

Myokine secretion in cultured human skeletal muscle cells

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Abstract

It is well established that exercise protect againsts chronic disorders such as cardiovascular diseases (CVD), type 2 diabetes (DM2), dementia and depression. It is unclear how contracting skeletal muscles mediate metabolic positive effects on health. One of the possible explanations for the health benefit of exercise can be that regular muscle contractions mediate important messengers; myokines. Experiments have shown an increased IL-6 concentration in plasma following skeletal muscle contraction. IL-6 is being released from skeletal muscle following a meal. This suggest that myokine release can also be modulated with diet.

In the present study cultured human myotubes (an *in vitro* model for skeletal muscle) were incubated in serum-free medium with and without energy-carrying metabolites for different periods of time. The purpose was to investigate the release of peptides and proteins by cultured human myotubes and further study the effect of energy-carrying metabolites on the release. The release of proteins was analyzed by the use of ELISAs and proteomic analysis performed on conditioned media from the cultured human myotubes. The inhibitor Brefeldin A was used to explore whether the release of proteins was due to secretion or leakage. The results show that cultured human myotubes release the proteins interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8) and interleukin-13 (IL-13), and that the release perhaps can be influenced by different energy-carrying metabolites. The mean concentration of the different proteins measured in the media was generally lower when the myotubes were incubated in media containing the inhibitor Brefeldin A (BFA), indicating that the release of proteins from myotubes is due to secretion. Our studies support the theory that myotubes release proteins with hormonal functions, and that the release might be influenced by energy-carrying metabolites.

Abbreviations

BC	Bicinchoninic acid
BSA	Bovine serum albumin
CVD	Cardiovascular diseases
DHA	Docosahexaenoic acid
DM2	Diabetes Mellitus type 2
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethyl sulphoxide
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaic acid
ER	Endoplasmatic reticulum
FCS	Foetal Calf Serum
FFA	Free fatty acids
GM-CSF	Granulocyte macrophage- colony stimulating factor
IFN- γ	Interferron- γ
IL-1 β	Interleukin-1 β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-7	Interleukin-7
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13

IL-15	Interleukin-15
LDH	Lactate dehydrogenase
MALDI-TOF MS	Matrix-assisted laser desorption/ionization-time of flight mass spectrometer
MQW	Milli-Q Water
OA	Oleic acid
PA	Palmitic acid
SPDI	Secreted Protein Discovery Initiative
TNF- α	Tumor necrosis factor- α
2-D PAGE	Two-dimensional Polyacrylamid Gel Electorphoresis

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1. Introduction

1.1 Health effects of exercise

It is well established that exercise protect againsts chronic disorders such as cardiovascular diseases (CVD), type 2 diabetes (DM2), dementia and depression [1]. Physical activity has been proven effective in the treatment of these and many other different medical disorders. Some of these beneficial effects are mediated through an improved lipid profile, elevated insulin sensitivity and lower blood pressure [2]. Striated skeletal muscle is one of the body's largest tissues, ca 40 % of the body mass in a normal slim individ. However, it is unclear how contracting skeletal muscles mediate metabolic positive effects on health. One of the possible explanations for the health benefit of exercise can be that regular muscle contractions mediate important messengers; myokines [3]. It might be that released myokines can explain how normal muscle activity can influence mood, physical performance and cognitive function [1].

1.2 Myokines

In the last years we have seen accumulating evidence that skeletal muscle release hormone-like substances. Secreted proteins play an important regulatory role in intercellular communication. When the muscle tissue communicate with other tissues and organs, we believe that it does so by releasing myokines [4]. Myokines are signal peptides with potential hormonal effect, that are being released by muscle cells [5]. Different tissues throughout the body have been proven to communicate with each other via hormonal signals synthesized and secreted into the circulation as demonstrated for adipose tissue [6].

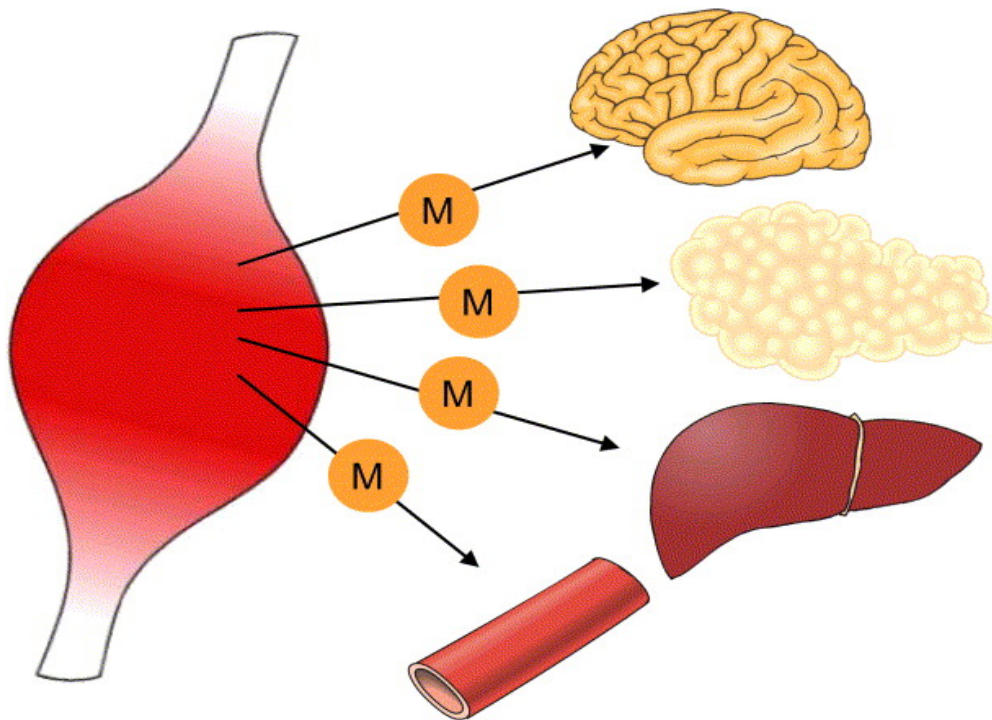


Illustration1. Muscle cells release myokines that affect other tissues throughout the body.

1.3 Skeletal muscle

Muscle is a very specialized tissue that has the ability to contract and conduct electrical impulses. Muscles are classified functionally as voluntary or involuntary, and structurally as striated or smooth muscle. A skeletal muscle is composed of bundles of muscle fibers that generally extend the entire length of the muscle. The cells are called fibers because of their threadlike shape [7].

In the adult skeletal muscle there is a pool of undifferentiated mononuclear cells, called satellite cells. They are quiescent in normal adult muscle and can be activated by stimuli such as myotrauma. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate, before differentiating and fusing to form multinucleated myotubes, which then mature into myofibers [8]. When the satellite cells are grown in culture they are seeded in a special medium, and the change from one type of medium to another initiate the differentiation of the myoblasts.

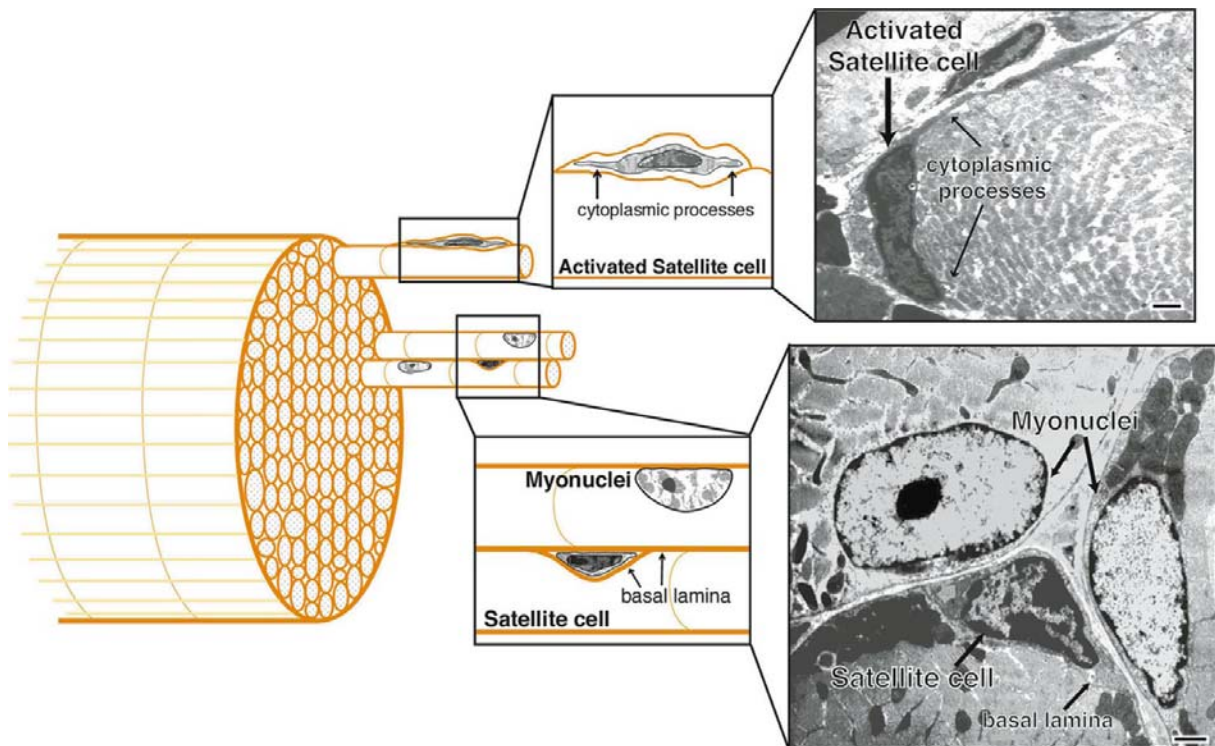
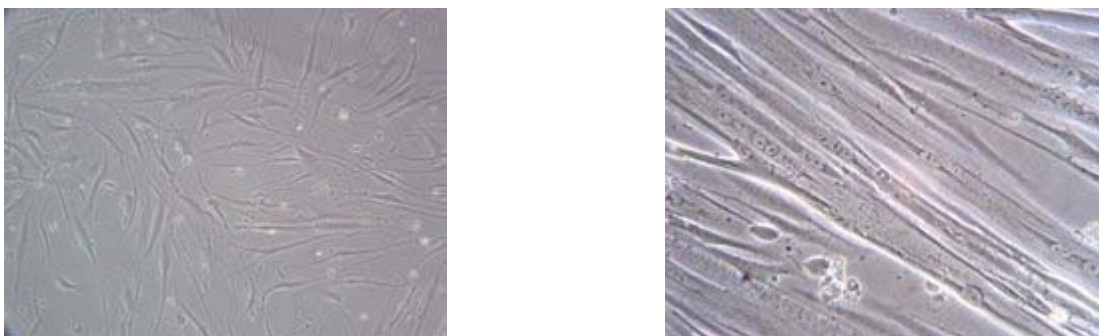


Illustration 2. Satellite cells occupy a sublaminar position in adult skeletal muscle. In the uninjured muscle fiber, the satellite cell is quiescent and rests in an indentation in the adult muscle fiber. The satellite cells can be distinguished from the myonuclei by a surrounding basal lamina and more abundant heterochromatin. When the fiber becomes injured, the satellite cells become activated and increase their cytoplasmic content. The cytoplasmic processes allow for chemotaxis of the satellite cell along the myofiber. Bar 5 μ m.



Picture 1 Human skeletal satellite cells. To the left; undifferentiated satellite cells. To the right; differentiated myotubes. Photo by Vigdis Aas.

