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Insulin and Contraction Directly Stimulate UCP2 and UCP3 mRNA Expression in Rat Skeletal Muscle *in Vitro*

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To study the regulation of the mitochondrial uncoupling protein 2 and 3 (UCP2 and UCP3), we studied the effect of insulin and muscle contraction on UCP mRNA expression in rat skeletal muscle *in vitro*. Insulin dose-dependently increased skeletal muscle UCP2 and UCP3 mRNA expression in m. extensor digitorum longus (EDL) with maximal stimulation obtained at around 0.6–6 nM. The concentration of insulin giving half-maximal stimulation was 60 pM for the UCP2 and 48 pM for the UCP3 mRNA expression. The effect of insulin was maximal after 2 h and the effect was sustained during the whole study period (6 h). The insulin-induced increase in UCP mRNA was independent of the glucose uptake (as UCP mRNA was stimulated even in incubations without glucose). In addition, electrically induced contractions (*in vitro*) increased UCP2 and UCP3 mRNA expression 60–120 min after a single bout of contraction (for 10 min). Both the increment of UCP2 and UCP3 mRNA were sustained throughout the study period (4 h) (153 ± 62 and 216 ± 71% above basal, $P < 0.05$ respectively). Finally, 5-aminoimidazole-4-carboxamid-ribosid (AICAR), an activator of the AMP-activated proteinkinase (AMPK), that is activated during exercise, was able to mimic the increase in UCP2 and UCP3 mRNA expression. In conclusion, UCP2 and UCP3 mRNA expression in skeletal muscle are stimulated rapidly by insulin and contraction *in vitro*, thus the stimulation is direct and not caused by changes in other hormones or metabolites. Even a brief bout of contraction induces an increase in UCP2 and UCP3 expression, an effect that could be mimicked by activation of the AMP-activated proteinkinase by AICAR.

The recently discovered mitochondrial uncoupling proteins UCP2 and UCP3 are highly expressed in skeletal muscle (1, 2) and based on the similarity with the UCP1 gene (1, 2), which uncouples respiration from ATP production and thus promotes heat formation (3), they have been implicated in the control of energy expenditure (2). Support for this notion is obtained from transfection studies in yeast and different cell-lines, as cells transfected with UCP2 or UCP3 have decreased cell mitochondrial membrane potential compared nontransfected cells (4–6). However, a number of observations have suggested other roles for the UCPs as recently reviewed (7). This is based on the findings that skeletal muscle UCP3 mRNA is increased during fasting (6, 8), where the energy expenditure actually is decreased. During fasting free fatty acids (FFA) are elevated and a later study convincingly demonstrated that FFA could increase skeletal muscle UCP3 mRNA levels (8). Furthermore, acute exercise (*in vivo*) has been shown to increase UCP3 mRNA expression quickly (9, 10) under circumstances where uncoupling of ATP synthesis might be inappropriate.

As the function of UCP in skeletal muscle is not fully understood, trying to determine factors that regulate UCP mRNA expression in skeletal muscle is still important. Most studies have been performed *in vivo* and therefore the direct effects of the hormones/manipulations are difficult to observe, as several compensatory mechanisms and changes in hormones and substrates might blur the picture.

It is known that hyperinsulinemia increases the energy expenditure (11), however, the precise cellular mechanism is still unknown. Part of the increase has been explained by increase in the sympathetic nervous system, however, even after beta-adrenergic receptor blockade insulin still seems to increase whole body energy expenditure although to a lesser degree (12) suggesting that other pathways than the sympathetic nervous system may be involved.

In a recent publication it was demonstrated that the changes in skeletal muscle UCP3 mRNA were similar to the changes in GLUT-4 mRNA levels in response to

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cold exposure (13). Another study demonstrated that overexpression of GLUT-4 (by transfection) was accompanied by an increase in UCP3 mRNA (14). Thus, these two studies indicate that the glucose flux or metabolism might regulate skeletal muscle UCP3 mRNA expression.

