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Cytokine response of primary human myotubes in an in vitro exercise model

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Scheler M, Irmeler M, Lehr S, Hartwig S, Staiger H, Al-Hasani H, Beckers J, Hrabé de Angelis M, Häring HU, Weigert C. Cytokine response of primary human myotubes in an in vitro exercise model. *Am J Physiol Cell Physiol* 305: C877–C886, 2013. First published August 7, 2013; doi:10.1152/ajpcell.00043.2013.—Muscle contraction during exercise is a major stimulus for the release of peptides and proteins (myokines) that are supposed to take part in the beneficial adaptation to exercise. We hypothesize that application of an in vitro exercise stimulus as electric pulse stimulation (EPS) to human myotubes enables the investigation of the molecular response to exercise in a clearly defined model. We applied EPS for 24 h to primary human myotubes and studied the whole genome-wide transcriptional response as well as the release of candidate myokines. We observed 183 differentially regulated transcripts with fold changes >1.3. The transcriptional response resembles several properties of the in vivo situation in the skeletal muscle after endurance exercise, namely significant enrichment of pathways associated with interleukin and chemokine signaling, lipid metabolism, and antioxidant defense. Multiplex immunoassays verified the translation of the transcriptional response of several cytokines into high-secretion levels (IL-6, IL-8, CXCL1, LIF, CSF3, IL-1B, and TNF) and the increased secretion of further myokines such as angiopoietin-like 4. Notably, EPS did not induce the release of creatine kinase. Inhibitor studies and immunoblotting revealed the participation of ERK1/2-, JNK-, and NF- κ B-dependent pathways in the upregulation of myokines. To conclude, our data highlight the importance of skeletal muscle cells as endocrine cells. This in vitro exercise model is not only suitable to identify exercise-regulated myokines, but it might be applied to primary human myotubes obtained from different muscle biopsy donors to study the molecular mechanisms of the individual response to exercise.

myokine; electric pulse stimulation; IL-8; ANGPTL4; ERK; human myotubes

IT IS COMMONLY ACCEPTED THAT regular exercise plays a key role in the prevention and treatment of metabolic and cardiovascular diseases and has also beneficial effects on neurodegeneration and cancer progression (5, 26). Cumulative evidence over the last decades leads to the conclusion that skeletal muscle is an endocrine organ that releases a diversity of biological active peptides and proteins that can have paracrine or endocrine

functions, the so-called myokines (29). Muscle contraction during exercise is one major stimulus of this endocrine function and activates the expression and secretion of proteins belonging to different families such as the interleukin 6 (IL-6) family members IL-6 and leukemia inhibitory factor (LIF; Refs. 6, 38), the C1q/tumor necrosis factor (TNF)-related protein myonectin (34), the CXC motive chemokine IL-8 (36), and matricellular glycoprotein secreted protein acid and rich in cystein (SPARC; Ref. 2). It is supposed that some of these exercise-regulated myokines take part in the beneficial effects of regular physical activity by autocrine/paracrine activation of their specific receptor-activated signaling cascades located in the muscle but also other organs (28).

Although over the last years many attempts have been made to characterize the complex nature of the muscle secretome, knowledge on myokines and their expression and release especially upon contraction-induced activation of intracellular signaling cascades is still limited. A major challenge here is to provide convincing evidence that the skeletal muscle cells per se are the source of circulating exercise factors.

Progress has been made in recent years in developing a cell culture model that enables the investigation of molecular events in contracting differentiated skeletal muscle cells. Application of electric pulse stimulation (EPS) to murine C2C12 myotubes resembles several properties of skeletal muscle contracting in vivo: increased energy expenditure with activation of the AMP kinase (AMPK), activation of MAPK signaling cascades, improved insulin-dependent and independent glucose uptake, upregulation of peroxisome proliferator-activated receptor- γ coactivator 1 (*PPARGC1A*; also known as PGC1 α), and release of chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL5, and IL-6 (7, 20–22, 42). In this model the in vivo motor nerve activation is replaced by electric pulses resulting in Ca²⁺ transients that induce de novo sarcomere assembly and contractile activities in the C2C12 myotubes (9). A recent study showed that EPS can also be applied to human myotubes to activate the sarcomere formation and contractile activity (16). The contraction induces a metabolic shift towards increased glucose uptake and glycolysis as well as enhanced expression of proteins involved in oxidative phosphorylation and expression and release of IL-6 (16, 23). These data indicate that EPS is suitable to study the secretome of myotubes as an in vitro exercise model.

We took advantage of this model to investigate the expression of myokines in exercising primary human myotubes by

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genome-wide expression arrays. Muscle biopsies were taken from 12 young and lean volunteers with normal glucose tolerance. Satellite cells were isolated, cultivated, and differentiated to myotubes, which serve as a well-established cell culture model to study multiple cellular functions of skeletal muscle (45, 48). We used EPS conditions established for C2C12 myotubes that should resemble the challenge of an acute endurance exercise bout (7). We focused on the myokines that were transcriptionally upregulated by EPS for 24 h and studied their release by multiplex immunoassays. Moreover, we investigated the activation of MAPK and their impact on the transcriptional response to EPS. Our results supported the concept that skeletal muscle cells per se are endocrine cells that release several myokines in response to EPS.

EXPERIMENTAL PROCEDURES

Materials. Cell culture media and supplements were from Lonza (Basel, Switzerland), chicken embryo extract was from Seralab (West Sussex, UK), amphotericin B was from Sigma (München, Germany). Antibodies against phospho-ERK1/2 (no. 9101), phospho-JNK (no. 9251), phospho-p38 (no. 9211), phospho-ERK5 (no. 3371), ERK1/2 (no. 9102), p38 (no. 9212), and ERK5 (no. 3372) were from Cell signaling (Frankfurt, Germany), and antibodies against JNK (610627) were from BD Biosciences (Heidelberg, Germany).

Cell culture. Primary skeletal muscle cells obtained from percutaneous needle biopsies performed on the lateral portion of quadriceps femoris (vastus lateralis) muscle of 12 subjects were used for microarray analysis and multiplex immunoassays. A subset was used for the other experiments. All subjects (3 females, 9 males) were young

(25.6 ± 4.4 yrs), lean (body mass index of 22.7 ± 2.0), and insulin sensitive (ISI-Matsuda 25.0 ± 2.0). They gave informed written consent to the study, and the protocol was approved by the Ethics Committee of the University of Tuebingen. Experiments were performed on the first and second passages of subcultured cells. Cells were grown in a 1:1 mixture of α -MEM and Ham's F-12 supplemented with 20% FBS, 1% chicken embryo extract, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.5 μ g/ml amphotericin B till 70–80% confluency. Cells were fused for 6–7 days to myotubes in α -MEM containing 5.5 mM glucose with 2% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.5 μ g/ml amphotericin B.

Electric pulse stimulation. EPS was applied to differentiated human primary myotubes in six-well dishes using C-Pace EP system from IonOptix (Dublin, Ireland). Directly before start of EPS medium was changed (fusion medium, see above). In case of inhibitor studies 10 μ M U0126 (Cell Signaling, Danvers, MA), 10 μ M JNK Inhibitor VIII (Calbiochem, Darmstadt, Germany) or 5 μ M Bay 11-7082 (Sigma-Aldrich, St. Louis, MO) was added. Cells were stimulated for a given time with 14 V, 5 Hz, and 2 ms unless otherwise noted.