1.4 Myokines are released from skeletal muscle cells

Experiments have shown an increased IL-6 concentration in plasma following skeletal muscle contraction [3]. Physical activity is associated with a systemic cytokine response comparable with levels observed during severe infections, except for that the increase of Tumor necrosis factor- α (TNF- α) and Interleukin 1- β (IL-1 β) is minimal if at all present after concentric exercise without muscle damage. This indicates that in non-traumatic exercise models, the cytokine cascade differs markedly from the classical acute-phase response studied during infections [3]. Skeletal muscle accounts for the majority of insulin-stimulated glucose use and is therefore the major site of insulin resistance [9]. Insulin resistance is a major characteristic of type 2 diabetes mellitus and is also associated with obesity, hypertension and cardiovascular disease. Circulating IL-6, insulin and free fatty acid concentrations are associated with impaired insulin action in obese and type 2 diabetic individuals [10]. IL-6 production is modulated by the carbohydrate availability in skeletal muscle, suggesting that IL-6 functions as an energy-sensor. The IL-6 response can be a signal that the muscle glycogen stores reach critically low levels. IL-6 is being released following exercise and it seems a paradox that working muscle should release a factor that inhibits insulin signaling when insulin action is increased in the immediate postexercise period [4]. IL-6 induces strong anti-inflammatory effects, inhibits low-grade TNF- α production and may inhibit TNF- α -induced insulin resistance. IL-6 might thereby have an important role in mediating the beneficial effects of exercise [11].

Production of IL-6 in contracting human skeletal muscle cells can account for the exercise-induced increase in plasma IL-6 [12]. Muscle contractions induce IL-6 mRNA-production in skeletal muscle in rats, showing that muscle tissue is the origin of IL-6 [13]. Further, it has been documented that the muscle cells are the cells responsible for the release of IL-6 [14-16].

Plasma concentration of interleukin-8 (IL-8) increases as a response from eccentric exercise, whereas concentric exercise with moderate intensity does not increase the

plasma concentration. High local expression and small release suggests that it perform its effect locally. Because of this IL-8 is classified as potential muscle derived myokine [4]. IL-15 (Interleukin-15) is constitutively expressed and might play a role in adipose-muscle cross-talk, and is therefore classified as a potential muscle derived myokine [4].

1.5 Myokines and diet

IL-6 is being released from skeletal muscle following a meal [17]. This combined with the fact that palmitate increase IL-6 from skeletal muscle cells in vitro [10] suggest that myokine release can also be modulated with diet.

1.6 The skeletal muscle secretome

Around 1000 new genes that probably encode secreted proteins are described by the Secreted Protein Discovery Initiative (SPDI). Bortoluzzi identified by computational approach 319 proteins putatively secreted by skeletal muscle during normal conditions. The skeletal muscle secretome can vary with different conditions, some proteins are secreted only during development or regeneration. The interleukin-6 (IL-6) gene, for example, is quiet in resting muscle, but activated by physical activity [2].

1.7 Proteomics

Proteomics is the study of proteins, their structure, expression, function and interactions with other molecules. The proteome includes all proteins produced by the human genome at any one time. The genome is the same in most cells, whereas the proteome varies with cell type. The environment and what is going on inside the cell e.g. cell division also influence the proteome. In our search for myokines we used different techniques considered essential for proteomics research like two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and mass spectrometry (MS) [18].

1.8 Aims

1.8.1 Study release of peptides and proteins by human myotubes in culture

The first object of this study was to investigate if we could detect proteins released from cultured human myotubes with our model.

1.8.2 Study the effect of energy-carrying metabolites on the release of proteins from human myotubes in culture

The second object was to incubate myotubes with energy-carrying nutrients and examine how this will influence the release of myokines.

2. Materials and methods

2.1 Materials

Dulbecco's modified eagle medium with L-Glutamine (DMEM), 10 % foetal calf serum (FCS) and penicillin-streptomycin 5000IU/mL - 5mg/mL was obtained from Sigma Chem, St.Lois, MO, USA. Ultrosor G was obtained from BioSeptra, CIPHERGEN Biosystem Inc, Paris, France. Extracellular matrix gel (ECM), essentially fatty acid free bovine serum albumin, bovine serum albumin (used for protein standard) Sigma Chem. Co, palmitic acid (PA), oleic acid (OA), eicosapentaic acid (EPA), DHA was obtained from Nu-Chek prep, Inc, Elysian, MN, USA. ¹⁴C labelled BSA was delivered by Perkin Elmer, Waltham, Massachusetts 02451 USA. Insulin Actrapid was obtained from Novo Nordisk, Bagsværd, Denmark. Trizol reagent was purchased from Invitrogen, Carlsbad, CA, USA. Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories, CA, USA. 12-well plates were obtained from Corning, NY, USA. Total protein was measured with Titertek Multiskan, Labsystems Oy, Helsinki, Finland. Cell Bind Flasks Corning, NY, USA. DMEM w/o glucose, phenol red, L-Glutamine, Sigma Chem. Co and IL-6 immunoassay, came from R&D Systems, MN, USA. Multiplex Immunoassay was from Lincoplex, St. Charles. Missouri, USA, and glucose was delivered by Sigma Chem. Co, the LDH-kit was delivered by Roche Diagnostics, Roche Applies Science, Mannheim, Germany, whereas RD 193058100 Human Myostatin ELISA came from BioVendor. For protein assay, the BC Assay protein kit was obtained from Uptima Interchim, Montluçon, France.

2.2 Cell culture

A cell bank of satellite cells was established from muscle biopsy samples taken from musculus vastus lateralis or m. obliquus internus abdominis from healthy volunteers, aged 24.7 ± 0.7 years with body mass index 23.6 ± 1.1 kg/m². The biopsies were

obtained with informed consent and approval by the National Committee for Research Ethics (Oslo, Norway). Muscle cell cultures free of fibroblasts were established by the method of Henry et al. with minor modifications. The cells were cultured in a humidified 5% CO₂ atmosphere at 37°C, and medium was changed every 2–3 days.

Medium 1 (M1) for seeding. 500 mL DMEM with low glucose (SIGMA, #D6046), 50 mL FCS (Foetal Calf Serum (SIGMA, #F7524), 5 mL L-glutamine (SIGMA, #G7513, 100 mL), 5 mL Penicillin/Streptomycin (GIBCO BRL, #15140-114, 100 mL)

M2 for proliferation. 500 mL DMEM with low glucose (SIGMA, #D6046), 10 mL FCS (Foetal Calf Serum (SIGMA, #F7524), 10 mL Ultrosor G, 5 mL L-glutamine (SIGMA, #G7513, 100 mL, 5 mL Penicillin/Streptomycin (GIBCO BRL, #15140-114, 100 mL)

M3 for differentiation. 500 mL DMEM with low glucose (SIGMA, #D6046), 10 mL FCS (Foetal Calf Serum (SIGMA, #F7524), 5 mL L-glutamine (SIGMA, #G7513, 100 mL), 5 mL Penicillin/Streptomycin (GIBCO BRL, #15140-114, 100 mL, 25 pmol Insulin (\Rightarrow 21 μ l of 1000 x diluted Insulin Actrapid[®], Novo Nordisk, 0,6 mM) Ultrosor-G (Bio Serpa, #P/N 15950-017) should be re-constituted by adding of 20 mL MQW to the container. Wait for 15 minutes, and transfer 10 mL to M2-medium. The rest (10 mL) is frozen (-20 °C) for further use.

2.3 Methods

2.3.1 Seeding satellite cells

Primary skeletal muscle cells were grown from satellite cells. 1 vial containing the satellite cells (approximately 1.5 million cells) was picked up from the tank containing liquid nitrogen. The suspension was thawed in 37 ° C until only a tiny piece of ice was left. In a LAF bench the cell suspension was added to a 50 mL tube containing 9 mL or 15 mL prewarmed M1 or 15 mL prewarmed SKBM depending on

the format used (12 well plate or 75 cm² flask). This was performed to avoid the cells to be exposed to the high concentration of DMSO which is poisonous for the cells when they are not frozen. The cell suspension was well mixed with M1 by a pipette. The cells were seeded with 750 μ L medium in each well in the 12 well plate and 15 mL in the 75 cm² flask and incubated in 37°C and 5% CO₂ over night. The day after the medium was changed from M1 to 1 mL M2 for the plates, SKGM (SKBM + bullet kit without the insulin + 2,5 mL Fungibact + 5 mL Glutamine) + 2 % FCS for the flasks. Bullet kit was added to increase the proliferation but inhibit differentiation. M2 and SKGM was changed every second or third day. When the cells had reached 80% confluence the medium was changed to either 1 mL M3 for the 12 well plate or 15 mL for the flask. When changing medium to M3 at this point the cells differentiated into multinucleated myotubes. The M3-medium was changed every second or third day. The experiments were performed either before M3-medium were given (undifferentiated cells) or after 2 or 5 days. Different substances were added to either M3 or DMEM according to the experiment performed. The cells were visually inspected under microscope during growth, differentiation and experiments. Trypsination was performed on the flasks to loosen the cells from the bottom for making possible the moving of the cells to bigger flasks. Medium was removed by vacuum. The cells were washed by adding and removing 5 mL roomtempered PBS in each flask to avoid trypsin from inactivation. 5 mL trypsin were added to each flask and incubated for 5 min by 37° C in 5% CO₂. The cells were checked in the microscope to verify that they had loosened from the bottom. The suspension was transferred to a vial. The flasks were added 5 mL DMEM with 2 % FCS to inactivate trypsin, this was also transferred to the same vial. The vial was centrifuged 3 minutes on 1500 rpm to get rid of trypsin and FCS. The supernatant was removed. The pellet was resuspended in 3 mL SKGM in the same vial. The new flasks were added 20 mL SKGM. The cells were shared between the new flasks

2.3.2 Monitoring cell damage by LDH activity

The myotubes were exposed to various potentially harmful substances in the different experiments. To monitor their well being we used Cytotoxicity Detection Kit that

measures how much of the intracellular enzyme lactate dehydrogenase (LDH) is leaking through the membrane. This can be used as a general marker on whether the cells are intact or not. Conditioned media was centrifuged on 3000 rpm for 5 min (on the day of harvesting to avoid dead cells from leaking LDH), 25 uL supernatant was transferred from each vial to a flat bottom 96-well plate. To each well 25 uL milliQ water and 50 uL LDH reagents were added. The muscle cells were lysated in 100-1000 uL 2% Triton X-100, 20 uL from the lysate were transferred to a flat bottom 96-well plate. To each well 30 uL MQW and 50 uL LDH reagents were added. The 96-well plates were incubated in 37°C for 15 minutes, wrapped in foil. The absorbance was measured by a spectrophotometer (Titertec) on 492 nm. Blank background samples consisting of either only DMEM or lysis-solution were measured in parallel and the value were subtracted from the respective values from the samples

2.3.3 Lysing muscle cells with Triton X-100

Lysing the cells was done to obtain 100 % LDH-leakage. 0,5 mL 2 % Triton X-100 in MQW was added to each well. The cells were incubated at 37 C in 5 % CO₂ for 5 min, and lysate collected in vials. 20 uL lysate from each vial was transferred to 96-well plates. The absorbance was measured by a spectrophotometer (Titertec) on 492 nm.

2.3.4 Protein quantification of the lysates

BC (bicinchoninic acid) Assay and Bio-Rad Assay were used to determine the amount of protein in samples. The BC-reagent reduces Cu²⁺ in alkaline solutions to Cu⁺ and two molecules of BCA interact with each molecule of Cu⁺ forming a water soluble red complex. The Bio-Rad Protein Assay is a dye-binding assay in which a differential colour change occurs in response to the concentration of protein.

Coomassie blue dye binds to primarily basic amino acid residues, especially arginine. Cell lysates were homogenized and 25 uL of each homogenate was transferred to separate wells on a flat bottom 96-wells plate. Protein standards made from BSA by serial dilution were likewise added to separate wells. Each well was added 200 uL

reconstituted coomassie reagent or BC-reagent, and the plate was then incubated at room temperature (5 or 30 min for Coomassie and BC-reagent, respectively). The absorbance values were determined by a spectrophotometer (Titertec) at either 590 nm (Coomassie) or 490 nm (BC). Sample protein concentrations were determined by plotting the absorbance values into the linear function obtained from the protein standards.

2.3.5 IL-6 measurements

ELISA is a sensitive immunoassay that uses an enzyme linked to an antibody as a marker for the detection of a specific protein. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples were pipetted into the wells, and any IL-6 present was bound by the immobilized antibody. The samples were compared against the standards curve to determine the amount of IL-6 present. Results were expressed in pg/mL. The lower detection limit was 0.07 pg/mL. 100 uL of Assay Diluent RD1W was added to each well. 100 uL of Standard, sample, or control was added to each well. The plate was incubated for 2 hours at room temperature. Each well was aspirated and washed, the process was repeated three times for a total of four washes. Each well was washed by filling Wash Buffer (400 uL) using a manifold dispenser. After the last wash, any remaining Wash Buffer was removed by aspirating or decanting. 200 uL of IL-6 Conjugate was added to each well. The plate was incubated for 2 hours at room temperature. The aspiration/wash was repeated as in step 5. 200 uL of Substrate Solution was added to each well. The plate was incubated for 20 minutes at room temperature protected from light. 50 uL of Stop Solution was added to each well. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm.

