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Novel methods for cold exposure of skeletal muscle *in vivo* and *in vitro* show temperature-dependent myokine production

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ABSTRACT

Proteins secreted from skeletal muscle serving a signalling role have been termed myokines. Many of the myokines are exercise factors, produced and released in response to muscle activity. Cold exposures affecting muscle may occur in recreational, occupational and therapeutic settings. Whether muscle temperature independently affects myokine profile, is still to be elucidated. We hypothesized that manipulating muscle temperature by means of external cooling would change myokine production and release. In the present study we have established new models for cold exposure of muscle *in vivo* and *in vitro* where rat hind limb or cultured human myotubes were cooled to 18 °C. After a recovery period, muscle tissue, cells and culture media were harvested for further analysis by qPCR and immunoassays.

Expression of several myokine genes were significantly increased after cold exposure in both models: in rat muscle, mRNA levels of CCL2 ($p = 0.04$), VEGFA ($p = 0.02$), CXCL1 ($p = 0.02$) and RBM3 ($p = 0.02$) increased while mRNA levels of IL-6 ($p = 0.03$) were decreased; in human myotubes, mRNA levels of IL6 ($p = 0.01$), CXCL8 ($p = 0.04$), VEGFA ($p = 0.03$) and CXCL1 ($p < 0.01$) were significantly increased, as well as intracellular protein levels of IL-8 (CXCL8 gene product; $p < 0.01$). The corresponding effect on myokine secretion was not observed, on the contrary, IL-8 ($p = 0.02$) and VEGF (VEGFA gene product) $p < 0.01$) concentrations in culture media were reduced after cold exposure *in vitro*.

In conclusion, cold exposure of muscle *in vivo* and *in vitro* had an effect on the production and release of several known exercise-related myokines. Myokine expression at the level of mRNA and protein was increased by cold exposure, whereas secretion tended to be decreased.

1. Introduction

Cold exposure may impact locally in the extremities and reduce the temperature of underlying skeletal muscle (Barcroft and Edholm 1943, 1946; Costello et al., 2012). Muscle cooling applied for shorter times, such as Whole Body Cryotherapy (WBC) and ice/winter swimming, has been suggested to lead to favourable adaptive changes in the antioxidant enzymes activity, however, only after a period of acclimatization (Lubkowska, Dołęgowska et al. 2012, 2013). Cold exposure treatment is thought to have beneficial effect also in relation to exercise recovery in terms of exercise-induced muscular pain relief, decrease in inflammation, mental well-being and enhanced quality of sleep (Bouzigon et al., 2016). On the other hand, cold exposures may cause adverse effects. For example, extended cold exposures in occupational settings have been associated with musculoskeletal complaints and chronic pain (Vale et al., 2017; Farbu et al., 2019; Farbu et al., 2020). The prevalence of this exposure in the general population is mostly unknown, however, most subjects experiencing cold report one or several cold-related complaints (Raatikka et al., 2007). The effect of cold on workers is moderated by factors such as physical activity, clothing, climate, socioeconomic and individual factors (Kinen and Hassi 2009).

Muscle has a role in maintaining thermal homeostasis. Muscle activity result in contraction and locomotion and also produces heat. The thermoneutral zone is defined as the ambient temperature range that allows core body temperature to be maintained without energy expenditure beyond basal metabolic rate (Kingma et al., 2014). In cold

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environments, voluntary muscle activity may contribute to shift the thermoneutral zone to tolerate better the cold surroundings (Kingma et al., 2014). Further cooling is compensated by adaptive muscle thermogenesis, which occurs mainly by repetitively contraction, known to most as cold-induced shivering (Eyolfson et al., 2001; Rowland et al., 2015; Betz and Enerback 2018). Within the first few seconds of repeated contraction muscle temperature can rise by several degrees (Kenny et al., 2003). Apart from muscle contraction, several genetically modified mouse models conclude that also non-shivering mechanisms in muscle must be activated to sustain heat production (Rowland et al., 2015). Thermogenic capacity, involving both shivering and non-shivering mechanisms, highlights the important role of muscles in temperature homeostasis.

Another important aspect of skeletal muscles and their impacts on whole body homeostasis, became evident as the cytokines and other proteins produced and released by skeletal muscle cells were discovered (Pedersen et al., 2003). Biomolecules secreted from muscle are termed myokines, and a subset were found to be excreted from contracting muscles working in a paracrine, autocrine and endocrine way (Pedersen et al., 2007; Pedersen 2011; Catoire and Kersten 2015). Since the discovery of the first myokine, several hundred have been identified (Hoffmann and Weigert 2017; Lee and Jun 2019). Multiple approaches for identifying myokines and their role have been pursued, mainly using model systems and focusing on the impact of physical exercise (Pedersen et al., 2003; Norheim et al., 2011; Raschke et al., 2013). One of the most extensively studied myokines is interleukin-6 (IL-6), however skeletal muscle also secretes: interleukin-8, (IL-8), formally called C-X-C motif chemokine ligand 8 (CXCL8); monocyte chemoattractant protein-1 (MCP-1), also referred to as C–C motif chemokine ligand 2 (CCL2); melanoma growth stimulating activity, alpha (GROα), now called C-X-C motif chemokine ligand 1 (CXCL1); stromal cell-derived factor 1 alpha (SDF-1α), called C-X-C motif chemokine ligand 12 (CXCL12); and vascular endothelial growth factor A (VEGFA) (Pourteymour et al., 2017). Locally, myokines have been identified to be involved in proliferation, differentiation and growth to maintain the muscle mass, strength and function (Lee and Jun 2019). On a systemic level myokines allow for cross talk between muscle and other organs and induce effects on bone formation, lipid and glucose metabolism, browning of white fat, tumour growth and more (Severinsen and Pedersen 2020).

