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

Christine Skagen [a,*], Tuula A. Nyman [b], Xiao-Rong Peng [c], Gavin O'Mahony [d], Eili Tranheim Kase [a], Arild Chr Rustan [a], G. Hege Thoresen [a,e]

[a] Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, Norway
[b] Department of Immunology, Institute of Clinical Medicine, University of Oslo and Oslo University Hospital, Norway
[c] Bioscience Metabolism, Research and Early Development Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden
[d] Medicinal Chemsitry, Research and Early Development Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden
[e] Department of Pharmacology, Institute of Clinical Medicine, University of Oslo, Norway

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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).

* Corresponding author. Section for Pharmacology and Pharmaceutical Biosciences, Department of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, 0316, Oslo, Norway.
E-mail address: Christine.skagen@farmasi.uio.no (C. Skagen).

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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).

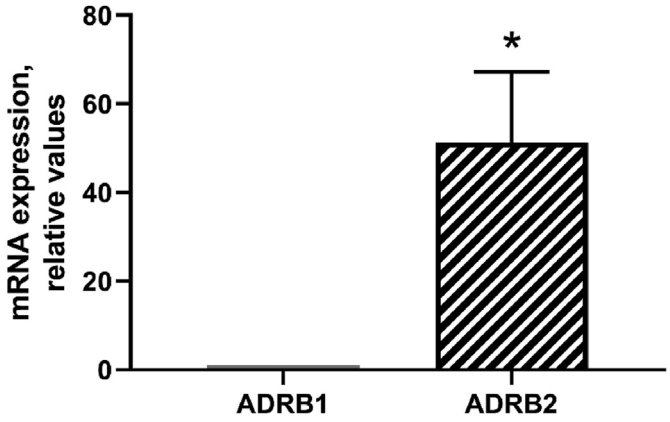

Fig. 1. mRNA expression of β-adrenergic receptors in human primary myotubes. Human myoblasts were seeded out, proliferated, and differentiated into myotubes on 6-well tissue culture plates. On day 7 of differentiation, the cells were harvested for PCR. mRNA was isolated and expression assessed by qPCR. 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).

