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The small molecule SERCA activator CDN1163 increases energy metabolism in human skeletal muscle cells

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ABSTRACT

Background and objective: A number of studies have highlighted muscle-specific mechanisms of thermogenesis involving futile cycling of Ca^{2+} driven by sarco (endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and generating heat from ATP hydrolysis to be a promising strategy to counteract obesity and metabolic dysfunction. However, to the best of our knowledge, no experimental studies concerning the metabolic effects of pharmacologically targeting SERCA in human skeletal muscle cells have been reported. Thus, in the present study, we aimed to explore the effects of SERCA-activating compound, CDN1163, on energy metabolism in differentiated human skeletal muscle cells (myotubes).

Methods: In this study, we used primary myotube cultures derived from muscle biopsies of the *musculus vastus lateralis* and *musculi interspinales* from lean, healthy male donors. Energy metabolism in myotubes was studied using radioactive substrates. Oxygen consumption rate was assessed with the Seahorse XF24 bioanalyzer, whereas metabolic genes and protein expressions were determined by qPCR and immunoblotting, respectively.

Results: Both acute (4 h) and chronic (5 days) treatment of myotubes with CDN1163 showed increased uptake and oxidation of glucose, as well as complete fatty acid oxidation in the presence of carbonyl cyanide 4-(trifluromethoxy)phenylhydrazone (FCCP). These effects were supported by measurement of oxygen consumption rate, in which the oxidative spare capacity and maximal respiration were enhanced after CDN1163-treatment. In addition, chronic treatment with CDN1163 improved cellular uptake of oleic acid (OA) and fatty acid β-oxidation. The increased OA metabolism was accompanied by enhanced mRNA-expression of carnitine palmitoyl transferase (*CPT*) *1B*, pyruvate dehydrogenase kinase (*PDK*) 4, as well as increased AMP-activated protein kinase $(AMPK)^{Thr172}$ phosphorylation. Moreover, following chronic CDN1163 treatment, the expression levels of stearoyl-CoA desaturase (*SCD*) 1 was decreased together with *de novo* lipogenesis from acetic acid and formation of diacylglycerol (DAG) from OA.

Conclusion: Altogether, these results suggest that SERCA activation by CDN1163 enhances energy metabolism in human myotubes, which might be favourable in relation to disorders that are related to metabolic dysfunction such as obesity and type 2 diabetes mellitus.

Abbreviations: SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; OA, oleic acid; OCR, oxygen consumption rate; T2DM, type 2 diabetes mellitus; FCCP, 4-(trifluromethoxy)phenylhydrazone; FA, fatty acid; DAG, diacylglycerol; CE, cholesteryl ester; SCD1, stearoyl-CoA desaturase 1; AMPK, AMP-activated protein kinase; ASM, acid-soluble metabolites.

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1. Introduction

Skeletal muscle represents about 40–50% of the body weight of adults and is a major site for fatty acid (FA) and glucose disposal (Egan and Zierath, 2013; Thoresen et al., 2011). Both glucose and FA metabolism in skeletal muscle are impaired in type 2 diabetes mellitus (T2DM) (Kelley et al., 2002). Since skeletal muscle is the largest insulin-sensitive organ, accounting for ~80% of insulin-stimulated glucose disposal (R. A. DeFronzo, 2004; Ralph A. DeFronzo and Tripathy, 2009), hyperglycaemia due to the reduced ability of insulin to increase glucose uptake is a key feature implicated in the pathogenesis of T2DM (R. A. DeFronzo, 2004). Being the largest metabolic organ in the body, skeletal muscle also contributes greatly to temperature homeostasis by both shivering and non-shivering thermogenic mechanisms (Bal et al., 2012; Block, 1994). Therefore, skeletal muscle plays a key role in regulation of whole-body metabolic rate.

It is well known that muscle contractile activity (shivering) results in heat liberation. However, prolonged muscle activity cannot be sustained as it results in overheating and muscle fatigue. On the other hand, muscle non-shivering thermogenesis (NST), which is independent of muscle contractions, is also suggested to play an important role in thermoregulation (Block, 1994; Silva, 2011). Recent studies have shown that futile calcium cycling between cytosol and sarcoplasmic reticulum, specifically sarcolipin-mediated uncoupling of Ca^{2+} transport by sarco (endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), could be an important mechanism for muscle-based NTS in mammals (Bal et al., 2012; Bombardier et al., 2013; de Meis, 2001; Smith et al., 2002). At the same time, free cytoplasmic Ca^{2+} ions can act as second messengers and elicit a stimulatory effect of oxidative processes and substrate catabolism (Block, 1994). SERCA is a member of family P-type Ca^{2+}-ATPase with more than 10 isoforms encoded by three separate genes; *ATP2A1* (SERCA1), *ATP2A2* (SERCA2) and *ATP2A3* (SERCA3), through alternative splicing (Periasamy and Kalyanasundaram, 2007; Rossi and Dirksen, 2006). Furthermore, SERCA1 and SERCA2 isoforms are expressed in fast-twitch and slow-twitch skeletal muscle respectively, whereas SERCA3 isoforms are mainly expressed in non-muscle tissues such as hematopoietic cell lines and epithelial cells (Periasamy and Kalyanasundaram, 2007). In cultured human myotubes, we have recently reported the expression of SERCA1 and SERCA2 (Jan et al., 2021).

Accumulating evidence shows that reduced expressions and function of SERCA is associated with development of various disease conditions. Using a rat model, Sayen et al. demonstrated that hypothyroidism led to a marked decrease in SERCA1 and SERCA2 mRNA levels in soleus muscle, whereas SERCA1 mRNA levels were decreased in extensor digitorum longus muscle (Sayen et al., 1992). Diminished activity and expression of SERCA2 in liver (Park et al., 2010), islets (Kulkarni et al., 2004), and heart (Wold et al., 2005) have also been described in animal models of obesity/diabetes, highlighting the imperative pathological role of SERCA dysfunction in disturbance of metabolic homeostasis. These findings have led to the search for pharmacological agents that increase SERCA activity as a potential approach in therapeutics for the treatment of obesity, T2DM, as well as cardiac dysfunction.

CDN1163 is a quinoline derivative that has been reported to be a SERCA activating compound (Cornea et al., 2013; Fisher et al., 2020; Gruber et al., 2014) which directly binds SERCA and upturn the activity at saturating [Ca^{2+}] (V_{max}) in a concentration-dependent manner (Gruber et al., 2014). The ability of CDN1163 to activate SERCA has been demonstrated in liver endoplasmic reticulum microsomes purified from *ob/ob* mice (Kang et al., 2016), sarcoplasmic reticulum (SR) membrane vesicles from pig hearts, skeletal muscle SR obtained from New Zealand white rabbits (Cornea et al., 2013), SERCA2 transfected HEK cells (Gruber et al., 2014) and reconstituted vesicles system of SERCA1 from rabbit skeletal muscle (Fisher et al., 2020). Furthermore, pharmacological activation of SERCA2 by CDN1163 has been shown to have a potential therapeutic effect in different animal models of oxidative stress-related diseases. These include amelioration of muscle impairment in CuZnSOD1-deficient mice (Qaisar et al., 2019), alleviating endoplasmic reticulum stress-induced cell death in the rat 6-hydroxydopamine model of Parkinson's disease (Dahl, 2017), rescuing injured neurons from apoptosis in APP/PS1 double transgenic mouse model of Alzheimer's disease (Krajnak and Dahl, 2018), and restoration of Ca^{2+} homeostasis in diabetic hearts by suppressing resistin expression in *ob/ob* diabetic mice (Singh et al., 2018). CDN1163 has also been shown to enhance Ca^{2+} transport activity in the liver of *ob/ob* mice (Kang et al., 2016). Pharmacological restoration of SERCA activity in this animal model was shown to improve glucose homeostasis and hepatic lipid metabolism as well as reduction in whole body fat mass.