Human studies suggest that gene expression changes occur in muscle due to environmental or locally applied cooling (Shute et al., 2018; Zak et al., 2018), and may underlie health effects, but not all studies have confirmed this (Zak et al., 2017; Opichka et al., 2019). More studies have looked into the impact of cold exposure on fat tissue, as it is thought to stimulate brown adipose tissue activity which results in metabolic benefits (Coolbaugh et al., 2019; Peres Valgas da Silva et al., 2019). Also, cold exposure has been reported to impact levels of serum cytokines individually, but also as a whole, i.e. the cytokine profile (Dulian et al., 2015; Bal et al., 2017; Jaworska et al., 2018). After exposing a group of volleyball players to a training program over two weeks, where half of the participants also followed a WBC program, the cryo-treated group had a significant drop in IL-6 after a single session of WBC (Jaworska et al., 2018). No change in the irisin level was found in this study (Jaworska et al., 2018), though irisin was reported to increase after extreme cold exposure in other studies (Dulian et al., 2015). By adapting mice to severe cold (4 °C) several humoral factors were induced, e.g. fibroblast growth factor 21 (FGF21), IL-1α, peptide YY (PYY), tumour necrosis factor (TNF), and IL-6 (Bal et al., 2017), and some of these are potentially myokines of muscle origin. In these and other studies, the cellular source of cold-induced factors in circulation is largely unknown, and to our knowledge, no studies in this field pinpoint the cellular origin to muscle and myokine production there.

In this study, we established novel experimental models for *in vivo* and *in vitro* cold exposure of muscle, and we used these models to investigate if cold exposure and low muscle temperatures had an impact on myokine expression and release.

2. Methods

2.1. Animal experiments

The animal studies were executed in accordance with regulations and approved by the Norwegian Food Safety Authority (FOTS, ID9483). Nine healthy 20 weeks old Lewis female rats were purchased from Janviers Lab, France. The rats weighed 150–200 g at purchase and housed in groups of 2–3 in plastic cages with steel top grill and free admission to food (SDS RM1, Scanbur AS, Norway) and water. The animals were initially sedated with 5% isoflurane gas (Baxter International Inc., USA) at 2.0 L/min O_2 flow for 3 min. Thereafter, the rats were maintained anesthetized through an inhalation mask with 2–3% isoflurane gas at 0.5 L/min O_2. Every 15 min, adequate anaesthesia was controlled through absence of withdrawal reflexes. Shavers were used to remove the fur from the posterior side of the hind limbs. The core temperature of the animals, assessed by a rectal probe, was maintained at 36–37 °C by a feedback heating pad (Homeothermic blanket control unit, Harvard Apparatus Ltd. Kent, UK).

During the experiment the anesthetized rat was placed on its back on the heating pad holding 37 °C. To achieve a local muscle cooling, the left hind limb was fixed with surgical tape so that the shaved skin of the posterior side (calf) was in direct contact with a cooling plate (Echo-Therm IC25, Torrey Pines, USA) holding 10 °C (Fig. 1A). The right hind limb was fixed in a similar fashion to the 37 °C heating pad and served as a control, and maintained 32–33 °C (Fig. 1B). The intramuscular temperature was measured during the cooling procedure for 3 rats using a probe thermocouple (Pico Technology Ltd; UK) inserted through a small incision of the skin and fasciae into the calf muscles of both limbs (Fig. 1B), and these rats were not subjected to further analyses. Six naïve rats were subjected to tissue harvesting: shaved, but otherwise intact, left hind limbs were similarly exposed in skin-contact with the cooling plate for 1 h. The duration of the cold exposure was limited to avoid respiratory complications and systemic effects, ensuring survival of the rat for the whole procedure. Following cold exposure, the hind limb was allowed to recover for 2 h while the rat was still under anaesthesia. This was done by removing the cooling plate and placing the exposed limb on the heating pad holding 37 °C. The right hind limb was kept at 37 °C throughout. The aim of the recovery phase after cold exposure was to normalize circulation in the tissue and balance conditions for the cold-exposed and control limb at the point of sample harvesting. After the thermal exposure, *m. gastrocnemius* from both cold-exposed and control hind limbs were harvested for further molecular analyses (Fig. 1C). This was done by a 100 μL heparin (5000 IE/mL, LEO Pharma AS, Denmark) injection into left ventricle, followed by transcardial perfusion with 100 mL HBSS. (HyClone Laboratories, Inc., USA); calf muscles were dissected out; *m. gastrocnemius* was isolated, snap-frozen and crushed into a fine powder with a mortar in liquid N_2; and the tissue was stored at −80 °C.

2.2. Culturing and cold exposure of human myotubes in vitro

Satellite cells, muscle resident stem cells, were isolated and cultured as previously described (Gaster et al., 2001; Hessvik et al., 2010) from *m. vastus lateralis* from healthy male donors. The biopsies were obtained with informed consent from all participants, and all research was performed in accordance with all relevant guidelines and regulations with approval from the National Committee for Research Ethics, Norway (ref. no. 2011/2007 REK sør-øst B). Aliquots of cells were stored in liquid N_2 until they were thawed and seeded out in 6-well cluster plates (CELL-BIND®, Corning). Multinucleated human myotubes were established by proliferation of satellite cells (passage 3 and 4) in DMEM-Glutamax™ (5.5 mM glucose) medium supplemented with 2% FBS and 2% Ultroser G, 25 IU penicillin, 25 μg/mL streptomycin, 1.25 μg/mL amphotericin B. At approximately 80% confluence, the culture medium was changed to DMEM-GlutamaxTM (5.5 mM glucose) medium supplemented with 2%

