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UCP3 overexpression neutralizes oxidative stress rather than nitrosative stress in mouse myotubes

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ABSTRACT

The deleterious effects of oxidants on proteins may be modified by overexpression of uncoupling protein 3 (UCP3) in skeletal muscle cells exposed to hyperoxia or H_2O_2. UCP3 overexpression significantly attenuated the increase in protein carbonylation in response to hyperoxia and H_2O_2 exposures. However, antioxidant enzyme content and activity (superoxide dismutases, peroxiredoxins, glutathione peroxidase-I, and catalase) were reduced or not modified in UCP3-overexpressing myotubes exposed to oxidants. Protein nitration increased in UCP3-overexpressing cells exposed to hyperoxia, but not to H_2O_2. We conclude that protein oxidation rather than nitration is neutralized by UPC3 overexpression in mouse myotubes exposed to abundant reactive oxygen species.

1. Introduction

Cancer cachexia is the commonest systemic manifestation of advanced malignant diseases. The presence of cachexia always implies a poor prognosis, having a great impact on the patients' quality of life and survival [1]. In cancer-induced cachexia, skeletal muscle hypercatabolism is mainly due to the activation of the ubiquitin-proteasome-dependent proteolysis, to apoptosis, and to the activation of uncoupling proteins [2–5]. These mechanisms seem to be responsible for the process of muscle mass loss by promoting protein and DNA breakdown, and energy inefficiency.

Excessive production of reactive oxygen and nitrogen species (ROS and RNS, respectively) has been shown to be involved in cancer-induced cachexia [6–9] as well as in models of immobilization-induced muscle atrophy [10]. Proteins were the major targets of oxidative stress-derived effects on tissues in several chronic conditions [9–13]. In keeping with, increased oxidative stress was also observed in the limb muscles of animals with cancer-induced cachexia [9], leading to increased muscle protein breakdown [7].

Uncoupling proteins (UCPs) are members of a family of mitochondrial carriers located in the inner mitochondrial membrane. Studies have clearly shown that UCP overexpression is associated with uncoupling in isolated culture systems [14,15]. However, UCPs have also been shown to participate in several processes other than the energy mismatching characteristic of hypercatabolic states [16–18]. For instance, UCPs were shown to counteract the damaging effects of ROS on tissues via a mechanism of hydroxynonenal (HNE) activation of UCP3 [19]. In fact, UCP3, a protein selectively localized within skeletal muscle fibers and brown adipose tissue, was shown to attenuate endogenous superoxide anion production by the mitochondrial electron transport chain [20]. Interestingly, hyperoxia-mediated oxidative stress also induced an increase in both protein and mRNA UCP3 levels in C2C12 myotubes [21], and mice lacking UCP3 exhibited increased mitochondrial protein and lipid oxidation in their muscles [22,23]. Nevertheless, in an in vivo study conducted on mice overexpressing UPC3, no significant differences were found in either protein oxidation or lipid peroxidation in their muscle mitochondria [22].

In view of these apparent discrepancies, we attempted to explore whether the deleterious effects of ROS and/or RNS on cell proteins may be modified by overexpression of UCP3 in skeletal muscle cells exposed to different conditions of oxidative stress induction: (1) stimulation of endogenous ROS production

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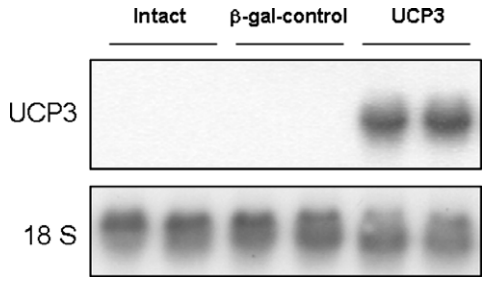

Fig. 1. Gene expression of UCP3 in intact C2C12 myotubes and in cells following transduction with AdCMV-βgal (β-gal) and AdCMV-UCP3 (UCP3). Expression of UCP3 gene in C2C12 was detected after hybridization with cDNA probes. Autoradiographs were subjected to scanning densitometry.