To the best of our knowledge, no studies have been conducted to examine the metabolic impact of targeting SERCA in human skeletal muscle cells. Therefore, in the current work we wanted to explore the potential effect of CDN1163 on energy metabolism in primary human myotubes.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM)-Glutamax™ low glucose, Dulbecco's Phosphate Buffered Saline (DPBS; without Ca^{2+} and Mg^{2+}), heat-inactivated foetal bovine serum (FBS), penicillin-streptomycin (10 000 IE/ml), amphotericin B, human epidermal growth factor (hEGF), trypsin-EDTA, Restore™ PLUS Western Blot stripping buffer, Super Signal™ West Femto Maximum Sensitivity substrate, Pierce™ BCA Protein Assay Kit, Power SYBR® Green PCR Master Mix, TaqMan reverse transcription kit reagents, High-Capacity cDNA Reverse Transcription Kit, MicroAmp® Optical 96-well Reaction Plate, MicroAmp® Optical Adhesive Film, and primers for TaqMan PCR were purchased from Thermo Fisher Scientific (Waltham, MA, US). Insulin (Actrapid® Penfill® 100IE/ml) was from NovoNordisk (Bagsvaerd, Denmark). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), β-mercaptoethanol, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), dexamethasone, gentamicin, L-glutamine, L-carnitine, protease inhibitor, phosphatase II inhibitor, trypan blue 0.4% solution D-glucose, oleic acid (OA, 18:1, n-9), and carbonyl cyanide 4-(trifluromethoxy)phenylhydrazone (FCCP) were obtained from Sigma-Aldrich (St. Louis, MO, US). QIAshredder and RNeasy Mini Kit were from QIAGEN (Venlo, the Netherlands). Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Precision Plus Protein™ Dual Color standard, bromophenol blue, Trans-Blot® Turbo™ Mini-size Transfer nitrocellulose membranes, Trans-Blot® Turbo™ Mini-size Transfer Stacks, Mini-Protean® TGX™ gels (4–20%), Goat anti-rabbit IgG (H+L)-HRP Conjugate (#170-6515), Clarity Western ECL substrates, Tris-HCl, sodium dodecyl sulfate (SDS), Tris/glycine/SDS buffer, and Tween 20 were from Bio-Rad (Copenhagen, Denmark). Antibodies against total and phosphorylated AMP-activated protein kinase (AMPK) α at Thr172 (#2531 and #2532, respectively), total and phosphorylated TBC1 domain family member 4 (TBC1D4) at Thr642 (#2670 and #4288) were from Cell Signalling Technology® Inc. (Beverly, MA, US). Antibody against human total oxidative phosphorylation (OXPHOS) complexes I to V (#110411) was from Abcam (Cambridge, UK). D-[^{14}C]glucose (3.0 mCi/mmol), [1–^{14}C]oleic acid (OA, 59.0 mCi/mmol) and [1–^{14}C]acetic acid (50.5 mCi/mmol) were from PerkinElmer NEN® (Boston, MA, US). Ultima Gold™ XR, Pico Prias 6 ml PE vials, 96-well Isoplate®, UniFilter®-96 GF/B microplates, and TopSeal®-A transparent film were obtained from PerkinElmer (Shelton, CT, US). 6-well, 12-well and 96-well Corning® CellBIND® tissue culture plates were from Corning (Schiphol-Rijk, the Netherlands). Thin layer chromatography plates were purchased from Merck (Darmstadt, Germany). Free fatty acids (FFA, 2 mg/ml), cholesterol ester (CE, 2 mg/ml) and mono-, di-, triglyceride mic (4 mg/ml) were from Supelco (Bellefonte, PA, US). CDN1163 (4-(1-Methylethoxy)-N-(2-methyl-8-quinolinyl)-benzamide) was provided by the AstraZeneca compound library.

2.2. Ethics statement

The human skeletal muscle biopsies were obtained after informed written consent and approval by the Regional Committee for Medical and Health Research Ethics South East, Oslo, Norway (reference number: 2011/2207). The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

2.3. Methods

2.3.1. Cell culture

Human satellite cells were isolated from muscle biopsy samples from *musculus vastus lateralis* and *musculi interspinales* from lean, healthy male donors as previously described (Lund et al., 2018). Isolation of satellite cells was performed based on the method of Henry et al. (Henry et al., 1995) with the modifications described by Gaster et al. (M. Gaster, Kristensen, Beck-Nielsen and Schrøder, 2001). The isolated cells were cultured and proliferated in DMEM-Glutamax (5.5 mM glucose) supplemented with 10% FBS, HEPES (25 mM), gentamicin (50 ng/ml), penicillin (25 IU), streptomycin (25 μg/ml), amphotericin B (1.25 μg/ml), hEGF (10 ng/ml), dexamethasone (0.39 μg/ml) and 0.05% BSA. Differentiation of myoblasts into multinucleated myotubes was induced at 80–90% confluence by changing the medium to DMEM-Glutamax (5.5 mM glucose) supplemented with 2% FBS and 25 pM insulin. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2, and the medium was changed regularly every 2–3 days. Experiments were carried out 7–8 days after the induction of cell differentiation.

2.3.2. Substrate oxidation assay

Myoblasts were seeded in 96-well culture plates at a density of 7000 cells/well, proliferated and differentiated into myotubes. The cells were preincubated with CDN1163 (0.01 μM, 0.1 μM or 1 μM) for 5 days (chronic) or for 4 h (acute) in the presence or absence of a mitochondrial uncoupler (FCCP, 1 μM) during the substrate oxidation assay, a method used for measurement of substrate uptake and oxidation. This assay was performed as previously described (Wensaas et al., 2007). In brief, a 96-well filter plate (UniFilter® GF/B) was first activated by NaOH (1 M) and 50 μl of substrate medium consisting of D-[^{14}C(U)]glucose (0.5 μCi/ml, 200 μM) or [1–^{14}C]oleic acid (0.5 μCi/ml, 100 μM) was added to each well of 96-well CellBIND® microplate cultured with myotubes. The filter plate was then mounted on top of the CellBIND® microplate forming a "sandwich" with a silicon gasket between them. After 4 h of trapping, the amounts of $^{14}CO_2$ produced from cellular respiration and cell-associated (CA) radioactivity was measured using a 2450 MicroBeta^2 liquid scintillation counter (PerkinElmer). CO_2 and CA data were related to cellular protein concentration measured using the Bio-Rad assay on a VICTOR™ *X*4 Multilabel plate reader (PerkinElmer). Total cellular uptake was quantified as the sum of oxidized substrate, acid-soluble metabolites (ASMs), and the remaining CA radioactivity in myotubes, whereas oxidative spare capacity was calculated by subtracting basal oxidation from oxidation of substrate in the presence of 1 μM FCCP.

2.3.3. Acid-soluble metabolites

Incomplete fatty acid oxidation (β-oxidation) was assessed by measuring ASMs from [1–^{14}C]OA (0.5 μCi/ml, 100 μM) released by the myotubes using the method described by Skrede et al. (Skrede et al., 1994). In brief, a volume of 100 μl radiolabelled incubation medium from the substrate oxidation assay was transferred to a new Eppendorf tube and precipitated with 30 μl BSA (6%) and 300 μl ice cold perchloric acid ($HClO_4$, 1M). Thereafter, the tube was centrifuged at 10.000 rpm for 10 min at 4 °C. Then, 200 μl of the supernatant was transferred to a scintillation vial before 3 ml of Ultima Gold scintillation fluid was added and counted by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer).

2.3.4. Lipid distribution - incorporation from acetate and oleic acid

Skeletal muscle cells were cultured on 12-well culture plates and treated with 0.01 μM, 0.1 μM or 1 μM CDN1163 the last 5 days of differentiation before the medium was replaced with DMEM-Glutamax (5.5 mM glucose) medium containing [1–^{14}C]OA (0.5 μCi/ml, 100 μM) and incubated for 4 h. Alternatively, the cells were incubated with [1–^{14}C]acetic acid (1 μCi/ml, 100 μM) in DMEM-Glutamax (5.5 mM glucose) in presence or absence of CDN1163 for the last 24 h of the differentiation period. Following incubation, the myotubes were washed twice with 0.5 ml PBS and harvested in 0.1% SDS (250 μl/well). Cellular lipids were extracted with chloroform-methanol (2:1, v/v, Folch extraction) and 0.9% sodium chloride solution (pH 2) as previously described (Folch et al., 1957; Michael Gaster, Rustan, Aas and Beck-Nielsen, 2004). After evaporation of the organic phase by N_2, the extracted lipids were re-dissolved in 130 μl hexane and separated by thin layer chromatography (TLC) using a non-polar solvent mixture of hexane:diethyl ether:-acetic acid (65:35:1, v/v/v). Iodine vapour was used to visualize the lipid bands prior to radioactivity quantification by liquid scintillation (Packard Tri-Carb 1900 TR, PerkinElmer).

2.3.5. RNA isolation and analysis of gene expression by qPCR

Total RNA from cultured myotubes was extracted using the RNeasy mini kit according to manufacturer's protocol, and the quality and concentration of RNA was determined by a microvolume spectrophotometer (Nanodrop Lite, Thermo Fisher Scientific). The RNA was reversely transcribed to cDNA with TaqMan Reverse Transcription Reagents using a PerkinElmer Thermal Cycler 9600 (25 °C for 10 min, 37 °C for 80 min and 85 °C for 5 min). cDNA content was determined by qPCR using StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) with SYBR® Green Master-mix and gene-specific primers designed using Primer Express® (Thermo Fisher Scientific). All experiments were run for 44 cycles (95 °C for 15 s followed by 60 °C for 60 s). The primer sequences for each gene used in this study are listed in Table 1. The relative RNA expression was determined by $2^{-\Delta\Delta Ct}$-method and normalisation to the housekeeping gene ribosomal protein lateral stalk subunit P0 (*RPLP0*). The housekeeping gene glyceralderhyde-3-phosphate dehydrogenase (*GAPDH*) was also analysed; there were no differences between normalising for *GAPDH* and *RPLP0*.

