Pharmacological and genetic approaches to modulate energy metabolism in skeletal muscle cells

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Table of contents

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List of publications

Paper I

Skagen C, Nyman TA, Peng XR, O'Mahony G, Kase ET, Rustan AC, Thoresen GH.

Chronic treatment with terbutaline increases glucose and oleic acid oxidation and protein synthesis in cultured human myotubes

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Paper II

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Expression and metabolic functions of the thermally activated transient receptor potential channels TRPA1 and TRPM8 in human myotubes

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Paper III

Løvsletten NG, Vu H*, **Skagen C***, Lund J, Kase ET, Thoresen GH, Zammit VA, Rustan AC.

Treatment of human skeletal muscle cells with inhibitors of diacylglycerol acyltransferases 1 and 2 to explore isozyme-specific roles on lipid metabolism

*These authors contributed equally to this work

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Paper IV

Skagen C, Nyman TA, Janovska P, Horakova O, Kopecky J, Rustan AC, Thoresen GH.

Loss of AMPKα2 increases substrate oxidation, but decreases relative maximal oxidative capacity and incorporation of exogenous fatty acids into lipids in cultured myotubes

Manuscript

Abbreviations

ACBP	Acyl-CoA binding protein
ACC	Acetyl-CoA carboxylase
ACL	ATP citrate lyase
ACSL	Acyl-CoA synthetase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	Amp-activated protein kinase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
β-AR	Beta adrenergic receptor
BAT	Brown adipose tissue
BMI	Body Mass Index
Ca^{2+}	Calcium
CAMKKβ	Ca^{2+}/Calmodulin-dependent protein kinase kinase β
cAMP	Cyclic adenosine monophosphate
CGI-58	Comparative gene identification 58
CoA	CoA
CPT	Carnitine palmitoyltransferase
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
ETC	Electron transport chain
FA	Fatty acid
FABPc/FABPpm	cytoplasmic/plasma membrane-associated fatty acid binding protein
FASN	Fatty acid synthase
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FATP	Fatty acid transporter protein
FFA	Free fatty acid
FCCP	Carbonyl cyanide p-trifluoromethoxyphenylhydrazone
G052	G0/G1 switch genes 2
G-6-P	Glucose-6-phosphate
GLUT	Glucose transporter
GPCR	G protein-coupled receptor
GS	Glycogen synthase

GSV	Glucose transporter storing vesicles
HK	Hexokinase
HSL	Hormone sensitive lipase
IMTG	Intramuscular triacylglycerol
IPA	Ingenuity pathway analysis
IRS	Insulin receptor substrate
KO	Knockout
LD	Lipid droplet
LKB1	Liver kinase B1
MAG	Monoacylglycerol
MGAT	Monoacylglycerol acyltransferase
MGL	Monoacylglycerol lipase
MHC	Myosin heavy chain
mTOR	Mammalian target of rapamycin
MYH	Myosin heavy chain, gene
NADH	Nicotinamide adenine dinucleotide
OA	Oleic acid
P13-kinase	Phosphatidylinositol 3-kinase
PDC	Pyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase kinase 4
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKA	Protein kinase A
PKB/Akt	Protein kinase B
PL	Phospholipid
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
SPA	Scintillation proximity assay
T2D	Type 2 Diabetes
TAG	Triacylgycerol
TCA	Tricarboxylic acid
TRP	Transient receptor potential
UCP	Uncoupling protein
WHO	World health organisation

Abstract

Over the last decades, the prevalence of obesity has nearly tripled. In 2016, it was estimated that approximately 39% of the world's adult population were overweight, while 13% were obese. With a rapidly increasing prevalence, there is also a rapid increase in the number of individuals suffering from metabolic disorders, such as type 2 diabetes (T2D) and cardiovascular disease. Lifestyle factors such as changes in diet and increased physical activity has shown to be both preventive and therapeutically beneficial for these metabolic disorders. However, it has shown to be difficult for the affected population to make these changes. Skeletal muscle, which constitutes 40% of total body mass, is the main site for the metabolism of glucose and lipids. At rest, the breakdown of lipids is the primary source of energy, but during insulin-stimulated conditions, glucose is the preferred energy source. Therefore, skeletal muscle has to be able to switch rapidly between lipid and glucose metabolism, making it one of the main regulators of energy metabolism. However, in the setting of insulin resistance this ability of metabolic switching is impaired. Approximately 55-60% of all free fatty acid taken up by skeletal muscle cells end up being stored as triacylglycerol (TAG) in lipid droplets. Lipid droplets are dynamic organelles whose dysregulation have shown to be an important problem when it comes to the alterations in skeletal muscle lipid metabolism seen in subjects with obesity and T2D. The four studies presented in this thesis aimed to investigate how different pharmacological and genetic approaches can modulate energy metabolism in skeletal muscle cells.

The metabolic effects of different pharmacological agonists were investigated in cultured human myotubes. It was found that activating the β_2 adrenergic receptor (β_2-AR) and the cold-sensing transient receptor potential (TRP)M8 had beneficial effects on energy metabolism. Myotubes treated with the β_2-AR agonist terbutaline had an increased glucose uptake, glucose and fatty acid oxidation, an upregulation of mitochondrial and oxidative pathways and an increased protein synthesis. Activation of TRPM8 by ligustilide resulted in an increased glucose uptake and oxidation, which was likely to be the result of an increased calcium flux into myotubes.

TAG dynamics were studied in myotubes by using selective inhibitors of diacylglycerol acyltransferase (DGAT)1 and DGAT2, the two isozymes that catalyzes the final step of TAG synthesis. We found that the two distinct enzymes exhibited different roles in skeletal muscle lipid metabolism. Whereas DGAT1 was shown as the main enzyme responsible for the re-synthesis of TAG, DGAT2 mainly contributes to *de novo* synthesis of TAG. Interestingly, it

was found that ablation of the α2 subunit of adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) resulted in altered TAG dynamics. Similar to what was found in human myotubes treated with a selective DGAT1 inhibitor, myotubes from *AMPKα2*$^{-/-}$ mice had a reduced accumulation and incorporation of exogenous lipids into diacylglycerol (DAG) and TAG. In addition, *AMPKα2*$^{-/-}$ myotubes exhibited a higher basal substrate oxidation compared to *AMPKα2*$^{+/+}$ cells, which was likely the result of an upregulation in oxidative pathways, but *AMPKα2*$^{-/-}$ myotubes also had a reduced relative maximal mitochondrial capacity and metabolic flexibility.

In conclusion, the results presented in this thesis has contributed to the new knowledge about the effects of pharmacological and genetic approaches on skeletal muscle energy metabolism. Cultured human myotubes established from satellite cells have shown to be a useful model for studying the biochemical and molecular effects of pharmacological agents, whereas myotubes generated from knockout animals have shown to be useful for investigating the role of different regulatory enzymes in skeletal muscle energy metabolism. Some of the effects described in this thesis, such as increased substrate oxidation, reduced lipid accumulation, increased protein synthesis and improved mitochondrial function, are desirable in the treatment of T2D and other metabolic disorders.

Introduction

Obesity, metabolic syndrome and type 2 diabetes

Over the last decades, the prevalence of obesity has rapidly increased. According to world health organization (WHO), the worldwide obesity has nearly tripled since the mid-1970s [1]. In 2016, it was estimated that approximately 39% (1.9 billion) of the world's adult population were overweight, while 13% (650 million) were obese [1].

Obesity is classified by Body Mass Index (BMI), which is calculated as the weight in kilograms divided by the square of height in meters. The WHO defines a BMI equal or more than 25 kg/m^2 as overweight, while a BMI equal or more than 30 kg/m^2 is classified as obese [1]. In most cases, obesity is caused by an imbalance between energy intake and expenditure as a result of excessive food intake, sedentary lifestyle and physical inactivity, although both stress and genetics are considered as contributing factors. Moreover, in the recent years the gut microbiota has shown to influence metabolic processes and can therefore be considered an environmental factor [2]. An increase in BMI puts mechanical stress on the body, which causes an increased risk of joint and musculoskeletal pain [3]. In addition, mechanical stress can cause inflammation, which can result in an increased risk of chronic pain [3]. Obesity is also associated with a cluster of risk factors called metabolic syndrome. The risk factors include high blood glucose, abnormal triglyceride and cholesterol levels, high blood pressure and excess fat around the waist [3]. The criteria for the diagnosis of metabolic syndrome is a collection of unhealthy body measurements and abnormal laboratory test results, such as glucose intolerance, hypertension, atherogenic dyslipidaemia, and proinflammation [4]. Someone suffering from metabolic syndrome are prone to developing cardiovascular disease, type 2 diabetes (T2D) and stroke [3]. The onset of metabolic syndrome gives a 5-fold increase in the risk of developing T2D, 2-fold risk in developing cardiovascular disease, 3- to 4-fold risk of myocardial infarct, and 2- to 4-fold increased risk of stroke [5, 6].

Although there are many risk factors and metabolic diseases associated with obesity, approximately 10-25% of obese individuals are metabolically healthy and have a reduced risk of cardiovascular events and all-cause mortality [7, 8]. However, the location of body fat is more important than over-all adiposity, as visceral fat and ectopic lipid accumulation in the liver and skeletal muscle are associated with disease development [9-11]. The increased storage of ectopic fat has been shown to contribute to insulin resistance in peripheral tissue [12, 13], whereas excessive visceral fat is associated with an increased fatty acid (FA) release

and raised plasma levels of free fatty acids (FFAs) [14, 15], which results in lipotoxicity [14]. Moreover, adipose tissue also functions as an endocrine organ by secreting bioactive peptides, known as adipokines, which plays an important role in dynamic cross-talk between metabolic organs [16]. However, in individuals living with obesity, the adipose tissue is prone to inflammation. Therefore, the combination of infiltrated macrophages and the production of inflammatory adipokines and/or cytokines can lead to a chronic low-level inflammation [16, 17]. In addition, the imbalance between the secretion of pro- and anti-inflammatory adipokines is suggested to contribute to the induction of insulin resistance. Despite obesity being an important clinical health burden and strongly associated with metabolic syndrome, cardiovascular disease and T2D [3, 5, 6]. The complications of these diseases are preventable and may be delayed by lifestyle interventions and pharmaceutical agents [18].

Insulin resistance is a condition that develops when cells become less sensitive to insulin leading to increased glucose production from the liver and reduced glucose uptake in skeletal muscle and adipose tissue, resulting in hyperglycemia [19, 20]. T2D is a chronic metabolic disease characterized by chronic hyperglycemia, reduced insulin production and insulin resistance [14, 21], which ultimately affects how the body utilizes energy. Multiple factors are reported to contribute to the onset of T2D, including insulin resistance in several organs (liver, skeletal muscle and adipose tissue), increased gluconeogenesis in the liver, pancreatic β-cell dysfunction, increased lipolysis from adipose tissue [14, 21], and accumulation of intramyocellular tricylglycerol (IMTG) in skeletal muscle [22-24]. It is estimated that approximately 420 million people worldwide are living with diabetes, and the majority of these have T2D [25]. Moreover, the international diabetes federation estimated that 4.3 million global deaths were related to diabetes in 2019 [26]. This T2D “epidemic” is caused by a mixture of both genetic and epigenetic predisposing factors, as well as environmental and behavioural risk factors [27]. However, the majority of people living with T2D are classified as being obese or overweight [28]. In Norway, approximately 230.000 people are living with a diabetes diagnosis, however, taken into account that many are living with undiagnosed diabetes, the real number is around 300.000 – 400.000 [29]. It has been reported that diabetes management is costing the Norwegian society, annually, billions of NOK [30]. It is also important to note that T2D is a major cause of blindness, lower limb amputations, heart attacks, kidney failure and stroke. With a rising number of cases, it has become important to develop more effective, specific, and cost-effective medication.

The role of skeletal muscle in obesity and type 2 diabetes

Skeletal muscle, which makes up about 40-50% of total body mass, is the largest organ in the human body and an important contributor to whole-body energy homeostasis [31]. Skeletal muscle accounts for more than 80% of insulin-stimulated glucose disposal, making it quantitatively the most important organ for insulin resistance, followed by the liver and adipose tissue [32-34]. Metabolic disturbances in skeletal muscle, such as decreased glucose uptake (as a result of insulin resistance), mitochondrial dysfunction, metabolic inflexibility (further discussed under "**Metabolic flexibility in skeletal muscle**") and dysregulation of lipid metabolism, has been associated with obesity and T2D [35-37]. Out of these, insulin resistance is considered the primary impairment as it leads to pancreatic β-cell dysfunction, hyperglycaemia and ultimately T2D [38]. Moreover, as mentioned previously, lipids stored as IMTG in skeletal muscle fibres are also associated with obesity and the development of T2D [39]. Insulin-resistant individuals have increased levels of IMTG within their muscles [40], however, this is also seen in insulin sensitive endurance-trained athletes, a phenomenon called the "athletes paradox" [40, 41]. IMTG is an essential source of fuel for skeletal muscle during physical activity, and exercise has shown to increase IMTG level, which positively correlates with insulin sensitivity and FA oxidation function [40, 42]. Therefore, the balance between storage and efficient utilization of IMTG (also known as lipid turnover) can potentially aid in the understanding of dysregulated glucose and lipid metabolism in skeletal muscle [40, 42, 43]. Due to the critical role skeletal muscle has in regulating whole-body energy homeostasis, it is an organ of particular interest when it comes to further understand metabolic conditions, such as obesity and T2D.

Energy metabolism in skeletal muscle

Skeletal muscle is a major contributor to basal metabolic rate, which can through several mechanisms increase energy metabolism [44]. It is the main tissue involved in the oxidation of lipids and glucose. Skeletal muscle accounts for 30% of metabolic rate at rest, and is the main site for the metabolism of lipids. During maximal exercise, the metabolic rate of skeletal muscle increases, as it is responsible for transforming chemical energy to mechanical work [45]. Moreover, it is the principal site for glucose disposal under insulin-stimulated conditions (80%) and the largest glycogen-storing organ [46]. Skeletal muscle has a remarkable flexibility in substrate oxidation. Thus, during the postprandial state, glucose dominates

substrate oxidation [47], while during fasting and prolonged exercise, fatty acid oxidation is increased. Therefore, skeletal muscle has to be able to switch rapidly between lipid and glucose metabolism [48]. However, in the setting of insulin resistance this ability is impaired.

During rest, glucose uptake is the rate-limiting step for the utilization of this substrate [49]. Glucose in skeletal muscle cells has three faiths; to be oxidized in the mitochondria for ATP production, stored as glycogen or become a precursor for lipid synthesis. Glucose uptake is facilitated by a group of membrane proteins, known as glucose transporters (GLUT). In skeletal muscle, glucose uptake is mainly mediated by GLUT1 and GLUT4 [50], but several other isoforms are expressed including the fructose transporter GLUT5 [51]. GLUT1 is responsible for basal glucose uptake in skeletal muscle [52], while GLUT4 increases glucose uptake in response to several stimuli [53]. In response to muscle contraction and insulin stimulation GLUT4 is translocated to the cell surface from intracellular vesicles, resulting in an increased glucose uptake [54-56]. The insulin receptor tyrosine kinase is activated by the binding and stimulation of insulin, which leads to the phosphorylation of several substrate proteins aiding in the recruitment and activation of phosphatidylinositol 3-kinase (PI3-kinase) [57]. P13-kinase triggers the phosphorylation and activation of protein kinase B (PKB/Akt), which is essential for the translocation of GLUT4 to the cell surface [58]. After glucose has been transported across the cell membrane by GLUT4, glucose is phosphorylated by hexokinase (HK) to glucose-6-phosphate (G-6-P), where it proceeds to enter glycolysis in order to generate pyruvate, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) (**Figure 1**) [58]. Moreover, glycogen synthase can convert G-6-P into glycogen for storage [58]. In some cases, such as excessive energy supply and limited ability to store glycogen, excess glucose is converted to lipids through lipogenesis [59]. Pyruvate, both from stored glycogen and plasma glucose, can by the pyruvate dehydrogenase complex (PDC) be decarboxylated in the mitochondria to acetyl coenzyme A (acetyl-CoA) [60].

FFAs from the circulation are taken up into skeletal muscle by passive diffusion or by protein-mediated transport. The major proteins mediating FA uptake in skeletal muscle are FA translocase (CD36), FA transport proteins (FATP1-6), and plasma membrane-associated FA-binding protein (FABPpm) [61, 62]. The quantitatively most important proteins are CD36 and FATP4 [63]. Once the FAs have entered the cell they are reversible bound to cytoplasmic FABP, which protects the FAs from lipotoxic accumulation and chaperones the imported FAs through cellular compartments (**Figure 1**) [64]. Acyl-CoA synthetase (ACL) mediates a reaction that activates the transported FAs to be broken down and converted into acyl-CoA

[65]. Acyl-CoA-binding protein (ACBP) transports acyl-CoA to the mitochondria, where it can either be oxidized for ATP production, esterified to monoacylglycerol (MAG) and diacylglycerol (DAG) to be stored as triacylglycerol (TAG) in lipid droplets (LDs), or be incorporated into phospholipids (PLs) to be used in cellular membranes or as lipid second messengers [66, 67]. The fate of the imported FA are dependent on many factors, including the concentration and type of incoming FAs, muscle fibre type, hormonal milieu, and energy demand of the muscle [64]. Acyl-CoA is transported into the mitochondria by carnitine palmitoyltransferase (CPT) 1 and 2, which is located on the outer and inner mitochondrial membrane, respectively [68]. CD36 is also found on the outer mitochondrial membrane, and appear to play a role in the regulation of mitochondrial FA transport [69, 70]. Once inside the mitochondrial matrix, acyl-CoAs are metabolized through three major metabolic pathways; β-oxidation, tricarboxylic acid (TCA) cycle and electron transport chain (ETC). β-oxidation metabolizes acyl-CoA to acetyl-CoA, a process which is regulated by CPT1 and acetyl-CoA carboxylase (ACC) via the intermediate malonyl-CoA (derived from acetyl-CoA from the glycolytic pathway) [68]. Acetyl-CoA enters the TCA cycle, where it is further metabolized to NADH, which is passed on to the ETC for ATP production [64, 71]. For long, the regulation of FA oxidation has been attributed to transport of FAs across the mitochondrial membranes and reduced inhibition of CPT1 by malonyl-CoA [68]. However, it is now believed that the regulation of skeletal muscle FA oxidation is a much more complicated process involving several regulatory sites. These regulatory sites include; FA transport across the cell membrane, binding and transport of FAs in the cytoplasm, formation and degradation of lipid droplets, FA transport across mitochondrial membranes, and potential regulators within β-oxidation, TCA cycle and ETC [72-74].

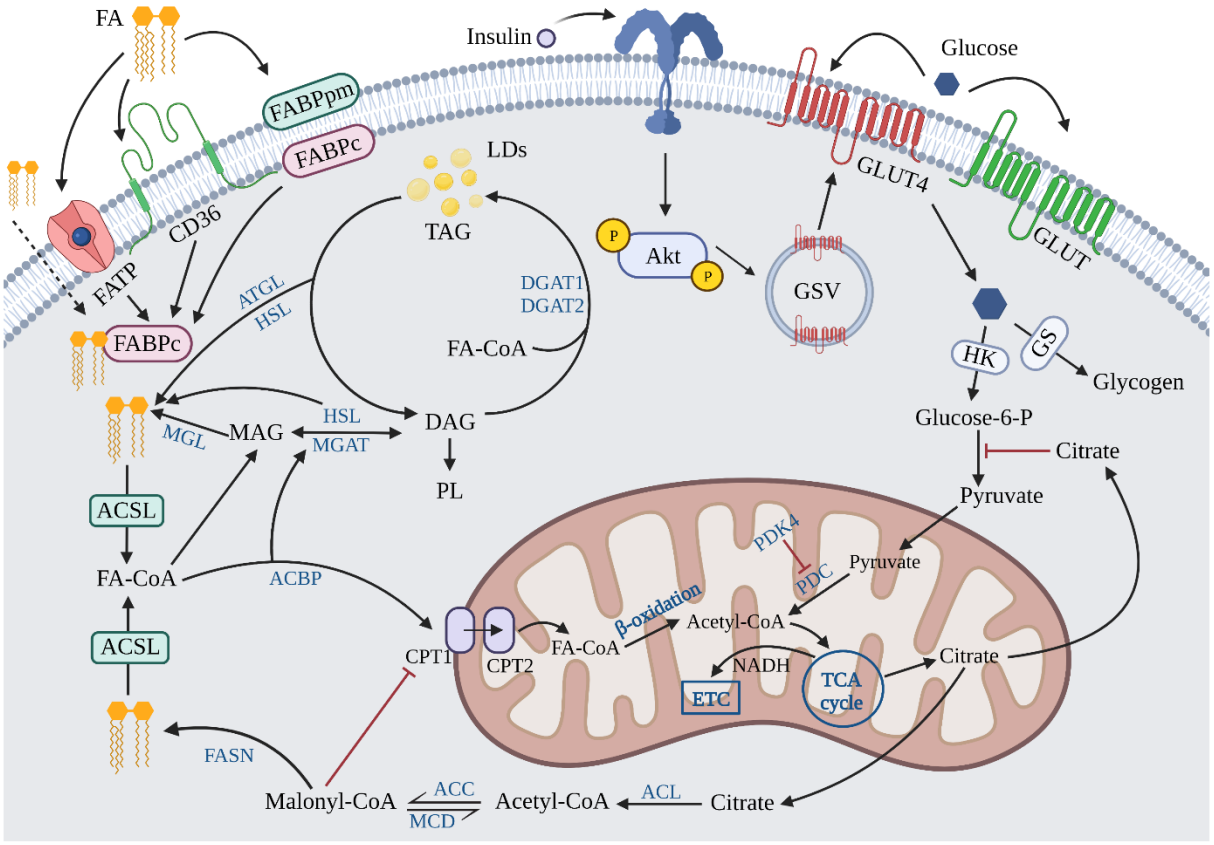


Figure 1: Energy metabolism in skeletal muscle. Glucose transport into the cell is facilitated by glucose transporters (GLUT). Once inside the cell, the glucose is either stored as glycogen or go through glycolysis in order to produce pyruvate. Following activation of the insulin signalling pathway, GLUT4 is translocated from intracellular vesicles, also known as glucose transporter storing vesicles (GSV), to the cell membrane. Fatty acids (FA) are taken up by three different transporter proteins; fatty acid transporter protein (FATP), fatty acid translocase (CD36) and plasma membrane associated fatty acid binding protein (FABPpm). The newly transported fatty acids bind to cytosolic cytoplasmic FABP (FABPc), which is activated by acyl-CoA synthetase (ACSL) to form FA-coenzyme A (CoA) before being transported intracellularly by acyl-CoA binding protein (ACBP). When there is an excessive energy supply, FA-CoA may be incorporated into complex lipids as phospholipids (PL), diacylglycerol (DAG) and triacylglycerol (TAG). These complex lipids are then being congregated and stored into lipid droplets (LDs) by the action of monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase 1 and 2 (DGAT1 and DGAT 2). When there is an energy demands, DAG and TAG are hydrolysed by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), and MAG is hydrolysed by MGAT. The resulting FAs from TAG, DAG and MAG are activated by ACSL to form FA-CoA, which can be used as fuel for energy. FA-CoA, from both exogenous and endogenous FAs, are transported into the mitochondria by carnitine palmitoyltransferase (CPT) 1 and 2. Inside the mitochondria, FA-CoA is metabolized through β-oxidation to generate acetyl-CoA which then enters the tricarboxylic acid (TCA) cycle. The pyruvate generated from glucose metabolism also enters the mitochondria where it is decarboxylated by pyruvate dehydrogenase complex (PDC) into acetyl-CoA which then enters the TCA cycle. In the TCA cycle, acetyl-CoA is metabolized to NADH, which is then passed on to the electron transport chain (ETC) for ATP production. Citrate, an intermediate from the TCA cycle, can "escape" the TCA cycle to be converted to acetyl-CoA by ATP citrate lyase (ACL), and thereafter to malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA can be conversed back to acetyl-CoA by malonyl-CoA

decarboxylase (MCD). Malonyl-CoA can further be converted to FAs by fatty acid synthase (FASN). Further, malonyl-CoA is a potent inhibitor of CPT1, and can thereby inhibit the entry and oxidation of FAs. FAs can supress glucose oxidation by inhibiting PDC by the action of pyruvate dehydrogenase kinase 4 (PDK4) and acetyl-CoA. Glycogen synthase (GS); hexokinase (HK), protein kinase B (AKT).

Metabolic flexibility in skeletal muscle

Skeletal muscle adapts to two very different physiological conditions; reduced energy intake during fasting and increased energy expenditure during exercise. Therefore, the energy demands of skeletal muscle cells are quite variable. As previously described, both carbohydrates and FAs are used as fuel by skeletal muscle cells. In the fed state, the increased availability of plasma glucose stimulates glucose oxidation and FA synthesis [47, 75], whereas the oxidation of FA is increased during fasting and sustained exercise, but shifts to glucose metabolism when exercise intensity increases [76, 77]. The ability to switch from FAs to glucose as the primary source of fuel is termed metabolic flexibility and is an important feature of healthy skeletal muscle [78, 79]. The inhibition of glucose oxidation by FAs is termed the "Randle cycle" [80], and is mediated by the inhibition of several glycolytic steps. Pyruvate dehydrogenase kinase isozyme 4 (PDK4) inhibits PDC by phosphorylation, which results in switching fuel source from glucose to FA (**Figure 1**) [81]. The increased citrate production from the enhanced FA oxidation escapes the mitochondria and inhibits the rate-limiting enzyme of glycolysis, 6-phosphofructo-l-kinase [82]. This leads to an increase in G-6-P, which inhibits HK, resulting in a reduced glucose uptake and oxidation [82]. The opposite situation, where glucose suppresses FA oxidation, [83], is referred to as the "reverse Randle cycle" [84]. Citrate, which has escaped from the oxidation of glucose, is transported back to the cytosol, where it is converted by ATP citrate lyase (ACL) to acetyl-CoA [83]. Acetyl-CoA is further converted to malonyl-CoA by ACC [83]. As previously described, malonyl-CoA inhibits CPT1, thereby blocking the entry and oxidation of FAs in the mitochondria [83, 85]. Thus, citrate plays an important role in the signalling during both fasted and fed state.

Loss of the ability to easily switch between glucose and FA as fuel is termed metabolic inflexibility [79], and is associated with a reduced lipid oxidation resulting in accumulation of lipids in skeletal muscle [86]. This accumulation can interfere with insulin function and signalling [86]. For instance, numerous studies has shown an association between DAG accumulation and the degree of insulin resistance [86].

Obesity, insulin resistance and T2D are associated with reduced FA oxidation during fasting and impaired postprandial switch from FA to glucose oxidation [87]. This inflexibility is also reported in individuals with impaired glucose tolerance [35]. Thus, suggesting that metabolic inflexibility plays a role in the development of T2D. Interestingly, it has been observed that cultured myotubes established from subjects with T2D and/or obesity have a reduced capacity to oxidize FAs compared to lean subjects [88-90]. Since metabolic flexibility of substrate oxidation is preserved when cells are grown in culture, metabolic flexibility might be an intrinsic property of skeletal muscle [91]. *In vitro* metabolic flexibility can be studied by measuring the ability of the cells to reduce FA oxidation when there is an acute addition of glucose, as well as the ability of the cells to reduce glucose oxidation when there is an acute addition of FAs. Moreover, metabolic inflexibility are likely to be due both intrinsic and extrinsic factors, but the molecular mechanisms remains to be established.

