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Hyperoxia-mediated oxidative stress increases expression of UCP3 mRNA and protein in skeletal muscle

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Abstract The uncoupling protein-3 (UCP3) is a mitochondrial protein expressed mainly in skeletal muscle. Among several hypotheses for its physiological function, UCP3 has been proposed to prevent excessive production of reactive oxygen species. In the present study, we evaluated the effect of an oxidative stress induced by hyperoxia on UCP3 expression in mouse skeletal muscle and C2C12 myotubes. We found that the hyperoxia-mediated oxidative stress was associated with a 5-fold and 3-fold increase of UCP3 mRNA and protein levels, respectively, in mouse muscle. Hyperoxia also enhanced reactive oxygen species production and UCP3 mRNA expression in C2C12 myotubes. Our findings support the view that both in vivo and in vitro UCP3 may modulate reactive oxygen species production in response to an oxidative stress.

Keywords: Uncoupling protein; UCP3; Hyperoxia; Oxidative stress; Reactive oxygen species; Antioxidant enzyme

1. Introduction

The uncoupling protein-3 (UCP3) belongs to the mitochondrial carrier family. This protein is mainly expressed in skeletal muscle and in brown adipose tissue of rodents. It has also been shown to be present in the skeletal muscle of other species, including human [1] and chicken [2]. Despite intensive work since its identification in 1997 by our group and other, its physiological function remains still unknown. UCP3 was originally proposed to uncouple the oxidative phosphorylation by dissipating the mitochondrial proton gradient, but its uncoupling activity is still debated. An overall view of studies on UCP3 function to date suggests a role for this protein in the regulation of energy metabolism, energy partitioning, mitochondrial production of reactive oxygen species (ROS) and in the detoxification of oxidant molecules by exporting peroxidized fatty acid out of the mitochondrial matrix [3–7]. ROS regroup molecules such as superoxide anion, hydroxyl radical and hydrogen peroxide and possess strong oxidative capacity. The potential role for UCP3 in modulating ROS formation is supported by in vivo and in vitro studies. For instance, mice lacking UCP3 exhibited an increase in ROS level in their muscles [8], and UCP3 overexpression in primary culture of neurons resulted in an inhibition of hyperglycemia-induced oxidative stress [9]. ROS, at their turn, might regulate UCP3 activity, as suggested by the fact that in isolated mitochondria the GDP sensitive-uncoupling activity of UCP3 depends on the presence of ROS [10].

Production of ROS during exposure to hyperoxia is widely held to be responsible for the lung injury seen in oxygen-exposed animals [11]. Hyperoxia has also been used as an experimental procedure to induce oxidative stress in other organ such as heart and skeletal muscle [12,13]. For instance, it has been shown that, in aged rats, exposure to high concentration of oxygen for 60 h resulted in an increased antioxidant activity of catalase and superoxide dismutase in skeletal muscle [14].

As mentioned above, it has been reported that manipulating UCP3 gene expression influenced ROS production. In the present study, we used a complementary approach to determine the relationship between UCP3 and ROS. We investigated the effect of hyperoxia-induced oxidative stress on muscle UCP3 expression in both in vivo and in vitro models. To determine the in vivo impact of hyperoxia on UCP3 expression, we exposed C57BL/6 mice in 100% oxygen for 72 h, assessed markers of oxidative stress and measured UCP3 mRNA and protein levels. In parallel, we examined whether in vitro hyperoxia induced ROS formation and regulated UCP3 expression in the murine skeletal muscle C2C12 cell line. Our results show that a well-defined condition of oxidative stress, namely hyperoxia, induced an increase in UCP3 expression both in mouse skeletal muscle and C2C12 myotubes, suggesting that this protein might participate to ROS metabolism.