Glucose, lactate, lactate dehydrogenase, and creatine kinase measurements. Cell supernatant was collected after EPS, spun for 4 min with 13,000 rpm at 4°C to remove nonadherent cells, and stored at 4°C. Afterwards, concentrations of glucose, lactate, creatine kinase, and lactate dehydrogenase activities in the supernatant were analyzed with the ADVIA 1800 clinical chemical analyzer (Siemens Healthcare Diagnostics, Fernwald, Germany).

RNA isolation and quantitative PCR analysis. Cells were homogenized by QIAshredder and total RNA was isolated using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual including digestion of remaining genomic DNA. One micro-

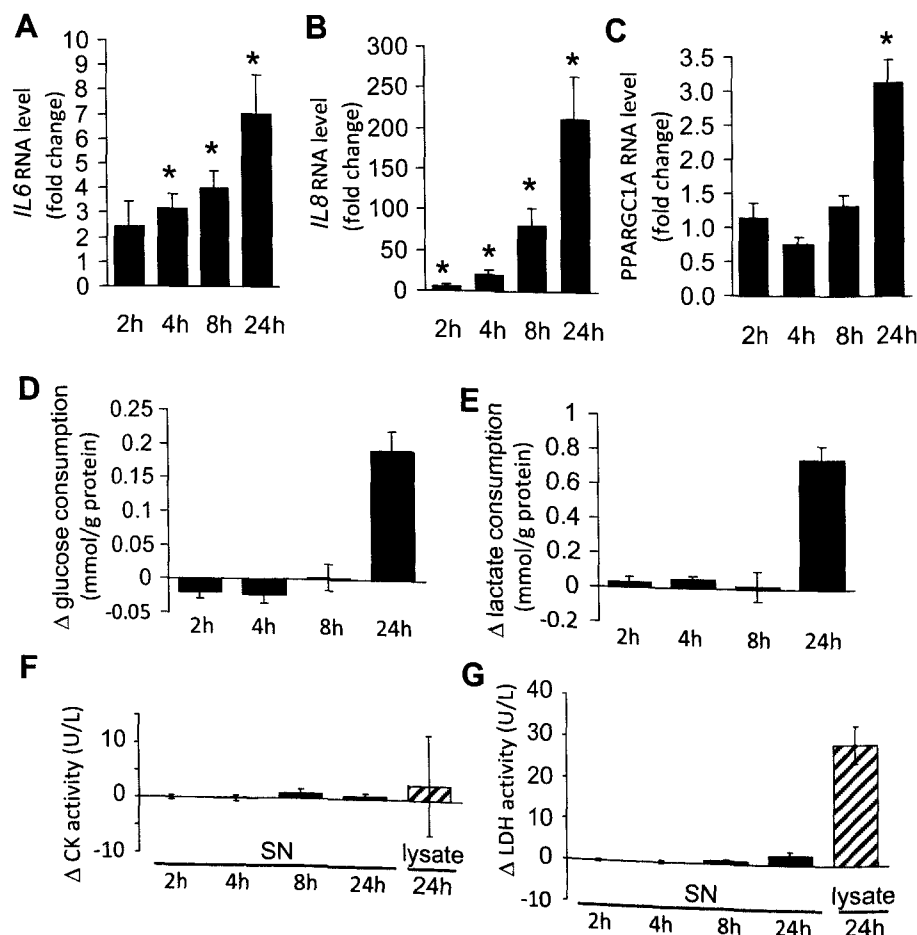


Fig. 1. Electrical pulse stimulation (EPS) for 24 h induced glucose consumption and consistent *IL6* and *IL8* as well as peroxisome proliferator-activated receptor- γ coactivator 1 (*PPARGC1A*) RNA expression. *IL6* (A), *IL8* (B), and *PPARGC1A* (C) mRNA levels were determined in myotubes that were EP stimulated for 2, 4, 8, and 24 h with 14 V, 5 Hz, and 2 ms. RNA was isolated from cultured myotubes after EPS and quantitative qRT-PCR was performed. Values are normalized to levels of house-keeping gene *TBP* and are shown as fold increase to control cells (means $\pm$ SE; $n = 8$). Statistical analysis was performed between mRNA levels of EP-stimulated and control cells (* $P < 0.05$ significant increase). Glucose consumption (D) and lactate production (E) of human primary myotubes EP stimulated for 2, 4, 8, and 24 h. Glucose and lactate levels were measured in the supernatant and are shown as difference of EPS to control. Values are shown as mmol/g protein (means $\pm$ SE; $n = 8$). Creatine kinase (CK; F) and lactate dehydrogenase (LDH; G) activities were measured in the supernatant (SN; filled bars) and in Triton X-100 lysates of the cells (striped bars). Values are shown as difference of EPS to control in U/L (means $\pm$ SE; $n = 5-8$).

gram of RNA was used for reverse transcription PCR with random hexamer primers using the Transcript First Strand cDNA Synthesis kit (Roche, Mannheim, Germany). Quantitative real-time PCR (qRT-PCR) was performed on a Roche Lightcycler 480 using QuantiTect Primer Assays Hs-TBP (QT00000721), Hs-IL6 (QT00083720), Hs-IL8 (QT00000322), Hs-PPARGC1A (QT00095578), Hs-NAMPT (QT00087920), Hs-ANGPTL4 (QT00003661), and MYOD1 (QT-00209713; Qiagen, Hilden, Germany).

Microarray analysis. The Agilent 2100 Bioanalyzer was used to assess RNA quality, and only high-quality RNA (RIN >8) was used for microarray analysis. Total RNA (150 ng) was amplified using the Ambion WT Expression Kit and the WT Terminal Labeling Kit (Affymetrix). Amplified cDNA was hybridized on Affymetrix Human Gene 1.0 ST arrays containing ~28,000 probe sets. Staining (Fluidics script FS450_0007) and scanning were done according to the Affymetrix expression protocol. Expression console (Affymetrix) was used for quality control and to obtain annotated normalized RNA gene-level data (standard settings including sketch-quantile normalization, annotation file mogene-1_0-st-v1.na32.mm9). Statistical analyses were performed by utilizing the statistical programming environment R (R Development Core Team; Ref. 32) implemented in CARMAweb (33). Genewise testing for differential expression was done employing the paired limma *t*-test in combination with the Benjamini-Hochberg multiple testing correction (false discovery rate <10%). Heatmaps were generated with CARMAweb and Gene Ontology (GO) term and pathway enrichment analyses ($P < 0.01$; adj $P < 0.05$) were done with GePS (Genomatix) or Ingenuity software ($P < 0.05$).

Array data was submitted to GEO (accession no. GSE44051 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tpajpygyemsefw&acc=GSE44051>).