2.3.6. Determination of lactate concentration in cell media

Skeletal muscle cells were cultured and treated with different

Table 1
Description of forward and reverse primers.

Gene	Acc.no.	Forward sequence	Reverse sequence
RPLP0	M17885	CCA TTC TAT CAT CAA CGG GTA CAA	AGC AAG TGG GAA GGT GTA ATC C
GAPDH	NM002046	TGC ACC ACC ACC TGC TTA GC	GGC ATG GAC TGT GGT CAT GAG
PPARGC1A	NM013261.3	AAA GGA TGC GCT CTC GTT CA	TCT ACT GCC TGG AGA CCT TGA TC
SCD1	NM_005063	CCG CTG GCA CAT CAAC	ATG GCG GCC TTG GAG ACT
CPT1B	D1852C12	GAG GCC TCA ATG ACC AGA ATG	GTG GAC TCG CTG GTA CAG GAA
CD36	L06850	AGT CAC TGC GAC ATG ATT AAT GGT	CTG CAA TAC CTG GCT TTT CTC AA
ACC2	U89344	CCA TCT TCG ACG TCC TGA ATA CT	CTG TTT AAC ACA TAG GCG ATG TAA GC
CYC1	NM001916	CTG CCA ACA ACG GAG CAT T	CGT GAG CAG GGA GAA GAC GTA
FABP4	J02874	TGG TGG TGG AAT GCG TCA T	CAA CGT CCC TTG GCT TAT GC
PDK4	BC040239	TTT CCA GAA CCA ACC AAT TCA CA	TGC CCG CAT TGC ATT CTT A
FAS	U26644	GAA CTC CTT GGC GGA AGA GA	GTT CTG AGA AAG GTC GAA TTT GC
FOXO1	NM002015.4	GTG TTG CCC AAC CAA AGC TT	CTC AGC CTG ACA CCC AGC TAT

concentrations of CDN1163 for 5 consecutive days during differentiation. Culture media were collected from the last 48 h of differentiation and CDN1163-treatment. The concentrations of lactic acid in culture media were measured using Biosen C-Line glucose and lactate analyser (EKF Diagnostics, London, UK), according to the manufacturer's protocol, where a volume of 20 μl media from each treatment was transferred into an EKF safe-lock tube prefilled with 1 ml of haemolysing solution prior to the measurement.

2.3.7. Immunoblotting

Myotubes were harvested in Laemmli buffer (0.5 M Tris-HCl, 10% SDS, 20% glycerol, 10% β-mercaptoethanol, and 5% bromophenol blue). The proteins were electrophoretically separated on 4–20% Mini-Protean TGX™ gels with Tris/glycine buffer (pH 8.3) followed by blotting to nitrocellulose membranes and incubation with antibodies. Immunoreactive bands were visualized with enhanced chemiluminescence (Chemidoc XRS, Bio-Rad) and quantified with Image Lab (version 6.0.1) software.

2.3.8. Mitochondrial stress test

Measurement of the mitochondrial oxygen consumption rate (OCR) was performed using a Seahorse XF24 bioanalyzer (Agilent, Wilmington, DE, US). Skeletal muscle cells from six donors were mixed and plated on a XF24-well cell culture plate at a cell density of 25 000 cells/well, proliferated and differentiated into myotubes prior to measurement of OCR and extracellular acidification rate (ECAR). One hour before the measurement, culture medium was replaced with unbuffered DMEM (Agilent) supplemented with 5.5 mM glucose and 1 mM sodium pyruvate. The "mitochondrial stress test" was performed with addition of oligomycin (5 μM) to determine proton leak, FCCP (3 μM) to determine maximal respiration, and rotenone (1 μM) to assess complex 1-independent respiration/non-mitochondrial respiration. OCR was measured over time following successive injection of the modulators in the cell assay plate. The OCR parameters of non-mitochondrial respiration (NMR i.e., minimum rate measurement after rotenone injection), basal respiration (BR i.e., last rate measurement before oligomycin injection – NMR), maximal respiration (MR i.e., maximum rate measurement after FCCP injection – NMR), proton leak (PL i.e., minimum rate measurement after oligomycin injection – NMR), spare respiratory capacity (SRC i.e., MR – BR), and ATP production (last rate measurement before oligomycin injection – minimum rate measurement after oligomycin injection) were calculated.

2.3.9. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.0.1 for Windows (GraphPad Software Inc., San Diego, CA, US). All data were presented as mean ± SEM unless stated otherwise. The value *n* represents number of individual experiments with 3–8 wells for each condition in each experiment unless stated otherwise. Comparisons between groups were performed using unpaired Student's *t*-test. A p value < 0.05 was considered statistically significant.

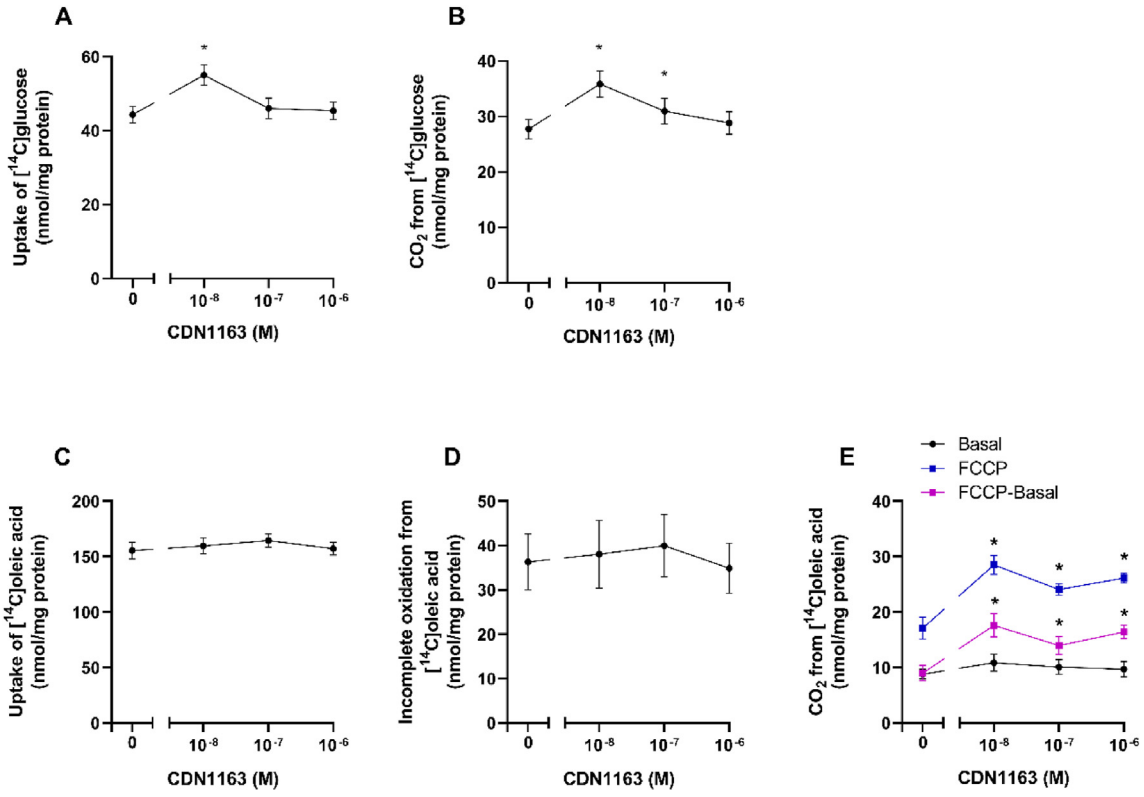

Fig. 1. Enhanced glucose metabolism and spare capacity of oleic acid oxidation in myotubes after acute CDN1163 exposure. Myotubes were incubated with 0.01 μM, 0.1 μM, or 1 μM CDN1163 for 4 h in the presence of [^{14}C]glucose (0.5 μCi/ml, 200 μM) before oxidation (trapped CO_2) and cell-associated (CA) radioactivity of [^{14}C]glucose were measured. (A) Glucose uptake was quantified as the sum of oxidized glucose, acid-soluble metabolites (ASM) and the remaining CA radioactivity in myotubes. (B) Complete oxidation of glucose by the cells. (C–E) Effects of acute CDN1163 on oleic acid (OA) metabolism after myotubes were exposed to CDN1163 together with [^{14}C]OA (0.5 μCi/ml, 100 μM) in presence or absence of carbonyl cyanide 4-(trifluromethoxy)phenylhydrazone (FCCP, 1 μM) for 4 h. (C) Total cellular uptake of OA (CO_2 + CA + ASM). (D) Formation of ASMs in myotubes after 4 h treatment with CDN1163. (E) Complete OA oxidation in presence or absence of FCCP. Spare capacity (E) was calculated by subtracting basal OA oxidation from oxidation of oleic acid in the presence of FCCP. Values are presented as mean ± SEM in nmol/mg cell protein from eight ($n = 8$, A and B) or four ($n = 4$, C-E) individual experiments. *Statistically significant compared to control ($p < 0.05$, unpaired *t*-test).

