Downregulation of uncoupling protein-3 in vivo is linked to changes in muscle mitochondrial energy metabolism as a result of capsiate administration

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Faraut B, Giannesini B, Matarazzo V, Marqueste T, Dalmasso C, Rougon G, Cozzone PJ, Bendahan D. Downregulation of uncoupling protein-3 in vivo is linked to changes in muscle mitochondrial energy metabolism as a result of capsiate administration. Am J Physiol Endocrinol Metab 292: E1474–E1482, 2007. First published January 30, 2007; doi:10.1152/ajpendo.00292.2006.—Although it has been suggested that the skeletal muscle mitochondrial uncoupling protein-3 (UCP3) is involved in regulating energy expenditure, its role is still poorly understood. In the present study, we aimed at investigating noninvasively, using magnetic resonance techniques, metabolic changes occurring in exercising muscle as a result of capsiate treatment, which has been previously linked to UCP3 upregulation. We showed that capsiate ingestion strongly reduced UCP3 gene expression in rat gastrocnemius muscle. This large underexpression was accompanied by a significant increase in the rate of mitochondrial ATP production and phosphocreatine level both at rest and during muscle stimulation. Similarly, the stimulation-induced ATP fall and ADP accumulation were significantly less after capsiate administration than in untreated rats. The larger oxidative ATP production rate could not be explained by a proportional decrease in the anaerobic component, i.e., glycolysis and phosphocreatine breakdown. In addition, the mechanical performance was not affected by capsiate administration. Finally, the plasma free fatty acid (FFA) level increased in capsiate-treated rats, whereas no significant change was observed after muscle stimulation in the control group. Considering the corresponding enhanced UCP3 mRNA expression occurring in the control group after muscle stimulation, one can suggest that changes in FFA level and UCP3 mRNA expression are not mechanistically correlated. Overall, we have shown that capsiate administration induced a UCP3 downregulation coupled with an increased mitochondrial ATP synthesis, whereas the muscle force-generating capacity was unchanged. This suggests that a decrease in muscle efficiency and/or additional noncontractile ATP-consuming mechanisms result from UCP3 downregulation.

UNCOUPLING PROTEINS (UCPS) are coded by nuclear DNA, transported to the inner mitochondrial membrane, and can function as proton channels draining protons from the intermembrane space directly into the mitochondrial matrix. In that respect the ATP synthase is bypassed, and the driving proton force occurring at the mitochondrial level is not coupled with ATP production. In addition to that, UCPS have been shown to reduce mitochondrial transmembrane potential and electron transfer, thereby limiting reactive oxygen species production.

One member of this family, UCP3, is a mitochondrial inner membrane protein that is predominantly expressed in skeletal muscle (40). On the basis of its closed sequence homology with UCP1, a protein that uncouples respiration from ATP production in brown adipose tissue, thereby promoting heat production in response to cold or overfeeding, UCP3 has been proposed to be involved in the control of energy expenditure, but its exact function still remains unclear. It has also been reported (31) that UCP3 would facilitate mitochondrial FFA oxidation due to its capacity of exporting fatty acids out of the mitochondrial matrix, thereby preventing their intramitochondrial accumulation.

To elucidate the role of UCP3 in muscle energetics, a few studies have been conducted in isolated mitochondria and in vivo. On the one hand, isolated mitochondria lacking UCP3 have a decreased state 4 oxygen consumption and have been considered as more coupled compared with control mitochondria. On the other hand, muscle mitochondria from mice overexpressing UCP3 have an increased state 4 oxygen consumption, indicating mitochondrial uncoupling (5, 39). However, indications about the role of UCP3 in muscle energetics are still lacking. In the present study, we aimed at investigating the physiological function of UCP3 in an integrative approach in vivo. This issue has actually been very scarcely addressed. In vivo 31P magnetic resonance spectroscopy (31P-MRS) experiments have suggested that the efficiency of mitochondrial ATP synthesis would be increased in UCP3-knockout mice (9). However, this acceleration in the rate of mitochondrial ATP synthesis is inconsistent with the lack of phenotypic changes at the whole body level (i.e., global energy metabolism and thermoregulation) (9, 39). Conversely, it has been reported (17) that UCP3 overexpression led to a decrease in mitochondrial energy coupling that could also explain the hypermetabolic status in hyperthyroidism (10). Overall, the metabolic changes associated with UCP3 expression are still unclear, particularly in relation to muscle energy consumption and fat metabolism.

