lead to an upregulation of PGC1-α and GLUT4 mRNA expression as well as increased mitochondrial staining and basal metabolic rate. However, this study used a combined ω-3 treatment which prevented an in-depth analysis of their differential effects[19]. By studying the ω-3 fatty acids independently, we found that DHA consistently increased the rates of glucose and palmitate oxidation, whereas EPA increased lactate production and only transiently elevated palmitate oxidation in skeletal muscle cells. Furthermore, DHA promoted AMPK phosphorylation and increased PGC1-α and CPT1b contents, which are compatible with enhanced oxidative capacity in skeletal muscle cells. These findings are in agreement with previous studies demonstrating that the administration of ω-3 fatty acids to HF-fed mice reversed hepatic insulin resistance in wild-type, but not in AMPKα2 knockout mice[135]. Thus, it appears that AMPK and its downstream proteins are important targets for DHA in both skeletal muscle and liver. Previous studies have also demonstrated that treatment of C6 glioma cells with EPA led to the upregulation of transcription factors responsible for mitochondrial biogenesis such as TFAM, PGC1α, and NRF1[136]. Indeed, our study found an increased content of PGC-1α in L6 cells treated with EPA. Together, our findings suggest that EPA and DHA operate through PGC-1α-mediated enhancement of skeletal muscle mitochondrial oxidative capacity.

It has been reported that obese and type-2 diabetic patients have fewer mitochondria with impaired bioenergetic capacity in their skeletal muscles[137]. This makes it particularly challenging for these subjects to reverse the damaging effects of lipid accumulation and inflammation within skeletal muscle tissue. The whole-body insulin-sensitizing effect of ω-3 fatty acids have been

attributed to a variety of factors including enhanced oxidative capabilities of adipose[138], hepatic[139], and muscle tissue[10], as well as promoting effective cross-talk between these peripheral tissues[140,141]. Our study provides additional support for the powerful and direct impact that DHA, and to some extent, EPA, have on skeletal muscle cell oxidative metabolism[19,142]. We have also found that EPA and DHA's effects were not mediated through alterations in the insulin signaling pathway. Interestingly, Aas et al. found that glucose uptake and oxidation as well as GLUT1 mRNA expression was increased in human myotubes; however, this study used a supraphysiological EPA concentration of 0.6 mM for 1 day[21]. On the contrary, our study explored a physiological 50µM concentration and a treatment time of up to 5 days that is more representative of long term ω-3 fatty acid consumption. We found that neither AKT, GSK3α, and GS phosphorylation nor glucose uptake and glycogen synthesis were affected in L6 myoblasts exposed to either DHA or EPA. Indeed, ω-3 fatty acids have been shown to reverse insulin resistance in Goto-Kakizaki diabetic rats[143] and in palmitate-induced insulin resistant C2C12 myotubes[144]. Thus, it is possible that DHA and EPA can improve insulin signaling under pathological conditions (*e.g.* type 2 diabetes), but not in intact normal-functioning skeletal muscle cells, such as the ones used in this study. Importantly, ATP production did not change with the administration of DHA and EPA and cell viability was preserved under all conditions. This indicates that the lack of an effect on insulin signalling was not due to a potential toxic effect of these fatty acids on skeletal muscles cells.

As mentioned previously, proinflammatory mediators can cause dysregulation of glucose and fat metabolism and can lead to tissue-specific insulin resistance. Indeed, individuals with type-2 diabetes or glucose intolerance have higher rates of IL-6, TLR4, and NF-κB expression in skeletal muscle as compared to healthy controls[145]. Rats that were fed a high fructose diet had

higher levels of TNF-α within skeletal muscle which triggered mitochondrial impairment and insulin resistance[146]. Additionally, when TLR is activated within skeletal muscle it leads to the translocation of NF-κB to the nucleus and the transcription of inflammatory genes such as TNF-α and IL-6[147]. Besides being activated by bacterial agonists as part of the innate immune response, polyunsaturated fatty acids can also serve as ligands for TLR4[148]. Most cell culture studies that explored the anti-inflammatory effects of ω-3 fatty acids have done so using an inflammation-induced model. For instance, when C2 mouse skeletal myoblasts were treated with 50 µM EPA for 72 hours, the detrimental effects of palmitate and TNF-α on cell viability and differentiation were mitigated[128]. DHA treatment of C2C12 myotubes has also been shown to attenuate the production of palmitate-induced pro-inflammatory cytokines such as MCP-1 and IL-6[149]. Although EPA and DHA's anti-inflammatory effects are evident, there is limited evidence to support the notion that these effects could be maintained under non-inflammatory conditions. In a separate study, C2C12 myotubes that were treated with either EPA or DHA at varying concentrations (400 µM – 600 µM) showed decreased IκBα phosphorylation and therefore reduced NF-κB translocation to the nucleus[23]. Although this is indicative of an anti-inflammatory effect, this study used non-physiological ω-3 fatty acid concentrations. Thus, in our study, we found the mRNA expression of key inflammatory genes such as NF-κB, CD40, TNF-αR, TLR4, and FAS to be significantly downregulated with 50 µM DHA treatment. On the other hand, 50 µM EPA treatment resulted in reduced expression in IL-6, CD40, TNF-α, TLR4, and FAS, but not in NF-κB. Evidently, these ω-3 fatty acids exert a powerful anti-inflammatory effect on L6 skeletal muscle cells, which may be of value for conditions that are characterized by chronic inflammation such as in obesity.