Another stimulus for UCP2 and UCP3 mRNA expression is exercise. It has been shown that acute exercise is accompanied by an increase in skeletal muscle UCP2 and UCP3 expression (9, 15) and that the UCP expression normalizes within 24 h (10). These studies have used exercise protocols where the animals have been forcefully exercised which is a very stressful condition and therefore the effect of exercise on UCP expression cannot be separated from the effect of stress.

In the present study we evaluate the direct effect of insulin, glucose and contractions on skeletal muscle UCP2 and UCP3 mRNA. To be able to study the direct effect of the individual agents isolated rat muscle strips are used *in vitro* thus allowing specific manipulation of each component separately.

MATERIALS AND METHODS

Animals. All experiments were approved by the Danish Animal Experiments Inspectorate and conducted in conformity with the "European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes" (Council of Europe No.123, Strasbourg, France, 1985). Male Wistar rats weighing approximately 70 g (Møllegaard's Breeding Center, Denmark) were deprived of food overnight. After decapitation EDL muscles were rapidly and carefully dissected out avoiding stretching of the muscle fibers. The muscles were incubated in 4 ml oxygenated Krebs–Henseleit bicarbonate buffer (KHB buffer: 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, pH 7.4) containing 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 12 mM mannitol, 8 mM glucose unless stated otherwise and 0.1% bovine serum albumin (RIA Grade; Sigma, St. Louis, MO) as previously described (16). Insulin was present throughout the experiment at the indicated concentrations and every 30 min the incubation medium was changed. At the indicated time points muscles were removed, snap-frozen in liquid nitrogen and stored at −80°C until RNA isolation.

The effect of muscle contraction was investigated using a pulse generator as described previously (16). Briefly, muscles were mounted on two platinum electrodes positioned 3 mm apart and surrounding the central part of the muscle. The mounted muscle was then immersed in 4 ml oxygenated KHB containing the same constituents as described above. Muscles were stimulated to contract for 10 min at 10 Hz with square-wave pulses of 0.5 ms duration and 10 V amplitude. After 30, 60, 120, and 240 min muscles were removed, snap-frozen in liquid nitrogen and stored at −80°C until RNA isolation.

All incubations were carried out at constant temperature (30°C) under continuous gassing with 95% O_2/5% CO_2 in a shaking water bath.

Isolation of RNA. Total RNA was isolated from the biopsies using the TriZol reagent (Gibco BRL, Life Technologies, Roskilde, Denmark), RNA was quantitated by measuring absorbency at 260 and 280 nm and the ratio was 1.8 or higher. Finally, the integrity of the RNA was checked by visual inspection of the two ribosomal RNA's 18S and 28S on an agarose gel.

Reverse transcription PCR (RT-PCR) assay for detection of UCP2 and UCP3 mRNA. Reverse transcriptions were performed using random hexamer primers as described by the manufacturer (GeneAmp RNA PCR Kit from Perkin–Elmer Cetus, Norwalk, CT) at 23°C for 10 min, 42°C for 60 min and 95°C for 10 min. Then, PCR-mastermix containing the specific primers and AmpliTaq GOLD DNA polymerase was added.

A linear increase in PCR product was observed when using RNA ranging between 1 and 300 ng (data not shown), all subsequent PCRs were performed using 25 ng RNA. A second set of PCRs were designed to determine the appropriate number of cycles to be run and the protocol was further optimized in order to coamplify the target and the housekeeping gene by multiplex PCR with primer dropping (17). Semiquantitative multiplex PCR (primer dropping) estimates the relative amount of target RNA to a known housekeeping gene (β-actin) and eliminates the sample to sample variability of the RT-step, as well as the PCR step. The lower expression of the target compared to the housekeeping mRNA (β-actin) was managed by preamplification of the target cDNA by performing a few PCR cycles before the housekeeping primers were added to the PCR tube (primer dropping) (17, 18). The number of initial cycles before dropping the housekeeping primers were 10 for UCP2 and 8 for UCP3. The β-actin primers were in all cases coamplified with the target genes for the remaining 24 cycles.