Skeletal muscle fibre types

Skeletal muscle is a complex heterogeneous tissue composed of different muscle fibre types with different contractile and metabolic properties [92, 93]. The metabolic capacity of the muscle fibres are dependent on the type of fibre and level of stimulation. The flexibility of skeletal muscle is based on the heterogeneity of the muscle fibres, which allows them to be used for various tasks; from continuous low-intensity activity, to repeated sub-maximal contraction, to fast and strong maximal contractions [94]. Muscle fibre types are metabolically, structurally and functionally different, and are classified into different phenotypes based on their speed of contraction (ATPase activity) and the aerobic/anaerobic production of ATP. Muscle fibre types are defined as either being slow- or fast-twitch, which is based on the their contractile property and the histochemical staining for myosin ATPase as type 1 (slow-twitch) and type II (fast-twitch) [94]. The slow-twitch type I fibres are associated with a higher mitochondrial content [95], and a higher expression of GLUT4 protein compared to fast-twitch type II fibres [96, 97]. Type I fibres are mainly dependent on oxidative (aerobic) pathways for ATP production, while type IIx fibres are dependent glycolytic (anaerobic) pathways [98]. Type IIa fibres display an intermediate phenotype [98]. Human skeletal muscle expressed three isoforms of myosin heavy chain (MHC): MHCβ, MHC2A and MHC2X, which are encoded by the MYH7, MYH2 and MYH1 genes, respectively [98]. MHCβ fibres are classified as type I muscle fibres, which are characterized as slow, oxidative and fatigue resistant, while MHC2A fibres are characterized as fast

oxidative type IIa muscle fibres, and MHC2X fibres are classified as fast glycolytic type IIx fibres [99]. Rodent skeletal muscle expresses the same fibre types as human skeletal muscle, but in addition expresses the type IIb fibre type, which is encoded by the MYH4 gene [98]. The type IIb fibres resembles IIx fibres as they are both fast-twitch glycolytic fibres, but IIb fibres has a lower mitochondrial content compared to both type IIa and IIx fibres [98, 100]. Interestingly, skeletal muscles with a higher composition of type I fibres are associated with an increased insulin responsiveness [101]. Individuals with insulin resistance and/or T2D exhibits a distinct muscle phenotype with decreased amounts of type I fibres [102, 103], and reduced expression of GLUT4 within the type I fibres [104]. However, one study performed on mice showed an improved glucose homeostasis and insulin action when the fibre composition in muscle was altered towards type IIb fibres in obese mice [105]. Furthermore, is important to note that muscle phenotype is also highly influenced by exercise, and muscle cells change their fibre type and enzymatic properties in response to altered functional demands [106]. The mechanisms involved in fibre type switching are complex and not fully understood, but the transcription factor myocyte enhancer factor 2 [107], and its target gene, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [108], has shown to be involved in regulation of the slow fibre type program.

Dynamics of skeletal muscle lipid pools

Skeletal muscle is subjected to various mechanical and energetic demands, which requires a continuous turnover of lipid stores that can serve as energy depots [109]. These lipid stores, also called lipid droplets (LDs), are not only a source of energy, but has shown to be dynamic organelles which plays a role in intracellular signalling and lipid transfer [43]. It has been shown that approximately 50-60% of the fatty acids taken up by skeletal muscle cells are incorporated and stored as TAG in LDs, these TAG containing LDs are usually referred to as IMTG [110]. However, LDs do not only contain TAG, but also DAG, free cholesterol and cholesteryl ester. LDs are surrounded by a monolayer of PLs and LD-associated proteins, such as the perilipin (PLIN) family of proteins which are important in LD biogenesis [43, 109]. There are five PLIN genes encoded in the mammalian genome, and with additional splice variants, these proteins exhibits different tissue expression patterns [111]. The five PLINs differs in size, affinity to LDs, stability when unbound and in transcriptional regulation. Since the PLINs are bound to the LD surface, they play an important role in managing access of lipases to lipids inside the LD core, and thereby also plays a role in the

regulation of LD size and turnover [112]. All of the five PLINs are expressed at various levels in human skeletal muscle cells [113]. TAG is a neutral lipid that consists of a glycerol backbone and three FAs. TAG is metabolically inert, whereas other metabolites, such as DAG, acyl-CoA and ceramides, have shown to promote insulin resistance in skeletal muscle [114, 115]. There are two major pathways known for TAG biosynthesis; the MAG pathway and the glycerol-3-phosphate pathway [116]. Common for these pathways are that they both utilize FA-CoA, the active form of FA, as acyl donors [117]. The glycerol-3-phosphate pathway is a *de novo* TAG synthesis pathway and is active in most tissues [118, 119]. The MAG pathway plays an important in dietary fat absorption in the small intestine, but is also the main TAG synthesis pathway in muscle [120]. In muscles, MAG is re-acylated to DAG which is further acylated to form TAG [109]. The conversion of MAG to DAG is catalysed by monoacylglycerol acyltransferase (MGAT), whereas the esterification of FA-CoA to DAG, which is further acylated to TAG, is catalysed by diacylglycerol acyltransferase (DGAT) 1 and 2 (**Figure 1**) (discussed in further details under "**The role of diacylglycerol acyltransferases in energy metabolism**") [109, 119, 120].

When there is a high energy demand, for instance during exercise, FAs are released from LDs through the lipolysis of neutral lipids. The first step in lipolysis, or the catabolism of TAG, is initiated by adipose triglyceride lipase (ATGL) which hydrolyses TAG to DAG [121, 122]. The following step, the conversion of DAG to MAG is catalysed by hormone-sensitive lipase (HSL), which also contributes to the lipolysis of TAG and MAG molecules. The final step of lipolysis, the degradation of MAG to glycerol and FA, is catalysed by monoacylglycerol lipase (MGL) (**Figure 1**). The generated FAs from lipolysis can then undergo mitochondrial oxidation, but can also be re-esterified back into neutral lipids. ATGL activity is regulated by the co-activator comparative gene identification 58 (CGI-58) and the inhibitory G0/G1 switch genes 2 (G052), which makes CGI-58 and G052 regulators of skeletal muscle TAG dynamics [121, 123, 124]. Moreover, it has been shown that both ATGL and CGI-58 are strongly associated during contraction-induced muscle lipolysis, and that they work together with PLIN proteins [125]. HSL is regulated through phosphorylation at serine residues [121].

β-adrenergic receptors in skeletal muscle

The β-adrenergic receptors (β-AR) belong to a class of G protein-coupled receptors (GPCR), which are activated by endogenous catecholamines (adrenaline and noradrenaline), and by synthetic β-AR agonists [126]. There are three isoforms of β-AR; β_1, β_2 and β_3. These three isoforms have different expression patterns in various tissues and regulate different physiological functions. β_1-AR is known to increase cardiac output [127], while β_3-AR increases lipolysis in adipose tissue and relaxes smooth muscle in the human bladder [128]. β_2-AR is known to have many functions, including skeletal muscle anabolism and increasing lipolysis [129, 130]. The most abundant subtype in skeletal muscle is the β_2-AR, but it is reported that β_1-AR accounts for approximately 10% of the AR population with a low population of β_3-AR and α_1-AR [131]. Previous research, both *in vivo* and *in vitro*, has shown that stimulation of the β_2–AR results in an increased glucose uptake, fatty acid metabolism and muscle hypertrophy [132-136].

When activated by a ligand, the β-AR causes activation of adenylyl cyclase through the G protein subunit $G\alpha_s$, and adenylyl cyclase in turn catalyses the synthesis of cyclic AMP (cAMP) [137]. The increased intracellular cAMP concentration activates the protein kinase A (PKA), which regulates metabolism through phosphorylation of target proteins. One of the proteins phosphorylated is mammalian target of rapamycin (mTOR), which is phosphorylated at serine 2481 (Ser2481) in skeletal muscle, thereby activating mTORC2 [132]. Moreover, also the Gβγ complex is thought to mediate effects of β-AR activation through the PI3K-Akt pathway, which activates mTORC1 by phosphorylation of mTOR at serine 2448 (Ser2448) [132, 137, 138]. mTORC1 plays an important role in regulating protein and lipid synthesis, but it is also reported to have a role in limiting autophagy [139]. The role of mTORC2 is not as well-defined as the one of mTORC1, but is reported to play a part in cytoskeletal organization [139].

Although, β_2-AR agonists are mostly used for their bronchodilating effects [140], previous studies has also shown that stimulation of the β_2-AR has several beneficial metabolic effects, including glucose uptake, fatty acid metabolism and muscle hypertrophy [132-136, 141, 142]. These studies were performed both *in vivo* and *in vitro*, where most of them showed that these effects are found in skeletal muscle [132-134, 136, 142].

The role of TRPs in skeletal muscle

The transient receptor potential (TRP) channels are a group of ion channels located on the cell surface. Altogether there are 28 different TRP channels, grouped into six different families, expressed in humans [143]. Although these ion channels are expressed in many types of cells, they are mainly expressed on the dorsal root and trigeminal ganglia neurons [144]. Cold temperatures are sensed through two of the TRP ion channels, TRPA1 and TRPM8, which are activated by temperatures below 17℃ and 26℃, respectively [143]. TRPA1 and TRPM8 are Ca^{2+}-permeable cation channels, which allow the entry of Ca^{2+} when activated, but can also induce calcium release from intracellular Ca^{2+} stores through intracellular Ca^{2+} release channels [145, 146]. Several studies performed in mice and the mouse myoblast cell line, C2C12, has described the expression of TRPA1 [147-149] and TRPM8 [150]. However, only one study has found functional expression of TRPA1 in human primary muscle cells [151]. No study has yet reported expression of TRPM8 in human skeletal muscle cells *in vitro*.

Cold temperatures activate the TRPA1 and TRPM8 ion channels of sensory nerves in peripheral tissues. The sensory nerves send information to the hypothalamus, which signals the sympathetic nervous system to release norepinephrine onto brown adipocytes [152]. In brown adipocytes, norepinephrine acts on the β-adrenergic receptors, which activates the cAMP/PKA/cAMP response element-binding signalling cascade, resulting in transcription of the thermogenic gene program [152]. In humans, there are two organs known to be thermogenic; brown adipose tissue (BAT) and skeletal muscle. As a cold response, skeletal muscle induces thermogenesis through small, repetitive muscle contractions called shivering, which ultimately results in heat generation and increases in energy expenditure [44]. Thermogenesis increases energy expenditure by inducing transcription of the thermogenic gene program, which include the mitochondrial modulators uncoupling proteins (UCPs) and PGC-1α [152]. The UCPs are found in the inner mitochondrial membrane, where they enhances proton conductivity when activated, resulting in uncoupling of the ETC and subsequent heat production [153]. Evidence suggests that there are five different UCPs expressed in human tissues, all of which exhibits different expression patterns [153, 154]. UCP1 is mainly expressed in BAT, where it is responsible for inducing non-shivering following activation of an upstream activator, for instance TRPM8 [153, 155]. UCP3 is primarily expressed in skeletal muscle and is reported to have a role in FA metabolism [153]. Interestingly, mice overexpressing UCP3 are resistant to diet-induced obesity and diabetes, which is believed to be the result of an energy-dissipating mechanism [156]. In accordance

with this, a study looking at obese diet-resistant women found a decreased proton leak associated with lower expression levels of UCP3 in skeletal muscle [157]. PGC-1α is a transcription coactivator, which plays an important in cellular energy metabolism by regulating mitochondrial biogenesis [158]. PGC-1α also promotes skeletal muscle remodelling by changing the fibre type composition from glycolytic type II fibres to the more oxidative type I fibres [158].

Moreover, TRP ion channels also have a chemosensory role. While TRPM8 is activated by compounds producing a cold sensation, for instance menthol and icilin [159-161], TRPA1 is activated by pungent compounds containing allyl isothiocyanate, such as ligustilide and cinnamaldehyde [143, 151, 162].

The role of diacylglycerol acyltransferases in metabolism

Diacylglycerol acyltransferases, DGAT1 and DGAT2, catalyse the terminal step of TAG synthesis, and thereby are important regulators of metabolism [118, 163, 164]. Due to their important roles in remodelling and *de novo* pathway of TAG synthesis, DGATs are expressed in various tissues [165]. The isozymes are located in the endoplasmic reticulum, but DGAT2 is also reported to co-localize with mitochondria and LDs in cultured adipocytes and fibroblasts [166, 167]. Despite catalysing the same step in TAG synthesis, the isozymes share no sequence homology and belong to unrelated families of proteins [163]. However, the phenotypes of DGAT1 and DGAT2 knock-out (KO) mice suggests that the isozymes are non-redundant in some functions. *DGAT1*$^{-/-}$ mice have a favourable metabolic phenotype with an increased insulin and leptin sensitivity, and resistance diet-induced obesity [168, 169], while *DGAT2*$^{-/-}$ mice die shortly after birth as a result of a defect in the skin barrier leading to rapid dehydration [170]. Moreover, studies have found beneficial effects of DGAT1 overexpression in mouse skeletal muscle, such as an increased TAG synthesis and protection against high-fat induced insulin resistance [171], whereas upregulation of DGAT2 resulted in an increased lipid accumulation and insulin resistance in glycolytic muscle [172].

Based on the phenotype of *DGAT1*$^{-/-}$ mice, the pharmaceutical industry sought to explore whether DGAT1 is a potential drug target for the treatment of metabolic diseases. In fact, several DGAT1 inhibitors reached clinical trials [173], two of these were LCQ908 and AZD767 [174, 175]. Despite treatment of these compounds led to reduced levels of fasting TAG [174, 175], they also resulted in gastrointestinal side effects, which impacted the further

development of DGAT1 inhibitors [173]. Although KO studies suggested DGAT2 inhibition to be harmful, DGAT2 has emerged as a potential target following studies looking at DGAT2 overexpression in mice. Overexpression of DGAT2 led to the development of hepatic steatosis and associated complications [176-178]. However, the role of DGAT2, and the transferability between rodents and higher species has been disputed [179].

The role of AMPK in skeletal muscle energy metabolism

The AMPK enzyme is a heterotrimeric protein complex, which consists of a catalytic α subunit, and two non-catalytic regulatory β and γ subunits. Two isoforms has been identified for the α (α1 and α2) and the β (β1 and β2) subunits, while three isoforms has been detected for the γ (γ1, γ2, and γ3) subunit [180]. These isoforms can give rise to 12 different versions of the AMPK enzyme, each with different tissue expression patterns and varying functions under different conditions [181].

There are two main biochemical triggers known to activate AMPK; an increased AMP:ATP ratio and alterations in intracellular Ca^{2+} concentrations. These two biochemical triggers activates AMPK through distinct upstream kinases [182]. The increased AMP:ATP ratio is a direct result of increased intracellular levels of AMP and decreased levels of ATP [183], which causes allosteric activation of the protein kinase, liver kinase B1 (LKB1) [184]. LKB1 forms a complex with the pseudokinase, STRAD, and an adaptor protein, MO25, and it is this LKB1-STRAD-MO25 complex that activates AMPK through phosphorylation [184]. Although the LKB1-AMPK pathway is known for its function as an energy sensing cascade, it has also shown to have an important role in cell growth, where it serves a metabolic checkpoint and arrests cell growth when there are low intracellular ATP levels [183]. The other kinase known to activate AMPK is Ca^{2+}/Calmodulin-dependent protein kinase kinase β (CaMKKβ), which is a part of a calcium-dependent pathway [182, 185]. In response to increased intracellular Ca^{2+} levels, CaMKKβ activates AMPK and together they form a unique signalling complex [186], independently of alterations in the AMP:ATP ratio [185]. Although LKB1 and CAMKKβ are the most studied kinases known to activate AMPK, it has been found that AMPK can also be activated in response to the actions of different cytokines and several hormones [187, 188].

LKB1, CaMKKβ and other potential upstream kinases activates AMPK by phosphorylating of threonine on position 172 (Thr172) on the catalytic α subunit. Phosphorylation of Thr172

causes adenosine monophosphate (AMP) to bind to the regulatory γ subunit, which ultimately leads to allosteric activation of AMPK [185, 189, 190]. The binding of AMP to the γ subunit also protects Thr172 from dephosphorylation by protein phosphatase, and thus, maintaining the enzyme in its activated state [187, 188].

AMPK plays an important role in the regulation of metabolic homeostasis and acts as a major cellular energy sensor. For instance, AMPK has shown to be important in modulating insulin sensitivity in skeletal muscle, and aids in the regulation of glucose and lipid metabolism in several mammalian tissues [187]. There are several physiological stimuli known to activate AMPK, for instance nutritional depravation, hypoxia and exercise [187, 188, 191]. The AMPK activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), is perhaps the most common compound used to study AMPK activation, but AMPK is also indirectly activated by the antidiabetic agent, metformin [187, 188, 191]. Activation of AMPK has shown to mediate glucose uptake through a phosphatidylinositol 3-kinase independent pathway, and are therefore not dependent on insulin [192, 193]. Similar to the effects of exercise, long-term activation of AMPK has shown to increase HK activity, GLUT4 protein levels, and the amount of glycogen in rat skeletal muscle [194]. Further, AMPK activation has also shown to increase cellular NAD(+) levels and sirtuin 1 activity, ultimately leading to PGC-1α activation and subsequent increases in expression of several mitochondrial genes [195, 196]. Moreover, one of the more profound effects of AMPK activation is an increased FA oxidation and decreased lipolysis. AMPK increases FA oxidation by inhibition of ACC, thereby decreasing the concentration of malonyl-CoA, allowing the entry of long-chain-acyl-CoA into the mitochondria for β-oxidation [187, 197, 198].

Several studies have been performed where one or more genes encoding the AMPK subunits have been altered in order to generate dysfunctional AMPK enzymes. One of the most commonly knocked out subunit of AMPK is the α2 subunit [188]. In AMPKα2 KO studies, it has been established that this subunit is important for whole-body insulin action, AICAR-mediated whole-body and skeletal muscle uptake, impaired skeletal muscle mitochondrial biogenesis and reduced skeletal muscle glycogen content [199-202].

Aims

The overall aim of the present thesis was to investigate the regulation of energy metabolism in human skeletal muscle cells. More specifically, the aims of the studies presented in this thesis were:

1) Discover the effects of β_2 adrenergic receptor activation, by using the agonist terbutaline, on energy metabolism and protein synthesis in cultured human primary skeletal muscle cells (**paper I**).

2) Investigate whether the thermosensing ion channels TRPA1 and TRPM8 are expressed in human myotubes, and study the effects of inhibiting and activating these channels on glucose metabolism (**paper II**).

3) Investigate the roles of the isozymes DGAT1 and DGAT2 on lipid metabolism in cultured human myotubes by using small-molecule inhibitors of the enzymes (**paper III)**.

4) Investigate the metabolic effects of ablating the α2 subunit of the AMPK enzyme in mice skeletal muscle (**paper IV**).

Summary of papers

Paper I: *Chronic treatment with terbutaline increases glucose and oleic acid oxidation and protein synthesis in cultured human myotubes*

In this study, the effects of the β_2 adrenergic signalling on skeletal muscle metabolism was explored. Previous *in vivo* studies has reported of several beneficial metabolic effects of β-adrenergic receptor agonist administration, including increased glucose uptake, fatty acid metabolism, lipolysis and mitochondrial biogenesis. In order to investigate whether these effects are promising pharmacological tools in the treatment of obesity, we treated cultured human myotubes with the β_2-AR agonist, terbutaline, for 4 and 96 hours. It was found that an acute, 4 hours treatment with terbutaline increased glucose uptake, while a chronic, 96 hours treatment increased both glucose and fatty acid oxidation, and increased protein synthesis. Moreover, proteomics analysis revealed an upregulation in protein related to the mitochondrial pathways and fatty acid β-oxidation. Interestingly, both PCR and proteomic analysis found an increase in the expression of the fructose transporter, GLUT5.

In conclusion, long-term activation of the β_2-AR in skeletal muscle resulted in an increased substrate oxidation, mitochondrial biogenesis and protein synthesis. These results suggests that β_2-ARs have direct effects in skeletal muscle that might be favourable for both T2D and degenerative muscle diseases. However, a deeper understanding of the signalling pathways underlying these effects is necessary for future pharmacological development.

Paper II: *Expression and metabolic functions of the thermally activated transient receptor potential channels TRPA1 and TRPM8 in human myotubes*

The thermally activated transient receptor potential channels (TRP)A1 and TRPM8 are cold-sensing ion channels, while TRPV1 is a heat-sensing ion channel. Although studies have found expression of TRPA1 and TRPV1 in human skeletal muscle, there are no reports of TRPM8 expression in human myotubes. The aim of this study was to investigate whether these ion channels are expressed in human skeletal muscle cells, and if their activation by chemical agonists had an effect on energy metabolism. PCR analysis confirmed the expression of TRPA1, TRPM8 and TRPV1 in cultured human skeletal muscle. Activation of TRPA1 by ligustilide resulted in an increased glucose uptake and oxidation in human myotubes, whereas activation of TRPM8 by menthol and icilin significantly decreased glucose uptake and oxidation. Activation of TRPV1 by capsaicin had no effect on glucose metabolism. Agonist-induced increases in intracellular Ca^{2+} levels by ligustilide and icilin in human myotubes confirmed direct activation of TRPA1 and TRPM8. The mRNA expression of some genes involved in thermogenesis were downregulated in human myotubes following TRPA1 activation, while the mRNA expression of TRPM8 and TRPA1 were downregulated following TRPM8 activation by menthol and icilin, respectively. Cold exposure of the cultured myotubes followed by a short recovery period had no effect on glucose uptake and oxidation in the basal situation, however cold exposure reduced glucose metabolism in the presence of inhibitors to TRPA1 and TRPM8.

In conclusion, mRNA of TRPA1, TRPM8 and TRPV1 was expressed in cultured human myotubes. Activation of the TRPA1 ion channel increased glucose uptake and oxidation, while activation of the TRPM8 ion channel decreased glucose metabolism. The metabolic effects seen following agonist TRPA1 and TRPM8 treatment cannot be explained by changes in the expression of the thermogenic gene program. Cold exposure had no effect on glucose metabolism, but when TRPA1 and TRPM8 channels were inhibited following cold exposure the glucose uptake was significantly decreased.

Paper III: *Treatment of human skeletal muscle cells with inhibitors of diacylglycerol acyltransferases 1 and 2 to explore isozyme-specific roles on lipid metabolism*

The aim of this study was to investigate the respective roles of diacylglycerol acyltransferases (DGAT)1 and DGAT2 on lipid metabolism in human skeletal muscle cells. Previously, it has been shown that these isozymes catalyses the final step of TAG synthesis, and therefore are important modulators of energy metabolism. By using specific small-molecule inhibitors of DGAT1 and DGAT2, it was found that the two isozymes have distinct roles in lipid metabolism. Both DGAT1 and DGAT2 are involved in *de novo* synthesis of TAG, and activating of these isozymes are important in determining the rate of FA oxidation. This indicates that they have a key role in balancing FAs between storage in TAG and efficient utilization of FAs for oxidation. Moreover, it was found that DGAT1 is the major enzyme responsible for the incorporation of endogenously generated and exogenously supplied FAs into cellular lipids.

In conclusion, when inhibiting DGAT1 and DGAT2 there were distinct responses on lipid metabolism in myotubes. Due to their distinct roles, they could give rise to heterogeneous pools of TAG in skeletal muscle. However, more studies are needed to investigate these effects and determine the mechanisms underlying the possible beneficial or deleterious effects of the DGAT isozymes.

Paper IV: *Loss of AMPKα2 increases substrate oxidation, but decreases relative maximal oxidative capacity and incorporation of exogenous fatty acids into lipids in cultured myotubes*

The adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) enzyme plays an important role in metabolic homeostasis and acts a major cellular energy sensor. The AMPK protein is a heterotrimeric enzyme consisting of a catalytic α subunit and two regulatory β and γ subunits. There are two isoforms of the α subunit (α1 and α2), where the α2 isoform is the most expressed form in skeletal muscle. The aim of this study was to investigate how the metabolism was altered when the catalytic α2 subunit of the AMPK enzyme was ablated. Myotubes from *AMPKα2*$^{-/-}$ mice had a higher basal oxidative capacity, but a lower relative mitochondrial maximal capacity and a metabolic flexibility compared to myotubes from *AMPKα2*$^{+/+}$ mice. *AMPKα2*$^{-/-}$ myotubes also had a lower accumulation of lipids and a decreased incorporation of exogenous lipids into DAG and TAG. Proteomics analysis were performed in order to compare the proteome between *AMPKα2*$^{+/+}$ and *AMPKα2*$^{-/-}$ myotubes. In myotubes from *AMPKα2*$^{-/-}$ mice there were an upregulation in proteins involved in oxidative pathways and calcium signalling. Proteomics also revealed a downregulation of the lipid droplet coating proteins Plin3 and Plin4. Moreover, qPCR confirmed decreased expression of Plin3, and also showed decreased expression of ATGL and increased expression of CPT1b.

In conclusion, loss of AMPKα2 results in higher basal substrate oxidation, but lower relative mitochondrial maximal capacity and metabolic flexibility. Moreover, these cells also had a decreased accumulation and incorporation of lipids into DAG and TAG, and a reduced expression of proteins involved in lipid droplet coating and lipolysis.

Methodological considerations

Primary human myotubes as an *in vitro* model of skeletal muscle

Cultured human myotubes have proven to be a useful *in vitro* model of human skeletal muscle. Compared to rodent cell cultures, it has a more relevant genetic background making it suitable for studying metabolic processes and pathways in human skeletal muscle [99]. The human myotubes used in the work presented in this thesis are not immortalized, thereby allows the investigation of the innate characteristics of the donors of which they are established. Since the extracellular environment of the cells can be precisely monitored, it is possible to study the biochemical and molecular mechanisms, and their underlying signalling pathways, under controlled conditions.

The human skeletal muscle cells used in the studies presented in this thesis were obtained from biopsies taken from *musculus vastus lateralis*, and were from a cohort previously described in *Lund et al.* (**paper I-III**) [203]. Satellite cells were isolated, activated and proliferated into myoblasts before being differentiated into multinucleated myotubes based on a method described by Henry *et al.* and modified according to Gaster *et al.* and Bourlier *et al.* [204-206]. During the differentiation from myoblasts to myotubes, the expression of key proteins involved in glucose [207] and lipid metabolism [208] were increased. Since the expression pattern in myotubes resembles adult muscle, they are preferred for experimental use [209].

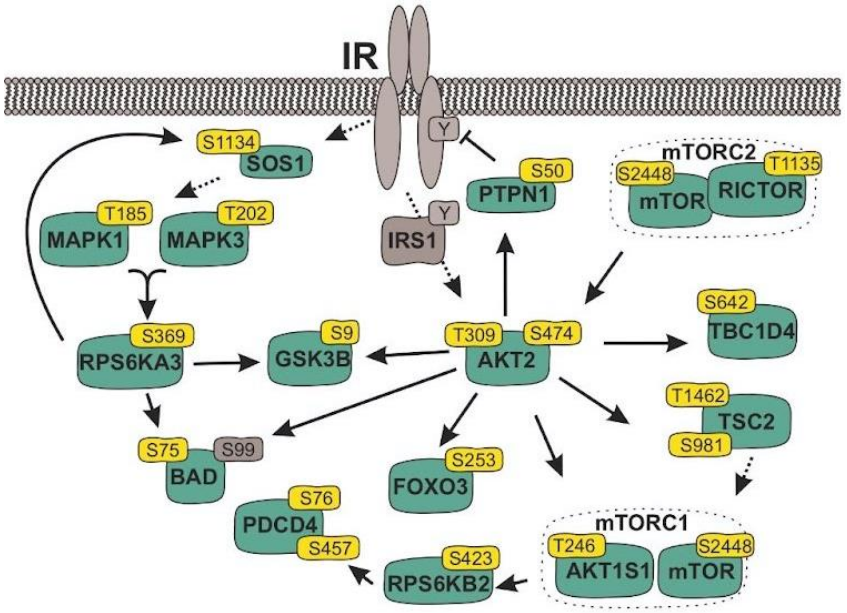
In order to study the role of AMPK in skeletal muscle energy metabolism, muscle myoblast cultures were established from the *m. gastrocnemius* and *m. soleus* muscles from the hind leg of *AMPKα2*$^{+/+}$ and *AMPKα2*$^{-/-}$ mice (**paper IV**). The AMPKα2 gene was disrupted by using standard homologous recombination in embryonic stem cells. In short, the exon encoding the catalytic AMPKα2 domain (amino acids 189-260) was flanked and cleaved by *Hind*III restriction enzyme, ultimately resulting in deletion of the catalytic domain [201]. Using cells from KO animals, allows us to study the roles of a specific protein at a time, however, the silencing of that specific gene may elicit compensatory cellular responses by up-regulating genes with overlapping functions.