Protein lysates, Western blotting, and NF- κ B assay. Cells were lysed with 100 μ l lysis buffer/well (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, and 1% Triton X-100, containing phosphatase inhibitors). Protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA). Cell extracts were separated by sodium dodecyl sulfate polyacrylamide (10%) gel electrophoresis, and proteins were transferred onto a nitrocellulose membrane by semidry electroblotting. Immunodetection was performed as recently described (44). The NF- κ B activity was determined with TransAM NF- κ B p65 assay (Active Motif, Carlsbad, CA). Preparation of nuclear extracts was performed according to manufacturer's manual, and 2 μ g nuclear extract were used for the assay.

Multiplex immunoassays and ELISA. The cell supernatant was collected after EPS, spun for 4 min with 13,000 rpm at 4°C to remove nonadherent cells, and stored at -80°C. Commercially available human Bio-Plex Pro multiplex bead-based immunoassays (Bio-Rad, Hercules, CA) were used to determine protein levels of IL-1B, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-15, IL-17A, colony stimulating factor 3 (CSF3), CSF2, IFNG, chemokine ligand 2 (CCL2), CCL4, TNF, vascular endothelial growth factor A (VEGFA), CXCL1, LIF, NAMPT, and IL-33. Analysis was performed using a Bioplex 200 suspension array system (Bio-Rad) according to the manufacturer's instructions. Protein concentrations were calculated from the appropriate optimized standard curves using Bio-Plex Manager software version 6.0 (Bio-Rad, Hercules). For analysis the amount of analyte was normalized to total protein amount per cell culture dish. The human ANGPTL4 ELISA was from BioVendor (Heidelberg, Germany).

Cell proliferation assay. Cell viability after EPS was determined by XTT cell proliferation kit (A8088, 1,000; AppliChem, Darmstadt, Germany). Medium was changed before start of EPS (2 ml/well fusion media). Directly after stimulation ended, 1 ml of XTT reagent solution with activation solution was added per well. After 40–50 min, 150 μ l of supernatant were transferred to a 96-well microtiter plate and absorbance was measured as described in the manufacturer's manual.

For analysis obtained absorptions were normalized to protein amount per well and results were calculated as fold increase to control value.

Immunostaining. Primary human myotubes on coverslips were washed twice in PBS, fixed in PBS containing 4% formaldehyde (pH 7.4) for 20 min, quenched with 150 mM glycine in PBS for 10 min, and treated with 0.1% Triton X-100 for 10 min. Blocking was performed in 1% NGS, 0.05% Tween 20 in PBS for 30 min. Coverslips were incubated with antibody recognizing fast-type skeletal myosin MyHC II (M4276; Sigma-Aldrich) diluted 1:100 in blocking solution for 1 h at room temperature, washed three times in PBS, and incubated with the Alexa 488-labeled secondary antibody (Invitrogen, Karlsruhe, Germany) diluted 1:200 in blocking solution for another 2 h and washed again. Nuclei were stained using TO-PRO3 (Invitrogen, Karlsruhe, Germany) diluted 1:1,000 in PBS for 1 h before being mounted in PermaFluor (Beckman Coulter, Krefeld, Germany).

Statistics. Values shown are means $\pm$ SE. A two-sided Student's *t*-test was used to compare the two groups.

RESULTS

EPS for 24 h induced glucose consumption and consistent IL-6 and IL-8 as well as PPARGC1A expression. First, we set out to define the EPS conditions suitable for primary human myotubes that induce a metabolic shift towards higher glucose consumption and lactate production as well as a significant transcriptional response of the known myokines IL6, IL8, and the exercise-regulated transcriptional coactivator PPARGC1A. Myotubes were stimulated for 2, 4, 8, and 24 h at 14 V, 5 Hz, and 2 ms. Increased RNA expression of IL6 and IL8 was already induced after 4 h of EPS resulting in an accumulation of IL6 and IL8 RNA after 24 h of stimulation (Fig. 1, A and B).

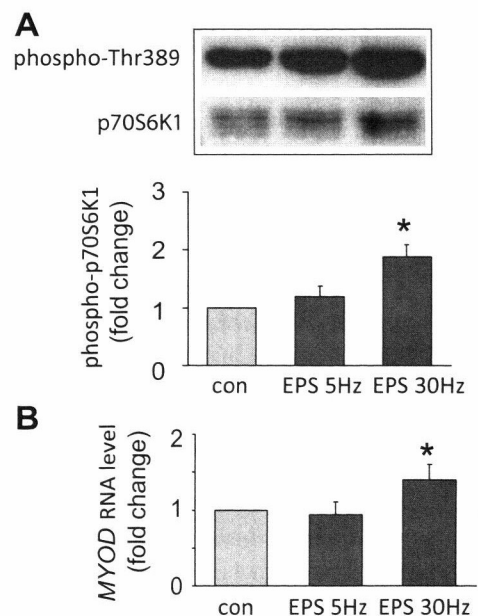


Fig. 2. High-frequency EPS mimics resistance exercise. A: phosphorylation of p70S6K1 was detected by immunoblotting of myotube cell lysates obtained after EPS with either 5 Hz or 30 Hz, 2 ms, and 14 V for 4 h. Membrane was reprobed for p70S6K1 protein. Histogram shows the result of the densitometric quantification as means $\pm$ SE ($n = 3-5$) * $P < 0.05$ vs. EPS 5 Hz. B: relative myogenic differentiation 1 (MYOD) mRNA expression level was measured in human primary myotubes that were EP stimulated with either 5 or 30 Hz, 2 ms, and 14 V for 4 h. RNA was isolated from cultured myotubes after EPS and qRT-PCR was performed. Values are normalized to levels of housekeeping gene TBP and shown as fold increase to control cells (means $\pm$ SE; $n = 4$). Statistical analysis was performed between mRNA levels of 5 and 30 Hz EP-stimulated cells (* $P < 0.05$).

The RNA expression of *PPARGC1A* was threefold induced after 24 h with no observable increase at the earlier time points (Fig. 1C). Increased glucose consumption and lactate production were also found after 24 h (Fig. 1, D and E). EPS did not result in increased release of creatine kinase into the medium (Fig. 1F). The activity of lactate dehydrogenase (LDH) in the supernatant of EPS-treated myotubes was slightly increased after 24 h, but this can be due to the higher total LDH activity found in the cellular lysates rather than cytotoxic effects induced by EPS (Fig. 1G). Moreover, cell viability after 24 h of EPS as determined by reduction of a tetrazolium dye (XTT assay) was not reduced (95 ± 7 vs. 100% of control cells, data not shown). Since we intended to use low-intensity, but long-duration stimulation to simulate nonexhaustive endurance exercise, we used the EPS conditions of 14 V, 5 Hz, and 2 ms for 24 h to study the whole genome transcriptional response. Application of higher frequency conditions (14 V, 30 Hz, and 2 ms) for 4 h activated the phosphorylation of p70S6 and the RNA expression of the myogenic regulator *MYOD*, both of which reflect high-intensity resistance training leading to increased muscle protein synthesis and muscle hypertrophy (Fig. 2, A and B).