Interestingly, capsiate administration has been reported to affect both UCP3 gene expression and whole body energy metabolism (24). Capsiate is actually a chemical analog of capsaicin, with an ester bond replacing the amide bond between the vanillyl moiety and the fatty acid chain (20). Capsaicin specifically binds the cell surface receptor “vanilloid receptor-1” (VR-1), a nociceptive cationic channel present on sensory nerve endings and activated by noxious heat (7). In
VR-1 knockout mice, the impaired nociception strongly supports a correlation between the pungency properties of capsaicin and the VR-1 receptor activation in vivo (6). Similarly to capsaicin, its analog (capsiate) can also bind and activate the VR-1 receptor and induce nociceptive responses when injected subcutaneously. However, neither neurotoxic nor pungent property has been reported when capsiate has been administered orally (16, 25). This analogy has also been reported regarding the metabolic effects. Similarly to capsaicin, oral administration of capsiate can elevate basal oxygen consumption and body temperature (25, 26). Also, capsiate administration in mice has been shown to increase the mitochondrial UCP1 and UCP3 expression in brown adipose tissue and skeletal muscle, respectively, and to suppress body fat accumulation (24, 25). However, the possible cause-effect relationship between capsiate-induced changes in UCP3 gene expression and muscle energy metabolism has not been documented so far.

We aimed in the present study at investigating the metabolic changes occurring in rat exercising muscle as a result of capsiate administration, which has been previously linked to an increase in UCP3 gene expression (24). We used an original experimental setup, allowing a strictly noninvasive investigation of skeletal muscle function in rat gastrocnemius muscle using magnetic resonance (MR) techniques (11, 12). These energy metabolism changes were analyzed with respect to the capsiate-induced changes in UCP3 gene expression and free fatty acid (FFA) metabolism.

**MATERIALS AND METHODS**

**Animal care and feeding.** Thirty-eight Sprague-Dawley female rats (Charles River Laboratory, L’Arbresle, France) weighing 275–300 g were used for these experiments according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals and the French Law–Related Animals Protection. Rats were housed in an environmentally controlled facility (12:12-h light-dark cycle, 22°C) and received water and standard food ad libitum until the time of experiment.

**Experimental protocols.** The capsiate solution obtained from Ajinomoto (Tokyo, Japan) consisted of capsiate and its dihydroderivatives (63% capsiate, 30% dihydrocapsiate, 7% nordihydrocapsiate) and was synthesized as reported previously (20). As a control solution, we used the capsiate vehicle, i.e., a solution containing 0.9% NaCl, 3% ethanol, and 10% Tween-80, as described elsewhere (25).

Animals were orally administrated either vehicle or 100 mg/kg body wt capsiate, and measurements were performed 2 h after the treatment. Basal energy metabolism and UCP3 expression changes have been already reported after a similar period of time in mice (24, 25). The first set of experiments was designed to evaluate the effect of capsiate on energy metabolism and force production in gastrocnemius muscle throughout a rest-stimulation-recovery protocol. For this purpose, 14 rats were randomly assigned either to the vehicle (n = 7) or to the capsiate (n = 7) group.

The effects of capsiate on gastrocnemius UCP3 gene expression and FFA plasma level were investigated in a second set of experiments in four groups (n = 6 in each group) of rats. Measurements were performed at rest (groups 1 and 2) and after muscle stimulation (groups 3 and 4). Groups 1 and 3 were orally given vehicle, whereas groups 2 and 4 ingested capsiate.

**Animal preparation.** Rats were initially anesthetized in an induction chamber (Equipevement Vétérinaire Minerve) with 4% isoflurane (Forene, Abbot Laboratories) mixed in 33% O₂ (0.5 l/min) and 66% N₂O (1 l/min). The right lower hindlimb was shaved, and electrode cream commonly used for electromyogram measurements was applied at the knee and heel levels to optimize electrical stimulation. The anesthetized rat was placed in supine position in a home-built cradle that was especially designed for the strictly noninvasive functional investigation of the right gastrocnemius muscle (12). This cradle integrates an hydraulic ergometer and two rod-shaped transcutaneous electrodes connected to an electrical stimulator (Stimulator I series, Hugo Sachs Elektronik; Harvard Apparatus) so that, when the rat is placed inside the cradle, one electrode is located above the knee and the other one under the heel. The foot was positioned on the ergometer pedal, and the hindlimb was immobilized in the cradle so that the lower hindlimb was centered inside a 30-mm-diameter ¹H Helmholtz imaging coil and the belly of the gastrocnemius muscle was located above an elliptical (10 × 16 mm) ³¹P-MRS surface coil. The gastrocnemius muscle was passively stretched at rest by adjusting the pedal position to modify the angle between the foot and the lower hindlimb to give maximum isometric twitch tension in response to supramaximal square wave pulses (6–8 mA, 1-ms duration). Throughout the experiment, anesthesia was maintained by gas inhalation with a facemask, which was continuously supplied with 2.5% isoflurane in 33% O₂ (0.4 l/min) and 66% N₂O (0.8 l/min). Corneas were protected from drying by application of an ophthalmic cream (Lacregril, Europharm, Monaco). The facemask was connected to an open-circuit gas anesthesia machine (Isotec 3; Ohmeda Medical). Exhaled and excess gases were removed through a canister filled with activated charcoal (Smiths Industries Medical System) mounted on an electrical pump extractor (Equipevement Vétérinaire Minerve). During anesthesia, body temperature was kept constant (physiological temperature) with an electric heating blanket (Prang & Partner) in a feedback loop with a temperature control unit (reference no. 507137; Harvard Apparatus) connected to a rectal probe (reference no. 507145; Harvard Apparatus).