When comparing metabolic studies using different species, it is important to consider the differences in metabolic regulation between species [210]. For instance, mice have a ~7.5 fold

greater basal metabolic rate than that of human. Moreover, mice exhibits a glucose tolerance that is more related to hepatic insulin than that of skeletal muscle.

Cultured human myotubes are characterized by fuel preference for glucose over lipids and for their low mitochondrial capacity [99]. Compared to adult human skeletal muscle, cultured human myotubes have a lower GLUT1:GLUT4 ratio, resulting in a lower insulin responsiveness [207, 211, 212]. Usually, insulin increases glucose uptake by 40-50% *in vivo* [207]. However, despite the reduced insulin-induced glucose uptake the mechanisms underlying glucose uptake *in vivo* are conserved *in vitro* [207]. In one unpublished study from our group, we explored phosphorylation events in the insulin signalling pathway in human skeletal muscle cells by phosphoproteomics. We found that the insulin signalling pathway is fully functional in our *in vitro* cell model of human skeletal cells (Figure 2A and 2B).

A



B

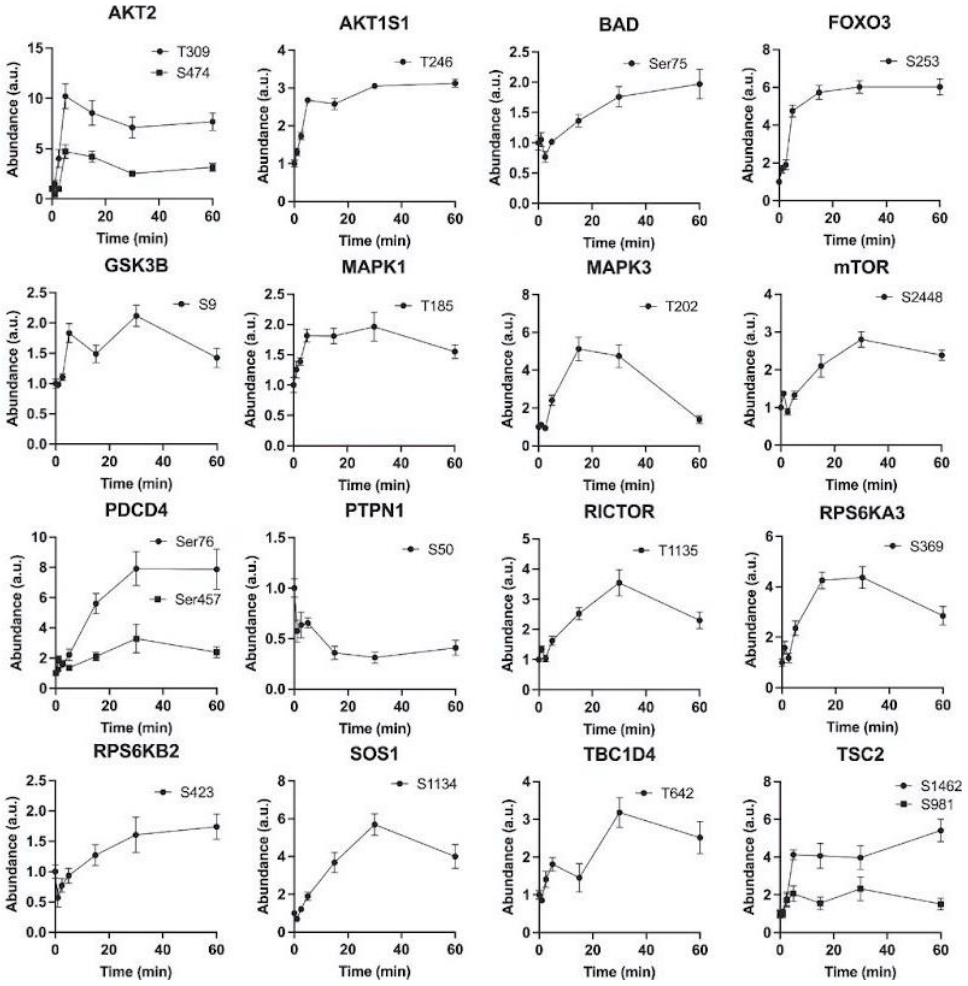


Figure 2. Time course for the phosphorylation of insulin targets. A: The overview of the targets in the insulin signalling pathways, most of which are substrates of main insulin-regulated kinase in skeletal muscle, AKT2. **B:** The phosphorylation kinetics of selected targets over a 60 minutes time course. RAC-beta serine/threonine-protein kinase (AKT2); proline-rich AKT1 substrate 1; Bcl2-associated agonist of cell death (BAD); forkhead box protein O3 (FOXO3); glycogen synthase kinase-3 beta (GSK3B); mitogen-activated protein kinase 1 (MAPK1); mitogen activated protein kinase 3 (MAPK3); mammalian target of rapamycin (mTOR); pyruvate decarboxylase 4 (PDC4); protein tyrosine phosphatase non-receptor type 1 (PTPN1): rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR); ribosomal protein S6 kinase alpha-3 (RPS6KA3); ribosomal protein S6 kinase beta-2 (RPS6KB2); son of sevenless homolog 1 (SOS1); TBC1 domain family member 4 (TBC1D4); tuberin (TSC2).

When performing research on donor-derived myotubes from various sources of muscle satellite cells, it is important to consider the heterogeneity of muscle fibres. However, research has shown that cultured myotubes co-express both slow- and fast-twitch muscle fibres independently of the fibre type they were isolated from [213]. This demonstrates that cultured myotubes are different from their donor muscle when it comes to MHC expression. Research from our group has described that fully differentiated myotubes expresses both

slow- and fast-twitch fibre types [99, 203]. In addition, it has been found that cultured myotubes have a plasticity potential as electric pulse stimulation (EPS), an *in vitro* model of exercise, can increase the cellular content of slow-twitch MHCI fibres [214]. Murine satellite cells are usually isolated from various major muscles, but have shown to uniform and after differentiation mainly express the intermediate MHCIIa fibre type regardless of muscle origin [215].

Several of the donor characteristics found *in vivo* are conserved *in vitro*. For instance, several studies has found that diabetic phenotype are conserved in cultured myotubes [216, 217]. Two of the donor effects that are conserved in myotubes from diabetic and obese subjects are the altered lipid metabolism [89, 90, 218, 219] and defects in glucose metabolism [204, 216]. The exact mechanisms by which myotubes are able to retain their *in vivo* characteristics are not fully understood. However, a review paper has suggested it to be due to a combination of genetic and epigenetic mechanisms [99]. The epigenetic regulations of skeletal muscle satellite cells are well-studied [220, 221]. Whereas several factors, such as family history of T2D, exercise and diet, have shown to influence DNA methylation in human myotubes [222-225]. All of these factors are traits which might accompany isolated satellite cells when they grow into myotubes. However, it is important to consider that the proliferative potential of myoblasts decreases and the metabolic properties myotubes differentiated from these cells changes with an increasing passage number. In order to overcome this potential problem, the experiments presented in this thesis were performed on cells from passage three or four, which exerted unchanged responses [226]. One of the weaknesses with cultured myotubes as a cell model of skeletal muscle is the lack of neural input. Therefore, the cultured myotubes are more a model of denervated skeletal muscle and will under normal conditions not spontaneously contract [227]. However, EPS can be used to mimic the neural input seen *in vivo* and therefore induce responses similar to those seen in exercising muscle [214, 228, 229]. Despite some minor limitations, the cell model of human myotubes is a valuable model for studying skeletal muscle energy metabolism.

Donor characteristics

Although cultured myotubes retain some of the phenotypic traits of their donors, there are several factors known to affect the metabolic processes of skeletal muscle [230]. Examples of these are age, BMI and gender. Increasing age *in vivo* has been associated with reduced

skeletal muscle mass [231], impaired insulin sensitivity [232, 233] and ectopic lipid accumulation [233, 234], in addition to decreased mitochondrial content and function. Recent work has suggested that donor age might have an effect on the substrate oxidation capacity of cultured human myotubes [230]. Obesity and an increased BMI is associated with alterations in skeletal muscle energy metabolism *in vivo* [23, 90, 235, 236]. In addition, work from our lab have found that donor BMI affects several metabolic processes in cultured myotubes [228, 230]. Gender is also a major source of variations in body composition, hormonal regulation and physiological function [237, 238], but these gender specific differences does not appear to be present in cultures myotubes, unless the cells are incubated in the presence of sex hormones [239, 240]. In the experiments presented in the thesis we used a relative homogenous cohort, only myotubes from male donors were used, the age varied from 21 to 34 years old, and the BMI ranged from 20-27 kg/m^2, thus possible variations due to age, gender and BMI are not relevant in these studies.

Methods measuring energy metabolism in cultured human myotubes

In the results presented in this thesis, the metabolic processes were described by a combination of functional studies using radiolabelled substrates with gene expression analyses (qPCR), calcium signalling measurements, and protein expression (proteomics). Substrate oxidation assay was used in all of the papers to measure substrate oxidation and accumulation [242]. One of the many benefits with the substrate oxidation assay is that it can be used to measure metabolic flexibility and maximal oxidative capacity (**paper IV**). Metabolic flexibility, the ability to suppress oxidation of glucose or FA by the opposite substrate, was measured by measuring the oxidation of radiolabelled glucose and the FA oleic acid (OA) in the presence and absence of acutely added unlabelled OA and glucose, respectively. The maximal mitochondrial oxidative capacity can be measured with the use of the mitochondrial uncoupler, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). Scintillation proximity assay (SPA) [241] was used to study the real time accumulation and decay of leucine in **paper I**. The accumulation of radiolabelled leucine, a measurement of leucine into cellular protein, was monitored over 24 h with measurements at different time points. Following the 24 h accumulation period, the experimental medium was changed to a basal medium containing no radiolabelled leucine, and the decline in radioactivity, quantified as efflux of labelled leucine, was measured at different time points over a 6 h period as a measure of protein decay. Proteomics, which generates a big amount of data, is the large-scale

study of proteins and has proven to be a valuable tool for the analysis of changes in the proteome. In **paper I**, proteomics was used to analyse the changes in protein expression following long-term treatment with terbutaline, while in **paper IV** this method was used to compare the proteome from skeletal muscle cells of *AMPKα2*$^{+/+}$ and *AMPKα2*$^{-/-}$ mice. One of the weaknesses with proteomics analysis is that not all proteins in the cells are detected due to the detection limits. Thin layer chromatography was used in **paper III** and **IV** to study the lipid distribution of incorporated radiolabelled precursors. Real time qPCR is useful for the investigation of a small number of anticipated regulated genes (**paper I-IV**). One important issue regarding the studies presented in this thesis, are the use of pharmacological active activators and inhibitors. In **paper I**, the well-studied β_2-AR agonist terbutaline was used to study the metabolic effects of β-adrenergic signalling, while in **paper II**, both activators and inhibitors of the cold-sensing TRP ion channels, TRPA1 and TRPM8, were used to investigate the effects of these ion channels on energy metabolism. In **paper III**, selective inhibitors of DGAT1 and DGAT2 were used to explore their role in lipid metabolism. The use of these compounds involves the risk of toxicity and non-specific effects, related to the concentrations being used in the experiments.

In **paper I**, we used the β_2-AR agonist terbutaline to investigate whether β-AR signalling has an effect on energy metabolism in human skeletal muscle cells. Terbutaline is a compound that has been studied extensively, both *in vivo* and *in vitro*, and is a selective β_2 agonist with an EC_{50} value of 13.8 nM *in vitro* [242]. In accordance with previous studies using β_2-AR agonists, we used a range of concentrations (0.01-30 μM) in order to study the dose-dependent effects of terbutaline on energy metabolism [243, 244]. For further experiments, we used the two most effective concentrations of terbutaline, 1 and 10 μM. In **paper II**, we used both activators and inhibitors of the cold-sensing thermoreceptors, TRPA1 and TRPM8. Menthol is a well-recognised cooling agent with local anaesthetic properties, which acts through the TRPM8 ion channel. The EC_{50} value of menthol ranges from 4 to 80 μM, and despite being classified as a TRPM8 agonist, it has shown to activate other thermosensitive TRP ion channels at higher concentrations (reviewed in [245]). A menthol concentration of 10 μM was used in our experiments. Icilin is a TRPM8 activator [246], which is shown to be more potent and efficacious than menthol [247]. Icilin has an estimated EC_{50} value of 125 ± 30 nm for the TRPM8 ion channel *in vitro* [161]. Despite many studies using icilin to study the TRPA1 ion channel, we were unable to find reports on the EC_{50} value for TRPA1. We used a concentration of 10 μM icilin in our experiments. Ligustilide is the major bioactive

component of *Angelica sinensis*, which is reported to acting as an activator of the TRPA1 ion channel [162, 248]. Ligustilide has an EC_{50} value of 44 μM [162], but previous *in vitro* studies have used a concentration ranging from to 10 to 300 μM [162, 248]. Based on this, we tested a wide range of concentrations of ligustilide, and found that a dose of 20 and 200 μM was effective in our cell model. HC-030031, is a selective TRPA1 inhibitor, which antagonizes calcium influxes evoked by the TRPA1 agonists AITC and formalin, with an IC_{50} value of 6.2 and 5.3 μM, respectively [249]. A previous study using HC-030031 to study the human TRPA1 channel used a range of concentrations, starting from 0.1 to 50 μM [250]. Based on the findings of this study, we used a HC-030031 concentration of 25 μM, which was shown to be effective in cultured skeletal muscle cells. The selective TRPM8 inhibitor, PF-05105679, was discovered by Pfizer in 2014 and has an IC_{50} value of 1173 nM [251]. Since its discovery, only 3 studies, all of which are *in vivo* studies, using this compound has been published. Therefore, the concentration of 25 μM, PF-05105679 used in our experiments, are based on preliminary studies performed in our lab. In **paper III**, the DGAT1 inhibitor, A922500, and the DGAT2 inhibitor, JNJ-DGAT2-A, was used. A922500 is commercially available and with an IC_{50} value of 7 nM *in vitro* [252], has shown excellent selectively over DGAT2 (IC_{50} = 53 μM) [253]. Based on previous studies, we used an A922500 concentration of 1 μM [254, 255]. JNJ-DGAT2-A has an IC_{50} value of 0.14 μM and exhibits selectively over DGAT1 (IC_{50} value of more than 10 μM) [255]. Previous studies using JNJ-DGAT2-A, has used a concentrations up to 50 μM [256]. However, based on preliminary studies performed in our lab, we utilized a concentration of 10 μM. In **paper IV**, the mitochondrial uncoupler, FCCP, was used to measure maximal mitochondrial reserve capacity [257].

General discussion

The studies presented in this thesis were performed on primary human myotubes and on cultured myotubes from mice (*AMPKα2*$^{+/+}$ and *AMPKα2*$^{-/-}$). In **paper I**, the β_2 agonist, terbutaline, was used to study the metabolic effects of β_2-AR activation and signalling in human myotubes. In **paper II**, the expression and activation of cold-sensing thermoreceptors TRPA1 and TRPM8 was explored in human skeletal muscle cells. In **paper III**, inhibitors of DGAT1 and DGAT2 was used to explore the roles of these enzymes in lipid metabolism. In **paper IV**, myotubes from *AMPKα2*$^{+/+}$ and *AMPKα2*$^{-/-}$ mice was used to investigate the role of AMPKα2 in energy metabolism in skeletal muscle.

Glucose and fatty acid metabolism

Energy metabolism in skeletal muscle was studied in all of the papers presented. Different experimental approaches were able to increase the uptake of glucose (**paper I** and **II**), the oxidation of glucose (**paper I**, **II** and **IV**) and/or OA (**paper I**, **III** and **IV**). The effects of these interventions are important when it comes to determining mechanisms and elucidating the potential beneficial effects targeting the altered skeletal muscle metabolism associated with obesity and T2D [85, 90, 258].

In **paper I** and **II**, the myotubes were treated with either with a β_2-AR agonist or chemical activators of the cold-sensing TRPA1 and TRPM8 ion channels, showing that activation of β_2-AR, TRPA1 and TRPM8 had an impact on glucose uptake. Myotubes treated with terbutaline for 4 and 96 hours (representing acute and chronic effects, respectively) had an increased uptake of 2-deoxyglucose (Figure 3) (**paper I**). Increased glucose uptake following acute and chronic treatment with a β_2-AR agonist is a finding that previously has been documented *in vivo* and *in vitro* [126, 131-134, 259]. Interestingly, in our study we found increased gene and protein expression of the fructose transporter GLUT5, which has previously shown to mediate uptake of 2-deoxyglucose with low efficacy (Figure 3) [260]. In **paper II**, there was an increased glucose uptake following activation of TRPA1 by ligustilide (Figure 3). TRPA1 activation by cinnamaldehyde has shown to accompany increased expression of GLUT4 [147-149], however, in our study there was a decreased mRNA expression of GLUT4 in ligustilide-treated myotubes. Therefore, the increased glucose uptake seen following TRPA1 activation by ligustilide seemed not to be mediated by an increased

expression of GLUT4. Moreover, TRPM8 activation by menthol and icilin resulted in reduced glucose uptake (Figure 3) (**paper II**).

AMPK is reported to have a crucial role in mediating both whole-body and muscle glucose uptake in response to physiological and pharmacological stimuli [187, 188, 191]. However, we found that the glucose uptake in *AMPKα2*$^{-/-}$ myotubes was not significantly different from the glucose uptake in *AMPKα2*$^{+/+}$ myotubes (**paper IV**). This was surprising, as previous studies using *AMPKα2*$^{-/-}$ mice has found that AICAR mediated whole-body and skeletal muscle glucose uptake was abolished in these mice [199-202]. However, one study reported that the activity of AMPKα1 in skeletal muscle was higher in *AMPKα2*$^{-/-}$ mice than in wild-type mice [261]. Therefore, it is possible that the loss of AMPKα2 is compensated in parts by an increased activity of AMPKα1.

Proteomics was used to study the proteome of human myotubes treated with terbutaline (**paper I**) and to compare the proteomes of myotubes obtained from *AMPKα2*$^{+/+}$ and *AMPKα2*$^{-/-}$ mice (**paper IV**). In both these studies, Ingenuity Pathway Analysis (IPA) was performed on the dataset from the proteomics analysis in order to get an overview of how different canonical pathways are affected by these interventions. In **paper I**, it was found that treatment with the β_2-AR agonist, terbutaline, for 96 hours increased both glucose and OA oxidation in cultured human myotubes. In **paper IV**, the myotubes from *AMPKα2*$^{-/-}$ mice exhibited an increased basal oxidation of glucose and OA compared to myotubes from *AMPKα2*$^{+/+}$ mice. The IPA analysis performed in these studies showed an upregulation of proteins involved in oxidative phosphorylation and β-oxidation both in terbutaline-treated myotubes (Figure 3) and in *AMPKα2*$^{-/-}$ mice (Figure 4). In addition, proteins in the TCA cycle were upregulated in terbutaline-treated myotubes (**paper I**). Therefore, the increased substrate oxidation seen in **paper I** and **IV** can be explained by an upregulation of oxidative pathways. Although the increased glucose and OA oxidation was only found in myotubes treated with terbutaline for 96 hours (**paper I**), this is in line with previous reports stating that an increased substrate oxidation is only seen after several days of β_2-AR agonist treatment [135, 244, 262]. This might be explained by evidence suggesting that long-term stimulation of β-ARs is necessary for inducing mitochondrial biogenesis [133]. In line with this, the mRNA expression of PGC-1α was increased in myotubes treated with terbutaline for 96 hours. To sum up the findings in **paper I**; treatment with β_2-AR agonists have beneficial metabolic effects as increased mitochondrial biogenesis results in an upregulation of oxidative pathways which ultimately results in an increased substrate oxidation (Figure 3).

PGC-1α, along with UCP1 and UCP3, makes up the thermogenic gene program, whose transcription increases in response to cold exposure [148, 150, 152]. Activation of the cold-sensing TRPA1 and TRPM8 ion channels by cold temperatures and chemical agonists increases the expression of the thermogenic gene program, which is suggested to be responsible for the increased substrate oxidation seen during thermogenesis [148, 150, 152]. However, in **paper II**, the increased glucose oxidation seen following activation of TRPA1 by ligustilide was not accompanied by an increased mRNA expression of any of the genes in the thermogenic gene program (Figure 3) [147]. Thus, the increased glucose oxidation seen following TRPA1 activation cannot be explained by an increased expression of UCP1, UCP3, and PGC-1α. Further, in contrast to previous reports [150], we found a decreased glucose oxidation following TRPM8 activation by menthol and icilin (**paper II**). TRPA1 and TRPM8 are Ca^{2+}-permeable cation channels that increases intracellular Ca^{2+} levels when activated. Ligustilide and icilin did increase the intracellular Ca^{2+} levels and induced membrane depolarization, however, icilin induced membrane depolarization to a much lesser extent than ligustilide (Figure 3) (**paper II**). Taken into account that free cytoplasmic Ca^{2+} ions can act as second messengers and can therefore elicit a stimulatory effect of oxidative processes and substrate catabolism [263], it is possible that increased glucose metabolism seen following TRPA1 activation by ligustilide is caused by the increased Ca^{2+} flux (Figure 3). The decreased glucose uptake and oxidation seen following activation of TRPM8 was unexpected, however. One study has found that TRPM8 activation by icilin is dependent on the presence of cytosolic Ca^{2+} [264]. Even though icilin was able to increase intracellular Ca^{2+} levels, the increase was modest compared to the increase induced by ligustilide. Therefore, it is possible that the cytosolic Ca^{2+} levels in cultured, non-innervated human myotubes are too low for full TRPM8 activation. No previous studies have described the functional expression of TRPM8 in cultured human myotubes, however, one previous study performed on the mouse skeletal muscle cell line, C2C12, reported a Ca^{2+}-dependent increased expression of UCP1 and PGC-1α following treatment with menthol [150]. In short; the findings in **paper II** show that both TRPA1 and TRPM8 are functionally expressed in cultured human myotubes and that activation of these ion channels increases the intracellular Ca^{2+} which may impact the glucose metabolism (Figure 3).

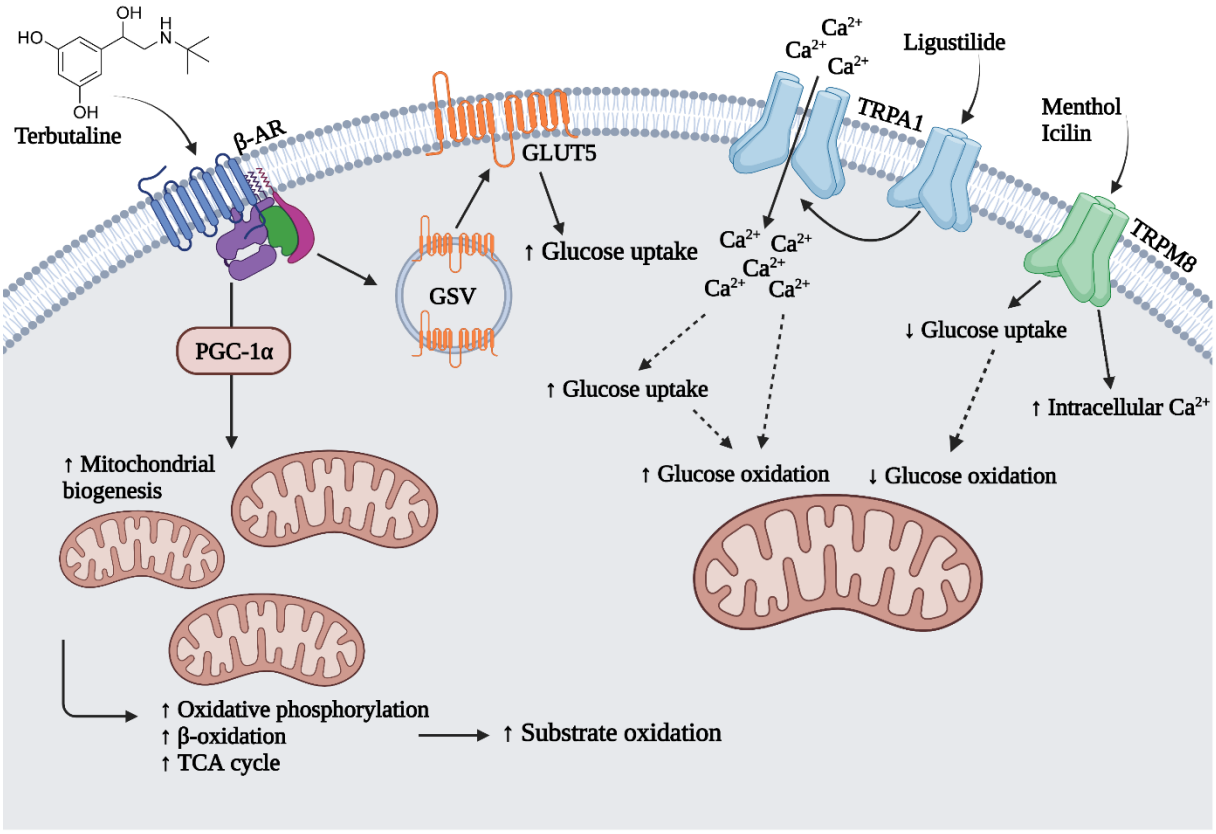


Figure 3. Effects of β_2-adrenergic receptor (AR) stimulation and activation of thermally activated transient receptor potential channels (TRP)A1 and TRPM8 on skeletal muscle metabolism. Chronic treatment (96 hours) with the β_2-AR agonist terbutaline increases the mRNA expression of PGC-1α resulting in mitochondrial biogenesis which upregulates several oxidative pathways in the mitochondria, ultimately resulting in an increased substrate oxidation. Treating cultured human myotubes with chemical activators of TRPA1 and TRPM8 ion channels increases the intracellular Ca^{2+}. Treating the myotubes with the TRPA1 activator, ligustilide, increased the uptake and oxidation of glucose which may be explained by the increased flux of Ca^{2+} into the cells. Moreover, activation of TRPM8 by menthol and icilin resulted in a modest increase in intracellular Ca^{2+} and a decreased glucose uptake and oxidation.