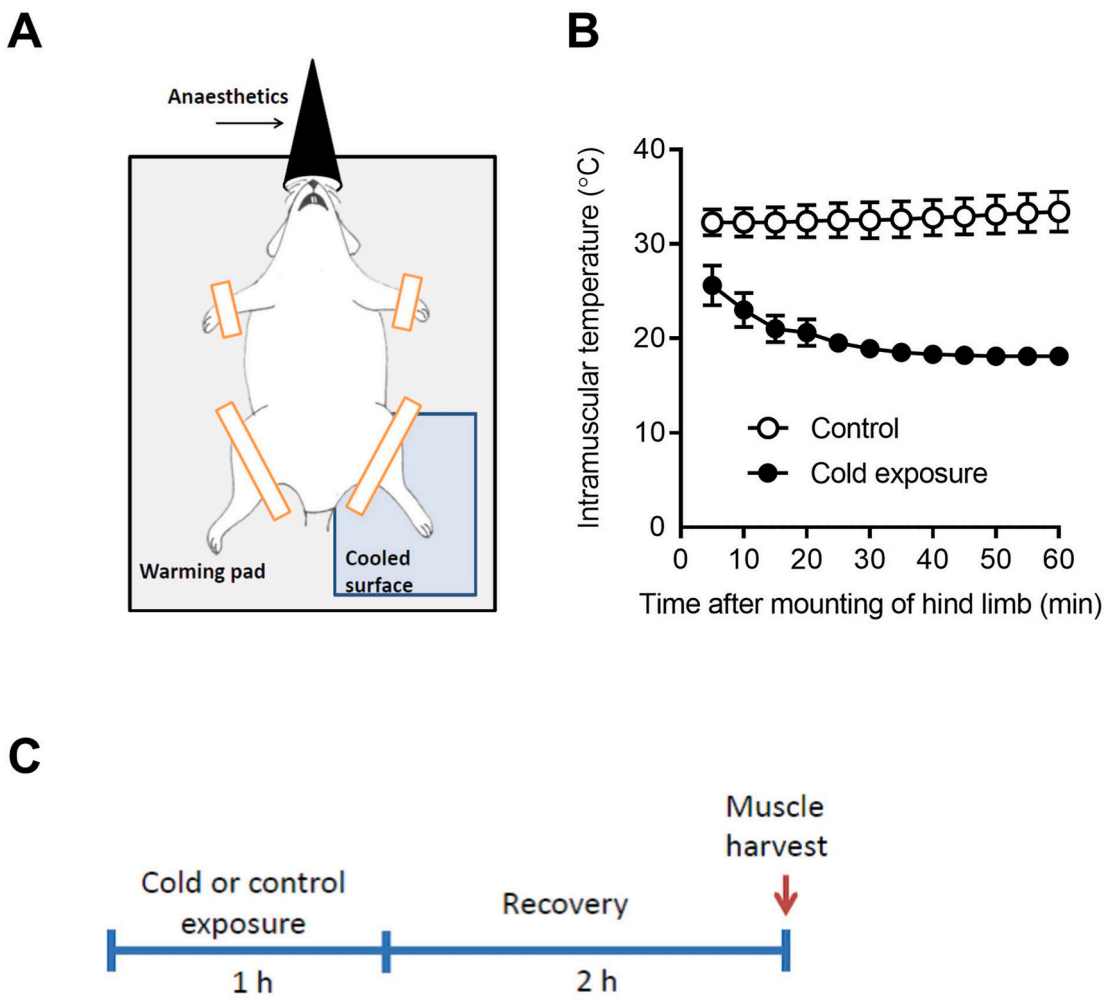

Fig. 1. Unilateral cold exposure of hind limb *in vivo*. A) Layout of cold exposure experiment: the anesthetized rat was placed on its back on a warming pad; while the left hind limb was fixed for 1 h to a cooled surface holding 10 °C; the right hind limb served as a contralateral control and was fixed to the warming pad (holding 37 °C). B) During the experiment the intramuscular (calf) temperature was measured in the control and cold-exposed limbs. Values are presented as mean ± SEM (n = 3). C) Timeline of experiment; after 1 h of cold or control exposure, the rat was allowed to recover at 37 °C for 2 h.

FBS and 25 pM insulin, 25 IU penicillin, 25 μg/mL streptomycin, 1.25 μg/mL amphotericin B to allow for differentiation into multinuclear myotubes. The medium was changed every 2–3 days, and the cells were kept in an incubator with humidified 5% CO_2 atmosphere at 37 °C. The differentiation procedure lasted for 6–7 days. Fresh media was added to each well before cold exposure started.

To expose the muscle cells to cold (18 °C) or control temperature (37 °C) the culture dishes were placed on temperature-regulated aluminium blocks strung with perfusion tubes of copper (Aavid Thermoalloy SRL, Italy), and isolated with Styrofoam. The blocks were cooled and heated by perfusion with water coming through isolated silicone tubing from refrigerated and heated water circulators, respectively (Heto-Holten AS, Denmark) (Fig. 2A and B). Temperatures of the perfused metal blocks were logged to ensure correct temperature exposure during experiments. Furthermore, we validated the direct thermal exposures to the cells, i.e. temperatures of the culture media, for a subset of experiments (Fig. 2C). The thermal exposures used for myotubes were either 18 °C or 37 °C for 18 h.

2.3. Recovery and harvesting of cells and media

Thermally exposed myotubes were harvested after a subsequent recovery at 37 °C. Thus, at cells harvest, cold-exposed cells and control cells were under similar temperature conditions. The recovery phase, or lag between cold exposure and cell analyses, pertains to the inert relationship between mRNA expression response to ambient temperature and the intracellular and extracellular accumulation of protein. In our experiments, cell culture medium and cells were harvested from designated wells at different time-points of the recovery phase. Medium was harvested at time-points 0, 3, 24 h of recovery. After harvesting, medium was spun at 1000×*g* for 15 min at 4 C° before supernatant was transferred to new tube and frozen at - 80 °C for further analysis. As the cell medium covering the cells was exchanged once with fresh medium, at the onset of cold exposure, it was predicted that an accumulation of myokines in the media would occur over time (Fig. 2D). At time-point 3 h of recovery, we also prepared homogenates of the cells and stored them at - 80 °C until mRNA and intracellular protein analysis.