Whole genome-wide transcriptional response to EPS. EPS for 24 h was applied to primary human myotubes obtained from 12 donors, and gene regulation was assessed by microarray analysis on Affymetrix Gene 1.0 ST arrays. Cluster analysis of the expression data grouped samples mainly according to the donors and not according to treatments (data not shown). Consequently, we used a paired approach for the statistical analysis. We observed 2,220 significantly regulated probe sets (false discovery rate <10%) corresponding to about 2,118 genes. Most of the 2,220 probe sets showed small expression changes, with 368 probe sets having fold changes >1.2 times

and 183 probe sets with fold changes >1.3 times. Among the 2,220 probe sets we observed more downregulated genes upon EPS (961 up, 1,259 down), whereas probe sets with higher ratios were mostly upregulated upon this treatment [>1.2-fold: 268 up, 100 down; >1.3-fold (153 up, 30 down)].

To evaluate the biological functions of the observed transcriptomic changes we searched for enriched GO terms and enriched pathways associated with regulated genes. We analyzed the 183 probe sets with the highest ratios and observed significant enrichment ($P < 0.01$; adj $P < 0.05$) of terms and pathways related to inflammation, leucocyte migration, lipid metabolism, smooth muscle cell adaption, and signaling by interleukins and chemokines as well as by NF- κ B and PPARG (Table 1). The vast majority of the genes were upregulated upon EPS indicating that the associated processes are likely to be activated. Among the strongest induced genes were *IL8* (13.5 times), *CXCL1* (6.7 times), and *IL6* (2.7 times). Other strongly upregulated cytokines and chemokines were *IL1B* (5.5 times) and *CXCL6* (5.6 times). Pronounced increases were also found in the expression of metalloproteinase genes and matrix metalloproteinases. PPARG coactivators were either increased at transcript level (*PPARGC1A*, 1.3 times) or predicted to be activated by Ingenuity software (*PPARGC1B*, data not shown).

EPS induced the release of cytokines. The translation of the transcriptional upregulation of cytokines and chemokines into enhanced release of peptides after 24 h of EPS from the myotubes of the 12 donors was measured by multiplex immunoassays (Table 2). The highest increase was found for IL-8 (44 ± 9 vs. 2 ± 0.3 pg/ μ g total protein). A pronounced increase was also found for CXCL1 (43 ± 9 vs. 5 ± 1 pg/ μ g total protein) and IL-6 (72 ± 19 vs. 19 ± 3 pg/ μ g total protein). Significant increases in protein secretion from EPS-

Table 1. GO term and pathway analysis

GO Term or Pathway Association	GO Term ID	P Value	Genes Observed	Gene Symbols of Observed Genes
Inflammatory response	GO:0006954	4.66E-10	20	CCL2, CCL5, C3, CXCL6, IL8, VCAM1, NFKB1Z, TNFAIP6, IRAK2, CCL11, IL1B, HMOX1, CXCL10, CXCL1, CXCL2, BDKRB1, TNFAIP3, CEBPB, AGTR1, IL6
Leukocyte migration	GO:0050900	6.16E-10	15	CCL2, CCL5, IL8, VCAM1, ANGPT1, SLC7A11, EDNRB, CCL11, IL1B, HMOX1, CMKLR1, MMP1, ICAM1, BDKRB1, IL6
Lipid metabolic process	GO:0006629	3.20E-06	26	LDLR, LPIN1, AKR1C2, ME1, CYP7B1, INSIG1, ABCG3, ELOVL2, SCD, FADS1, FADS2, FASN, ANGPTL4, DHCR7, IL1B, NPC1, PLIN2, LRP8, ABCA1, CRABP2, HSD11B1, TXNRD1, AKR1B10, AKR1C3, AGTR1, PTGES
Regulation of smooth muscle cell proliferation	GO:0048660	1.19E-04	5	CCL5, IGF1, HMOX1, TNFAIP3, IL6
Negative regulation of muscle cell apoptosis	GO:0010656	2.62E-03	2	<i>IGF1, NRG1</i>
NF- κ B		1.40E-11	27	CCL2, CCL5, CXCL6, IL8, VCAM1, NFKB1Z, NAMPT, SPP1, IRAK2, NFKB1A, CCL11, <i>ID1</i> , SOD2, IL1B, HMOX1, IER3, NFKB2, BIRC3, MMP1, CXCL10, CXCL1, CXCL2, ICAM1, TNFAIP3, CEBPB, IKBKE, IL6
Interleukin 1		1.52E-10	17	CCL2, LIF, MMP3, IL8, VCAM1, IRAK3, IRAK2, NFKB1A, SOD2, IL1B, MMP1, ZC3H12A, CXCL10, ICAM1, BDKRB1, IL6, PTGES
Chemokine (CC motif) ligand 2		2.98E-09	12	CCL2, CCL5, IL8, VCAM1, NAMPT, IL1B, ZC3H12A, CXCL10, CXCL1, CXCL2, ICAM1, IL6
Peroxisome proliferator activated receptor- γ		1.64E-08	12	LPIN1, SCD, NAMPT, FADS2, FASN, NFKB1A, PLIN2, CXCL10, ABCA1, CEBPB, AGTR1, PTGES
Matrix metalloproteinase		5.58E-06	12	CCL2, CCL5, MMP3, IL8, TIMP4, NAMPT, SPP1, SOD2, IL1B, MMP1, CXCL10, PTGES

Shown are selected significantly ($P < 0.01$; adj $P < 0.05$) enriched Gene Ontology (GO) terms and pathways associated with the set of 183 significant probe sets, which were significantly regulated >1.3-fold by electric pulse stimulation (EPS) stimulation. Genes downregulated by this treatment are shown in italics (*CRABP2*, *IGF1*, *NRG1*, and *ID1*); all other genes were upregulated. See text for definitions.

Table 2. Summary of secreted proteins detected by multiplex immunoassays in cell supernatant after 24 h of EPS and corresponding microarray expression data