2. Materials and methods

2.1. Exposure of mice to hyperoxia

Two- to three-month old C57BL/6 male mice (Charles River, France) were exposed to room air or hyperoxic conditions by delivery of 100% oxygen to a sealed Plexiglas chamber for 72 h as previously described [15]. The mice were given free access to food and water and were exposed to a 12-h light–dark cycle. At the end of oxygen exposure, mice were killed and skeletal muscles were rapidly dissected,

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Abbreviations: CM-H_2DCFDA, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester; CuZn-SOD, copper/zinc-superoxide dismutase; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solutions; PCR, polymerase chain reaction; ROS, reactive oxygen species; UCP2, uncoupling protein-2; UCP3, uncoupling protein-3

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frozen in liquid nitrogen and stored at −80 °C until further analysis. All procedures used were approved by the Office Vétérinaire Cantonal of Geneva, Switzerland.

2.2. Exposure of cells to hyperoxia

C2C12 myoblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose (4.5 g/L) supplemented with 10% fetal bovine serum until they reach 90% confluence. In order to differentiate the cells into myotubes, the growth medium was replaced by low glucose (1 mg/L) DMEM supplemented with 2% horse serum. After 5 additional days of culture, a large proportion of long multinucleated myotubes was observed among the myoblasts. These differentiated cells were placed under 95% oxygen/5% CO_2 (hyperoxia) or maintained in normal air condition (normoxia) for 48 h. After exposure, cells were used either for measurement of ROS production or RNA extraction (see below).

2.3. Measurement of ROS production

ROS were detected using fluorescence assay on C2C12 cells plated in 96 microwell optical bottom plates (Nunc GmBH&Co. KG, Wiesbaden Germany). Myotubes under hyperoxia or normoxia were quickly washed in Hank's balanced salt solutions (HBSS) and incubated with 10 μM of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) membrane-permeable dye (Molecular Probes, Europe BV, Leiden, The Netherland) at 37 °C for 10 min. Then, cells were washed twice with HBSS to remove excess dye. After addition of HBSS, cellular ROS accumulation was determined by recording the fluorescence (excitation wavelength: 485 nm and emission wavelength: 535 nm) for 10 min using a thermostate controlled (37 °C) microplate reader.

2.4. Northern blotting

Total RNA from mouse skeletal muscle was isolated by the method of Chomczynski and Sacchi [16]. Northern blot analysis was performed as described previously [17]. Full length cDNAs were used as probes for the detection of UCP3, CuZn-superoxide dismutase (CuZn-SOD) and catalase mRNAs. Human CuZn-SOD and human catalase cDNAs were kindly provided by the Dr. Lan Jornot. Human β-actin probe was used to ensure that equivalent RNA amounts were blotted on the membrane. The signals were quantified by scanning photodensitometry and normalized using the corresponding actin mRNA values.

2.5. Real-time PCR

The determination of UCP3 mRNA levels in myotubes was performed by quantitative polymerase chain reaction (PCR). Total RNA was prepared using the NucleoSpin RNA II kit mRNA (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Oligo-dT first strand cDNA were synthesized using the Superscript™ II RNase H Reverse Transcription kit (Invitrogen™, Life technologies, Basel, Switzerland) according to the manufacturer's instructions. Real-time PCR was performed using ABI rapid thermal cycler system and a SYBR Green PCR master mix according to the manufacturer's instructions. Cyclophilin A was used as a control to account for any variations due to the efficiencies of the reverse transcription and PCR. UCP3 oligonucleotide primers used were: upstream 5′-GGAGTCTCACCTGTTTACTGACAACT-3′ and downstream 5′-GCACAGAAGCCAGCTCCAA-3′ (GenBank Accession No. NM009464). Cyclophilin A oligonucleotide primers used were: upstream 5′-AGCACTGGGGAGAAAGGATT-3′ and downstream 5′-CATGCCTTCTTTCACCTTCC-3′ (GenBank Accession No XM355936). The conditions of PCR were a step at 50 °C for 2 min followed by a denaturing step at 95 °C for 10 min and by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. After each run, a relative quantification of amplified PCR products in the different samples was performed. For this purpose, standard curves were constructed for the gene of interest as well as for cyclophilin. The results are expressed as the ratio between the concentration of the target gene and that of cyclophilin A.