**Stimulation protocol and force measurement.** The stimulation protocol consisted of 5.7 min of repeated isometric contractions at 3.3 Hz, which were electrically induced with square-wave pulses (6–8 mA, 1-ms duration). Electrical signal coming out from the pressure transducer was amplified (reference no. 13-4515-50; Gould), converted to a digital signal, and processed on a personal computer using ATS software (Cyisma, Aix-en-Provence, France). Isometric force production was calculated every 14.25 s of stimulation by integrating isometric tension (N) relative to time (s).

**MR spectroscopy and data processing.** Investigations were performed in a 4.7-Tesla horizontal superconducting magnet (47/30 Biospec Avance; Bruker, Karlsruhe, Germany). ³¹P-MRS spectra (30 μs rectangular pulse, 16 accumulations, 1.8 s repetition time, 8 kHz spectral width, 512 data points) from the gastrocnemius muscle region were continuously acquired in 28.5-s blocks throughout the experimental protocol, i.e., 5.7 min of rest, 5.7 min of stimulation, and 16.6 min of recovery. Magnetic resonance data acquisition was gated to muscle stimulation to reduce motion artefacts due to contraction. MR data were processed using proprietary software developed on IDL (Interactive Data Language; Research Systems).

Relative concentrations of phosphocreatine (PCr) and ATP were obtained by a time domain fitting routine using the AMARES-MRUI Fortran code (38) and appropriate prior knowledge of the ATP multiplets. Signal areas were corrected for magnetic saturation effects using fully relaxed spectra collected at rest with a repetition time of 20 s. ATP concentration was measured using HPLC in both vehicle and capsiate groups. Absolute concentrations of phosphorylated compounds were expressed relative to ATP concentration measured by HPLC in extracts of freeze-clamped resting gastrocnemius muscle. Muscular intracellular pH (pHᵢ) was calculated from the chemical shift difference between Pᵢ and PCr signals (2). Time points for the time course of pHᵢ and phosphorylated metabolite concentrations were assigned to the midpoint of the acquisition interval.

**Calculations.** The rates of ATP production from PCr degradation, oxidative phosphorylation, and glycolysis were calculated at any point
of the stimulation period using the method proposed by Kemp et al. (18). The rate of ATP production from PCr degradation (D, in mM/min) was directly calculated from the time course of [PCr] throughout the stimulation period: D = −dPCr/dt.