With respect to the ability of skeletal muscle cells to respond to inflammatory mediators, our gene expression data also indicates that EPA had a milder anti-inflammatory effect than did DHA. When treated with EPA, muscle mRNA expression of IL-6, CD40, TNF-α, and FAS was diminished for only 1 day and NF-κB remained entirely unaffected. On the other hand, DHA induced long-term downregulation of the mRNA expression of NF-κB, CD40, TNF-α, TLR4, and FAS. The distinct effects that DHA and EPA exert on substrate metabolism and the expression of inflammatory genes in L6 muscle cells can be at least partially attributed to DHA having a greater degree of unsaturation relative to EPA, which can potentially facilitate its utilization by peripheral tissues. In fact, as compared to EPA and other polyunsaturated fatty acids, DHA has shown higher levels of incorporation into skeletal and cardiac muscle membranes[150]. A greater degree of unsaturation within the phospholipid bilayer can also result in greater membrane fluidity among cells treated with DHA. These differences can directly impact ω-3 fatty acid bioavailability and can explain the higher relative potency of DHA on L6 myoblast metabolism. Therefore, on a molecular level, EPA, and especially DHA, exert their effects on L6 muscle cells by downregulating inflammatory pathways as well as directly upregulating proteins involved in oxidative metabolism. The increased rate of substrate utilization that is provoked mainly by DHA could lead to the decreased accumulation of harmful intramuscular lipid intermediates, which may be of therapeutic value against the development of skeletal muscle and whole-body insulin resistance.

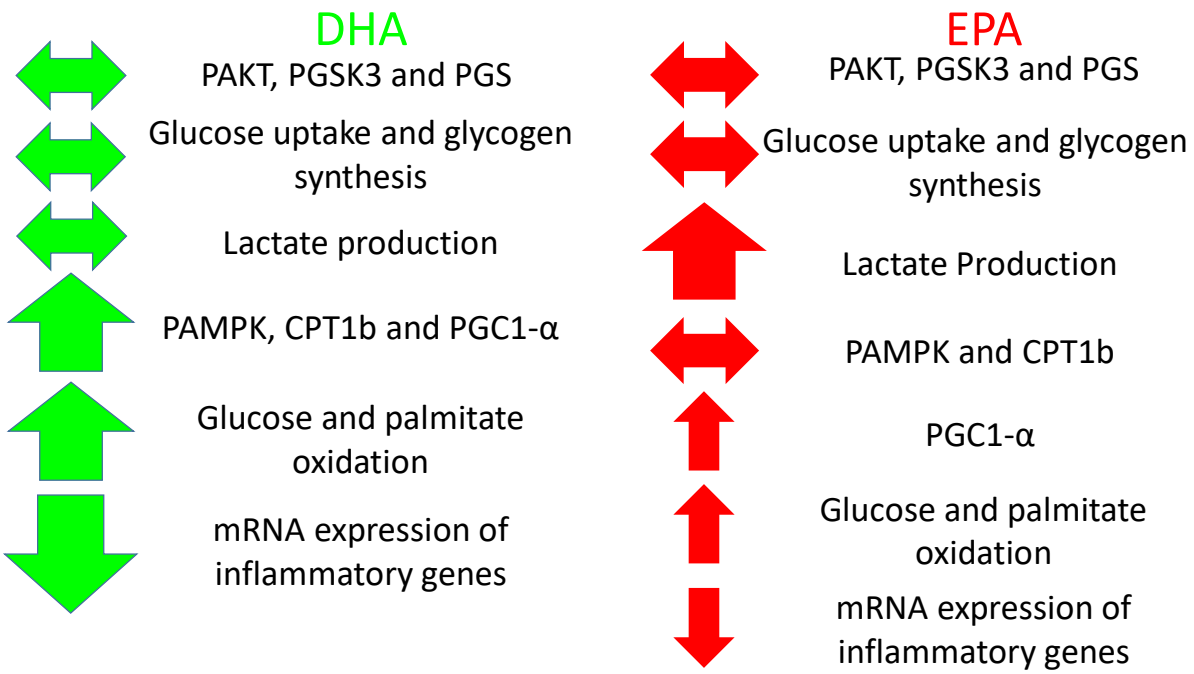

Figure 4: Summary of findings. Phosphorylation of AKT, GSK, and GS as well as glucose uptake and glycogen synthesis showed no changes with EPA and DHA. Lactate production was unchanged with DHA, but showed a sustained increase with EPA treatment. PAMPK, CPT1b, and PGC1-α are all upregulated with DHA treatment which reflects the sustained increase in glucose and palmitate oxidation. On the contrary, there was no change in PAMPK and CPT1b, but a transient increase in PGC1-α lasting for 1 day which reflected the blunted glucose and palmitate oxidation with EPA treatment. DHA led to a long-term reduction in the mRNA of inflammatory genes, whereas EPA had a short-term reduction.

