Journal of Thermal Biology 116 (2023) 103623

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Functional expression of the thermally activated transient receptor potential channels TRPA1 and TRPM8 in human myotubes

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ARTICLE INFO

Keywords:
Transient receptor potential
Glucose metabolism
Cold exposure
Skeletal muscle
In vitro

ABSTRACT

Transient potential (TRP) ion channels expressed in primary sensory neurons act as the initial detectors of environmental cold and heat, information which controls muscle energy expenditure. We hypothesize that non-neuronal TRPs have direct cellular responses to thermal exposure, also affecting cellular metabolism. In the present study we show expression of TRPA1, TRPM8 and TRPV1 in rat skeletal muscle and human primary myotubes by qPCR. Effects of TRP activity on metabolism in human myotubes were studied using radiolabeled glucose. FURA-2 was used for Ca^{2+} imaging.

TRPA1, TRPM8 and TRPV1 were expressed at low levels in primary human myotubes and in *m. gastrocnemius, m. soleus,* and *m. trapezius* from rat. Activation of TRPA1 by ligustilide resulted in an increased glucose uptake and oxidation in human myotubes, whereas activation of TRPM8 by menthol and icilin significantly decreased glucose uptake and oxidation. Activation of heat sensing TRPV1 by capsaicin had no effect on glucose metabolism. Agonist-induced increases in intracellular Ca^{2+} levels by ligustilide and icilin in human myotubes confirmed a direct activation of TRPA1 and TRPM8, respectively. The mRNA expression of some genes involved in thermogenesis, i.e. peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), uncoupling protein (UCP) 1 and UCP3, were downregulated in human myotubes following TRPA1 activation, while the mRNA expression of TRPM8 and TRPA1 were downregulated following TRPM8 activation by menthol and icilin, respectively. Cold exposure (18 °C) of cultured myotubes followed by a short recovery period had no effect on glucose uptake and oxidation in the basal situation, however when TRPA1 and TRPM8 channels were chemically inhibited a temperature-induced difference in glucose metabolism was found.

In conclusion, mRNA of TRPA1, TRPM8 and TRPV1 are expressed in rat skeletal muscle and human skeletal muscle cells. Modulation of TRPA1 and TRPM8 by chemical agents induced changes in Ca^{2+} levels and glucose metabolism in human skeletal muscle cells, indicating functional receptors.

1. Introduction

Obesity is the result of an imbalance between energy intake and energy expenditure, and the its global prevalence has increased massively over the last decades. Therefore, strategies that could aim in increasing energy expenditure is one of the tools that could be used to prevent and combat obesity (Frühbeck et al., 2009). In this context, it has been speculated whether cold exposure could have a beneficial effect (Manfredi 2021). Cold temperatures activate the cold-sensing thermoreceptors of sensory nerves in peripheral tissues. Thermogenesis, the process of generating heat through the dissipation of energy, is highly dependent on the sympathetic nervous system (SNS) and central nervous system (CNS) (described in (Ye et al., 2013)). Skeletal muscle is the largest organ in the human body and a major contributor to basal

Abbreviations: BAT, Brown adipose tissue; Ca^{2+}, Calcium; CNS, Central Nervous System; PGC-1α, Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha; SNS, Sympathetic Nervous System; TRPA1, Transient Receptor Potential, subfamily Ankyrin 1; TRPM8, Transient Receptor Potential cation channel subfamily Melastatin 8; TRPV1, Transient Receptor Potential cation channel subfamily Vanilloid 1; UCP, Uncoupling protein.

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https://doi.org/10.1016/j.jtherbio.2023.103623
Received 7 February 2022; Received in revised form 29 April 2023; Accepted 11 June 2023
Available online 17 June 2023

metabolic rate. In cold environments, muscles have the ability to increase energy expenditure through shivering and non-shivering thermogenic mechanisms (Periasamy et al. 2017). However, it is unknown whether human muscle cells also may recognize temperature directly, bypassing the nervous system, to set energy metabolism.

The transient potential channel (TRP) channels are a group of ion channels located on the cell surface that senses changes in temperature. These ion channels are mainly expressed on the dorsal root and trigeminal ganglia neurons, but their expression has also been reported in other tissues (Nilius and Owsianik 2011). One of the most studied TRP ion channel is the TRP melastatin 8 (TRPM8), which is categorized as a cold-sensing thermoreceptor. This ion channel is activated by temperatures below 26 °C and by agents inducing a cold sensation such as menthol and the synthetic agonist icilin (Peier et al., 2002; McKemy et al. 2002). Another cold-sensing thermoreceptor is the TRP ankyrin 1 (TRPA1) ion channel. TRPA1 is activated by temperatures below 17 °C, and by pungent compounds such as mustard oil, cinnamaldehyde and ligustilide (Schepers and Ringkamp 2010; Osterloh et al., 2016; Zhong et al., 2011). TRPA1 and TRPM8 ion channels are Ca^{2+}-permeable cation channels, which allow the entry of Ca^{2+} when activated, but can also induce calcium release from intracellular Ca^{2+} stores through the intracellular Ca^{2+} release channels (Pan et al., 2016; Pedersen et al. 2005). The TRP vanilloid 1 (TRPV1) ion channel is a heat-sensing thermoreceptor activated by temperatures above 43 °C and by its chemical agonist capsaicin (Schepers and Ringkamp 2010).

Activation of the cold-sensing TRP channels, TRPA1 and TRPM8 in afferent sensory neurons of the dorsal root and trigeminal ganglia, increases whole-body energy expenditure by inducing thermogenesis (reviewed in (McKemy 2013)). There are two types of thermogenesis; shivering and non-shivering (Betz and Enerbäck 2018). Shivering is caused by small and repetitive skeletal muscle contractions, while brown adipose tissue (BAT) is responsible for non-shivering thermogenesis (Periasamy et al. 2017; Betz and Enerbäck 2018). However, there are also evidence suggesting that skeletal muscle might also be responsible for non-shivering thermogenesis (reviewed in (Periasamy et al. 2017)). Thermogenesis increases energy expenditure through activation of the mitochondrial modulators; peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and mitochondrial uncoupling proteins (UCPs) (Ye et al., 2013). The UCPs are found in the inner mitochondrial membrane, where they enhances proton conductivity when activated, resulting in uncoupling of the electron transport system and subsequent heat production (Rousset et al., 2004).