The rate of oxidative ATP synthesis (Q, in mM/min) was calculated considering the fact that oxidative ATP synthesis is controlled by [ADP] throughout a hyperbolic relationship: Q = Qmax/(1 + KADP), in which KADP (the [ADP] at half-maximal oxidation rate) is ~50 μM in rat skeletal muscle and Qmax is the maximal rate of oxidative ATP synthesis. [ADP] was calculated from [PCR], [ATP], and pH using the creatine kinase equilibrium constant (K = 1.67 × 10⁶ M⁻¹). Qmax (in mM/min) was calculated using the initial rate of PCR resynthesis (VPCRrec) during the poststimulation period and the concentration of free cytosolic ADP measured at the end of the stimulation period: Qmax = VPCRrec (1 + [ADP] / [ADP]ead). VPCRrec was calculated at the start of the stimulation period, since VPCRrec = k[PCR]constant, in which [PCR]constant indicates the amount of PCR consumed at the end of the stimulation period. The first-order PCR recovery rate constant (k) was determined from a fitting of the PCR time-dependent changes during the poststimulation period to a single exponential curve described by the equation: [PCR] = [PCR]rest − [PCR]constant e⁻kt, where [PCR]rest is the concentration of PCR measured at rest. The glycolytic rate of ATP synthesis (L, in mM/min) was inferred considering that glycolytic ATP production, when coupled to ATP hydrolysis, is related to proton synthesis (P) with a stoichiometry of 1.5 mol ATP per proton (L = 1.5 P) (15). L was calculated from the proton balance, i.e., pH changes and oxidative phosphorylation (HADP) on the one hand and PCR degradation (HPCR) buffered protons (H⁺), and proton efflux (H⁺flux) on the other hand (18, 41): P = HPCR + HADP − H⁺flux − H⁺CK (in mM/min) was calculated from the time-dependent changes in [PCR] and from the stoichiometric coefficient: P = 1/[1 + pH⁵⁻⁶.575] (Roche, Penzberg, Germany), which represents the proton balance associated with PCR consumption (43): HPCR = −d[PCR] / dt. HADP was calculated from the apparent buffering capacity βtotal (in Stykes, millimoles acid added/unit change in pH) and from the variations in pH (ΔpH = pHobserved − pHrest): HADP = −βtotal ΔpH, where βtotal takes into account the buffering capacity of Pi (βPi) and the buffering capacity of tissue (βtissue): βtotal = βPi + βtissue. βPi was calculated as previously described (43): βPi = 2.3 × 10⁻⁷ [Pi] / {1 + 10⁶⁴⁻⁶.755 [Pi] + 10⁵⁻⁶.755 [Pi]³}. It has been demonstrated (1) that βPi varies linearly between pH = 7 (16 Stykes) and pH = 6 (37 Stykes) in rat gastrocnemius muscle. According to these data, βtissue was calculated as follows: βtissue = −21 pH + 163. H⁺flux (in mM/min) was calculated for each time point of muscle stimulation using the proportionality constant λ-relating proton efflux rate to ΔpH: H⁺flux = −λΔpH. This proportionality constant λ (in mM·min⁻¹·pH unit⁻¹) was calculated at the start of the poststimulation recovery period: λ = −d[H⁺ flux] / dt. During this period, PCR is regenerated throughout the creatine kinase (CK) reaction as the consequence of oxidative ATP synthesis in mitochondria, and H⁺flux can be calculated from the rates of proton production from the CK reaction (H⁺CK, in mM/min) and mitochondrial ATP synthesis (H⁺OX, in mM/min) on one side and the rate of pH changes on the other side. At this time ATP production is exclusively aerobic, and lactate production is considered as negligible: H⁺flux = H⁺CK + H⁺OX + βtissue ΔpH / dt. H⁺OX (in mM/min) was calculated from the factor m = 0.16/[1 + 10⁶⁴⁻⁶.755 [Pi] + 10⁵⁻⁶.755 [Pi]³], which accounts for the amount of protons produced through oxidative ATP synthesis: H⁺OX = mVPCRrec.

Oxidative ATP cost of contraction [in mM·(N·s)⁻¹] was calculated throughout the whole stimulation period as the ratio between the total amount of oxidative ATP (Q) produced during the 5.7 min of stimulation and the total isometric force (Fisotonic in N·s) produced during the same period of time. Nonoxidative ATP cost of contraction [in mM·(N·s)⁻¹] was calculated similarly from ATP production by creatine kinase reaction and glycolysis (D + L).

Quantitative real-time RT-PCR analysis. Gastrocnemius muscle was dissected and immediately frozen in liquid nitrogen and then homogenized. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA was then treated with 1 unit of DNase I (Promega) to remove genomic DNA and reverse transcribed to cDNA using the Superscript III first-strand RT-PCR system kit (Invitrogen). Briefly, 4 μg of RNA were added with 50 ng of random hexamer primers, 1 mM dNTP mix, 40 units of RNaseOUT, and 1 unit of Superscript retrotranscriptase. Two units of RNase H were added at the end of the reactions. Real-time PCR was carried out using an Applied Abi prism 7000 cycler (Applied Biosystems) with SYBR Green as the fluorescent dye (Bio-Rad), a cycler (19). The cycle used for the PCR reaction was as follows: 95°C for 180 s on-site, 95°C for 30 s, 60°C for 30 s, 72°C for 30 s 50 times, and 95°C for 60 s on-site. Samples were subjected to a melting curve analysis to confirm the amplification specificity. Changes in SYBR Green fluorescence were monitored throughout each cycle, and the threshold cycle (CT) was determined as the value above the background signal for each reaction. For each cDNA sample, a ratio between the relative amounts of UCP3 and 18S was calculated to compensate for variations in quantity or quality among the mRNA samples, as well as for differences in reverse transcriptase efficiency. The relative change in the target gene with respect to the 18S endogenous control gene was determined as follows: 2ΔΔCT, where ΔCT = CTUCP3 − CT18S and Δ(ΔCT) = ΔCTcondition − ΔCTvehicle. RT-PCRs were run for each rat in triplicate.