2.3.6 Myostatin measurements

In the BioVendor Human Myostatin ELISA (Prodomain Specific), Calibrators, Quality Controls (QCs) and samples of sera or plasma are incubated in microtitration

wells coated with monoclonal anti-human myostatin prodomain antibody. After one hour incubation and washing, biotin labelled polyclonal anti-human myostatin prodomain antibody is added and incubated with captured myostatin prodomain or prodomain complex. 100 µl of diluted Calibrators (0.1-10.0 ng/ml), Dilution Buffer (=Blank), Quality Controls and samples, were pipetted into the appropriate wells. The plate was incubated at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker. The wells were washed 5-times with Wash Solution (0.35 mL per well). All liquid was aspirated from the wells. 100 µl of Biotin Labelled Anti-Myostatin Prodomain Antibody Solution was added into each well. The plate was incubated at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker. The wells were washed 5-times with Wash Solution (0.35 mL per well). 100 µl of Streptavidin-HRP Conjugate was added into each well. The plate was incubated at room temperature (ca. 25°C) for 30 minutes, shaking at ca. 300 rpm on an orbital microplate shaker. The wells were washed 5-times with Wash Solution (0.35 mL per well). 100 µl of Substrate Solution was added into each well. The plate was incubated at room temperature for 10 minutes. The colour development was stopped by adding 100 µl of Stop Solution into each well. The absorbance was determined by reading the plate at 450 nm.

2.3.7 Cytokine measurements

High sensitivity human cytokine multiplex immunoassay kit may be used for analyses of many cytokines in culture supernatants simultaneously. The medium from myotubes incubated with and without BFA were collected. 13 cytokines in the conditioned media were analysed by Luminex multiplex technology. The concentrations of the cytokines were measured and the total amounts of the cytokines in the media were estimated. The total amount of the cytokines from the well or flask were related to the total amount of protein in the cell lysate from the same well or flask (Table 1). The amount of protein in the cell lysate indicated the amount of cells remaining after washing and incubation in the well or flask. By relating the amount of cytokines to the amount of protein we adjusted for the eventual differences in myotube density in the different wells or flasks. The ratio of media volumes (mL) to

the growth area (cm²) were 0.11 mL/cm² for the wells and 0.07 mL/cm² for the flasks. The wells had 1.6 times higher volume per cm² than the flasks. The cytokines tested were IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IFN γ , GM-CSF and TNF α . The filter was pre-wet by pipetting 200 μ L of 1X Wash Buffer into each well of the microtiter plate. The plate was sealed and put on a plate shaker for 10 minutes at room temperature. Wash Buffer was removed by vacuum. The bead bottle was sonicated for 30 seconds and vortexed for 1 minute. 25 μ L of the mixed beads were added to each well. Liquid was removed from the wells by vacuum. 50 μ L Assay Buffer were added to the 0 Standard and the sample wells. 50 μ L of each Standard and Control were added to appropriate wells. 50 μ L matrix solution were added to the Background, Standards, and Control wells. The plate was covered with aluminium foil and incubated with agitation on a plate shaker overnight (19 hr). The fluid was removed by vacuum aspiration. The plate was washed with 200 μ L Wash Buffer 2 times. The removing of Wash Buffer was performed by vacuum. 50 μ L of Detection Antibody Cocktail were added each well. The plate was sealed with aluminium foil, and incubated on a plate shaker for 1 hour at room temperature. 50 μ L Streptavidin-Phycoerythrin were added each well. The plate was covered with aluminium foil, and incubated with agitation on a shaker for 30 minutes at room temperature. The content was removed by vacuum. The plate was washed with 200 μ L Wash Buffer two times and the Wash Buffer was removed by vacuum. 100 μ L of Sheat Fluid were added each well. The plate was covered with aluminium foil and resuspended the beads on plate shaker for 5 minutes. The plate was read on Luminex Instrument.

2.3.8 Brefeldin A (BFA) and protein secretion

The myotubes were added incubation medium with or without 1 μ g/mL BFA. By comparing the amount of protein measured in the media from the two treatments we can explore the secretion of different peptides and proteins derived from myotubes.

2.3.9 SDS and two-dimensional electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a method for separating complex mixtures of proteins. The proteins are separated in two steps, first by their charge and then by their mass. After separation and staining, the spots are excised from the gel and digested using trypsin and the peptide fragments are characterized by MS. Proteins (17 μ l) were separated according to their molecular weight in SDS-PAGE using NuPAGE 4-12 % Bis-Tris gels (Invitrogen). In two-dimensional electrophoresis the proteins were first separated by isoelectric focusing using Immobiline DryStrip pH 3-10, 7 cm (GE Healthcare) which was rehydrated in 7 M urea, 2 M thiourea, 4 % CHAPS, 0.2% Pharmalytes and 1.5 mg/ml DDT. The samples (10 μ l) were added rehydration buffer (115 μ l) and applied along the whole strip and rehydrated over night. The focusing was run with a Bio-Rad Protein IEF Cell (Bio-Rad). After the focusing the strips were equilibrated in LDS sample buffer (Invitrogen) containing reducing and alkylating solutions and the proteins were applied to SDS-PAGE as described above. Proteins were visualized by staining either with Coomassie or silver. Magic Mark protein standard (Invitrogen) was used to estimate molecular weights. The proteins from the gel were further characterized by Matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF).

2.3.10 MALDI-TOF MS

After isolation of proteins by gel electrophoresis, the unknown proteins can be identified by the use of MALDI-TOF MS. First the proteins are being digested with an enzyme and then the masses of the proteolytic peptides are being matched with a database. The biomolecules are ionized and then they are being accelerated in an electric field and go into a flight tube. In the tube the molecules are separated according to their mass to charge ratio and they come to the detector at different times, so that every molecule gives a special signal.

2.3.11 Experiment 1 – Lactate dehydrogenase (LDH) activity in media from human myotubes at different stages of differentiation after incubation in serum-free medium for 6 or 18 hours

Human satellite cells were seeded and differentiated for 0, 2 and 5 days in three 12 well plates as described above. On the day of the experiment the cells were being washed twice by adding and removing 1 mL preheated DMEM. The cells were added 450 uL DMEM and incubated for 6 or 18 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. 400 uL supernatant was being frozen for later experiments. The wells were washed by adding and removing 1 mL PBS. The myotubes were lysed as described above. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above.

2.3.12 Experiment 2a – The effect of increasing the number of washing procedures on removal of radioactively labeled BSA from cultured human myotubes

Human satellite cells were seeded and differentiated for 4 days in three 12 well plates as described above. The wells were added 1 mL M3 with 0.01 uCi/mL ¹⁴C-BSA and incubated for 24 hours in 37° C in 5% CO₂. On the day of the experiment the radiolabelled media were transferred to vials, one per well. The cells were being washed 1-12 times by adding and removing 1 mL preheated DMEM that was collected and transferred to vials, one per well. The wells were added 1 mL serum-free medium and incubated for 24 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The cells were lysed as described above. The radioactivity in the media and the lysates were measured using Wallac Microbeta Trilux. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above. Proteins were measured as described above.

2.3.13 Experiment 2b – The effect of increasing the number of washing procedures on removal of radioactively labeled BSA from cultured human myotubes

Human satellite cells were seeded and differentiated for 4 days in a 12 well plate as described above. 3 wells were used in the experiment. The wells were added 1 mL M3 with 0.086 uCi/ml (190000dpm/ml) ¹⁴C-BSA and incubated for 24 hours in 37° C in 5% CO₂. On the day of the experiment the radiolabelled media were transferred to vials, one pr well. The wells were being washed 9 times by adding and removing 1 mL preheated DMEM that was collected and transferred to vials, one pr well. The wells were added 1 mL serum-free medium and incubated for 24 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The cells were lysed as described above. The radioactivity in the media and the lysates were measured using Wallac Microbeta Trilux. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above. Proteins were measured as described above.

2.3.14 Experiment 3 – The effect of varying the number of repeated washing procedures on LDH activity in cultures of human myotubes

Human satellite cells were seeded and differentiated for 4 days in a 12 well plate as described above. 9 of the wells were used. On the day of the experiment the cells were being washed 3, 6 or 9 times by adding and removing 1 mL preheated DMEM. The wells were added 500 uL DMEM and incubated for 24 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The cells were lysed as described above. Proteins were measured on the lysates as described above. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above.

2.3.15 Experiment 4 - Myostatin concentration in media from cultured human myotubes at different stages of differentiation after incubation in serum-free medium for 6 or 18 hours

Myostatin was measured in frozen samples from experiment 2. Myostatin concentrations were measured using Human Myostatin ELISA as described above.

2.3.16 Experiment 5 - IL-6 concentration in media from human myotubes at different stages of differentiation after incubation in serum-free medium for 6 or 18 hours

IL-6 was measured in frozen samples from experiment 2. IL-6 concentrations were measured using ELISA as described above.

2.3.17 Experiment 6 - IL-6 concentration in media from human myotubes after incubation in medium with and without the inhibitor Brefeldin A

Human satellite cells were seeded and differentiated for 2 or 5 days in four 12 well plates as described above. On the day of the experiment the cells were being washed six times by adding and removing 1 mL preheated DMEM. The wells were added 400 uL DMEM or DMEM with Brefeldin A (5 ug/ml) and incubated for 0, 1, 2, 3, 4 or 6 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The myotubes were lysed as described above. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above. Proteins in the lysate was measured as described above. IL-6 in the supernatant was measured as described above.

2.3.18 Experiment 7 - IL-6 concentration in media from human myotubes after incubation in medium with increasing concentrations of Brefeldin A

Human satellite cells were seeded and differentiated for 5 days in two 12 well plates as described above. On the day of the experiment the cells were being washed six times by adding and removing 1 mL preheated DMEM. The wells were added 400 uL DMEM with 0, 1, 2, 3, 4, or 5 ug/ml Brefeldin A and incubated for 6 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The myotubes in one of the plates were lysed as described above. The myotubes in the second plate were being washed twice by adding and removing 1 mL preheated DMEM. The myotubes in the second plate were added 0,4 mL DMEM and incubated for 3 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The myotubes in the second the plate were lysed as described above. LDH in the supernatants was measured and related to the LDH measured in the cell lysis as described above. Proteins in the lysates was measured as described above. IL-6 in the supernatants was measured as described above.

2.3.19 Experiment 8 - IL-6 concentration in media from human myotubes after incubation in serum-free medium for different periods of time

Human satellite cells were seeded and differentiated for 5 days in two 12 well plates as described above. On the day of the experiment the cells were being washed six times by adding and removing 1 mL preheated DMEM. The wells were added 400 uL DMEM and incubated for 0, 1, 3, 6, 9, 24, 48 or 72 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The myotubes were lysed as described above. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above. Proteins in the lysate was measured as described above. IL-6 in the supernatant was measured as described above.

2.3.20 Experiment 9 - Cytokine secretion in media from human myotubes after incubation in serum-free medium with or without Brefeldin A for 6 hours

Human satellite cells from donor 29 were seeded and differentiated for 5 days in two 12 well plates as described above. Human satellite cells from donor 29, 30 and 37 were seeded out in two 75 cm² flasks each, as described above. On the day of the experiment the cells were being washed six times by adding and removing preheated DMEM, 1 mL in each well of the plates and 20 mL for the flasks. The cells were added DMEM or DMEM with Brefeldin A (1ug/ml), 0.4 mL in each well of the plates and 20 mL in each flask and incubated for 6 hours. The media was removed and centrifuged for 10 minutes by 4000 rpm. 3 and 3 wells were pooled (donor 29). Cytokines in the conditioned media from the flasks (donor 29, 30 and 37) were measured by Luminex multiplexing technology as described above. Samples from the plates (donor 29) and from the flasks (donor 30 and 37) were frozen at 20 ° C and sent to Hormonlaboratoriet at Aker University Hospital for proteomic analysis.

