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RESEARCH ARTICLE

Advances in GPCRs: Structure, Mechanisms, Disease, and Pharmacology

The lactate receptor GPR81 is predominantly expressed in type II human skeletal muscle fibers: potential for lactate autocrine signaling

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Abstract

G_i-coupled protein receptor 81 (GPR81) was first identified in adipocytes as a receptor for L-lactate, which upon binding inhibits cyclicAMP (cAMP)-protein kinase (PKA)-cAMP-response element binding (CREB) signaling. Moreover, incubation of myotubes with lactate augments expression of GPR81 and genes and proteins involved in lactate- and energy metabolism. However, characterization of GPR81 expression and investigation of related signaling in human skeletal muscle under conditions of elevated circulating lactate levels are lacking. Muscle biopsies were obtained from healthy men and women at rest, after leg extension exercise, with or without venous infusion of sodium lactate, and 90 and 180 min after exercise (8 men and 8 women). Analyses included protein and mRNA levels of GPR81, as well as GPR81-dependent signaling molecules. GPR81 expression was 2.5-fold higher in type II glycolytic compared with type I oxidative muscle fibers, and the expression was inversely related to the percentage of type I muscle fibers. Muscle from women expressed about 25% more GPR81 protein than from men. Global PKA activity increased by 5%–8% after exercise, with no differences between trials. CREB^{S133} phosphorylation was reduced by 30% after exercise and remained repressed during the entire trials, with no influence of the lactate infusion. The mRNA expression of vascular endothelial growth factor (VEGF) and peroxisome-proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) were increased by 2.5–6-fold during recovery, and that of lactate dehydrogenase reduced by 15% with no differences between trials for any gene at any time point. The high expression of GPR81-protein in type II fibers suggests that lactate functions as an autocrine signaling molecule in muscle; however, lactate does not appear to regulate CREB signaling during exercise.

CREB; HCAR1; PGC-1α; PKA; resistance exercise

INTRODUCTION

The cell surface located G_i-coupled protein receptor 81 (GPR81; 1), also known as hydroxycarboxylic acid receptor 1 (HCAR1), was first shown to be highly expressed in adipose tissue and to exhibit a high affinity for L-lactate (2, 3). Although GPR81 can be activated by several agonists, the most physiologically relevant one is L-lactate, which has an EC₅₀ for GPR81 ranging from 1.5 to 5 mM depending on experimental conditions (2–5). Moreover, Liu et al. (3) showed that although the mRNA expression of human GPR81 is predominantly expressed in adipose tissue, GPR81 mRNA is also detected in skeletal muscle, albeit at 10-fold lower levels. Subsequently, GPR81 protein was found to be present in myotubes and that prolonged lactate incubation of these myotubes resulted in an up-regulation of both GPR81 mRNA and protein (6). The expression of

GPR81 in human skeletal muscle has, to our knowledge, never been studied.

Upon ligand binding, GPR81 signals to reduce intracellular levels of cyclicAMP (cAMP), thus inhibiting cAMP-dependent protein kinase (PKA) signaling and lipolysis. The lactate-induced inhibition of lipolysis was first illustrated in adipose tissue (3), an effect that was confirmed using GPR81-knock-out mice (4), and later also shown in myotubes (6) as well as mouse skeletal muscle (7). One well-defined target of PKA is the cAMP-response element binding (CREB) protein, where activated PKA phosphorylates CREB at Ser133, promoting the binding of the latter to DNA (8). Accordingly, lactate binding to GPR81 is considered to result in lower PKA activity, which would lead to reduced CREB^{Ser133} phosphorylation (6). Exercise is known to increase lactate production; however, the effects on CREB phosphorylation are unclear, as no effects, increases or decreases have been reported (9–12).



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In addition to regulating lipolysis, lactate has also been shown to stimulate the expression of genes involved in lactate- and energy metabolism in skeletal muscle cells. This was first illustrated by Hashimoto et al. (13) where incubation of L6 myotubes with 10–20 mM of lactate increased mRNA expression of peroxisome-proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α) and monocarboxylate transporter 1 (MCT-1). Similar effects were also shown in mouse skeletal muscle following an intraperitoneal lactate injection (14). At present, the gene regulatory effects of lactate in muscle tissue have not been confirmed to be GPR81-dependent. Although other pathways may also be involved, a GPR81-dependence has been described in other cell types. For example, lactate-induced vascular endothelial growth factor (VEGF) expression in mouse brain (15) as well as PGC-1 α and MCT expression in cancer cells (16) are inhibited by GPR81 silencing or knock out.

Collectively, a growing body of literature shows that lactate possesses signaling properties that control metabolic processes in skeletal muscle tissue. However, at present, there is a lack of in vivo studies on human skeletal muscle exploring this notion. Hence, the objective of this study was initially to characterize the expression of GPR81 in human skeletal muscle. Next, we assessed the potential role of lactate in GPR81 signaling by elevating circulating lactate levels during resistance exercise with and without venous infusion of sodium lactate.

METHODS

Experimental Design

The experimental design and other relevant descriptive data are presented elsewhere (17). Briefly, the study involved 8 female and 8 male training accustomed participants with the following characteristics (means \pm SE): age 28 \pm 5 yr, height 173 \pm 10 cm, weight 69 \pm 11 kg, one leg maximal strength 88 \pm 20 kg, Wingate peak power 11 \pm 1.9 W/kg, Wingate mean power 7 \pm 1.3 W/kg, type II myosin heavy chain (MyHC) distribution 60 \pm 4%. All participants gave their written consent to participate after being fully informed about the purpose and the execution of the study with all associated risks. The study protocol was preapproved by the Regional Ethical Review Board in Stockholm (2017/1139-31/4) and was performed in accordance with the principles outlined in the Declaration of Helsinki.

Preliminary Testing

The subjects performed three preliminary test sessions, which are described in detail in Liegnell et al. (17). The first session involved a medical screening, surface measurements of leg muscle volume and area, a one-legged maximal strength test (1-RM) in a leg extension machine and finally a 30 s (Wingate) all-out cycling sprint to determine peak anaerobic power and mean anaerobic capacity. These tests were performed in the nonfasted state at various times of day. The second and third visits involved familiarization of the resistance exercise protocol that was subsequently performed during the experimental trials. These sessions were performed in the morning in the overnight fasted state and separated by approximately by 1 wk.