Free fatty acid levels. Transcardiac blood (0.25 ml) was sampled in anesthetized rats at rest or after muscle stimulation. Plasma was immediately isolated using blood centrifugation (15 min at 4000 rpm) in EDTA-treated tubes. Plasma FFA levels were quantified using a colorimetric acyl-CoA synthetase- and acyl-CoA oxidase-based method (Roche, Penzberg, Germany).

Statistics. For variables evolving with respect to time during the stimulation period (force production, metabolite concentrations, and pH), the effect of capsaicin on the overall time course was determined using a one-way ANOVA with repeated measurements (JMP software; SAS Institute). For each time point, post hoc repeated comparisons (Fisher’s test) were used to compare values between vehicle and capsaicin conditions. Other variables were compared using two-way ANOVA, and a post hoc comparison test was performed when a significant interaction between the two factors was observed (Student-Newman-Keuls test). Values are means ± SE.

RESULTS

Phosphorylated compounds and intracellular pH. As illustrated in Fig. 1. [PCR], pH, [ATP], and [ADP] were 17.6 ± 0.7 mM (Fig. 1A), 7.08 ± 0.02 (Fig. 1B), 6.2 ± 0.1 mM (Fig. 1C), and 7.5 ± 0.4 μM (Fig. 1D), respectively, in the vehicle group at rest. Capsaicin administration resulted in a significant PCr increase ([PCr] = 23.9 ± 2.1 mM) and a pH decrease (pH = 7.00 ± 0.01), whereas [ATP] (5.9 ± 0.2 mM) and [ADP] (6.6 ± 0.5 μM) remained unchanged (Fig. 1, A–D).

During the stimulation period, capsaicin administration modified the overall time course of [PCR] (Fig. 1A) and [ADP] (Fig. 1D) but did not change pH, and [ATP] time-dependent changes (Fig. 1, B and C). For both groups, [PCR] rapidly decreased in the early stage of the stimulation period and reached a steady state that was maintained from the second minute onward. Interestingly, the initial difference regarding the resting [PCR] between vehicle and capsaicin groups was kept approximately constant throughout the stimulation period. At the same time, capsaicin administration led to a lower stimulation-induced ADP accumulation (Fig. 1D). For both groups, [ATP] slightly decreased during the stimulation period but to a lesser extent in the capsaicin group (Fig. 1B). The pH time course during the stimulation period was strongly similar.
between both groups, with a rapid acidosis early in the stimulation period (slightly larger in the capsiate group) followed by a steady state during the remaining stimulation period (Fig. 1D).

ATP production rate. The rates of ATP production with respect to time are displayed in Fig. 2. The inferred basal rate of oxidative ATP production ($Q$) was faster in the capsiate group compared with the vehicle group ($2.3 \pm 0.2$ vs. $1.5 \pm 0.2$ mM/min; Fig. 2A). In both groups, $Q$ increased in the early stage of the stimulation period and continuously decreased until the end of the stimulation period. Similarly to the observation at rest, the inferred rate of oxidative ATP production during the stimulation period was significantly faster after capsiate administration compared with the vehicle group (at the maximum rate: capsiate, $8.3 \pm 1$ vs. vehicle, $5.0 \pm 0.5$ mM/min). In addition, the maximum oxidative capacity ($Q_{max}$) was 68% higher after capsiate administration, whereas the oxidative ATP cost of contraction was 43% larger (Table 1). During the poststimulation period, the initial rate of PCr resynthesis was faster in capsiate-treated animals ($3.7 \pm 0.2$ vs. $2.8 \pm 0.2$ mM/min; Table 1). On the contrary, capsiate administration did not affect the total anaerobic rate of ATP production, i.e., from anaerobic glycolysis and PCr breakdown (Fig. 2, B and C). Furthermore, the nonoxidative ATP cost of contraction and the total ATP cost of contraction did not significantly differ between both groups (Table 1).

Mechanical measurements. Changes in isometric force production during the stimulation protocol are presented in Fig. 3. In both groups, isometric force transiently increased in the early stage of the stimulation period and reached a maximal value. From that time, isometric force progressively decreased until the end of the stimulation period, illustrating the fatigue phenomenon. One-way ANOVA with repeated measurements indicated that capsiate administration did not affect the overall time course of isometric force. At the end of the stimulation period, the extent of isometric force reduction did not differ between both groups, reaching $39.5 \pm 11.1$ and $35.1 \pm 2.8\%$ of the initial value for the vehicle and capsiate groups, respectively.

Quantitative measurements of muscle UCP3 gene expression. A real-time quantitative RT-PCR was used to quantify UCP3 mRNA level in gastrocnemius muscle at rest and following muscle stimulation in capsiate and vehicle groups. As illustrated in Fig. 4, capsiate administration reduced UCP3 mRNA expression at rest and after muscle stimulation. On the contrary, muscle stimulation increased UCP3 transcript level in vehicle and capsiate groups. Given that we did not find any significant interaction between capsiate administration and muscle stimulation, the effects of capsiate cannot be compared between the resting and the stimulation periods. Similarly, stimulation-induced changes in UCP3 transcript level cannot be compared between vehicle and capsiate groups.