3. Results

3.1. Effects of acute CDN1163 treatment on substrate metabolism

Since glucose and FA are important fuel sources in skeletal muscle, we aimed to examine the effect of CDN1163 on metabolism of these substrates. After acute exposure of human myotubes to different concentrations of CDN1163 in the presence of ^{14}C-glucose or ^{14}C-OA for 4 h, total uptake and oxidation of the substrates by the cells were determined. The total uptake of glucose was increased in myotubes treated with 0.01 μM CDN1163 compared to control cells (Fig. 1A). Moreover, incubation of myotubes with 0.01 μM and 0.1 μM CDN1163 induced an increase in glucose oxidation (Fig. 1B). There was FCCP-response in the range of 2.5- to 3-fold increase; however, there was no significant changes in glucose metabolism in the presence of FCCP for any of the CDN1163 concentrations (data not shown).

The CO_2 liberated from ^{14}C-OA was also measured in presence or absence of the mitochondrial uncoupler FCCP. Assessment of cellular FA uptake, calculated as the sum of the remaining ^{14}C-OA accumulated (i.e., CA), CO_2 liberated, and ASMs showed no difference between control and CDN1163-treated myotubes (Fig. 1C). The results also showed that there were no changes in complete FA oxidation (CO_2) or incomplete β-oxidation (ASM) under basal conditions after acute treatment with CDN1163 (Fig. 1D and E). However, when we additionally supplied FCCP (1 μM) together with CDN1163, OA oxidation (Fig. 1E) and oxidative spare capacity (Fig. 1E) were observed to be significantly increased by CDN1163.

Based on the effects of CDN1163 observed on substrate oxidation, cellular metabolism was assessed by extracellular flux analyser (Seahorse instrumentation) in acute CDN1163-treated human myotubes. The oxygen consumption rate (OCR) at a concentration of 0.1 μM CDN1163 was higher compared to control cells after addition of the mitochondrial uncoupler FCCP (Fig. 2A). Acute treatment with this concentration of CDN1163 also increased maximal respiration and spare respiratory capacity (Fig. 2B). Extracellular acidification rate (ECAR) measurements also showed increased acidification upon CDN1163 exposure at baseline (Supplementary Fig. 1).

3.2. Effects of chronic CDN1163 treatment on substrate utilisation

In order to determine whether prolonged treatment with CDN1163 affected glucose and FA utilisation, myotubes were pretreated with various concentrations of CDN1163 for 5 days during differentiation. The results revealed a modest increase in both cellular uptake (Fig. 3A and B) and oxidation (Fig. 3C and D) of glucose when data were normalised to untreated control myotubes. In addition, both total cellular uptake of OA (Fig. 3E) and incomplete FA β-oxidation (Fig. 3F) were increased following chronic CDN1163-treatment.

In accordance with the findings for OA metabolism from acute CDN1163 treatment, there was no difference in the extent of complete OA oxidation induced by CDN1163 compared to control (Fig. 3G). However, CO_2 liberated from ^{14}C-OA in the presence of FCCP (Fig. 3H) were significantly enhanced by CDN1163 at a concentration of 0.1 μM when data were normalised to control under basal conditions. The oxidative spare capacity was also increased by CDN1163 (Fig. 3I).

We investigated further the metabolism of myotubes following 5 days of chronic CDN1163-treatment using Seahorse bioanalyzer. Mitochondrial stress test revealed that the OCR was consistently higher in myotubes chronically exposed to CDN1163 compared to control cells (Fig. 4A). Furthermore, spare respiratory capacity was also significantly increased (Fig. 4B), whereas maximal respiration tended to be increased for the lowest concentration of CDN1163 ($p = 0.058$). The other parameters (i.e., basal respiration, proton leak, non-mitochondrial oxygen consumption, and ATP production) were unchanged. Although ECAR measurements showed no significant differences between CDN1163-treated and untreated myotubes, acidification upon CDN1163 exposure was observed to be higher at baseline as well as in the presence of oligomycin, FCCP and rotenone (Supplementary Fig. 2).

As there was an alteration in cellular uptake of glucose both after acute and chronic CDN1163-exposure, we also wanted to determine the effect of CDN1163 on lactate production. Differentiated myotubes were cultured in the presence of CDN1163 for 5 days before lactate concentration was measured in conditioned medium collected from the last 48 h of differentiation and CDN1163-treatment. Compared to media collected from untreated control myotubes, a small increase in lactate concentration was observed in media from myotubes treated with CDN1163 (Fig. 4C and D).

3.3. Changes in expressions of genes and proteins after chronic treatment of cultured myotubes with CDN1163

In order to explore whether the observed metabolic changes in myotubes by CDN163-treatment could be due to changes in gene expression, mRNA expression of selected mitochondria-related genes were evaluated

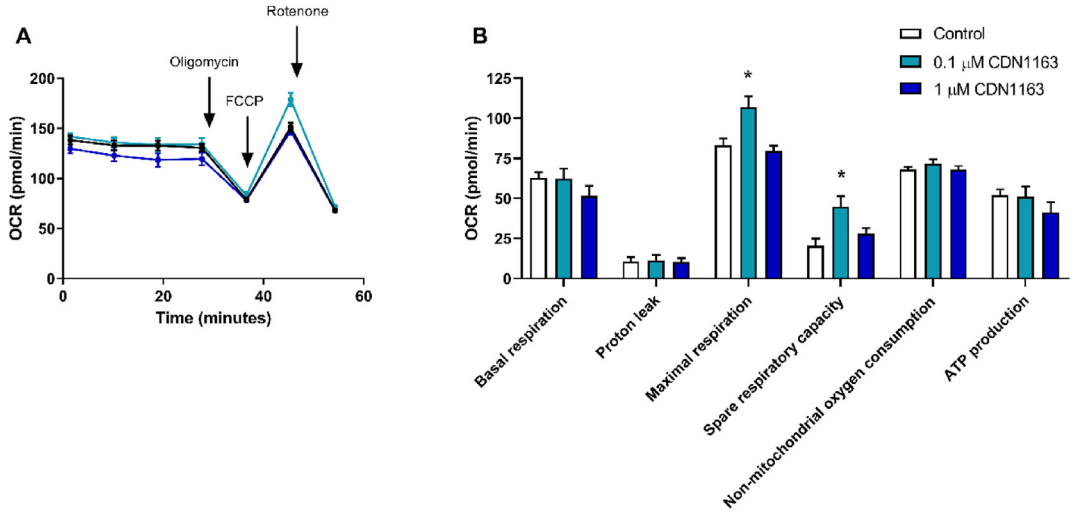

Fig. 2. Acute CDN1163 exposure increased mitochondrial function in human myotubes. Human myotubes grown in XF24-well cell culture plate were incubated for 1 h with CDN1163 prior to measurements. Oxygen consumption rate (OCR) was assessed with the Seahorse XF24 bioanalyzer following a sequential addition of oligomycin, carbonyl cyanide 4-(trifluromethoxy)phenylhydrazone (FCCP) and rotenone. (A) OCR time curve and assessment of mitochondrial function (B) following acute (1 h) CDN1163 incubation. Results are presented as mean ± SEM in pmol/min from three to four observations ($n = 3$–4 wells from a mix of myotubes from six donors). *Statistically significant compared to untreated control cells ($p < 0.05$, unpaired *t*-test).