Analyte	Protein Amount, pg analyte/ μ g protein		<i>P</i> Value	Fold Change Protein (EPS vs. Control)	Linear Ratio RNA (EPS vs. Control)	Concentration in Range of the Immunoassay, pg/ml
	Control	EPS				
IL8	1.93 $\pm$ 0.29	43.9 $\pm$ 11.9	0.0019*	20.8 $\pm$ 3.5	13.5	1.3 to 23,662
CXCL1	4.73 $\pm$ 1.21	42.7 $\pm$ 8.57	0.0002*	10.4 $\pm$ 1.7	6.7	8.8 to 7,619
IL6	18.9 $\pm$ 3.47	72.2 $\pm$ 19.3	0.0126*	3.8 $\pm$ 0.6	2.7	2.0 to 30,250
LIF	0.15 $\pm$ 0.03	0.36 $\pm$ 0.05	0.0019*	3.8 $\pm$ 1.1	2.2	8.4 to 33,926
IL4	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	0.0350*	1.7 $\pm$ 0.1	NS	0.4 to 5,246
IL13	0.02 $\pm$ 0.01	0.04 $\pm$ 0.01	0.0025*	1.7 $\pm$ 0.2	NS	2.3 to 9,169
IL17A	0.11 $\pm$ 0.01	0.20 $\pm$ 0.03	0.0138*	2.3 $\pm$ 0.3	NS	9.6 to 37,676
IL1B	0.008 $\pm$ 0.001	0.015 $\pm$ 0.002	0.0256*	1.8 $\pm$ 0.1	5.5	2.4 to 9,381
CSF3	0.25 $\pm$ 0.04	1.19 $\pm$ 0.42	0.0389*	4.8 $\pm$ 1.2	NS	7.1 to 34,427
TNF	0.17 $\pm$ 0.03	0.27 $\pm$ 0.04	0.0294*	1.8 $\pm$ 0.1	NS	7.0 to 88,192
CCL2	19.5 $\pm$ 2.28	25.3 $\pm$ 1.28	0.0369*	1.5 $\pm$ 0.1	1.6	2.1 to 7,940
IL2	†	0.04 $\pm$ 0.01	†	†	NS	3.6 to 14,420
IL5	0.009 $\pm$ 0.001	0.012 $\pm$ 0.002	0.1907	NS	NS	2.8 to 9,942
IL7	0.027 $\pm$ 0.004	0.033 $\pm$ 0.004	0.3290	NS	NS	1.6 to 18,824
IL12	0.11 $\pm$ 0.02	0.13 $\pm$ 0.02	0.4519	NS	NS	3.4 to 42,103
IFNG	0.96 $\pm$ 0.13	1.27 $\pm$ 0.13	0.0989	NS	NS	42.5 to 27,191
IL10	0.018 $\pm$ 0.003	0.021 $\pm$ 0.002	0.3480	NS	NS	1.6 to 21,265
VEGFA	7.83 $\pm$ 0.82	9.35 $\pm$ 0.86	0.2128	NS	NS	2.9 to 37,025
IL-1RA	ND	ND			NS	55.7 to 12,821
IL15	ND	ND			NS	9.2 to 9,000
IL33	ND	ND			NS	5.3 to 89,287
CCL4	ND	ND			NS	2.2 to 6727
NAMPT	ND	ND			1.8	84.8 to 1,393,973
CSF2	‡	‡			NS	4.1 to 16,001

Absolute protein amount in the supernatant of control and EPS cells in relation to total protein amount of the cells (means $\pm$ SE) with corresponding *P* value (**P* < 0.05). Protein amount as fold change of EPS cells to control cells is shown as means $\pm$ SE. Significantly regulated RNA expression is shown as linear ratio (data of array analysis, false discovery rate <10% and ratio >1.3-fold). †IL2 was not detectable in the supernatant of control cells but in 9 of 12 samples of EPS cells. ‡CSF2 concentration in samples was below CSF2 concentration in medium. ND, no detectable concentration in >75% of all samples; NS, not significant. See text for other definitions.

treated myotubes that correspond to increased RNA expression were also measured for IL-1B, CCL2, and LIF. The interleukins IL-2, IL-4, IL-13, IL-17A, TNF- α , and CSF3 were found to be elevated in the supernatant without a detectable increase in gene expression after 24 h of EPS. IL-5, IL-7, IL-10, IL-12, interferon- γ (IFNG), and VEGFA were detected by the multiplex assay in the supernatant but not elevated by the EPS treatment (Table 2). IL-1RA, IL-15, IL-33, and CCL4 protein levels were below detection limit in the supernatant of the myotubes, while CSF2 levels were below the concentration found in the medium.

Of note, we observed a wide range of the inducibility of cytokine release in the 12 different myotube cultures obtained from the 12 different donors. It is exemplarily shown for IL-8, CXCL1, and IL-6 (Fig. 3, A–C). The fold change of secreted protein showed a strong correlation with the fold change of transcripts of the respective gene after 24 h of EPS (Fig. 3, D–F). We wanted to exclude that the differences in the cytokine response depend on a high variability in myotube differentiation and formation. Analysis of the fusion index of myotube cultures with a low or high upregulation of IL8 RNA expression and IL-8 protein release in response to EPS revealed a comparable myotube formation and no significant difference in the fusion index between the groups (Fig. 3, G–I). Moreover, the RNA expression of the differentiation markers *CKM*, *MYH2*, and *MYH7* did not correlate with the upregulation of IL8 RNA expression (Fig. 3, J–L).

To conclude, the data indicate that the EP-stimulated upregulation of transcription is a major determinant for the increased release of these cytokines. Moreover, it suggests individual dif-

ferences in the cytokine response of the myotube cultures that might be due to individual characteristics of the donors that are conserved in the primary cells.

Different kinetics of cytokine, *PPARGC1A*, *NAMPT*, and *ANGPTL4* expression. Microarray analysis revealed a significant increase in *ANGPTL4* (1.6 times) and *NAMPT* (1.8 times) transcript levels. We studied the kinetics of the transcriptional response of these novel EPS target genes. *NAMPT* showed an accumulative response with significant increase after 8 and 24 h (Fig. 4A). The increase in *ANGPTL4* expression peaked after 4 h (Fig. 4B). Well in accordance, *ANGPTL4* protein levels in the supernatant were increased after 8 h of EPS (1.8 $\pm$ 0.3 vs. 1.1 $\pm$ 0.2 ng/ml). *NAMPT* protein levels were below detection limit in the supernatant of the myotubes.

The different kinetics of expression of the investigated genes *NAMPT* and *ANGPTL4* (Fig. 4) and *IL6*, *IL8*, and *PPARGC1A* (Fig. 1) in EP-stimulated myotubes suggests the involvement of distinct EPS-activated signal transduction pathways.

Activation of MAPK and NF- κ B in response to EPS. Previous studies have shown that EPS treatment of myotubes led to the phosphorylation of ERK1/2 as well as phosphorylation of JNK (16, 22). EPS conditions of 14 V, 5 Hz, and 2 ms induced a significant increase in the phosphorylation of ERK1/2 and JNK in human myotubes after 2 and 4 h of stimulation, respectively (Fig. 5, A and B), whereas the phosphorylation of p38 MAPK or ERK5 was not upregulated (Fig. 5, C and D). In our transcriptome analysis we found in addition that NF- κ B is likely to be activated (Table 1). In the following experiments, the involvement of these activated pathways in the observed