Experimental Trials

Full details about the experimental trials are provided in Liegnell et al. (17). In brief, all trials were initiated at 07:00 AM with participants being in the overnight fasted state. First, cannulas were placed in the antecubital vein of each arm, one for venous infusion and one for repeated blood sampling. Then, a resting skeletal muscle biopsy was taken from the vastus lateralis muscle under local anesthesia, using a Bergström needle with applied suction. Thereafter, a venous infusion of sodium lactate (50 μ mol/kg/min at pH 6.4) or volume-matched isotonic saline was initiated. The resistance exercise protocol was commenced after 20 min of baseline infusion and involved a total of nine sets with 10 repetitions at a maximal load corresponding to 75% of the subject's 1-RM. After completion of the exercise bout, the infusion was terminated resulting in a total infusion time of \sim 55 min. In the Lactate trial, this corresponded to an average of 216 \pm 8 mmols of infused lactate.

Immediately after cessation of the exercise and infusion a second muscle biopsy was taken from the exercised leg, with additional biopsies obtained after 90 and 180 min of supine rest. All biopsies in one trial were taken from the same exercised leg, all from a new incision in the proximal direction. After collection, biopsies were rapidly blotted free from blood and frozen in liquid nitrogen. A total of 15 venous blood samples were taken during each trial; at rest, approximately every 10th min during infusion and then repeatedly during the 180 min of recovery [see Liegnell et al. (17) for schematic protocol]. Blood samples were analyzed immediately after collection for levels of lactate, glucose, sodium, potassium, base excess, and pH. After the final samples were obtained, subjects left the laboratory, returning for the next trial 7–10 days later where the infusion and leg used for exercise and biopsies were randomized in a counter-balanced manner.

Exercise performance did not differ between the trials and no subject could identify or show any side effects of the sodium lactate infusion. The sodium lactate infusion was successful in significantly elevating blood lactate levels compared with placebo. Peak blood levels of lactate noted immediately postexercise was 3.0 \pm 0.2 mmol \times l⁻¹ in the Saline trial, whereas it was 130% greater (6.8 \pm 0.3 mmol \times l⁻¹) in the Lactate trial. The range of peak blood lactate levels in the Lactate trial was 5.0–10.2 mmol \times l⁻¹. Significant differences in blood lactate levels between trials were evident up until 90 min of recovery.

Protein Extraction and Immunoblotting

The methodology for sample preparation and immunoblotting are described in detail in Liegnell et al. (17) but explained briefly here. Approximately 3.5 mg of lyophilized muscle tissue, carefully dissected, free from blood and connective tissue into small fiber bundles were used for immunoblotting. Samples were homogenized in a HEPES-based buffer (pH 7.4) containing several protease and phosphatase inhibitors and 1%-TritonX-100 as detergent, using a BulletBlender (NextAdvance, New York). The homogenates were then rotated and centrifuged to obtain supernatants, of which the protein concentrations were determined and diluted to a final concentration of

1.5 $\mu\text{g} \times \mu\text{L}^{-1}$ in 4 \times Laemmli sample buffer (LSB; Bio-Rad Laboratories, Richmond, CA).

As a positive control for GPR81 protein expression, human subcutaneous adipose tissue from the periumbilical area of one male and one female were used. Approximately 100 mg of frozen adipose tissue was used for protein extraction according to the principles described by Diaz Marin et al. (18). In brief, the tissue was homogenized in RIPA-buffer using a BulletBlender (3 \times 1 min at max speed), followed by 1 h incubation at 4°C. Samples were subsequently centrifuged three times at 20,000 g for 15 min, with the removal of lipids after each centrifugation. The protein concentration was determined, and samples were subsequently diluted in 4 \times LSB to a final concentration of 1.5 $\mu\text{g} \times \mu\text{L}^{-1}$.

For determination of the influence of skeletal muscle fiber type on GPR81 protein expression, we first examined muscle samples from 16 individuals who participated in a separate project that involved subjects with extreme variations in muscle fiber type composition, where biopsies were taken after an overnight fast (19). Their type I% ranged from 28% to 90% and were grouped as type II dominant (36% type I, $n = 8$) and type I dominant (65% type I, $n = 8$). These muscle samples were prepared for immunoblotting as described earlier. Second, fiber-type specific pools of single fibers were prepared. Approximately, 100 type I and 100 type II fibers each were isolated from five baseline muscle samples (three males and two females). Following fiber type determination with the THRIFTY method (20) all type I and type II fibers were pooled separately, weighed, and then prepared for immunoblotting as described earlier.

For analysis of phosphorylated proteins 22.5 μg of protein were loaded onto 26-well Criterion TGX gradient gels (4%–20% acrylamide; Bio-Rad Laboratories), whereas 15 μg of protein was loaded for the analysis of total protein expression. All samples for each subject were loaded onto the same gel. Electrophoresis was performed at 300 V for 30 min on ice, with subsequent transfer to polyvinylidene fluoride membranes at a constant current of 300 mA for 3 h at 4°C, followed by control of equal loading and transfer by staining with MemCode Reversible Protein Stain Kit (Thermo Scientific, Rockford, IL). Membranes were subsequently destained, blocked for 1 h at room temperature, and then incubated overnight at 4°C with commercially available primary antibodies (Table 1). The next day, after serial washing, the membranes were incubated with suitable secondary horseradish peroxidase (HRP)-conjugated antibodies (Table 1) for 1 h at room temperature. Following serial washing, proteins were visualized by the application Super Signal West Femto Chemiluminescent Substrate (Thermo Scientific) and

subsequently detected in the molecular imager ChemiDoc MP. Quantification of the detected bands was performed using the Image Lab software (Bio-Rad Laboratories). Total protein expression and phosphorylation of PKA substrates were normalized to the total protein stain, whereas phosphorylation of CREB was normalized to total CREB protein expression.

Gene Expression

Skeletal muscle mRNA levels were analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from 2.5 mg lyophilized clean dissected muscle using PureZOL RNA isolation reagent (Bio-Rad Laboratories), a Bullet Blender bead beater, and RNase-free ZrO_2 beads. Total RNA was subsequently isolated from the homogenates according to the manufacturer's instructions. The pelleted RNA was dissolved in 30 μL RNase-free water and heated at 55°C for 5 min to facilitate dissolution. RNA concentration and purity (260/280 nm ratio) were measured on a NanoDrop Lite spectrophotometer (Thermo Scientific). The average RNA yield was 187 ng/ μL with an average purity of 1.87. Subsequently, 2 μg of RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories) in accordance with the manufacturer's instructions.