A similar setup was used for negative controls except that the reverse transcriptase was omitted and no PCR- products were detected under these conditions. The following primer pairs were used: UCP2 primers: 5′-TTCAAGGCCACAGATGTGCC and 5′-TCGGGCAATGGTCTTGTAGGC. UCP3 primers: 5′-CCCTGACTCCTTCCTCCCTG and 5′-GCACTGCAGCCTGTTTTGCTGA. β-Actin: 5′-TGTGCCCATCTACGAGGGGTATGC and 5′-GGTACATGGTGGTGCCGCCAGACA. PCR protocol: 95°C in 10 min, then each cycle comprising 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s. Finally, the PCR products were extended for 5 min at 72°C.

The PCR-products were loaded on a 2% agarose gel, stained with ethidium bromide, and analyzed using the Bio-Rad Gel-Doc 1000 system and the ratio between target PCR product and the β-actin PCR product was calculated. The coefficient of variation for the determination of the UCP/β-actin ratio was 12% ($N = 15$).

Statistical analysis. When multiple comparisons were made ANOVA followed by Duncan's post hoc analysis was used. For the time course studies and dose response studies, a GLM Repeated Measures analysis was used, finally Student's t test was used to analyze for difference between two mean values, a P value less than 0.05 was considered as significant. Data are given as means ± SEM. All analyses were performed with the SPSS 10.0.7 statistical package (SPSS, Inc., Chicago, IL).

RESULTS

Regulation of UCP2 mRNA and UCP3 mRNA in Skeletal Muscle in Vitro

Effects of different hormones (Fig. 1). Stimulation of EDL muscles for 4 h with triiodothyronine (T3) (100 mM) isoproterenol (200 nM), leptin (50 nM), growth hormone (GH) (80 ng/ml) did not influence UCP2 mRNA expression (Fig. 1A) or UCP3 expression (Fig. 1B). As shown insulin (6 nM) increased both UCP2 and UCP3 expression during the 4-h incubation period.

Time course of insulin (Fig. 2). As shown in Fig. 2A (UCP2 mRNA in EDL) and Fig. 2B (UCP3 mRNA in EDL) the UCP2 and UCP3 mRNA expression did not change during the 6 h incubation under baseline conditions. Addition of insulin (6 nM) time-dependently