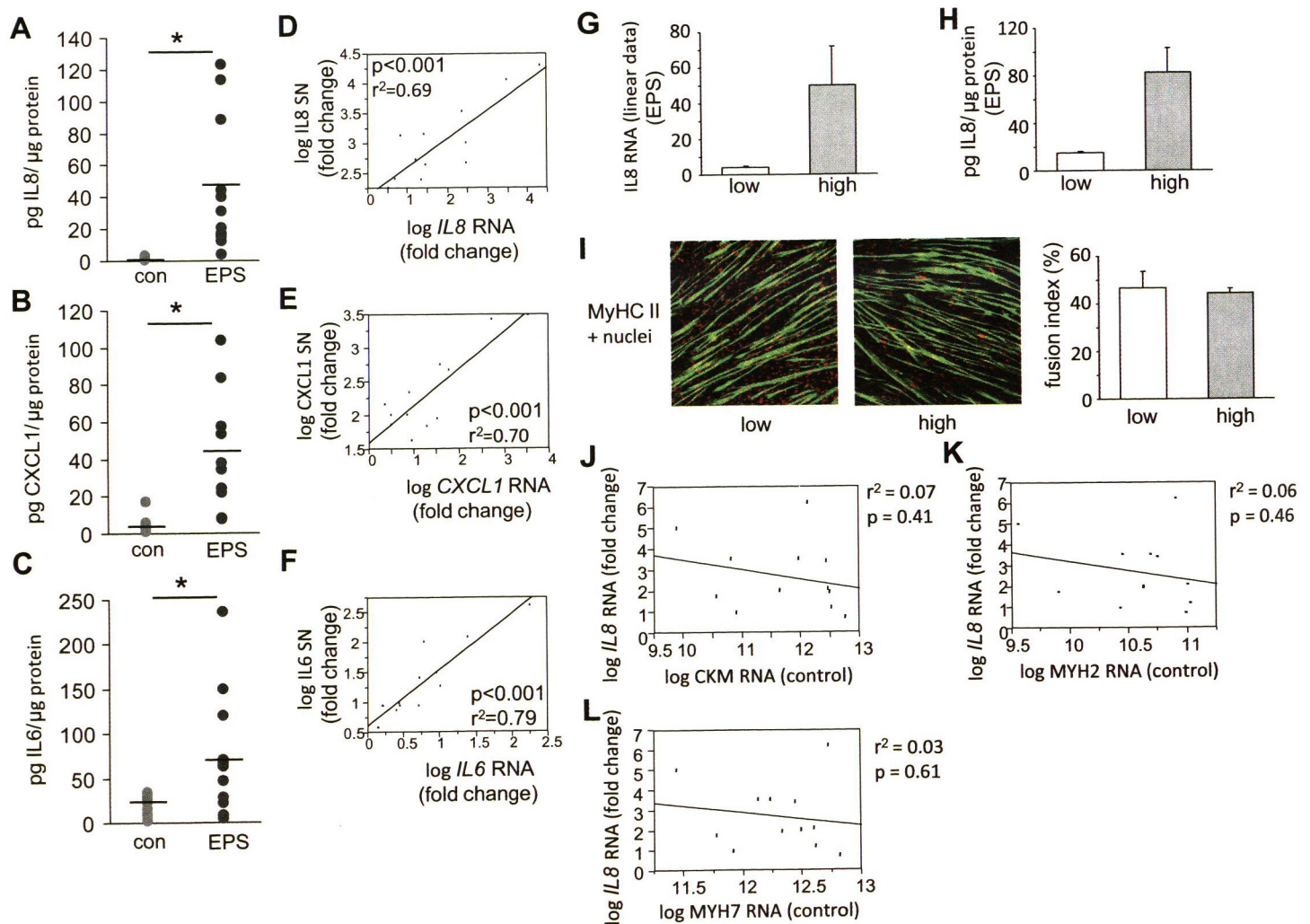


Fig. 3. EPS induced the release of cytokines. A–C: supernatant of 12 human primary myotubes was analyzed with Bio-Plex Pro multiplex bead-based immunoassays for IL-8, chemokine (C-X-C motif) ligand 1 (CXCL1) and IL-6. Cells were EP stimulated for 24 h (14 V, 5 Hz, and 2 ms) and cell supernatant was collected afterwards. Values of the individual myotube cultures are shown as dots as pg analyte/μg protein, the mean value is indicated by the black line (means $\pm$ SE is depicted in Table 2). Statistical analysis was performed between supernatant of EPS and control cells (* $P < 0.05$). D–F: correlation of secreted protein (SN as fold change to control cells) and mRNA level (RNA level as fold change to control cells) for IL-8, CXCL1 and IL-6 after 24 h of EPS ($n = 12$). G–I: immunostaining of MyHCII protein (I, $\times 10$ magnification) in human myotubes showing a low or high IL8 RNA expression (G) and IL8 protein release (H) in response to EPS ($n = 3$ per group, data are shown as means $\pm$ SE). Nuclei are shown in red. Fusion index was determined by dividing the number of nuclei in MyHCII-positive myotubes by the total number of nuclei analyzed. J–L: correlation of basal CKM or MYH2 or MYH7 RNA expression (RNA levels of control cells) and EPS-induced IL8 RNA expression (RNA levels as fold change to control cells after 24 h of EPS; $n = 12$).

transcriptional response to EPS was exemplarily studied at the expression levels of *IL8*, *PPARGC1A*, and *ANGPTL4*.

Inhibitors directed against activation of ERK1/2 (U0126), JNK (JNK VIII), and NF- κ B (Bay11-7082) were added to the myotubes during EPS treatment for 4 or 24 h. The induction of *IL8* was reduced by U0126 (after 4 and 24 h), by JNK VIII (after 4 h), and by Bay11-7082 (after 24 h; Fig. 5E). The induction of *PPARGC1A* expression after 24 h was inhibited by U0126 and JNK VIII (Fig. 5F), while the induction of *ANGPTL4* expression after 4 h was only blocked by U0126 (Fig. 5G). Together, these data showed that inhibition of the ERK1/2 pathway had a strong effect on the EPS-induced gene expression of *IL8*, *PPARGC1A*, and *ANGPTL4*. Involvement of the transcription factor NF- κ B was demonstrated by the pronounced transcriptional repression of *IL8* after 24 h in the presence of Bay11-7082. Notably, the inhibition of the ERK1/2 pathway also prevented the activation of NF- κ B after 24 h of EPS

(Fig. 5H), suggesting a role for ERK1/2 in the EPS-induced activation of NF- κ B.

DISCUSSION

The major aim of our study was to investigate the regulation of gene expression of primary human myotubes upon EPS with a specific focus on potentially secreted peptides and proteins and to determine the release of these myokines. Now we will discuss our data by considering the in vivo response of the working muscle. The advantage of a skeletal muscle cell culture model hereby is the clear assignment of the identified secreted factors as myokines. Among the 153 significantly upregulated transcripts with fold changes >1.3 times are 35 assigned as secreted products and 7 of the 10 genes with the highest fold increase are encoding secreted proteins and peptides [IL-8, CXCL1, CXCL6, IL-1B, tissue factor pathway inhibitor (TFPI2), matrix metalloproteinase 1 (MMP1), and

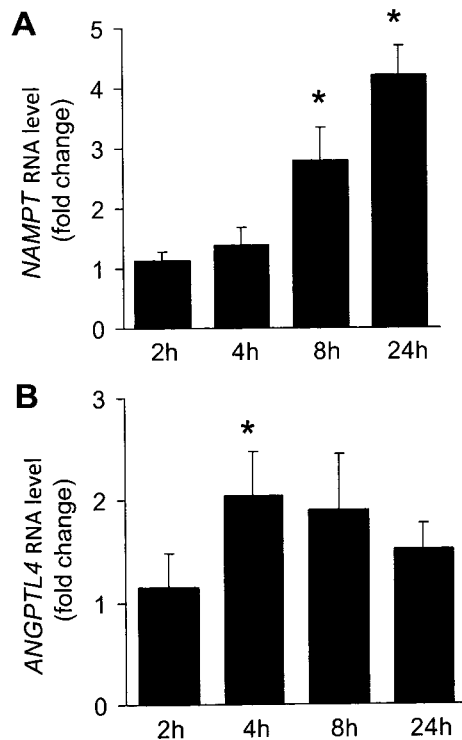


Fig. 4. EPS of human primary myotubes increases nicotinamide phosphoribosyltransferase (*NAMPT*) and angiopoietin-like 4 (*ANGPTL4*) RNA expression levels. Relative *NAMPT* (A) and *ANGPTL4* (B) mRNA expression levels were measured in human primary myotubes that were EP stimulated for 2, 4, 8, and 24 h. RNA was isolated from cultured myotubes after EPS and qRT-PCR was performed. Values are normalized to levels of housekeeping gene *TBP* and shown as fold increase to control cells (means $\pm$ SE; $n = 8$). Statistical analysis was performed between mRNA levels of EP stimulated and control cells (* $P < 0.05$).