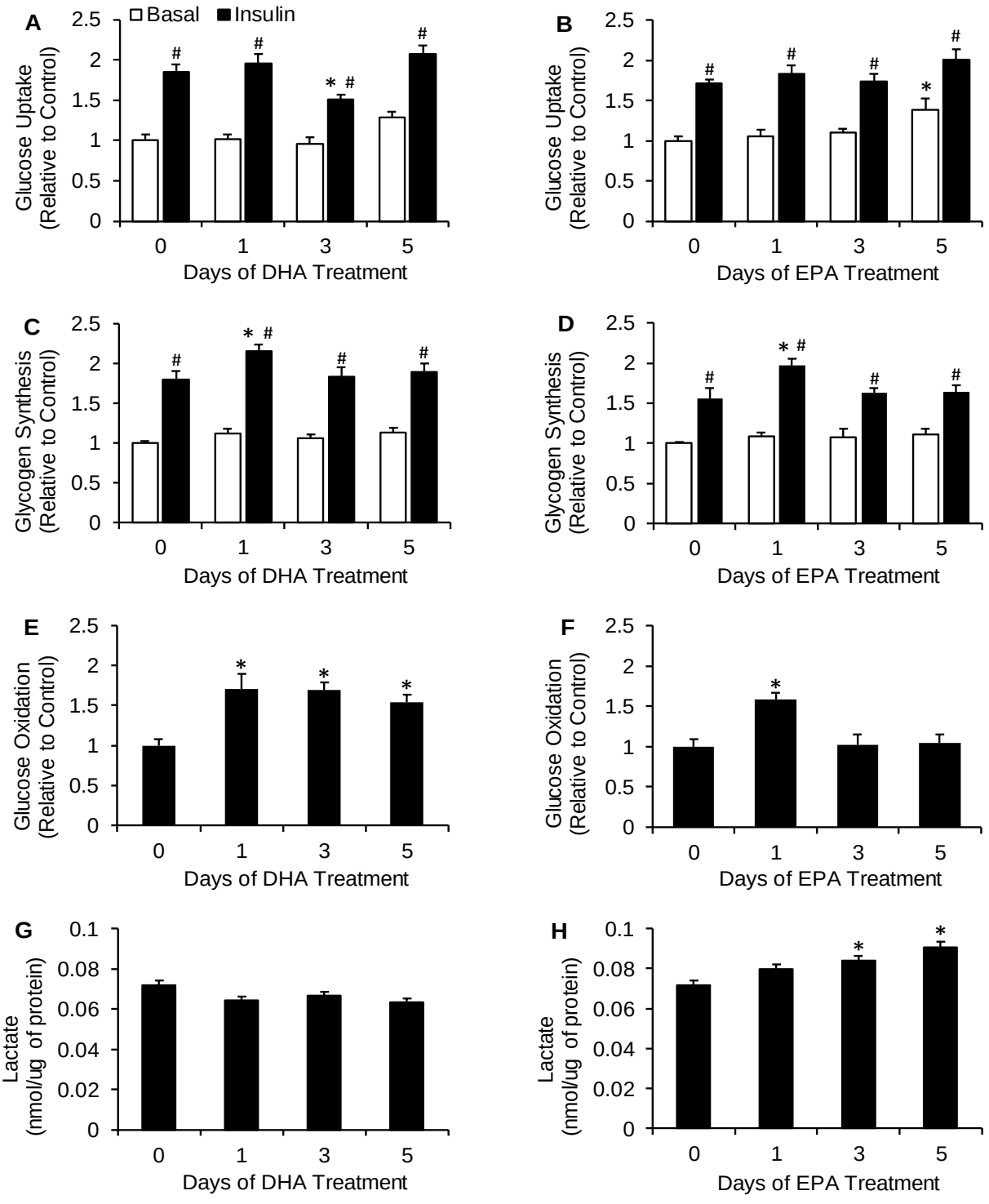

Figure 1: Effects of DHA and EPA on glucose metabolism in L6 skeletal muscle cells. After treating cells with EPA, DHA, or control for 1, 3, and 5 days, glucose uptake (A and B) and glycogen synthesis (C and D) was measured and showed no change under basal and insulin stimulated conditions. Glucose oxidation increased with 1, 3, and 5 days of DHA treatment (E), but only increased with 1 day of EPA treatment (F). Lactate media content was found to increase with EPA (H), but not DHA (G). *P<0.05 vs. 0 days of treatment, #P<0.05 vs. basal. Two-way and one-way analysis of variance (ANOVAs), n=6-12.