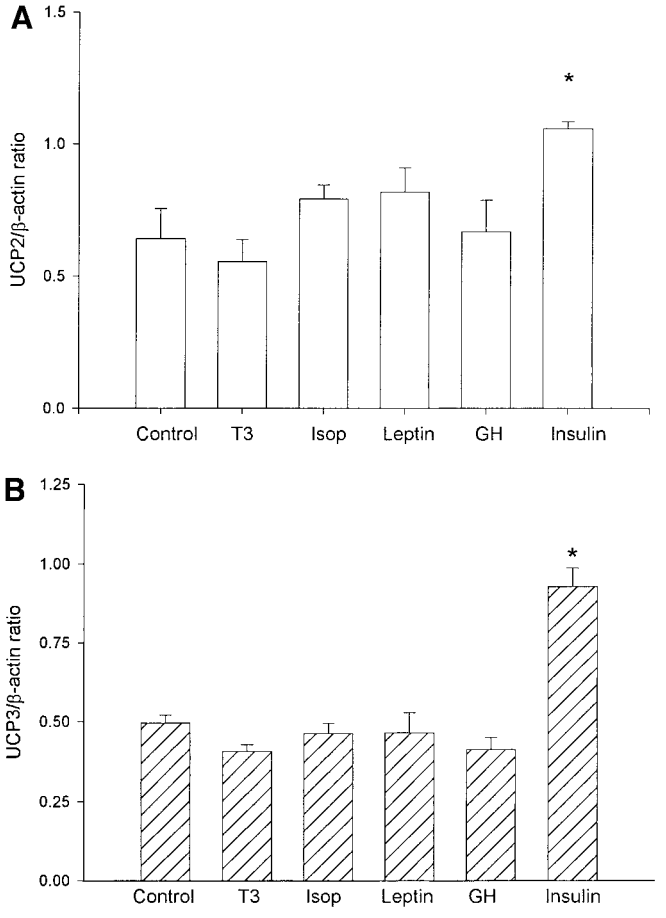

FIG. 1. Effect of triiodothyronine (T3) (100 mM), isoproterenol (200 nM), leptin (50 nM), growth hormone (GH) (80 ng/ml), and insulin (6 nM) on UCP2 mRNA (A) and UCP3 mRNA (B). EDL muscles were incubated for 4 h with the indicated hormone before UCP mRNA expression was quantitated by RT-PCR (*$P < 0.05$, $N = 6$).

increased both UCP2 and UCP3 mRNA expression in EDL. Already after 2 h the stimulatory effect of insulin on the UCP mRNA expression was maximal, and the expression remained elevated during the rest of the study period (6 h) (similar results were obtained in m. soleus, data not shown).

Insulin dose response (Fig. 3). Insulin dose-dependently increased UCP2 and UCP3 mRNA expression. For both UCP's the maximal increase was observed with insulin concentrations around 0.6–6 nM and insulin at 60 nM did not further increase the UCP expression (Figs. 3A and 3B). For the UCP2 stimulation the insulin concentration giving half maximal stimulation (EC_{50}) was 60 pM and for the UCP3 expression the EC_{50} value was in the same range, 48 pM.

Interactions between insulin and glucose on the UCP mRNA expression (Fig. 4). To discriminate between a direct insulin effect and an effect mediated through increased glucose uptake and metabolism in the muscle, EDL muscles were incubated at different glucose concentrations (0, 8, and 20 mM) with or without insulin (6 nM) for 4 h. As shown in Figs. 4A and 4B insulin stimulated both UCP2 and UCP3 mRNA in incubations performed without glucose in the medium, indicating that the insulin effect on UCP mRNA is not mediated by an increase in glucose uptake. In addition, high concentrations of glucose (20 mM) reduced both the basal and the insulin stimulated UCP3 expression to 50-60% of the UCP3 expression observed at 8 mM glucose ($P < 0.05$).

Effects on Contraction on UCP mRNA Expression in Vitro (Fig. 5)

As exercise and contraction is known to affect skeletal muscle UCP mRNA expression *in vivo,* we used the *in vitro* incubation system to investigate whether the effects of contraction were direct or mediated through changes in the hormonal milieu during exer-