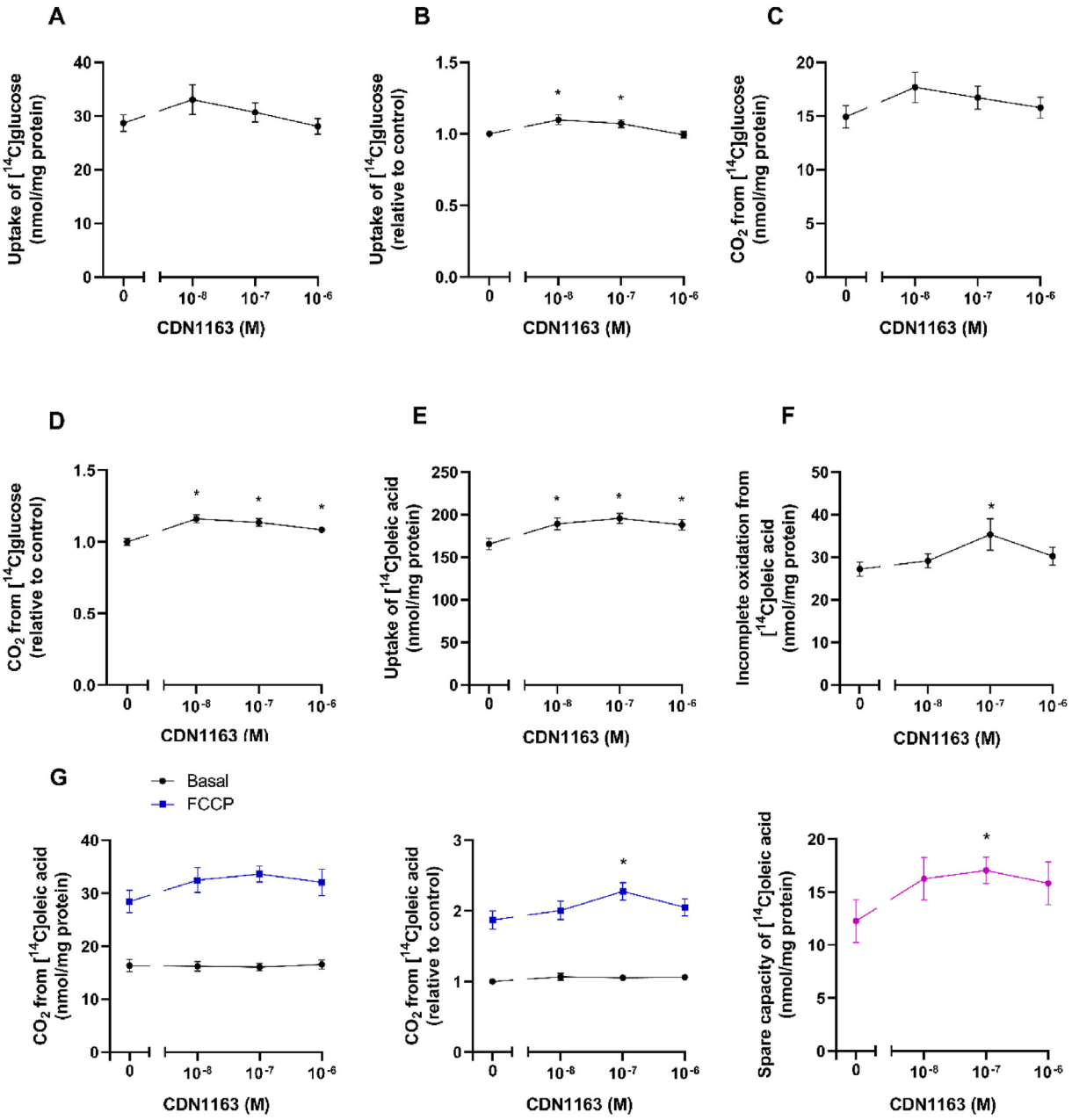

Fig. 3. Improved glucose and oleic acid metabolism in myotubes following prolonged CDN1163-treatment. Myotubes were pretreated with different concentrations of CDN1163 (0.01 μM, 0.1 μM or 1 μM) for 5 days followed by incubation with [^{14}C]glucose (0.5 μCi/ml, 200 μM) or [^{14}C]oleic acid (OA, 0.5 μCi/ml, 100 μM) in presence or absence of carbonyl cyanide 4 (trifluromethoxy)phenylhydrazone (FCCP, 1 μM) for 4 h. Cellular uptake of glucose (A and B) was calculated from the amount of CO_2 produced from [^{14}C]glucose oxidation (C and D) and the remaining cell-associated (CA) radioactivity in myotubes. (E) Cellular uptake of OA calculated as CO_2+CA and (F) incomplete OA oxidation based on formation of acid-soluble metabolites (ASM). Oxidation of OA in presence or absence of FCCP (G and H) was obtained by measuring the amount of CO_2 captured from [^{14}C]OA. Spare capacity (I) was calculated by subtracting basal OA oxidation from FCCP-uncoupled OA oxidation. Data are shown as mean ± SEM in nmol/mg cell protein (A, C, E, F, G and I) or normalised to control (B, D and H) from seven ($n = 7$, A – D) and seven to nine ($n = 7$–9, E – I) individual experiments. *Statistically significant compared to respective control cells ($p < 0.05$, unpaired *t*-test).

after 5 days of CDN1163-treatment. Real-time quantitative PCR analysis of peroxisome proliferator-activated receptor γ coactivator 1α (*PPARGC1A*), cytochrome *c*1 (*CYC1*), and forkhead box O1 (*FOXO1*) showed no difference in expression between CDN1163-treated and untreated myotubes. However, the expression levels of carnitine palmitoyltransferase 1B (*CPT1B*) and pyruvate dehydrogenase kinase 4 (*PDK4*) were observed to be increased in CDN1163-treated myotubes (Fig. 5A).

Further investigations at protein level were also performed to elucidate the molecular mechanisms by which CDN1163 induced beneficial effects on mitochondrial efficiency in primary human myotubes. There were no changes in expressions of oxidative phosphorylation (OXPHOS) complex (Fig. 5B), whereas phosphorylation of AMPKα^{Thr172} was observed to be increased in CDN1163-treated myotubes (Fig. 5C).

3.4. Effect of SERCA activation on incorporation of oleic and acetic acid in to various lipid classes

As chronic CDN1163-exposure during cell differentiation increased cellular uptake of OA (Fig. 3E), we aimed to assess the effect of CDN1163 on lipid distribution after adding ^{14}C-OA for the last 4 h prior to analysis. We observed that treatment of myotubes with CDN1163 for 5 days promoted a concentration-dependent reduction in OA incorporation into diacylglycerol

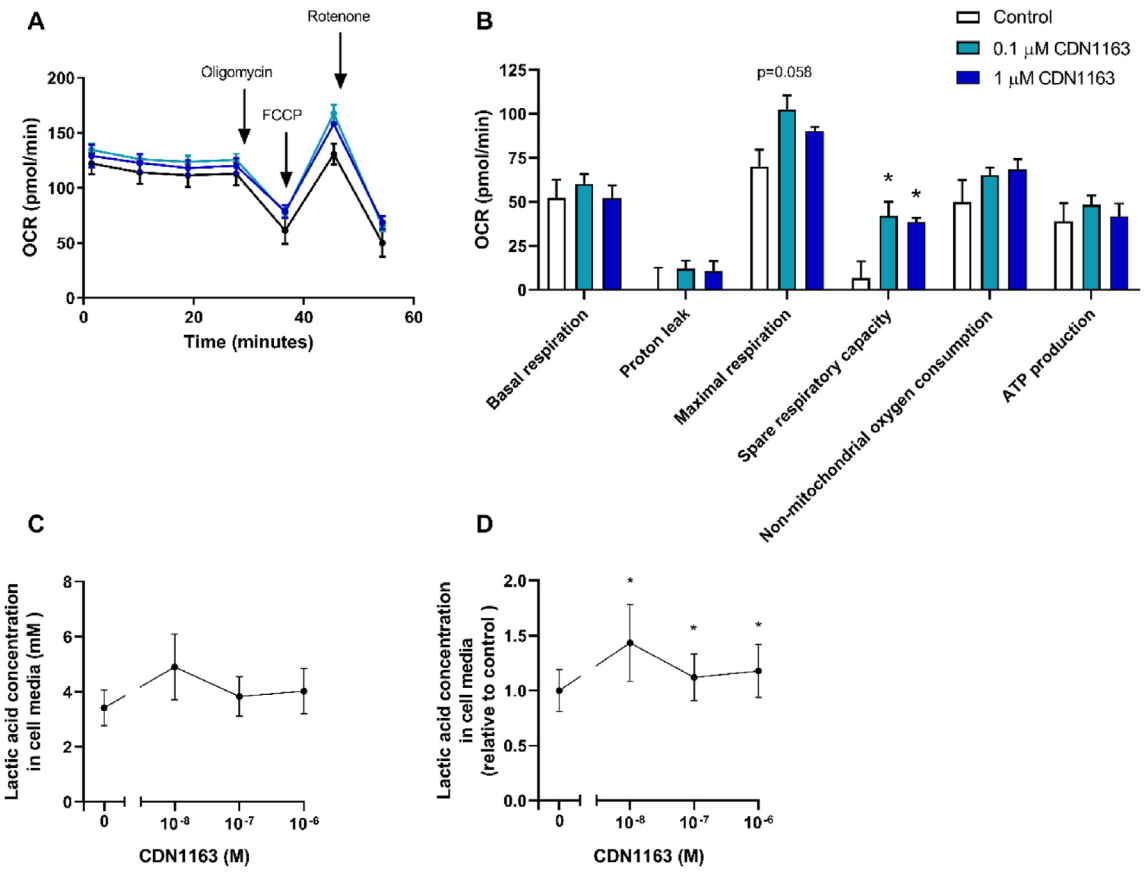

Fig. 4. Increased mitochondrial spare capacity and production of lactic acid in CDN1163 treated myotubes. Myotubes were cultured on a XF24-well cell culture plate and incubated for 5 days with CDN1163. Oxygen consumption rate (OCR) was measured in the presence of oligomycin, carbonyl cyanide 4-(trifluromethoxy)phenylhydrazone (FCCP) and rotenone. Changes in OCR time curve (A) and OCR parameters (B) were calculated as described in methods. Results are presented as mean ± SEM in pmol/min from three to four observations ($n = 3$–4 wells from a mix of myotubes six donors). (C and D) Measured lactic acid concentration in media. Myotubes were cultured and pretreated with CDN1163 for 5 days. Cell culture media were collected from the last 48 h of differentiation period, as described in methods. Biosen C-Line glucose and lactate analyser was used to measure the level of lactic acid concentration. Data are presented as mean ± SEM from eight to 13 individual samples ($n = 8$–13) in absolute values (C) and as normalised to medium from untreated control (D). *Statistically significant compared to control ($p < 0.05$, unpaired *t*-test).