(hyperoxia exposure), and (2) direct exposure of mouse myotubes to ROS (H_2O_2 model). Accordingly, our objectives were to explore the levels of both protein carbonylation and nitration, and of several antioxidant mechanisms in an in vitro model of mouse myotubes exposed to these two different oxidizing conditions.

2. Materials and methods

(See the online Supplementary data for additional information).

2.1. C2C12 cell cultures

C2C12 mouse skeletal muscle cells were used. Construction of recombinant adenoviruses containing the human UCP3 (AdCMV-UCP3), under the control of the cytomegalovirus promoter, was conducted as previously described [24]. UCP3 gene was successfully transfected in C2C12 cells as shown in Fig. 1, in which mRNA expression levels of UCP3 were very high, while undetectable levels were observed in either intact or β-gal control transduced myotubes.

2.2. In vitro models of oxidative stress

Oxidative stress was induced using two different models: hyperoxia and H_2O_2 exposures following identical procedures previously published [21,25]. AdCMV-β-galactoside (β-gal)-myotubes were designated as the appropriate control groups in both hyperoxia and H_2O_2 models, while non-transduced myotubes were designated as intact cells.

2.3. RNA isolation and Northern blot analysis

These analyses were conducted as described elsewhere [26,27].

2.4. Immunoblotting

Redox balance was evaluated following methodologies published elsewhere [9,13,28].

2.5. Total SOD activity assay

A commercially available SOD assay kit was used according to the corresponding manufacturer's instructions to determine total SOD activity.

2.6. Statistical analysis

Data are presented as mean (S.D.). One-way analysis of variance (ANOVA) together with Tukey's test to adjust for multiple comparisons was employed in order to compare results between UCP3-overexpressing cells and β-gal control myotubes under normoxia and oxidative stress conditions. A *P*-value of 0.05 or less was considered to be significant.

3. Results

(See the online Supplementary data for additional information).

3.1. Protein oxidation

Protein carbonylation levels were greater in intact myotubes (hyperoxia), as well as in control (β-gal-transduced) and UCP3-overpressing cells exposed to both oxidizing conditions (hyperoxia and H_2O_2, Fig. 2). UCP3 overexpression induced a significant reduction in protein carbonylation in normoxia, attenuating the increase in this oxidative stress marker in cells exposed to both hyperoxia and H_2O_2 (Fig. 2).

3.2. Antioxidant mechanisms

3.2.1. Superoxide dismutases (SOD)

Mn-SOD content significantly increased in both intact and control myotubes exposed to hyperoxia (Fig. 3A). Moreover, UCP3 overexpression induced a significant increase in Mn-SOD content in normoxia. This effect was blunted when these cells were exposed to hyperoxia and H_2O_2, although in the former model, it did not reach the statistical significance (Fig. 3A). The content of cytosolic CuZn-SOD was reduced in UCP3-transduced cells exposed to hyperoxia compared to normoxic cells (Fig. 3B). UCP3 overexpression induced a significant increase in SOD activity in normoxia, which was not observed when cells were exposed to either hyperoxia or H_2O_2 (Fig. 3C). Interestingly, hyperoxia induced a