2.6. Mitochondria preparation

Mitochondria of skeletal muscle were prepared as previously described [18]. Mitochondrial protein concentrations were determined according to Bradford et al. [19], using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin as a standard.

2.7. Western blotting

Muscle mitochondria were prepared and Western blot performed as previously described [18], using a rabbit polyclonal primary antibody raised against human UCP3 protein (CabrX; Research Diagnostics Inc., San Antonio, LA, USA). Membranes were subsequently reblotted with a monoclonal antibody specific for prohibitin (Santa Cruz) to ensure that equivalent amount of mitochondria proteins was loaded onto the gel. The signals were detected by chemiluminescence using a standard ECL kit, and developed on a Hyperfilm ECL film. They were quantified by scanning photodensitometry of the autoradiograms using ImageQuant Software version 3.3 of Molecular Dynamics (Sunnyvale, CA).

2.8. Aconitase activity

To measure aconitase activity, we used the method described by Hausladen and Fridovich [20] with some modifications. Briefly, 200 μg of mitochondrial proteins from anterior leg muscles were disrupted by 3 times frozen–thaw in aconitase buffer containing Tris–HCl 50 mM, pH 7.4, sodium citrate 5 mM, cysteine 1 mM and $MnCl_2$ 0.6 mM. Mitochondrial extracts were then added to an aconitase buffer containing 10 units of $NADP^+$-dependent isocitrate dehydrogenase. The reaction started by adding $NADP^+$ 0.2 mM. Basal NADPH absorbance was measured every minute in a spectrophotometer and when stable values were obtained $NADP^+$ was injected. Calculation of aconitase activity was based on quantity of NADPH produced in the first 10 min after injection of $NADP^+$. Aconitase activity was expressed as nmoles NADPH produced per mg of mitochondrial protein per minute. To convert OD_{340} in nmoles, we used a standard curve with 0.5, 5, 25 and 50 nmol of NADPH.

2.9. Statistical analysis

Data are expressed as means ± S.E.M. Significance was evaluated using the unpaired Student's t test. A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Hyperoxia induces an oxidative stress in skeletal muscle of exposed mice

We first assessed the oxidative stress level in skeletal muscle of mice exposed to hyperoxia for 72 h (hyperoxia) or maintained in normoxic conditions (normoxia). As an in vivo indicator of mitochondrial ROS accumulation we determined the aconitase activity in muscle mitochondria. Loss in aconitase activity is interpreted as a measure of oxidative stress. As shown in Fig. 1A, aconitase activity was significantly decreased by 38% in hyperoxic mice compared with normoxic controls. The maximal aconitase activities were similar between normoxic and hyperoxic mitochondria. Then, we determined the mRNA levels of CuZn-SOD, catalase and β-actin in skeletal muscle. Fig. 1B show that hyperoxia induced a significant 3.0-fold and 2.6-fold increase in mRNA levels of CuZn-SOD and catalase, respectively. β-Actin mRNA level was found to be unchanged by hyperoxia. These data indicate that exposure to 100% oxygen for 72 h causes an oxidative stress in the mouse skeletal muscle.

3.2. UCP3 mRNA and protein are increased in skeletal muscle of hyperoxic mice

To examine the effect of oxidative stress on UCP3 expression, we compared the UCP3 mRNA level in skeletal muscle of hyperoxic mice with those of normoxic controls. As shown in Fig. 2A, oxygen exposure produced a 5-fold increase in UCP3 mRNA. Then, we determined the UCP3 and prohibitin protein levels in muscle mitochondria. Fig. 2B shows that UCP3 protein expression was also upregulated in muscle