One study described functional expression of the TRPA1 channel in human primary myoblasts (Osterloh et al., 2016). In this study, the authors describe a role of TRPA1 activation in skeletal muscle repair, such as myoblast fusion and cell migration (Osterloh et al., 2016). The expression of the TRPA1 channel, along with inducible Ca^{2+} transients, declined during myoblast differentiation (Osterloh et al., 2016). Studies looking at the effects of the TRPA1 agonist cinnamaldehyde, both in mice and the murine myoblast cell line C2C12, found that treatment with this compound improved glucose metabolism by increasing the expression of the glucose transporter, GLUT4 (reviewed in (Zhu et al., 2017)). Several studies using C2C12 cells reported a dose-dependent increase in GLUT4 mRNA expression following treatment with cinnamaldehyde (Nikzamir et al., 2014; Gannon et al., 2015).

It has previously been reported that the C2C12 myoblasts are cold-responsive and expresses the TRPM8 channel when fully differentiated into myotubes (Li et al., 2018). In addition, it has been shown that these cells can increase the expression of genes in the thermogenic gene program (PGC-1α and UCPs) independently of the CNS (Li et al., 2018). Furthermore, when menthol was administered to C2C12 myotubes there was an upregulation in the expression of PGC-1α and UCP1 (Li et al., 2018). However, there is currently no evidence supporting a role for TRPM8 in human skeletal muscle cells.

The heat-sensing thermoreceptor TRPV1 has shown to be expressed in both rat and mouse skeletal muscle and in C2C12 myocytes (Luo et al., 2012; Lotteau et al., 2013). There is evidence of TRPV1 expression in human skeletal muscle, and although it has been shown to play a role in vascular function of the skeletal muscle feed arteries, its role in skeletal muscle metabolism remains unclear (Ives et al., 2017). However, many clinical studies looking at the effects of the TRPV1 agonist capsaicin has shown an increase in energy expenditure, as a result of increases in both lipid oxidation (Lejeune et al. 2003; Janssens et al., 2013; Yoshioka et al., 1998) and carbohydrate metabolism (Lim et al., 1997; Yoshioka et al. 1995, 1998). Although no studies have looked at the molecular processes underlying the effects of TRPV1 activation in human skeletal muscle, studies on mice reported an increased expression of PGC-1α and several genes related to mitochondrial respiration and ATP production following TRPV1 activation (Luo et al., 2012; Lotteau et al., 2013; Xin et al., 2005). These studies found that the TRPV1 ion channel was primarily expressed in the sarcoplasmic reticulum and was involved in Ca^{2+} homeostasis (Lotteau et al., 2013; Xin et al., 2005).

The aim of this study was to investigate the functional expression in human skeletal muscle cells of the putative cold sensors TRPA1 and TRPM8, and, in comparison, of the heat sensor TRPV1.

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}, Collagen I, foetal bovine serum (FBS), penicillin-streptomycin (10000 IE/mL), Epidermal growth factor human (hEGF), amphotericin B, Power SYBR® Green PCR Master Mix, SYBR® Green PCR primers, MicroAmp® Optical Adhesive Film, MicroAmp® Optical 96-well Reaction Plate, TaqMan® Reverse Transcription Reagents, D-glucose monohydrate and Fura-2 AM were from ThermoFisher Scientific (Waltham, MA, US). Insulin (Actrapid®) was obtained from NovoNordisk (Bagsvaerd, Denmark). Bovine serum albumin (BSA, essentially FA-free), L-carnitine, D-glucose, HEPES, Dimethyl sulfoxide (DMSO), Menthol, Capsaicin, PF-051065679, Potassium chloride, dexamethasone, and gentamicin were from Sigma-Aldrich (St. Louis, MO, US). Calcium chloride dehydrate, Disodium hydrogen phosphate dihydrate, Magnesium chloride hexahydrate, Magnesium sulfate heptahydrate, Sodium hydrogen carbonate, Sodium chloride, and Potassium dihydrogen phosphate were from Merck (Darmstadt, Germany). Icilin, and Ligustilide were from Alomone labs (Jerusalem, Israel) and dissolved in DMSO. D-[^{14}C(U)]glucose (107.3 mCi/mmol) were purchased from PerkinElmer NEN® (Boston, MA, US). 96-well and 6-well Corning® CellBIND tissue culture plates were from Corning (Schiphol-Rijk, the Netherlands). 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). Bio-Rad Protein Assay Dye Reagent Concentrate was from Bio-Rad (Copenhagen, Denmark). Total RNA Purification Kit was from Norgen (Ontario, Canada). Glass Bottom Microwell Dishes (35 mm petri dish, 14 mm microwell, No. 1.5 cover glass) were obtained from MatTek (Ashland, MA, US).

2.1.1. 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). In total nine different donors were included, all healthy men, 24.9 (± 1.15) years old with a body mass index of 23.65 (± 1.05) kg/m^2.

The donors used in this study were obtained from a previous study and 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 (m.) vastus lateralis*. The satellite cells were used to establish a biobank of myoblasts, where the cells were upscaled at different passages and cryopreserved. In this study, all experiments were performed on cells from passages 3 or 4.

The cells were cultured on multiwell plates in a growth medium containing DMEM-Glutamax TM (5.5 mM glucose) supplemented with 10% FBS, 25 IU penicillin, 25 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 50 ng/mL gentamycin, 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 containing DMEM-Glutamax TM (5.5 mM glucose) supplemented with 2% FBS, 25 IU penicillin, 25 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 50 ng/mL gentamycin, 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 every 2–3 days.

2.1.2. Animal experiments

The animal studies were executed in accordance with regulations and approved by the Norwegian Food Safety Authority (FOTS, ID9483). Three healthy 20 weeks old Lewis female rats were purchased from Janviers Lab, France. The rats weighed 150–200 g at arrival to our animal facility and housed in groups of 2–3 in plastic cages with steel top grill and free admission to regular feed (SDS RM1, Scanbur AS, Norway) and tap water.

The major muscles *m. gastrocnemius, m. soleus,* and *m. trapezius* were harvested for PCR analysis. To this end, isoflurane anesthetized rats were subjected to thoracotomy and injection of 100 μl heparin (5000 IE/mL, LEO Pharma AS, Denmark) into left ventricle to avoid coagulation, followed by transcardial perfusion with 100 mL HBSS (HyClone Laboratories, Inc., USA). The three different muscle tissues were then isolated, snap-frozen and crushed into a fine powder with a mortar in liquid N2 and stored at – 80 °C.