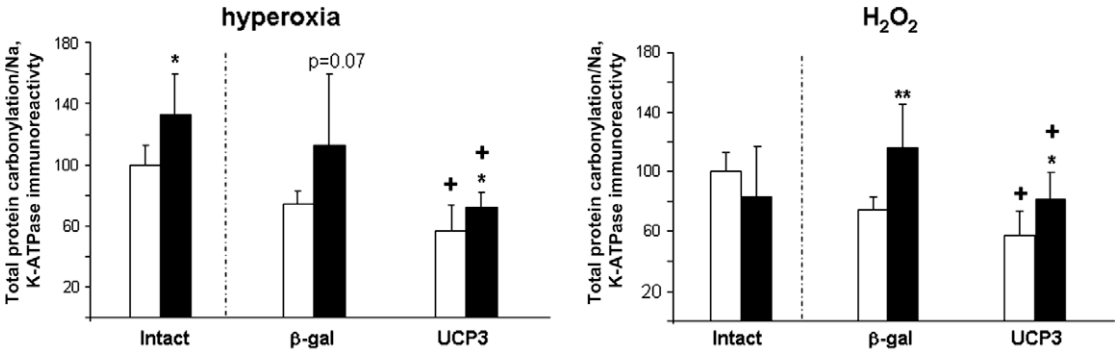

Fig. 2. Optical densities (OD) in the histograms are expressed as the ratio of the OD of total reactive carbonyl groups to those of Na^+, K^+-ATPase alpha-subunit. The values are indicated as mean (SD) for six samples in each experimental group. White bars: normoxia. Black bars: hyperoxia or H_2O_2. Statistical significance is expressed as follows: normoxia vs. hyperoxia or H_2O_2: $^{*}P < 0.05$, $^{**}P < 0.01$; control vs. UCP3: $^{+}P < 0.05$.

significant rise in SOD activity in control cells compared to normoxic myotubes (Fig. 3C).

3.2.2. Peroxiredoxins, glutathione peroxidase-I, and catalase

Peroxiredoxin-II levels were reduced in UCP3-transduced cells exposed to H_2O_2 compared to control cells (Fig. 4A). Peroxiredoxin-III levels were significantly reduced in UCP3-overexpressing cells exposed to both hyperoxia and H_2O_2 compared to normoxic myotubes and to control cells exposed to hyperoxia (Fig. 4B). Importantly, exposure to H_2O_2, but not to hyperoxia, induced a significant increase in glutathione peroxidase (GPx)-I in intact and control myotubes (Fig. 4C). However, this effect was almost significantly blunted in UCP3-overexpressing cells exposed to H_2O_2 (Fig. 4C). Catalase content was significantly reduced in intact and control cells exposed to both hyperoxia and H_2O_2 as well as in UCP3-overexpressing cells exposed to hyperoxia, whose levels were, in turn, lower than those in the hyperoxic control myotubes (Fig. 4D).

3.3. Protein tyrosine nitration

UCP3 overexpression induced a significant increase in protein tyrosine nitration in myotubes exposed to hyperoxia, but not to H_2O_2, compared to normoxic myotubes and an almost significant increase when compared to hyperoxia-exposed control cells (Fig. 5). Protein nitration was significantly reduced in intact myotubes exposed to hyperoxia (Fig. 5).

4. Discussion

The main findings in this study are the following: (1) Protein oxidation increased in control and UCP3-overexpressing cells exposed to both hyperoxia and H_2O_2. (2) UCP3 overexpression attenuated the increase in protein oxidation in cells exposed to both hyperoxia and H_2O_2. (3) Mn-SOD content increased in response to hyperoxia, but not to H_2O_2, in control and intact cells. (4) Overexpression of UCP3 induced an increase in Mn-SOD content in normoxia, while this effect was blunted under hyperoxia and H_2O_2 conditions. (5) UCP3 overexpression, however, did not modify CuZn-SOD content, and exposure to hyperoxia lowered its levels compared to normoxia. (6) UCP3 overexpression induced an increase in SOD activity in normoxia. (7) UCP3-overexpressing cells exposed to both hyperoxia and H_2O_2 exhibited a reduction in peroxiredoxin-III levels compared to normoxia. (8) GPx-I content was increased in intact and control cells exposed to H_2O_2, and this effect was almost blunted in UCP3-overexpressing cells exposed to