Plasma FFA levels. Capsiate administration increased plasma FFA level at rest ($0.59 \pm 0.11$ mM) compared with the vehicle group ($0.37 \pm 0.02$ mM) (Fig. 5). Conversely, a 2.3-fold decrease in plasma FFA concentration after muscle stimulation was measured in the capsiate group ($0.35 \pm 0.08$ mM) compared with the vehicle group ($0.37 \pm 0.02$ mM at rest and $0.35 \pm 0.08$ mM after the stimulation period) and reduced FFA level in the capsiate group ($0.59 \pm 0.11$ mM at rest and $0.15 \pm 0.05$ mM after the stimulation period).

DISCUSSION

This study demonstrates for the first time that oral administration of capsiate downregulates UCP3 gene expression and
enhances mitochondrial ATP production in rat skeletal muscle both at rest and in exercising.

Experiments conducted at rest in knockout mice for the UCP3 gene have suggested that UCP3 is involved in mitochondrial proton leakage, thereby contributing to the uncoupling between mitochondrial proton gradient and ATP production. In vivo experiments in skeletal muscle isolated from UCP3 knockout mice (9) have shown that UCP3 gene disruption causes a fourfold increase in the ratio of ATP synthesis rate to tricarboxylic acid cycle flux (i.e., the mitochondrial energy coupling) compared with wild type, suggesting that the efficiency of mitochondrial ATP synthesis would be increased in UCP3 knockout animals. A similar approach aimed at measuring the degree of mitochondrial energy coupling in the rat hindlimb muscles has shown that a 10-day oral administration of triiodo-L-thyronine resulted in an eightfold increase in UCP3 mRNA expression, whereas mitochondrial energy coupling as measured from the ratio of ATP production to the tricarboxylic acid cycle flux was reduced by factor 2 (17). However, these results were not consistent with the measurements performed at the whole body level, showing that the global energy expenditure, determined from the turnover of doubly labeled body water, was unchanged in UCP3 knockout mice. Overall, these results would suggest that UCP3 gene disruption altered muscle energetics in isolated muscles, but, paradoxically, not at the whole body level (9). It is noteworthy that mice overexpressing UCP3 had a significantly lower body weight, providing evidence supporting the hypothesis that UCP3 can affect whole body energetics (8). This issue actually warrants further investigation to clearly determine the links between UCP3 expression and the corresponding changes in muscle energetics as-

| Table 1. Effects of capsiate on energy metabolism using noninvasive \(^3\)P-MRS |
|-------------------------------|-----------------|-----------------|-----------------|
|                               | Control         | Capsiate        |
| Oxidative ATP cost, mM \((\text{N} \cdot \text{s})^{-1}\) | 9.6±1.0         | 13.8±1.0        | \((P=0.011)\)   |
| Nonoxidative ATP cost, mM \((\text{N} \cdot \text{s})^{-1}\) | 29.2±5.7        | 36.3±2.7        | \((P=0.146)\)   |
| Total ATP cost, mM \((\text{N} \cdot \text{s})^{-1}\) | 38.8±4.7        | 50.1±3.6        | \((P=0.071)\)   |
| \(Q_{\text{max}}\), mM/min | 11.8±1.2        | 19.9±2.0        | \((P=0.005)\)   |
| VPCR_{\text{rec}}, mM/min  | 2.8±0.2         | 3.7±0.2         | \((P=0.003)\)   |

Values are means ± SE. \(^3\)P-MRS, \(^3\)P-magnetic resonance spectroscopy; \(Q_{\text{max}}\), maximal oxidative capacity; VPCR\(_{\text{rec}}\), initial rate of phosphocreatine resynthesis during the poststimulation period. Oxidative, nonoxidative, and total ATP cost were calculated during the stimulation period by scaling ATP production rate to isometric force production. \(P\) value indicates the result of 2-tailed Student’s \(t\) test.

Fig. 2. Changes in the rate of oxidative ATP production (A), ATP production from creatine kinase (B), and glycolytic ATP production (C) during 5.7 min of gastrocnemius muscle stimulation in vehicle (○) and capsiate (●) groups. Values are presented as means ± SE. The first time point \((t = 0)\) indicates the resting value. \(P\) value indicates the overall result of the 1-way ANOVA with repeated measurements. *Significant difference between vehicle and capsiate values for a given time point \((P < 0.05)\) by Fisher’s post hoc test.