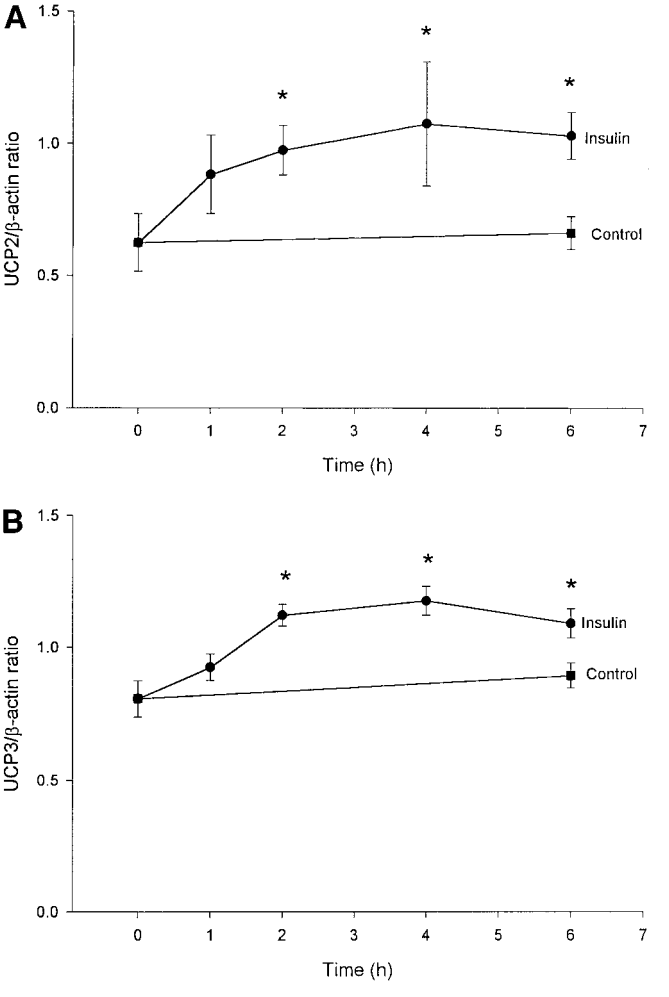

FIG. 2. Time course of the insulin effect on UCP mRNA expression. Rat skeletal muscles (EDL) were incubated without or with insulin (6 pM) for the indicated time, then the UCP2 mRNA (A) and UCP3 mRNA (B) levels were quantitated (*$P < 0.05$, $N = 6$).

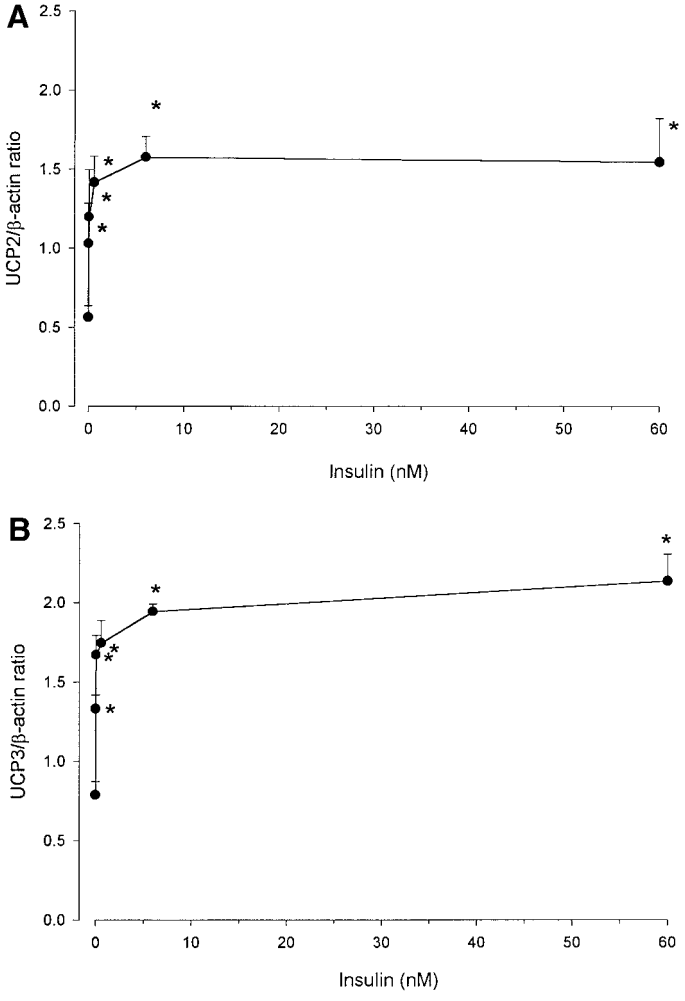

FIG. 3. Insulin dose–response curves for UCP2 (A) and UCP3 (B). Rat skeletal muscles (EDL) were incubated for 4 h with the indicated concentrations of insulin. UCP mRNA expression was quantitated by RT-PCR (*$P < 0.05$, $N = 6$).

cise. Contractions were induced by electrical stimulation *in vitro* (continuously contractions at 10 Hz for 10 min) and the UCP mRNA expression was analyzed after 30, 60, 120, and 240 min. As shown in Fig. 5. even contraction for this short period (10 min) induced UCP mRNA expression. UCP2 mRNA was quickly increased and 1 h after the last contraction it was increased 139 ± 35% ($P < 0.05$) above basal, and the UCP2 mRNA level remained elevated throughout the study period (4 h) (153 ± 63, $P < 0.05$). The increase in UCP3 mRNA seemed slower in onset than UCP2 (Fig. 5B), UCP3 mRNA was significantly increased 2 h after the stimulation and remained elevated for at least 4 h (216 ± 71% above basal level, $P < 0.05$).