The mRNA was quantified using real-time quantitative PCR (qRT-PCR). To determine a suitable cDNA concentration and annealing temperature for each primer, pooled cDNA obtained from all the participants was diluted (45, 22.5, 12.25, 6.125, and 3.06 ng/reaction), and RT-qPCR was performed on a Bio-Rad CFX96 (Bio-Rad Laboratories, Sweden). The standard curves of the primers exhibited good efficiency and single melting peaks were observed during melt curve analysis, confirming the presence of only a single product. For the qRT-PCR, 10 μL amplification mixtures containing the template cDNA in RNase-free water (22.5–45 ng/reaction), SYBR Green Supermix (Bio-Rad Laboratories), and 2.5 pg of corresponding primers were used. PCR primers were synthesized by Eurofins Genomics (Luxembourg, see Table 2 for primer sequences used). The mRNA content corresponding to the following genes was quantified: *MCT1*, *MCT4*, *PGC-1 α* total, *PGC-1 α 1*, *PGC-1 α 4*, *VEGF*, *LDHA*, *GPR81*, and *UCP3*. Geometric mean between *GAPDH* and *CYP11A* was used as a stable control, and gene expression was calculated using the $2^{-\Delta\text{CT}}$ method.

Statistical Analysis

The data were analyzed using TIBCO Statistica 13 for Windows (TIBCO Software Inc., Palo Alto, CA). The data are presented as the means \pm standard error (SE) unless otherwise noted. The Shapiro–Wilk test was applied to test the

Table 1. Details of antibodies used for immunoblotting

Protein	Manufacturer	Dilution	Species	ID No.
Phospho-CREB Ser 133 (10E9)	SCBT	1:500	Mouse	sc-81486
Total CREB (48H2)	CST	1:1,000	Rabbit	9197
Phospho-PKA Substrates (100G7E)	CST	1:1,000	Rabbit	9624
MCT1 (H-1)	SCBT	1:500	Mouse	sc-365501
LDH (H-10)	SCBT	1:1,000	Mouse	sc-133123
GPR81	Thermo Scientific	1:1,000	Rabbit	PA5-67873
Anti-Rabbit IgG, HRP-linked antibody	CST	1:10,000	N/A	7074
Anti-Mouse IgG, HRP-linked antibody	CST	1:10,000	N/A	7076

CST, Cell Signaling Technology; HRP, horseradish peroxidase; SCBT, Santa Cruz Biotechnologies.

Table 2. Primer sequences used for RT-qPCR

Gene	Forward Primer	Reverse Primer
LDHA	AGCCCGATTCCGTTACCT	CACCAGCAACATTTCATTCCA
GPR81 (HICAR1)	TGCCAGCGTGTCTGCTAGACT	TACACCAGGGGATCCAGCATGC
MCT1 (SLC16A1)	TGACCATTGTGGAATGCTGT	GAGCCGACCTAAAAGTGGTG
MCT4 (SLC16A3)	GCATCTCCTACGGCATGGTG	CAGGAGTTTGCCCTCCCGAA
PPARGCA1A tot	CAAGCCAAACCAACAACCTTTATCTCT	CACACTTAAGGTGCGTTCAATAGTC
PPARGCA1A iso1	ATGGAGTGACATCGAGTGTGCT	GAGTCCACCCAGAAAGCT GT
PPARGCA1A iso4	TCACACCAAACCCACAGA GA	CTGGAAGATATGGCACAT
VEGF	ACTGCCATCCAATCGAGACC	GATGGCTTGAAGATGTACTCGATCT
UCP3	GACTATGGACGCTACAGAACC	CTCCTTGAGGATGTCTAGGTC
GAPDH	AACCTGCCAAATATGATGAC	TCATACCAGGAAATGAGCTT
CYPA	GGTCTCTTTGGGCGGAAGAC	CTCTCCCAGATGATGCCTT

normality of the data and variables that did not meet the assumption of normality were log transformed. A Student's paired *t* test was performed for evaluating potential differences in GPR81 expression between the type II and type I dominant groups. Correlative analysis for type I fiber % and GPR81 expression was determined with Pearson's product-moment correlation (*r*). A two-way repeated measures analysis of variance (ANOVA; trial \times time) was used for the data analyses on protein phosphorylation, protein expression, and mRNA expression. Fisher's least significant difference post hoc test was performed for any of these analyses if significant main effects appeared. For assessing potential sex differences in protein and mRNA expression a three-way ANOVA (sex \times trial \times time) was used. Data were considered statistically significant if $P < 0.05$.

RESULTS

Human Skeletal Muscle GPR81 Expression

GPR81 protein was clearly detected in human skeletal muscle using immunoblotting. The expression in relative terms was 25% and 40% of that than in human subcutaneous adipose tissue when related to total tissue protein and control proteins, respectively (Fig. 1A). However, when related to milligram of tissue the expression was fourfold higher in human skeletal muscle (Fig. 1A). Next, we assessed whether the expression of GPR81 is fiber type dependent. We found that GPR81 protein expression was 15% higher ($P < 0.05$, Fig. 1B) in a group of type II fiber-dominant individuals (36% type I) compared with a group of type I fiber dominant individuals (65% type I). In addition, there was a negative relationship between the percentage of type I fibers and expression of GPR81 protein ($P < 0.05$, $r = 0.69$, Fig. 2C). Finally, we compared the GPR81 protein expression in pools of type I and type II fibers derived from five subjects and found that the GPR81 expression was ~150% greater in the type II fiber pool (Fig. 2D).

GPR81 mRNA was readily detected using RT-qPCR, with an average Ct value of 24.8 across samples when using 45 ng cDNA per reaction, which in relative terms was 1/256 of the expression of the control genes. For comparison to other analyzed genes, the relative expressions of *PGC-1 α* total, *LDH*, and *MCT-4* were 1/128, 1/4, and 1/2,048, respectively. The mRNA expression of *GPR81* (Fig. 2A) remained stable over time in the saline trial, but there was a trend for an increase in the lactate trial ($P = 0.10$). In addition, there were no sex

differences in the mRNA expression, although men tended to exhibit lower values at baseline (Fig. 2B).

Similar to the mRNA expression, the protein expression of GPR81 was not influenced by exercise or lactate infusion (Fig. 2C), but there was a main effect ($P < 0.05$) for a lower skeletal muscle GPR81 protein content in the males compared with the females (Fig. 2D).