(DAG) (Fig. 6A). The level of unesterified OA (FFA) and incorporation of OA into triacylglycerol (TAG), cholesteryl ester (CE) and phospholipids (PL), on the other hand, were not affected by CDN1163-treatment.

In addition to study OA incorporation into cellular lipids, we also evaluated *de novo* lipogenesis from ^{14}C-acetic acid. Here, myotubes were exposed to different concentrations of CDN1163 for 5 days and co-incubated with ^{14}C-acetic acid for the last 24 h prior to analysis of cellular lipid distribution. The results showed that CDN1163 promoted a reduction in acetate incorporation into the lipid classes in a concentration-dependent manner (Fig. 6B). Intracellular content of FFA, DAG, TAG, CE, as well as PL from acetic acid were simultaneously reduced by the treatment of 0.1 μM CDN1163.

We hypothesized that the reduction in *de novo* lipogenesis, as well as formation of DAG from OA were consequent to alteration genes related to lipid storage following CDN1163-treatment. Results from RT-qPCR analysis showed that CDN1163 induced a significant reduction in mRNA expression of stearoyl-CoA desaturase 1 (*SCD1*) (Fig. 6C). However, no difference in mRNA expression of fatty acid transporter (*CD36*), acetyl-CoA carboxylase 2 (*ACC2*), fatty acid-binding protein 4 (*FABP4*), or fatty acid synthase (*FAS*) were observed (Fig. 6C).

3.5. Changes in insulin response after chronic treatment of cultured myotubes with CDN1163

To examine whether CDN1163 modified the insulin response as a result of the observed metabolic changes, myotubes were treated with 0.1 μM CDN1163 for 5 days and exposed to 100 nM insulin for 15 min before harvesting protein samples. TBC1D4^{Thr642} phosphorylation (pTBC1D4) and total TBC1D4 were detected by immunoblotting. The results revealed no difference in pTBC1D4/total TBC1D4 ratio, suggesting no changes in insulin response after CDN1163 treatment (Fig. 7).

4. Discussion

In the current study we have investigated the effect of a small molecule SERCA activator, CDN1163, on glucose and FA handling in differentiated human skeletal muscle cells. To the best of our knowledge this is the first study to demonstrate metabolic effects of pharmacological SERCA activation by CDN1163 in primary human myotubes.

Findings from both acute (4 h) and chronic (5 days) treatment of human myotubes with CDN1163 showed enhanced cellular uptake and oxidation of glucose. Additionally, our data demonstrated that complete OA oxidation (CO_2) in the presence of the mitochondrial uncoupler FCCP was significantly increased after acute CDN1163-treatment. Chronic treatment with CDN1163 for 5 days during differentiation of the cells, on the other hand, markedly enhanced incomplete FA β-oxidation, but no effect was found on complete OA oxidation. Simultaneously, CO_2 from OA was significantly increased in the presence of FCCP as also observed after acute CDN1163-treatment. These findings are important as they indicate that the presence of a mitochondrial uncoupler under both acute and chronic treatment with CDN1163 enhanced the spare capacity for OA oxidation in CDN1163 exposed myotubes. Mitochondrial stress test

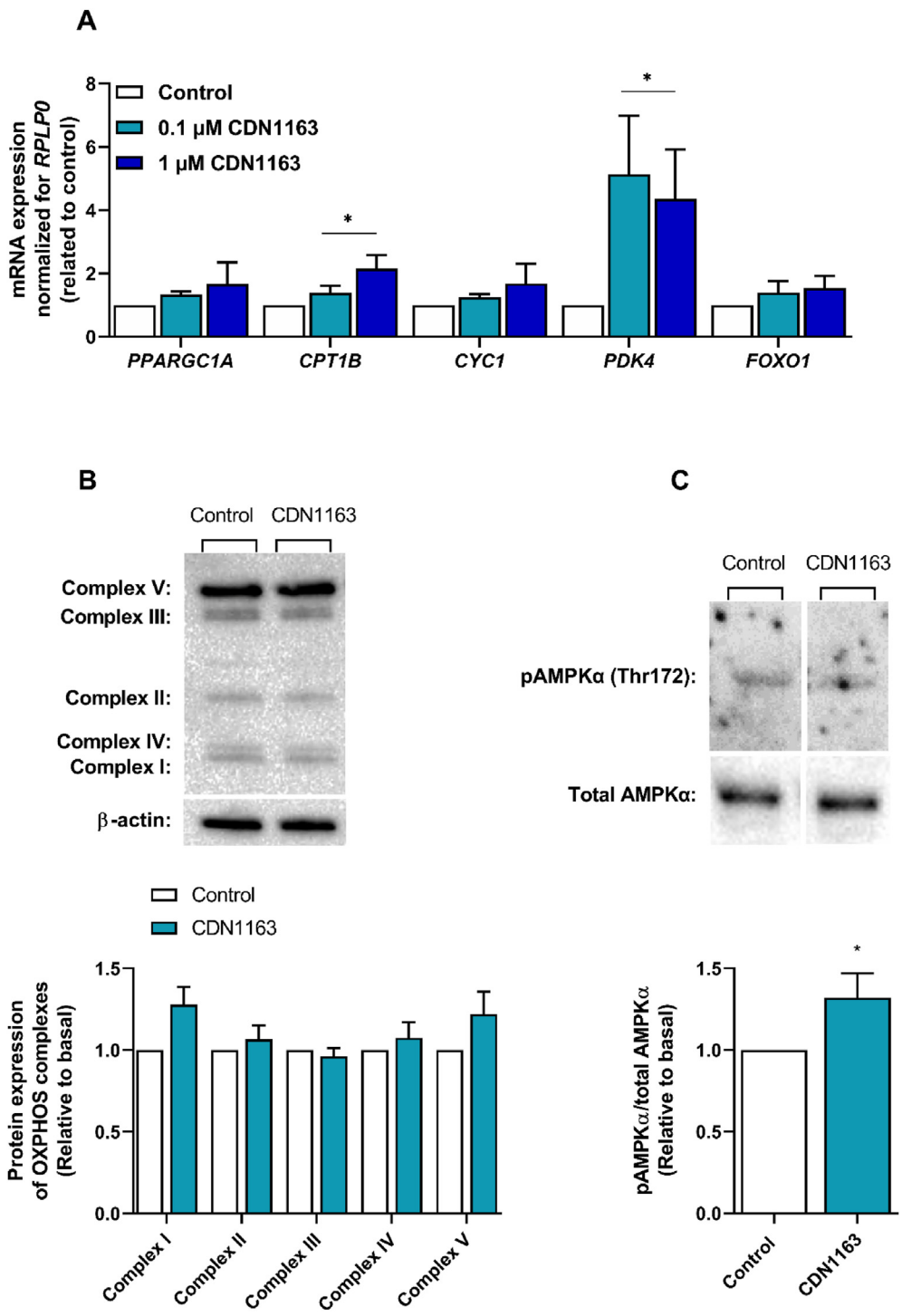

Fig. 5. CDN1163-induced changes in mRNA and protein expression levels of mitochondria-related genes and AMPKα^{Thr172} phosphorylation. After 5 days of 0.1 μM CDN1163-treatment during differentiation, mRNA and proteins were isolated from the myotubes. Gene and protein expressions were assessed by qPCR and immunoblotting, respectively. (A) mRNA expression of selected mitochondria-related genes. (B and C) Representative immunoblots and quantified protein expression of oxidative phosphorylation (OXPHOS) complexes (B) and AMP-activated protein kinase (AMPK) α^{Thr172} phosphorylation (C). Results are presented as mean ± SEM from three ($n = 3$, A) and eight ($n = 8$, B and C) individual experiments. *Statistically significant compared to control cells ($p < 0.05$, unpaired *t*-test). *PPARGC1A*, peroxisome proliferator-activated receptor γ coactivator 1α; *CPT1B*, carnitine palmitoyltransferase 1B; *CYC1*, cytochrome *c*1; *FOXO1*, forkhead box O1; *PDK4,* pyruvate dehydrogenase kinase 4.

confirmed these effects of CDN1163 on mitochondrial function. Furthermore, cellular uptake of OA was increased after chronic CDN1163-treatment and a lower incorporation of OA into DAG was observed. Moreover, formation of FFA and complex lipids from acetic acid (*de novo* lipogenesis) was reduced as a result of chronic exposure of myotubes to CDN1163. Here, we also demonstrated that CDN1163 increased AMPKα^{Thr172} phosphorylation, indicating increased AMPKα activity in CDN1163-exposed myotubes, whereas mRNA expression level of *SCD1* was reduced following 5 days of CDN1163-treatment.