2.1.3. Cold exposure of human myotubes in vitro

Cold exposure of the cells were done according to Krapf et al. (2021) (Krapf et al., 2021). The myotubes were exposed to cold (18 °C) or control temperatures (37 °C) by placing the culture dishes on temperature-regulated aluminium blocks (Aavid Thermoalloy SRL, Italy). The blocks were temperature-regulated by perfusion with water circulators (Heto-Holten AS, Denmark). Temperatures of the perfused metal blocks were logged to ensure correct temperature exposure during experiments. The thermal exposures used for myotubes were either 18 °C or 37 °C for 18 hours (h). The myotubes were allowed to recover for 3 h at 37 °C prior further experimentation.

2.1.4. Substrate oxidation assay for measurement of glucose metabolism

Skeletal muscle cells (7000 cells/well) were cultured on 96-well CellBIND® microplates. On the 6th day of differentiation, the myotubes were treated with either 10 μM menthol, 10 μM icilin, 10 μM capsaicin, 25 μM HC-030031, 25 μM PF-051065679, or different concentrations of ligustilide for 24 h. The total content of cellular protein in each experiment was determined. There were no changes in protein content following 24 h treatment with these modulators indicating no cell toxic effects of the treatment. At day 7 of differentiation, the cells were given [^{14}C]glucose (0.5 μCi/mL, 200 μM) as previously described (Wensaas et al., 2007). The substrates were added in DPBS solution containing 10 mM HEPES and 10 μM BSA. A 96-well UniFilter® microplate, soaked with NaOH (1 M), was mounted on top of the CellBIND® plate and the produced CO_2 by the cells was trapped during the 4 h at 37 °C. Cell-associated labeled glucose 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.

2.1.5. RNA isolation and analysis of gene expression by qPCR

Total RNA was isolated from approximately 10 mg frozen rat muscle by extraction using Isol Lysis reagent (5 PRIME, Germany) according to the manufacturer's protocol. Total RNA was isolated from human myotubes was done by using Total RNA purification kit (Norgen, Ontario, Canada) according to the manufacturer's protocol. Except, Isol Lysis reagent was used for the samples in Fig. 1B. The quality and quantity of RNA was determined using a Nanodrop ND-1000 (Thermo Scientific).

Reverse transcription of 500 ng RNA to cDNA was performed with Maxima™ First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc, US) on a PerkinElmer 2720 Thermal Cycler according to instructions.

qPCR was performed using a real-time instrument (StepOnePlus or QuantStudio, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with appropriate reagents (PerfeCTa SYBR Green, Quantabio, QIAGEN Beverly Inc, Beverly, MA, USA) according to the supplier's protocol. The following human forward and reverse primers were used at a concentration of 30 μmol/l: P0 (36B4/RPLP0, CGGTTTCTGATTGGCTAC and ACGATGTCACTTCCACG); Solute carrier family 2, facilitated glucose transporter member 1 (GLUT1/SLC2A1, ACCTCAAATTTCATTGTGGG and GAAGATGAAGAACAGAACCAG); Solute carrier family 2, facilitated glucose transporter member 4 (GLUT4/SLC2A4, CCATTGTTATCGGCATTCTG and ATTCTGGATGATGTAGAGGTAG); Transient Receptor Potential cation channel, subfamily V, member 1 (TRPV1, GGATGGCTTGCCTCCCTTTA and CCGCCTCTGCAGGAAATACT); Transient Receptor Potential cation channel, subfamily A, member 1 (TRPA1, ACCATGCTTCACAGAGCTTCA and AGTGGAGAGCGTCCTTCAGA); Transient Receptor Potential cation channel, subfamily M, member 8 (TRPM8, TGGGAGGGTGTCATGAAGGA and AGACCCTTGAGATCATTAAGCTTTG); Uncoupling protein 1 (UCP1, ACAGCACCTAGTTTAGGAAG and CTGTACGCATTATAAGTCCC); Uncoupling protein 3 (UCP3, CATCATGAGGAATGCTATCG and TGGAGGTGAGTTCATATACC); Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha (PGC-1α/PPARGC1A, GCAGACCTAGATTCAAACTC and CATCCCTCTGTCATCCTC). Rat primers were: TRPV1 (CAGCTACTACAAGGGCCAGACA and CATCTGCTCCATTCTCCACCAA); TRPA1 (ACAATGGCTGGACTGCTTT and GTGCTGTGTTCCCTTCTTCATC); TRPM8 (GAGTCTTCTGCCTGCTGTTTC and ATCTCCTCTGCGTTGTCGTT); and ACTB (CTAAGGCCAACCGTGAAAAGA and ACAACACAGCCTGG ATGGCTA).

2.1.6. Calcium imaging

We measured intracellular calcium responses in human myotubes during TRPM8 and TRPA1 agonist stimulation with ratiometric imaging of Fura-2 AM. The calcium imaging experiments were done using an inverted Zeiss Axio Observer Z1 microscope. The microscope was equipped with a Fluar 20x/0.75 M27 Objective. Fura-2 AM excitation was performed at 340 and 380 nm by an ultra-high-speed wavelength switcher Lambda DG-4 plus (Sutter Instrument), and the fluorescence signal was collected by an ORCA-Flash 4.0 V3 Digital CMOS camera C13440–20CU through a bandpass 510 nm filter (Hamamatsu Photonics).

Myotubes were cultured on MatTek glass bottom microwell dishes coated with collagen I (0.5%). At day 7 of differentiation, the myotubes were washed once with HBSS before being washed three times with HBSS +0.1% BSA (fatty acid free). The cells were then labeled with 5 μM Fura-2 AM in HBSS +0.1% BSA for 45 min in cell incubator (37 °C; 5% CO_2). Following the incubation, the cells were washed four times with Low-KCl solution (132 mM NaCl, 5 mM KCl, 1 mM MgCL2, 5 mM CaCL2, 12 mM HEPES, 10 mM Glucose). Thereafter, 250 μL Low-KCl solution was added to the dishes, followed by incubation for 45 min at 37 °C under 5% CO_2 to de-esterify and trap Fura-2 intracellularly. For every experiment, a culture dish with cells was placed on the microscope stage for data acquisition taking images every 2–5 seconds (s), lasting for 120 s. During the first 30 s of the experiments, the baseline fluorescent signal was acquired, corresponding to the initial level of intracellular Ca^{2+}. Experiments to stimulate cells with agonists was initiated 30 s into the