2.3.21 Experiment 10 - Proteomic analysis on media from human myotubes after incubation in serum-free medium with or without Brefeldin A for 6 hours

At Aker University Hospital: Centrifugation through membranes with a cut-off of 10 kDa (Nanosep 10K OMEGA, Pall Corporation) was used to concentrate the proteins. The samples were concentrated from 2 mL to 60 ul, 100 ul, 50 uL and 100 uL for the samples from donor 29 with BFA, donor 29 without BFA, donor 37 with BFA and donor 37 without BFA, respectively. SDS-PAGE and 2-dimensional electrophoresis was performed as described above.

2.3.22 Experiment 11 - Proteomic analysis on media from human myotubes after preincubation in medium with high and low glucose and with and without Brefeldin A

Human satellite cells were seeded and differentiated for 3 days in a 12 well plate as described above. The wells were added 400 uL M3 or M3 with 20 mM glucose and preincubated for 4 days in 37° C in 5% CO₂, media changed after 2 days. On the day of the experiment the cells were being washed six times by adding and removing 1 mL preheated DMEM. The wells were added 400 uL DMEM or DMEM with Brefeldin A (1 ug/ml) and incubated for 6 hours in 37° C in 5% CO₂. The media were centrifuged for 40 minutes by 100 000 rpm in 10 ° C. The supernatants were frozen in 20 ° C and sent to Hormonlaboratoriet at Aker University Hospital for proteomic analysis. At Aker University Hospital the samples were concentrated by centrifugation through a membrane with a cut-off of 10 kDa. 10 uL from each sample was added to the gel SDS-PAGE performed as described above. Western blot was performed with IL-6 antibody and an antibody towards a specific sugarmodification. 1 mL sample was concentrated to 80 ul, 10 uL was mixed with SDS-applying buffer and 80 % applied to the gel. Western blot was performed with anti-albumin. 2-dimensional gel electrophoresis with silverstain was performed.

2.3.23 Experiment 12 - IL-6 concentration in media from human myotubes after incubation in medium with different fatty acids bound to BSA in different concentrations for 6 hours

Human satellite cells were seeded and differentiated for 5 days in two 12 well plates as described above. On the day of the experiment the cells were being washed six times by adding and removing 1 mL preheated DMEM. The wells were added 400 uL DMEM with either palmitate, linoleate or eicosapentaenate in the concentrations 0, 50, 100, 175, 250 or 500 uM, or BSA in concentrations 0, 20,40, 70, 100 or 200 uM and incubated for 6 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The myotubes were lysed as described above.

LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above. Proteins in the lysate were measured as described above. IL-6 in the supernatant was measured as described above.

2.3.24 Experiment 13 - IL-6 concentration in media from cultured human myotubes after incubation in medium with different fatty acids dissolved in ethanol for 6 hours

Human satellite cells were seeded and differentiated for 5 days in a 12 well plate as described above. On the day of the experiment the cells were being washed six times by adding and removing 1 mL preheated DMEM. The wells were added 400 uL DMEM with either palmitate, linoleate or eicosapentaenate solved in 0.5 % ethanol in the concentrations 1, 5 or 50 and incubated for 6 hours in 37° C in 5% CO₂. The media was removed and centrifuged for 5 minutes by 3000 rpm. The myotubes were lysed as described above. LDH in the supernatant was measured and related to the LDH measured in the cell lysis as described above. Proteins in the lysate were measured as described above. IL-6 in the supernatant was measured as described above.

3. Results

3.1 Lactate dehydrogenase (LDH) activity in media from human myotubes at different stages of differentiation after incubation in serum-free medium for 6 or 18 hours

To find out if the myotubes tolerated serum-free conditions over time we let them incubate in serum free medium for 6 and 18 hours (see **Materials and Methods, Experiment 1**). The enzymatical LDH activity was measured in the conditioned media as an indicator of plasma membrane integrity/cell survival and related to LDH activity measured in the lysate (100% cell death). The cells were tested at different stages of differentiation.

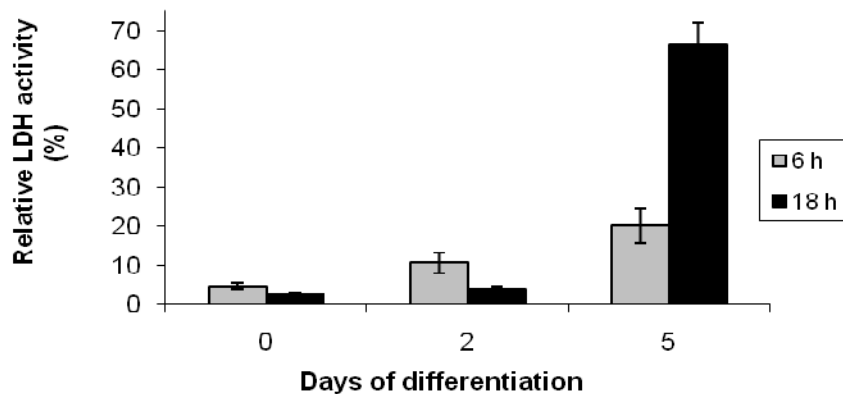


Figure 1. LDH activity in media from myotubes incubated under serum-free conditions.

Undifferentiated myoblasts and myotubes differentiated 2 or 5 days were incubated in serum-free medium for 6 or 18 hours. The bars show mean relative LDH activity \pm SEM (n=5) measured in the conditioned media.

After incubation for 6 and 18 hours the undifferentiated and 2-days differentiated cells showed a relative LDH-activity that was less than 10 % (Fig. 1). For the 5-days

differentiated cells the relative LDH-activity was 20 % after 6 hours incubation and 66 % after 18 hours incubation (Fig. 1). For these cells the relative LDH-activity was thus 3 times higher after 18 hours compared to 6 hours. We observed that the LDH activity in the media increased with increasing myotube differentiation status for cells incubated in serum free medium, both after 6 and 18 hours (Fig. 1). Myoblasts and myotubes that had differentiated 2 days showed generally less LDH activity than the myotubes that had differentiated 5 days. There was more LDH activity in the media from 5-days differentiated cells that had incubated for 18 hours than for 6 hours. Assuming that serum-free medium would represent stress for the cells we expected an increase in LDH activity with increasing time in serum-free medium from 6 to 18 hours independent of differentiation status. On background of these results we chose to incubate the cells for 6 hours.

3.2 The effect of increasing the number of washing procedures on removal of radioactively labeled BSA from cultured human myotubes

To investigate how many times we needed to wash the cells to remove an adequate amount of proteins from the growth-medium, ¹⁴C-BSA was used as a marker for macromolecules (see **Materials and Methods, Experiment 2a**). We made the assumption that the amount of ¹⁴C-BSA removed can be compared with the amount of the total of proteins removed. Hence, a known concentration of ¹⁴C-BSA, which was quantified by scintillation counting, was added to the medium and ¹⁴C-BSA removal was monitored. The myotubes were washed by adding and removing serum-free medium to the culturing dish repeatedly. After removal, ¹⁴C-BSA was estimated by scintillation counting in the wash solutions.

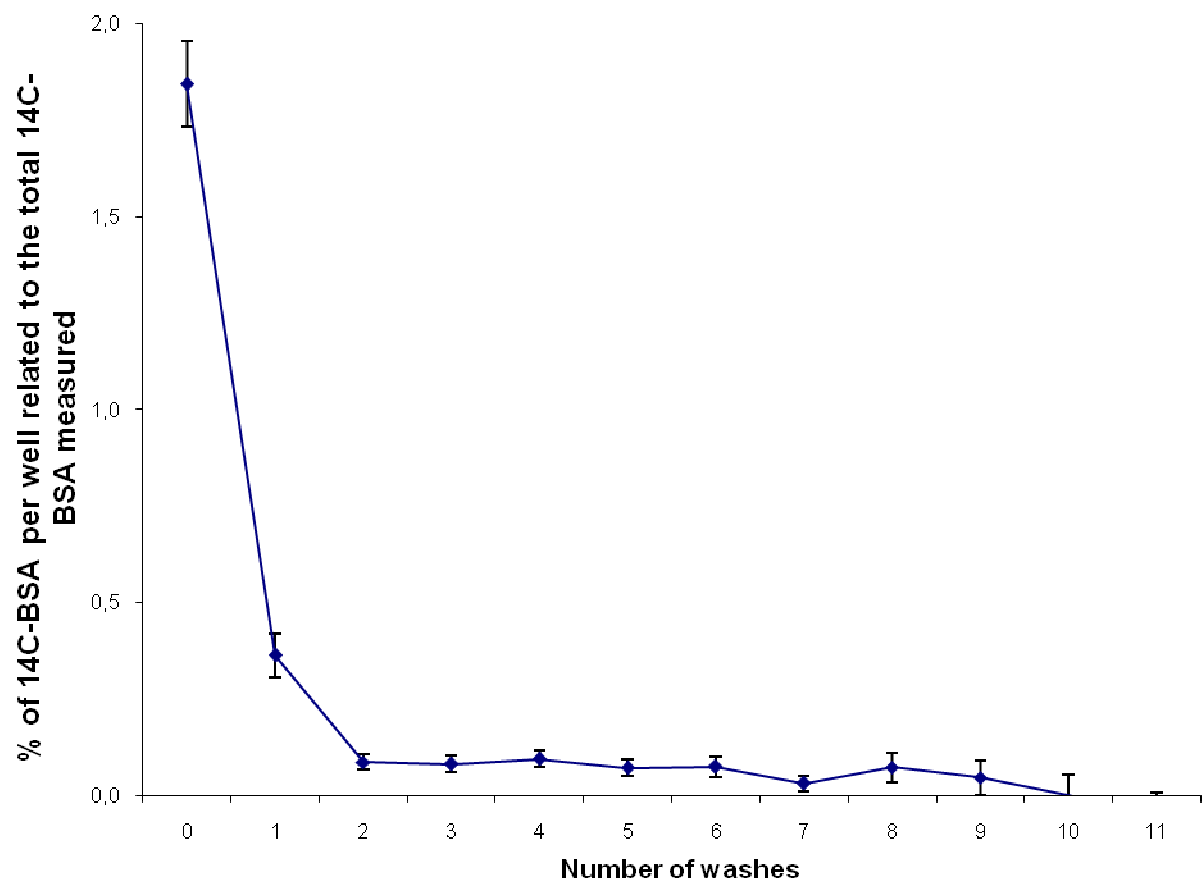


Figure 2. Level of radioactivity in rinsings of cultured human myotubes incubated with radiolabeled BSA. After 4 days of differentiation human myotubes were incubated with ¹⁴C-BSA and then washed 1-12 times. The diagram shows radioactivity, as a measure of ¹⁴C-BSA, in washing solutions 1-12 relative to the total in all washing solutions. The experiment was performed in triplicates and each point represents mean ± SEM.

Amount of radioactivity decreases from 1st to 2nd (2) washing medium (Fig. 2). After the 2nd washing the media contain less than 0.1 % radioactive BSA of what was initially added (Fig. 2). We conclude that there was too little radioactivity added to compare the small differences between the washes following the 2nd washing.

3.3 The effect of increasing the number of washing procedures on removal of radioactively labeled BSA from cultured human myotubes

To further investigate the removal of BSA by washing we performed the same experiment as above, but this time with a higher total amount of ^{14}C -BSA (see **Materials and Methods, Experiment 2b**).