Effects of AICAR on the UCP Expression in Rat Skeletal Muscle Strips (in Vitro) (Fig. 6)

An important enzyme concerning glucose metabolism in exercising skeletal muscle is the 5′-AMP activated protein kinase (AMPK) which is activated during exercise, and previously it has been shown that the exercise induced increase in glucose uptake can be mimicked by AICAR an activator of the AMPK. Therefore, we incubated rat EDL muscles for 4 h with 0.5 mM AICAR and as shown in Fig. 5. AICAR stimulated both the UCP2 and UCP3 mRNA expression in isolated skeletal muscle strips.

DISCUSSION

In the present study we demonstrated that UCP2 and UCP3 mRNA expression are increased in skeletal muscle after stimulation with insulin (1–2 h). As UCP2 and UCP3 are suggested to uncouple the respiratory chain from ATP formation and thus dissipate energy as heat (4–6), our findings might help to understand some of the mechanisms behind the thermogenic effect of insulin (19). The precise nature of the insulin/

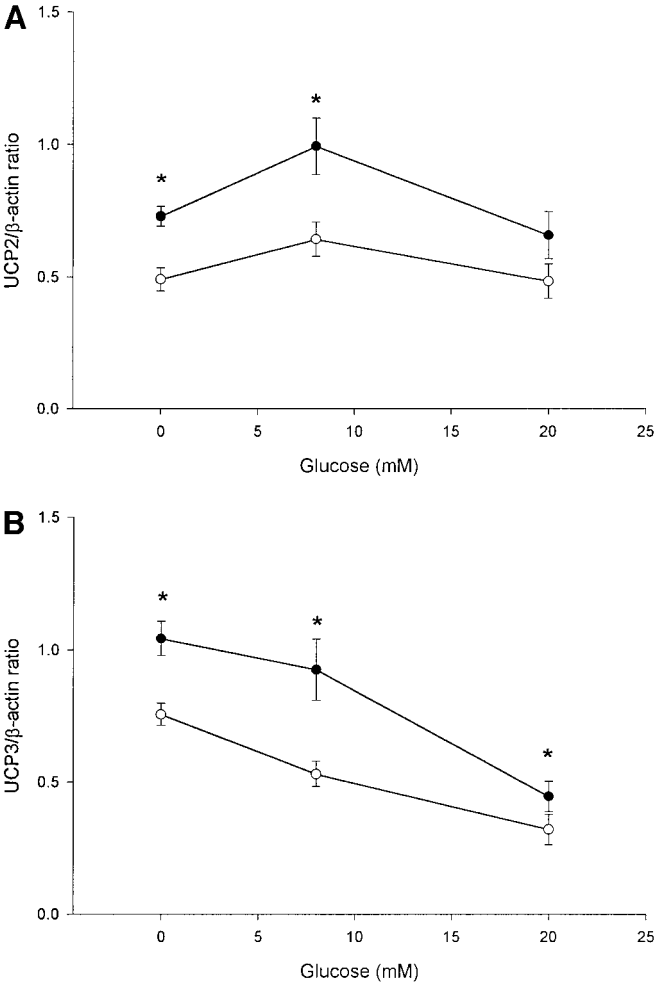

FIG. 4. Effects of glucose and insulin on the UCP2 mRNA expression (A) and UCP3 mRNA expression (B). Rat skeletal muscles (EDL) were incubated without insulin (○) or with insulin (●) in media containing 0, 8, and 20 mM glucose (*, $P < 0.05$, $N = 8$).