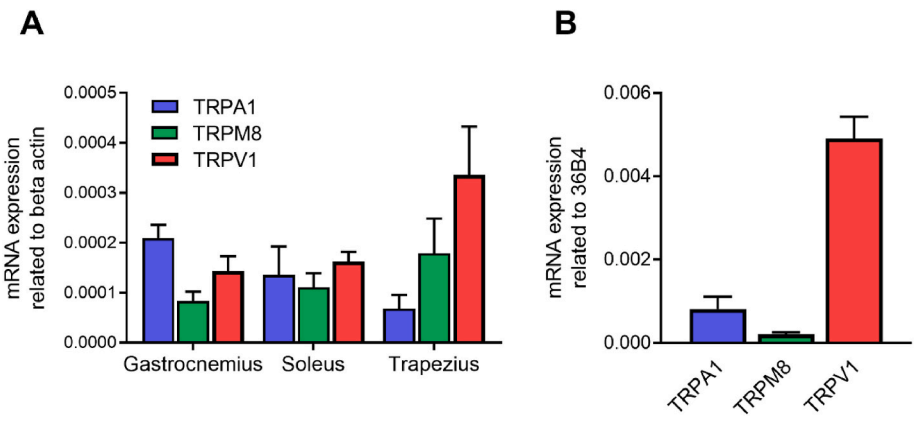

Fig. 1. mRNA expression of the TRP ion channels in skeletal muscle. A: RNA was isolated from rat *m. gastrocnemius*, *m. soleus*, and *m. trapezius*. **B**: Human myotubes were grown and differentiated for 7 days in 6-well tissue culture plates before being harvested for PCR. All values were corrected for the housekeeping control beta actin (**A**) or large ribosomal protein P0 (36B4) (**B**). The results are presented as mean ± SEM from n = 3 animals (**A**) or n = 3 individual experiments on myotubes derived from 3 different donors (**B**). Transient receptor potential cation channel, subfamily V, member 1 (TRPV1), Transient receptor potential cation channel, subfamily A, member 1 (TRPA1), Transient receptor potential cation channel, subfamily M, member 8 (TRPM8).

experiment by pipetting onto the cells an additional 250 μL of either 400 μM ligustilide or 20 μM icilin solution (agonist in DMSO vehicle dissolved in Low-KCl solution), giving a final concentration on the cells of 200 μM ligustilide or 10 μM icilin, plus 0.05% DMSO vehicle. Control experiments lasted 180 s and were performed by agonist-free sham-stimulation at 30 s using Low-KCl solution with the 0.05% DMSO vehicle alone, followed by addition of 1 mL of High-KCl solution (17 mM NaCl, 120 mM KCl, 1 mM MgCL2, 5 mM CaCL2, 12 mM HEPES, 10 mM Glucose) onto the cells at 120 s to cause cell depolarization at an extracellular concentration of 80 mM K^+. The fluorescent signal intensities were recorded, and the raw data was obtained by image analysis with the Physiology Module of the ZEN 2.3 pro software. One cell-culture dish was considered as one experiment and up to 18 cell recordings from each dish were used for further analysis.

2.2.7 Presentation of data and statistics.

For the calcium imaging experiments, the background fluorescence intensity was subtracted from the raw signal intensities of each biological replicate. The recorded fluorescence intensity of Fura-2 in the Ca^{2+} bound state ($F_{Ca\text{-}bound}$) and fluorescence intensity of Fura-2 in the Ca^{2+} unbound state ($F_{Ca\text{-}free}$) was used to calculate the $F_{Ca\text{-}bound}/F_{Ca\text{-}free}$ ratio, which was used for data analysis.

Data are presented as mean ± SEM unless specified in the figure legends. Each experiment was performed with myotubes from at least 2–3 different donors, with at least 3 biological replicates in each experiment. For the calcium imaging experiments, the results are presented as mean ± SEM of fluorescence intensities from cells in one or several individual imaging experiments. Statistical analysis was performed using GraphPad Prism 8.0.1 Software (GraphPad Software Inc., La Jolla, CA, US). Unpaired or paired *t*-test was performed to determine effects of the treatments, where $p < 0.05$ was considered significant.

3. Results

3.1. mRNA expression of the TRP ion channels in rat skeletal muscle and human myotubes

First, we explored the expression of the TRPA1, TRPM8 and TRPV1 in different muscles. The mRNA levels of these ion channels were initially measured in muscle biopsies isolated from three different skeletal muscles in rat; *m. gastrocnemius*, *m. soleus* and *m. trapezuis* (Fig. 1A). The three TRP ion channels were expressed in all three types of muscles.

Next, we measured the expression of these channels *in vitro* in cultured myotubes isolated from *m. vastus lateralis* from young healthy males. Also in this cell model, all three TRP ion channels were expressed, however, TRPV1 seemed to be expressed at a higher level than TRPA1 and TRPM8 (Fig. 1B).

3.2. Activation of the TRPA1 ion channel increased glucose uptake and oxidation

Ligustilide is the major bioactive component of *Angelica sinensis*, which is reported to have multiple pharmacological properties including acting as an activator of the TRPA1 ion channel (Zhong et al., 2011; Yang et al., 2019). To explore the metabolic effects of TRPA1 activation in human skeletal muscle cells, the myotubes were treated with different concentrations of ligustilide for 24 h before glucose uptake and oxidation were measured. All concentrations of ligustilide examined significantly increased both glucose uptake and oxidation (Fig. 2A and B).

3.3. Activation of the TRPM8 ion channel decreased glucose uptake and oxidation following a 24 h treatment

Several studies have reported an increase in energy expenditure following TRPM8 activation by menthol (Li et al., 2018; Ma et al., 2012; Sanders et al. 2021). The TRPM8 agonist icilin is reported to both be more potent and efficacious than menthol (Wei and Seid 1983). However, glucose metabolism was decreased in myotubes treated with either of the two agonists for 24 h (Figs. 3A and 4B).

3.4. Activation of the TRPV1 ion channel with capsaicin had no effect on glucose metabolism

Capsaicin, the active component of chili pepper, acts as a TRPV1 agonist. Many *in vivo* studies investigating the metabolic effects of chili pepper consumption have reported an increase in energy expenditure as a result of increases in oxidation of both carbohydrates (Lim et al., 1997; Yoshioka et al., 1995) and fatty acids (Yoshioka et al., 1998; Lejeune et al. 2003; Janssens et al., 2013).