GPR81-Signaling

As a primary readout of GPR81-signaling, we determined the phosphorylation status of CREB at Ser133. Total CREB protein levels did not change over time in any of the two trials (Fig. 3A); however, the phosphorylation of CREB^{Ser133} was reduced by ~30% compared with baseline immediately after exercise, with no differences between trials ($P < 0.05$, Fig. 3B). The phosphorylation of CREB^{Ser133} remained below baseline throughout the 180 min of recovery in both trials. As a readout of global PKA activity, we determined the phosphorylation status of PKA-substrates at baseline and immediately after exercise in both trials. When analyzing all detected substrates (whole lane), the global phosphorylation status of PKA-substrates was increased by 5%–6% after exercise in both trials ($P < 0.05$, Fig. 3C). When selectively analyzing all substrates at 75 kDa or higher (Fig. 3D) there was no change in phosphorylation status, whereas the selective analysis of substrates below 75 kDa exhibited an exercise-induced increase of 8% in both trials ($P < 0.05$, Fig. 3E).

Protein Levels

To evaluate if lactate infusion altered the levels of key proteins involved in lactate metabolism, we analyzed the protein levels of lactate dehydrogenase (LDH) and MCT-1. However, neither exercise nor lactate infusion affected total protein levels of LDH or MCT-1 (Fig. 4, A and B). Finally, although there was a trend toward lower MCT-1 expression in the males, there were no statistically significant differences between the sexes regarding LDH and MCT-1 protein content (data not shown).

mRNA Expression

In general, lactate infusion did not influence the expression of any of the analyzed genes, whereas exercise effects were noted for several genes. The average exercise-induced increase above baseline for *PGC-1 α* total, *PGC-1 α 1*, and *PGC-1 α 4* was sixfold, twofold, and 10-fold, respectively ($P < 0.05$, Fig. 5, A–C), with no differences between the sexes at any

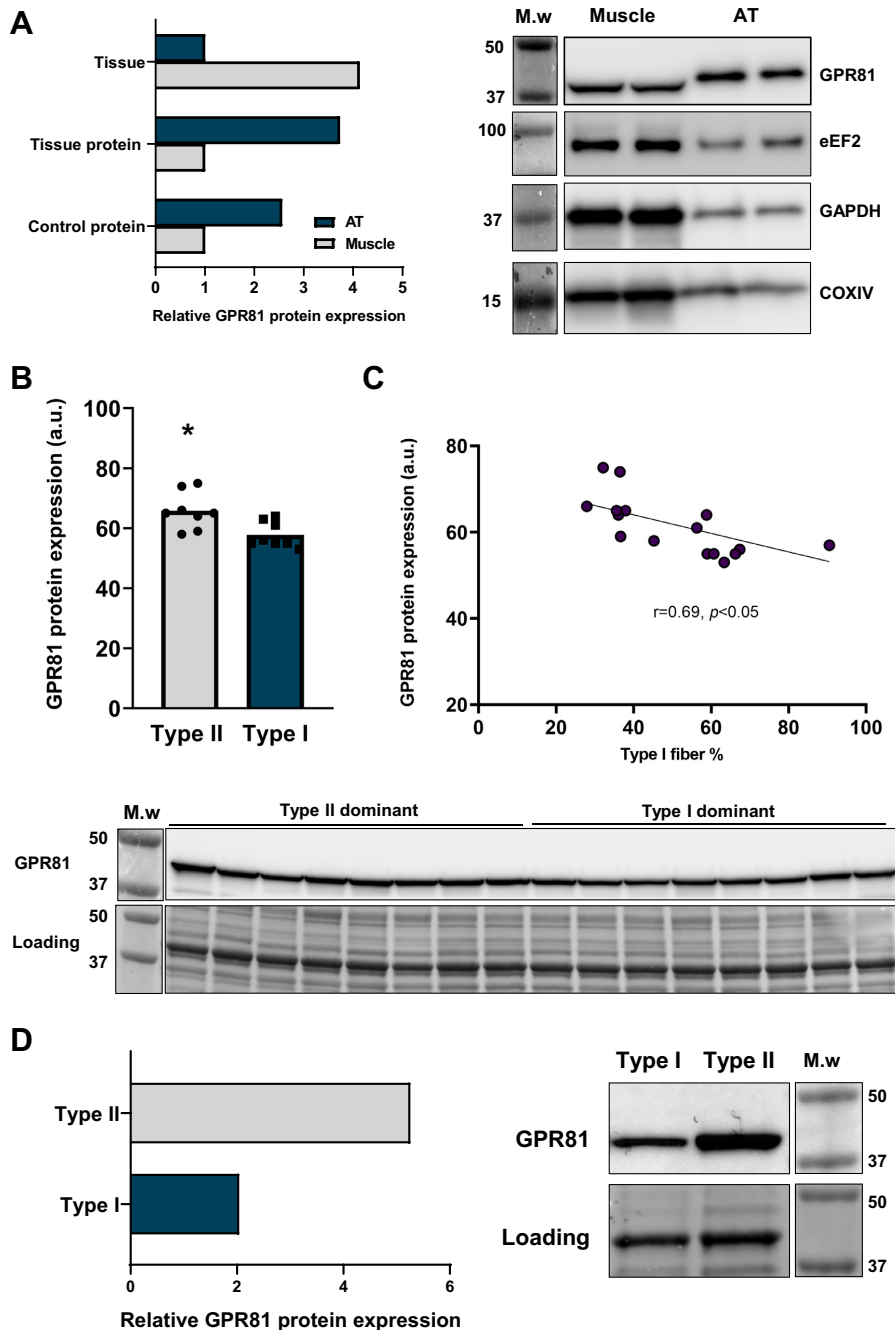


Figure 1. A: blots and quantification for the expression GPR81 and three housekeeping proteins (eEF2, GAPDH, and COX IV) in 10 μ g of protein human skeletal muscle or human subcutaneous adipose tissue (AT). The relative expression of GPR81 per "Tissue" is related to corresponding amount of tissue in milligram, "Tissue protein" is related to total protein content in the tissue homogenates and "Control protein" is related to the average expression of eEF2, GAPDH, and COX IV in that tissue. B: GPR81 protein expression in groups of type II fiber dominant (36% type I, $n = 8$ subjects) and type I fiber dominant (65% type I, $n = 8$ subjects) individuals. The symbols represent the individual data points, and the * represents $P < 0.05$ compared with the type I dominant group. C: the relationship between GPR81 protein expression and type I fiber % for the individuals quantified in B. Above B and C are the blots for GPR81 and the loading control for which the data are based upon. D: blots and quantification for the expression GPR81 in pools of type I and type II fibers, as well as a visual comparison with the expression in a mixed fiber sample. For the fiber pools 5 μ g of total protein was loaded, whereas 10 μ g of protein was loaded for the mixed fiber sample. The relative expression of GPR81 in the fiber pools were normalized to the loading control (total protein stain). a.u., Arbitrary units; GPR81, G α -coupled protein receptor 81; M.w, molecular weight.

time point. The mRNA expression of *VEGF* was increased by ~ 2.5 -fold at 90 and 180 min after exercise in both trials ($P < 0.05$, Fig. 5D), with no differences in expression between the males and the females. The mRNA expression of *LDH* was reduced by an average of 13% in both trials after 180 min of recovery ($P < 0.05$, Fig. 5E). The male subjects exhibited an $\sim 60\%$ higher *LDH* expression compared with the female subjects independent of time point ($P < 0.05$, data not shown). The mRNA expression of *LDH*, *MCT-1*, and *MCT-4* remained unaltered in both trials, but *MCT-1* mRNA levels were $\sim 20\%$ lower in the male subjects compared with the females independent of time point ($P < 0.05$, data not shown).