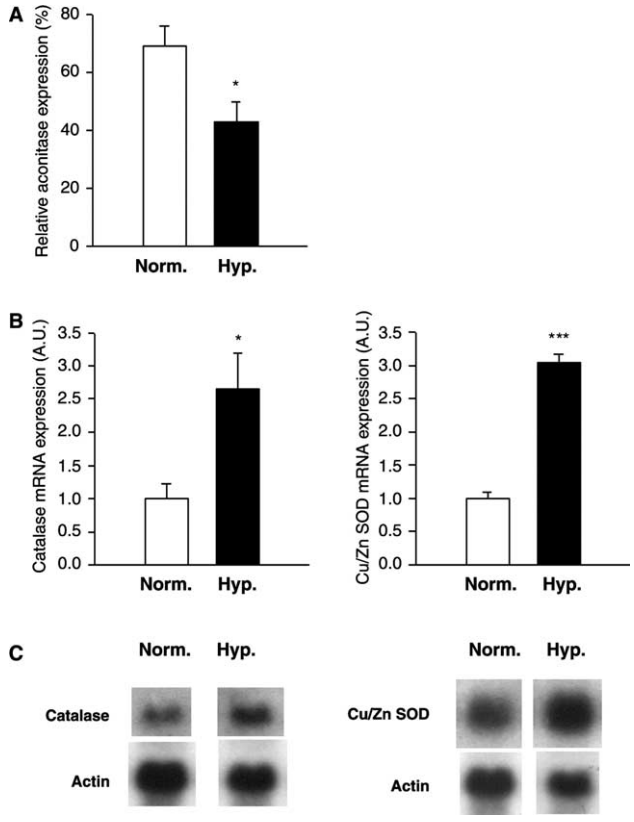

Fig. 1. Oxidative stress evaluation in skeletal muscle of hyperoxic mice. (A) Aconitase activity in mouse muscle of normoxic and hyperoxic mice. Aconitase activity was measured as described under Section 2. Results are expressed as the percent of the maximal aconitase activity. Values are means ± S.E.M.; $n = 10$. (B) Catalase and CuZn-SOD mRNA levels in normoxic and hyperoxic mice. The expressions of catalase and superoxide dismutase are shown relative to that of β-actin. The ratio of the normoxic values is considered as 1.0. Results are expressed as means ± S.E.M. of arbitrary units; $n = 5$. (C) Representative catalase, CuZn-SOD and actin mRNA signals (Norm. = normoxia and Hyp. = hyperoxia). *, $P < 0.05$; ***, $P < 0.0005$, unpaired Student's t test.

mitochondria of hyperoxic mice. No difference was observed in the level of prohibitin between normoxia and hyperoxia conditions. These results show that 72 h hyperoxia induced a marked increase of UCP3 mRNA and protein expression in mouse skeletal muscle.

3.3. 48 h hyperoxia induces UCP3 mRNA expression and oxidative stress in C2C12 myotubes

To examine the direct effect of hyperoxia on UCP3 expression in muscle cells, we performed in vitro experiment on confluent C2C12 myotubes exposed to either normoxic (control) or hyperoxic conditions for 48 h. The UCP3 mRNA expression relative to that cyclophilin, which is considered to be a reference housekeeping gene, was significantly increased by 1.9-fold in hyperoxic myotubes compared to control cells (Fig. 3A). We also evaluated the level of oxidative stress in C2C12 myotubes after 48 h exposure to hyperoxia. Using a dichlorofluorescein probe (CM-H_2DCFDA) to detect ROS, we found a 40% increase in oxidative stress level of hyperoxic myotubes compared to controls (Fig. 3B). Thus, in C2C12 myotubes, hyperoxia generates an augmentation of ROS production associated to an increase in UCP3 mRNA expression, suggesting that UCP3 expression could be control by the level of cellular oxidative stress.

4. Discussion

Here, we report that hyperoxia induced an increase in UCP3 mRNA and protein expression in skeletal muscle and in UCP3 mRNA level in C2C12 myotubes. In parallel to the increase in UCP3 expression by hyperoxia, we also observed an increase in the level of oxidative stress. Our data suggests that the oxidative stress produced by hyperoxia could be the stimulator of UCP3 expression.