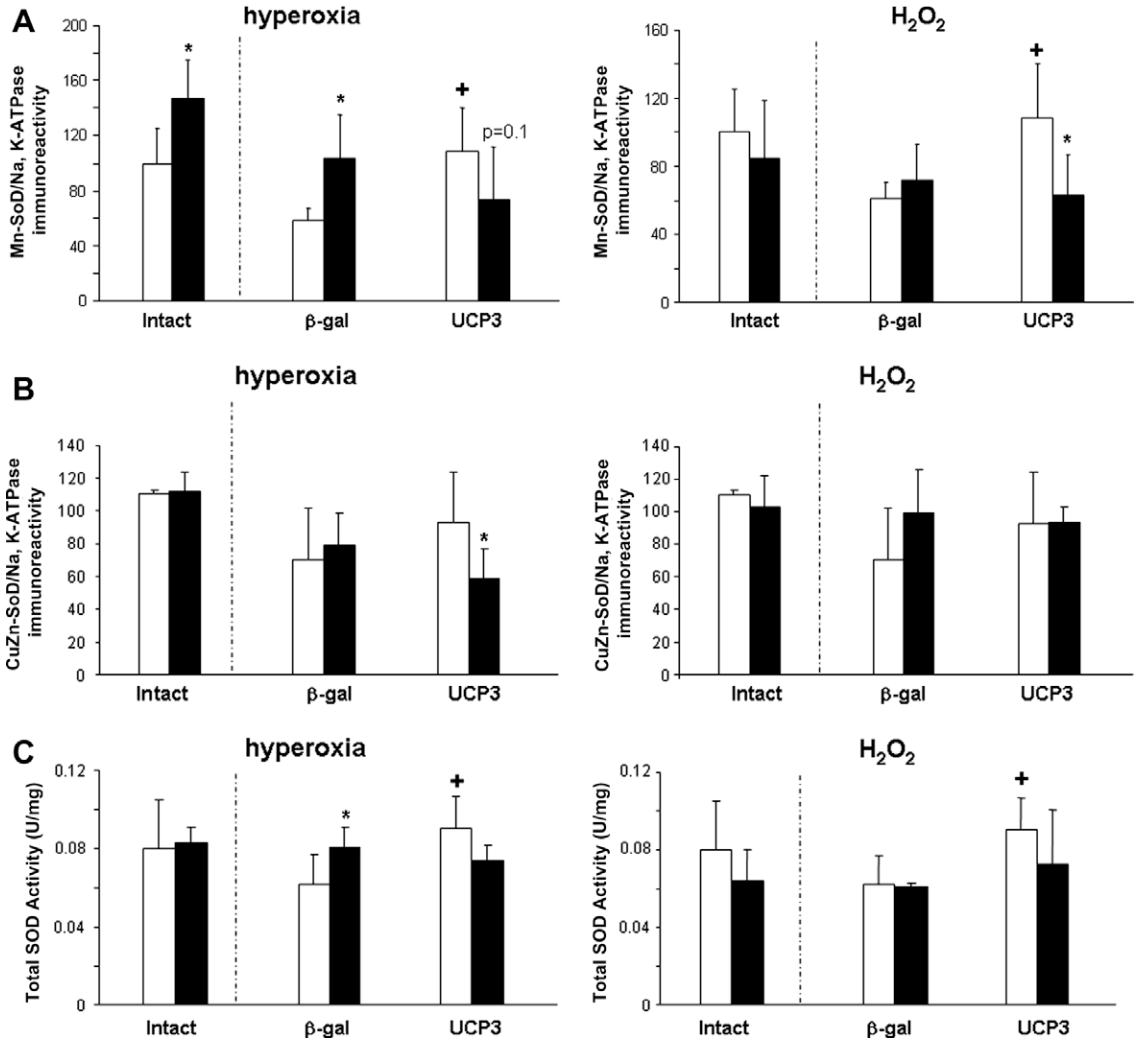

Fig. 3. Histograms correspond to: the ratio of the OD of Mn-SOD protein to those of Na^+, K^+-ATPase alpha-subunit (panel A), the ratio of the OD of CuZn-SOD protein to those of Na^+, K^+-ATPase alpha-subunit (panel B), and to total SOD activity (U/mg) (panel C). The values are indicated as mean (S.D.) for six samples in each experimental group. White bars: normoxia. Black bars: hyperoxia or H_2O_2. Statistical significance is expressed as follows: normoxia vs. hyperoxia or H_2O_2: $^*P < 0.05$; control vs. UCP3: $^+P < 0.05$.