DISCUSSION

In the present study, we examined GPR81 expression, GPR81-signaling, and the expression of genes and proteins involved in lactate- and energy metabolism in human skeletal muscle after resistance exercise performed with or without infusion of sodium lactate. The key findings were first that GPR81 protein is readily detected in human skeletal muscle and is predominantly expressed in type II glycolytic muscle fibers. Furthermore, resistance exercise reduced the phosphorylation of CREB^{Ser133} but was not altered by the elevated lactate levels induced by the infusion. Finally, the elevated systemic lactate levels did not alter the mRNA

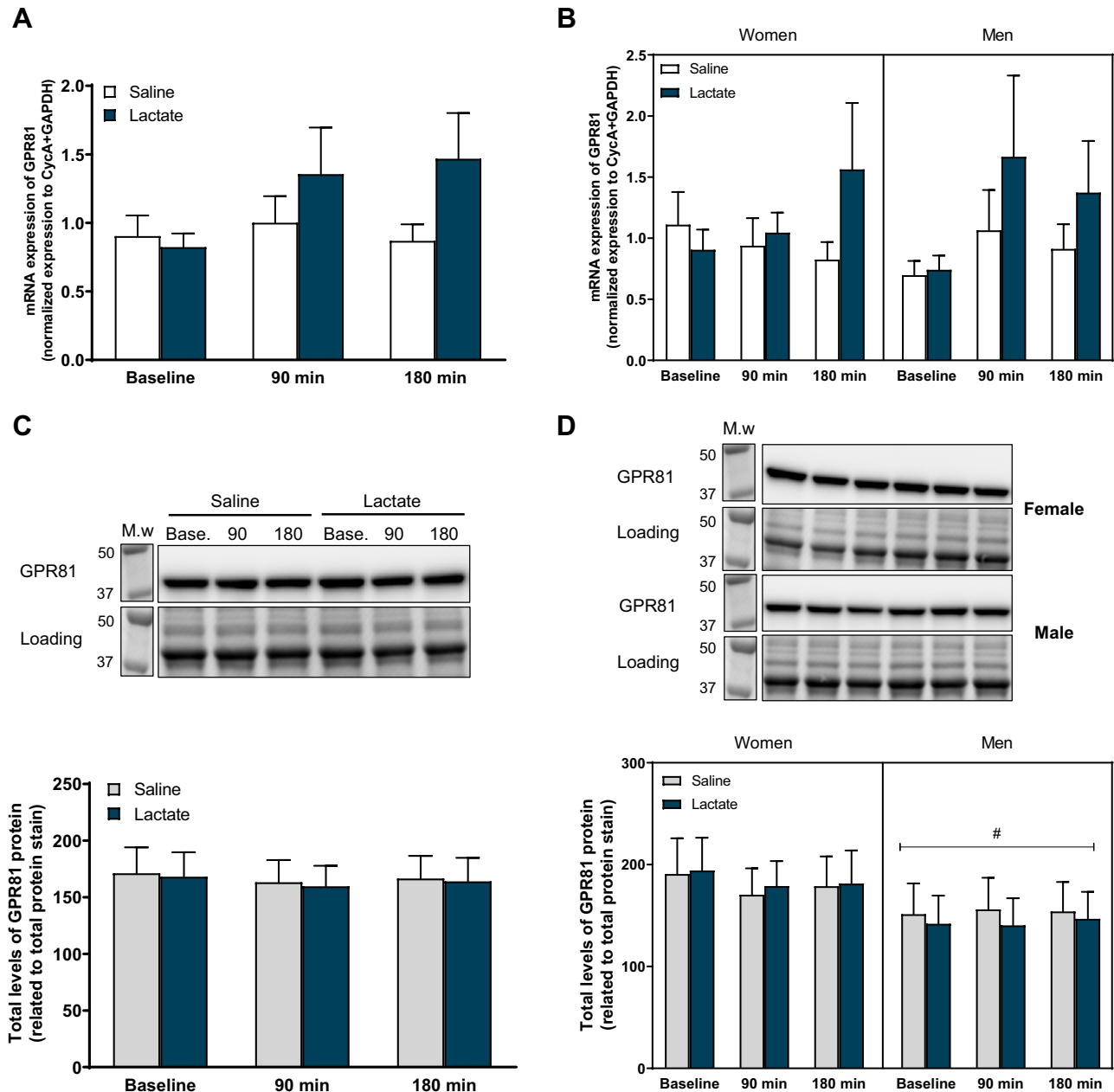


Figure 2. The mRNA expression of GPR81 in muscle samples taken in the rested state at baseline as well as after 90 and 180 min of recovery from exercise, illustrated for the whole group ($n = 16$ subjects, **A**) and for the women and the men separately ($n = 8$ subjects each, **B**). White bars represent the Saline trial and dark blue bars the Lactate trial. The protein expression of GPR81 in muscle samples taken in the rested state at baseline as well as after 90 and 180 min of recovery from exercise, illustrated for the whole group ($n = 16$ subjects, **C**) and for the women and the men separately ($n = 8$ subjects each, **D**). Light gray bars represent the Saline trial and dark blue bars the Lactate trial. Representative blots of GPR81 are provided from one individual in **C**, and for one male and one female in **D**. In **C**, representative bands (at ~ 40 kDa) from the total protein staining (loading control) are provided from the same samples as illustrated for GPR81. The data presented are the means \pm SE for the given group of subjects. #Main effect of sex. M.w, molecular weight.

expression of exercise-sensitive (*PGC-1 α* , *VEGF*, and *LDHA*) or exercise-insensitive (*MCT-1*, *MCT-4*, and *UCP3*) genes.