Although exposure to 100% oxygen is well known to induce a marked oxidative stress and oxidative cell damages in lung, the effect of hyperoxia on oxidative stress levels in skeletal muscle has been rarely studied [14]. We therefore determined the level of oxidative stress generated in skeletal muscle of hyperoxic mice by two indirect but commonly used methods; the measurements of aconitase activity and antioxidant enzymes mRNA expression. Aconitase is an enzyme in the tricarboxylic acid cycle that is inhibited by superoxide radicals. Loss of aconitase activity is a widely used index of oxidative stress. We observed that the relative aconitase activity (% of the maximal activity) in hyperoxia represents 60% of the activity in normoxic condition. In line with our observation, similar decrease in aconitase activity was reported in mitochondria of superoxide dismutase-2 heterozygous knockout mice [21]. We also observed an increase in the gene expression of CuZn-SOD and catalase mRNA after 72 h hyperoxia suggesting an increase in ROS level. Hyperoxia seems to induce ROS production in mouse skeletal muscle in a time-dependent manner as 24 h hyperoxia exposure did not affect catalase and CuZn-SOD mRNA expression (data not shown). UCP3 mRNA and protein levels were also unchanged in 24 h hyperoxia compared to normoxia (data not shown).

The observation that 72 h hyperoxia induced gene expression of CuZn-SOD and catalase, suggest that some ROS-dependent signaling pathways have been turn on. This is in contrast with the observation of Amicarelli et al [14], who reported that 60 h hyperoxia did not result in significant changes of antioxidant enzymes activities in muscle homogenate of young rats. It is possible that the difference between their results and ours might be due to the use of two different animal models, i.e., exposure to oxygen for 60 h in rat vs. 72 h in mouse. However, the fact that there is not a systematic correlation between activities of antioxidant enzymes and their mRNA expression make difficult the comparison between the two studies. To support data obtained in hyperoxic muscle, we demonstrated by measuring ROS production that hyperoxia generated an oxidative stress in cultured myotubes. Taken together, our data indicate that 72 h hyperoxia is a condition that creates production of free radicals in skeletal muscle.

Hyperoxia is known to turn on several ROS-dependent signaling pathways such as MAPK, NF-κB or AP-1 pathways in endothelial and epithelial cells [22]. It is unknown whether UCP3 expression could be directly induced by one or many of these pathways. However it can be mentioned that TNF-α, which induces mitochondrial ROS production and activates NF-κB signaling in C2C12 myotubes [23], upregulates UCP3 mRNA expression in rat skeletal muscle [24]. Based on these reports, one may hypothesize that the NF-κB pathway