2.4. RNA isolation and qPCR

Total RNA was isolated from approximately 10 mg frozen rat muscle tissue or human myotubes by extraction with Isol Lysis reagent (5 PRIME GmbH, Germany) in accordance with the protocol supplied by the manufacturer. RNA integrity and concentration was examined using BioAnalyzer™ (Agilent Technologies, USA) and Nanodrop™ 2000 (Thermo Scientific™, Denmark), respectively. RNA concentrations for the rat samples ranged between 85 and 1000 ng/μL and RNA values for human myotubes ranged from 45 to 100 ng/μL (20 μL final volume), and

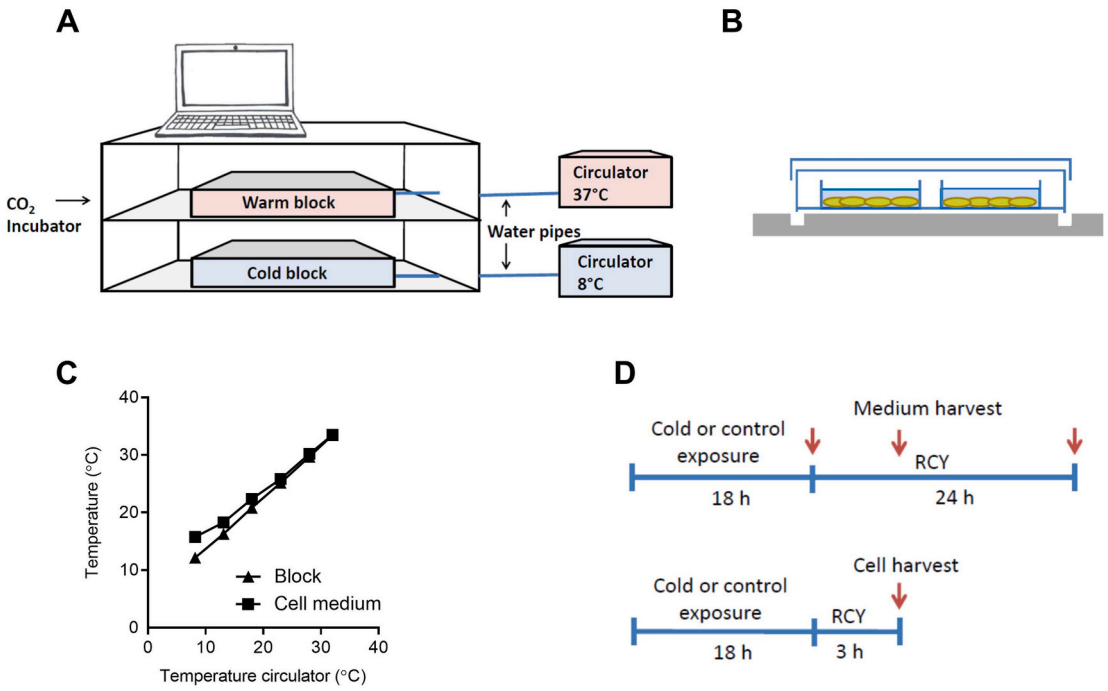

Fig. 2. Cold exposure *in vitro*. A) and B) Layout of experiment: cold exposure of cultured human primary myotubes in CO_2 incubator; 6-well cell culture plates rested on metal blocks perfused with water supplied by temperature-controlled circulators. C) Temperatures within the cell culture media in the wells of the culturing plate, as well as temperature on the perfused metal block was logged with a computer and aligned with the temperature of the circulators (mean ± SEM, n = 3). D) Timelines of experiments for cell culture medium harvest for immunoassay (upper), and cell homogenate harvest for qPCR and immunoassay (lower). RCY, recovery.

verification of the method by random sampling gave RNA integrity numbers (RIN) between 7 and 9. Reverse transcription of 500 ng to cDNA was performed with Maxima™ First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc, USA). qPCR was performed on a StepOnePlus™ instrument; human cDNAs were analysed using PerfeCTa™ SYBER Green Fastmix™ (Quantabio) and pre-designed KiCqStart® primers (Sigma-Aldrich). qPCR reactions for human myotube samples were run in duplicate at the following schedule: 95 °C for 30 s followed by 40 cycles (95 °C for 5 s, 60 °C for 15 s and 70 °C for 10 s). A premade TaqMan gene array was used for the rat cDNAs, which were run in duplicate at 50 °C for 120 s, 95 °C for 20 s followed by 40 cycles (95 °C for 1 s and 60 °C for 30 s). Rat primers are listed in Supplementary Table S1 and human primers in Supplementary Table S2. Ribosomal protein P0 (RPLP0) was used as reference gene for relative ΔΔCt quantification of transcripts. The housekeeping 18S rRNA was also detected in rat samples, and normalization of gene expression with 18S gave the same results as when normalizing against RPLP0. The human samples were only normalized against RPLP0, its suitability as a housekeeping gene in human myotubes has been documented in several publications, including Lund et al. (Lund et al., 2017; Lund et al., 2018a, 2018b).

2.5. Luminex immunoassay

Multiplex immunoassays, Bio-Plex Pro™ magnetic bead sets, targeting IL-6, IL-8, CCL2, VEGFA, CXCL1, CXCL12 were used to measure myokine protein concentrations in cell culture media and cell lysates. Media were collected and spun down at 1000×*g* and 4 °C for 15 min before the supernatant was collected and kept frozen at −80 °C. Cells were lysed using Tissue extraction Reagent I (Invitrogen™) supplemented with a protease inhibitor cocktail (cOmplete Tablet Mini EASYpack, Roche) after the manufacturer's description. Lysates were then kept frozen at −80 °C. Upon analyses, lysates and media were thawed on ice and the myokine content was measured as indicated by the manufacturer's protocol. This includes firstly a calibration of the Bio-Plex System within the Bio-Plex Manager™ Software, we used Bio-Plex MAGPIX multiplex reader (BIO RAD™). A standard dilution series in 7 steps (1:4 per step) was prepared according to guidelines. Magnetic beads with capturing antibody were diluted and added to each well of the Bio-Plex Pro, flat bottom 96 well plate (BIO-RAD™) followed by two washing steps, using a handheld Magnetic Separator Block (Merck Millipore). Undiluted samples (50 µL), standard and control, were added in duplicates to their designated wells on the plate. The plate was then covered with sealing tape and incubated on a MTA 2/4 digital microplateshaker (IKA ™) at 850+/-50 rpm at room temperature for 30 min. The plate was then washed three times, using the Magnetic Separator Block, before 25 µL biotinylated detection antibody was added to each well, followed by shaker for 30 min at 850+/-50 rpm and three new washing steps before adding 50 µL streptavidin-PE (SA-PE). Then, the plate was shaken for 10 min at 850+/-50 rpm, followed by three new washing steps before adding 125 µL assay buffer. The plate was kept on the shaker for 30 s at 850+/-50 rpm straight before inserting it into the Bio-Plex MAGPIX multiplex reader for bead analysis.