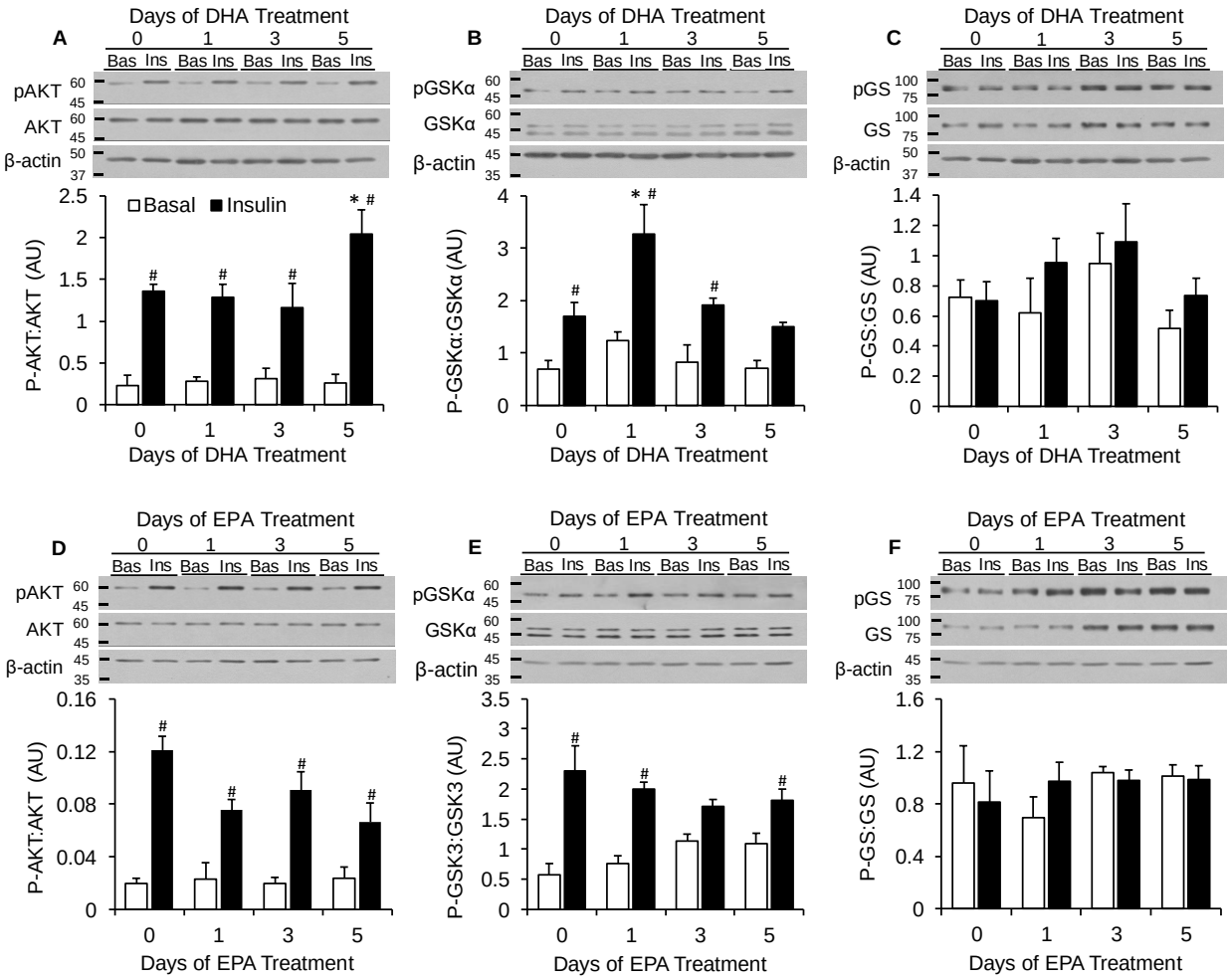

Figure 2: Effects of DHA and EPA on insulin signalling in L6 skeletal muscle cells. Phosphorylation and contents of AKT (A and D), GSKα (B and E), and GS (C and F) with DHA and EPA treatment. *P<0.05 vs. 0 days of treatment, #P<0.05 vs. basal. Two-way ANOVA, n=4-6.

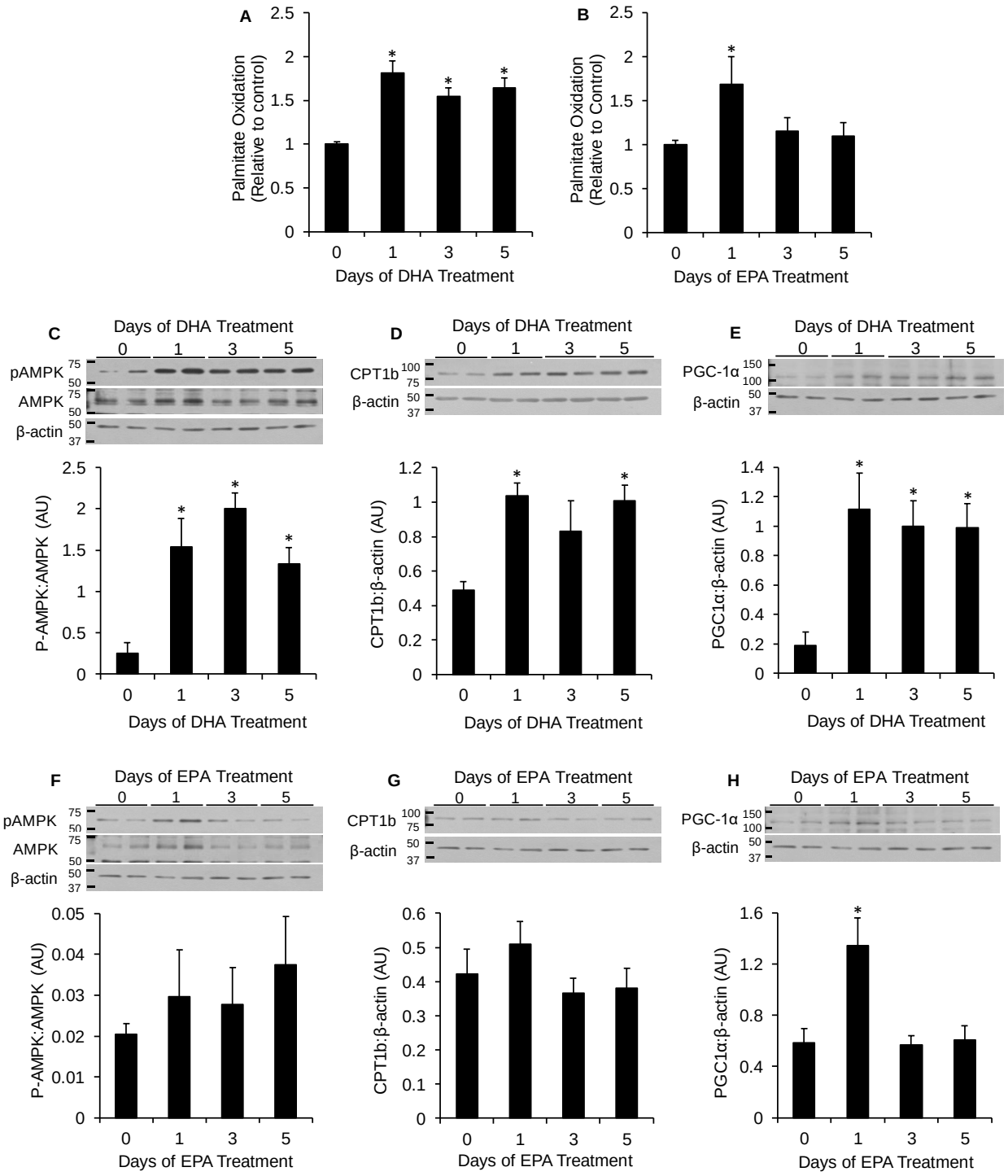

Figure 3: Effects of DHA and EPA on palmitate oxidation, AMPK phosphorylation, and contents of CPT1b and PGC1α in L6 skeletal muscle cells. Palmitate oxidation increased with 1, 3, and 5 days of DHA treatment (A), but only with 1 day of EPA treatment (B). AMPK phosphorylation (C) and CPT1b (D) and PGC1α (E) contents also increased under all time points with DHA treatment. EPA treatment led to no change in AMPK phosphorylation (F) and CPT1b content (G) and an increase in PGC1α content after 1 day (H). *P<0.05 vs. 0 days of treatment. One-way ANOVA, n=4-6.