Myotubes were treated with 10 μM capsaicin for 24 h. However, the capsaicin treatment had no effects on glucose uptake and oxidation (Fig. 4A and B).

3.5. Activators of the TRPA1 and TRPM8 ion channels increased intracellular calcium levels in human myotubes

TRPA1 and TRPM8 are Ca^{2+}-permeable cation channels that increases the intracellular Ca^{2+} levels when activated (Pan et al., 2016; Pedersen et al. 2005). To examine whether TRPA1 and TRPM8 are functional in human myotubes, we aimed to investigate the effects of ligustilide and icilin on Ca^{2+} influx. DMSO, the solvent of ligustilide and icilin, failed to increase intracellular Ca^{2+} levels, while KCl induced membrane depolarization did (Fig. 5A). Both ligustilide and icilin increased intracellular Ca^{2+} levels, indicating presence of functional TRPA1 and TRPM8 ion channels in human myotubes (Fig. 5B).

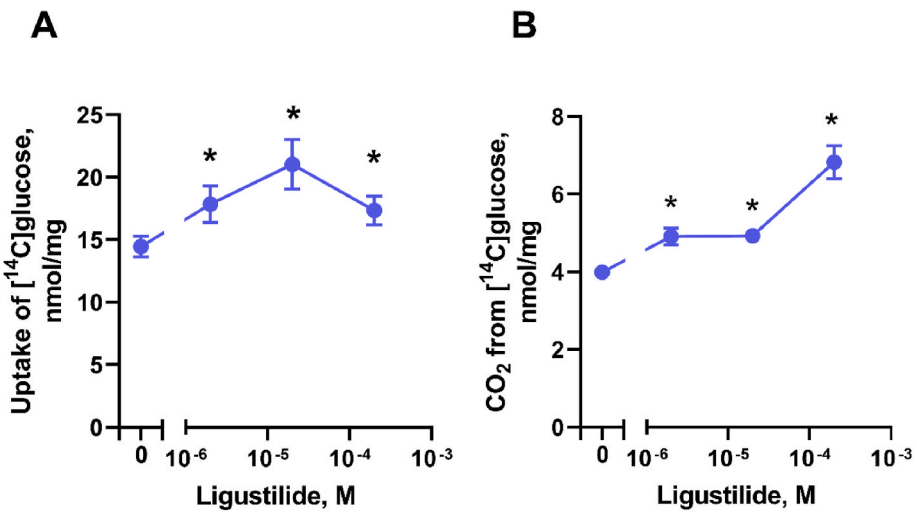

Fig. 2. Effects of 24 h treatment with ligustilide on glucose metabolism. Human myoblasts were grown and differentiated into myotubes in 96-well tissue culture plates. On day 6 of differentiation, the myotubes were either treated with control or different concentrations of ligustilide for 24 h. Thereafter, the cells were incubated with 200 μM [^{14}C]glucose (0.5 μCi/mL) together with ligustilide for 4 h. Oxidation, the CO_2 production, and cell-associated (CA) radioactivity of [^{14}C]glucose, and cellular uptake (CO_2 + CA) was determined. Cellular uptake of [^{14}C]glucose (**A**) and oxidation, measured as CO_2 production from [^{14}C]glucose (**B**) were determined after 4 h trapping. Results are presented as means ± SEM of 4 experiments on myotubes derived from 2 to 3 donors, each with 8–16 biological replicates in each experiment. *p < 0.05 vs control, unpaired *t*-test.

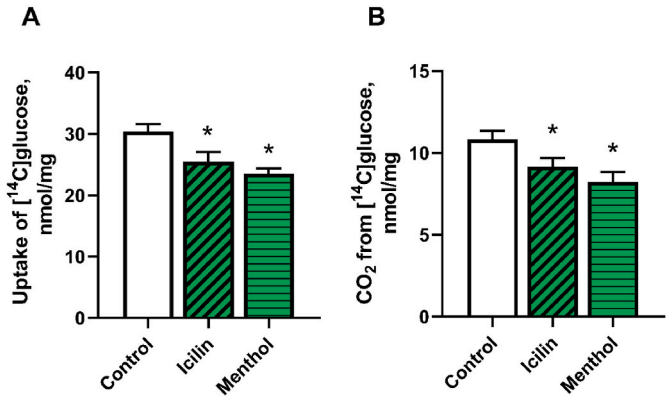

Fig. 3. Effect of 24 h treatment with icilin and menthol on glucose metabolism. Human myoblasts were grown and differentiated into myotubes in 96-well tissue culture plates. On day 6 of differentiation, the myotubes were either treated with control, icilin or menthol for 24 h. Thereafter, the cells were incubated with 200 μM [^{14}C]glucose (0.5 μCi/mL) together with menthol or icilin for 4 h. Oxidation, the CO_2 production, and cell-associated (CA) radioactivity of [^{14}C]glucose, and cellular uptake (CO_2 + CA) was determined. Cellular uptake of [^{14}C]glucose (**A**) and oxidation, measured as CO_2 production from [^{14}C]glucose (**B**) were determined after 4 h trapping. Results are presented as means ± SEM of 3 experiments on myotubes derived from 3 donors with 8 biological replicates in each experiment.*p < 0.05 vs control, unpaired *t*-test.

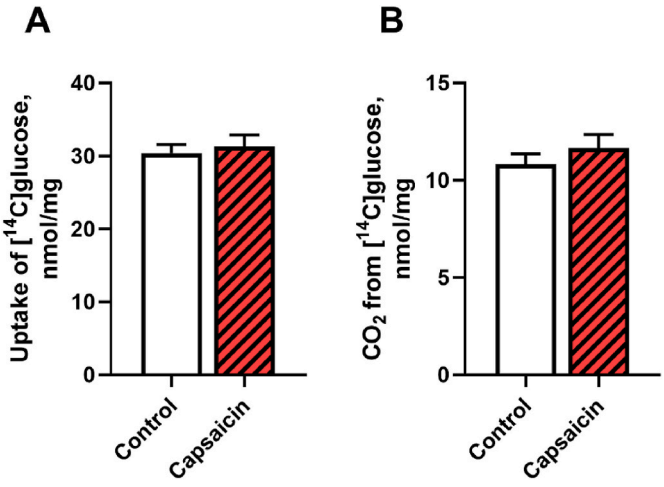

Fig. 4. Effect of 24 h treatment with capsaicin on glucose and metabolism. Human myoblasts were grown and differentiated into myotubes in 96-well tissue culture plates. On day 6 of differentiation, the myotubes were treated with control or 10 μM capsaicin for 24 h. Thereafter, the cells were incubated with 200 μM [^{14}C]glucose (0.5 μCi/mL) together with capsaicin for 4 h. Oxidation, the CO_2 production, and cell-associated (CA) radioactivity of [^{14}C]glucose, and cellular uptake (CO_2 + CA) was determined. Cellular uptake of [^{14}C]glucose (**A**) and oxidation, measured as CO_2 production from [^{14}C]glucose (**B**) were determined after 4 h trapping. Results are presented as means ± SEM of 3 experiments performed on myotubes derived from 3 donors, with 8 biological replicates in each experiment.*p < 0.05 vs control, unpaired *t*-test.