2.6. Presentation of data and statistics

All values are reported as mean ± SEM. The value n represents the number of different donors or animals used. Microsoft Excel 2010 and GraphPad Prism version 8.01 was used for calculations. Student's t-test was used for comparisons between the different exposures. Linear mixed model (SPSS version 20, IBM SPSS Statistics, Armonk, NY, US) was used to compare levels of myokines in media over time. A p-value ≤ 0.05 was considered significant.

2.7. Data availability

All data generated or analysed during this study are included in this article.

3. Results

3.1. Expression of myokines in rat muscle after cold exposure

To study the myokine response to cold exposure, we chose to analyse a panel of myokines previously found to be elevated after skeletal muscle contraction *in vivo* and/or *in vitro* (Pedersen 2011; Catoire et al., 2014; Feng et al., 2015; Puchert et al., 2016; Pourteymour et al., 2017; Chen et al., 2019; Opichka et al., 2019). In rat, we measured the mRNA expression of IL6, CCL2, VEGFA, CXCL1, and CXCL12 in *m. gastrocnemius* from cold-exposed and contralateral control hind limbs (Fig. 3). We also measured the mRNA level of RNA-binding motif protein 3 (RBM3), an evolutionarily conserved RNA-binding protein that is transcriptionally upregulated in response to low temperature (Zhu et al., 2016). RBM3 mRNA expression was significantly increased by 39% in the cold-exposed limb compared to the contralateral control limb (Fig. 3).

As shown in Fig. 3, mRNA expression of IL6 ($p = 0.03$) was significantly reduced, while the expression levels of CCL2 ($p = 0.04$), VEGFA ($p = 0.02$), CXCL1 ($p = 0.02$) and RBM3 ($p = 0.02$) were significantly increased in the cold-exposed limb compared to control limb. Gene expression of CXCL12 ($p = 0.07$) was not significantly changed by temperature.

3.2. Expression and secretion of myokines in human myotubes after cold exposure

Cold-exposed human myotubes in culture were analysed using the same panel of myokines as in rat muscle, except for excluding RBM3 and including IL8/CXCL8. After 18 h of cold exposure at 18 °C, and 3 h of recovery at 37 °C, exposed cells showed a significant increase in mRNA levels of IL6 ($p = 0.01$), VEGFA ($p = 0.03$) and CXCL1 ($p < 0.01$), as compared to control cells kept at standard conditions at 37 °C. Levels of IL-8 ($p = 0.08$), CCL2 ($p = 0.07$) and CXCL12 ($p = 0.1$) mRNA, on the other hand, were unchanged under cold circumstances (Fig. 4A). As myokine genes were affected by the cold exposures, the corresponding intracellular protein levels were also measured. Protein expression level of IL-8 ($p < 0.01$) was significantly increased. However, CCL2 ($p = 0.09$), CXCL12 ($p = 0.07$), IL-6 ($p > 0.1$) and CXCL1 ($p > 0.1$) were unchanged in the cold-exposed cells (Fig. 4B). Intracellular VEGFA was under the detection limit.

As myokines are secreted proteins, we measured levels in the cell culture media from myotubes exposed to cold (18 °C) for 18 h. Media was harvested immediately after cold exposure (0 h), and after recovery at 37 °C for 3 h and 24 h (Fig. 4C–H). Many of the secreted myokines showed a tendency to be reduced by cold exposure compared to control conditions (constant 37 °C), however, only IL-8 ($p = 0.02$) and VEGFA ($p < 0.01$) were significantly reduced by cold. The levels of IL-6 ($p = 0.09$), CCL2 ($p > 0.1$), CXCL1 ($p > 0.1$) and CXCL12 ($p > 0.1$) were unchanged after cold exposure (Fig. 4D and F). At the 3 h recovery time-point, myokine concentrations differed by more than 2-fold between cold exposure cells and controls. The cold induced effect on myokine concentrations was diminished at the 24 h recovery time-point (Fig. 4C–F), indicating that myokine release rates were accentuated in cold-exposed cells during the 3–24 h portion of the recovery period (Fig. 4A). This is in accordance with the elevated intracellular myokine levels in lysates from cold-exposed cells harvested after 3 h of recovery, as we expect a lag between protein synthesis and release (Fig. 4B).

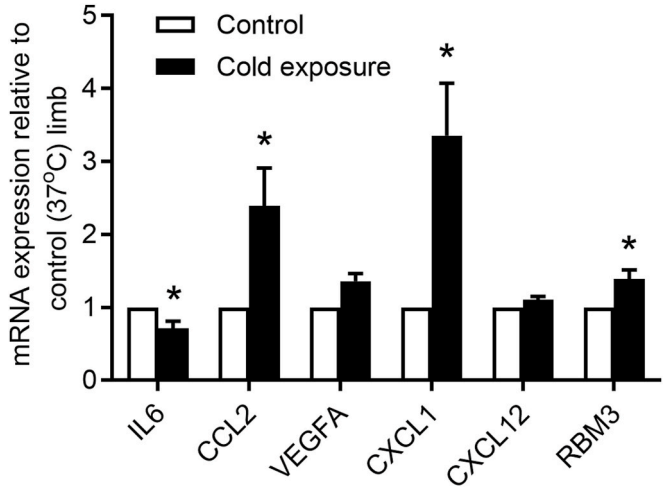

Fig. 3. Effects of cold exposure on gene expression in rat skeletal muscle. Rat hind limbs were exposed to cold or control conditions, as described in Fig. 1, before RNA was isolated from *m. gastrocnemius*. A) mRNA levels of interleukin-6 (IL6), C–C motif chemokine ligand 2 (CCL2), vascular endothelial growth factor A (VEGFA), C-X-C motif chemokine ligand 1 (CXCL1), CXCL12 and RNA-binding motif protein 3 (RBM3) in cold-exposed muscle are shown relative to contralateral control muscle (mean ± SEM, n = 6), *Statistically significant vs. control ($p < 0.05$, Student's t-test).