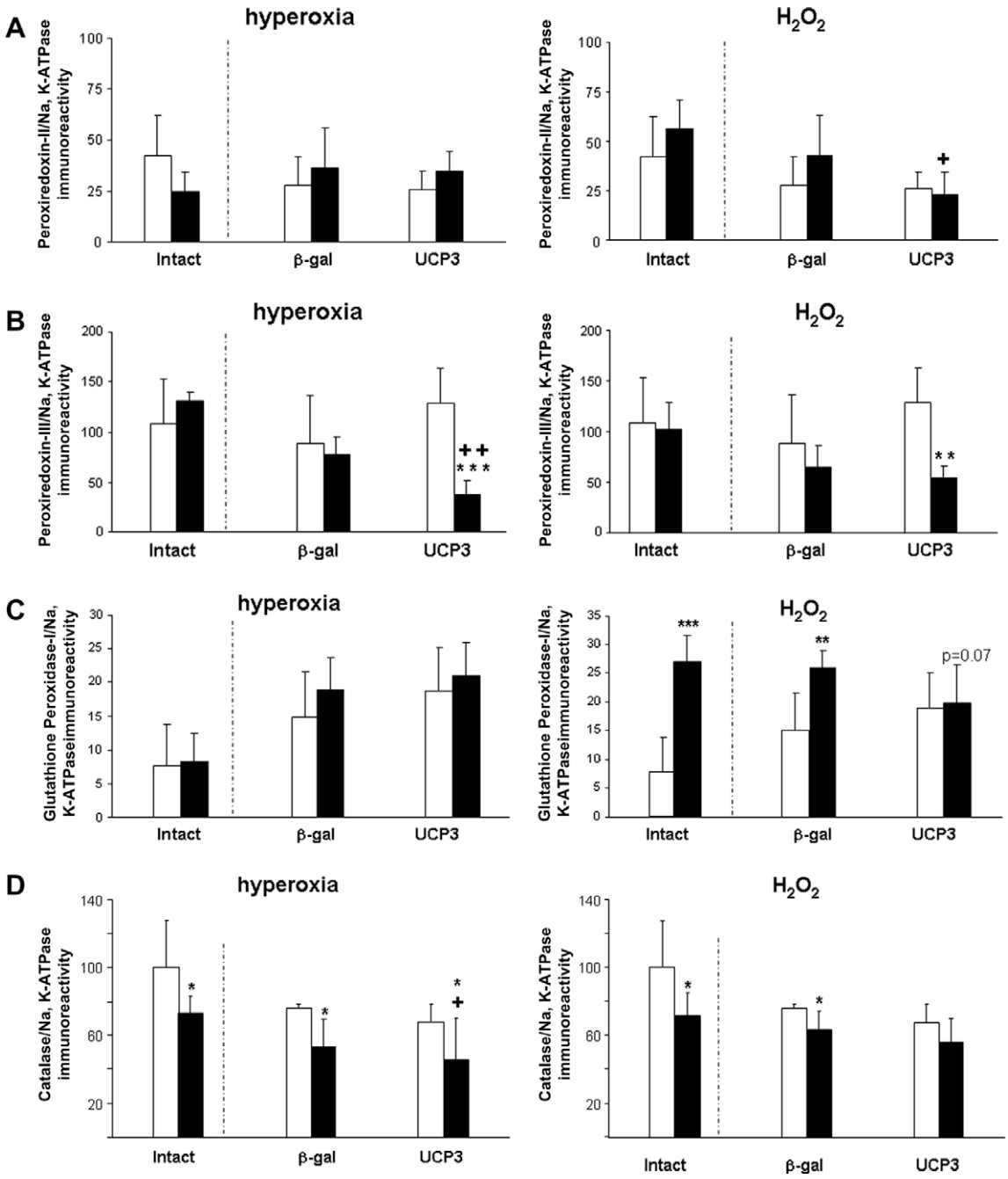

Fig. 4. OD in the histograms are expressed as: the ratio of the OD of peroxiredoxin-II protein to those of Na^+, K^+-ATPase alpha-subunit (panel A), the ratio of the OD of peroxiredoxin-III protein to those of Na^+, K^+-ATPase alpha-subunit (panel B), the ratio of the OD of GPx-I protein to those of Na^+, K^+-ATPase alpha-subunit (panel C), and the ratio of the OD of catalase protein to those of Na^+, K^+-ATPase alpha-subunit (panel D). The values are indicated as mean (S.D.) for six samples in each experimental group. White bars: normoxia. Black bars: hyperoxia or H_2O_2. Statistical significance is expressed as follows: normoxia vs. hyperoxia or H_2O_2: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; control vs. UCP3: $^{+}P < 0.05$, $^{++}P < 0.01$.

this ROS. (9) UCP3 overexpression induced a greater reduction in catalase content in the myotubes exposed to hyperoxia than in control cells. 10) Finally, protein nitration increased in UCP3-transduced myotubes in response to hyperoxia but not to H_2O_2 exposure.

4.1. Oxidative stress