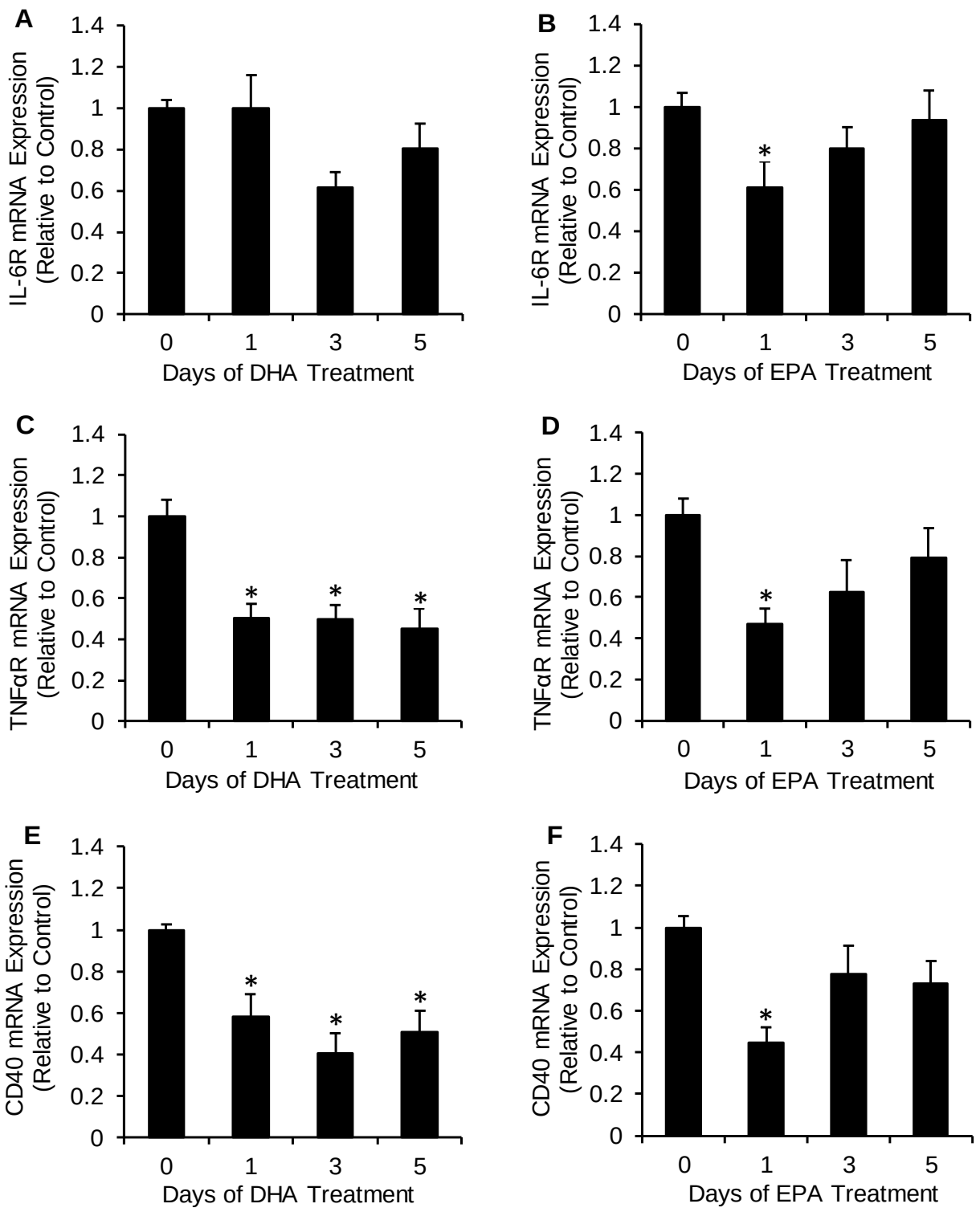

Figure 4: Effects of DHA and EPA on mRNA expressions of IL-6R, TNFαR, and CD40 in L6 skeletal muscle cells. IL-6R mRNA expression decreased with 1 day of EPA treatment (B), but no change occurred with DHA treatment (A). TNFαR (C) and CD40 (E) mRNA expression decreased with 1, 3, and 5 days of DHA treatment, however, EPA treatment led a decrease only after 1 day (D and F). *P<0.05 vs. 0 days of treatment. One-way ANOVA, n=4-6.