3.6. mRNA expression of genes of interest following activation of the TRPA1 and TRPM8 ion channels

To further understand the molecular mechanisms underlying the effects seen on glucose metabolism, we examined the effects of the TRP agonists ligustilide, menthol and icilin on the mRNA expression levels of TRPA1,TRPM8, the glucose transporters GLUT1 and GLUT4, as well as some genes involved in the thermogenic gene program. Icilin significantly downregulated the expression of TRPA1, while the expression of TRPM8 was significantly decreased by menthol (Fig. 6A). None of the compounds altered the expression of GLUT1, while ligustilide significantly downregulated the expression of the insulin-dependent GLUT4 transporter (Fig. 6B). Moreover, ligustilide was also the only compound that affected the thermogenic gene program, where the expression of PGC-1α (PPARGC1A), UCP1 and UCP3 were downregulated (Fig. 6C).

3.7. Cold exposure altered the effects of TRPA1 and TRPM8 inhibitors on glucose metabolism

We also wanted to study the effects of cold exposure on glucose metabolism, and whether TRPA1 and TRPM8 were involved in the cold response. In a previous study the TRPA1 inhibitor HC-030031 inhibited cinnamaldehyde-induced currents of human TRPA1 expressed in *X. laevis* oocytes (Gupta et al., 2016). The selective TRPM8 inhibitor PF-05105679 has been described to exhibit analgesic properties in relation to cold-related pain (Andrews et al., 2015).

Based on a previous study, where live rat hind limbs were exposed to a cooled surface of 10 °C and the intramuscular temperature in the limb was about 18 °C after 60 min, and human skeletal muscle cells were exposed to the same temperatures (Krapf et al., 2021), the myotubes were subjected to control conditions (37 °C) or cold (18 °C)

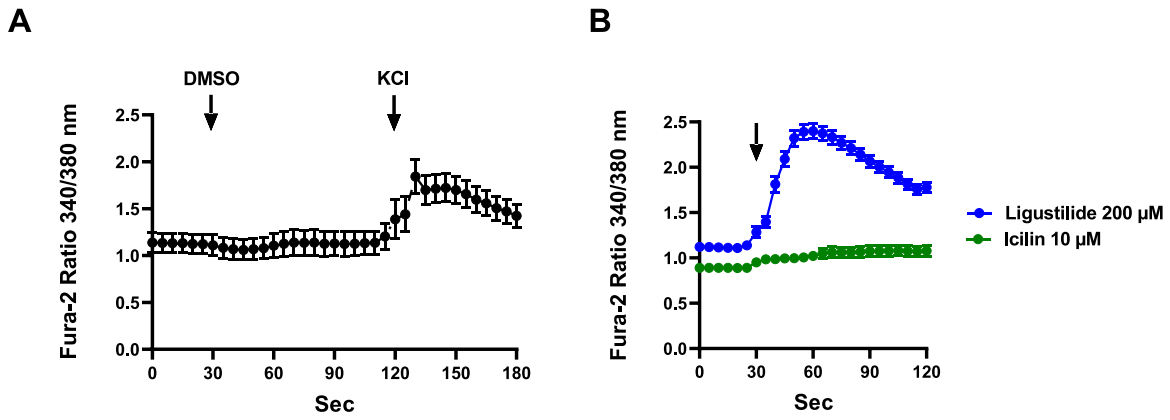

Fig. 5. Effects of ligustilide and icilin on intracellular calcium levels. Human myotubes were grown and differentiated into myotubes on glass bottom microwell dishes. On day 7 of differentiation, the cells were loaded with Fura-2 AM and placed in an inverted microscope to trace intracellular Ca^{2+}. In control experiments myotubes were stimulated with vehicle alone (0.05% DMSO) at 30 s and 80 mM KCl at 120 s (**A**). Myotubes stimulated with 200 µM ligustilide or 10 µM icilin at 30 s (**B**). The results are presented as means of $F_{Ca\text{-}bound}/F_{Ca\text{-}free}$ of 3 (**A**) and 6–8 (**B**) experiments on myotubes derived from 2 donors, each experiment with up to 18 cell recordings.

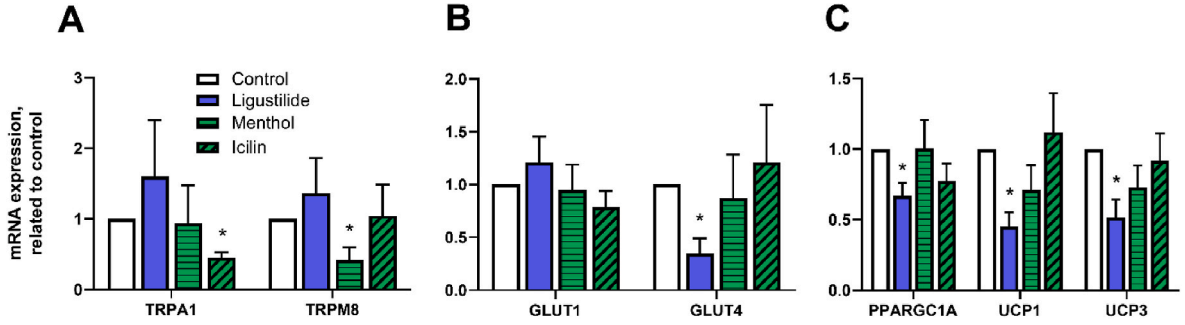

Fig. 6. Effects of TRPA1 and TRPM8 activators on gene expression in human myotubes. Human myotubes were grown and differentiated in 25 cm^2 cell culture flasks. On day 6 of differentiation, the myotubes were either treated with the TRPA1 activator, ligustilide, or one of two different TRPM8 agonists, icilin or menthol for 24 h. The cells were then harvested and mRNA expressions were assessed by qPCR. mRNA expression levels were related to the housekeeping gene 36B4 and normalized to control. **A**: Expressions of transient receptor potential cation channel, subfamily A, member 1 (TRPA1) and transient receptor potential cation channel, subfamily M, member 8 (TRPM8), **B**: Expressions of solute carrier family 2, facilitated glucose transporter member 1 (GLUT1) and solute carrier family 2, facilitated glucose transporter member 4 (GLUT4). C: Expressions of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1A), uncoupling proteins; UCP1 and UCP3. Results are presented as mean ± SEM from n = 6 individual experiments. *p<0.05 vs control, paired *t*-test.