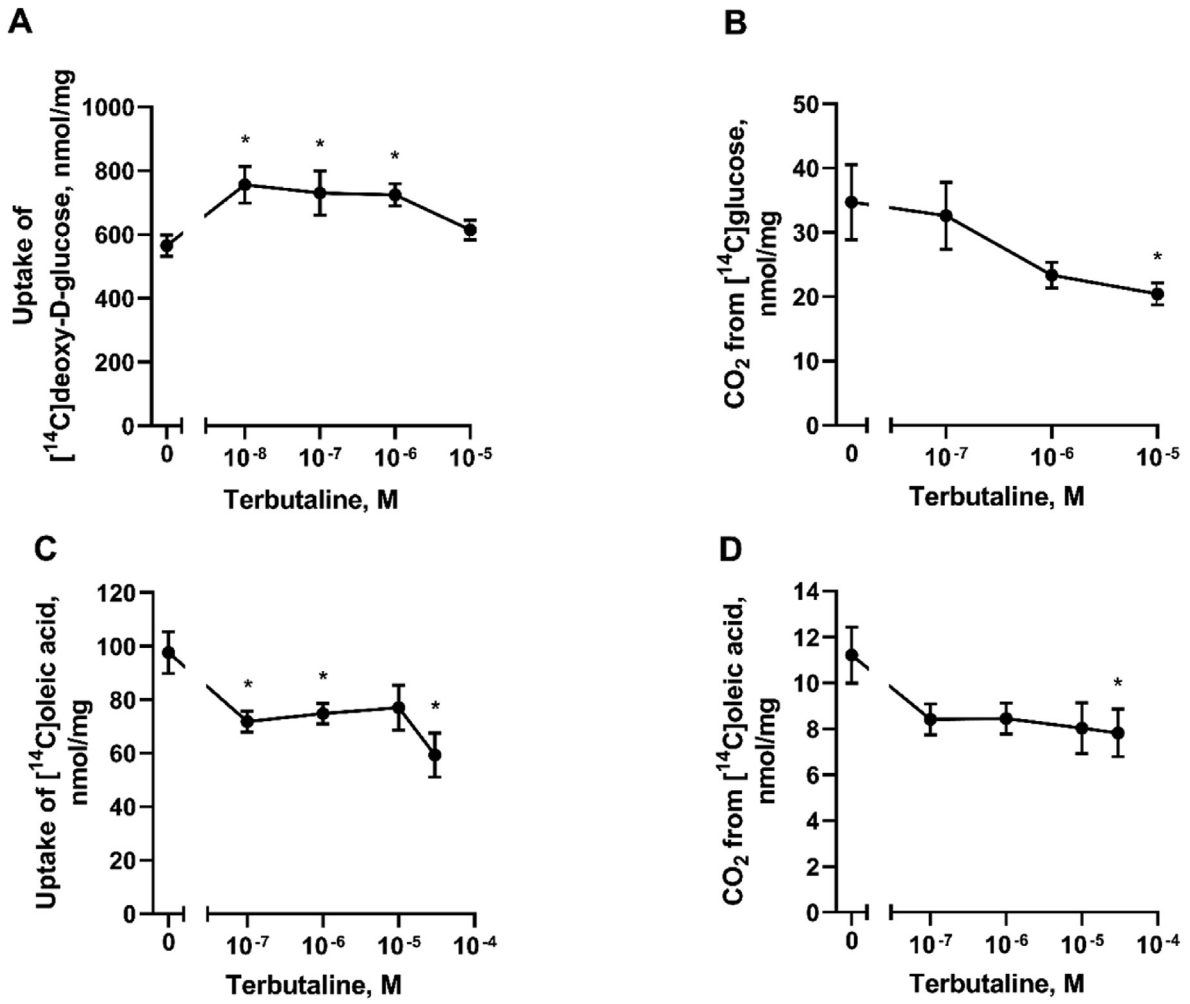

Fig. 2. Effect of 4 h treatment with terbutaline on glucose and oleic acid metabolism in human myotubes. Human myoblasts were grown and differentiated into myotubes for 7 days. **A:** The cells were treated with DMSO (0.01%) control or terbutaline for 4 h before the uptake of [^{14}C]deoxy-D-glucose (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