4. Discussion

Multiple studies suggest exercise-regulated release of myokines from muscle (Pedersen et al., 2003; Pedersen et al., 2007; Pedersen 2011). However, a relationship between muscle temperature and myokine secretion has not yet been identified. In humans, skeletal muscles contribute to approximately 40% of the total body weight (Zurlo et al., 1990), thus even small changes in myokine secretion may potentially have systemic impact.

The purpose of this study was primarily to demonstrate two innovative approaches of muscle cooling: an *in vivo* model with anesthetized rats, and an *in vitro* model with cultured human myotubes. Secondly we wanted to explore the effects of localized low temperature on muscle expression and secretion of myokines. We designed an *in vivo* layout for unilateral cold exposure in rat using the contralateral hind limb as control. Exposure to cold conditions (10 °C) for 1 h induced an intramuscular temperature of 18 °C, and this was followed by a recovery for 2 h, before *m. gastrocnemius* was harvested for molecular analysis. For the *in vitro* experiments, we exposed human myotubes to cold for 18 h, and keeping the cell media temperature stable at approximately 18 °C. Control myotubes were kept at 37 °C for this period. After that, both groups were left to rest at 37 °C through a recovery phase. Cell culture media were harvested and analysed for myokine content at different time-points of recovery (0–24 h), whereas cells for myokine expression analyses were harvested after 3 h of recovery.

A strength of this study was the tight control of the thermal exposure, i.e. the cellular temperature. Environmental temperature was maintained and controlled using thermostats, and temperature logging applied to ensure correct exposure at the cellular level, both *in vivo* and *in vitro*. Not unexpectedly, some results differed between the models considering that intact muscle of a rat and isolated human muscle cells were studied. The models originate from two species with differences in thermal biology, although humans and rats exhibit similar skin and core temperature at thermoneutral conditions (Wanner et al., 2015). However, the high surface area to body volume found in rats compared to larger animals, such as humans, is linked to a higher exchange of heat to the environment and thereof follows a metabolic rate which is 3–5 fold greater compared to humans (Wanner et al., 2015). Also, as the diameter of the rat limb is smaller than of a human leg, muscle cooling is more likely to occur, and so, adaptive response mechanism to cooling of muscles in rats and humans may differ. Nonetheless, in this study we used an *in vivo* and an *in vitro* model with the limitations that comes with each of these. The rats were unilaterally exposed to cold under anaesthesia. Cold exposure was set to 1 h and recovery to 2 h, hence the rats were anesthetized a total of 3 h. Duration was limited to avoid impairing respiration, observed with longer lasting anaesthetic treatments. A longer time course could have been preferred as well, however would