temperatures for 18 h followed by a 3 h recovery period with and without treatment with the TRPA1 inhibitor HC-030031 or the TRPM8 inhibitor PF-05105679 (Fig. 7). Cold exposure had no significant effect on glucose uptake and oxidation in the basal situation. However, under control conditions, the TRPA1 inhibitor HC-030031 treatment significantly increased glucose uptake and oxidation, an effect that was not found after cold exposure (Fig. 7A and B). Treatment with the TRM8 inhibitor PF-05105679 had no effect, however when comparing cells treated with PF-05105679 at control and after cold exposure, glucose uptake was lower after cold exposure compared to control conditions (Fig. 7A).

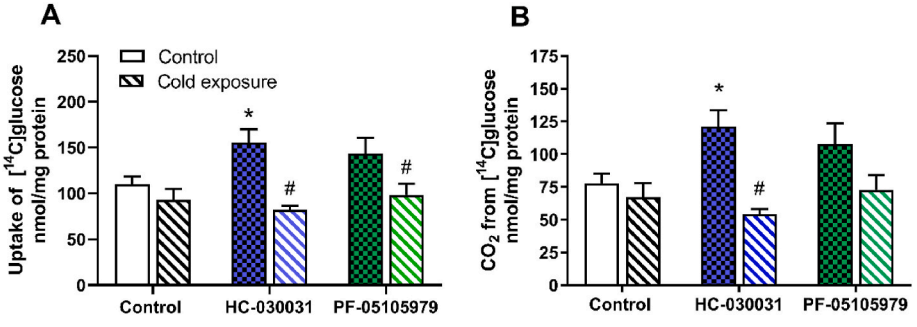

Fig. 7. Effect of cold exposure and TRP inhibitors on glucose metabolism. Human myoblasts were grown and differentiated into myotubes in 96-well tissue culture plates. On day 6 of differentiation, the myotubes were exposed to cold (18 °C) for 18 h plus 3 h of recovery at 37 °C or control conditions (37 °C) while being treated with either 25 µM HC-030031 or 25 µM PF-05105679 for 21 h. Thereafter, the cells were incubated with 200 µM [^{14}C]glucose (0.5 µCi/mL) for 4 h. Oxidation (CO_2), and cell-associated (CA) radioactivity of [^{14}C]glucose were measured, and cellular uptake (CO_2 + CA) was determined. **A**: Cellular uptake of [^{14}C]glucose, **B**: CO_2 production from [^{14}C]glucose. Results are presented as means ± SEM of 4 experiments performed on myotubes derived from 3 donors, with up to nine biological replicates in each experiment.*p < 0.05 vs untreated control, #p < 0.05 vs 37 °C the same inhibitor, unpaired *t*-test.

4. Discussion

We hypothesized that non-neuronal TRPs play a role in responses to thermal exposures. The aim of this study was to investigate if the thermally activated ion channels TRPA1, TRPM8 and TRPV1 are functionally expressed in human skeletal muscle cells.

It was confirmed that all three TRP ion channels of interest were expressed in cultured human myotubes, as well as in skeletal muscles from rats. In human myotubes, the TRPA1 activator ligustilide significantly increased glucose uptake and oxidation. However, this compound reduced the mRNA expression of the thermogenic genes UCP1, UCP3 and PGC-1α (PPARGC1A). When treating the cells with menthol and icilin, agonists of the TRPM8 ion channel, glucose uptake and oxidation were significantly decreased. Treatment with menthol also decreased the expression of TRPM8, while icilin decreased expression of TRPA1. Activation of TRPV1 by capsaicin had no effect on glucose metabolism. Activation of TRPA1 and TRPM8 with ligustilide and icilin, respectively, increased intracellular Ca^{2+} levels and induced membrane depolarization, however icilin to a much lesser extent that ligustilide. Under control conditions (37 °C) treatment with the TRPA1 antagonist HC-030031 significantly increased glucose uptake and oxidation, while treatment with the TRPM8 agonist PF-05105679 had no effect. Cold exposure had no effect on glucose uptake and oxidation under basal conditions, however when TRPA1 and TRPM8 channels were chemically inhibited a temperature-induced difference in glucose metabolism was found.

The expression of TRPA1 (Osterloh et al., 2016) and TRPV1 (Cavuoto et al., 2007) in human skeletal muscle cells have been demonstrated previously, while the expression of TRPM8 has only been shown in mice skeletal muscle (Krüger et al., 2008) and in C2C12 myotubes (Li et al., 2018). Thus, to our knowledge this is the first report of expression of TRPM8 in differentiated human skeletal muscle cells.

TRPA1 have a clear chemosensory role, whereas its role as a thermosensitive ion channel remains controversial. For instance, one study found that the cold-sensitivity of TRPA1 knockout (KO) mice were indistinguishable from the cold response in wild-type mice (Bautista et al., 2006). Moreover, another study found deficits in behavioral response to cold exposure and noxious cold sensing in TRPA1 KO mice (Kwan et al., 2006). A study using TRPM8 KO, TRPA1 KO and double KO (dKO) mice reported of similar deficits of TRPM8 KO and dKO mice when it came to cellular cold responses in a two-temperature preference assay (Knowlton et al., 2010). In the same study, TRPA1 KO and wild-type mice exhibited similar results, with no significant differences between the two, in both the cellular cold sensitivity and two-temperature preference assay (Knowlton et al., 2010). This might indicate that acute cold-sensing and cold mimetics are dependent on the TRPM8 ion channel, and that TRPA1 serves an inferior role in acute cold-sensation and response.