When GPR81 was first characterized in adipocytes (3, 21), it was shown that human GPR81 mRNA was expressed in skeletal muscle cells, although at a level only 10% of that in adipose tissue (3). However, to the best of our knowledge, a characterization of GPR81 protein expression in human skeletal muscle has not been reported. Using a commercially

available, highly specific, antibody we readily detected the expression of GPR81 protein in human skeletal muscle and found that the relative expression was $\sim 35\%$ of that in human subcutaneous adipose tissue when related to control proteins or total tissue protein (22). However, when related to tissue weight, the expression of GPR81 protein was approximately fourfold higher in muscle compared with adipose tissue. Provided the well-documented role of GPR81 in adipose tissue signaling, it is likely that the high protein

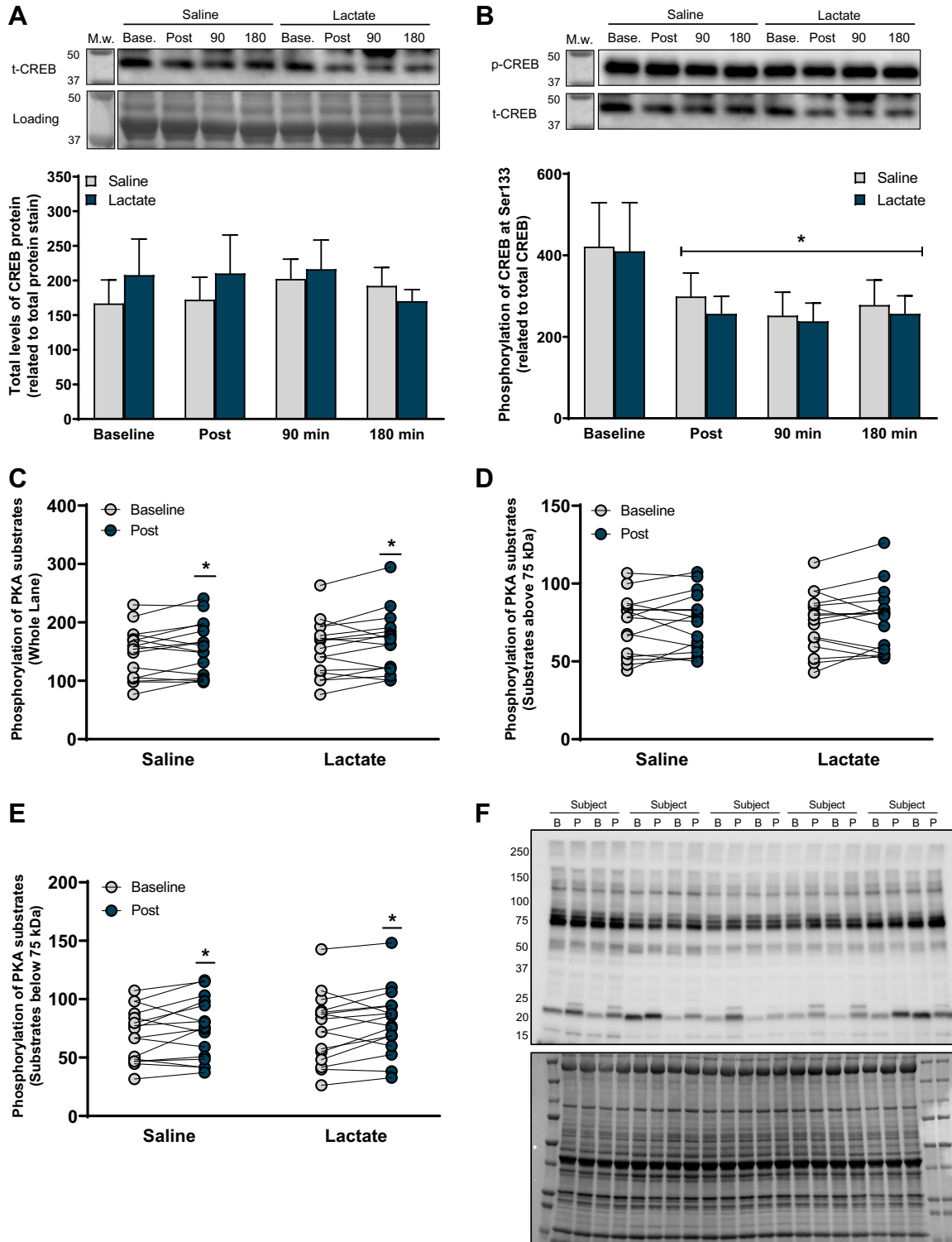


Figure 3. Total levels of CREB protein (A) and phosphorylation of CREB at Ser133 (B) in muscle samples taken in the rested state at baseline, immediately after exercise, as well as after 90- and 180-min of recovery. Light gray bars represent the Saline trial and dark blue bars the Lactate trial. CREB^{Ser133} was normalized to the total protein levels of CREB in each sample. Representative blots from the same subject are provided above the graphs for both total and phosphorylated CREB. Phosphorylation of PKA-substrates at baseline and immediately after exercise quantified for whole lane (C), for substrates above 75 kDa (D), and for substrates below 75 kDa (E). Light gray symbols represent the Saline trial and dark blue symbols the Lactate trial. F: a representative blot for phosphorylated PKA-substrates and the corresponding total protein stain where the membrane contains samples from five subjects where B = Baseline and P = Post. The * represents $P < 0.05$ compared with baseline. In A and B, the data are presented as means \pm SE and in C, D, and E as individual data points, all $n = 16$ subjects. CREB, cAMP-response element binding; M.w., molecular weight; PKA, protein kinase.