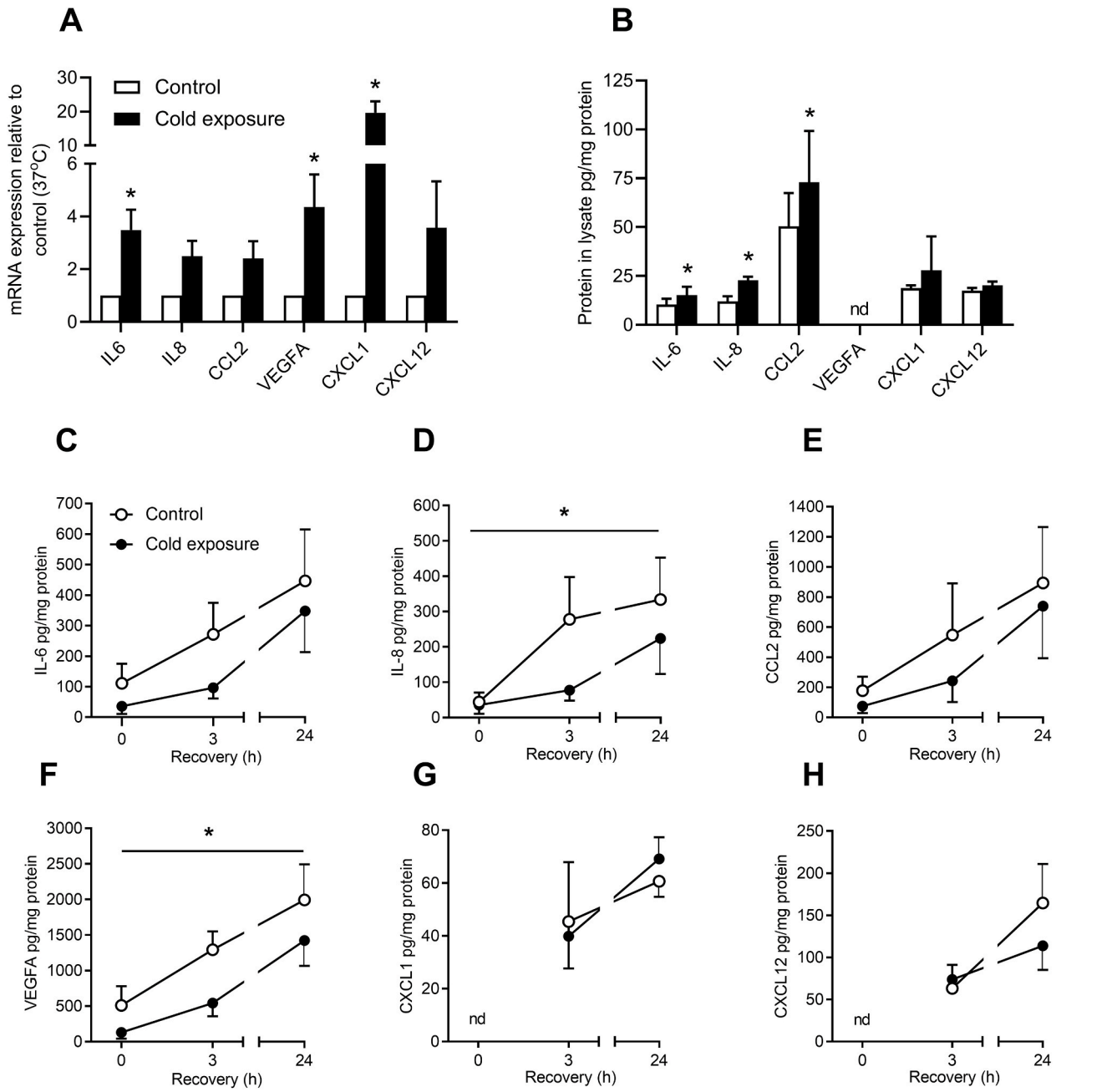

Fig. 4. Effects of cold exposure on mRNA and protein expression of myokines in human myotubes, and on myokine concentrations in cell media. Human myotubes were grown and differentiated in 6-well tissue culture plates, and then exposed for 18 h to either cold (18 °C) or control conditions (37 °C) as described in Materials and Methods. A) Cell homogenates for RNA extraction were harvested after another 3 h of recovery at control conditions for both groups. Bars display mRNA expression of genes encoding interleukin-6 (IL6), interleukin-8 (IL8/CXCL8), C–C motif chemokine ligand 2 (CCL2), vascular endothelial growth factor A (VEGFA), C-X-C motif chemokine ligand 1 (CXCL1) and CXCL12 in cells, as determined by qPCR. Data are presented as relative to control conditions and as mean ± SEM (n = 4). B) Similarly, intracellular proteins were extracted after 18 h exposure to cold or control conditions followed by 3 h recovery at control conditions. Bars display protein expression levels of IL-6, IL-8, CCL2, VEGFA, CXCL1 and CXCL12 in cells under cold conditions relative to control conditions, as quantified in cell homogenates with a multiplex immunoassay. Values were normalized to total protein levels in each sample and presented as mean ± SEM (n = 4). *Statistically significant vs. control ($p < 0.05$, Student's t-test); nd, not detected. C–H) Extracellular protein levels were quantified in cell culture media conditioned by cells exposed to either cold or control conditions for 18 h as a function of different lengths of recovery at control conditions (0–24 h at 37 °C). Graphs display concentrations measured by multiplex immunoassay of IL-6; IL-8; CCL2; VEGF; CXCL1; CXCL12; nd, not detected; *statistically significant all-over vs. control ($p < 0.05$, linear mixed model).

have introduced stress in the rat and potentially impacting the outcome. We also allowed for a recovery time of 2 h to have restored blood-flow and similar temperature in cold-exposed and control limbs at the time of harvesting. This could, however, have created another uncertainty in terms of whether the effect we see is due to the cooling or the recovery period *per se*. With this experimental set-up *in vivo* we cannot separate effects of lowering muscle temperature from normal conditions, or elevating temperature from cold conditions. We can, however, draw conclusions regarding this particular sequence of intramuscular thermal states. The *in vitro* experiments allowed us to extend the duration of cold exposure. Here we also chose to include a recovery period, with equivalent temperatures in cold-exposed and control cells at the time of

harvesting, so that the cell model and the rat model would be comparable. Analyses of cell culture media harvested after multiple lengths of recovery from cold exposure (0, 3 and 24 h recovery) showed that myokine levels increased steadily during recovery, but also that myokines differed in their secretion patterns after cold exposure. For instance, VEGFA levels were affected by cold exposure alone, i.e. before recovery (0 h), whereas the effect of cold on IL-8 levels only became evident after 3 and 24 h of recovery.

Despite differences, both models allowed us to observe an effect of cold exposure on the production and release of several myokines. In the cold-exposed rat muscle, mRNA levels of CCL2, VEGFA and CXCL1 were significantly increased, while in the cold-exposed cultured human myotubes, mRNA levels of IL6, VEGFA and CXCL1 increased. In the myotube experiments, protein levels of the myokines were also measured, both in cell lysates and in cell culture media. In cell lysates, IL-8 protein was significantly increased by cold, in accordance with the mRNA levels. The other myokines whose protein levels were measured in in the cell lysates, were not significantly increased, but there was a tendency ($P < 0.08$) for CXCL12. There is often reported discrepancies between mRNA and protein levels which may be explained by complex post-transcriptional processes such as alternative splicing, mRNA modification, and mRNA decay, steps occurring before the RNA is translated to protein (Greenbaum et al., 2003; Krapf et al., 2020). Further, protein levels are also determined by synthesis and decay rates. Methodological aspect may also contribute to a gap in mRNA and protein measurements, since immunoassays used to determine protein levels are not as sensitive as the qPCR reactions used to determine mRNA expression (Greenbaum et al., 2003).