Protein carbonylation constitutes one of the best characterized markers of protein oxidation in several conditions [9–13]. In the present study, UCP3 protection against oxidative stress was explored in mouse myotubes overexpressing this protein using two different experimental approaches of oxidative stress induction (Fig. 6). Interestingly, normoxic UCP3-transduced myotubes were shown to induce activation of proteolytic systems involved in myofibrillar protein degradation, suggesting a possible link between UCP3 and the main mechanisms involved in muscle wasting during cancer cachexia [27]. Oxidative stress, as measured by protein carbonylation, increased in response to oxidants in control and UCP3-overexpressing cells in these two in vitro models of oxidative stress: stimulation of endogenous ROS production (hyperoxia) and direct exposure to ROS (H_2O_2). This is consistent with previous

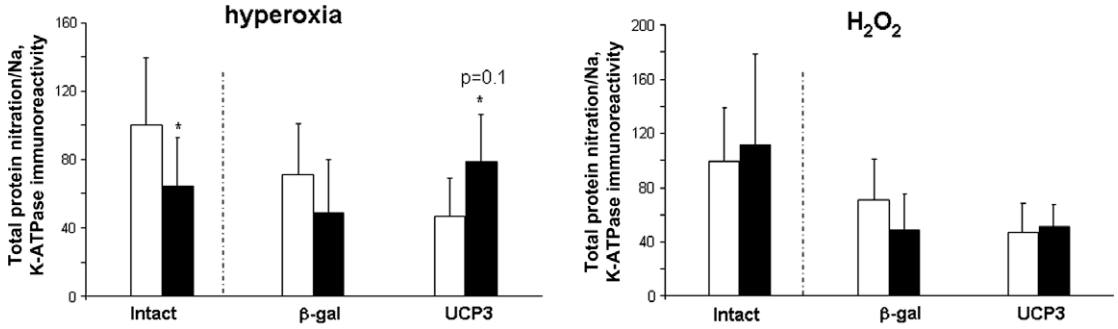

Fig. 5. OD in the histogram are expressed as the ratio of the OD of total protein tyrosine nitration to those of Na^+, K^+-ATPase alpha-subunit. The values are indicated as mean (S.D.) for six samples in each experimental group. White bars: normoxia. Black bars: hyperoxia or H_2O_2. Statistical significance is expressed as follows: normoxia vs. hyperoxia or H_2O_2: $^*P < 0.05$; control vs. UCP3: $P = 0.1$.