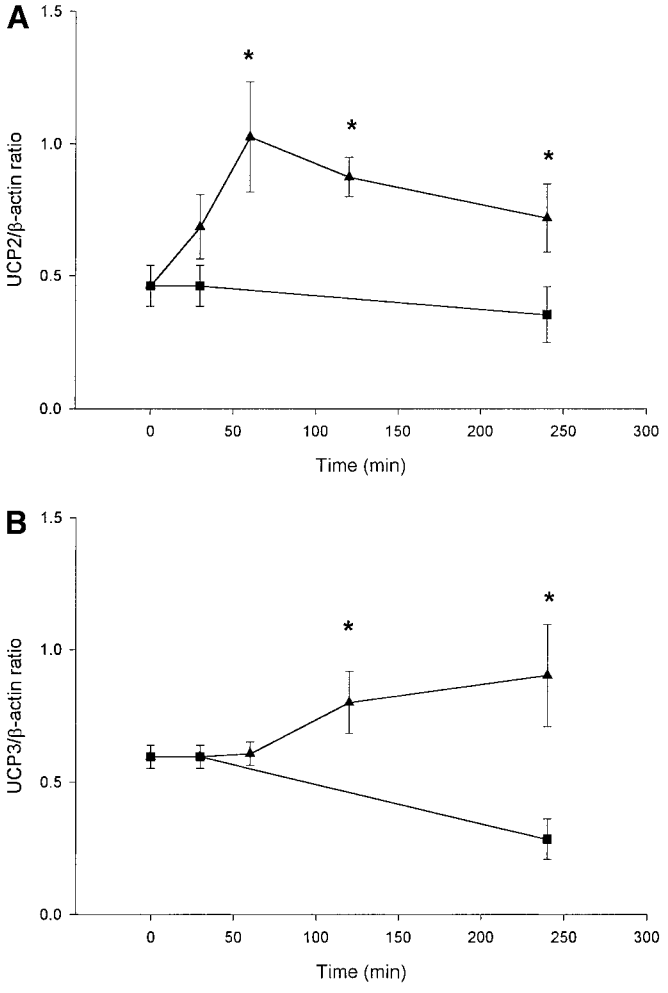

FIG. 5. Effects of electrically induced contractions on skeletal muscle UCP expression. Skeletal muscle (EDL) was made to contract for 10 min (10 Hz) and then the UCP2 mRNA expression (A) and UCP3 mRNA (B) expression were investigated immediately after the contraction ($t = 0$), 30, 60, 120, and 240 min after stopping the contractions (*$P < 0.05$, $N = 8$ at each time point).

glucose mediated increase in energy expenditure *in vivo* probably have several components, a part which may be mediated through an increase in the sympathetic nervous system as the increase in thermogenesis could be partly blocked (64–70%) by beta-adrenergic blockade (12, 20, 21), and smaller part that is independent of the sympathetic nervous system. The latter component is not fully characterized but based on our findings it may involve insulin mediated upregulation of UCP2 and UCP3 in skeletal muscle. The earlier *in vivo* investigations on the thermogenic effect of insulin have used either euglycemic hyperinsulinemic clamp (11, 20) or glucose ingestion (22) and therefore it has been impossible to separate the effects of increased glucose flux into the muscle from any direct insulin effect on the thermogenesis.

To our knowledge the effects of insulin on skeletal muscle UCP expression have not been tested directly previously. However, Yoshitomi *et al.* (23) found a close correlation between changes in serum-insulin and changes in skeletal muscle UCP2 and UCP3 mRNA in rats (treated wit a β_3 adrenergic agonist) and suggested that insulin might regulate UCP mRNA expression in skeletal muscle. Recently, it was demonstrated that transgenic mice overexpressing the GLUT-4 glucose transporter in skeletal muscle and adipose tissue had an increase in UCP3 mRNA expression in the same tissues (14) and it was suggested that the increased glucose flux in these tissues was responsible for the increase in UCP3 mRNA expression. This was also suggested in the study by Lin *et al.* (13) based on the similar regulation of UCP3 mRNA and GLUT-4 mRNA in skeletal muscle from rats after cold exposure. However, in these earlier studies it is difficult to evaluate the direct effect of either insulin or glucose on the UCP mRNA expression as *in vivo* treatment with insulin and/or glucose is associated with changes in the sympathetic nervous system and an increase in skeletal muscle uptake of glucose. Our results clearly show that insulin increase both UCP2 and UCP3 mRNA expression in skeletal muscle independent of the glucose transport as the insulin effect was similar in skeletal muscle incubated without glucose.