In **paper III**, cultured human myotubes were treated with specific inhibitors of DGAT1 and DGAT2. It was found that activity of the two DGAT isozymes had an impact on FA β-oxidation and complete oxidation (CO_2) which resulted in altered FA rates for oxidation. When inhibiting DGAT1, the major storage pathway of FAs in myotubes is blocked, and thus resulted in more lipids being available for FA oxidation (Figure 4). This finding has previously been shown in mouse cardiac muscle cells and another study using human myotubes [99, 265]. On the other hand, treatment with a DGAT2 inhibitor resulted in a decreased FA oxidation. As described in **paper III**, this could be the result of competition between CPT1, mitochondrial glycerol-3-phosphate and DGAT1, which is unaffected by the inhibited DGAT2. Interestingly, in brown adipocytes DGAT2 has been linked to channelling

de novo synthesized FA into a rapidly mobilised TAG pool, and thus providing substrate for mitochondrial FA oxidation [256].

Metabolic flexibility and maximal mitochondrial oxidative capacity

As discussed under "**Glucose and fatty acid metabolism**", it was found that ablation of the catalytic AMPKα2 subunit resulted in a higher oxidation of both glucose and OA **(paper IV**). In addition, *AMPKα2*$^{-/-}$ myotubes also had a decreased response to mitochondrial uncoupling and a reduced metabolic flexibility. The decreased response to mitochondrial uncoupling suggest that *AMPKα2*$^{-/-}$ myotubes might have a reduced relative maximal mitochondrial oxidative capacity compared to *AMPKα2*$^{+/+}$ myotubes. This can possibly be explained that most of the substrates taken up by the *AMPKα2*$^{-/-}$ myotubes are oxidized rather than stored. However, it is important to note that one study performed on human myocytes suggested that AMPK is involved in the regulation of mitochondrial capacity through the transcription factor peroxisome proliferator-activated receptor alpha (PPARα) [266]. Therefore, it is possible that the ablation of the AMPKα2 subunit results in a discrepancy in mechanisms regulating substrate storage which might affect the maximal mitochondrial oxidative capacity (**paper IV**). Metabolic flexibility can experimentally be measured by measuring the oxidation of radiolabelled glucose and OA in the presence of unlabelled OA and glucose, respectively. Under normal conditions there would be a suppressed oxidation of the radiolabelled substrates, which was found in the *AMPKα2*$^{+/+}$ myotubes. However, in *AMPKα2*$^{-/-}$ myotubes the oxidation of glucose and OA was not affected by the presence of unlabelled OA and glucose, which suggest that *AMPKα2*$^{-/-}$ myotubes are metabolically inflexible, and that AMPKα2 subunit may play a potential role in metabolic flexibility (**paper IV**). The role of AMPK in regulating lipid oxidation is well-known and has therefore been suggested to be an important contributor to metabolic flexibility [197]. When AMPK is activated in response to an increased AMP:ATP ratio, it ultimately results in a decreased concentration of malonyl-CoA, which causes an increased activity of CPT1 and an increased entry of FA into the mitochondria for β-oxidation [187, 197, 198]. In our study the mRNA expression of CPT1b was increased in the *AMPKα2*$^{-/-}$ myotubes, which may explain the increased OA oxidation seen in these cells. Thus, suggesting that AMPKα2 may play a role in regulating metabolic flexibility in skeletal muscle cells.

Lipid storage and turnover

LDs are considered to be highly dynamic organelles and are the major storage form of lipids. Dysregulation of LDs pose an important problem when it comes to the alterations in skeletal muscle lipid metabolism seen in subjects with obesity and T2D [39]. LDs mainly consists of DAG, TAG and cholesterol [109]. Due to their dynamics, LDs alternate between storage and breakdown by lipases in order to generate FAs for mitochondrial β-oxidation, and thereby can adapt to the metabolic demand of the cell [121]. Furthermore, the FAs generated from lipolysis can also be re-incorporated back into TAG molecules [267].

In **paper III**, the effects of inhibiting the enzymes responsible for catalysing the final step of TAG synthesis, DGAT1 and DGAT2, were studied in cultured human skeletal muscle cells. It has previously been shown that DGAT1 and DGAT2 exhibits different roles in various tissues [256, 268, 269], but their role in skeletal muscle has not been well-defined. In this study, the isozyme-specific roles of the DGAT enzymes in skeletal muscle lipid metabolism was explored by using selective inhibitors of DGAT1 and DGAT2. It was found that DGAT1 is the dominant enzyme responsible for incorporating exogenous and endogenously generated FAs into TAG. Whereas, DGAT2 is predominately involved in *de novo* synthesis of TAG. These results suggests that the two DGAT enzymes gives rise to different pools of TAG in skeletal muscle. Interestingly, inhibition of the two DGAT enzymes did not result in DAG accumulation (Figure 4), which is a lipid intermediate that has previously been linked to skeletal muscle insulin resistance [14, 114]. The specific roles of the two DGAT enzymes might be a contributing factor to the biphasic relationship between IMTG and insulin sensitivity; IMTG is associated with both high insulin sensitivity in endurance-trained individuals and low insulin sensitivity in individuals with obesity and T2D [40, 270, 271]. In short, in **paper III** we found that inhibition of DGAT1 resulted in an increased oxidation of FAs, but a reduced accumulation and incorporation of exogenous lipids into DAG and TAG (Figure 4). Whereas, inhibiting DGAT2 altered the FA oxidation rates but had no profound effects on FA accumulation and incorporation into complex lipids.

Using myotubes generated from *AMPKα2*$^{-/-}$ mice, we demonstrated that AMPKα2 plays an important role in lipid storage in skeletal muscle cells. In **paper IV,** we found that ablation of AMPKα2 resulted in a decreased accumulation and incorporation of lipids into TAG and DAG (Figure 4). Similar to the findings in *AMPKα2*$^{-/-}$ myotubes, human myotubes treated with a DGAT1 inhibitor exhibited a reduced DAG and TAG accumulation (**paper III)**. Although these findings are similar, the mechanisms underlying these effects in *AMPKα2*$^{-/-}$

myotubes are not fully understood. In **paper III**, an enzyme (DGAT1) with known function was inhibited, whereas in **paper IV** the regulatory subunit of an upstream kinase was ablated. Due to AMPK being an upstream kinase known to regulate many metabolic factors, it is difficult to estimate which of the downstream targets of AMPK were affected by the ablation of AMPKα2. It is also important to consider that human myotubes were only treated with DGAT1 inhibitor for up to 24 hours, while the *AMPKα2*$^{-/-}$ mice were born with this defect. Therefore, we are looking at a relatively short time effect (**paper III**) versus a long time effect (**paper IV**) and it is unlikely that the decreased incorporation and accumulation of DAG and TAG are caused by inhibition of DGAT1 in the *AMPKα2*$^{-/-}$ myotubes. Moreover, the mRNA expression of the LD coating protein, PLIN3 was significantly downregulated in the *AMPKα2*$^{-/-}$ myotubes (**paper IV**). Proteomics analysis also revealed a downregulation of PLIN3 and PLIN4. A previous study in our lab found that the loss of PLIN2 in myotubes resulted in reduced accumulation of lipids and decreased incorporation of lipids into TAG and DAG, which was explained by an increased lipolysis [272]. Although lipolysis was not measured in **paper IV,** the *AMPKα2*$^{-/-}$ myotubes had a decreased expression of ATGL, the enzyme catalysing the first step of lipolysis. Thus, the decreased lipid accumulation and incorporation into TAG and DAG seen in *AMPKα2*$^{-/-}$ myotubes cannot be explained by an increased lipolysis. Moreover, one study using *Plin4*$^{-/-}$ mice found a decreased cardiac accumulation of TAG [273]. Although the reduced TAG accumulation was not found in soleus muscle of *PLIN4*$^{-/-}$ [273], it is interesting due to our finding of a reduced lipid incorporation into TAG in *AMPKα2* $^{-/-}$ myotubes. It is therefore possible that AMPK and Plin4 are important for the utilization of exogenous lipids for the synthesis of TAG. To summarise the findings in **paper IV**, ablation of the catalytic AMPKα2 subunit resulted in an increased substrate oxidation, but a reduced maximal mitochondrial oxidative capacity and a decreased metabolic flexibility. *AMPKα2*$^{-/-}$ myotubes also exhibited a reduced accumulation of FAs and a decreased incorporation of exogenous FAs into DAG and TAG (Figure 4).

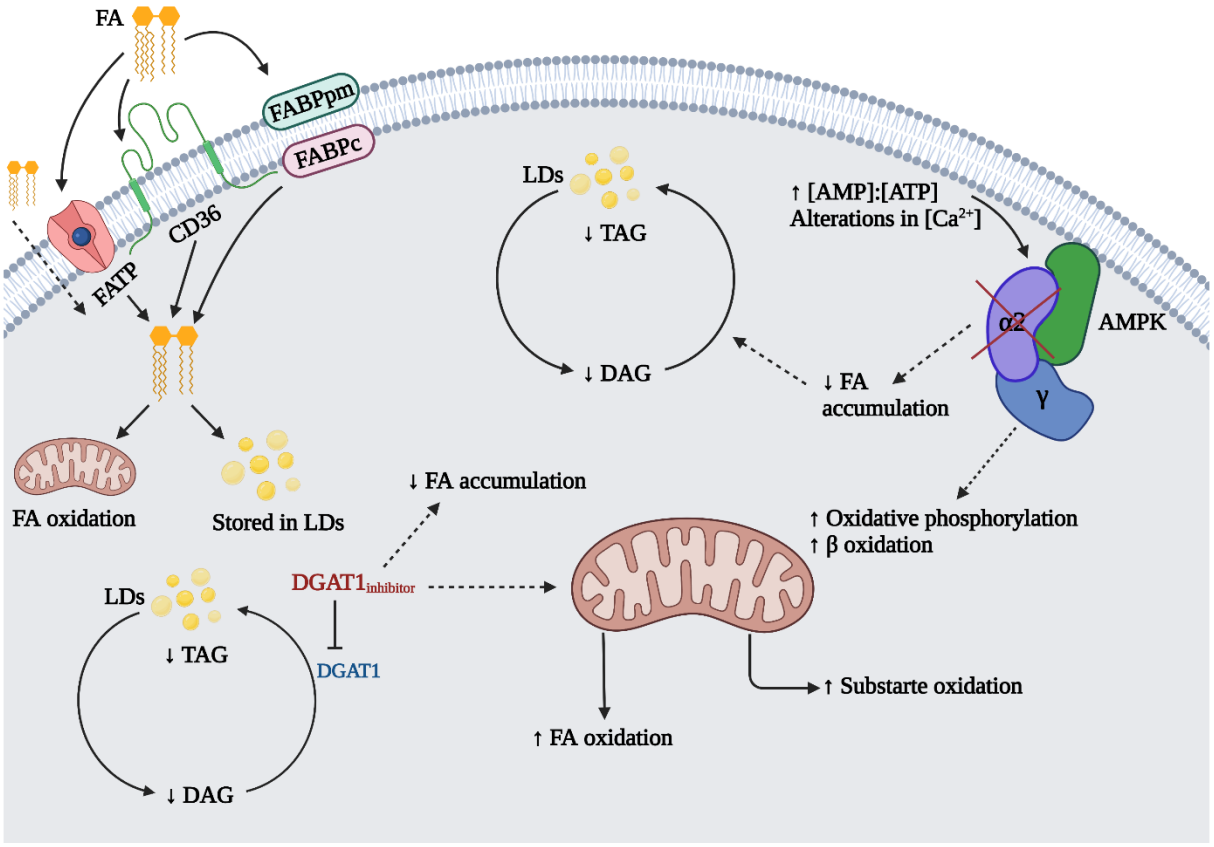


Figure 4. Effects of diacylglycerol acyltransferase (DGAT)1 inhibition and ablation of the α2 subunit of the adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) on energy metabolism in skeletal muscle cells. Fatty acids (FA) are taken up into cells by three different transporter proteins; FATP, CD36 and FABPpm. Intracellularly, FAs in skeletal muscle can either be oxidized or be incorporated into diacylglycerol (DAG) and triacylglycerol (TAG) and stored as lipid droplets (LDs). Inhibiting DGAT1, the enzyme catalysing the terminal step of TAG synthesis, resulted in an increased FA oxidation, but a reduced accumulation of FA and a decreased incorporation of FAs into DAG and TAG. AMPK is an enzyme which has shown to be an important regulator of several metabolic processes, including glucose uptake and lipid oxidation. Ablation of the catalytic α2 subunit of the AMPK enzyme resulted in an upregulation of oxidative pathways in the mitochondria which further increased the substrate oxidation. *AMPKα2*$^{-/-}$ myotubes also exhibited a decreased FA accumulation and a reduced incorporation of exogenous FAs into TAG and DAG compared to *AMPKα2*$^{+/+}$ myotubes. Fatty acid transporter protein (FATP); fatty acid translocase (CD36); plasma membrane-associated fatty acid binding protein (FABPpm)

Hypertrophic effects in skeletal muscle

The hypertrophic effects of β_2-AR agonists are well-known, and are believed to be the result of an increased protein synthesis and a decreased protein breakdown resulting in a positive net protein balance [274-276]. Because of these properties, β2-AR agonists have previously been applied in livestock production, physiological and pharmacological studies, as well as being misused for growth-promoting purposes [130, 137, 277, 278]. In **paper I**, we did find an

increased incorporation of leucine into cellular protein in terbutaline-treated myotubes, which suggests that protein synthesis in these cells were increased. Moreover, it was found that the mRNA and protein level of MYH7 were downregulated in terbutaline-treated myotubes. Several studies that have reported a transition from slow- to fast-twitch phenotype following chronic β_2-AR agonist treatment, and therefore the downregulation of slow-twitch associated MYH7 is in line with previous reports [136, 279-281].

Conclusion

The main findings in the work presented in this thesis are summarized as follows:

- Both short- and long-term treatment with the β_2 adrenergic agonist, terbutaline, resulted in several beneficial metabolic effects in human skeletal muscle (**paper I**).

  I. A) 4 hours treatment with terbutaline increased glucose uptake in human myotubes.

  B) Long-term treatment with terbutaline (96 hours) increased protein synthesis, as well as glucose and oleic acid oxidation in skeletal muscle cells.

  C) Proteomics analysis revealed an increase in mitochondrial proteins following 96 hours treatment with terbutaline.

- The cold-sensing ion channels TRPA and TRPM8 were found to be functionally expressed in cultured human skeletal muscle. Activation of these ion channels had varying effects on glucose metabolism (**paper II**).

  II. A) Activation of TRPA1 and TRPM8 by chemical agonists increased intracellular Ca^{2+} levels.

  B) Activation of TRPA1 by ligustilide increased glucose uptake and oxidation.

  C) Activation of the TRPM8 ion channel with icilin and menthol decreased glucose uptake and oxidation.

  D) The effects seen on glucose metabolism did not seem to be mediated by genes involved in inducing thermogenesis.

- The two isozymes of diacylglycerol acyltransferases (DGAT) 1 and 2 exhibited distinct roles in lipid metabolism in human myotubes (**paper I**).

  III. A) DGAT1 was responsible for the utilization of exogenous and endogenous FAs for TAG re-synthesis.

  B) The primary role of DGAT2 was to contribute to *de novo* synthesis of TAG in skeletal muscle.

- Deleting the α2 subunit of AMPK enzyme resulted in several alteration of metabolic functions and pathways (**paper IV).**

IV. A) The oxidation of glucose and oleic acid was higher in myotubes generated from *AMPKα2*$^{-/-}$ mice than in myotubes from *AMPKα2*$^{+/+}$ mice, but *AMPKα2*$^{-/-}$ myotubes exhibited a decreased response to mitochondrial uncoupling and abolished metabolic flexibility.

B) There was a decreased incorporation of exogenous lipids into TAG and DAG in myotubes generated from *AMPKα2*$^{-/-}$ mice.

C) Proteomics analysis revealed an upregulation of oxidative pathways in the *AMPKα2*$^{-/-}$ myotubes compared to *AMPKα2*$^{+/+}$ myotubes.

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Chronic treatment with terbutaline increases glucose and oleic acid oxidation and protein synthesis in cultured human myotubes

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ABSTRACT

Objective: *In vivo* studies have reported several beneficial metabolic effects of β-adrenergic receptor agonist administration in skeletal muscle, including increased glucose uptake, fatty acid metabolism, lipolysis and mitochondrial biogenesis. Although these effects have been widely studied *in vivo*, the *in vitro* data are limited to mouse and rat cell lines. Therefore, we sought to discover the effects of the β_2-adrenergic receptor agonist terbutaline on metabolism and protein synthesis in human primary skeletal muscle cells.

Methods: Human cultured myotubes were exposed to terbutaline in various concentrations (0.01–30 μM) for 4 or 96 h. Thereafter uptake of [^{14}C]deoxy-D-glucose, oxydation of [^{14}C]glucose and [^{14}C]oleic acid were measured. Incorporation of [^{14}C]leucine, gene expression by qPCR and proteomics analyses by mass spectrometry by the STAGE-TIP method were performed after 96 h exposure to 1 and 10 μM of terbutaline.

Results: The results showed that 4 h treatment with terbutaline in concentrations up to 1 μM increased glucose uptake in human myotubes, but also decreased both glucose and oleic acid oxidation along with oleic acid uptake in concentrations of 10–30 μM. Moreover, administration of terbutaline for 96 h increased glucose uptake (in terbutaline concentrations up to 1 μM) and oxidation (1 μM), as well as oleic acid oxidation (0.1–30 μM), leucine incorporation into cellular protein (1–10 μM) and upregulated several pathways related to mitochondrial metabolism (1 μM). Data are available via ProteomeXchange with identifier PXD024063.

Conclusion: These results suggest that β_2-adrenergic receptor have direct effects in human skeletal muscle affecting fuel metabolism and net protein synthesis, effects that might be favourable for both type 2 diabetes and muscle wasting disorders.

1. Introduction

Skeletal muscle makes up 40% of total body weight and is the main site for metabolism of glucose and lipids. During fasting phase, the breakdown of lipids is the primary source of energy, however during fed state or insulin-stimulated conditions, glucose is the preferred energy source. Therefore, skeletal muscle cells has to be able to switch rapidly between glucose and lipid metabolism (Thoresen et al., 2011).

The β-adrenergic receptors (AR) are G protein-coupled receptors which are activated by the endogenous catecholamines adrenaline and noradrenaline, and by synthetic β-AR agonists. There are three isoforms of β-AR; β_1, β_2 and β_3. The reported homology between these receptors are 65–70% (Koziczak-Holbro et al., 2019). These three isoforms have different expression patterns in various tissues and regulate different physiological functions. β_1-AR is known to increase cardiac output, while β_3-AR increases lipolysis in adipose tissue, mostly in rodent. β_2-AR has many functions, including skeletal muscle anabolism and increasing lipolysis in adipose tissue (Choo et al., 1992; Large et al., 1997). In

Abbreviations used: Adrenergic receptor (AR), Cyclic AMP (cAMP); Deoxyglucose (DOG), protein-coupled receptor (GPCR); Mammalian target of Rapamycin (mTOR), Oleic acid (OA); Protein Kinase A (PKA), Scintillation Proximity Assay (SPA); Type 2 diabetes (T2D), Trichloroacetic acid (TCA).

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skeletal muscle, the most abundant subtype is the β_2-AR, followed by β_1-AR, which accounts for approximately 10% of the adrenergic receptor population with a low population of β_3-AR (Ito et al., 2019).

Insulin increases glucose uptake in skeletal muscle through a canonical pathway, ultimately leading to GLUT4 translocation (Sato et al., 2014). However, in the setting of insulin resistance and type 2 diabetes (T2D) this pathway is impaired. Studies both *in vivo* and *in vitro* have suggested that stimulation of the β-AR mediates glucose uptake in skeletal muscle, possibly through an alternative pathway (Nevzorova et al., 2006; Sato et al., 2014; Ziegler et al., 2012). β-AR agonists are also reported to play a role in muscle anabolism by increasing protein synthesis while decreasing protein degradation (Hesketh et al., 1992; Maltin et al., 1989; Yimlamai et al., 2005).

Synthetic β_2-AR agonists are widely used in the treatment of chronic obstructive pulmonary disease and asthma by inducing bronchodilation (Solis-Cohen, 1990). However, previous research has shown that stimulation of the β_2-AR increased glucose uptake, fatty acid metabolism and muscle hypertrophy both *in vivo* and *in vitro* (Acheson et al., 1988; Hostrup et al., 2020; Hostrup et al., 2015; Nevzorova et al., 2006; Sato et al., 2014; Ziegler et al., 2012). Many β_2 agonists and their optical isomers are on WADAs list of prohibited substances due to these properties (Agency, 2020; Morten Hostrup et al., 2020). However, these properties are also what possibly could make them suitable as potential drugs to treat T2D and muscle wasting conditions (Fan and Evans, 2017; Guerrieri et al., 2017).

T2D develops when pancreatic beta cells fail to produce sufficient insulin to compensate for insulin resistance. Skeletal muscle is responsible for 75% of whole body glucose uptake and utilization mediated by insulin and therefore improvement of glucose disposal in skeletal muscle likely has great impact on diabetes (reviewed in Thoresen et al., 2011). Earlier studies have shown that glucose uptake could also be mediated through an insulin-independent pathway involving β-AR signalling (Koziczak-Holbro et al., 2019; Sato et al., 2014; Ziegler et al., 2012). A study by Nevzorova et al. suggested three mechanisms by which the glucose uptake is controlled in rat skeletal muscle cells and these include; insulin-dependent pathway involving PI3K, a pathway involving both PI3K and cAMP mediated by β_2-AR activation, and a β-AR independent pathway suggested by cAMP analogues (Nevzorova et al., 2006).

Although the effects of β_2-AR stimulation have been well studied in human skeletal muscle *in vivo*, it is not established whether the effects seen *in vivo* are direct effects on skeletal muscle or whole body effects. The benefit of using an *in vitro* human muscle cell model in this study is that it would be possible to dissect mechanisms observed *in vivo* from those on muscle itself. The metabolic and hypertrophic effects of the β_2-AR agonist terbutaline have been well studied *in vivo* (Morten Hostrup et al., 2015; M. Hostrup, Onslev, Jacobson, Wilson and Bangsbo, 2018), and therefore it would be interesting to see whether its metabolic and hypertrophic effects are caused by direct metabolism in skeletal muscle. The purpose of this study was to investigate the acute and chronic metabolic effects of the β_2-AR agonist terbutaline on energy metabolism and protein synthesis in cultured human myotubes and to explore the cellular and molecular mechanisms underlying these effects.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM-Glutamax™) low glucose with sodium pyruvate, Dulbecco's phosphate buffered saline (DPBS, without Mg^{2+} and Ca^{2+}), Dulbecco's phosphate buffered saline (DPBS, with Mg^{2+} and Ca^{2+}), foetal bovine serum (FBS), penicillin-streptomycin (10000 IE/ml), Epidermal Growth Factor human (hEGF), amphotericin B, Pierce™ BCA Protein Assay Kit, Power SYBR® Green PCR Master Mix, SYBR® Green PCR primers, MicroAmp® Optical Adhesive Film, MicroAmp® Optical 96-well Reaction Plate, TaqMan® Reverse Transcription Reagents, and Halt Protease and phosphates inhibitor cocktail were from ThermoFisher Scientific (Waltham, MA, US). Terbutaline was a gift from AstraZeneca, Mölndal, Sweden. Insulin (Actrapid®) was obtained from NovoNordisk (Bagsvaerd, Denmark). Bovine serum albumin (BSA, essentially FA-free), L-carnitine, D-glucose, oleic acid (OA, 18:1, n-9), HEPES, DMSO, dexamethasone, and gentamicin were from Sigma-Aldrich (St. Louis, MO, US). [^{14}C]oleic acid (OA, 56–59 mCi/mmol), D-[^{14}C(U)]glucose (107.3 mCi/mmol), 2-[1–^{14}C] deoxy-D-glucose (45–60 mCi/mmol) and L-[^{14}C(U)]leucine (300 mCi/mmol) were purchased from PerkinElmer NEN® (Boston, MA, US). 96-well, 24-well and 6-well Corning® CellBIND tissue culture plates were from Corning (Schiphol-Rijk, the Netherlands). UniFilter®-96 GF/B microplates, Isoplate®-96 scintillation microplates, TopSeal®-A transparent film, and Ultima Gold were obtained from PerkinElmer (Shelton, CT, US). QIAshredder and RNeasy Mini kit were from QIAGEN (Venlo, the Netherlands). hEGF was from Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad (Copenhagen, Denmark).

2.2. Donor characteristics and culturing of human myotubes

All procedures performed in studies involving human participants were in accordance with the ethical standards of Regional Committee for Medical and Health Research Ethics (REK) South East, Oslo, Norway (reference number 2011/2207). The donors were healthy men, 25.8 (±1.9) years old with a body mass index of 24.2 (±1.1) kg/m^2 (Table 1), from a cohort previously described in Lund et al. (2018).

The method of isolating satellite cells has been well-described earlier (Lund et al., 2018). Satellite cells were isolated from muscle biopsies taken from *musculus vastus lateralis* from healthy young men. The satellite cells were used to establish a biobank of myoblasts, where the cells were up-scaled at different passages and cryopreserved. In this study, all experiments were performed with cells from passages 3 or 4.

The cells were cultured on multiwell plates in DMEM-Glutamax™ (5.5 mM glucose) supplemented with 10% FBS, 25 IU penicillin, 25 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 50 ng/ml gentamicin, 0.05% BSA, 10 ng/ml hEGF, 0.39 μg/ml dexamethasone and 25 mM HEPES. When the cells had grown to approximately 80% confluence, the growth medium was replaced by a differentiation medium (DMEM Glutamax™ (5.5 mM glucose) supplemented with 2% FBS, 25 IU penicillin, 25 μg/ml streptomycin, 1.25 μg/ml, amphotericin B, 50 ng/ml gentamicin, 25 mM HEPES and 25 pM insulin). The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere, and the medium was changed very 2–3 days. Acute experiments with terbutaline (0.01–30 μM) were performed after 7 days of differentiation. For chronic experiments, the cells were given differentiation medium containing terbutaline (0.01–30 μM) for 96 h on day 3 of differentiation. Terbutaline was dissolved in DMSO. Therefore, all control cells were treated with 0.01% DMSO. The total content of cellular protein in each experiment was determined. There were no changes in protein content with increasing terbutaline concentrations indicating no cell toxic effects of the treatment.

2.3. Deoxyglucose uptake

Myotubes were cultured in 24-well tissue culture plates. For chronic experiments, the cells were treated with terbutaline for the last 4 days

Table 1
Donor characteristics.