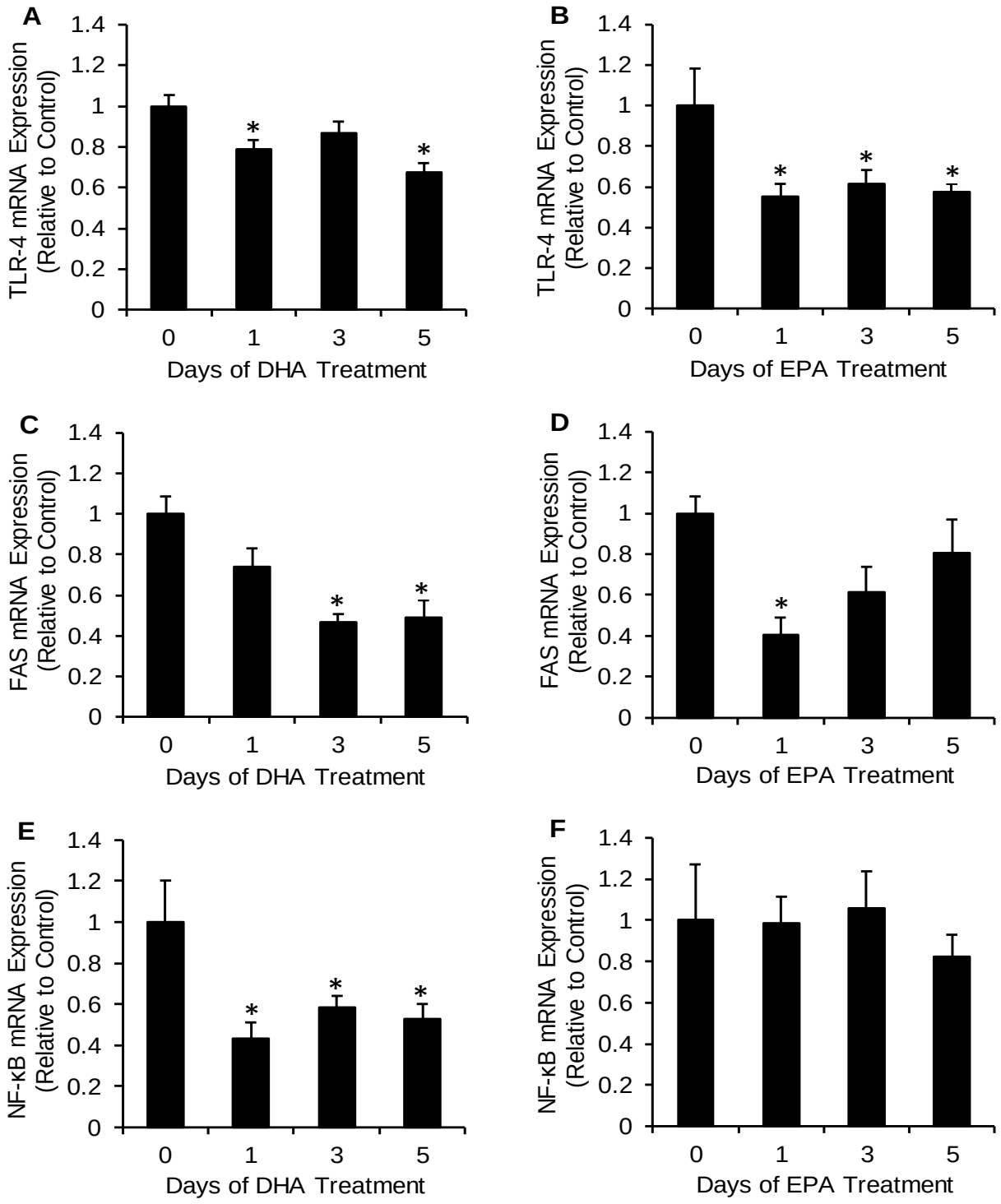

Figure 5: Effects of DHA and EPA on mRNA expressions of TLR-4, FAS, and NF-κB in L6 skeletal muscle cells. TLR-4 mRNA expression decreased with 1 and 5 days of DHA treatment (A) and 1, 3, and 5 days of EPA treatment (B). DHA treatment reduced mRNA expression of FAS after 3 and 5 days (C), but EPA only did so after 1 day (D). NF-κB mRNA expression decreased under all time points with DHA treatment (E), and had no change with EPA treatment (F). *P<0.05 vs. 0 days of treatment. One-way ANOVA, n=4-6.

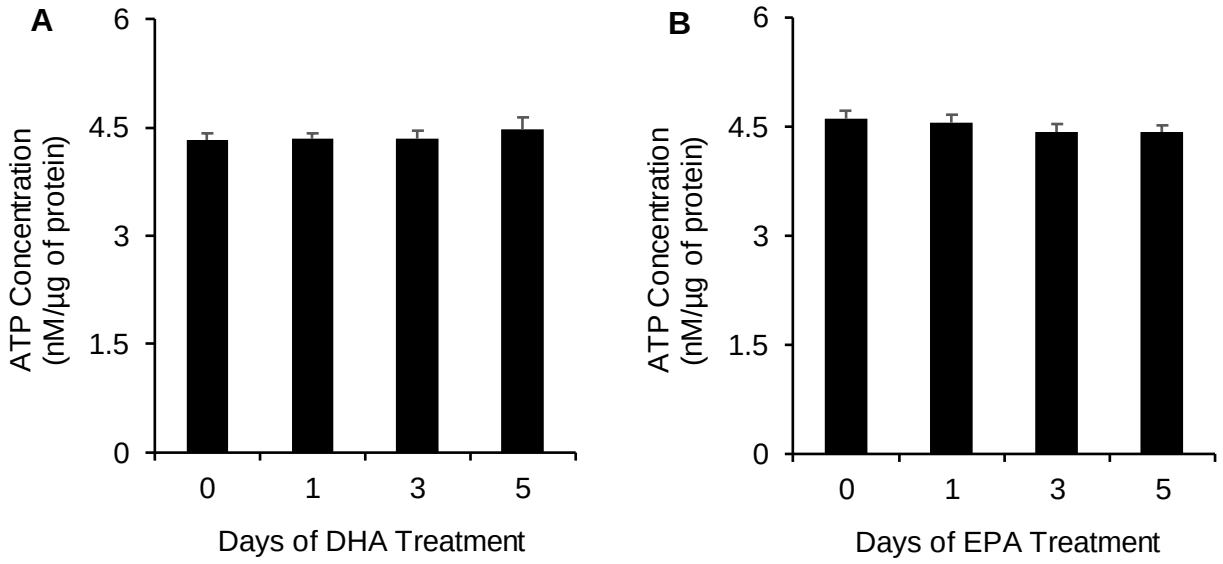

Figure 6: ATP concentration in media of L6 skeletal muscle cells treated with DHA and EPA. *P<0.05 vs. 0 days of treatment. One-way ANOVAs, n=6.

Conclusion and future directions

In our study, we show that besides exerting anti-inflammatory effects, DHA and EPA directly regulated glucose and fat metabolism in L6 skeletal muscle cells. This was demonstrated through increased glucose and palmitate oxidation as well as upregulation of proteins that are involved in these processes. We also observed that, relative to EPA, DHA had a more potent anti-inflammatory effect and was better able to upregulate skeletal muscle energy metabolism. Thus, by directly enhancing glucose and fat oxidation, DHA may increase glucose disposal and protect skeletal muscle cells against lipotoxicity.

By testing DHA and EPA separately, we were able to identify their differential effects. However, as mentioned previously, in nature, polyunsaturated fatty acids do not exist in isolation, but rather in a mixed form. For this reason, future experiments should address whether combining EPA and DHA together would produce an additive effect or if they interact in an alternative manner. To expand this experiment, another treatment condition should include a mixture that combines EPA, DHA, and an ω-6 fatty acid, such as arachidonic acid. There is extensive literature that recommends a dietary ω-6: ω-3 ratio of 1:1. It would be important to understand whether ω-3 fatty acids can effectively mitigate the proinflammatory and insulin resistant states that are associated with ω-6 fatty acids.