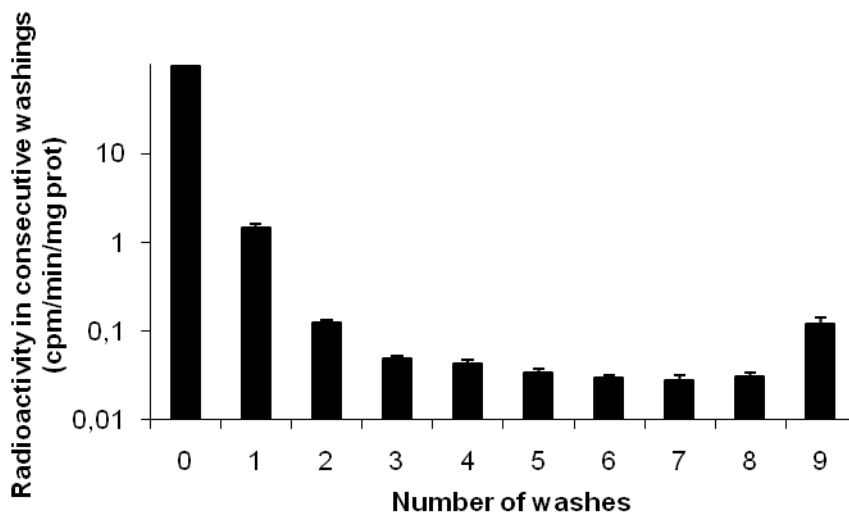


Figure 3. Level of radioactivity in rinsings of cultured human myotubes incubated with radiolabeled BSA. After 4 days of differentiation human myotubes were incubated with ^{14}C -BSA and then washed 9 times. The diagram shows radioactivity, as a measure of ^{14}C -BSA, in washing solutions 1-9 relative to the total in all washing solutions. The experiment was performed in triplicates and each point represents mean \pm SEM.

The 1st washing contained 1.5 % of total radioactive BSA (Fig. 3). The 2nd washing contained 0.1 % of total radioactive BSA (Fig. 3). Washing solution 3-9 contained less than 0.05 % of total radioactive BSA (Fig. 3). There was a gradual decrease in radioactive BSA in the washing solutions of the first 6 washing procedures (Fig. 2.2).

We conclude that it is necessary to wash the cells 6 times to remove an adequate amount of serum.

3.4 The effect of varying the number of repeated washing procedures on LDH activity in cultures of human myotubes

To investigate how many times we could wash the wells without harming the cells we used LDH-activity as a measurement of cell integrity (see **Materials and Methods, Experiment 3**). We suspected that the washings flushed away the cells and measured proteins in the lysate as an indication of how many cells there are left after the washings. We washed the myotubes by adding and removing serum-free medium (DMEM).

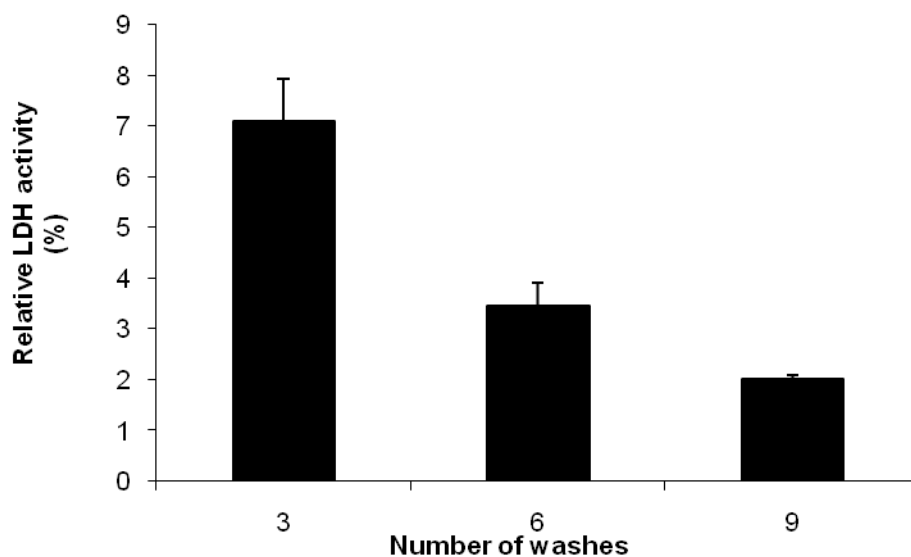


Figure 4. The effect of the number of repeated washing procedures on LDH activity in cultured human myotubes. Human myotubes were washed by adding and removing washing solution 3, 6 or 9 times and incubated in medium (DMEM) for 24 hours. The conditioned media were collected and the figure shows the LDH activity measured in the media related to the total LDH activity measured in lysed cells (relative LDH activity). The experiment was done once in triplicates and each point represents mean \pm SEM.

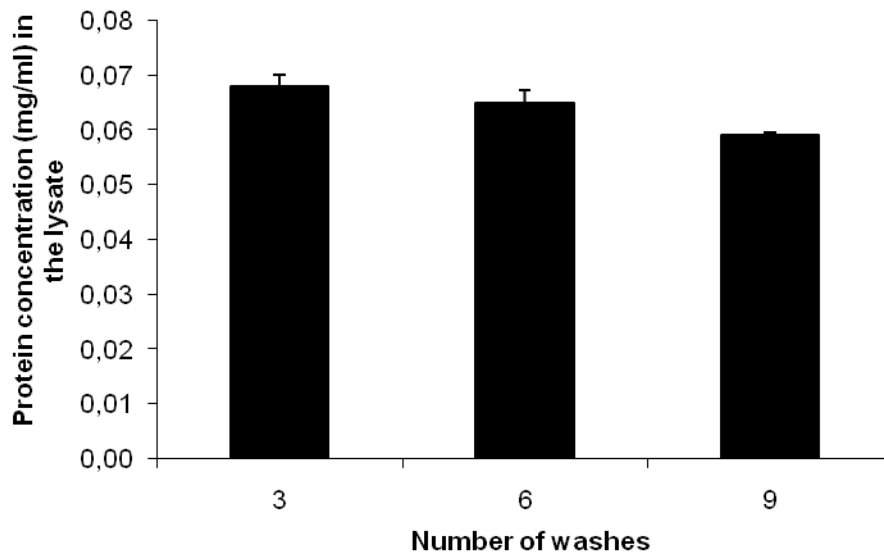


Figure 5. Protein concentration in the lysate of the myotubes after exposure of different number of washings. The myotubes were washed 3, 6 and 9 times with washing solution and incubated for 24 hours. The bars represent protein concentration measured in total cell lysates (mean \pm SEM) of one experiment performed in triplicates.

After 3, 6 and 9 washes the relative LDH-activity in the media is 6.6, 3.4 and 2.0 %, respectively. Increasing the number of washes from 3 to 9 reduced the LDH-activity significantly ($P = 0.03$). The protein concentrations measured in the lysates after exposure of 3, 6 and 9 washes were 68.0, 64.9 og 59.1 $\mu\text{g/mL}$, respectively. Lysates from myotubes exposed to 9 washes had statistically less protein content than lysates from myotubes exposed to 3 washes ($P = 0.037$).

The LDH activity decreased significantly from 3 to 9 washings as opposed to our expectations. We consider damage of the cells relatively small and the need to remove serum from the media necessary to a degree that the necessity to wash the cells many times exceeds the need to protect the cells. We suggest that the cells should be washed 6 times.

3.5 Myostatin concentration in media from cultured human myotubes at different stages of differentiation after incubation in serum-free medium for 6 or 18 hours

Because we wanted to explore whether skeletal muscle cells released proteins we searched for myostatin, which is a protein known to be produced in skeletal muscle (see **Materials and Methods, Experiment 4**). Frozen supernatants from experiment 1 were thawed and myostatin concentration was determined by ELISA.

Probably the myostatin concentrations in the conditioned media from the myotubes were below the detection limit for our ELISA assay.

3.6 IL-6 concentration in media from human myotubes at different stages of differentiation after incubation in serum-free medium for 6 or 18 hours

We investigated whether different differentiation status affected the release of IL-6 and whether the release of IL-6 increased with increasing time in serum free medium (see **Materials and Methods, Experiment 5**). Frozen supernatants from experiment 1 were thawed and IL-6 concentration was determined by ELISA.

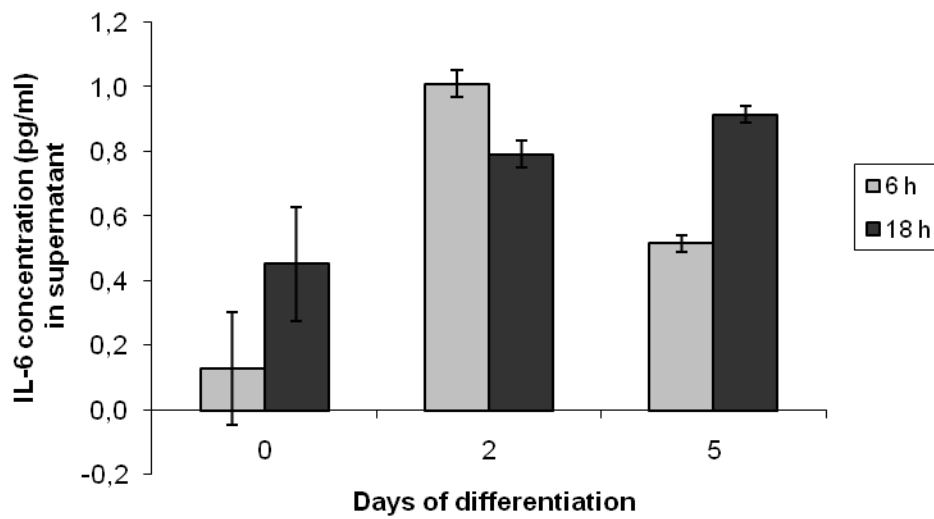


Figure 6. IL-6 concentration in the media from myotubes incubated without FCS. IL-6 concentration was measured in the media from muscle cells differentiated 0, 2 and 5 days and incubated in medium without serum for 6 or 18 hours. The bars show mean concentration of IL-6 and standard deviation for duplicates from each well (n = 2).

The concentrations of IL-6 in the media of undifferentiated myoblasts were 0.13 pg/mL and 0.45 pg/mL after 6 and 18 hours of incubation, respectively.

Undifferentiated and 5-days differentiated myotubes had more than 3 and almost 2 times, respectively, higher concentration of IL-6 in the media from 18 hours of incubation compared to 6 hours of incubation. The myotubes that had been differentiated for two days had 1.0 pg/mL IL-6 in the media after 6 hours of incubation. This was the highest in this experiment.

3.7 IL-6 concentration in media from human myotubes after incubation in medium with and without the inhibitor Brefeldin A

To investigate whether IL-6 was released as a result of secretion or leakage, we measured IL-6 concentration in media from myotubes following incubation for 6 hours in medium containing the inhibitor Brefeldin A (see **Materials and Methods, Experiment 6**).

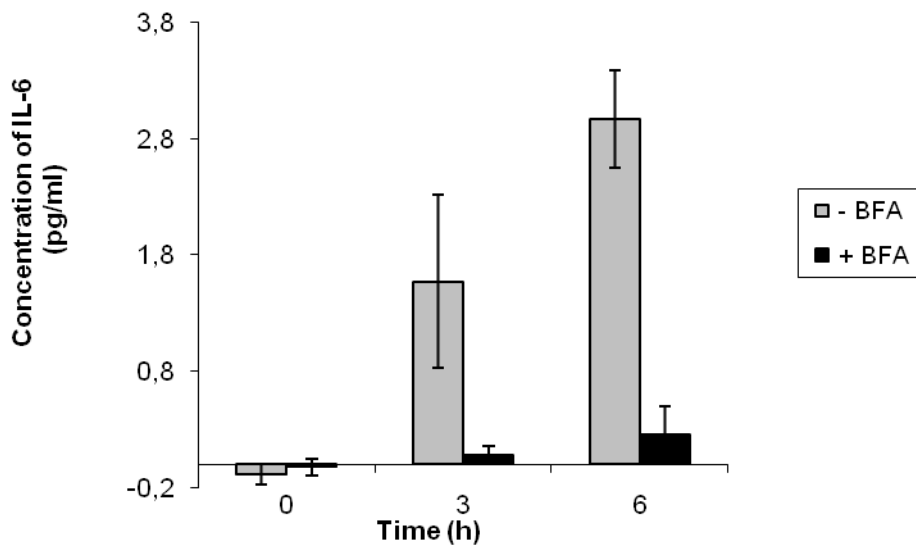


Figure 7. IL-6 concentration in the supernatants in media from myotubes incubated with or without BFA (5 ug/mL). The bars show means and SEM from two wells in duplicate (n = 2).