studies [21,25], in which an increase in ROS synthesis was demonstrated both in vivo and in vitro after exposure of mice and cells, respectively, to oxidative stress conditions (hyperoxia and H_2O_2). Importantly, UCP3-overexpressing myotubes exhibited significantly lower levels of protein carbonylation in response to either hyperoxia or H_2O_2 than control cells, suggesting that UCP3 overexpression plays a major role in the defense against oxidative stress, at least in vitro. In an in vivo study, in which mice overexpressing UCP3 were used, no significant differences were found in skeletal muscle mitochondria protein oxidation or lipid peroxidation compared to wild type animals [22]. Conversely, it was also shown that transgenic mice lacking UCP3 exhibited increased mitochondrial protein and lipid oxidation levels in their muscles [22,23], concluding that endogenous UCP3 protects muscle mitochondria against oxidative damage in vivo [22]. Interestingly, in intact myotubes, protein carbonylation was not increased in response to H_2O_2, suggesting that transduction may have sensitized myotubes to H_2O_2-mediated protein modifications.

The mitochondrial enzyme Mn-SOD catalyzes the conversion of two superoxide anions into hydrogen peroxide and molecular oxygen (Fig. 6). In the current study, the content of mitochondrial Mn-SOD increased in response to hyperoxia in intact and control myotubes. The fact that SOD activity selectively scavenges superoxide anion may account for the observed increase in Mn-SOD content in response to hyperoxia, but not to H_2O_2. In keeping with, previous studies have also shown an increase in either Mn-SOD or

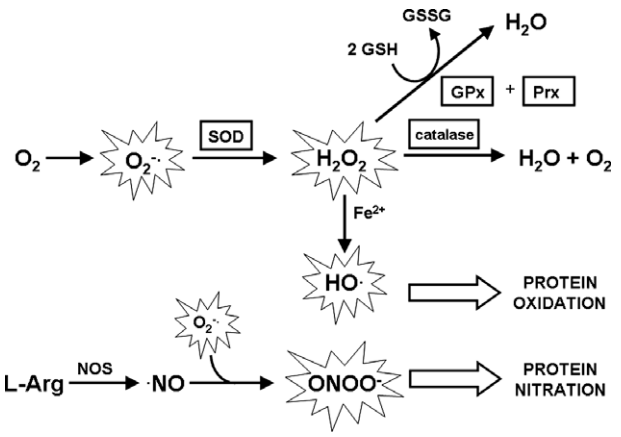

Fig. 6. Schematic representation of the different molecular reactions involved in the study experiments. Abbreviations: O_2, molecular oxygen; $O_{2\cdot-}$, superoxide anion; L-Arg, L-arginine; ·NO, nitric oxide; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; Fe^{2+}, iron, HO·, hydroxyl radical; H_2O, water; $ONOO^-$, peroxynitrite; GSH, reduced glutathione, GSSG, oxidized glutathione; GPx, glutathione peroxidases, Prx, peroxiredoxins.

CuZn-SOD in muscles exposed to hyperoxia [21] or in response to glucose-mediated oxidative stress [29]. Importantly, in normoxia, UCP3 overexpression induced a significant rise in Mn-SOD content, which was blunted when cells were exposed to both hyperoxia and H_2O_2. Likewise, under normoxia conditions, but not in response to oxidative stress, SOD activity levels were also higher in UCP3-overexpressing myotubes than in control cells. Moreover, levels of the cytosolic enzyme CuZn-SOD were also decreased in UCP3-overexpressing cells exposed to hyperoxia. Taking together, it could be concluded that while UCP3 overexpression conferred protection against oxidative damage in both in vitro models, it exerted a two-fold effect on SOD activity: induction and attenuation of SOD content and activity in normoxia and oxidative stress conditions, respectively. In fact, UCP3 has been shown to cause mild uncoupling [30]. These results are in agreement with a potential role of UCP3 as a part of a negative feedback mechanism to limit endogenous superoxide anion production in the mitochondria, which could account for the reduced levels of SOD content observed in the UCP3-overexpressing myotubes exposed to oxidants. Clearly, the design of future studies will be required in order to further explore this hypothesis.