Two of the myokines, IL-8 and VEGFA were secreted at a lower level in the cold exposed cells compared to the controls. In addition, IL-6 showed a tendency ($P < 0.1$), but was not significantly decreased. It is intriguing, though, that myokine secretion was reduced by cold exposure, despite increased mRNA and/or protein levels in the cells. We expect to find a lag between expression and secretion, so it is possible that a compensatory mechanism of enhanced expression is active in the cold exposed cells without manifesting itself as increased media concentrations at the 3 h recovery time-point. After that, between 3 h and 24 h of recovery, cold exposed cells displayed an elevated secretion (steeper slopes), compared to controls, suggesting that expressional changes observed at 3 h only became evident at later time-points. However, myokine concentrations in the cell media from cold exposed cells had not fully caught up with levels in controls after 24 h of recovery. IL-8 is produced by most tissues (Baggiolini et al., 1994) and traditionally seen as a pro-inflammatory cytokine (Pedersen 2011; David et al., 2016), and is also associated with insulin resistance and obesity (Bruun et al., 2002). The release of IL-8 have been measured during several kinds of exercises and seem to vary depending on the concentric and eccentric part of the exercise (Nieman et al., 2001; Ostrowski et al., 2001; Suzuki et al., 2003; Pourteymour et al., 2017). Whole-body cryotherapy resulted in a significant reduction in serum IL-8 level in a group of athletes (Banfi et al., 2010), however the source of IL-8 was not investigated. VEGFA is a major regulator of blood vessel formation, have neuroprotective function, and was also shown to stimulate skeletal muscle regeneration *in vivo* (Arsic et al., 2004). In our experiments VEGFA mRNA expression increased in skeletal muscle after cold exposure both in the *in vivo* and the *in vitro* model. These results are in accordance with the findings of Joo et al., (2016) (Joo et al., 2016), where cold water immersion increased VEGF mRNA expression in human skeletal muscle. Chronic cold exposure increased capillary density skeletal muscle in rats (Suzuki et al., 1997), possibly through increased VEGF production.

Our study shows that CXCL1 mRNA expression increased after cold exposure both in the *in vivo* and the *in vitro* model, but protein levels were unchanged intra- and extracellularly. CXCL1 is a chemotactic cytokine produced during inflammation and has also a role in development and maintenance of pathological pain (Silva et al., 2017). Increased expression of CXCL1 has been found in skeletal muscle after exercise in mice *in vivo* and *in vitro* (Pedersen et al., 2011; Chen et al., 2019). Furthermore, CCL2 expression was increased after cold exposure in the *in vivo* model, our data suggest, but unchanged *in vitro*. In addition to be an exercise-induced myokine (Catoire et al., 2014), CCL2 is a well-known inflammatory marker, important to mount an adequate inflammatory response to repair acute skeletal muscle injury. Previous studies suggest that CCL2 originates from both bone marrow cells and monocytes, but also injured muscle cells to recruit monocytes and macrophages to the injury (Lu et al., 2011). In human skeletal muscle biopsies, it has been shown that mRNA expression of CCL2 increased after acute resistance exercise, however, no effect of cold water immersion was found compared to active recovery (Peake et al., 2017). CCL2 may possibly be marker for acute injury, seen in both exercise and cold exposure to the muscle.

In our experiments, IL-6 mRNA expression was reduced after cold exposure in the rat *in vivo* model, however mRNA levels were increased by cold in the human myotubes. Protein levels were not affected by cold. IL-6 is the prototypic myokine; it was the first myokine described, and is the most extensively studied myokine (Pedersen et al., 2001; Febbraio and Pedersen 2002). IL-6 is found to be acutely released into the circulation during exercise in an intensity- and duration-dependent fashion (Ostrowski et al., 2001; Eckardt et al., 2014). Increased mRNA levels of IL-6, as well as of CCL2 and IL-8, were found after 45 min of acute exercise of 70% of VO_2max (Pourteymour et al., 2017). The increase was first thought to be related to muscle damage (Bruunsgaard et al., 1997), however, more recent studies classifies muscle-derived IL-6 as an anti-inflammatory cytokine and a potential protector against metabolic syndrome. The acute increase of IL-6, unlike the chronically elevated IL-6, increase the muscle insulin sensitivity (reviewed in (Eckardt et al., 2014)). IL-6 is therefore considered more as an exercise factor and higher exercise-induced increases of IL-6 are found especially in persons exercising with low muscular glycogen levels (Steensberg et al., 2001). IL-6 has also been put in the context of temperature regulation (Egecioglu et al., 2018). In mice, serum levels of IL-6 increased after cold exposure (Bal et al., 2017). Global IL-6 deficient mice exhibited significantly lower body temperature and oxygen consumption after 6 days at 4 °C compared to wild-type mice (Egecioglu et al., 2018). However, in a human study combining training and 3 min of whole body cryostimulation, the cryostimulation group experienced a significant reduction in serum IL-6 compared to the control group (Jaworska et al., 2018). Both in our *in vitro* myotube experiment, with 18 h cold exposure, and in a study where mice were exposed to 4 °C for 6 days, increases of IL-6 were found (Egecioglu et al., 2018). Similarly, with shorter cold exposure time, as we performed in our *in vivo* rat study, we found a decrease in the mRNA level in line with *Jaworska* et al. which found a decreased serum IL-6 in human participants after cryostimulation lasting only 3 min at −110 °C (Jaworska et al., 2018). In our experiment, unlike in the experiments of Egecioglu et al. (Egecioglu et al., 2018) and *Jaworska* et al. (Jaworska et al., 2018), cultured muscle cells were used. Therefore, all myokines in the media in our experiment are derived from muscle cells, excluding other organs or cell types as a potential source of these myokines. Also, differences in time and temperature in different models may explain divergent results.

Working in a cold environment or sustained exposure to cold temperatures has been associated with chronic pain in some studies (Vale et al., 2017; Farbu et al., 2019), while cold exposure for shorter time periods (e.g. cryotherapy) may relieve pain and inflammation, and induce physiological and psychological benefits (Bouzigon et al., 2016). Whether myokines play a role in any of these conditions are currently unknown. We believe that there is not sufficient research on this field yet, however, we are hoping that our contribution will increase the interest and research in the area.

In conclusion, we have designed and tested two models for cold exposure of skeletal muscle to investigate regulation of myokine production and secretion. We observed some discrepancies between the two

models used in this study. This was not unforeseen as the experiments were performed in two different model systems; muscles of anesthetized, but otherwise intact rats; and cultured muscle cells isolated from human donors. Our results, however, indicate that these novel methods can be used to study cold exposure in skeletal muscle, and that myokine production is regulated by intramuscular temperature.

Authors' contribution

FH conceived the idea, SK, FH, TS, LA and SKH analysed the data, SK, FH, HT and TS wrote the main manuscript and SK and HT prepared the figures.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2021.102930.

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