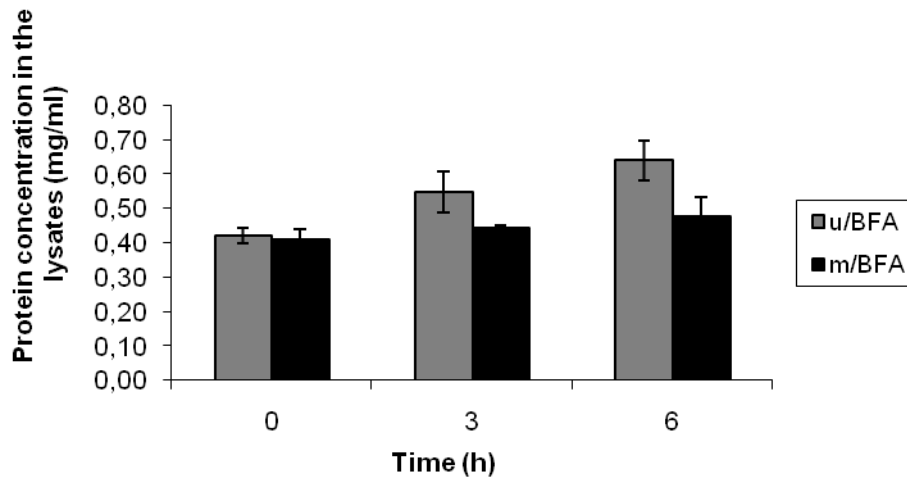


Figure 8. Protein concentration in the lysates of myotubes after incubation with medium with or without BFA. The myotubes were incubated with 400 μ L DMEM or DMEM with BFA (5 μ g/mL) for 0, 3 or 6 hours. The bars represent protein concentration measured in total cell lysates (mean \pm SEM) of one experiment performed in triplicates.

The IL-6 concentration in the media from cells incubated without BFA was higher than in the media from cells incubated with BFA. The IL-6 concentration in the media from all cells increased with increasing time.

The lower concentration of IL-6 in the supernatants from myotubes incubated in media with BFA compared to the incubation in media without BFA suggests inhibition of active secretion by BFA. IL-6 was thus used as a marker for protein secretion, because we assumed that if BFA inhibited secretion of IL-6 it would most probably inhibit secretion of other proteins as well.

3.8 IL-6 concentration in media from human myotubes after incubation in medium with increasing concentrations of Brefeldin A

To explore how different concentrations of BFA would influence the concentration of IL-6 in the media we incubated the myotubes for 6 hours in media with increasing concentrations of BFA (see **Materials and Methods, Experiment 7**).

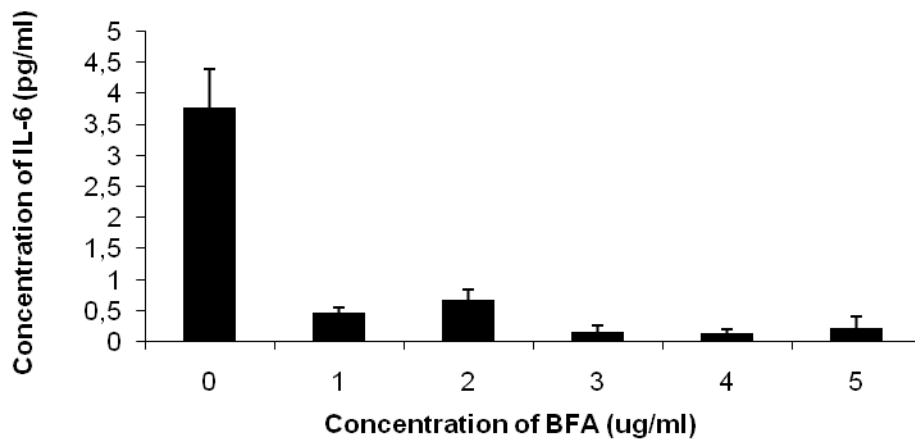


Figure 9. IL-6 in media from myotubes incubated with increasing concentrations of BFA. The five days differentiated myotubes were incubated in medium (DMEM) with 0, 1, 2, 3, 4, or 5 ug/mL BFA for 6 hours. IL-6 concentrations were measured in the supernatants. The bars show mean concentration \pm SEM (n=4) of IL-6 in the media from two wells in duplicate.

The IL-6 concentration was more than 3.5 pg/mL for the controls. The IL-6 concentration is approximately 0.5 pg/mL for all the cells incubated in BFA, independent of BFA concentration. The lowest concentration of BFA (1 ug/mL) inhibited secretion of IL-6 by 87.5 %.

There was almost complete inhibition of secretion of IL-6 even with only 1 ug/ml BFA in the medium. Because even the smallest concentration of BFA seemed to give the inhibition that we needed we chose to use it.

3.9 IL-6 concentration in media from human myotubes after incubation in serum-free medium for different periods of time

To optimize the chance of finding an appropriate amount of protein in the media we measured how increasing time would influence the release of IL-6 in the media (see **Materials and Methods, Experiment 8**). The myotubes were incubated in serum-free medium for different periods of time varying from 0 to 72 hours.

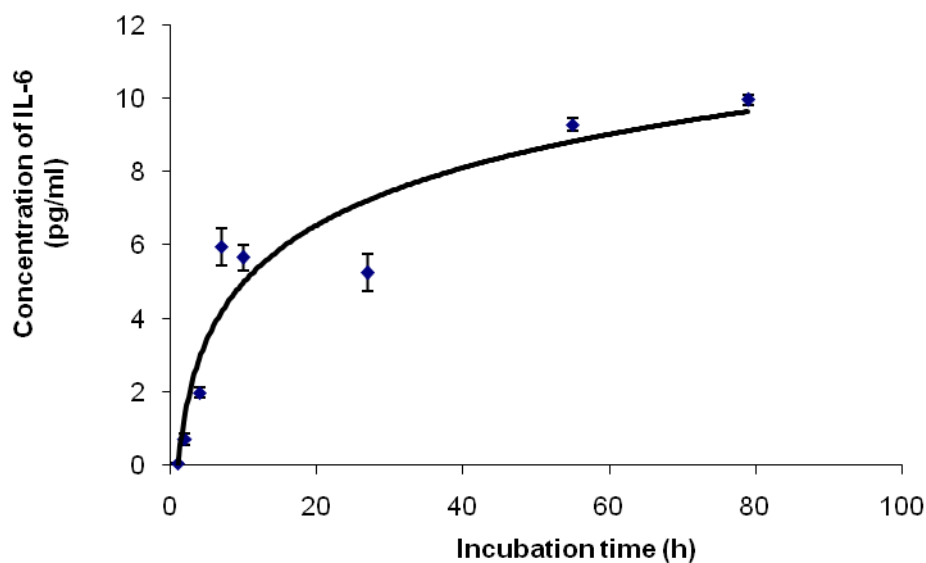


Figure 10. IL-6 concentration in media from myotubes incubated up to 72 hours in DMEM.

The spots show mean \pm SEM (n=6) concentrations of IL-6 in the media after the different incubation periods for the duplicates for three wells.

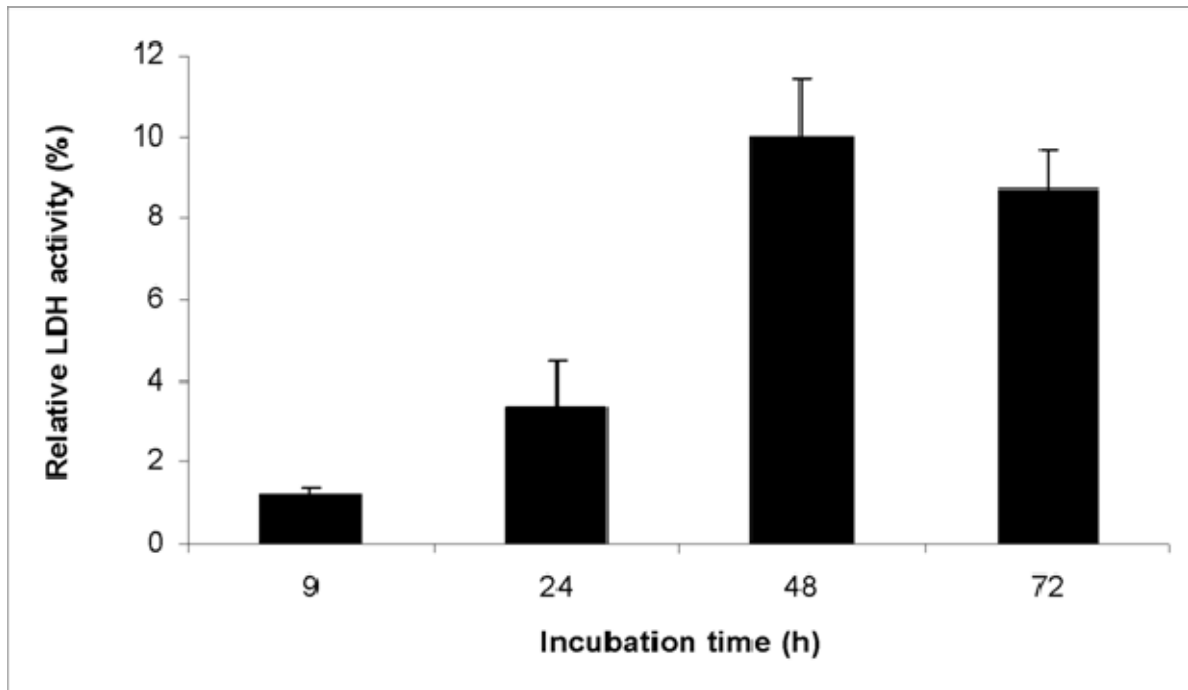


Figure 11. LDH activity in supernatants from myotubes incubated up to 72 hours in DMEM.

The five days differentiated human myotubes were incubated in medium (DMEM) for 9, 24, 48 or 72 hours. The conditioned media were collected and the diagram shows the LDH activity measured in the media related to the total LDH activity measured in lysed cells (relative LDH activity). The experiment was done once with three wells in duplicates and each point represents mean \pm SEM.

There was an immediate release of IL-6. The curve was almost linear the first 6 hours. There was a steep rise that flattened a little after 10 hours, but continued to rise although not so fast. The IL-6 concentration in the medium after 6 hours was 59.8 % of the concentration after 72 hours. The relative LDH-activity in the media from cells that had incubated in serum-free media for 9, 24, 48 and 72 hours were 1.5 %, 4 %, 10 % and 8 %, respectively. The LDH-activity doubled from 24 hours incubation to 48 hours incubation. Since we chose to measure IL-6 in our media for validation purposes we do not know whether other myokines may need longer or shorter time to be released and might need longer or shorter incubation time. The increasing LDH activity with increasing time indicates that the conditions for the cells were not optimal.

3.10 Cytokine secretion in media from human myotubes after incubation in serum-free medium with or without Brefeldin A for 6 hours

To search for novel myokines we measured the following cytokines with multiplexing technology: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IFN γ , GM-CSF, TNF- α (see **Materials and Methods, Experiment 9**). We seeded the cells in flasks to get more cells and possibly a larger amount of protein. The myotubes were incubated in medium with or without BFA for 6 hours before we analyzed the media. The concentrations of the suspected, secreted cytokines were measured and total amount of cytokines in the media was estimated. The total amount of cytokines from well or flask was related to total amount of protein in cell lysate from the same well or flask. The amount of protein in the cell lysate indicated the amount of cells left after washing and incubation. By relating the amount of cytokines to the amount of protein we adjusted for eventual differences in myotube density in the different wells/flasks. The ratio media volume (mL) to growth area (cm²) was 0.11 mL/cm² for the wells and 0.07 mL/cm² for the flasks. The wells had 1.6 times volume per cm² as the flasks.