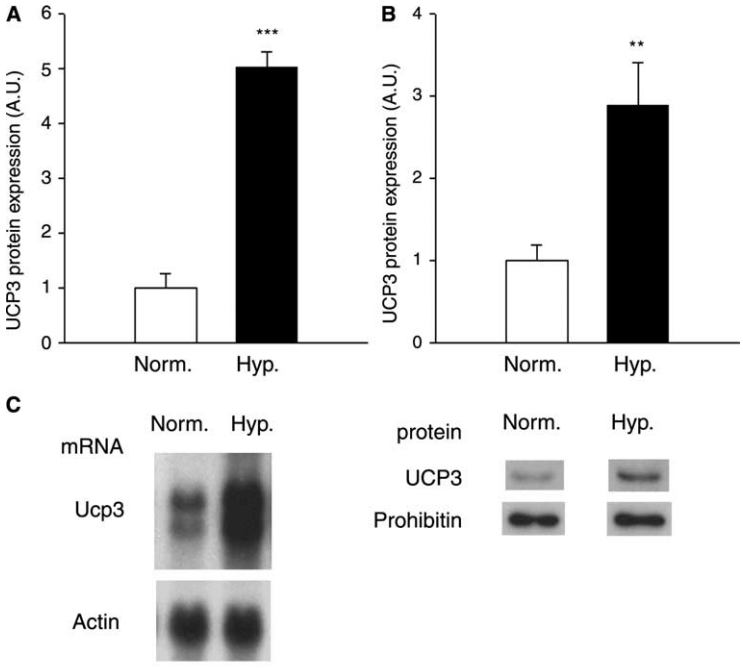

Fig. 2. UCP3 mRNA and protein expression in skeletal muscle of hyperoxic mice. (A) UCP3 mRNA levels in mouse muscle of normoxic and hyperoxic mice. The expression of UCP3 is shown relative to that of β-actin. The ratio of the control values is considered as 1.0. Results are expressed as means ± S.E.M. of arbitrary units; $n = 5$. (B) UCP3 protein levels in muscle mitochondria of normoxic and hyperoxic mice. Western blot was hybridized with UCP3 antibody and subsequently with prohibitin antibody as described under Section 2. The expression of UCP3 is shown relative to that of prohibitin. The ratio of the normoxic values is considered as 1.0. Results are expressed as means ± S.E.M. of arbitrary units; $n = 11$. (C) Representative UCP3 and actin mRNA signals, and UCP3 and prohibitin protein signals are shown under the respective graph (Norm. = normoxia and Hyp. = hyperoxia). **, $P < 0.005$; ***, $P < 0.0005$, unpaired Student's t test.

mediates the effect of ROS on UCP3 gene expression. Future studies will address this hypothesis.

The relationship between UCP3 and ROS has mostly been studied by quantifying the degree of ROS production in models lacking or overexpressing UCP3 [8,25]. Here, we determined the regulation of UCP3 mRNA and protein expression in response to an oxidative stress. We showed that hyperoxia increased UCP3 expression in mouse skeletal muscle and in C2C12 myotubes. As 72 h of hyperoxia significantly induced an oxidative stress in muscle cells both in vivo and in vitro, we might hypothesize that the induction of UCP3 expression would result from an enhanced ROS production. Recent studies, which have investigated the expression of uncoupling proteins in relation to oxidative stress, showed that the oxidative stress is an inducer of uncoupling protein-2 and -5 [26–28]. For instance, the increased production of mitochondrial ROS is associated with an increase in the mitochondrial content of UCP2 in hepatocytes [27]. Two hours exposure to hydrogen peroxide increased by 1.5-fold UCP2 mRNA expression in INS-1 cells [28]. In the present study, we showed that an oxidative stress can also increase the expression of another member of the uncoupling protein family that is UCP3, both in vivo, in mouse skeletal muscle, and in vitro, in C2C12 myotubes. The possibility that UCP3 may act as a regulator of mitochondrial free radical generation or an exporter of peroxidized fatty acids in muscle mitochondria remains to be investigated. It could be interesting in further study to test whether exposition to hyperoxia may induce an increase in the proton leak on isolated mitochondria or cells.

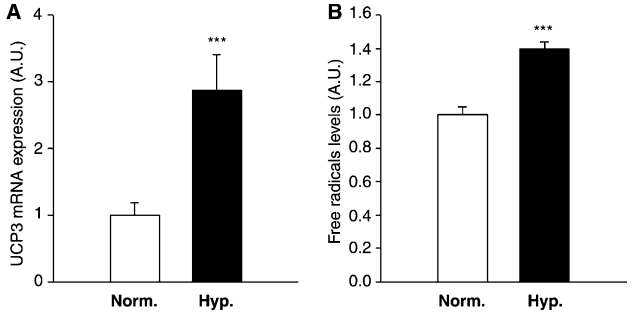

Fig. 3. UCP3 mRNA expression and oxidative stress level in C2C12 myotubes under hyperoxia. (A) UCP3 mRNA levels in C2C12 myotubes under normoxic or hyperoxic conditions for 48 h. The levels of UCP3 mRNA relative to those of cyclophilin were determined by real-time PCR as described under Section 2. The ratio of the normoxic values is considered as 1.0. (B) ROS level in C2C12 myotubes after 48 h of hyperoxia. Cells were loaded with 10 μM of CM-H_2DCFDA probe and ROS accumulation was measured as described under Section 2. Values are means ± S.E.M. of six different determinations. ***, $P < 0.0005$, unpaired Student's t test.

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