Another important direction that requires further exploration is DHA's potential capability of restoring muscle energy metabolism dysfunction. To accomplish this, insulin resistant skeletal muscle cells should be treated with DHA and subsequently assessed for insulin sensitivity, glucose uptake, and glucose oxidation. Alternatively, a combination DHA + saturated fat treatment can be implemented to determine whether DHA can prevent the onset of saturated fat-induced lipotoxicity. Therefore, future experiments should focus on pairing ω-3 fatty acids

with other fats to identify how they interact with one another in the context of skeletal muscle energy metabolism.

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Appendix
Appendix A: Detailed experimental methods

Determination of FFA concentration using Wako HR series NEFA-HR kit

1. Dissolve 5.8mg of reagent A for every 1ml of solvent A.
2. Dissolve 10.4 mg of reagent B for every 1ml of solvent B.
3. Pipette sample and standard into cuvettes.
4. Add 0.5 mL of the prepared solution A into each cuvette and incubate for 5 min in 37˚C water bath.
5. Remove cuvettes from water bath, add 0.25 ml of solution B into each cuvette, and incubate for 5 min in 37˚C water bath.

Cuvette #	**Name**	**1mEq/L NEFA Standard**	**H_2O**
1	Blank	0	12.5 µl
2	Low standard	6.25 µl	6.25 µl
3	Standard	12.5 µl	0
4	Sample	12.5 µl	0

6. Read the absorbance at 550 nm
7. NEFA concentration is calculated as follows: Cs = As/AStd*1 (mEq/L)
    a. Cs = sample concentration, As = absorbance of 245 sample at 550 nm, AStd = absorbance of standard solution at 550 nm. mEq/L = mmol/L.

Western blotting

Western blot buffers:

10X Running Buffer- pH 8.3:

- 144 g Glycine
- 10 g SDS
- 30.34 g Tris base
- 1 L ddH2O
- Store at room temperature

1X Running Buffer- pH 8.3:

- 200 ml 10X running buffer
- 1800 ml ddH2O
- Store at room temperature

10X Transfer Buffer- pH 8.3:

- 30.3 g Tris base
- 144 g Glycine
- 1 L ddH2O
- Store at 4°C

1X Transfer Buffer- pH 8.3:

- 150 ml 10X transfer buffer
- 300 ml Methanol
- 1050 ml ddH2O
- Store at -20°C

10X Wash Buffer:

- 60.57 g Tris base
- 87.66 g NaCl
- 1 L ddH2O
- Store at 4°C

1X Wash Buffer:

- 400 ml 10X wash buffer
- 3600 ml ddH2O
- Add 500 μL/L each of Tween-20 and NP-40
- Mix solutions and store at 4°C

Blocking Buffer:

- 3% BSA (3 g/100 ml) in 1X wash buffer
- Store at 4°C

Antibody Buffer:

- 1°antibody:
    - Usually 1:1000 (v/v) dilution of 1°antibody in blocking buffer
    - 0.02% (v/v) sodium azide.

- 2°antibody:
    - Usually 1:2000 (v/v) dilution of 2°antibody in blocking buffer

Resolving Gel Tris Buffer- 1.5 M, pH 8.8:

- 90.86 g Tris base
- 500 ml ddH2O
- Store at 4°C

Stacking Gel Tris Buffer- 0.5 M, pH 6.8:

- 30.3 g Tris base
- 500 mL ddH2O
- Store at 4°C

10% SDS Solution:

- 2 g SDS
- 20 ml ddH2O
- Store at room temperature

10% APS Solution:

- 1 g APS
- 10 mL ddH2O
- Store at -20°C

Lysis Buffer:

- 25 mM Tris-HCl
- 25 mM NaCl-pH 7.4
- 1 mM MgCl2
- 2.7 mM KCl
- 1% Triton-X
- 10% glycerol
- Aliquot and store at -20°C
- Add protease and phosphatase inhibitors prior to use

2X Laemmli Sample Buffer:

- 950 µl Laemmli sample buffer
- 50 µl βmercaptoethanol
- Dilute sample 1:1 with sample buffer, incubate at 95°C for 5 min.

Preparation of Cell Lysates:

1. Seed cells into 6-well plates prior to the start of the treatment.
2. On the day of lysis, aspirate cells and wash with PBS
3. 150 µl of lysis buffer is to be added to each well and further broken down mechanically with a 23-gauge needle and a 1 mL syringe.
4. Aliquot each sample for protein determination using Bradford method. Aliquot remainder and store at -80°C.

Western Blotting Protocol:

Prepare 10% resolving and 4% stacking gels using the recipe below:

Component	Quantity (2 resolving gels- 10%)	Quantity (2 stacking gels- 4%)
ddH_2O	6.91 ml	3.92 ml
30% Acrylamide- 37:5:1	5.67 ml	0.87 ml
Tris-HCL- 1.5 M, pH 8.8	4.25 ml	0 ml
Tris-HCL- 0.5 M, pH 6.8	0 ml	1.63 ml
10% SDS	0.17 ml	65 µl
TEMED	20 µl	10 µl
10% APS	100 µl	50 µl

Running gel:

1. Remove samples from -80°C freezer and briefly centrifuge them
2. Place gels into tanks and fill tanks with 1X running buffer
3. Remove the green combs and pipette 6 µl of protein ladder into the very left-most well
4. Pipette samples into the appropriate wells
5. Close the container and turn on the voltage 110 V for approximately 2 h

Transferring to membrane:

1. Have 1X transfer buffer read and in -20°C.
2. Fill a large dish with cold transfer buffer
3. Soak equal-sized PVDF membranes in methanol for 1 min
4. Have equal-sized filter papers foam pads ready
5. Take the gels out of the tank and soak in transfer buffer and remove the glass plates
6. Place the black side of the cassette down in the dish with transfer buffer Place a foam pad, 2 filter paper squares, followed by the gel, PVDF membrane, 2 more filter papers, and another foam pad. Close the cassette and place into transfer tank.
7. Fill the tank up with 1X transfer buffer and put an ice pack into the transfer tank to maintain cold water temperature. Surround the transfer tank with ice to prevent overheating
8. Turn on the voltage to 120 V for 2 h or 20 V for 18 h