Table 1. The amount of cytokines in the media related to the amount of protein in the cell lysate from the well or flask (pg cytokine/mg protein)

BFA	Donor 29		Donor 30		Donor 37	
	-	+	-	+	-	+
IL-6	21.36± 0.96	1.29±0.08***	2.65±0.25	0.41#±0.14**	200.56±12.18	2.87±0.11***
IL-8	100.22±2.60	2.64±0.30***	4.31±.027	0.36±0.05***	199.13±4.23	2.15±0.14***
IL-7	9.95±1.16	4.19±1.05*	6.73±0.47	6.39±0.84	5.72±0.05	5.05±0.25
IL-13	10.53±2.15	2.98±0.17*	2.46±0.55	1.93±0.35	1.74±0.00	1.44±0.02***
IL-10	0.15#±0.08	0.25#±0.10	0.21#±0.09	0.42#±0.17	0.47#±0.07	0.16#±0.05
GM-CSF	0.91#±0.13	0.63#±0.09	0.73#±0.13	0.42#±0.08	0.52#±0.05	0.33#±0.06
TNF α	ND	ND	ND	ND	ND	ND
IL-1 β	ND	ND	ND	ND	ND	ND
IFN γ	ND	ND	ND	ND	ND	ND
IL-12	ND	ND	ND	ND	ND	ND
IL-2	ND	ND	ND	ND	ND	ND
IL-4	ND	ND	ND	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND

The mean values \pm SEM are based on triplicate measurements (except for donor 37 without BFA which are based on duplicates), ND = not detectable, # extrapolated values, * < 0.05, ** < 0.01, *** < 0.001

IL-6, IL-8, IL-7 and IL-13 were detected. IL-6 and IL-8 had significant differences between the media with and the media without BFA. The medium from donor 29 had the highest concentration of IL-13 pr mg protein compared to the other donors. The mean IL-13 concentration in the medium without BFA from donor 29 was 10.5 pg/mL and had more than 3.5 times higher concentration than the mean IL-13 concentration in the medium with BFA.

The concentration of IL-6 in the supernatant was 80.6 pg/mL. This means that the concentration from 2 mL to 100 uL resulted in the concentration 1.6 pg IL-6/ul.

Media from hepatocytes and adipocytes were tested with the same multiplex analysis and media from myotubes were the only ones that showed IL-13 within the standard curve.

3.11 Proteomic analysis on media from human myotubes after incubation in serum-free medium with or without Brefeldin A for 6 hours

To get more proteins for the proteomic analysis we seeded the cells in flasks to get more cells and possibly a larger amount of protein (see **Materials and Methods, Experiment 10**). We sent the media to Aker for proteomic analysis.

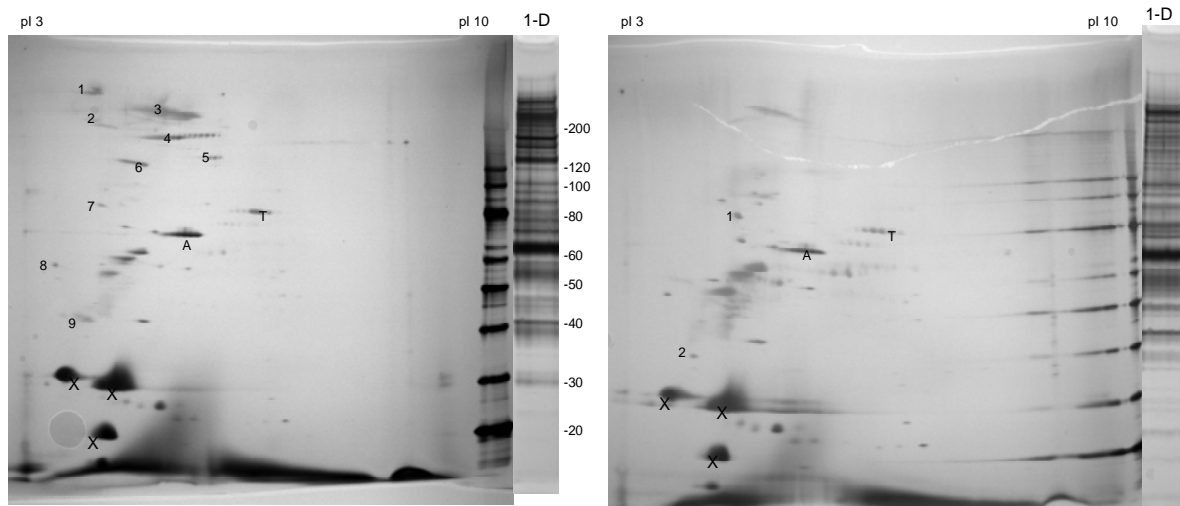


Figure 12. Two-dimensional electrophoresis of proteins from media of cultured human myotubes after incubation in serum-free medium with or without Brefeldin A for 6 hours.

Differentiated myotubes were incubated in media without BFA (left panel) and media with BFA (right panel). The proteins from the conditioned media were first separated by isoelectric focusing and then by size.

Comparing the pictures we can see 9 spots more when the myotubes were incubated without BFA. The media from myotubes incubated without BFA contained high concentrations of protein compared to the media derived from myotubes incubated with BFA. The high protein concentrations lead to clotting of the filters when the concentration of the proteins took place. This resulted in a poorer concentration and a higher final volume with the same cut-off value for media derived from myotubes incubated without BFA. The proteins from media from myotubes from donor 29 and 37 incubated with BFA were concentrated from 2 mL to 60 μ L and 50 μ L, respectively. For both donors incubated without BFA 2 mL media ended up with 100 μ L. Because of no adjustment before the electrophoresis was performed, the gel pictures showed weaker differences between media from myotubes incubated with and without BFA than there really were.

The gel picture show more spots for the media from myotubes incubated without BFA. This supports our earlier conclusion; that BFA inhibits secretion. 10 μ L and estimated 16,1 pg IL-6 applied on the gel was enough to visually detect proteins with 2-D PAGE, but not enough to further identify secretion products with MALDI-TOF.

3.12 Proteomic analysis on media from human myotubes after preincubation in medium with high and low glucose and incubation with and without Brefeldin A

To investigate whether nutrients would affect the release of proteins we incubated the myotubes with different glucose concentrations (**see Materials and Methods, Experiment 11**). The myotubes were preincubated in either 5 mM or 20 mM glucose for four days. They were then incubated for 6 hours with and without BFA. The

supernatants were frozen and sent to Aker University Hospital for proteomic analysis.

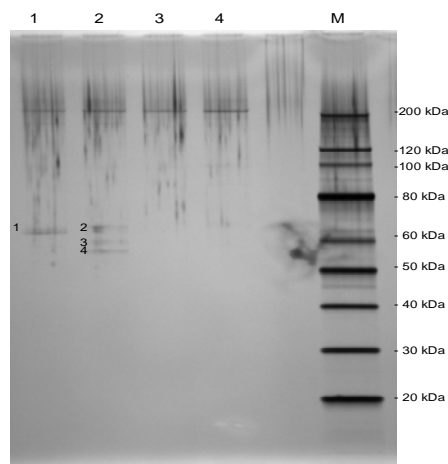


Figure 13. Silverstain of proteins derived from media of human myotubes after incubation in medium with high and low glucose and with and without Brefeldin A. Det må sies at det er 1D gelelektroforese. Row 1 and 2 show molecules from myotubes incubated without BFA and row 3 and 4 show molecules from myotubes incubated in BFA. Molecules in row 1 and 3 derive from control (5 mM glucose) and row 2 and 4 derive from myotubes incubated in high glucose (20 mM).

SDS-PAGE. Bands 1 (row 1) and band 2, 3 and 4 (row 2) all in the range around 60 kDa, do not exist in the samples with the inhibitor BFA (row 3 and 4) and are probably secretion products. Compared to the control (row 1) we can see two more bands from myotubes incubated with high glucose (row 2). Band 1 is albumin, band 2, 3 og 4 are probably sugar modified albumin (band 3 and 4 might be degrading products of modified albumin). Prealbumin exists theoretically in this secretome according to Bortoluzzi [19].

2-D and Western with albumin-antibody were done with the high glucose sample, to explore whether it was albumin being detected with silverstain. Western blotting with the albumin antibody provides a positive signal in this area, but not as strong as with intact albumin. Thus, it is still unclear what the two bands below albumin represent.

3.13 IL-6 concentration in media from human myotubes after incubation with different fatty acids bound to BSA in different concentrations for 6 hours

To further investigate whether nutrients would affect the release of proteins we incubated the myotubes for 6 hours with different concentrations of fatty acids bound to albumin (see **Materials and Methods, Experiment 12**). The fatty acids used in the experiment were palmitic acid (PA), linoleic acid (LA) and eicosapentaenoic acid (EPA) in the concentrations as follows: 50, 100, 175, 250 and 500 μM . PA stimulates the IL-6 secretion in the concentrations 50 μM and 100 μM . LA and EPA has a negative effect on the IL-6 secretion.

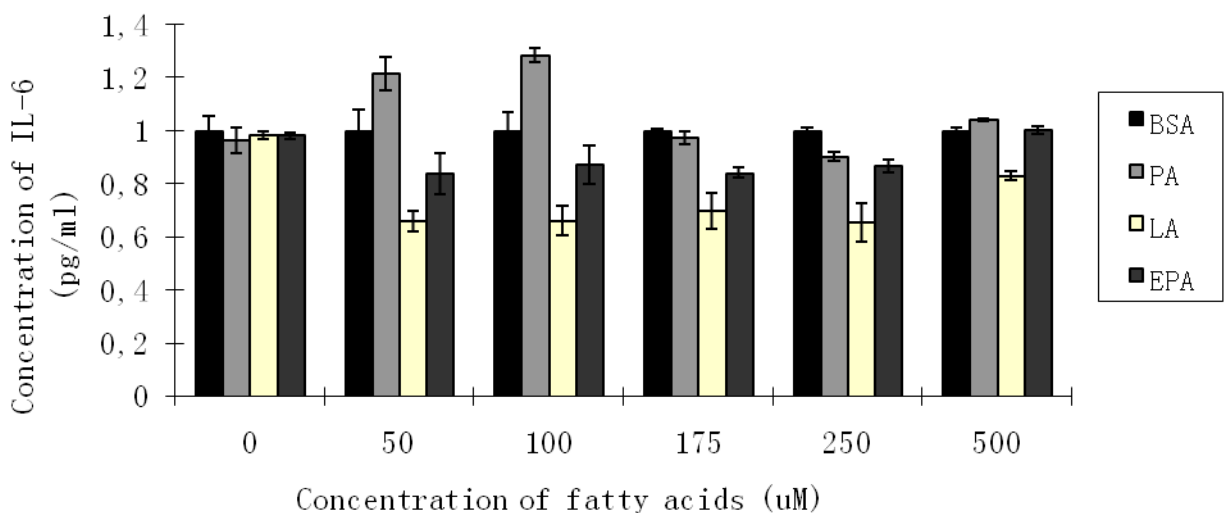


Figure 14. IL-6 in medium from myotubes incubated with increasing concentrations of different fatty acids. The five days differentiated myotubes were incubated in medium (DMEM) with PA, LA or EPA in 0, 50, 100, 175, 250 or 500 μM for 6 hours. IL-6 concentrations were measured in the supernatants. The bars show mean concentration \pm SEM for the concentrations of IL-6 in the media from two wells in duplicate.

3.14 IL-6 concentration in media from cultured human myotubes after incubation in medium with different fatty acids dissolved in ethanol for 6 hours

To investigate whether the variation of IL-6 release in the last experiment was due to BSA in the media or whether it was an effect of the different concentrations of the fatty acids we dissolved the fatty acids in ethanol (see **Materials and Methods, Experiment 13**). Ethanol provides an alternative way to get the fatty acids into the cells without albumin. We used the same fatty acids as above but in the following concentrations: 1, 5 and 50 μ M. PA has a negative effect on IL-6 secretion. LA and EPA stimulate the IL-6 secretion.