MMP3]. We could verify the functional relevance of the altered transcriptome as significant increase of cytokines and chemokines in the supernatant of the EPS-treated myotubes. In detail, we identified the interleukins IL-1B, IL-2, IL-4, IL-6, IL-8, IL-13, IL-17A, and CXCL1, LIF, CSF3, TNF, and CCL2 as EPS-induced human myokines. Some of these factors have already been described as myokines released from the exercising muscle of humans (IL-6, IL-8, and TNF; Refs. 28, 37), or plasma concentrations are found to be increased after an exercise bout but the origin is undefined (CSF3 and CCL2; Refs. 10, 24). Some factors do not reach measurable elevations in plasma levels, but muscular protein levels are increased after exercise (CCL2 and IL-1B; Refs. 13, 18). Based on our whole genome expression data and the measured levels of secreted peptides and proteins, we conclude that EPS induces the expression and release of several cytokines and chemokines in human myotubes and this at least partially reflects the in vivo situation in the working muscle.

The muscular cytokine response to exercise in vivo is strongly influenced by the mode and intensity of the exercise, and exercise protocols including eccentric exercise, downhill running, and resistance training induce a more robust activation of cytokine expression in the exercising muscle and also high systemic levels (25). The general assumption here is that exercise-induced muscle damage must occur to activate infiltration of immune cells leading to a robust cytokine expression and release from both muscle fibers and immune cells. This

kind of muscle damage is accompanied by a pronounced release of creatine kinase into the circulation. Our data obtained in an in vitro exercise model with primary human myotubes indicate that muscle cells are capable of upregulating the expression and release of cytokines and other myokines in response to EPS without concomitant release of creatine kinase and thus without severe cell damage and furthermore in the absence of immune cells. Following that our data suggest that skeletal muscle cells may contribute to the local and systemic cytokine response in a greater extent than generally assumed. One limitation of our experimental approach is that we did not perform a separation step of our primary skeletal muscle cells by fluorescent cell sorting. Thus we cannot exclude the presence of other cells than myoblasts in our cell culture system.

The biological function of the exercise-induced cytokine response is not completely understood, and the current status of research was reviewed recently (25, 27, 31). IL-6 is the best studied myokine and is important for muscle tissue hypertrophy, satellite cell proliferation, and myotube formation (12, 35, 50). CCL2 is involved in immune cell attraction and maintenance of the inflammatory phase in injured muscle (39). IL-8 can induce angiogenesis, which might support skeletal muscle adaptation to regular exercise (27). In a physiological context, the cytokine response of the exercised muscle appears to be important for muscle regeneration and muscle adaptation to high-intensity exercise. Of note, the upregulation of genes involved in anti-oxidant defense mechanism, e.g., heme oxygenase 1 (HMOX1) or metallothioneins is not only observed in EPS-treated myotubes but also in human skeletal muscle after different exercise protocols (15, 17, 30). An attractive part of the myokine concept is the hypothesis that the release of peptides from the exercised muscle takes part in the adaptation of metabolism to exercise. This may occur locally as increased glucose uptake and fat oxidation in the muscle, systemically by increasing hepatic glucose output and lipolysis, or as long-term adaptation by activating e.g., oxidative phosphorylation, vascularization, and muscle hypertrophy. The myokine candidates possibly involved herein include IL-6, IL-8, and LIF and were reviewed recently (29).

Over the last years further proteins in addition to the mentioned cytokines and chemokines are identified as exercise-inducible myokines. This list includes NAMPT (8), myonectin (CTRP15) (34), SPARC (2), IL-15 (40), and Irisin (4). The corresponding transcripts *CTRP15/FAM132B*, *SPARC*, *IL15*, and *FND5* (Irisin) were detected in the human myotubes by our microarray expression analysis but not increased by EPS for 24 h. Possible explanations are that regular training is needed for the upregulation as it is reported for Irisin, or that in addition to EPS systemic factors are needed to activate the expression, or that muscle cells are not the source of the elevated protein concentrations found after exercise. We also might have missed the transcriptional upregulation of myokines with a rapid and transient expression. It is also possible that EPS enhanced the translation or cleavage of a protein but not the expression of the respective gene. In the case of SPARC, the studies of Aoi et al. (2) showed that increased release of SPARC in mechanically stretched C2C12 myotubes does not require transcription but protein translation. The expression of *NAMPT* was significantly increased in the EPS-treated myotubes, but we could not detect NAMPT protein in the supernatant of the cells. Therefore, NAMPT might not be released from muscle cells, well in accor-