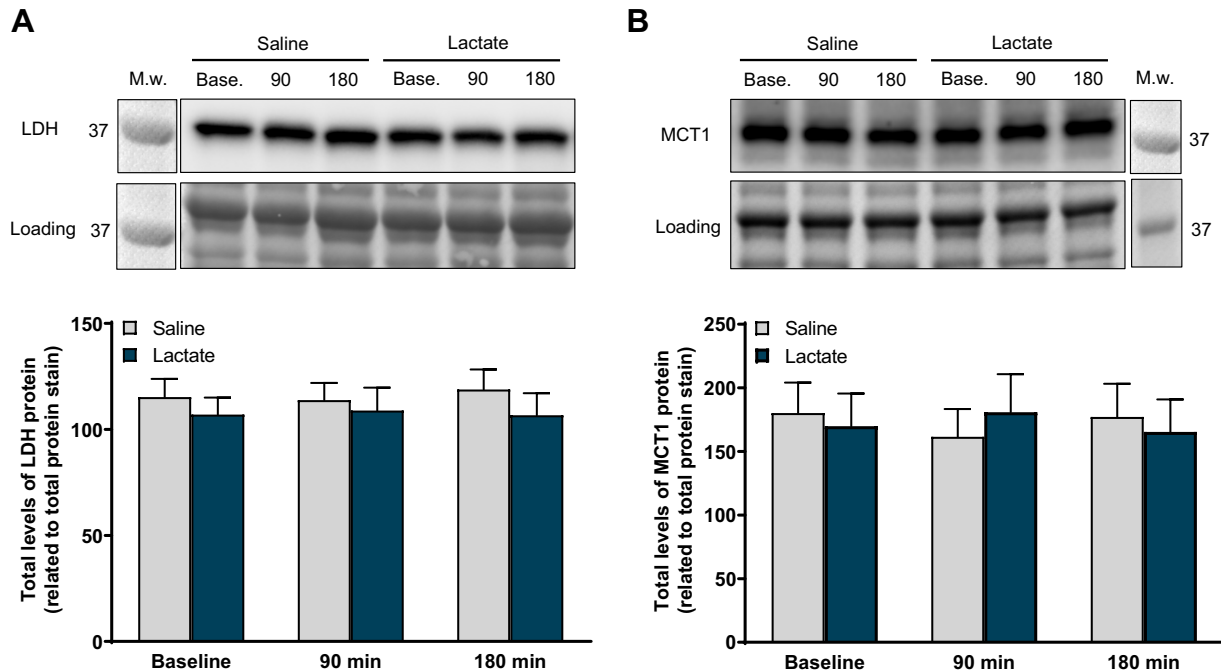


Figure 4. Total protein levels of LDH (A) and MCT-1 (B) in muscle samples taken in the rested state at baseline as well as after 90- and 180-min of recovery from exercise. Light gray bars represent the Saline trial and dark blue bars the Lactate trial. Representative blots from one subject with corresponding representative bands (at ~40 kDa) from the total protein staining (loading control) are shown above each graph. The data are presented as means \pm SE for $n = 16$. LDH, lactate dehydrogenase; MCT-1, monocarboxylate transporter 1.

expression also plays an important role in signaling in skeletal muscle.

Noteworthy, we made the novel observation that GPR81 protein is expressed primarily in type II glycolytic muscle fibers, those that produce the highest amounts of lactate. The difference at the single fiber level was large enough to significantly influence whole muscle levels where individuals who are type II fiber dominant expressed higher levels of GPR81 compared with type I dominant individuals. The higher expression of GPR81 in the lactate-producing glycolytic fibers is intuitively logical and suggests that GPR81 in skeletal muscle plays a role in autocrine lactate signaling. Interestingly, despite having a significantly higher type II composition (MHC type II 67 ± 3 vs. $54 \pm 4\%$), the males exhibited a significantly lower expression of GPR81 compared with the females. This argues for a sex-specific influence in the regulation of GPR81 expression. An obvious potential mechanism to explain this finding is that the higher testosterone levels in men may suppress GPR81 protein expression. However, when analyzing results from women who received exogenous administration of testosterone (23), no change in GPR81 expression was observed (data not shown). Thus, this explanation does not seem tenable. In this context, it would be of interest to assess whether GPR81 expression differs between the sexes in other tissues, including adipocytes.

The expression of GPR81 has been shown to be acutely up-regulated in C2C12 muscle cells by short-term (hours) lactate exposure (6) as well as in mouse gastrocnemius after 5 wk of repeated lactate injections (7). These data argue for a feed-forward mechanism where lactate is able to augment its own signaling properties. However, we found no support for this

mechanism during resistance exercise performed with low or high levels of circulating lactate. Possibly, higher levels or longer exposure to elevated lactate are required to significantly alter muscle GPR81 expression. Regardless, the present data indicate that GPR81 expression in human skeletal muscle is not subject to rapid change.

The best characterized effect of lactate binding to GPR81 is the suppression of the cAMP-PKA pathway and thus inhibition of CREB^{Ser133} phosphorylation (3, 4, 6). The importance of this mechanism in human skeletal muscle with exercise can be assessed as follows. During isometric contraction, when high levels of muscle lactate are obtained, there is no change in muscle levels of cAMP (24), likely because the circulation to the muscle is occluded and, therefore, adrenaline cannot reach its receptor on the cell surface. This clearly dissociates between lactate and accumulation of cAMP under these conditions. During heavy dynamic exercise, where the circulation is intact, cAMP increases together with large increases in lactate. However, when the same exercise is repeated in the presence of the β -blocker propranolol, accumulation of cAMP is abolished, whereas the increases in muscle lactate accumulation remain high (24). Finally, during moderate dynamic exercise, the activity of PKA increases 60%–70% regardless of whether exercise is performed with high glycogen levels (large increases in muscle lactate) or low glycogen levels (small increases in muscle lactate; 25, 26). Taken together, these results speak against an important role of lactate in the accumulation of cAMP and activation of PKA.

It is possible that the lack of additional effects of the elevated lactate levels in the present study derive from lactate levels after infusion that were too low or that exercise blunts