Donor	Age	Weight (kg)	Height (m)	BMI (kg/m^2)
1	21	86.4	1.79	27.0
2	24	78.3	1.81	23.9
3	28	90.0	1.86	26.0
4	34	92.8	1.91	25.4
5	24	78	1.84	23.0
6	24	70.8	1.90	19.7
Mean	25.8	82.7	1.85	24.2
SEM	1.9	3.4	0.01	1.1

(96 h) of differentiation. For acute experiments, the cells were treated with terbutaline for 4 h on day 7 of differentiation. On day 7, after incubation with terbutaline, the cells were given serum-free DMEM-Glutamax (5.5 mM glucose) containing [^{14}C]deoxy-D-glucose (1 μCi/ml, 5.5 mM glucose) and were allowed to incubate for 1 h (Vigdis Aas, Rokling-Andersen, Kase, Thoresen and Rustan, 2006). After incubation, the cells were washed twice with PBS before being lysed in 0.1 M NaOH. The amount of protein per well was determined according to Bradford (1976) using BSA as a reference protein. Radioactivity accumulated in the cells was measured by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer) and the amount of deoxyglucose accumulated was related to total cell protein content.|

2.4. Substrate oxidation assay for measurement of glucose and oleic acid metabolism

Skeletal muscle cells (7000 cells/well) were cultured on 96-well CellBIND® microplates. For chronic experiments, the cells were treated with different concentrations of terbutaline for 96 h. To measure glucose oxidation, the cells were given [^{14}C]glucose (0.5 μCi/ml, 200 μM) or [^{14}C]oleic acid (0.5 μCi/ml, 100 μM) with and without terbutaline on day 7 of differentiation. The substrates were added in DPBS containing 10 mM HEPES, 10 μM BSA and 1 mM L-carnitine. To measure fatty acid oxidation from pre-labelled intracellular lipid pools, myotubes were given [^{14}C]oleic acid (0.5 μCi/ml, 100 μM) for 24 h on day 6 of differentiation. Myotubes were then washed with PBS containing 0.5% BSA and given DPBS containing 10 mM HEPES, 10 μM BSA and 1 mM L-carnitine with and without terbutaline. The myotubes then followed a 4 h trapping procedure as previously described (Wensaas et al., 2007). A 96-well UniFilter® microplate, soaked with NaOH (1 M), was mounted on top of the CellBIND® plate and the produced CO_2 was trapped during 4 h at 37 °C. Cell-associated labelled oleic acid and CO_2 were measured by liquid scintillation using a PerkinElmer 2450 MicroBeta^2 scintillation counter (PerkinElmer). The amount of protein per well was determined using Bradford protein assay. The sum of $^{14}CO_2$ and the remaining cell-associated (CA) radioactivity reflects the total cellular uptake of the substrate.

2.5. Leucine incorporation into protein

Cells were cultured in 24-well tissue culture plates. After 3 days of differentiation, the cells were incubated with two different concentrations of terbutaline (1 or 10 μM) for 96 h. On day 7 of differentiation, the cells were incubated in differentiation medium containing terbutaline and [^{14}C]leucine (1 μCi/ml, 0.8 mM) for 24 h. The cells were then washed with PBS and lysed in 0,01% SDS. The amount of protein per well was determined by Pierce BCA protein assay. Protein from the cell lysates was precipitated with 1% BSA in 50% trichloroacetic acid overnight at −20 °C. The next day, the cell lysates were centrifuged to form a protein pellet. The pellet was washed in acetone before being centrifuged, air-dried and resuspended in SDS-NaOH. Radioactivity was measured by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer), and the amount of labelled protein was normalized to the total cell protein content.

2.6. Scintillation proximity assay

Scintillation proximity assay (SPA) is a method to measure real time accumulation of radiolabelled substrates by adherent cells. Radioactivity concentrated closer to the scintillator embedded in the plastic bottom of each well (ScintiPlate®-96 TC, PerkinElmer) provides a stronger signal than the radiolabelled substrate in the culture medium (Wensaas et al., 2007). Myotubes were cultured on 96-well ScintiPlate® tissue culture plates, and SPA was used to measure leucine incorporation into protein. The cells were treated with terbutaline for 96 h on day 3 of differentiation. On day 7 of differentiation the cells were given DMEM without phenol red supplemented with 5.5 mM glucose, 2% FBS, 25 IU penicillin, 25 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 25 pM insulin and [^{14}C]leucine (0.5 μCi/ml, 0.8 mM) in the presence or absence of terbutaline. The time course for [^{14}C]leucine incorporation was measured by a PerkinElmer 2450 MicroBeta^2 scintillation counter (PerkinElmer). The plate was counted at 0, 2, 3, 6, 8, 12 and 24 h during the 24 h incubation. Thereafter, the cells were washed twice with 10 μM BSA in DPBS (with Mg^{2+} and Ca^{2+}), before new media containing DPBS with 10 mM HEPES, 0.5% BSA, and 0.1 mM glucose were added. Liquid scintillation measurements were monitored at 0, 2, 4 and 6 h in order to investigate the decay of [^{14}C]leucine in the myotubes. The amount of protein per well was determined according to Bradford, and the amount of protein was normalized to total cell protein content.

2.7. RNA isolation and analysis of gene expression by qPCR

Total RNA was isolated from myotubes by using Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The quantity of RNA was determined using a Nanodrop ND-1000 (Thermo Scientific). RNA was reversely transcribed with a High-Capacity cDNA Reverse Transcription and TaqMan Reverse Transcription Reagents using a PerkinElmer 2720 Thermal Cycler (25 °C for 10 min, 37 °C for 80 min, 85 °C for 5 min). Primers were designed using Primer Express® (Applied Biosystems). qPCR was performed using a StepOnePlus Real-Time PCR system (Applied Biosystems). Both TaqMan and SYBR primers were used. Target genes quantified by SYBR was quantified in duplicates carried out in 25 μL reaction volume according to the supplier's protocol. While the target genes quantified by TaqMan was quantified in duplicates carried out in 18 μl reaction volume according to the supplier's protocol. All assays were run for 44 cycles *(95°C for* 15 s *followed by 60°C for* 60 s). TaqMan primers: *ADRB1* (Beta-1 adrenergic receptor), *ADRB2* (Beta-2 adrenergic receptor), *ADRB3* (Beta-3 adrenergic receptor) and *TBP* (TATA-binding protein). Expression levels were normalized to the average of the housekeeping gene *TBP*. The Taqman primers used were a gift from AstraZeneca, Mölndal, Sweden. The following forward and reverse primers, for SYBR quantification, were used at a concentration of 30 μmol/L: large ribosomal protein P0 (*RPLP0*, acc. no. M17885); matrix metalloproteinase-2 (MMP-2, acc. no. NM_004530.6); Myosin heavy chain 3 (MYH3, acc. no. NM_002470.4); Myosin heavy chain 7 (MYH7, acc. no. NM_000257.2); Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, acc. no. NM_013261.3); Solute carrier family 2 member 5 (GLUT5, acc. no. NM_001328619.2).

2.8. Proteomics analysis

Human skeletal muscle cells were seeded out, grown and differentiated in 25 cm^2 flasks. On day 3 of differentiation the cells were treated with either 0.01% DMSO or 1 μM terbutaline. At day 7 of differentiation the cells were washed in DPBS (with Mg^{2+} and Ca^{2+}), harvested by scraping, washed in DPBS and spun down at 1000 rpm at 4 °C for 15 min. The cells were then resuspended in DPBS, and frozen at −80 °C.

The cells were lysed using 150 μl of RIPA buffer containing protease inhibitor followed by protein aggregation CAPTURE (ref PMID: 30833379), and protein reduction, alkylation and digestion into peptides with trypsin. Resulting peptides were desalted and concentrated before mass spectrometry by the STAGE-TIP method using a 3M Empore™ C18 resin disc. Each peptide mixture was analyzed by a nEASY-LC coupled to QExactive HF with EASY Spray PepMap® RSLC column (C18, 2 μl, 100 Å, 75 μm × 25 cm) using 90 min LC separation gradient. Resulting MS raw files were submitted to the MaxQuant software version 1.6.7.0 for protein identification and label-free quantification. Carbamidomethyl (C) was set as a fixed modification and acetyl (protein N-term), carbamyl (N-term) and oxidation (M) were set as variable modifications. First search peptide tolerance of 20 ppm and main search error 4.5 ppm were used. Trypsin without proline restriction enzyme option was used, with two allowed

miscleavages. The minimal unique + razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. Label-free quantitation was employed with default settings. UniProt database with 'human' entries (September 2018) was used for the database searches. Known contaminants as provided by MaxQuant and identified in samples were excluded from further analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD024063.

2.9. Presentation of data and statistics

Data are presented as mean ± SEM unless specified in the figure legends. Each experiment was performed with myotubes from at least 3 different donors, with at least 3 biological replicates in each experiment. Statistical analysis was performed using GraphPad Prism 8.0.1 Software (GraphPad Software Inc., La Jolla, CA, US). Unpaired *t*-test was performed to determine effects of treatment, where $p < 0.05$ was considered significant.

MaxQuant data was further analyzed using Perseus version 1.6.1.3, where two-tailed paired *t*-test was performed to determine differences in protein expression caused by the treatment. Pathway analysis was done using Ingenuity Pathway Analysis (IPA). Principal component analysis (PCA) done in Perseus revealed that one of six donors was an outlier, and this donor was excluded from *t*-test and further bioinformatic analysis by IPA.

3. Results

3.1. Expression of the β-adrenergic receptors

First we determined the mRNA expression levels of the three β-ARs in our cell model. Both β_1-AR (ADRB1) and β_2-AR (ADRB2) were expressed in human myotubes, where β_2-AR was most abundant (Fig. 1). Moreover, β_3-AR mRNA was not detected.

3.2. Acute effects of terbutaline on glucose and oleic acid metabolism

The effects of terbutaline on deoxyglucose uptake (DOG) and glucose oxidation, and on oleic acid (OA) uptake and oxidation were studied in cultured human myotubes. DOG uptake by the cells was significantly increased after 4 h treatment with 0.01, 0.1 and 1 μM terbutaline (Fig. 2A). On the other hand, terbutaline treatment caused a concentration-dependent decrease in glucose oxidation at 10 μM (Fig. 2B). Uptake of OA was significantly decreased by myotubes after being treated for 4 h with 0.01, 1 and 30 μM terbutaline (Fig. 2C), while oxidation of OA was significantly decreased at 30 μM terbutaline (Fig. 2D).

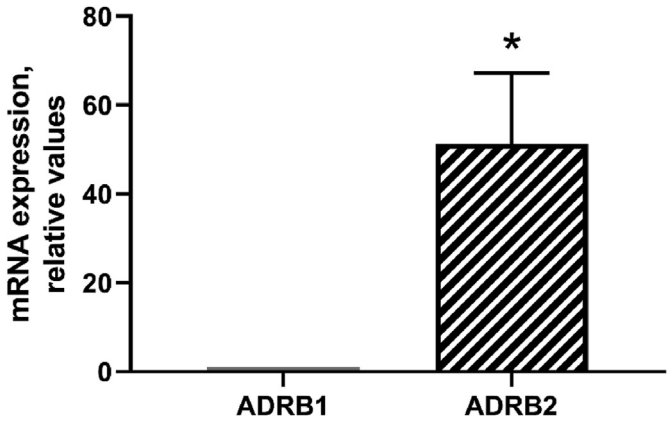

Fig. 1. mRNA expression of β-adrenergic receptors in human primary myotubes. Human myoblasts were seeded out, proliferated, and differentiated into myotubes on 6-well tissue culture plates. On day 7 of differentiation, the cells were harvested for PCR. mRNA was isolated and expression assessed by qPCR. Expression levels were normalized to the housekeeping gene TATA-binding box protein (TBP). ΔCt values were calculated and the expression of ADRB2 was related to the expression of ADRB1. Values are presented as mean ± SEM from n = 5 individual experiments with myotubes derived from 3 different donors. *p‹0.05 vs ADRB1, paired *t*-test.

3.3. Effects of chronic terbutaline exposure on glucose and oleic acid metabolism

To study the chronic effect of terbutaline on energy metabolism, the myotubes were treated with various concentrations of terbutaline for 96 h and glucose and oleic acid metabolism were studied. The DOG uptake was significantly increased after 96 h treatment with 0.01, 0.1 and 1 μM of terbutaline (Fig. 3A), and glucose oxidation was increased at 1 μM terbutaline (Fig. 3B and C). The cellular uptake of OA was not significantly changed by terbutaline (Fig. 3D). After chronic terbutaline treatment, oxidation of [^{14}C]oleic acid when added for 4 h or 24 h was studied. When [^{14}C]oleic acid was added for 4 h, OA oxidation was increased at the highest concentration tested, 30 μM (Fig. 3E and F). However, after pre-labelling with [^{14}C]oleic acid for 24 h, OA oxidation was significantly increased for all terbutaline concentrations examined (Fig. 3G).

3.4. Chronic terbutaline treatment increased leucine incorporation into cellular protein

To determine the effect of chronic terbutaline treatment on protein synthesis, incorporation of [^{14}C]leucine into cellular protein was measured. The myotubes were treated with 1 or 10 μM terbutaline for 96 h, before [^{14}C]leucine together with terbutaline was added for 24 h. It was observed that both concentrations of terbutaline significantly increased incorporation of [^{14}C]leucine into cellular protein (Fig. 4B). To further explore the effects on protein synthesis, a scintillation proximity assay (SPA) was used to study real-time accumulation of radiolabelled leucine in the cells. Myotubes were pre-treated with 1 μM terbutaline, before [^{14}C]leucine plus terbutaline was added for 24 h. Already after 2 h, accumulation of leucine was significantly increased by terbutaline and this continued for all time points up to 24 h (Fig. 4C). Following the 24 h accumulation of [^{14}C]leucine into cellular protein, the decay of [^{14}C]leucine was monitored for 6 h. Although, the amount of [^{14}C]leucine was initially higher in the cells treated with 1 μM terbutaline compared to the control cells, the decay of [^{14}C]leucine was similar for the two groups (Fig. 4D).

3.5. Proteomics revealed an increase in mitochondrial metabolism after chronic terbutaline treatment

To have a global overview of the effect of chronic terbutaline treatment, the proteomes of myotubes treated with terbutaline (1 μM) for 96 h or control (DMSO 0.01%) were examined using quantitative label-free proteomics. This analysis detected more than 3300 proteins, of which 101 were statistically significantly upregulated (Supplementary Tables 1) and 63 were downregulated (Supplementary Table 2) after treatment with terbutaline. The proteins that were more than two-fold up- or downregulated are presented in Table 2. The third most upregulated (3.7-fold) protein in the dataset was SLC2A5 (GLUT5), a fructose transporter. Several mitochondrial proteins (nudix hydrolase 9 (NUDT9), mitochondrial 39S ribosomal protein L44 (MRPL44), and NADH dehydrogenase 1 beta subcomplex subunit 10 (NDUFB10)) were upregulated at least 2-fold. Several proteins related to the contractile apparatus, e.g. myosin-binding protein H (MYBPH), myosin 3 and 7 (MYH3, MYH7), myosin light chain (MYL4) and myosin light chain 2, skeletal muscle isoform (MYLPF) were significantly downregulated by terbutaline treatment (Table 2).

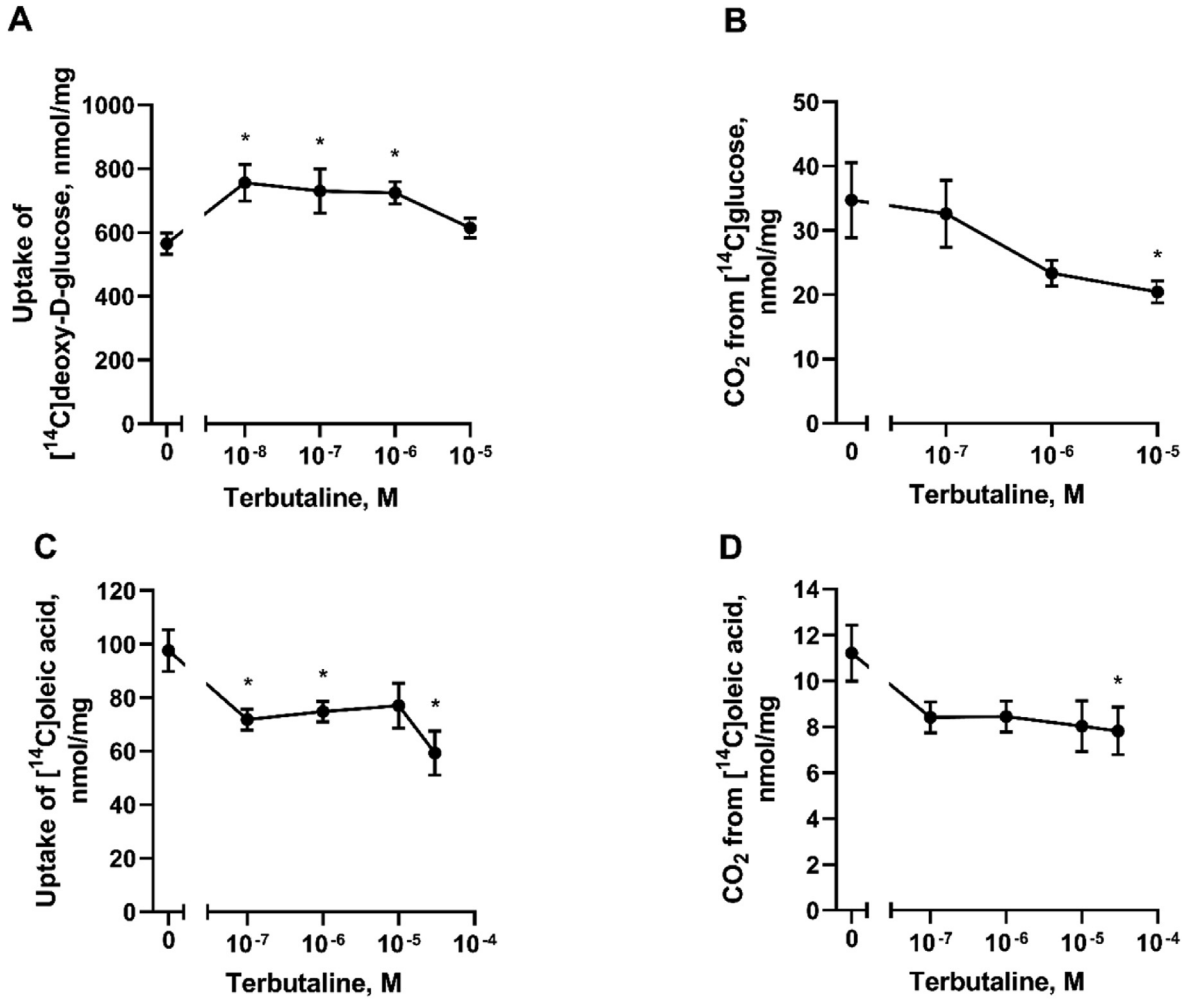

Fig. 2. Effect of 4 h treatment with terbutaline on glucose and oleic acid metabolism in human myotubes. Human myoblasts were grown and differentiated into myotubes for 7 days. **A:** The cells were treated with DMSO (0.01%) control or terbutaline for 4 h before the uptake of [^{14}C]deoxy-D-glucose (1 μCi/ml, 5.5 mM glucose) was measured for 1 h. **B:** The cells were incubated with 200 μM [^{14}C]glucose (0.5 μCi/ml) ± different concentrations of terbutaline for 4 h, and CO2 production from [^{14}C]glucose oxidation was measured. **C** and **D:** The cells were incubated with 100 μM [^{14}C]oleic acid (0.5 μCi/ml) ± different concentrations of terbutaline for 4 h, uptake **(C)** and oxidation **(D)** of [^{14}C]oleic acid were measured. Results are presented as means ± SEM of 3 experiments with myotubes derived from 3 different donors, with 3 (**A**) and 8 biological replicates in each experiment (**B**, **C** and **D**). *p˂0.05 vs control, unpaired *t*-test.

Pathway analysis revealed a significant upregulation of metabolic pathways related to the mitochondria and fatty acid β-oxidation in cells treated with terbutaline (Fig. 5A). The signalling pathways regulated include sirtuin signalling pathway, calcium signalling, protein kinase A (PKA) signalling, cardiac hypertrophy signalling and phospholipase C signalling (Fig. 5B). An overall upregulation was found for the sirtuin pathway, while an overall downregulation of the calcium signalling pathway was found, and the same was seen for protein kinase A, cardiac hypertrophy and phospholipase C signalling pathways.

3.6. Gene expression of selected genes of interest from the proteomic results

Following the findings from the proteomic data we wanted to study the mRNA expression of some genes of interest after treatment with terbutaline for 96 h. One gene of interest was SLC2A5 (GLUT5), which was the third most upregulated protein in the dataset. Most of the metabolic pathways that were upregulated were related to the mitochondria. Therefore, the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is considered the master regulator of mitochondrial biogenesis, was measured. Many of the downregulated proteins were related to the contractile apparatus, e.g. myosin heavy chain 3 (MYH3) and myosin heavy chain 7 (MYH7). Several studies have reported that the matrix metalloproteinase-2 (MMP-2) plays a major role in skeletal muscle hypertrophy (Q. Zhang et al., 2015). Therefore, we investigated gene expression of MMP-2 in response to terbutaline treatment as a marker of skeletal muscle hypertrophy. In line with the proteomics results, terbutaline treatment induced a significant an increase in the expression of GLUT5 and PGC-1α, as well as a decrease in the expression of MYH3 and MYH7 (Fig. 6). The expression of MMP-2 was not changed.

4. Discussion

In this study, we present the effects of a β-AR agonist, terbutaline, on energy metabolism and protein synthesis in primary human skeletal muscle cells. The acute (4 h) actions of terbutaline included an increased glucose uptake, which was accompanied by a decrease in OA uptake in myotubes. Oxidation of both glucose and OA were decreased. Moreover, chronic (96 h) treatment of myotubes with terbutaline increased oxidation of both glucose and OA, increased the uptake of glucose, while also increasing incorporation of leucine into cellular protein. We also performed proteomics analysis on cells chronically treated with terbutaline. To our knowledge this is the first study that has looked at changes in the proteome of primary human myotubes following chronic treatment with a β-AR agonist. The proteomics analysis revealed an upregulation of several proteins involved in mitochondrial oxidation and fatty acid

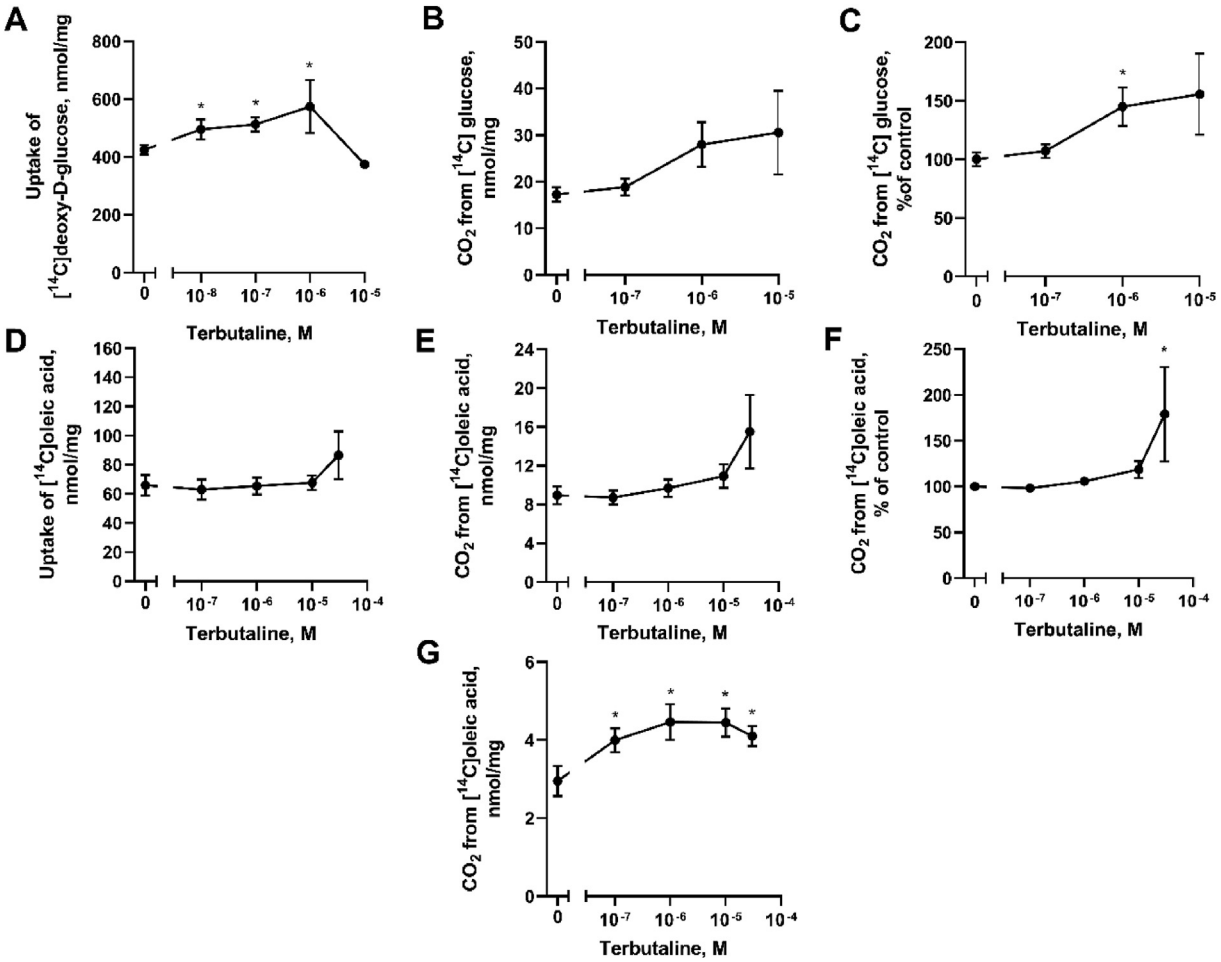

Fig. 3. Effect of 96 h terbutaline treatment with terbutaline on glucose and oleic acid metabolism in human myotubes. Myoblasts were grown and differentiated into myotubes for 7 days. On day 3 of differentiation the cells were treated with DMSO (0.01%) control or terbutaline for 96 h. On day 7 of differentiation: **A:** The uptake of [^{14}C]deoxy-D-glucose (1 μCi/ml, 5.5 mM glucose) was measured for 1 h. **B** and **C:** The cells were incubated with 200 μM [^{14}C]glucose (0.5 μCi/ml), and oxidation, the CO_2 production of [^{14}C]glucose, was measured for 4 h. In C, the results are presented as means ± SEM normalized to control. **D** and **E:** The cells were incubated with 100 μM [^{14}C]oleic acid (0.5 μCi/ml) for 4 h, and uptake (**D**) and oxidation (**E** and **F**) of [^{14}C]oleic acid was determined. In F, the results are presented as means ± SEM normalized to control. **G:** On day 6 the cells were pre-labelling with 100 μM [^{14}C]oleic acid (0.5 μCi/ml) for 24 h, and oxidation was measured for 4 h. Results are presented as means ± SEM of 3 experiments with myotubes derived from 3 different donors, with 3 (**A**) and 8 biological replicates in each experiment (**B**, **C**, **D**, **E**, **F** and **G**). *p‹0.05 vs control, unpaired *t*-test.

β-oxidation, and a downregulation of several proteins related to the contractile apparatus. The most predominately upregulated pathways were related to mitochondrial metabolism, in particular oxidative phosphorylation and TCA cycle, and fatty acid β-oxidation.

The expression of β-AR subtypes in human myotubes was confirmed by qPCR, which showed that β_2-AR is the predominant β-AR in myotubes. This is in line with what has been reported previously, however the relative expression of β_1-AR compared to β_2-AR was lower in this cell model compared to what has been reported from *in vivo* studies (Ito et al., 2019; Koziczak-Holbro et al., 2019). Although the expression of β_1-AR in our cell model is low, we cannot rule out a minor involvement from the β_1-AR in this study. In previous studies it has been shown that despite terbutaline having a lower affinity for β_1-ARs, the receptor can be stimulated by high concentrations of terbutaline (Baker, 2010).