Fig. 3. Changes in isometric force production during 5.7 min of gastrocnemius muscle stimulation in vehicle (○) and capsiate (●) groups. Values are presented as means ± SE. The first time point \((t = 0)\) indicates the resting value. \(P\) value indicates the overall result of the 1-way ANOVA with repeated measurements.
of muscle stimulation. A 2-way ANOVA was performed and no significant effect of capsiate administration (P > 0.001) was observed. Significant interaction between capsiate administration and muscle stimulation was found.

In the present study, the nearly 10-fold reduction in UCP3 gene expression associated with a 65% increase in the inferred basal mitochondrial ATP synthesis is consistent with these results and suggests a possible effect of UCP3 expression on mitochondrial energy coupling. Furthermore, we found that capsiate increased PCr level and reduced pH at rest. Considering that mitochondrial ATP generation is controlled by ADP through a feedback loop, the PCr increased could be understood as a compensatory mechanism aimed at keeping [ADP] constant in the face of a change in pH with unchanged mitochondrial function. On the contrary, the evidence from PCr recovery measurements is that mitochondrial function is increased by capsiate, and the unchanged ADP is therefore consistent with an increased ATP turnover at rest. In addition, considering that glycolytic rates of ATP production in the early stage of the stimulation period are similar in both groups, increased lactate production can be ruled out as a responsible factor of the reduced pH value measured at rest. The more acidic intracellular pH measured at rest in the capsiate group possibly resulted from a different set point for proton-efflux mechanisms. To ensure pH homeostasis, protons are actively transported against the negative membrane potential mainly by the sodium/proton antiport. These proton-efflux mechanisms are regulated by cellular pH, and consequently, the resting pH is set at the point where the transporters are active enough to maintain pH homeostasis through a balance between passive influx and active efflux. The pH fall measured at rest in the capsiate group could then be due to a decrease in the set point of the sodium/proton antiport.

Previously reported metabolic effects of capsiate, such as an increased oxygen consumption, could also be related to an increased ATP production (25). Uncoupling effects of capsiate have been already documented at rest as an increased basal oxygen consumption and an overexpression of UCP3 (24, 25). This increased oxygen consumption likely occurs to maintain ATP homeostasis despite increased energy utilization through uncoupling mechanisms. On the basis of these results, one could expect that a reduced uncoupling would decrease oxygen consumption and leave oxidative ATP synthesis unchanged. We showed that oxidative ATP production was likely enhanced as a result of a corresponding reduced efficiency of mitochondrial respiration, but the link with the reduced uncoupling is still to be determined. One can also hypothesize about some additional ATP-consuming mechanisms occurring as a result of capsiate treatment and/or reduction of the uncoupling mechanism ultimately responsible for an enhanced oxidative rate of ATP synthesis. Although this greater oxidative ATP production is clear, the determination of the exact stimulus would need further investigation.

The inferred rate of oxidative ATP production was also significantly increased during the stimulation period. During muscle contraction, ATP is continuously regenerated through the mitochondrial oxidative phosphorylation, anaerobic glycolysis, and PCr breakdown to maintain ATP homeostasis. The increased rate of aerobic ATP production measured as a result of capsiate administration could result from a proportional decrease of the anaerobic contribution to ATP production, i.e., PCr breakdown and glycolysis. However, we found ATP synthesis rates from PCr degradation and glycolysis similar in both groups. Also, the 43% increase in the oxidative ATP cost of contraction likely reflects the total ATP cost, which tended to increase. The increased mitochondrial function shown from inferred measurements at rest and in exercising muscle is further supported by direct measurements performed during the postexercise recovery period. PCr resynthesis is indeed exclusively aerobic, and its enhanced rate clearly illustrates this increased mitochondrial function coming with UCP3 down-regulation as a result of capsiate administration.

In contracting muscle, the increased ADP concentration is an important activator of mitochondrial respiration (18). In the present study, ADP accumulation throughout the stimulation period was significantly lower in the capsiate group compared with control, hence indicating that a lower mitochondrial activation was linked to a faster ATP production. This finding, together with the 68% increase in maximal oxidative capacity, suggests that capsiate administration alters the control of mi-
tochondrial energy production with respect to ADP. The ATP demand in working muscle is tightly coupled with the mechanical output (14). According to the common view of muscle energetics, the rate of ATP production is highly regulated to keep pace with large energy demand changes throughout muscular activity (14, 35). Surprisingly, we found that capsiate treatment was linked to an increased aerobic ATP production with no corresponding change of the mechanical performance. This would suggest that the oxidative ATP production found in excess in the capsiate group would not be devoted to contractile but to noncontractile activity, thereby indicating reduced muscle efficiency, i.e., higher energy consumption for a similar mechanical output. However, it has been shown that, under fatiguing conditions, oxidative ATP can be used to replenish PCr stores in contracting skeletal muscle (13). Considering the larger PCr level at rest and the faster PCr recovery rate measured during the poststimulation period in the capsiate group, one could suggest a cause-effect relationship between the 40% increase in aerobic ATP production and the 36% raise in PCr pool.