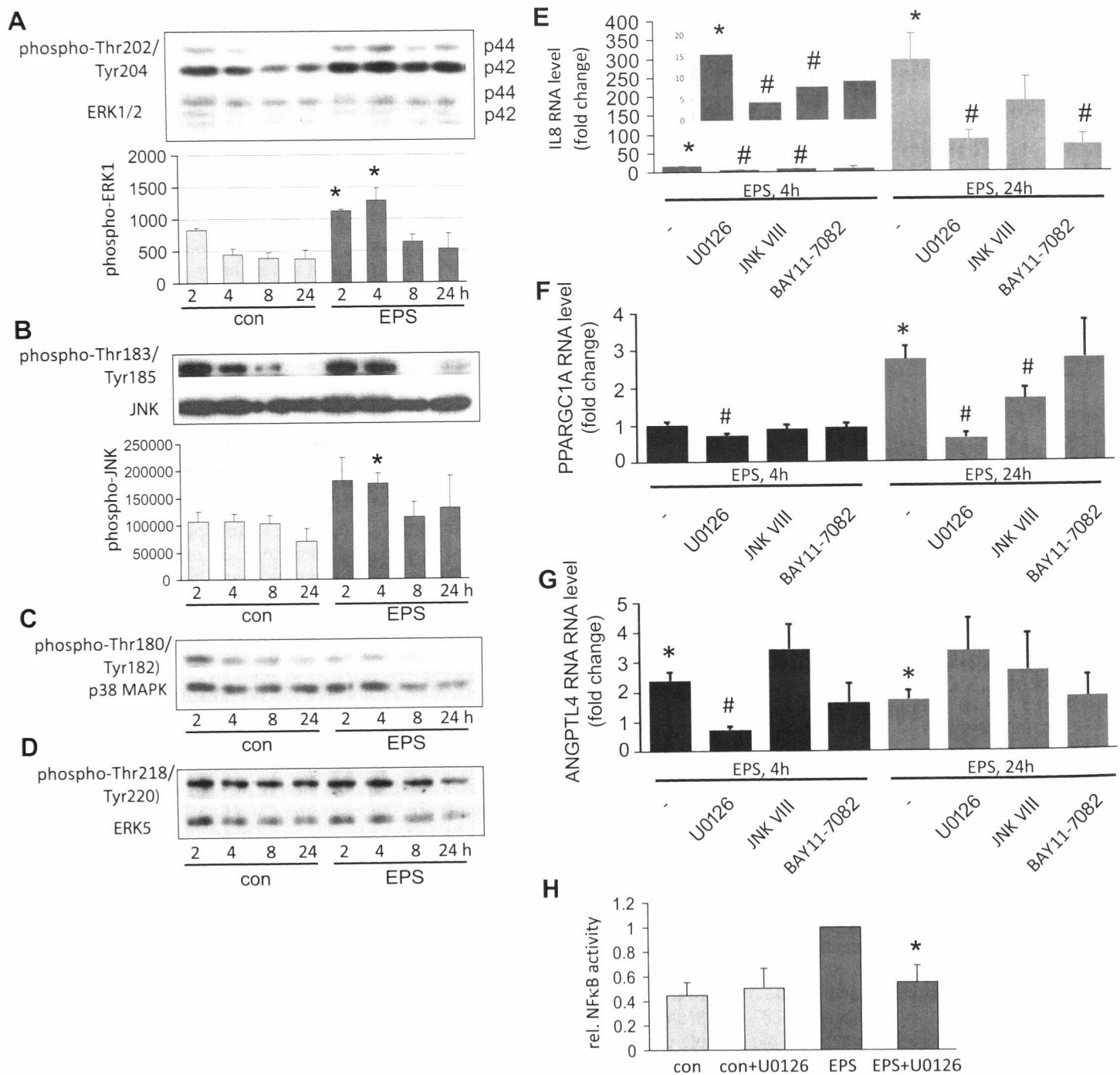


Fig. 5. Activation of MAPK and NF- κ B in response to EPS. Phosphorylation of ERK1/2 (A) and JNK (B) was detected by immunoblotting of myotube cell lysates obtained after the indicated time of EPS. Membranes were re probed for ERK1/2 protein and JNK protein respectively. Histogram shows the results of the densitometric quantification as means $\pm$ SE ($n = 3$). * $P < 0.05$ vs. control cells. Phosphorylation of p38 MAPK (C) and ERK5 (D) was detected by immunoblotting of myotube cell lysates obtained after the indicated time of EPS. Membrane was re probed for p38 MAPK and ERK5 protein respectively. *IL8* (E), *PPARGC1A* (F), and *ANGPTL4* (G) mRNA expression levels were measured in human primary myotubes that were EPS-treated for 4 and 24 h without inhibitor (—), MEK1/2-inhibitor (U0126), JNK-inhibitor (JNK VIII), or NF- κ B-inhibitor (BAY11-7082). Values are normalized to levels of housekeeping gene *TBP* and shown as fold increase to control cells without inhibitor of either 4 or 24 h (means $\pm$ SE; $n = 8-12$). Statistical analysis was performed between EP stimulated and control cells without inhibitor (* $P < 0.05$ significant increase) and between EP stimulated cells with and without inhibitor of the respective time point (# $P < 0.05$ significant decrease). H: NF- κ B activity was measured by the TransAM NF κ B p65 assay in nuclear extracts of myotubes obtained after 24 h of EPS in the absence or presence of 10 μ M U0126. EPS-treated samples were set as 1 (means $\pm$ SE; $n = 6$, $P < 0.05$ vs. EPS-treated cells without inhibitor).

dance with the study of Costford et al. (8), who described upregulation of the intracellular NAMPT protein amount in skeletal muscle after exercise.

We also studied the signaling pathways that are responsible for the EPS-induced cytokine response in the human myotubes.

In vivo, activation of NF- κ B in human skeletal muscle was shown as increased DNA binding activity and decreased I κ B α protein levels or nuclear localization of NF- κ B after resistance exercise (43), cycling (41), or eccentric exercise (14). Cycling and endurance exercise increase the phosphorylation of ERK1/2

in human skeletal muscle (3, 47, 49). EPS treatment of rodent and human myotubes activates these pathways (16, 19, 22, 46, and present study) underlining the suitability of this in vitro exercise model to investigate contraction-induced mechanisms. We found a striking effect of the ERK1/2 inhibitor U0126 on the EPS-induced expression of the NF- κ B-target gene *IL8* after both 4 and 24 h of EPS and on the upregulation of *PPARGC1A* after 24 h. The participation of ERK1/2 signaling in contraction-induced NF- κ B activation was also suggested in a previous study where the U0126 compound was applied to electric pulse-stimulated isolated rat muscles (11).

Altogether, our data underline the function of the skeletal muscle as an endocrine organ and highlight the importance of skeletal muscle cells per se as source of the elevated secretion of myokines from the working muscle during exercise. The results indicate that the EPS conditions used in our study are suitable to study the molecular response of myotubes to prolonged endurance exercise, since the transcriptional response of several exercise target genes is comparable with the regulation of genes found in the exercised muscle in vivo. One possible limitation of our cell culture model is that cultures of human myotubes have a rather glycolytic metabolism (1), which may retard the adaptation of the mitochondrial metabolism to EPS. While we did not detect transcriptional upregulation of respiratory genes, we did not perform functional tests of oxidative capacity, e.g., oxidation of fatty acids or glucose. A recent publication could demonstrate increased oxidation of oleic acid and glucose of human myotubes in response to EPS, which indicate that human myotubes are able to adopt their oxidative capacity in an in vitro endurance exercise model (23). A further difference to the in vivo situation is that muscle fibers are surrounded by connective tissue and extracellular matrix, which clearly can influence the transcriptional activation of genes in the contracting fibers but also the release and diffusion of secreted proteins.

Our data show an individual response of myotubes to EPS. Myotubes obtained from different muscle biopsy donors exhibited a quite different change in cytokine expression and amount of secreted protein, which could not be explained by differences in myotube fusion. The reason for this different response is not yet clear, but it suggests that individual genetic and epigenetic determinants of the muscle response in vivo might be conserved in the respective myotubes. Thus our in vitro exercise model is not only suitable to identify exercise-regulated myokines, but it can be applied to primary human myotubes obtained from different muscle biopsy donors to study molecular mechanisms of the individual outcome of exercise intervention.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.S., M.I., S.L., and S.H. performed experiments; M.S., M.I., S.L., and S.H. analyzed data; M.S. and M.I. prepared figures; M.S., M.I., and C.W. drafted manuscript; H.S., H.A.-H., J.B., M.H.d.A., H.-U.H., and C.W. interpreted results of experiments; H.S., H.A.-H., J.B., M.H.d.A., and H.-U.H. edited and revised manuscript; C.W. conception and design of research; C.W. approved final version of manuscript.

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