Probing the membrane:

1. Place the membranes in containers that have 6 ml blocking buffer and leave on shaker for at least 1 h
2. Replace blocking buffer with 1° antibody and leave on shaker for 2 h at room temperature or at 4°C overnight
3. Replace the 1° antibody with 1X wash buffer and have it shaking at room temperature for 50 minutes, replacing the wash buffer every 10 min
4. Remove the wash buffer and add the 2° antibody for 1 h at room temperature
5. Replace 2° antibody with the 1X wash buffer and repeat the washing procedure

Developing the membrane:

1. Remove the wash buffer and incubate the membrane in 3 mL of chemiluminescence Millipore Luminata Forte Western HRP Substrate at room temperature for 2 min, making sure to shake the containers.
2. Place the membranes inside the developing cassette.
3. In the dark room, expose a piece of film to the membrane inside the cassette
4. When ready, place film in developer solution, followed by a brief soak in water and then fixer solution to terminate the reaction.

Quantified Polymerase Chain Reaction (qPCR)

RNA Isolation

1. Seed cells into 6-well plates prior to the start of the treatment.
2. On the day of lysis, aspirate wells and wash with PBS.
3. Add 1 ml of Trizol™ reagent per well, pipette up and down to homogenize the samples.
4. Place the samples into 2 ml microtubes and incubate at room temperature for 5 min.
5. Add 0.2 ml of chloroform and vortex briefly.
6. Incubate the sample at room temperature for 2-3 min.
7. Centrifuge the samples at 13,000 rpm for 15 min. at 4°C.
8. The sample will separate into a lower red phenol-chloroform phase, an interphase and a colourless upper phase. Transfer the aqueous upper phase into a new tube.
9. Add 0.5 ml of 100% isopropanol to the aqueous phase and mix samples.
10. Allow samples to incubate at room temperature for 10 min. and then centrifuge the samples at 13,000 rpm for 20 min. at 4°C.
11. Discard the supernatant from the tube and wash the remaining pellet with 1 ml of 75% ethanol. Centrifuge the tube at 13,000 rpm for 20 min. at 4°C. Discard the ethanol.
12. Air-dry the RNA pellet for 5-10 min.
13. Resuspend the RNA pellet in RNAse-free water.
14. Incubate RNA on heat block for 10 min at 60°C and quantify yield using the NanoDrop™ machine. Store samples at -80°C.

cDNA Synthesis

1. Using the ABM EasyScript™ Reverse Transcriptase kit, combine 2 µg RNA with 1 µl of 10 µM random primers and 1 µl of 10 mM dNTP mix.
2. Incubate the mixture at 65°C for 5 min. and then place on ice for 1 min.
3. Add 4 µl of 5X RT buffer, 0.5 µl of RNAseOFF Ribonuclease inhibitor, and 1µl of EasyScript™ RTase to sample.
4. Mix components and centrifuge briefly.
5. Incubate the samples at 25°C for 10 min, 50°C for 50 min. and 85°C for 5 min.
6. Store the cDNA at -20°C.

Real-Time qPCR

1. Using an RNAse-free 96-well PCR plate, combine 4 µl cDNA, 0.4 µl primer solution, 10 µl ABM EvaGreen qPCR mastermix, and 5.6 µl of RNase-free water into each well.
2. Centrifuge the plate at 2500 rpm for 5 min. at 4°C.
3. Complete real-time PCR analysis using the following amplification conditions: 95°C (10 min); 40 cycles of 95°C (15 s), 60°C (60 s).
4. Use TBP as a control gene to normalize the genes you are testing.
5. Use the ΔΔCt method in order to determine relative differences in gene expression between treatment groups.

Lactate colorimetric assay

Lactate content in media was measured using the Lactate Colorimetric Assay Kit from BioVision:

1. Seed cells into 6-well plates prior to the start of the treatment.
2. On last day of media change, α-MEM media without phenol red is to be used instead (phenol red media would interfere with the microplate's absorbance reading)
3. On day of experiment, extract 0.5ml of media from each well, place in liquid nitrogen, and then store at -80°C for future use.
4. When ready, remove samples from freezer, and thaw on ice. Samples are to be deproteinized by centrifuging them through 10 kDa filters for 15 min at 4°C
5. To measure lactate, a 96-well clear plate that is compatible with the spectrophotometer is required.
6. To create standard curve: 10 µl of 100nmol/µl lactate standard must be added to 990 µl of lactate assay buffer. 0, 2, 4, 6, 8, and 10 µl of this standard solution should be placed into the 96-well plate and the volume adjust the volume to 50 µl with lactate assay buffer. To each standard well, add a reaction mix that contains 46 µl of lactate assay buffer, 2 µl of lactate enzyme mix, and 2 µl of probe.
7. Add 2.5 µl of sample into separate wells and as with the standards, adjust the volume to 50 µl and then add the reaction mix.
8. Incubate the plate at room temperature for 30 min. shielded from light.
9. Read the absorbance of the samples at 570nm.

Cell viability

ATP content in media was measured using the CellTiter-Glo® Luminescent Cell Viability Assay:

1. Seed cells into 96-well plates prior to the start of the treatment.
2. On last day of media change, 50 µM α-MEM media without phenol red is to be used instead (phenol red media would interfere with the microplate's absorbance reading).
3. Incubate at room temperature for 30 min.
4. Transfer 10 ml of CellTiter-Glo® buffer into the CellTiter-Glo® substrate to create a reaction mix
5. To create a standard curve, prepare 0.01, 0.1, 0.25, 0.5, and 1 µM of ATP in α-MEM media without phenol red and load 50 µl into each well.
6. Add 50 µl of the reaction mix to sample and standard wells
7. Place on a shaker for 5 min. and incubate at room temperature for 10 min.
8. Measure luminescence of the samples. (ATP media content is directly proportional to the number of metabolically active cells present).