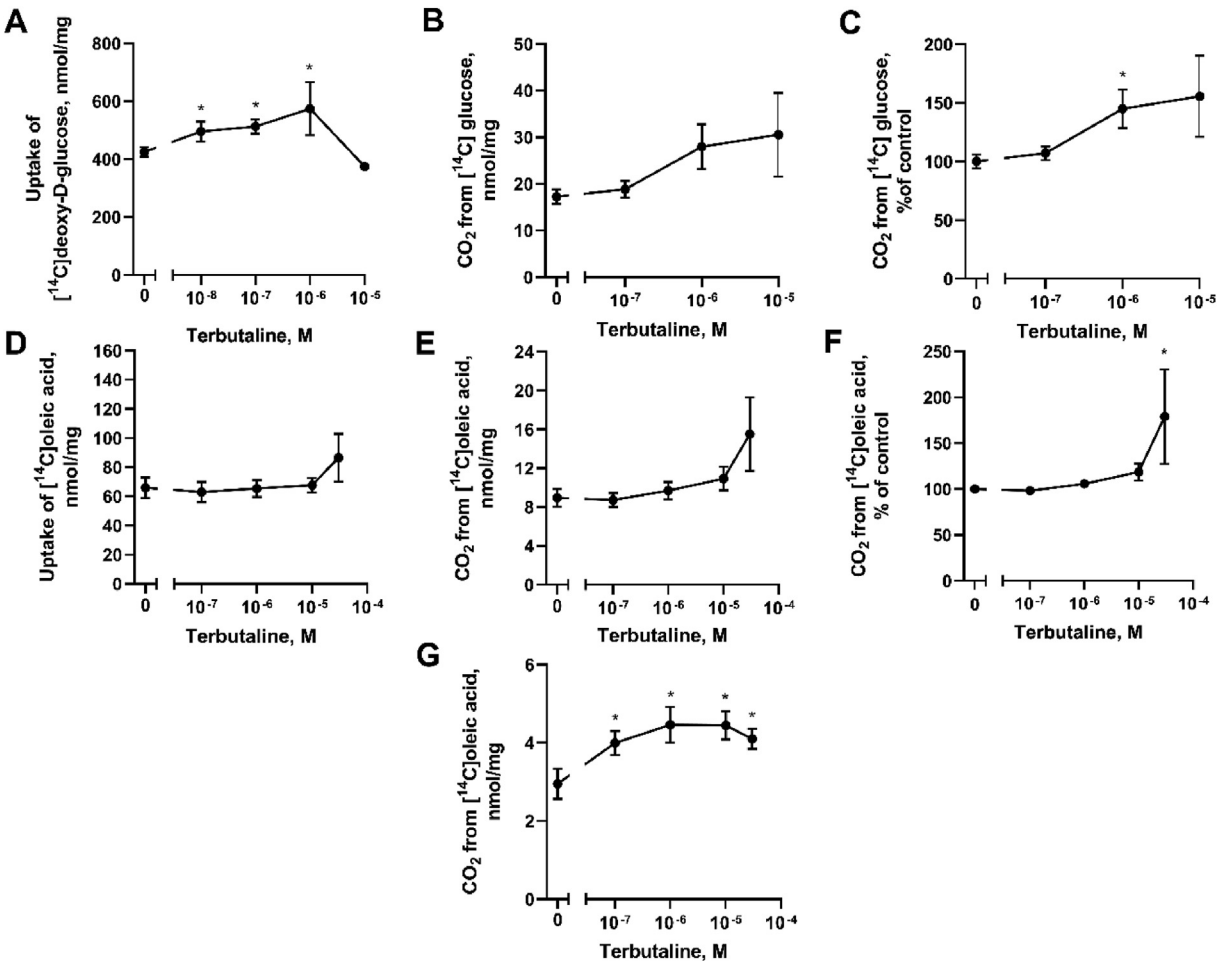

Fig. 3. Effect of 96 h terbutaline treatment with terbutaline on glucose and oleic acid metabolism in human myotubes. Myoblasts were grown and differentiated into myotubes for 7 days. On day 3 of differentiation the cells were treated with DMSO (0.01%) control or terbutaline for 96 h. On day 7 of differentiation: **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).

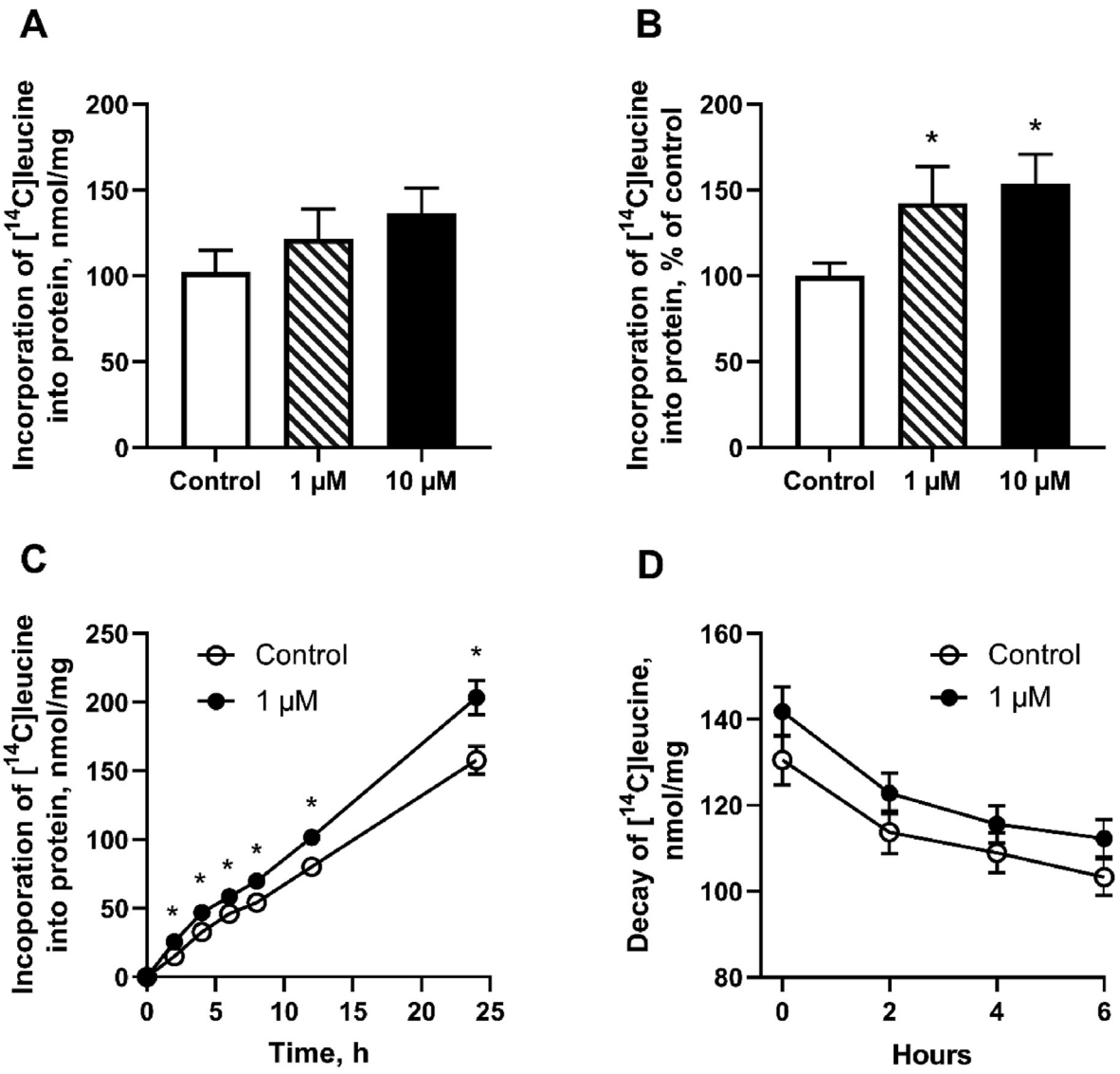

Fig. 4. Effect of 96 h terbutaline treatment on protein synthesis. Human myoblasts were grown and differentiated into myotubes. On day 3 of differentiation, the myotubes were treated with DMSO (0.01%) control or terbutaline (1 and 10 μM) for 96 h, before the cells were incubated with [^{14}C]leucine (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