Several reports have indicated the involvement of SERCA dysfunction in the development of different pathologies such as diabetes, heart failure, and neurodegenerative diseases and that restoration of SERCA expression or activity, on the other hand, could be a possible disease-preventive strategy (Adachi et al., 2004; Britzolaki et al., 2020; Gianni, Chan, Gwathmey, del Monte and Hajjar, 2005; Kulkarni et al., 2004; Morgan et al., 1990; Park et al., 2010; Roe et al., 1994; Zarain-Herzberg et al., 2014). Moreover, muscle-specific mechanisms of thermogenesis involving futile cycling of Ca^{2+} driven by SERCA and generating heat from ATP hydrolysis has been proposed to be a promising strategy to counteract obesity and metabolic dysfunction (Bal et al., 2012; Block, 1994; Maurya et al., 2018; Periasamy et al., 2017). The association between obesity and insulin resistance has been recognized as a central pathophysiological feature in the development of T2DM (B. B. Kahn and Flier, 2000). Circulating FFA concentrations are often elevated in obese

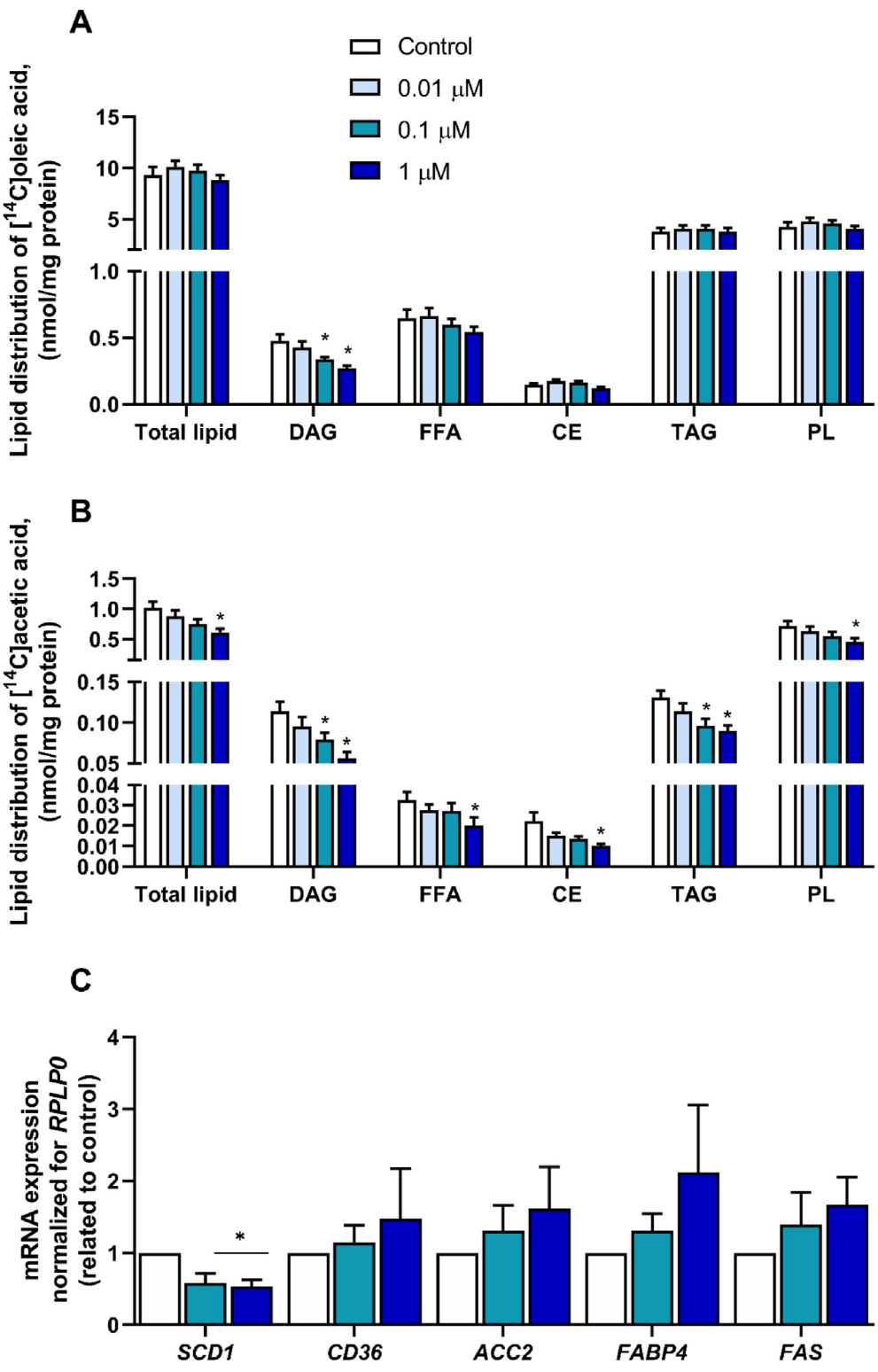

Fig. 6. CDN1163 reduced incorporation of fatty acids and acetate into cellular lipids and mRNA expression of *SCD1*. Myotubes were treated with different concentrations of CDN1163 (0.01 μM, 0.1 or 10 μM) for the last 5 days of the differentiation period. The cells were then incubated with 100 μM of [^{14}C]oleic acid (OA) or [^{14}C]acetic acid for 4 h or 24 h, respectively, as described in methods. Cells were harvested and lipids extracted and separated by thin layer chromatography prior to quantification by liquid scintillation. (A and B) Lipid distribution of [^{14}C]OA and [^{14}C]acetic acid after treatment with CDN1163, respectively. Results are expressed as mean ± SEM as nmol/mg cell protein from four individual experiments ($n = 4$). (C) qPCR analysis of genes related to lipid storage following 5 days of CDN1163 treatment. Data are presented as mean ± SEM from three individual experiments ($n = 3$), normalised to untreated (control) cells. *Statistically significant compared to control cells ($p < 0.05$, unpaired *t*-test). FFA, free fatty acids; DAG, diacylglycerol; TAG, triacylglycerol; CE, cholesteryl ester; PL, phospholipids; SCD1, stearoyl-CoA desaturase 1; CD36, fatty acid transporter; ACC2, acetyl-CoA carboxylase 2; FABP4, fatty acid-binding protein 4; FAS, fatty acid synthase.

subjects (Boden, 2008), which lead to excessive lipid flux into skeletal muscle and mediate intramyocellular lipid accumulation (Corcoran et al., 2007). The accumulation of intermediates of lipid metabolism, such as DAG and ceramides, accompanied by reduced mitochondrial β-oxidation and lipid turnover are thought to be factors contributing to disorders associated with lipid accumulation and impaired insulin signalling in skeletal muscle (Corcoran et al., 2007; Itani et al., 2002; Schmitz-Peiffer, 2000). Given the observed effects of CDN1163 to ameliorate FA uptake, improve β-oxidation and to reduce intracellular levels of certain FA metabolites after prolonged exposure, the current data imply a role of SERCA-activation in accounting for at least some of observed changes in lipid metabolism of CDN1163-treated myotubes.

Previous studies with animal models have shown that pharmacological activation or overexpression of SERCA is related to changes in gene expressions that affect lipid metabolic pathways (Kang et al., 2016; Park et al., 2010). Therefore, we examined expression of certain genes to gain

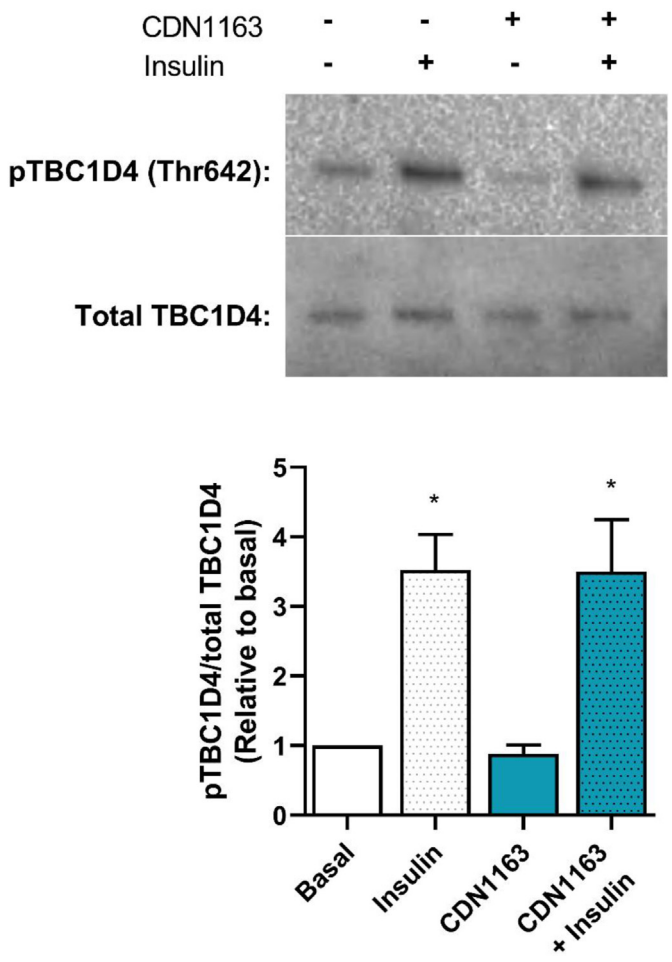

Fig. 7. CDN1163 showed no changes in TBC1D4^{Thr642} phosphorylation. Human myotubes were pretreated with 0.1 μM CDN1163 for 5 days. Prior to harvesting, half of the samples were treated with 100 nM insulin. Proteins were isolated and expressions of total TBC1D4 and TBC1D4 phosphorylated at Thr642 were assessed by immunoblotting. Expression of pTBC1D4 was normalised to total TBC1D4 expression. Data are presented as mean ± SEM from eight individual experiments ($n = 8$). *Statistically significant compared to basal (unstimulated) cells ($p < 0.05$, unpaired *t*-test).