Interestingly, previous studies (32) have also reported concomitant changes in aerobic capacity and UCP3 expression. In endurance-trained athletes, the higher maximal rate of oxygen consumption is correlated with a lower expression of UCP3 mRNA, further indicating an inverse correlation between aerobic metabolism and UCP3 expression. Overall, the increased oxidative cost of contraction could result either from a decreased muscle efficiency or from additional mechanisms of ATP consumption.

Previous studies have documented combined changes in plasma FFA and UCP3 mRNA levels, but the cause-effect relationship has not been clearly established. UCP3 expression can increase as a result from fasting (3), intralipid infusion (42), or after an acute exercise (37). This whole set of conditions is also coupled to an increase in FFA levels. A direct relationship has also been illustrated in vitro, i.e., addition of FFA to myocytes enhances UCP3 gene expression (30). These data suggest that FFA could be involved in the UCP3 gene expression induction, and the nuclear hormone peroxisome proliferator-activated receptors could possibly mediate this response (34). However, the opposite phenomenon has not been observed, i.e., a reduction in the circulating FFA level (using the antilipolytic agent nicotinic acid) does not alter UCP3 gene expression in the rat gastrocnemius (29). Moreover, it has recently been reported that the time-dependent changes in UCP3 mRNA expression are independent of plasma FFA time course in response to a moderate intensity exercise in the rat gastrocnemius muscle. This is not consistent with FFA acting as an inducible factor of UCP3 gene expression (21). In keeping with that, we have reported in the present study that muscle stimulation per se does not change FFA level but enhances UCP3 mRNA level. This is also consistent with the results of Kusuhara et al. (21) showing a rapid upregulation in UCP3 transcriptional activity after muscle exercise or following skeletal muscle contraction in vitro (27). Due to the short time period, we could reasonably hypothesize that exercise-induced changes in UCP3 gene expression probably do not result in altered protein expression during the time of exercise. Hence, the changes in muscle energetics during exercise most likely result from the capsiate-induced UCP3 downregulation prior to exercise rather than the stimulation-induced change in UCP3 gene expression. The plasma FFA level measured in the control conditions was within the range previously reported in rodents (4, 21), and the higher FFA level induced by capsiate at rest was also comparable with that found in a previous study of capsiate-treated mice (25). In that study, an increased epinephrine level was measured as a result of capsiate administration. This change could subsequently activate the hormone-sensitive lipase and then increase the circulating FFA level (22, 25). Furthermore, the FFA increase in capsiate-treated rats at rest has been associated with a major reduction in muscle UCP3 gene expression, further suggesting that changes in FFA might not be linked to changes in UCP3 expression.

The UCP3 expression downregulation reported in the present study as a result of capsiate administration is inconsistent with a previous study conducted in mice (24). Although this discrepancy remains unclear, several potential contributing factors could be proposed, such as the species differences, capsiate concentration used, and the presence of capsiate dihydroderivatives in the solution administered. Although measurements of UCP3 protein expression could be of interest, many studies devoted to the metabolic regulation of skeletal muscle UCP3 expression have been performed at the mRNA level arguably because of the difficulty in obtaining specific antibodies. Our in vivo measurements were performed in anesthetized animals. Although it is well known that all types of anesthetics could induce vasodilatation, hypotension, and depress respiration (23), these changes are unlikely to enhance aerobic ATP production. Furthermore, the utilization of proper control conditions clearly rules out these factors as responsible for the observed metabolic differences.

Further studies should specify whether the effects of capsiate reported in the present study are mediated by the activation of the VR-1 receptor expressed in skeletal muscle cells (44). Research activity on molecules from edible plants that can bind and activate the VR-1 receptor has long been limited to the pungent component capsaicin used as a neuropharmacological tool to characterize sensitive neurons. The biological action of capsiate, which does not show any pungency following ingestion, highlights the pharmacological potential of a VR-1 agonist from edible plants.

In conclusion, we have demonstrated that capsiate downregulates UCP3 gene expression in skeletal muscle and enhances oxidative ATP production at rest, during exercise, and after exercise. These changes were not associated with any improvement in muscle force-generating capacity, suggesting the contribution of additional noncontractile ATP-consuming process(es) and/or a decreased muscle efficiency. These results also demonstrate the ability of noninvasive 31P-MRS to study in vivo energy metabolism changes resulting from chemically-induced alteration in gene expression.

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