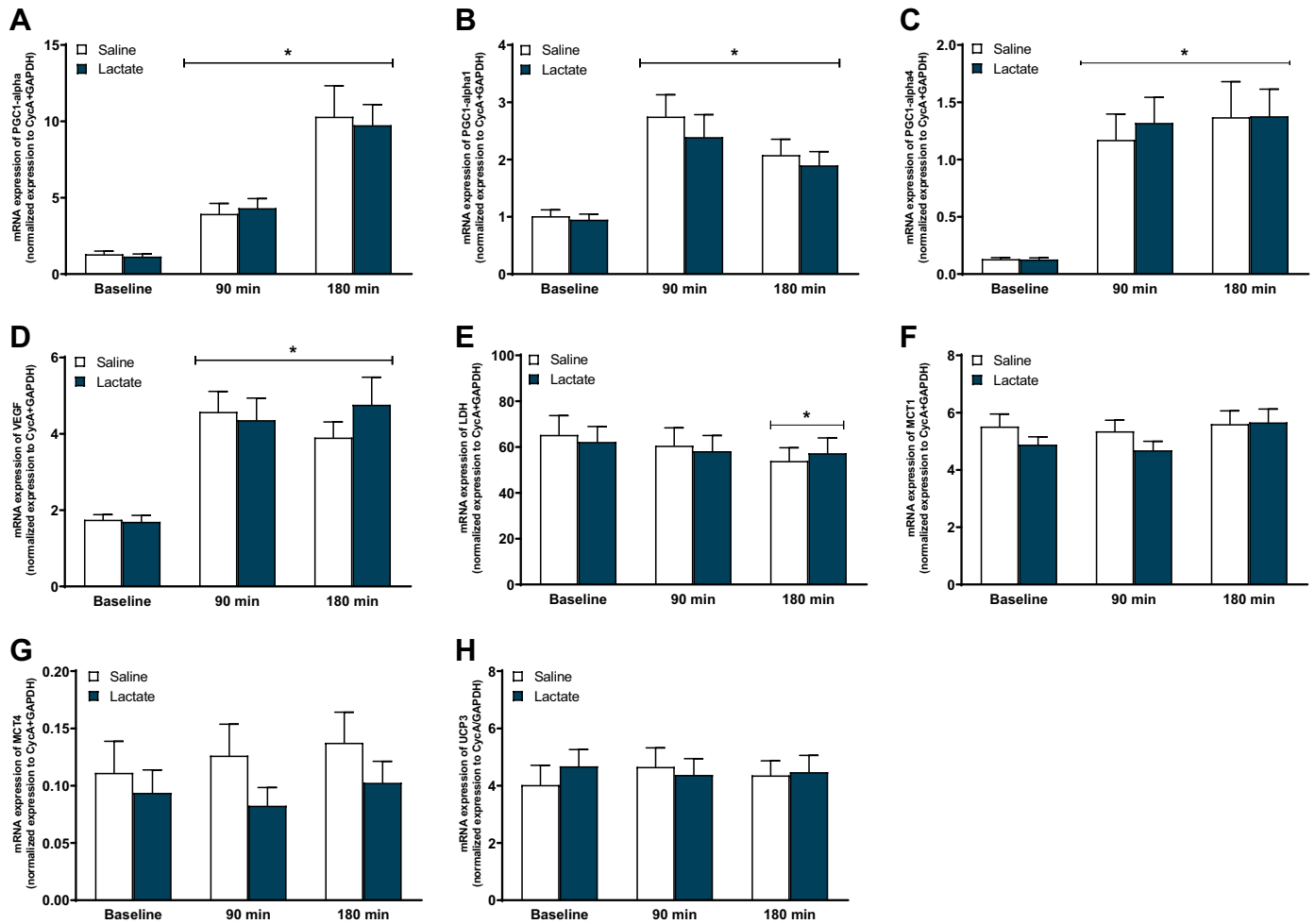


Figure 5. The mRNA expression of PGC-1 α total (A), PGC-1 α 1 (B), PGC-1 α 4 (C), VEGF (D), LDH (E), MCT1 (F), MCT4 (G), and UCP3 (H) in muscle samples taken in the rested state at baseline as well as after 90- and 180-min of recovery from exercise. White bars represent the Saline trial and dark blue bars the Lactate trial. The * represents $P < 0.05$ compared with baseline. The data are presented as means \pm SE for $n = 16$. MCT-1, monocarboxylate transporter 1; PGC-1 α , peroxisome-proliferator-activated receptor gamma coactivator 1 alpha; VEGF, vascular endothelial growth factor.

any influence of lactate. Regarding the first possibility, the described EC₅₀ value for lactate on GPR81 in vitro is 1.5–5 mM and accordingly, the plasma levels of lactate in the saline trial (average 4.4 mM) and the lactate trial (average 8.9 mM) should have activated the receptor in both conditions, and potentially enhanced the activity in the latter. Moreover, our observation that CREB^{Ser133} phosphorylation decreased during exercise, whereas PKA was activated, indicates that CREB phosphorylation during resistance exercise is primarily due to activation of protein phosphatases rather than kinases. It should, however, be noted that kinases other than PKA can phosphorylate CREB at the Ser133 residue (8), e.g., mitogen-activated protein kinases and calcium-sensitive kinases that can be affected by muscle contractile activity. This notion argues against exercise itself overriding any potential signaling effect of lactate alone in this setting. Similar reasoning applies to PKA, whose activity increases during muscle contraction (25, 27), but reduced after lactate administration in rodent skeletal muscle at rest (7). Collectively, the data suggest that lactate does not regulate CREB^{Ser133} phosphorylation through GPR81/PKA signaling during exercise.

The first evidence of lactate signaling in muscle tissue was provided by Hashimoto et al. (13) who showed that incubation of C2C12 muscle cells with 10 mM of lactate stimulates the mRNA expression of PGC-1 α and MCT-1. The effect on PGC-1 α transcripts was later also confirmed in an in vivo rodent model where intraperitoneal sodium lactate injections induced plasma lactate levels of 18 mM (14). Another consistent finding is lactate-stimulated VEGF mRNA and protein expression in nonmuscle tissue after exposure of lactate levels ranging from 5 to 15 mM (15, 28, 29). As expected, the exercise protocol in the present study induced a robust increase in the expression of VEGF, PGC-1 α total, PGC-1 α 1, and PGC-1 α 4 (30–33), but without any influence of the markedly elevated plasma lactate levels in the infusion trial. Despite over 100% difference in plasma lactate levels between trials the peak of ~ 9 mM in the Lactate trial is nevertheless lower than the 10–20 mM used in previous cell and rodent studies, which could explain the lack of significant impact here. In contrast, Gustafsson et al. (30) found a significant relationship between VEGF expression and exercise-induced plasma lactate levels, all below 10 mM. As noted previously, all previous studies on lactate signaling in

skeletal muscle tissue are performed on resting cells and, thus, it is possible that the strong stimuli evoked by the exercise here over-rides any influence evoked by lactate. This speculation recently gained support in an exercise/lactate injection mouse model (34).

In conclusion, GPR81 protein is significantly expressed in human skeletal muscle at markedly higher levels in type II glycolytic fibers compared with type I oxidative fibers, suggesting that lactate may function as an autocrine signaling molecule. Our data also indicate a sex-specific regulation of muscle GPR81 expression. Finally, it is unlikely that lactate-activated GPR81 signaling controls CREB^{ser133} phosphorylation during exercise. The pathway of lactate-dependent GPR81 signaling in skeletal muscle during exercise remains to be established.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.K. and M.M. conceived and designed research; F.N., R.L., W.A., and M.M. performed experiments; F.N., S.J.B., and M.M. analyzed data; W.A., S.J.B., A.K., and M.M. interpreted results of experiments; F.N. and M.M. prepared figures; M.M. drafted manuscript; F.N., W.A., A.K., and M.M. edited and revised manuscript; F.N., R.L., W.A., S.J.B., A.K., and M.M. approved final version of manuscript.

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