In our study, treatment with the TRPA1 activator, ligustilide, successfully increased intracellular Ca^{2+} levels, and increased glucose uptake and oxidation in a dose-dependent manner. However, the mRNA expression of GLUT4, PGC-1α, UCP1 and UCP3 were significantly decreased following treatment with ligustilide. Thus, the increased glucose metabolism seen with this compound cannot be explained by activation of these genes. In line with our findings, several studies have shown an increased glucose metabolism when activating TRPA1 with cinnamaldehyde (reviewed in (Zhu et al., 2017)). However, in contrast to our findings, an increased expression of GLUT4 (Nikzamir et al., 2014; Gannon et al., 2015) was seen following TRPA1 activation by cinnamaldehyde in C2C12 cells, and in one of these studies it was suggested that this effect was mediated by an increased expression of PGC-1α (Gannon et al., 2015). Moreover, as we did see an increase in intracellular Ca^{2+} levels following TRPA1 activation by ligustilide, it is possible that increased glucose uptake and oxidation is caused by the increased Ca^{2+} flux. Free cytoplasmic Ca^{2+} ions can act as second messengers and elicit a stimulatory effect of oxidative processes and substrate catabolism (reviewed in (Clapham 2007)).

The activity of the TRP ion channels increases when they are stimulated by their activation temperatures or agonists (Andrews et al., 2015; Caterina et al., 1997; Bandell et al., 2004). The TRPM8 agonist icilin was able to increase intracellular Ca^{2+} levels, but to a much lesser extent than ligustilide. However, it is reported that the activation of TRPM8 by icilin is dependent on the presence of cytosolic Ca^{2+} (Chuang et al. 2004). Therefore, it is possible that the cytosolic Ca^{2+} levels in cultured, non-innervated human myotubes were too low for full TRPM8 activation.

We found a decrease in glucose uptake and oxidation following treatment with the TRPM8 agonists, icilin and menthol, and no changes in expression of genes in the thermogenic gene program were detected. This was unexpected as one study performed in C2C12 mytotubesreported a Ca^{2+}-dependent increased expression of UCP1 and PGC-1α following treatment with menthol (Li et al., 2018). Therefore, it is possible that increases in energy metabolism in response to TRPM8 activation and subsequent increases in energy expenditure from resulting thermogenesis in human myotubes are dependent on neural input.

When the myotubes were treated with agonists of the TRPM8 channel we found that the expression TRPA1 and TRPM8 was significantly decreased following treatment with icilin and menthol, respectively. UCP1 is mainly expressed in BAT, where it is responsible for inducing non-shivering following activation of an upstream activator (Ma et al., 2012; Rousset et al., 2004). Due to UCP1 having an important role in non-shivering thermogenesis in BAT, it is expected that the expression of this protein would be downregulated following treatment with ligustilide in human myotubes. UCP3 is primarily expressed in skeletal muscle and is reported to have a role in fatty acid metabolism (Rousset et al., 2004). Interestingly, mice overexpressing UCP3 are resistant of diet-induced obesity and diabetes, which is believed to be the result of an energy-dissipating mechanism (Clapham et al., 2000). There are no evidence to suggest that UCP3 are involved in cold-induced thermogenesis, but it does seem to play a role in basal metabolic rate (Bouchard et al., 1997). Therefore, the decrease in the expression of UCP3 following TRPA1 activation by ligustilide can possibly be explained by the protein playing no role in thermogenesis.

The metabolic effects of inhibiting the TRPA1 and TRPM8 ion channels have, to our knowledge, not been investigated previously. We found that inhibition of TRPA1 by HC-030031 increased glucose uptake and oxidation under control (37 °C) conditions. This was unexpected, as also activation of TRPA1 by ligustilide increased glucose uptake and oxidation. In an attempt to activate cold-sensing receptors, we also measured glucose metabolism following 18 h exposure to cold temperatures (18 °C), a temperature previously found to be achieved in live rat limbs after 60 min of cold exposure (Krapf et al., 2021). After cold exposure and temperature recovery, glucose uptake and oxidation was not significantly altered when compared to control conditions (37 °C), and the effect of the TRPA1 inhibitor HC-030031 was abolished. When comparing cells treated with PF-05105679 at control and after cold exposure, glucose uptake was lower after cold exposure. Thus, it seemed like inhibiting TRPA1 and TRPM8 revealed a temperature-induced difference in glucose metabolism.

Based on previous studies, it is clear that HC-030031 exhibits antagonistic properties in terms of calcium handling (Memon et al., 2019; Koba et al. 2011). Although the effects of PF-05105679 on intracellular calcium concentrations have not been described before, one study found that the TRPM8 antagonist AMTB blocked the menthol-induced increase in intracellular Ca^{2+} (Li et al., 2018).

In conclusion, this study describes the functional expression of TRPA1, TRPM8 and TRPV1 in cultured human skeletal muscle cells. Activation of the TRPA1 ion channel increased glucose uptake and oxidation, while activation of the TRPM8 ion channel decreased glucose metabolism, and TRPV1 activation gave no effects on glucose metabolism. However, activation of both TRPA1 and TRPM8 increased intracellular Ca^{2+} levels. The metabolic effects seen following stimulation of TRPA1 and TRPM8 cannot be easily explained neither by changes

in intracellular Ca^{2+} levels nor by the mRNA expression of the thermogenic gene program. Cold exposure alone had no effect on glucose metabolism, but when TRPA1 and TRPM8 channels were chemically inhibited, a temperature-induced difference in glucose metabolism was found. More studies are needed to explore the links between TRP activation and energy metabolism in skeletal muscle.

Author contribution

FH conceived the idea. **FH** and **HT** administrated the project. **CS, NGL**, **LA**, **ZAL** and **FH** performed the experiments. **CS**, **NGL**, **AR**, **HT** and **FH** analysed the data. **CS** and **HT** prepared the figures. **CS** wrote the main manuscript. **CS**, **NGL**, **LA**, **ZAL**, **AR**, **HT** and **FH** review and edited the manuscript, and approved the final version to be submitted.

Funding

Fred Haugen has received grants for this research at the National Institute of Occupational Health in Norway (ICELAB, reference number: 2013 00396). This work was also funded by the University of Oslo, and supported by grants from the Norwegian Diabetes Foundation, Freia Chocolade Fabrik's Medical Foundation, and Anders Jahre's Foundation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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