Skeletal muscle is a major consumer of fatty acids and glucose, and shows a high metabolic flexibility where it has to switch rapidly between fatty acid and glucose metabolism under healthy condition. Glucose uptake, measured as DOG uptake, was increased both after acute and chronic treatment with terbutaline. This has previously been well-studied *in vivo*, but *in vitro* studies have mainly been limited to mouse (Ito et al., 2019) and rat (Kalinovich et al., 2020; Koziczak-Holbro et al., 2019; Nevzorova et al., 2006; Sato et al., 2014) skeletal muscle cells with only few reports from human myotubes (Koziczak-Holbro et al., 2019; Sato et al., 2014). It is important to note that there are issues of translation between studies performed in rodent cells and human cells due to rodents having a higher energy expenditure and metabolic flux than human cells (Wang et al., 2012). One study showed a similar concentration-dependent increase in glucose uptake in rat L6 myotubes after acute treatment with clenbuterol (Kalinovich et al., 2020). Although their results showed a higher increase in glucose uptake compared to results from our study, this could be explained by the differences in cell model, compounds, and concentrations used (Abdelmoez et al., 2020; Kalinovich et al., 2020).

Glucose uptake in skeletal muscle cells is reliant on GLUT transport proteins, one of which is GLUT4 and whose translocation to the plasma membrane increases in response to exercise and muscle contraction (Richter and Hargreaves, 2013). Moreover, GLUT4 is universally known as the insulin-regulated glucose transporter and it has been of great interest to pharmacologically increase its expression in order to increase glucose uptake. Previous research from our group has shown that human myotubes in culture express low levels of GLUT4 (V. Aas et al., 2013; Feng et al., 2014). This is line with what has been found in other studies comparing GLUT4 expression in human primary skeletal muscle cells and the skeletal muscle cell lines, L6 and C2C12 (Abdelmoez et al., 2020).

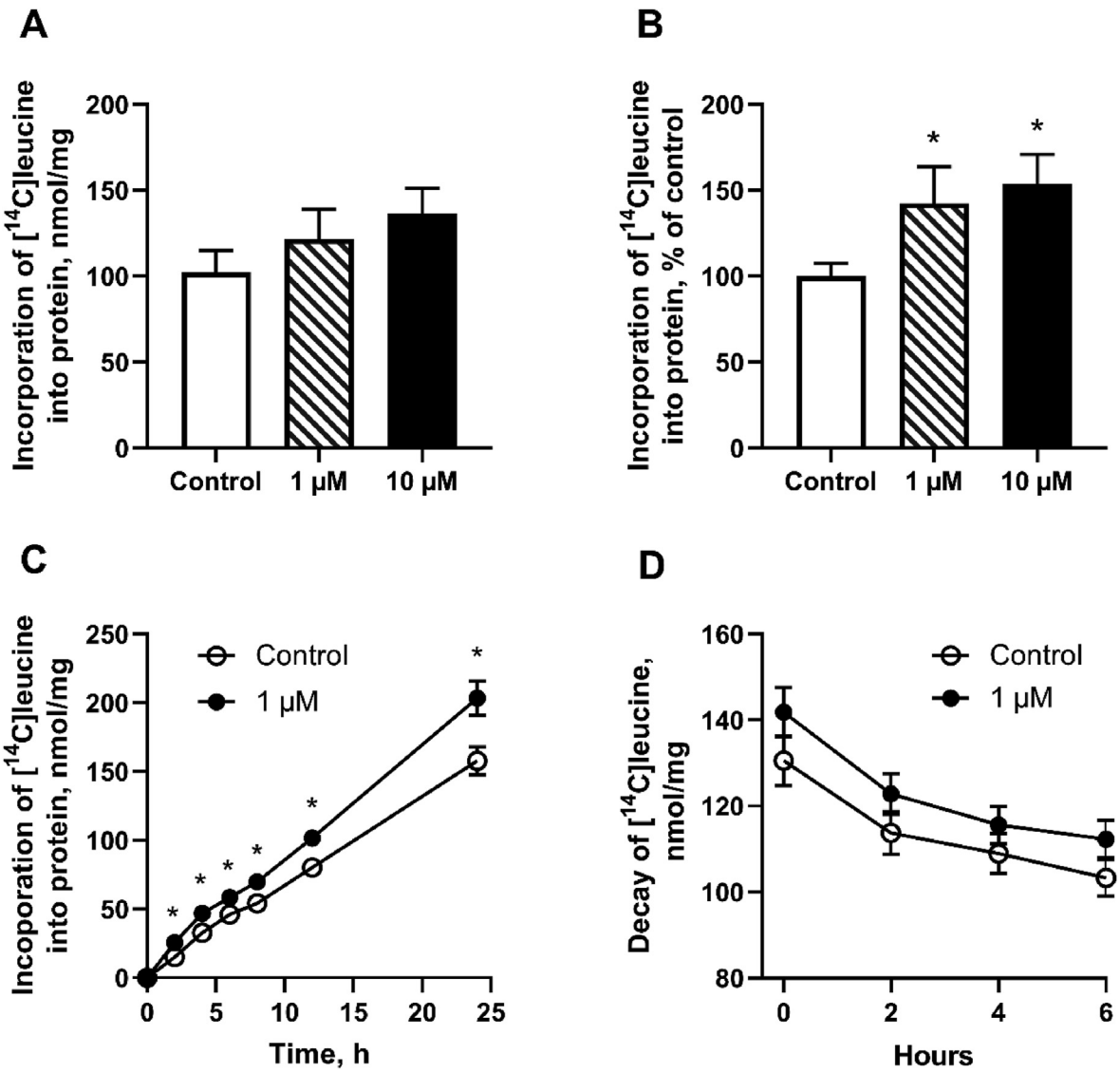

Fig. 4. Effect of 96 h terbutaline treatment on protein synthesis. Human myoblasts were grown and differentiated into myotubes. On day 3 of differentiation, the myotubes were treated with DMSO (0.01%) control or terbutaline (1 and 10 μM) for 96 h, before the cells were incubated with [^{14}C]leucine (1 μCi/ml, 0.8 mM) ± terbutaline for 24 h. **A** and **B**: Incorporation of [^{14}C]leucine. Results are presented as means ± SEM of 5 experiments on myotubes derived from 5 different donors, with 3 biological replicates in each experiment. In panel B, the results are presented as mean ± SEM normalized to control. *p˂0.05 vs control, unpaired *t*-test. **C**: Human myotubes were grown in ScintiPlates®, pre-treated with 1 μM terbutaline for 96 h, before [^{14}C]leucine ± 1 μM terbutaline was added, and the real-time accumulation of [^{14}C]leucine was monitored for 24 h. **D**: Following the 24 h accumulation of [^{14}C]leucine, the decay of [^{14}C]leucine in the myotubes was monitored for 6 h. Results are presented as means ± SEM of 4 experiments on myotubes derived from 3 different donors, with 24 biological replicates for each experiment. *p˂0.05 vs control, unpaired *t*-test.

Hence, the low terbutaline-induced increase in glucose uptake could be explained by the low expression of GLUT4 seen in human myotubes. Moreover, the proteomics analysis revealed that the fructose transporter GLUT5 was upregulated in terbutaline-treated cells, with a 3.7-fold increase in protein level. This was also confirmed by qPCR. Although this transporter has been reported to have a low activity for uptake of other monosaccharides (Nomura et al., 2015), a study by Kayano et al. showed that GLUT5 could mediate uptake of 2-deoxyglucose with low efficacy (Kayano et al., 1990). Therefore, it is possible that the increase in DOG uptake induced by chronic terbutaline treatment is mediated in part by increased GLUT5 transporter expression.

In vivo studies have reported that increased glucose uptake (Kalinovich et al., 2020), fatty acid metabolism (reviewed in Hostrup et al., 2020), and increased protein synthesis (Hostrup et al., 2018a,b; Koopman et al., 2010) are seen after several days with β2-AR agonist treatment. Interestingly, in this study both glucose and OA oxidation were increased after 96 h treatment with terbutaline. This is in line with other studies which have shown that long-term stimulation of the β-AR is necessary for increasing mitochondrial biogenesis (reviewed in Ziegler et al., 2012). These results are also supported by the proteomics results, which showed a significant terbutaline-induced increase in pathways related to mitochondrial metabolism, in particular oxidative phosphorylation, and by the increase in gene expression of PGC-1α, the master regulator of mitochondrial biogenesis (Cantó and Auwerx, 2009). The concentration of terbutaline required to significantly increase the oxidation of glucose and oleic acid, and affect the expression of related proteins seemed to vary in our study, possibly reflecting the sensitivity of different methods.

Both mRNA and protein levels of MYH3 and MYH7, were downregulated by terbutaline, MYH7 is related to slow-twitch muscle (Schiaffino, 2018), and the downregulation of this protein is in line with several studies that have reported a transition from slow-to fast-twitch

Table 2
Proteins upregulated or downregulated more than two-fold after treatment with 1 μM terbutaline for 96 h.

Name	Description	Fold change vs control
TPT1	Translationally-controlled tumor protein	7.0
RPS21	40S ribosomal protein S21	4.4
SLC2A5	Solute carrier family 2, facilitated glucose transporter member 5	3.7
NUDT9	ADP-ribose pyrophosphatase, mitochondrial	3.6
SLC12A7	Solute carrier family 12 member 7	2.8
NHP2	H/ACA ribonucleoprotein complex subunit 2	2.7
SRP14	Signal recognition particle 14 kDa protein	2.6
CD55	Complement decay-accelerating factor	2.3
PPP1CA	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	2.2
MRPL44	39S ribosomal protein L44, mitochondrial	2.1
SRRM1	Serine/arginine repetitive matrix protein 1	2.1
BCKDK	[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial	2.0
NDUFB10	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	2.0
PRAMEF20	PRAME family member 20	−6.2
TUBA1C	Tubulin alpha-1C chain	−5.3
CALM3	Calmodulin-3	−4.6
S100A6	Protein S100-A6	−3.6
RPLP1	60S acidic ribosomal protein P1	−3.6
ANKRD2	Ankyrin repeat domain-containing protein 2	−2.9
STMN1	Stathmin	−2.9
COX17	Cytochrome c oxidase copper chaperone	−2.7
MYBPH	Myosin-binding protein H	−2.6
FAM234A	Protein FAM234A	−2.5
PFN2	Profilin-2	−2.5
ATP6V1C1	V-type proton ATPase subunit C 1	−2.3
CAD	CAD protein	−2.3
TNNT3	Troponin T, fast skeletal muscle	−2.2
MELTF	Melanotransferrin	−2.3
MYH3	Myosin-3	−2.2
MYL4	Myosin light chain 4	−2.1
MYH7	Myosin-7	−2.0
MYLPF	Myosin regulatory light chain 2, skeletal muscle isoform	−2.0

phenotype following chronic β_2-AR agonist treatment (Dodd et al., 1996; Hostrup et al., 2015; M. Hostrup et al., 2018a,b; K. M. Zhang et al., 1996). On the other hand, there are also many reports of PGC-1α facilitating MYH7 expression (reviewed in Blaauw et al., 2013). An increase in MYH7 following the increase in expression of mitochondrial proteins and PGC-1α from the proteomics and PCR results in the present study, might have been expected. However, different splice variants or isoforms of PGC-1α has been described (Brandt et al., 2017), and β_2-AR activation was found to be involved in the exercise-induced increase of some, but not all, isoforms in skeletal muscle (Tadaishi et al., 2011). Thus, it is possible that different isoforms of PGC-1α have different effects on MYH7 expression.

The hypertrophic effects of β_2-AR agonists on skeletal muscle has been known for decades, and has been applied in the production of livestock, physiological and pharmacological studies, as well as being misused for growth-promoting purposes (Choo et al., 1992; Hinkle et al., 2002; Lynch and Ryall, 2008; Yang and McElligott, 1989). These effects have been widely studied *in vivo* in both animals and humans without understanding the underlying mechanism. Only a few studies have shown the hypertrophic effects *in vitro* (Ito et al., 2019; Koziczak-Holbro et al., 2019), and majority of them are from non-human cells (Wannenes et al., 2012). We observed hypertrophic effects in human myotubes upon treatment with terbutaline for 96 h, measured as increased incorporation of leucine into cellular protein. β_2-AR agonist treatment induces muscle hypertrophy by increasing protein synthesis while decreasing protein breakdown causing a positive net protein balance (Hesketh et al., 1992; Maltin et al., 1989; Yimlamai et al., 2005). Although we did see a clear increase in leucine incorporation into protein in the myotubes, and no difference in protein decay, the overall effect, seen from the IPA analysis, in pathways relating to hypertrophy was negative. In order to further investigate the effects of terbutaline on protein synthesis and possible effects on hypertrophy, we measured the mRNA expression of MMP-2, an enzyme that previously has been used as a marker of skeletal muscle hypertrophy (Aguilar-Agon et al., 2019; Q. Zhang et al., 2015). The PCR results showed no differences in the mRNA expression of MMP-2 between control and terbutaline-treated cells. This might be explained by

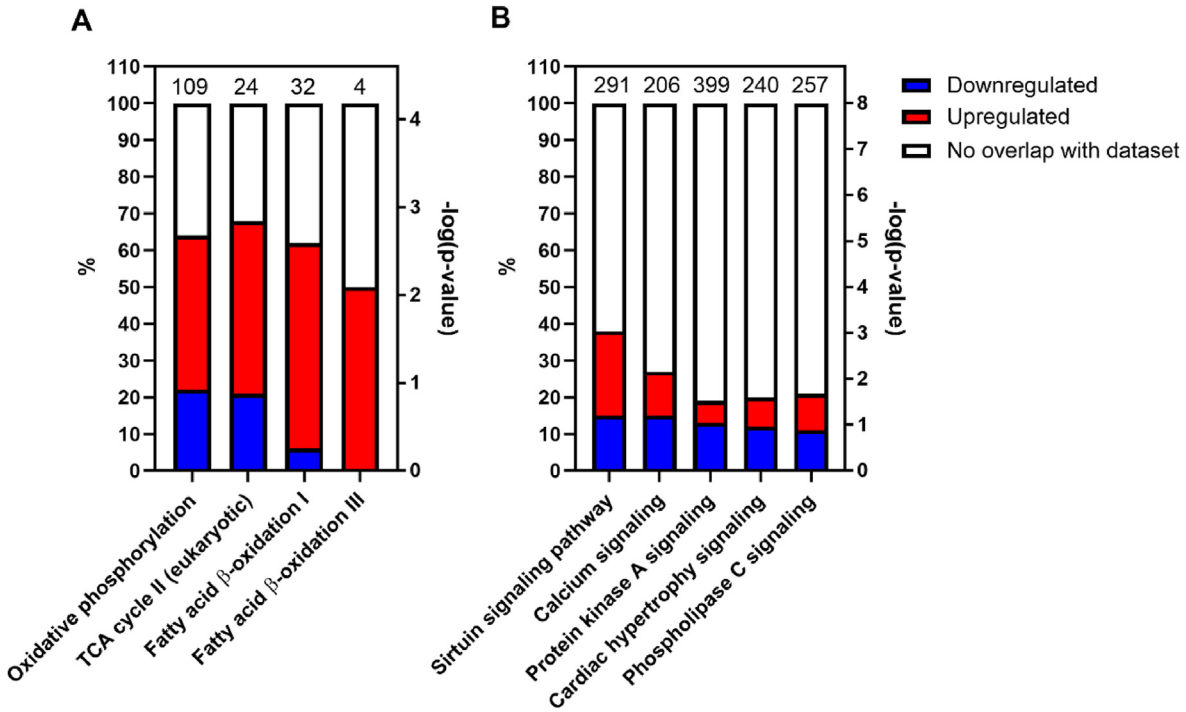

Fig. 5. Pathway analysis of myotubes after 96 h treatment with 1 μM terbutaline. Human myotubes were grown and differentiated in 25 cm^2 flasks. On day 3 of differentiation, cells were treated with DMSO control (0.01%) or 1 μM terbutaline for 96 h. On day 7 of differentiation the myotubes from 6 different donors were harvested for proteomic analysis. Selected differently regulated canonical pathways related to metabolism (**A**) and signalling pathways (**B**) in the proteome of cells treated with terbutaline compared to control cells revealed by Ingenuity Pathway Analysis.

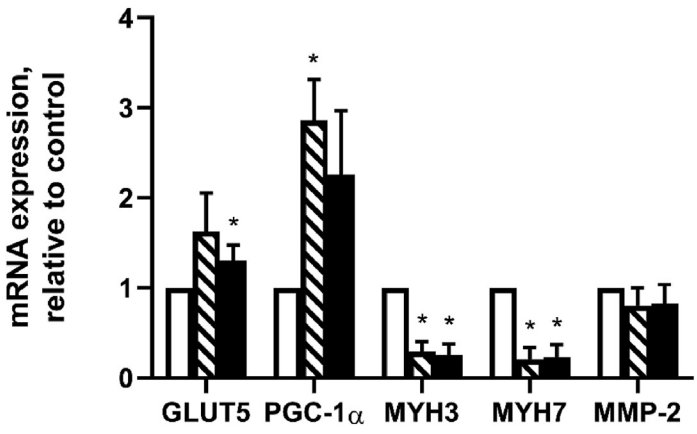

Fig. 6. mRNA expression of genes of interest after 96 h treatment with terbutaline. Human myotubes were grown and differentiated in 25 cm^2 cell culture flasks. On day 3 of differentiation, myotubes were treated with DMSO control (0.01%) or with 1 or 10 μM of terbutaline for 96 h. On day 7 of differentiation the cells were harvested for PCR. mRNA was isolated and expression assessed by qPCR. All values were corrected for the housekeeping control large ribosomal protein P0 (RPLP0), and presented as mean ± SEM from n = 6 individual experiments on myotubes derived from 6 different donors. *p˂0.05 vs control, paired *t*-test. Matrix metalloproteinase-2 (MMP-2), Myosin heavy chain 3 (MYH3), Myosin heavy chain 7 (MYH7), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), Solute carrier family 2 member 5 (GLUT5).

the fact that MMP-2 is normally seen in overload-induced skeletal muscle hypertrophy, an effect that is not pharmacologically inducible (Calve et al., 2012; Q. Zhang et al., 2015). A study by Hostrup et al. investigated the effects of the β_2-AR agonist salbutamol on protein synthesis during resistance exercise in young men. They found that treatment with salbutamol increased protein turnover rates and myofibrillar fractional synthetic rate. However, there was also an increase in protein breakdown, but the net protein balance was positive (Hostrup et al., 2018a,b). Thus, although their study was conducted *in vivo*, their results of increased protein synthesis and protein accumulation are in line with our findings from the present study.

In *in vivo* studies the effects of an intervention in a cell type or organ could be influenced by cross-talk between cells and organs, which makes it difficult to establish whether the effects seen are direct or indirect. Thus, this may explain some of the differences between our study and *in vivo* studies. The skeletal muscle cells in this study are from healthy young men only, with limited variation in age. In further studies also cells from women, other age groups and patients with e.g. diabetes and muscle wasting diseases should be included.

The IPA analysis also showed terbutaline-induced regulation of several signalling pathways; e.g. sirtuin signalling pathway, calcium signalling, PKA signalling, cardiac hypertrophy signalling and phospholipase C signalling. The role of adrenergic signalling in SIRT1 activation has previously been described for cardiac muscle (Corbi et al., 2013; Spadari et al., 2018). Indeed, this would provide an explanation for increased oxidation of energy substrates seen for the chronic terbutaline experiments. However, in rodents the expression of PGC1-α was increased after acute administration of β_2-AR agonists but abolished after chronic treatment (Koopman et al., 2010; Pearen et al., 2009). We had an overall downregulation of the calcium signalling pathway, the same was seen for the protein kinase A and phospholipase C signalling pathways. This downregulation trend is possibly due to these pathways sharing many of the same proteins. Phospholipase C signalling has been shown to play an important role for glucose transport through DAG and protein kinase C in skeletal muscle (Henriksen et al., 1989; Wright et al., 2002; Wright et al., 2003), and cAMP-regulated genes have been shown to stimulate hypertrophy and mitochondrial biogenesis, contribute to muscle repair, improve nutrient uptake and thereby have an effect on energy metabolism (Berdeaux and Hutchins, 2019; Hostrup et al., 2018a, b).

In conclusion, we found that terbutaline acutely increased glucose uptake, and that chronic exposure increased both glucose uptake and oxidation, as well as fatty acid oxidation, and protein synthesis. Thus, the results presented indicate that β_2-ARs have direct effects in skeletal muscle that might be favourable for both T2D and degenerative muscle diseases. However, a deeper understanding of the signalling pathways underlying these effects is necessary for future development.

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CRediT authorship contribution statement

Christine Skagen: Conceptualization, Project administration, Methodology, Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing, Final approval of the version to be submitted. **Tuula A. Nyman:** Methodology, Investigation, Visualization, Formal analysis, Writing – original draft, Writing – review & editing, and Final approval of the version to be submitted. **Xiao-Rong Peng:** Writing – original draft, Writing – review & editing, and Final approval of the version to be submitted. **Gavin O'Mahony:** Writing – original draft, Writing – review & editing, and Final approval of the version to be submitted. **Eili Tranheim Kase:** Conceptualization, Supervision, Writing – review & editing, and Final approval of the version to be submitted. **Arild Chr Rustan:** Conceptualization, Supervision, Methodology, Formal analysis, Writing – review & editing, and Final approval of the version to be submitted. **G. Hege Thoresen:** Conceptualization, Supervision, Methodology, Formal analysis, Writing – review & editing, and Final approval of the version to be submitted.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xiao-Rong Peng and Gavin O'Mahony are employees of AstraZeneca and may own stock or stock options.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphar.2021.100039.

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III

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Treatment of human skeletal muscle cells with inhibitors of diacylglycerol acyltransferases 1 and 2 to explore isozyme-specific roles on lipid metabolism

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Diacylglycerol acyltransferases (DGAT) 1 and 2 catalyse the final step in triacylglycerol (TAG) synthesis, the esterification of fatty acyl-CoA to diacylglycerol. Despite catalysing the same reaction and being present in the same cell types, they exhibit different functions on lipid metabolism in various tissues. Yet, their roles in skeletal muscle remain poorly defined. In this study, we investigated how selective inhibitors of DGAT1 and DGAT2 affected lipid metabolism in human primary skeletal muscle cells. The results showed that DGAT1 was dominant in human skeletal muscle cells utilizing fatty acids (FAs) derived from various sources, both exogenously supplied FA, *de novo* synthesised FA, or FA derived from lipolysis, to generate TAG, as well as being involved in *de novo* synthesis of TAG. On the other hand, DGAT2 seemed to be specialised for *de novo* synthesis of TAG from glycerol-3-posphate only. Interestingly, DGAT activities were also important for regulating FA oxidation, indicating a key role in balancing FAs between storage in TAG and efficient utilization through oxidation. Finally, we observed that inhibition of DGAT enzymes could potentially alter glucose–FA interactions in skeletal muscle. In summary, treatment with DGAT1 or DGAT2 specific inhibitors resulted in different responses on lipid metabolism in human myotubes, indicating that the two enzymes play distinct roles in TAG metabolism in skeletal muscle.

Skeletal muscle utilizes both carbohydrates and fat as energy sources. Approximately 50–60% of the free fatty acids (FFAs) taken up by skeletal muscle are stored as triacylglycerol (TAG) in lipid droplets (LDs)[1]. TAG, which is a neutral lipid, consists of a glycerol backbone and three FAs attached by ester bonds. The terminal and only committed step of TAG synthesis, the esterification of fatty acyl-CoA to diacylglycerol (DAG), is catalysed by the enzymes diacylglycerol acyltransferase (DGAT) 1 and 2[2–4]. Both DGAT enzymes reside at the endoplasmic reticulum[5,6], though DGAT2 is also found to co-localize with LDs and mitochondria in cultured fibroblasts and adipocytes, in contrast to DGAT1[5,6]. Although the two isozymes catalyse the same reaction, there are several differences between them. They share no sequence homology with each other, belong to unrelated families of proteins[4] and overexpression of the two isozymes in rat hepatoma cells give rise to LDs with markedly different morphology (size) and intracellular distribution[7]. In addition, they are non-redundant in some functions, which are reflected by the phenotype of mice lacking *DGAT1* or *DGAT2*. Whereas *Dgat1*$^{-/-}$ mice are viable with a favourable metabolic phenotype showing an increased insulin and leptin sensitivity and resistance to diet-induced obesity, *Dgat2*$^{-/-}$ mice die shortly after birth; they are lipopenic, have a defect in the skin barrier leading to rapid dehydration[8–10], and are possibly unable to utilize glucose in brown adipocytes for thermoregulation[11].

TAG formation can occur in two ways, namely from re-esterification of partial glycerides (derived from lipolysis of TAG) and through *de novo* incorporation of glycerol 3-phosphate into the glyceride entity followed by

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formation of DAG and TAG[12,13]. Several studies have been done to investigate and determine the roles of DGAT1 and DGAT2 in different tissues. For instance, the enzymes demonstrated to have non-redundant roles in intestinal lipid metabolism in mice enterocytes[14]. In liver and brown adipose tissue, DGAT1 seems to favour the incorporation of exogenous supplied FAs, whereas DGAT2 appears to be an enzyme of major importance for TAG synthesis of FAs derived from *de novo* lipogenesis[11,15,16]. Moreover, DGAT1 and DGAT2 have recently been shown to have distinct and overlapping functions for TAG synthesis in adipocytes[17], where DGAT1 have been linked to the lipolysis-re-esterification cycle of preformed FA, a process that may also protect the endoplasmic reticulum from lipotoxic stress and adipose tissue inflammation[18].

In muscle, almost all previous studies have focused on DGAT1. Human cardiomyocytes and cultured mouse myocytes treated with a specific DGAT1 inhibitor exhibited reduced mRNA expression of genes mediating FA uptake and oxidation[19]. Further, inactivation of *DGAT1* in a mouse cardiac model reduced TAG synthesis and increased FA oxidation, whereas co-inhibition of DGAT1/2 abrogated TAG synthesis and protected against high fat diet-induced lipid accumulation[20]. Interestingly, upregulation of *DGAT1* in mouse skeletal muscle increased TAG synthesis and protected against high-fat diet-induced insulin resistance[21], whereas overexpression of *DGAT2* in glycolytic muscle resulted in an increased amount of TAG, ceramides and long-chain fatty acyl-CoAs, followed by an impaired insulin signalling[22]. Overall, these reports emphasize the potential for specialized roles of DGAT1 and DGAT2 in various tissues. Moreover, skeletal muscle is an important site for metabolic disturbances[23] and the balance between storage and efficient utilization of TAG is a potential key to understand the interaction in dysregulated fat and glucose metabolism in skeletal muscle[24,25].