We also tested the direct effect of other hormones, but were unable to demonstrate any effect of T3, isoproterenol, leptin and GH in our *in vitro* incubation system. Previously *in vivo* treatment with leptin (4, 24), GH (18) and T3 (4, 5) are reported to increase in muscle UCP expression, the discrepancy might be caused by differences in the studies (in vivo versus *in vitro*) but also the time factor must be taken into consideration (hours versus days). Regarding the lack stimulation by isoproterenol our study is well in line

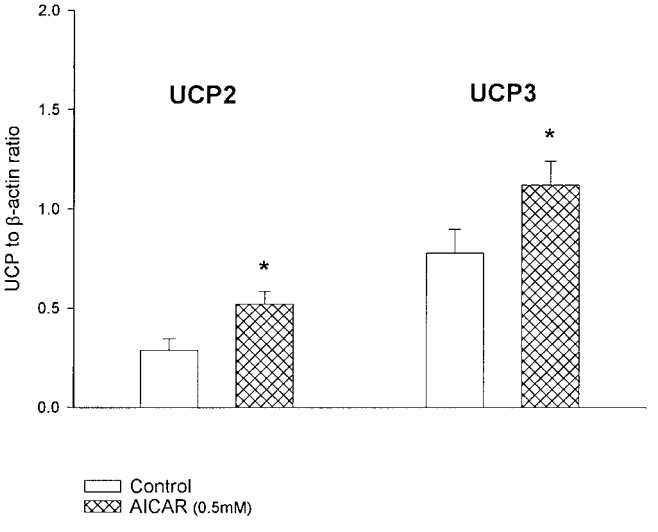

FIG. 6. Effect of 5-aminoimidazole-4-carboxamid-ribosid (AICAR) 0.5 mM on UCP2 mRNA. Isolated muscle strips (EDL) were incubated for 4 h (*$P < 0.05$, $N = 6$).

with the study by Yoshitomi demonstrating that the UCP2 promoter contained a cAMP-response element, but isoproterenol was unable to stimulate the UCP2 mRNA expression (25).

Finally, we studied the effect of muscle contraction *in vitro.* Previous studies have indicated that UCP mRNA is up-regulated acutely after exercise whereas UCP mRNA levels fall to values even below initial values after 12–24 h. Using our *in vitro* model system with isolated skeletal muscle we could demonstrate that electrical induced contractions were able to induce UCP mRNA expression shortly after a single burst of contraction, and this increase in UCP mRNA expression remained for at least 4 h. These results clearly demonstrate that exercise/contraction by itself can increase skeletal muscle UCP mRNA expression independent of any changes in the hormonal milieu.

Finally, we demonstrated that stimulation of the muscle strips with AICAR which, like contractions, stimulates the AMP-activated protein kinase (26, 27), resulted in an increase in both UCP2 and UCP3 mRNA expression. A similar finding was recently reported by Zhou *et al.* (15), indicating that the effects of contraction on UCP expression probably are mediated through activation of the AMP-activated proteinkinase.

In conclusion, even a short electrically induced contraction rapidly stimulates UCP2 and UCP3 mRNA expression *in vitro,* an effect that can be mimicked by an AMPK activator (AICAR). Furthermore, our findings prove that insulin directly stimulates skeletal muscle UCP2 and UCP3 mRNA expression, and that the insulin stimulation is fast and unrelated to the accompanying glucose influx in the muscle cells. These data might support the notion that an insulin-mediated increase in skeletal muscle UCP expression could play a role for the insulin-induced increase in thermogenesis observed *in vivo.*

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