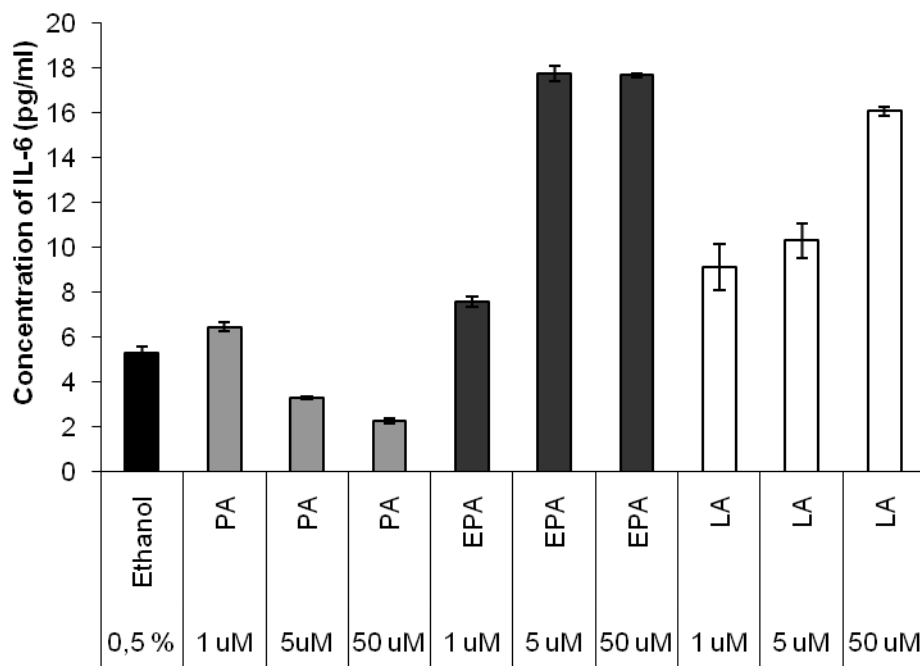


Figure 15. IL-6 in media from myotubes incubated with increasing concentrations of different fatty acids dissolved in ethanol. The five days differentiated myotubes were incubated in medium (DMEM) with PA, LA or EPA in concentrations 1, 5 or 50 μ M for 6 hours. IL-6 concentrations were measured in the supernatants. The bars show mean concentration \pm SEM for the concentrations of IL-6 in the media from two wells in duplicate.

4. Discussion

To study the *in vitro* secretion of myokines from human skeletal muscle cells, we had to use a model appropriate for our hypothesis. Examination of many different proteins and pathways in cultured muscle cells suggest, although some differences exist, that these cells contain much of the same proteins and signaling systems as mature muscle fibres [20]. Thus, this model of primary human skeletal muscle cells is a useful and physiological relevant model to use for the study of proteins secreted from human muscle tissue, although contractions are not present.

The medium routinely given cultured human myotubes contains abundant amounts of exogenous serum-derived proteins that had to be removed before analysis of myokine secretion could be performed. For the purpose of proteomics, we needed to generate conditioned media containing proteins derived from myotubes alone, without foetal calf serum (FCS) proteins (mainly bovine serum albumin (BSA)). Earlier experiments (personal communication, Dr. Vigdis Aas and colleagues at University of Oslo) indicated that it was important to remove as much protein from serum in the media as possible before the proteomics analysis. Thus, to find potential secretory proteins derived from myotubes in the medium, albumin and other proteins from the serum in the medium had to be removed before the samples could be analyzed by 2-dimensional (2D)-gel electrophoresis.

We had to incubate the myotubes in protein-free conditions to avoid contamination of proteins derived from FCS during proteomic analysis. Therefore we had to examine how the myotubes were influenced by serum free conditions. We wanted to maintain cell integrity and make sure we could reach detectable levels of secretory proteins in the conditioned medium. When assessing how long time the myotubes could be incubated without FCS, we explored the relative LDH activity (see below), and found that it was a 3 times higher relative LDH activity for 5 days differentiated myotubes incubated 18 hours compared to 6 hours. Thus, we chose to incubate the cells for 6 hours.

When changing from media with to media without FCS, the serum proteins needed to be properly removed by washing the cells. Extensive washing can be harmful to the cells, but it was also important to evaluate the washing procedure with respect to the removal of exogenous proteins. Therefore, the aim was to find the number of wash-cycles that sufficiently removed the albumin without significantly reducing cell viability.

Two experiments using ¹⁴C-labelled BSA were performed to estimate the amount of protein removed by the washes. The conclusion was that the more we washed the cells, the more proteins were removed. After 6 washes we did not detect any differences and we conclude that it is necessary to wash the cells 6 times to remove an adequate amount of serum.

In our search for new secretory proteins from myotubes it was important to monitor what condition the cells were in. In vitro culturing is closely associated with cell-death and damaged or dead myotubes might contaminate the conditioned media with intracellular proteins and thus masque the secreted proteins [21]. To explore how washing influenced plasma membrane integrity, we measured LDH activity in the media. LDH is an intracellular enzyme which may leak out of the cell when the membrane is damaged. The principle of measuring LDH activity of cellular culture supernatants as a measure of cytotoxicity have successfully been applied on different cell types. LDH release assays are an appropriate and possibly preferable means of measuring cellular cytotoxic reactions [22]. The LDH activity decreased significantly from 3 to 9 washings as opposed to our expectations. Because LDH activity was expected to rise with increasing number of washes we measured an additional variable to see if cells had been lost during the washing procedures. Protein content in cell lysates can be used as an indicator of the amount of cells. Therefore, we compared the amount of total cellular proteins in cells after different washing exposure. Our hypothesis was that the cells are being flushed away during washing. The decrease in protein concentration from 3 to 9 washings supports this theory. Another hypothesis is that some cells are more fragile, leak more LDH and are more easily washed away. If so the LDH activity will necessarily decrease together with

the protein concentration. A third hypothesis is that the washing flushed away fragile cells, and the cells that were left were robust and did not leak that much LDH. We suggest that the cells should be washed 6 times since the cells seemed to handle it well. In a previous study of myotubes, the cells were washed 4 times [23].

It was also necessary to investigate how many days the cells should be differentiated before experiments were performed. When mononuclear myoblasts fuse to make multinuclear myotubes the muscle specific gene expression changes [24]. To find out whether differentiation from myoblast to myotube has any effect on the myokine synthesis we let the cells differentiate from two to five days. We considered it likely that differentiated cells, as being more developed, have also developed their abilities to release myokines.

Before we began our search for novel myokines, we validated our muscle model system with regards to the secretion of known myokines. Myostatin is synthesized by skeletal muscle, circulates in the blood and functions as a negative regulator of muscle growth. There is increasing evidence that myoblasts and satellite cells are targets of myostatin signaling *in vivo*, although it is possible that also other cells can respond to myostatin. Overexpression of myostatin in mice have been shown to give a dramatic wasting syndrome (cachexia), characterized by loss of muscle and adipose tissue. This indicates that myostatin also has an effect on adipocytes [25]. Because of this, myostatin is regarded as a bona fide myokine and we wanted to explore this in our model system. We tried to measure myostatin but we could not trace any myostatin in the media from the myotubes with our methods. This tells us that our cells secrete myostatin in too small amounts to be detectable with our assay. Few commercial assays are available for measuring myostatin. Thus, instead of elaborating on this we chose to focus on a different myokine to validate our muscle model system. We chose to validate our cell model with respect to myokine secretion by measuring IL-6, a previously identified myokine. The aim was to examine if IL-6 could be found in media of our cultured myoblasts and myotubes. Therefore, we performed an experiment to explore whether we could trace IL-6 in the media from the myotubes at all. We detected IL-6 in the media from both myoblasts and

myotubes. The myotubes that had been differentiated for 2 days had 1.0 pg/mL IL-6 in the media after 6 hours of incubation. This was the highest result in this experiment.

We also wanted to explore whether the proteins released from the myotubes into the media were due to secretion per se or just a secondary effect of cell damage.

Brefeldin A (BFA) is a reversible inhibitor affecting intracellular protein transport [26]. It is a lactone antibiotic produced by fungal organisms, and BFA interferes specifically and reversibly with protein transport from endoplasmic reticulum to Golgi apparatus and leads to accumulation of proteins inside endoplasmic reticulum (ER) [27]. In mammalian cells BFA inhibits activation of a GTPase which is involved in formation of transport vesicles by recruiting coat proteins to intracellular membranes [28]. Thus, cells incubated with BFA might be used as a negative control to investigate whether release of proteins from myotubes was due to leakage or secretion. The lower concentration of IL-6 in the supernatants from myotubes incubated in media with BFA compared to the incubation in media without BFA suggests inhibition of active secretion by BFA. We started out giving the cells 5 ug/mL BFA, but assuming that it was damaging for the cells to be incubated in high BFA concentrations, we explored whether the inhibition was complete also with lower concentrations of BFA. Our experiment showed that there was almost complete inhibition of secretion of IL-6 with only 1 ug/mL BFA in the medium. Because the lowest concentration of BFA tested seemed to give the inhibition that we needed, we chose to use 1 ug/mL in later experiments.

Because the concentration of peptides and proteins in the media could be low and difficult to measure, we performed a time-course experiment of the IL-6 concentration in the media, to explore how long the increase was linear.

The IL-6 concentration in the media seemed to increase almost linearly up to 6 hours. The LDH-activity doubled from 24 hours incubation to 48 hours incubation. Other proteins might display different release patterns than IL-6, but as a trade-off between

myokine concentration and cell integrity we decided to incubate the myotubes for 6 hours, also when searching for novel myokines.

We then started our search for novel myokines using the model that we developed and validated. IL-6 is a known myokine [5]. Skeletal muscle cell cultures express several cytokines, such as IL-6, IL-8, interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) [29, 30]. Wanting to detect other known cytokines in the media from the myotubes, we tested 13 different cytokines by Luminex multiplexing technology. IL-6, IL-8, interleukin-7 (IL-7) and interleukin-13 (IL-13) were detectable with our assay. IL-8 is, as mentioned in the Introduction, classified as a potential myokine [4]. We observed that the concentration of IL-13 decreased after incubation in BFA, which indicates that the release might be due to a secretion.

Experiments have been performed searching for interleukin-1 α (IL-1 α), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-12 (IL-12) and IFN- γ [31]. Under all test conditions none were found from human myoblasts.

We sent samples to Hormonlaboratoriet at Aker University Hospital for proteomic analysis. Our ambition was to be able to “rediscover” IL-6 as a myokine.

Theoretically, based on quantification by ELISA, the amount of IL-6 applied on the gel was enough to visually detect proteins with 2-D PAGE and silver staining.

However, silver staining is not well compatible with further identifying secretion products with MALDI-TOF. We would need approximately 300 pg IL-6 protein to be able to characterize it further with MALDI-TOF. Therefore, we estimated that we would need 20 times more protein than in our experiments in order to “rediscover” IL-6.

Circulating IL-6, insulin and free fatty acid concentrations have been associated with impaired insulin action in obese and type 2 diabetic individuals. Skeletal muscle represents a target of impaired insulin action. Studies under several metabolically different conditions have shown that cultured myotubes are responsive to fatty acids as well as glucose [10]. We measured IL-6 as a representative myokine to see

whether free fatty acids (FFAs) and glucose could affect IL-6 expression in human myotubes.

First we performed one experiment where we let the myotubes incubate in medium containing different concentrations of glucose. We expected higher concentration of IL-6 in media from myotubes incubated with low glucose concentration than high, because previous work indicate that IL-6 is released from myotubes and that the release is enhanced by low glycogen stores [32]. We did not detect any IL-6 at all.

We performed one experiment where the myotubes were incubated in media containing fatty acids bound to BSA. Whereas palmitic acid (PA) seemed to stimulate the release of IL-6, we observed an inhibition of IL-6 release when the myotubes had been incubated with linoleic acid (LA) and eicosapentaenic acid (EPA) in different concentrations. Earlier experiments showed a significant increase in IL-6 production after incubation with PA. Unsaturated FFA did not activate IL-6 expression, whereas oleate reduced the secretion [9, 10].

Experiments have also shown a small, but significant effect of free fatty acid-free BSA alone on IL-6 gene expression [10].

Because of this finding we performed one experiment where the fatty acids were solved in ethanol instead of bound to BSA. We then observed an inhibition of IL-6 release when the myotubes were incubated in PA, and an increase of IL-6 concentration when the myotubes were incubated in LA and EPA.

The solving of the fatty acids in ethanol though, is a situation that is not physiological relevant.

5. Conclusion

We conclude from our studies that in the search for novel myokines, human myotubes in culture should be washed 6 times prior to incubation to remove proteins from the serum added to the media. The incubation period in serum free medium should be 6 hours, this seemed to suffice to give the cells time to release proteins into the media, while the cells are still in good condition. Based on our studies, 5 days of differentiation should ensure an optimal developmental stage for the cells to be able to secrete muscle proteins. In addition to IL-6 we detected some potentially new myokines; IL-7, IL-8 and IL-13. Our studies support the theory that myotubes release proteins with hormonal functions, and that the release might be influenced by energy carrying metabolites. Further studies are required to explore the role muscle tissue has in promoting good health and regulating metabolic pathways in the human body.

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