In the present study we wanted to explore whether the roles of DGAT1 and DGAT2 are also specialized in human primary myotubes and to determine if DGAT1 and DGAT2 in skeletal muscle follow the same patterns of lipid incorporation that has previously been shown in other cell types. Using highly specific small-molecule inhibitors of DGAT1 (A922500, D1i)[26] and DGAT2 (JNJ-DGAT2-A, D2i)[16] we investigated the effects of their specific inhibition on TAG synthesis in FA metabolism using labelled precursors. Further, we examined the effect of DGAT1 and DGAT2 inhibition on other parameters including FA turnover (oxidation, lipolysis and re-esterification). Additionally, we examined if inhibition of DGAT enzymes also could influence glucose metabolism in human myotubes.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM-Glutamax) low glucose with sodium pyruvate, Dulbecco's phosphate buffered saline (DPBS, without Mg^{2+} and Ca^{2+}), foetal bovine serum (FBS), penicillin-streptomycin (10000 IE/ml), amphotericin B, Collagen I, Hoechst 33258, Bodipy 493/503, Pierce BCA Protein Assay Kit, Power SYBR Green PCR Master Mix, MicroAmp Optical Adhesive Film, MicroAmp Optical 96-well Reaction Plate and TaqMan Reverse Transcription Reagents were from ThermoFisher Scientific (Waltham, MA, US). Ultroser G was purchased from Pall Life Sciences (Cergy-Saint-Christophe, France). Insulin (Actrapid) was obtained from NovoNordisk (Bagsvaerd, Denmark). Bovine serum albumin (BSA, essentially FA-free), L-carnitine, D-glucose, oleic acid (OA, 18:1, n-9), HEPES, DMSO, gentamicin, glycogen, etomoxir, A922500, and β-mercaptoethanol were from Sigma-Aldrich (St. Louis, MO, US). T0901317 was purchased from Cayman Chemical Company (Ann Arbor, MI, US). [^{14}C]oleic acid (OA, 56–59 mCi/mmol), D-[^{14}C(U)]glucose (107.3 mCi/mmol and 263 mCi/mmol), D-[^{14}C(U)]glycerol (142 mCi/mmol), and [^{14}C]acetate (50.5 mCi/mmol) were purchased from PerkinElmer NEN (Boston, MA, US). 96-well and 6-well Corning CellBIND tissue culture plates were from Corning (Schiphol-Rijk, the Netherlands). 96-well Scintiplate tissue culture plates, UniFilter-96 GF/B microplates, Isoplate-96 scintillation microplates, TopSeal-A transparent film, OptiPhase Supermix, and Ultima Gold were obtained from PerkinElmer (Shelton, CT, US). TG PAP 150-kit was from Biomérieux (Craponne, France). Thin layer chromatography plates were purchased from Merck (Darmstadt, Germany). QIAshredder and RNeasy Mini kit were from QIAGEN (Venlo, the Netherlands). Glass Bottom Microwell Dishes (35 mm petri dish, 14 mm microwell, No. 1.5 cover glass) were obtained from MatTek (Ashland, MA, US). Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad (Copenhagen, Denmark). JNJ-DGAT2-A was from Janssen Research and Development (High Wycombe, UK).

Methods. *Culturing of human myotubes.* Multinucleated myotubes were established by activation and proliferation of myoblasts obtained from satellite cells. Satellite cells were isolated, proliferated to myoblast that were stored in a cell bank and further cultured as previously described[27] from the *musculus vastus lateralis* of eight healthy young male subjects. Donors were 25.5 (±0.95) (mean ± SEM) years old with a body mass index of 22.1 (±0.8) kg/m^2. More details about the donor cohort used in this study, as well as characteristics of myotubes can be found in the study by Lund *et al.*[28]. Muscle biopsies were obtained after informed written consent and approval by the National Committee for Research Ethics, Oslo, Norway (2011/2207 REK South East B). The study adhered to the Declaration of Helsinki.

For most experiments, the cells were cultured on multiwell plates in DMEM-Glutamax (5.5 mM glucose) supplemented with 2% FBS, 25 IU penicillin, 25 µg/ml streptomycin, 1.25 µg/ml amphotericin B, 50 ng/ml gentamycin, and 2% Ultroser G. At approximately 80% confluency, the growth medium was replaced by DMEM-Glutamax (5.5 mM glucose) supplemented with 2% FBS, 25 IU penicillin, 25 µg/ml streptomycin, 1.25 µg/ml amphotericin B, 50 ng/ml gentamycin, and 25 pM insulin. During culturing, the cells were incubated in a humidified 5% CO_2 atmosphere at 37 °C, and the medium was changed every 2–3 days as previously described[29]. Experiments were performed on myotubes after 6–7 days of differentiation. Cells from all eight donors were not used in all experiments.

Lipid distribution – incorporation from OA and glycerol. The muscle cells were cultured on 12- or 24-well plates. After 6–7 days of differentiation, the myotubes were incubated with 100 µM [^{14}C]OA (0.5 µCi/ml) in DMEM-Glutamax (5.5 mM glucose) supplemented with L-carnitine (1 mM) and BSA (40 µM) for 4 h. Alternatively,

myotubes were given DMEM-Glutamax (5.5 mM glucose) supplemented with 10 µM D-[^{14}C(U)]glycerol (1 µCi/ml), L-carnitine (1 mM) and BSA (40 µM) with or without 100 µM OA. DGAT1 inhibitor (D1i, A922500, 1 µM) or DGAT2 inhibitor (D2i, JNJ-DGAT2-A, 10 µM) were added to the cells 30 min before the radiolabelled substrate was added and incubated for 4 h. Thereafter, the myotubes were washed twice with 0.5 ml PBS, harvested in 125 µl distilled water and frozen at −20 °C. Cellular lipids were extracted and separated as described earlier[30]. Briefly, lipids were extracted by addition of chloroform:methanol (2:1, v/v) and 0.9% sodium chloride solution (pH 2). The mixture was then separated into two phases and the organic phase was dried under nitrogen gas and lipids were re-dissolved in hexane. Lipids were separated by thin layer chromatography (TLC), and radioactivity was quantified by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer, US). The amount of lipids was related to total cell protein content measured according to Bradford[31]. For lipid distribution we have done 3 or more individual experiments (*n*) with triplicate repetitions (3 separate culture wells) for each condition, see legends to figures.

Imaging. Myotubes were cultured on glass bottom microwell dishes coated with Collagen I (0.01%). At day 7 of differentiation, the cells were incubated with 100 µM OA for 4 and 24 h in the presence or absence of DGAT inhibitors (D1i, 1 µM; D2i, 10 µM). Myotubes were then incubated with Bodipy 493/503 (2 µg/ml) to stain lipid droplets, and Hoechst 33258 (2.5 µg/ml) to stain nuclei, both for 15 min. Cells were then washed with PBS and fixated with 3% paraformaldehyde for 20 min. Pictures were taken with an UPLSAPO 60x objective (NA:1.35) and images were taken in 6–8 positions in each well, using a confocal microscope (Olympus FLUOVIEW FV1000 confocal microscope, Japan). The calculated optical thickness was 0.7 µm for the whole image. Images were analyzed using ImageJ version 1.51j8 (NIH, US)[32]. Briefly, the number of lipid droplets were counted by ImageJ before nuclei were accounted for manually. If there were several nuclei in the edge, they were calculated together (two half nuclei equal one nucleus in the analysis), as long as they had surrounding lipid droplets confirmed by visual observation and ImageJ.

Determination of total TAG content. Myotubes grown in 75 cm^2 cell culture flasks were incubated with 100 µM OA in presence or absence of D1i (1 µM) or D2i (10 µM) for 24 h. Thereafter, the myotubes were washed with PBS and harvested in 0.1% SDS. Measurement of total cellular TAG content after extraction, separation by TLC and redissolution in 50 µl 2-propanol, was performed with the TG PAP 150-kit according to the supplier's protocol (Biomérieux). Basal TAG content in myotubes without OA was approximately 30 µg/mg protein, and after 24 h with 100 µM OA TAG content increased 2–3-fold.

Lipid distribution - incorporation from acetate and glucose. Myotubes were cultured on 12-well tissue culture plates. After 6–7 days of differentiation, the myotubes were incubated in presence or absence of D1i (1 µM) or D2i (10 µM) together with 100 µM [^{14}C]acetate (2 µCi/ml) in DMEM-Glutamax (5.5 mM glucose) supplemented with BSA (40 µM) for 4 h. Thereafter, cells were harvested and cellular lipids extracted and quantified as described under section "Lipid distribution". Alternatively, myotubes were treated with liver X receptor agonist T0901317 (1 µM) the last 4 days of differentiation to promote FA synthesis[33]. Thereafter the cells were incubated for 24 h in DMEM-Glutamax (5.5 mM glucose) supplemented with D-[^{14}C(U)]glucose (2 µCi/ml), 2% FBS and 25 pmol/l insulin in presence or absence of D1i or D2i. Then, cells were harvested and quantified as described above.

Substrate oxidation assay and acid soluble metabolites for OA. Myotubes were cultured and treated as described above (sections "Culturing of human myotubes" and "Lipid distribution"). After 4 h of incubation with [^{14}C]OA (0.5 µCi/ml, 100 µM) in presence of D1i (1 µM) or D2i (10 µM), 100 µl (24-well) or 200 µl (12-well) of radiolabelled cell medium was transferred to a multiwell plate, sealed and frozen at −20 °C. To measure CO_2 production, 40 µl of 1 M perchloric acid ($HClO_4$) was added immediately to the frozen medium. A 96-well UniFilter-96 GF/B microplate was activated to capture CO_2 by addition of 20 µl 1 M sodium hydroxide and was mounted on top of the multiwell plate as previously described[34]. The mixture was then incubated at room temperature for 3 h to trap radiolabelled CO_2. The CO_2 produced during the 4 h of incubation with [^{14}C]OA was captured by the sodium bicarbonate buffer system in the cell medium. After adding $HClO_4$ to the frozen medium, CO_2 was released and captured in the UniFilter-96 GF/B microplate. Radioactivity was measured by liquid scintillation (2450 MicroBeta^2 scintillation counter, PerkinElmer).

Measurement of acid soluble metabolites (ASMs), which reflects incomplete FA oxidation (β-oxidation) and consists mainly of tricarboxylic acid cycle metabolites, was performed using a method modified from Skrede *et al.*[35]. From the radiolabelled incubation medium, 100 µl was transferred to a new Eppendorf tube and precipitated with 300 µl cold $HClO_4$ (1 M) and 30 µl BSA (6%). Then, the tube was centrifuged at 10 000 rpm/10 min/4 °C, before 200 µl of the supernatant was counted by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer).

Scintillation proximity assay (SPA) and lipolysis assay. SPA is a method to measure the amount of radiolabelled substrates inside the cells. The scintillator is embedded in the bottom of each well and provides a stronger signal when radiolabelled substrates are accumulated in the cells compared to in the medium alone[34]. Human myotubes were cultured on 96-well ScintiPlate tissue culture plates. After 6 days of differentiation the cells were given DMEM without phenol red supplemented with [^{14}C]OA (0.5 µCi/ml, 100 µM), 5.5 mM glucose, 2% FBS, 25 IU penicillin, 25 µg/ml streptomycin, 1.25 µg/ml amphotericin B, and 25 pM insulin. After 24 h the cells were washed twice with DPBS with 0.5% BSA, before fresh DPBS medium supplemented with 0.5% BSA, 10 mM HEPES and 0.1 mM glucose were added to the cells. The decline in [^{14}C]OA in the cells (taken as a measurement of lipolysis[36]) were measured after 0, 1, 2, 4, and 6 h in presence or absence of DGAT inhibitors (D1i, 1 µM; D2i, 10 µM) and etomoxir (10 µM). Etomoxir is a selective inhibitor of mitochondrial carnitine palmitoyltransferase-1, thereby inhibiting FA oxidation by 90% at 10 µM[34,37]. Remaining cell-associated radioactivity was measured by liquid scintillation.

Substrate oxidation assay for glucose. Myotubes were cultured on 96-well tissue culture plates. At day 7 of differentiation, cells were given [^{14}C(U)]glucose (0.5 µCi/ml, 200 µM) with D1i (1 µM) or D2i (10 µM) during 4 h of CO_2 trapping as previously described[34]. CO_2 production was measured in DPBS medium with 10 mmol/l HEPES, 1 mmol/l L-carnitine and 10 µM BSA. CO_2 and cell-associated labelled glucose were measured by liquid scintillation using a 2450 MicroBeta[2] scintillation counter (PerkinElmer).

Glycogen synthesis. Myotubes were cultured on 12-well tissue culture plates as described under section "Culturing of human myotubes". At day 7 of differentiation, the myotubes were given DMEM medium without glucose for 90 min to starve the cells. Then, the myotubes were incubated with D-[^{14}C(U)]glucose (1 µCi/ml, 5.5 mM) in presence or absence of D1i (1 µM) or D2i (10 µM) and with or without insulin (100 nM) for 3 h. Thereafter, the cells were washed twice with PBS and harvested in 1 M potassium hydroxide (KOH). Protein content was measured by Pierce BCA Protein Assay Kit. D-[^{14}C(U)]glucose incorporated into glycogen was measured as previously described[38] with some modifications. Briefly, glycogen and more KOH were added to the samples for a final concentration of 20 mg/ml and 19%, respectively. The samples were incubated for 20 min at 80 °C, before glycogen were precipitated in the samples by adding 100% ice-cold ethanol and centrifuged at 10 000 rpm for 20 min at 4 °C. The pellets were washed once with ice-cold 70% ethanol, centrifuged at 10 000 rpm for 20 min at 4 °C, air-dried, and re-suspended in distilled water. Radioactivity was measured by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer) and the amount of glycogen was related to total cell protein content.

Presentation of data and statistics. Data are presented as mean ± SEM if not stated otherwise, as absolute values and as % of control, where data are normalized to the mean value for the control within each separate experiment. The value *n* represents number of individual cell culture experiments normally with 3–8 cell culture wells for each condition in each experiment unless specified otherwise in figure legends. Statistical analyses were performed using GraphPad Prism 7.02 Software (GraphPad Software Inc., La Jolla, CA, US), where two-tailed paired t-test was performed to determine effects of treatment unless specified otherwise in figure legends. The number of experiments (*n*) was increased for some parameters studied when it was expected to observe small treatment effects. Linear mixed-model analysis (LMM, SPSS 25.0.0.1, IBM SPSS Inc., Chicago, IL, US) was used in the time-course lipolysis experiments (SPA). $p < 0.05$ was considered significant.

Results

Effect of DGAT inhibitors on distribution of exogenous OA in various lipid classes. We first wanted to explore the roles of DGAT1 and DGAT2 in metabolism of exogenously added [^{14}C]oleic acid (OA), with particularly emphasis on TAG synthesis in human myotubes. Initially, we confirmed that both DGAT enzymes were expressed in human myotubes, where *DGAT1* appeared to be most abundant (Fig. S-1). To determine the optimal concentrations of the specific inhibitors of DGAT1 (A922500, D1i, IC_{50} value of 7 nM)[26] and DGAT2 (JNJ-DGAT2-A, D2i, IC_{50} value of 140 nM)[16], myotubes were treated with labelled OA and different concentrations of the inhibitors. Two processes were measured: incorporation and complete oxidation of [^{14}C]OA into TAG and CO_2, respectively (Fig. S-2). Based on these results and available literature[11,16,36], concentrations for further experiments were determined to be 1 µM (D1i) and 10 µM (D2i). Furthermore, visual observation and measurement of cell protein content indicated none cell-toxic effects with neither D1i (1 µM) nor D2i (10 µM) up to 24 h treatment. Additionally, acute treatment with DGAT inhibitors did not influence expression of several genes involved in lipid metabolism (FA oxidation, storage, lipolysis, and turnover) in human myotubes (Fig. S-3).

We aimed to characterize how acute treatment with DGAT inhibitors influenced lipid distribution of OA in the myotubes. Treatment with D1i significantly decreased the level of total lipid generated from [^{14}C]OA. D1i reduced the levels of TAG by ~80% compared to control, while diacylglycerol (DAG) and FFAs were reduced to a lesser extent (Fig. 1A). The highest proportion of total lipids was recovered in phospholipids (PLs) and TAG, which showed an increased and decreased percentage after treatment with D1i, respectively (Fig. 1B). Treatment with D2i had little effect on absolute values, with only a minor reduction in the levels of cholesteryl ester (CE) (Fig. 1C). However, when calculated as percentage of total lipids, it was a paradoxical increase in the percentage of TAG, accompanied by decreased levels of PLs, DAG and CE (Fig. 1D). When combining the two inhibitors the effect on TAG reduction was similar to D1i alone (~80%). Further, D1i + D2i increased the levels of PLs, and reduced the levels of CE, DAG and TAG (Fig. 1E). A substantially increased percentage of total lipids was recovered as PLs, whereas the fraction of DAG and TAG was decreased, indicating a shift in the flux of OA from DAG and TAG into PLs (Fig. 1F).

The differences induced by DGAT inhibitors on incorporation of OA into various lipid classes were reflected in changes in the number of LDs after incubation with OA and either of the two inhibitors (Fig. 2). Treatment with D1i reduced the number of LDs, whereas the number of LDs in cells treated with D2i was slightly increased compared to the control cells (Fig. 2D). Furthermore, a 35% reduction in total TAG content was seen in the myotubes after 24 h treatment with D1i and OA (Fig. 2E).

Effect of DGAT inhibitors on incorporation of glycerol, acetate and glucose into TAG and total lipids. We next wanted to study the incorporation of a substrate involved in *de novo* synthesis of TAG, *i.e.* glycerol. Therefore, myotubes were incubated with D-[^{14}C(U)]glycerol with or without OA for 4 h in presence or absence of D1i or D2i. Both in absence and presence of OA, D1i and D2i had an inhibiting effect on TAG synthesis measured by incorporation of [^{14}C]glycerol. Interestingly, D2i appeared to reduce the TAG levels to a greater extent (~40% vs. ~60%) in the absence of OA, whereas D1i reduced TAG levels most strikingly in the presence of OA (~70% vs. 25%) (Fig. 3A,D).

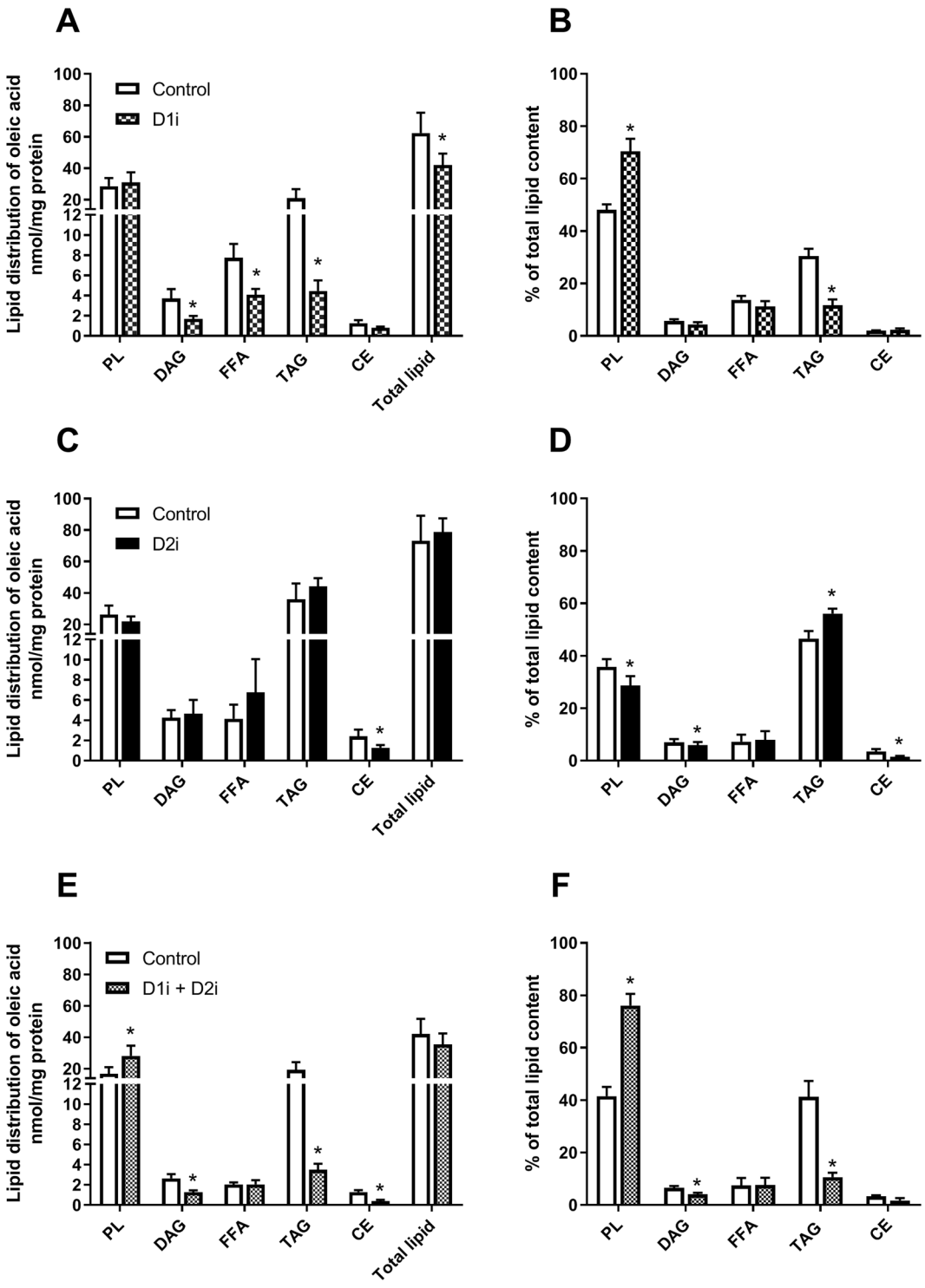

Figure 1. Effect of DGAT inhibitors on incorporation of oleic acid into various lipid classes. Human myotubes were grown and differentiated on 12- or 24-well tissue culture plates for 7 days. On day 7 of differentiation myotubes were incubated with 100 µM [^{14}C]oleic acid (0.5 µCi/ml) for 4 h in presence or absence of D1i (1 µM) and/or D2i (10 µM). Cellular lipids were extracted and separated by thin layer chromatography (TLC), and radioactivity was measured by liquid scintillation. The sum of all radioactivity on the TLC plate is presented as total lipids. (**A–F**) Lipid distribution of [^{14}C]oleic acid after treatment with D1i, D2i and combination of D1i and D2i. Results are presented as mean ± SEM as nmol/mg protein (**A,C,E**) or as % of total lipids in the cell (**B,D,F**). $n = 6$ (**A,B**), $n = 8$ (**C,D**) and $n = 5$ (**E,F**) individual experiments with 3 separate culture wells for each condition. *$p < 0.05$ vs. control, paired t-test. CE, cholesteryl ester; D1i, DGAT1 inhibitor; D2i, DGAT2 inhibitor; DAG, diacylglycerol; FFA, free fatty acid; PL, phospholipid; TAG, triacylglycerol.

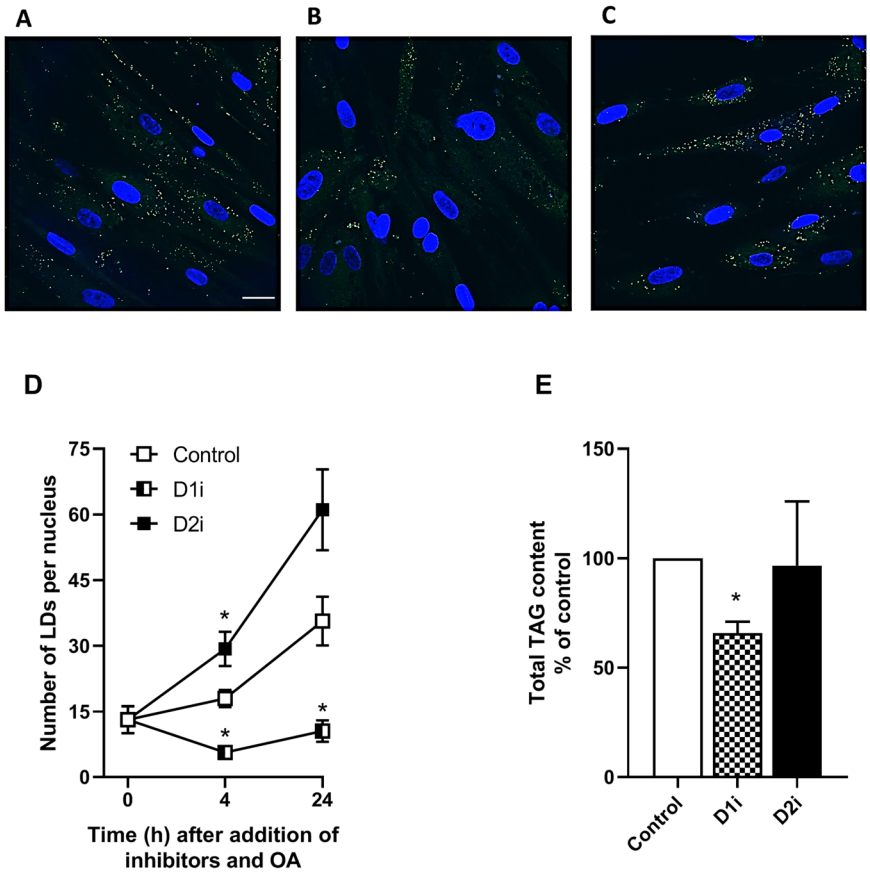

Figure 2. Effect of DGAT inhibition on lipid droplets and total cellular triacylglycerol content. Human myotubes were grown and differentiated on glass bottom microwell dishes. At day 7 of differentiation, myotubes were incubated with 100 µM OA for 4 (**A–C**) and 24 h (**D**) in presence or absence of DGAT inhibitors; D1i (1 µM) and D2i (10 µM). The cells were stained for lipid droplets (green) and nuclei (blue) using Bodipy 493/503 and Hoechst 33528, respectively, and images taken with a 60x objective on a confocal microscope. Scale bar 25 µm. (**A–C**) Representative images are presented for control (**A**), D1i (**B**) and D2i (**C**). (**D**) LDs were quantified (ImageJ) by relating number of LDs to number of nuclei. Results represent mean ± SEM from one experiment at 4 h and one experiment at 24 h where calculations are based on 6–8 different images for each condition, unpaired t-test, *$p < 0.05$ vs control at the same time-point. (**E**) Human myotubes were incubated with 100 µM OA in presence or absence of D1i (1 µM) and D2i (10 µM) for 24 h and total TAG content was measured. The results are presented as mean ± SEM as % of control from $n = 3$ individual experiments with one 75 cm^2 cell culture flask for each condition. *$p < 0.05$ vs. control, paired t-test. D1i, DGAT1 inhibitor; D2i, DGAT2 inhibitor; LDs, lipid droplets; OA, oleic acid; TAG, triacylglycerol.

To examine lipid utilization for TAG synthesis in more detail, we used [^{14}C]acetate as a marker for *de novo* synthesized FAs. Exposure to labelled acetate showed an inhibiting effect on TAG levels after treatment with D1i (Fig. 3B,E). Also, incorporation of acetate into phospholipids was decreased by DGAT1 inhibition (Supplemental Fig. S-4). Moreover, we also used [^{14}C]glucose as substrate for incorporation of ^{14}C-label into lipids. From experience we know that incorporation of [^{14}C]glucose into cellular lipids in myotubes is usually low, but treatment with the liver X receptor agonist T0901317 has been shown to increase incorporation of [^{14}C]glucose into lipids in this cell model[33,39]. Myotubes were therefore incubated with the liver X receptor agonist T0901317 (1 µM) for 96 h and then incubated for another 24 h with [^{14}C]glucose in the presence or absence of DGAT inhibitors. Incorporation of [^{14}C]glucose into labelled total lipids was significantly reduced for both D1i and D2i (Fig. 3C,F).