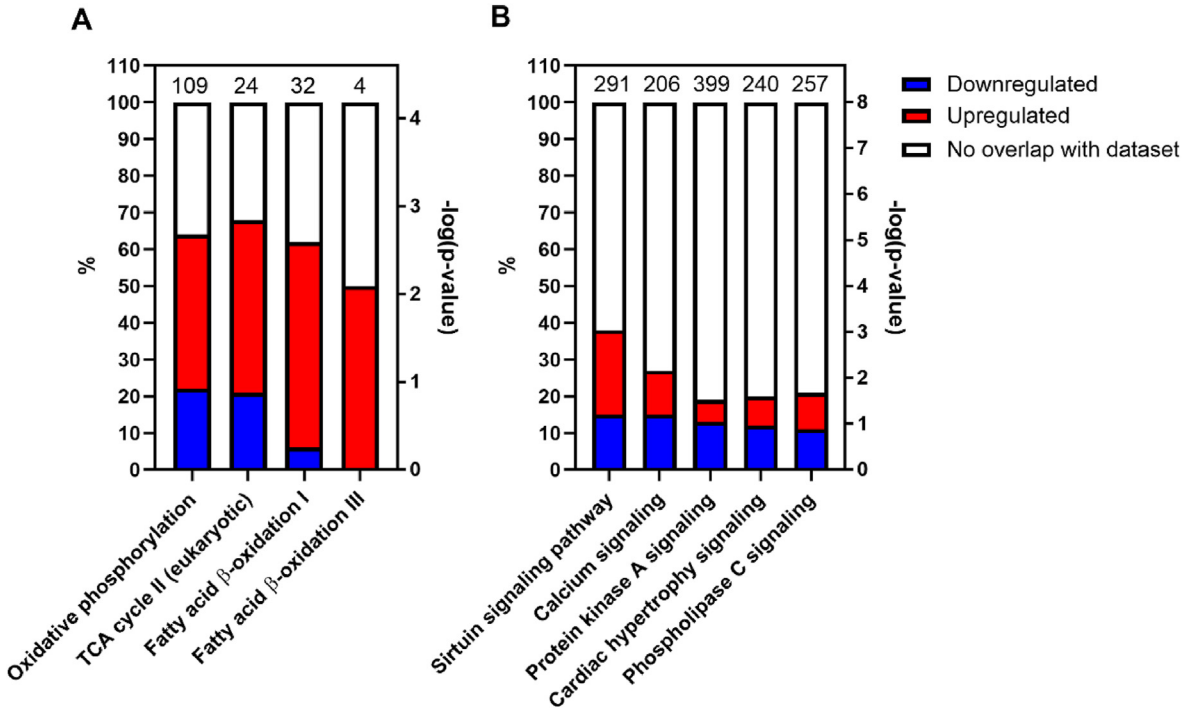

Fig. 5. Pathway analysis of myotubes after 96 h treatment with 1 μM terbutaline. Human myotubes were grown and differentiated in 25 cm^2 flasks. On day 3 of differentiation, cells were treated with DMSO control (0.01%) or 1 μM terbutaline for 96 h. On day 7 of differentiation the myotubes from 6 different 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.

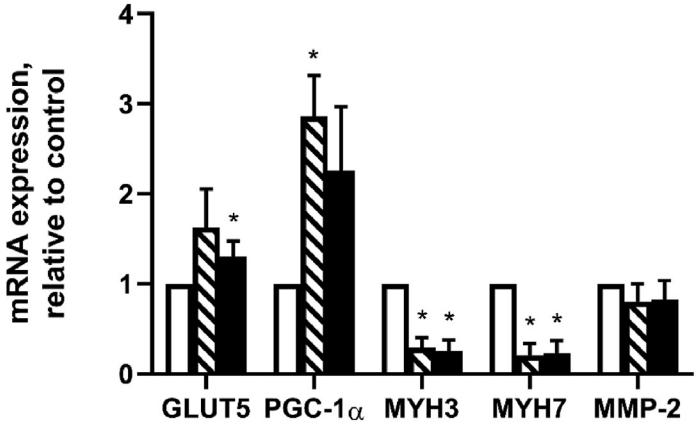

Fig. 6. mRNA expression of genes of interest after 96 h treatment with terbutaline. Human myotubes were grown and differentiated in 25 cm^2 cell culture flasks. On day 3 of differentiation, myotubes were treated with DMSO control (0.01%) or with 1 or 10 μM of terbutaline for 96 h. On day 7 of differentiation 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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