molecular insights related to the effects of CDN1163 on lipid handling. Our study indicated that the expression levels of mitochondria-related genes *CPT1B* and *PDK4* were upregulated in CDN1163-treated myotubes compared with untreated cells. The significance of CPT1β as a rate-limiting enzyme of mitochondrial FA oxidation has been established (McGarry and Brown, 1997; Stephens et al., 2007). It is also known that inactivation of pyruvate dehydrogenase complex by upregulation of PDK4 can induce a switch from glucose to FA utilisation as both glucose and FA compete with each other for oxidation (Pettersen et al., 2019; Zhang et al., 2014). Hence, the upregulation of these genes may provide an explanation to the observed effects of CDN1163 on increased OA uptake and oxidation in human myotubes.

Moreover, a key lipogenic gene, *SCD1*, was found to be downregulated by CDN1163-treatment. *SCD1* is an important rate-limiting enzyme in lipogenesis, catalysing the committed step in the synthesis of monounsaturated FA from saturated FA that are either synthesised *de novo* or derived from the diet (Dobrzyn and Ntambi, 2005; Flowers and Ntambi, 2008; Stamatikos and Paton, 2013). Research in human and animal models has shown that elevated *SCD1* activity in skeletal muscle is associated with increased intramuscular triacylglycerol synthesis and reduced FA oxidation (Dobrzyn and Ntambi, 2005), whereas *SCD1* deficiency decreases intracellular FA-CoAs by increasing the rate of β-oxidation and phosphorylation of AMPK in oxidative muscle (Dobrzyn et al., 2005). Thus, the data obtained in the current study support the possibility that CDN1163-suppression of *SCD1* could be responsible for the reduced lipid accumulation, as well as increased β-oxidation in the myotubes after chronic exposure.

In subjects with obesity and T2DM, the functional capacity of mitochondria is diminished and the mitochondrial electron transport chain in skeletal muscle is less efficient for electron transport (Kelley et al., 2002). This can contribute to reduced oxidative metabolism of FA and other energy substrates in these subjects. Although we found no difference in uptake or incomplete β-oxidation from OA after acute treatment of myotubes with CDN1163, the complete oxidation of OA in the presence of the mitochondrial uncoupler FCCP was found to be elevated. Thus, acute CDN1163-treatment together with FCCP enhanced the oxidative spare capacity in a similar fashion to chronically CDN1163-exposed myotubes in the presence of FCCP. Further investigation on mitochondrial function also revealed that the spare capacity and maximal respiration were enhanced in myotubes as a consequence of CDN1163-treatment. These results suggest that the mitochondrial activity is increased in both chronically and acutely CDN1163-treated myotubes.

FA oxidation is also regulated by AMPK, a sensor of the AMP level in cells, and has been shown to play a key role in maintaining cellular energy homeostasis (D. Grahame Hardie, 2003). AMPK is activated by high ratio of ADP:ATP or AMP:ATP and metabolic or cellular stress that either inhibits ATP production or accelerates ATP hydrolysis (D. G. Hardie, 2004; D. Grahame Hardie and Sakamoto, 2006). Upon its activation, AMPK promotes a switch from anabolic to catabolic state, which increases glucose and FA metabolism to restore ATP levels (D. Grahame Hardie and Sakamoto, 2006; Barbara B. Kahn, Alquier, Carling and Hardie, 2005). Moreover, evidence from *SCD1*$^{-/-}$ mice has demonstrated that SCD1 is involved in the regulation of AMPK-mediated thermogenesis by increasing expression of uncoupling protein 1 which uncouples oxidative respiration from ATP synthesis to induce higher energy expenditure and dissipation of energy as heat (Lee et al., 2004). We hypothesized that increased ATP demand from enhanced SERCA pump activity as a result of CDN1163-treatment accompanied by a significant reduction in *SCD1* mRNA expression could induce AMPK phosphorylation. This may in part be a possible mechanism to explain the improved substrate metabolism in CDN1163-treated myotubes. In support of our hypothesis, we observed that CDN1163 promoted a significant increase in AMPK^{Thr172} phosphorylation, indicating that the observed alteration in substrate metabolism could have been induced through increased AMPK activity concomitant with repression of *SCD1* in the CDN1163-treated myotubes. Further, with a higher demand for ATP or when the rate of ATP production by OXPHOS become insufficient, activation of the glycolytic pathway increases and muscle production of lactate is important to sustain a high rate of ATP regeneration from glycolysis (Baker et al., 2010). Interestingly, we found that prolonged treatment of human myotubes with CDN1163 induced a modest increase in lactate production. We also observed higher ECAR values upon CDN1163-stimulation of myotubes, suggesting that CDN1163 might contribute to generating ATP via modulation of the glycolytic pathway to meet the increased ATP demand.

Enhancing SERCA activity by overexpression of SERCA in an animal model of obesity (*ob/ob* mice) has been shown to improve glucose intolerance and insulin resistance (Park et al., 2010). In addition, Kang et al. (2016) demonstrated that activation of SERCA using CDN1163 reduced blood glucose and fasting insulin levels in a genetic animal model of insulin resistance and T2DM (*ob/ob* mice). In accordance with these findings, glucose uptake and oxidation after both acute and chronic CDN1163-treatment of myotubes were improved under basal conditions compared to untreated myotubes, which might be a consequence of improved insulin sensitivity. Although our data showed no changes in insulin-stimulated TBC1D4^{Thr642} phosphorylation, it is likely that repression of *SCD1* can also affect insulin signalling via activation of AMPK (Dobrzyn et al., 2005).

In summary, our findings demonstrated that pharmacological activation of SERCA by CDN1163 mediated an increase in FA metabolism and improved the lipid profile in primary human myotubes. We also showed increased glucose utilisation and lactate production in CDN1163-treated cells. It is possible that glucose taken up and metabolised by these cells might be used as a fuel to generate ATP in order to compensate for the increased ATP-consumption, possibly due to enhanced SERCA activity. The effects were accompanied by upregulation of the mitochondria-related genes *CPT1B* and *PDK4*. Furthermore, CDN1163-treatment led to enhanced phosphorylation and activation of AMPK that may promote mitochondrial biogenesis. More studies should be carried out to provide further insight into the mechanisms underlying the improved substrate metabolism in CDN1163-treated myotubes.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xiao-Rong Peng, Sven Göpel and Gavin O' Mahony are employees of AstraZeneca. The other authors had no conflict of interest to disclose.

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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.2021.100060.

Author contributions statement

AMM. Conceptualisation, project administration, methodology, investigation, formal analysis, visualisation, writing – original draft, writing – review and editing, final approval of the version to be submitted. JL. Methodology, investigation, formal analysis, visualisation, writing – review and editing, final approval of the version to be submitted. PK. Methodology, investigation, writing – review and editing, final approval of the version to be submitted. RG. Methodology, investigation, formal analysis, final approval of the version to be submitted. HGB. Methodology, investigation, final approval of the version to be submitted. PKL. Methodology, investigation, writing – review and editing, final approval of the version to be submitted. LE. Methodology, investigation, writing – review and editing, final approval of the version to be submitted. GO'M. Conceptualisation, writing – review and editing, final approval of the version to be submitted. SG. Conceptualisation, writing – review and editing, final approval of the version to be submitted. XRP. Conceptualisation, writing – review and editing, final approval of the version to be submitted. ETK. Writing – review and editing, final approval of the version to be submitted. GHT. Conceptualisation, funding acquisition, supervision, methodology, investigation, formal analysis, visualisation, writing – review and editing, final approval of the version to be submitted. ACR. Conceptualisation, funding acquisition, supervision, methodology, investigation, formal analysis, visualisation, writing – review and editing, final approval of the version to be submitted.

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