Effect of DGAT inhibitors on lipid turnover and oxidation. Since DGAT inhibition was shown to have different effects on incorporation of radioactive labelled ^{14}C from OA and glycerol into TAG, depending on which DGAT isozyme being inhibited, we further examined the roles of DGAT1 and DGAT2 on lipid turnover of accumulated lipids. We pretreated human myotubes with [^{14}C]OA for 24 h to prelabel the endogenous TAG pools. After 24 h of incubation, fresh medium containing D1i or D2i was added to the cells. Cell-associated radioactivity (presumed to be mostly ^{14}C-TAG or ^{14}C-PL, see Fig. 1) was measured 4 h after adding the inhibitors. Treatment with D1i for 4 h reduced the levels of cell-associated ^{14}C-labelled lipids compared to control. By contrast treatment with D2i showed no effect (Fig. 4A). When the experiments were performed in the presence of etomoxir (inhibitor of FA oxidation) during 6 h (Fig. 4B), cell-associated lipids were reduced in D1i-treated cells compared

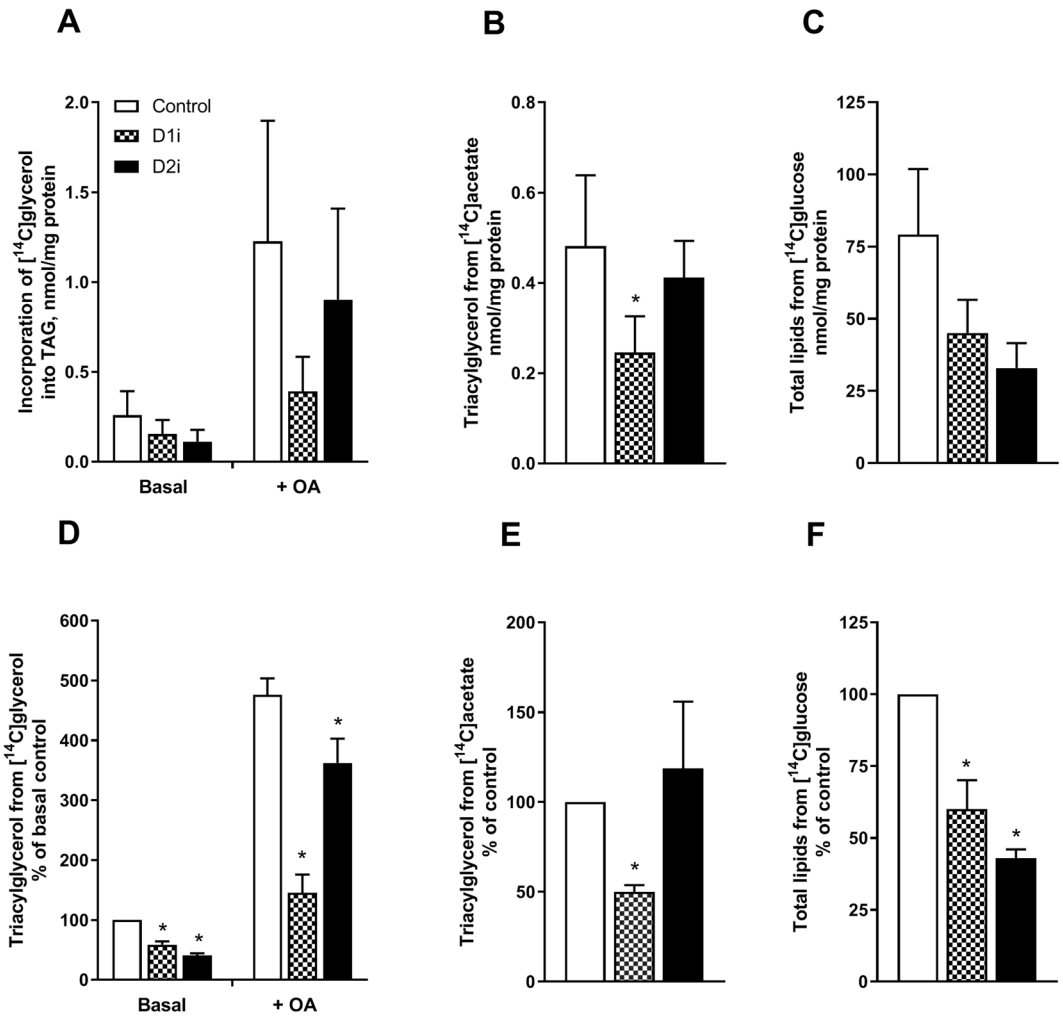

Figure 3. Effect of DGAT inhibition on incorporation of glycerol, acetate and glucose into TAG and total lipids. Human myotubes were grown and differentiated on 12-well tissue culture plates for 6–7 days. At day 7 of differentiation myotubes were incubated with D-[^{14}C(U)]glycerol (1 µCi/ml, 10 µM) supplemented with D1i (1 µM) or D2i (10 µM), in presence or absence of 100 µM oleic acid for 4 h (**A,D**) or with 100 µM [^{14}C] acetate (2 µCi/ml) in presence or absence DGAT inhibitors for 4 h (**B,E**). **C,F**) Cells were incubated with the liver X receptor agonist T0901317 (1 µM) for 96 h. Thereafter, myotubes were incubated for 24 h with D-[^{14}C(U)]glucose (2 µCi/ml, 5.5 mM) in presence or absence of DGAT inhibitors. Lipids were separated by thin layer chromatography and measured using liquid scintillation. The sum of all radioactivity on the TLC plate is presented as total lipids. Results represent mean ± SEM from $n = 4$ (**A,B**), $n = 5$ (**D,E**) or $n = 3$ (**C,F**) individual experiments with 3 separate culture wells for each condition presented at absolute values (**A, B, C**) or normalized to control (**D–F**). *$p < 0.05$ vs. control, paired t-test. D1i, DGAT1 inhibitor; D2i, DGAT2 inhibitor; TAG, triacylglycerol.

to control cells, indicating that only alterations in the rate of re-esterification were involved (Fig. 4C). Next, we examined the effects of the inhibitors on oxidation of [^{14}C]OA, where both CO_2 production (complete FA oxidation Fig. 5A–C) and acid-soluble metabolites (ASMs, FA β-oxidation, Fig. 5D–F) were measured. We observed a significant increased oxidation (ASMs and CO_2) in cells treated with D1i, whereas treatment with D2i showed a reduced oxidation (ASMs and CO_2).

Effect of DGAT inhibitors on glucose metabolism. Skeletal muscle is a major consumer of both FAs and glucose, and shows a high metabolic flexibility. Since DGAT enzymes play a central role in lipid metabolism, and our data also indicated the importance of DGAT in determining the flux of FAs to oxidation as well as storage (Fig. 5), we wanted to study possible effects of acute inhibition of DGAT enzymes on glucose metabolism in human myotubes. Although there was no effect of the DGAT inhibitors on glucose oxidation, we observed a small but significant increased glucose uptake after treatment with D1i (Fig. 6A,B). Moreover, treatment with D2i reduced the level of basal as well as insulin-stimulated glycogen synthesis compared to control (Fig. 6C).

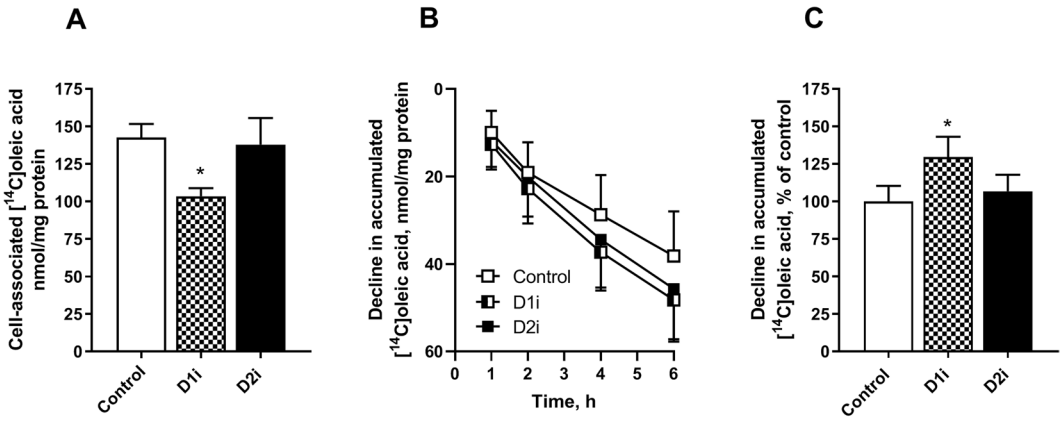

Figure 4. Effect on DGAT inhibition on turnover of accumulated lipids. Human myotubes were grown and differentiated on 96-well tissue culture plates, SPA plates were used for time-course (**B,C**). At day 6 of differentiation myotubes were incubated with [^{14}C]oleic acid (0.5 µCi/ml, 100 µM) for 24 h. After 24 h pre-treatment with [^{14}C]oleic acid myotubes were washed and re-incubated with D1i (1 µM) or D2i (10 µM). (**A**) Cell-associated radioactivity from [^{14}C]oleic acid was measured after 4 h treatment with DGAT inhibitors. (**B,C**) Decline in cell-associated radioactivity was measured over 6 h in presence of an inhibitor of carnitine palmitoyltransferase 1 (etomoxir, 10 µM) and DGAT inhibitors; D1i (1 µM) and D2i (10 µM). Results represent mean ± SEM as nmol/mg protein (**A,B**) and as all-over effects normalized to control (**C**) from $n = 4$ individual experiments with 8 separate culture wells for each condition. *$p < 0.05$ vs control, paired t-test (**A**), LMM statistical test (**C**). D1i, DGAT1 inhibitor; D2i, DGAT2 inhibitor; SPA; scintillation proximity assay; LMM, Linear mixed model.

Discussion and Conclusion

Discussion. In this study, we present a description of the respective roles of DGAT1 and DGAT2 on lipid metabolism in primary human skeletal muscle cells. It was found that inhibition of DGAT1 and DGAT2 had distinct effects on lipid metabolism in skeletal muscle cells. Through the use of specific inhibitors, DGAT1 was shown to be the major enzyme responsible for incorporation of both exogenously supplied and endogenously generated (through lipolysis) FAs into cellular lipids in human myotubes. In addition, FAs derived from *de novo* FA synthesis were utilized by DGAT1. In contrast, DGAT2 could not compensate for the loss of DGAT1 in synthesizing TAG from exogenous or endogenous FAs generated by lipolysis. However, results indicated that DGAT2 played a prominent role in *de novo* synthesis of TAG from glycerol-3-phosphate at lower substrate concentrations. Similarly, DGAT1 contributed to this process to a greater extent when the concentrations of substrates were higher. Interestingly, inhibition of the two DGATs affected FA oxidation differently and so it would appear that DGAT activities are important in determining the rates of FA oxidation. Finally, we observed possible roles of DGATs on glucose accumulation and glycogen synthesis.

Skeletal muscle utilize FAs which are mainly metabolised through oxidation, TAG synthesis (storage in LDs) and PL synthesis[40]. We observed that treatment with D1i or D2i gave distinct responses on the handling of preformed FAs. D1i reduced the levels of TAG by nearly 80%, whereas D2i did not show any reducing effect on TAG synthesis when using exogenous FAs as substrate (Fig. 1). Instead, we observed a relative increased proportion of TAG using D2i. Thus, whereas inhibition of DGAT1 reciprocally lowered relative flux into TAG synthesis and increased flux into PLs, inhibition of DGAT2 appeared to achieve the opposite and induced a shift in the flux of preformed FAs from PLs into TAG. This might be catalysed by DGAT1, which is still active as it could be a competition between these two pathways for a common DAG substrate. The same pattern was reflected in LDs and total TAG content generated from exogenous added FAs, where only D1i reduced the quantity of LDs and TAG (Fig. 2). The lack of effect of D2i on TAG synthesis and LD formation from exogeneous fatty acid may be related to much lower expression of this enzyme compared to DGAT1. Our data from skeletal muscle cells is consistent with studies done in cells from other tissues, hepatocytes and brown adipocytes, which show that incorporation of exogenous FA into TAG is predominantly mediated by DGAT1, and not DGAT2[11,15,16]. However, the enzymes have been found to partially compensate for each other for TAG storage in adipocytes[17,18]. We observed that D1i almost totally abolished incorporation of exogenous FAs into TAG and LDs (Fig. 2), which demonstrate the major role of DGAT1 for overall TAG content in muscle cells, which further is supported by DGAT1 being most abundant in skeletal muscle[2]. This essential role of DGAT1 is compatible with studies done in murine skeletal muscle that show increased intramyocellular lipid content and TAG synthesis after overexpression of *DGAT1*[21,41,42]. Moreover, inactivation of *DGAT1* in heart resulted in a moderate suppression of TAG synthesis and turnover[20]. Also, exercise has been shown to induce the expression of DGAT1, followed by increased TAG levels in the muscles of mice and humans[21,43,44]. Thus, DGAT1 function has been linked to the "athlete paradox", where endurance-trained athletes, in contrast to sedentary subjects, are highly insulin sensitive despite high levels of intramyocellular TAG[21].

Myotubes were incubated with glycerol, to label the backbone of the glycerolipids in order to distinguish between the formation of TAG by re-esterification of partial glycerides and *de novo* synthesis of TAG (Fig. 3). Labelled acetate was used for measuring incorporation of FAs derived from *de novo* FA synthesis, while glucose

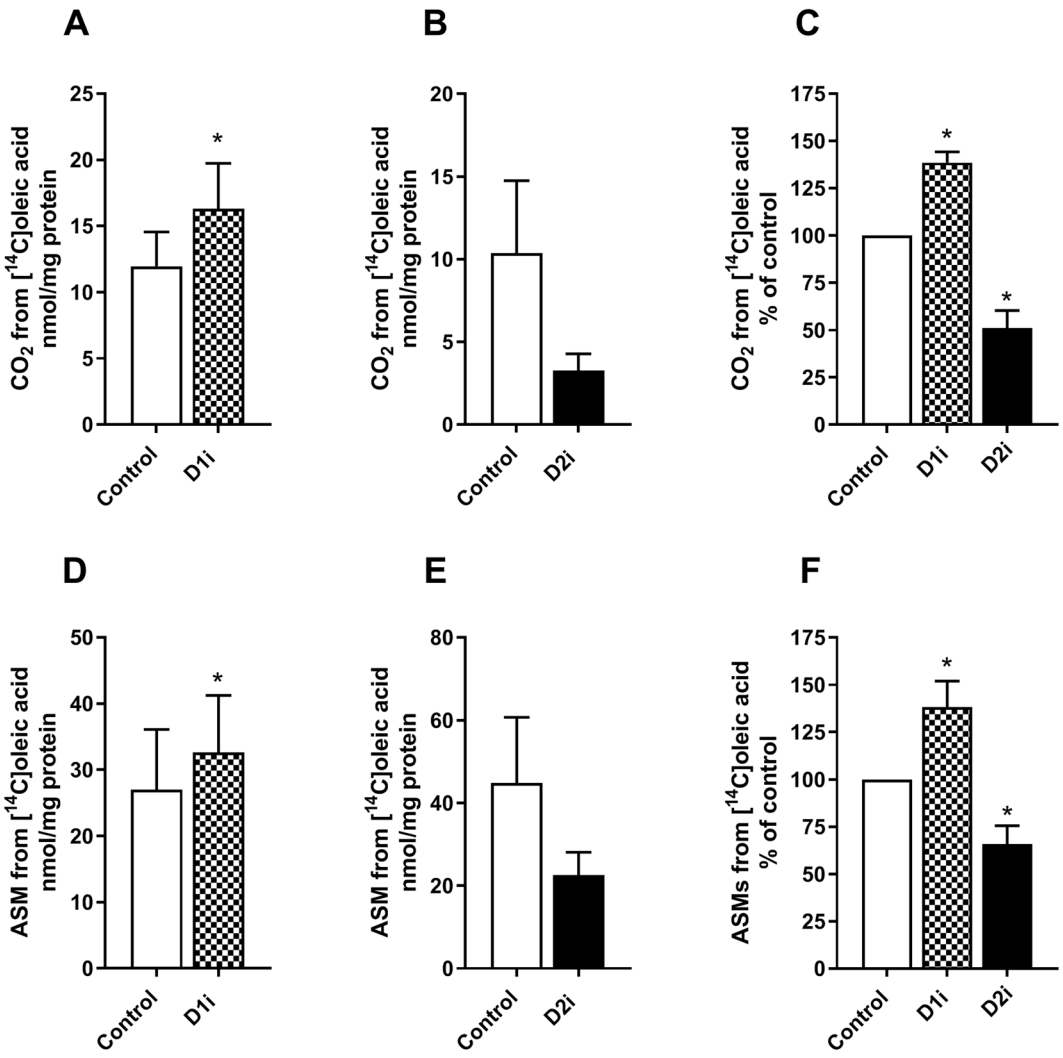

Figure 5. Effect of DGAT inhibition on oleic acid oxidation. Human myotubes were grown and differentiated on 12- or 24-well tissue culture plates. At day 7 of differentiation, myotubes were incubated with 100 µM [^{14}C] oleic acid (0.5 µCi/ml, 100 µM) for 4 h in presence or absence of D1i (1 µM) or D2i (10 µM), respectively. Complete oxidation (CO_2 production) and β-oxidation (ASMs) were measured. (**A–C)** Complete oxidation (CO_2) of oleic acid. (**D–F)** ASMs, which reflects incomplete oxidation (β-oxidation), of oleic acid. Results are presented as mean ± SEM from $n = 5$–6 (D1i) and $n = 8$ (D2i) individual experiments with 8 separate wells for each condition, as absolute values (nmol/mg protein) (**A,B** and **D,E**) and normalized to control (**C,F**). *$p < 0.05$ vs control, paired t-test. ASMs, acid soluble metabolites; D1i, DGAT1 inhibitor; D2i, DGAT2 inhibitor.

could be incorporated in both the glycerol backbone and Fas (Fig. 3). Interestingly, we observed a reduction of TAG by both D1i and D2i when glycerol or glucose was used as label. The effect of D2i was most striking without OA present, with a ~60% reduction in labelled TAG, whereas in the presence of OA a minor reduction (~25%) was observed. In contrast, treatment with D1i showed a marked inhibition of glycerol incorporation into TAG (~70%) in presence of OA, compared to without (~40%). These data suggest, as supported by studies in other tissues[15,16], that DGAT2 in skeletal muscle presumably contributes to *de novo* synthesis of TAG. Moreover, DGAT2 but not DGAT1 is co-localized with lipogenic enzymes like glycerol-3-phosphate acyltransferase and stearoyl-CoA desaturase 1[45,46]. Noteworthy, in the presence of exogenous FAs D1i was most effective in blocking TAG synthesis, even though the effect of D2i remained. This might be explained by substrate concentrations, as it has been reported that DGAT1 tends to be more active at higher substrate concentrations utilizing exogenous FA, whereas DGAT2 tends to be more active at lower FA concentrations[4]. Furthermore, DGAT2 has been suggested in some, but not all reports, to preferentially utilize endogenously synthesized FAs to form TAG in liver cells and brown adipocytes[11,15,16,47]. Our results did not indicate such a role of DGAT2 in skeletal muscle, where only DGAT1 inhibition reduced TAG synthesis from acetate, *i.e. de novo* FA synthesis. Also, incorporation of acetate into phospholipids was decreased by DGAT1 inhibition indicating that *de novo* FA synthesis was reduced when DGAT1 was inhibited, not only TAG synthesis (Fig. S-4). FA synthesis is, however, a complex process to measure in human primary muscle cells, since FA synthesis generally is limited[48], and time-aspects, substrate availability and experimental conditions may play important roles.

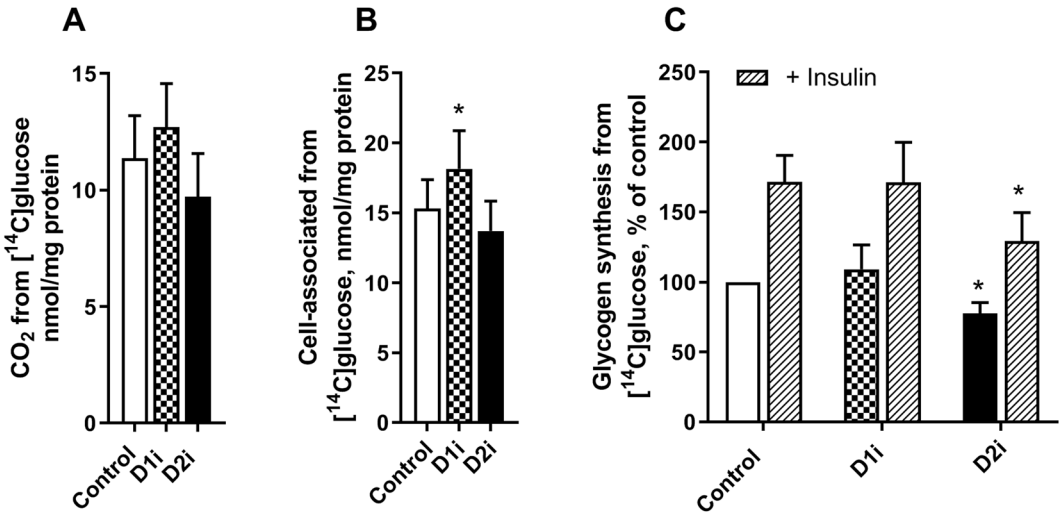

Figure 6. Effect of DGAT inhibitors on glucose metabolism. (**A–B**) Human myotubes were grown on 96-well tissue culture plates. At day 7, myotubes were incubated with [^{14}C(U)]glucose (0.5 µCi/ml, 200 µM) and D1i or D2i for 4 h. Oxidation, measured as CO_2 production from [^{14}C(U)]glucose (**A**) and cell-associated radioactivity from [^{14}C(U)]glucose (**B**) was measured after 4 h treatment with DGAT inhibitors; D1i (1 µM) and D2i (10 µM). (**C**) Alternatively, cells were grown in 12-well tissue culture plates. At day 7, myotubes were starved for 90 min (DMEM without glucose) before incubation for 3 h with D-[^{14}C(U)]glucose (1 µCi/ml, 5.5 mM) in presence or absence of D1i (1 µM) or D2i (10 µM) and with or without insulin (100 nM). Results are presented as mean ± SEM from $n = 9$ (**A,B**) individual experiments with 8 separate wells for each condition, or $n = 4$ (**C**) individual experiments with duplicate wells for each condition. Absolute values for glycogen synthesis: control 11.0 ± 4.5 and with insulin 22.4 ± 10.8 nmol/mg cell protein. *$p < 0.05$ vs control, paired t-test.

Skeletal muscle is central for utilizing lipids for energy production[49]. FAs stored as TAG in LDs are easily hydrolysed upon energy demand, and thereby are an important energy source[25]. Furthermore, turnover of TAG in LDs reflects the balance between lipolysis and esterification[50]. Our data showed that treatment with D1i, but not D2i, reduced the accumulation of [^{14}C]OA in cell lipids, and increased net lipolysis (Fig. 4). If it is assumed that the rate of lipolysis was the same between control and D1i-treated cells, there appears to have been a lower rate of re-esterification of FAs when DGAT1 was inhibited. Further, these data suggest that only DGAT1 is involved in the re-esterification of FA products of lipolysis in skeletal muscle. This is consistent with a previous observation in adipocytes, where DGAT1 has been shown to be the major enzyme involved in the re-synthesis of FAs derived from adipocyte lipolysis[18]. In our study we also observed an increased oxidation (complete and incomplete) after treatment with D1i, whereas D2i reduced both processes (Fig. 5). Increased oxidation after treatment with D1i confirmed previous observations, using the same D1i (1 µM) in human myotubes[36]. In accordance with this, a study in a cardiac-specific DGAT1 deletion mouse model showed an increased oxidation of exogenous FAs compared to wildtype[20]. We hypothesise that the increased oxidation we observed when DGAT1 was inhibited, is due to the diversion of FAs for oxidation. The reduced oxidation by D2i is on the other hand difficult to explain. It appears to be due to reduced substrate availability since both CO_2 and ASMs showed a similar reduction. This indicates a possible competition between utilization of oleoyl-CoA by carnitine palmitoyltransferase 1 and/or mitochondrial glycerol-3-phosphate acyltransferase and DGAT1. Moreover, DGAT2, but not DGAT1, is associated with LDs and co-localized to mitochondria in cultured fibroblasts and adipocytes[5,6], which may add a further dimension to the relationship between DGAT1 or DGAT2 activity and oxidation of exogenous or endogenous FAs.

As glucose and FA metabolism in skeletal muscle are tightly regulated[51,52], we also examined the effect of DGAT inhibitors on glucose metabolism (Fig. 6). Inhibition of DGAT enzymes could potentially alter glucose–FA interactions, as DGAT1 and DGAT2 are catalysing synthesis of TAG, which is the primary unit for storage of FAs and also has the potential of protecting cells from deleterious lipid intermediates[53]. We did not observe any effect on glucose oxidation by either of the DGAT inhibitors. However, treatment with D1i increased accumulation of glucose, whereas D2i decreased basal and insulin-stimulated glycogen synthesis. Even though these effects were relatively small, this indicates a link between DGAT-regulated processes and glucose metabolism in skeletal muscle. We also examined glucose metabolism with DGAT inhibitors in presence of oleic acid added acutely (that was shown to increase or decrease FA oxidation in Fig. 5), but no changes were found on glucose metabolism nor insulin action (data not shown). In support of our observations treatment with JTT-553 (DGAT1 inhibitor) in mice resulted in increased glucose uptake in adipose tissue[54]. Moreover, DGAT2 has been linked to the generation of acylceramides; its inhibition may raise intracellular ceramide levels[55]. Thus, inhibition of DGAT2 could potentially affect ceramide signalling. We also examined the effect of DGAT inhibitors on acylceramide formation from labelled FA which was very low and not changed by the inhibitors (data not shown). Although interesting, these data on glucose metabolism are presently difficult to interpret and requires more elaborate studies that are outside the scope of the present study.

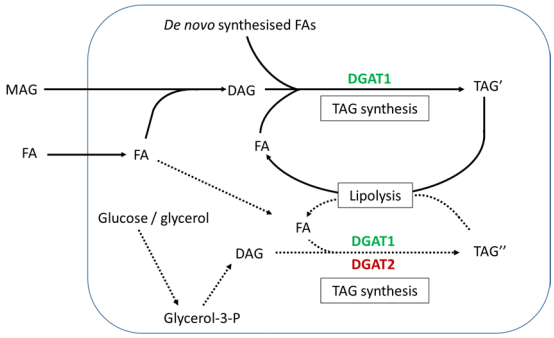

Figure 7. Model illustrating possible functions of DGAT1 and DGAT2 in human skeletal muscle cells. DGAT1 is dominant in human skeletal muscle cells utilizing FAs derived from various sources (exogenously supplied, *de novo* FA synthesis or FA derived from lipolysis) to generate TAG, both through the monoacylglycerol pathway and the *de novo* pathway from glycerol-3-P. DGAT2 seems to be specialised only for the synthesis of TAG, involving *de novo* incorporation of the glycerol moiety into TAG. Moreover, DGAT1 seems to operate at higher substrate concentrations, whereas DGAT2 may esterify substrates at lower concentrations for storage in TAG. Fatty acid oxidation from exogenous FA is regulated by DGAT activities. DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FA, fatty acid; Glycerol-3-P, glycerol-3-phosphate; MAG, monoacylglycerol; TAG, triacylglycerol; TAG′ and TAG″, heterogeneous pools of TAG.

Conclusion. In this study we demonstrated how inhibitors of DGAT1 and DGAT2 had distinct responses on lipid metabolism in skeletal muscle cells. DGAT1 seemed to be the major enzyme responsible for incorporation of both exogenously supplied and endogenously generated (through lipolysis) FAs into cellular lipids in human myotubes, as well as being involved in *de novo* synthesis of TAG (Fig. 7). On the other hand, DGAT2 seemed mainly to be contributing in *de novo* synthesis of TAG from glycerol-3-phosphate. Interestingly, DGAT activities were also important in determining the rates of FA oxidation, indicating a key role in balancing FAs between storage in TAG and efficient utilization through oxidation (Fig. 7). Because DGAT1 and DGAT2 may have distinct roles, they could give rise to heterogeneous pools of TAG in skeletal muscle. However, more studies are required to determine and further elucidate the mechanism and possible beneficial/deleterious effects of DGAT1 and DGAT2 on energy metabolism in skeletal muscle.

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Competing interests
The authors declare no competing interests.

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