Peroxiredoxins and glutathione peroxidases are present in the cytosol and mitochondria and are responsible for the detoxification of H_2O_2 (Fig. 6), especially in the mitochondrial matrix, in which catalase is lacking. In the current investigation, hyperoxia or H_2O_2 exposures did not modify peroxiredoxin-III levels in control cells. Furthermore, UCP3 overexpression induced a reduction in the content of peroxiredoxin-III in the myotubes exposed to both hyperoxia and H_2O_2 compared to normoxic cells. Importantly, GPx-I was significantly increased in response to H_2O_2 exposure in intact and control cells. Nevertheless, such an increase was not observed in UCP3-overexpressing cells exposed to H_2O_2, which, indeed, showed an almost significant reduction in GPx-I compared to controls.

Catalase is a ubiquitous heme protein with great catalytic activity, also responsible for the detoxification of H_2O_2 (Fig. 6). In the current investigation, catalase content was reduced in intact and control cells exposed to both hyperoxia and H_2O_2. Furthermore, UCP3 overexpression induced a greater reduction in catalase levels in myotubes exposed to hyperoxia, but not to H_2O_2. On this basis, it could be suggested that excessive exposure to H_2O_2 may have saturated the specific antioxidant defenses against this ROS, thus reducing their content within the cells, while UCP3 overexpression did not induce any of these heme enzymes.

4.2. Protein tyrosine nitration

Our study is the first to report the levels of protein tyrosine nitration in UCP3-transduced myotubes exposed to two different

experimental oxidizing conditions. Exposure of myotubes to hyperoxia, but not to H_2O_2, induced a significant reduction in protein tyrosine nitration in intact cells. Actually, this finding is consistent with the increase in Mn-SOD content observed in this group of myotubes. Surprisingly, protein tyrosine nitration was increased in the UCP3-transduced cells exposed to hyperoxia, but not to H_2O_2. This finding is also consistent with the concomitant decrease in Mn-SOD observed in the UCP3-transduced myotubes exposed to hyperoxia conditions. In line with this, excessive in vivo production of nitric oxide has been shown to exert damaging effects on tissues as a result of its diffusion-limited reaction with the superoxide anion to form the powerful species peroxynitrite [31,32] (Fig. 6), and protein tyrosine nitration in skeletal muscles is primarily mediated by peroxynitrite. Exposure to H_2O_2, however, did not result in any significant change in protein nitration levels in the myotubes.

4.3. Study critique

One limitation in this investigation is related to the potential biological and/or clinical relevance of the study findings using these experimental approaches of ROS induction in an in vitro model of UCP3 overexpression in skeletal muscle cells. However, we believe that the results shown in the current investigation serve as the basis for the design of future studies in which the specific effects of UCP3 overexpression on oxidative stress-mediated muscle cachexia will be explored using in vivo models. From an ethical point of view, it is highly recommended that a hypothesis should be first tested using in vitro studies in order to justify the use of in vivo models in further studies. The novelty in the current investigation is related to the demonstration that levels of protein oxidation, but not of protein nitration, were attenuated in UCP3-overexpressing myotubes exposed to two different oxidative stress conditions. Another aspect that deserves attention in this investigation has to do with the undetectable mRNA expression of endogenous UCP3 in either intact or control myotubes as opposed to the low, but detectable levels, of endogenous UCP3 in skeletal muscles of animals [23,33,34]. Nevertheless, UCP3 overexpression leads to a large increase in mRNA expression of UCP3 in a similar fashion in both in vivo [33,34] and in vitro models. Finally, it should also be mentioned that the findings encountered in this study are in keeping with previous reports [22,23], and that the two models of oxidative stress induction have already been well established in the literature [21,25].

5. Conclusions

We conclude from these findings that UCP3 attenuates oxidative stress rather than nitrosative stress in mouse myotubes exposed to two different models of abundant ROS generation. Further investigations using in vivo models of cachectic states will be designed in order to explore the specific role of UCP3 on the oxidative stress-mediated muscle wasting process.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.12.023.

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