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Original Contribution

Oxidative stress-induced proteome alterations target different cellular pathways in human myoblasts

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ABSTRACT

Although increased oxidative stress has been associated with the impairment of proliferation and function of adult human muscle stem cells, proteins either involved in the stress response or damaged by oxidation have not been identified. A parallel proteomics approach was performed for analyzing the protein expression profile as well as proteins preferentially oxidized upon hydrogen peroxide-induced oxidative stress. Fifteen proteins involved in the oxidative stress response were identified. Among them, protein spots identified as peroxiredoxins 1 and 6, glyceraldehyde-3-phosphate dehydrogenase, and α-enolase were shifted to a more acidic isoelectric point upon oxidative stress, indicating posttranslational modifications. Oxidized proteins were evidenced by immunodetection of derivatized carbonyl groups followed by identification by mass spectrometry. The carbonylated proteins identified are mainly cytosolic and involved in carbohydrate metabolism, cellular assembly, cellular homeostasis, and protein synthesis and degradation. Pathway analysis revealed skeletal and muscular disorders, cell death, and cancer-related as the main molecular networks altered. Interestingly, these pathways were focused on two distinct proteins: p53 for altered protein expression and huntingtin for increased protein carbonylation. This study emphasizes the importance of performing analysis addressing different aspects of the cellular proteome to have a more accurate view of their changes upon stress.

Skeletal muscle aging is characterized by a progressive and dramatic loss of muscle mass and strength leading to decreased muscular function resulting in muscle weakness, which is often referred to as sarcopenia [1]. Although under healthy conditions, skeletal muscle is a relatively stable postmitotic tissue compared to high-turnover tissues, such as skin or gut, in response to increased load or damage it has a remarkable regenerative capacity due to the presence of adult muscle stem cells, also known as satellite cells, which play a major role in postnatal muscle growth and repair [2,3]. In the adult, upon muscle injury or damage, satellite cells are activated and proliferate extensively, generating a pool of myoblasts, which subsequently differentiate and fuse to repair or replace damaged myofibers. The satellite cell population is maintained by self-renewal and appears to be self-sufficient as a source of new myonuclei [4,5], but the age-related loss of muscle mass and regenerative ability may reflect an impairment of this mechanism.

Increased oxidative stress in skeletal muscle during aging is now well documented and has been implicated in the development of sarcopenia [6–8]. Skeletal muscle is a highly oxygenated tissue and it is continually exposed to high concentrations of reactive oxygen species (ROS). ROS are generated during many normal physiological processes, including aerobic metabolism, and are known to play a dual role in biological systems resulting in either beneficial or harmful effects. Low concentrations or transient exposure to ROS induce cell proliferation and regulate the activation of several signaling pathways [9]. However, at high concentrations, ROS are important mediators of damage. Protein oxidation is of utmost importance because proteins are the largest group of cellular macromolecules. Various types of protein oxidative modifications are induced directly by ROS or indirectly by reactions with secondary products of oxidative stress [10]. Cysteine and methionine residues in proteins are particularly prone to oxidative attack by almost all ROS. However, these modifications are not always linked to protein damage, because it has been shown that they can participate in cellular signaling events [11]. Furthermore, the existence of repair mechanisms can compensate at least in part for the eventual induced damages.

Abbreviations: DIGE, differential in-gel electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; HD, Huntington disease; MS, mass spectrometry; ROS, reactive oxygen species; RMI, relative modification index; XTT, sodium 3,3′-[1((phenylamino)carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

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On the other hand, irreversible oxidation products of other residues result most frequently in their hydroxylation and carbonylation. Detection of protein-associated carbonyls represents a classic way of assessing protein oxidation after carbonyl derivatization with 2,4-dinitrophenyl hydrazine [12]. Because oxidative modifications that give rise to carbonyl groups generally cause loss of catalytic or structural function in the affected proteins, it is likely that the increased levels of these oxidized proteins during aging and age-related diseases would have serious deleterious effects on cellular and organ function [13,14]. In addition, accumulation of carbonylated proteins represents a hallmark of cellular aging that has been related to the occurrence of increased oxidative stress and impairment in oxidized protein degradation and repair [15]. Oxidized proteins in the cytosol and nucleus are preferentially degraded by the 20S proteasome in an ATP- and ubiquitin-independent manner [16]. Oxidized proteins represent good substrates for the 20S proteasome unless they become heavily oxidized and cross-linked [17]. In such situations, not only do these highly damaged proteins become resistant to proteolysis by the proteasome but they also can act as inhibitors of proteasome activity as demonstrated for proteins cross-linked after modification by the lipid peroxidation product 4-hydroxy-2-nonenal [18].

Hydrogen peroxide is one of the most abundant ROS in aerobic biological systems and it has also been widely used to induce oxidative stress in cellular systems in vitro. Previous studies in primary culture of human myoblasts have shown that a single treatment with exogenous hydrogen peroxide resulted in the loss of viability, a shorter population-doubling competence, and a significantly decreased proliferative capacity [19]. Furthermore, increased protein carbonylation and calcium-dependent proteolysis by calpains has been reported in human immortalized LHCN-M2 myoblasts [20]. However, proteins that are either targets of oxidation or involved in the oxidative stress response have not yet been identified. In this study, we have evidenced and identified protein targets of carbonylation as well as those undergoing changes in their expression level to gain further insights into the cellular pathways that are affected by oxidative stress in human myoblasts.

Material and methods

Material

Dulbecco's modified Eagle medium (4.5 mg ml^{-1} glucose), medium 199, gentamicin, trypsin solution for cell culture (0.05% (w/v)), and fetal bovine serum were purchased from Invitrogen (Cergy Pontoise, France). Cell culture plastics were purchased from BD Falcon (Le Pont de Claix, France). Cell Proliferation Kit II (XTT) was obtained from Roche (Meylan, France). Two-dimensional differential in-gel electrophoresis (2D-DIGE) materials and other 2D chemicals of analytical grade, as well as Western blot reagents, were purchased from GE Healthcare (Saclay, France). Coomassie Brilliant Blue G-250, BCA protein assay, and chemicals for SDS–PAGE were purchased from Bio-Rad (Marnes La Coquette, France). The OxyBlot protein oxidation detection kit was purchased from Millipore (Molsheim, France). Trypsin for mass spectrometry was obtained from Promega (Madison, WI, USA). All other reagents were obtained from Sigma–Aldrich (Saint-Quentin Fallavier, France), unless specified.

Cell culture and oxidative stress

Immortalized human LHCN-M2 myoblasts were grown as previously described [21]. Briefly, 3.9×10^5 cells were plated in 225-cm^2 cell culture flasks and cultivated in proliferation medium (four parts Dulbecco's modified Eagle medium to one part medium 199) with gentamicin (5 μg/ml) and dexamethasone (1 μM), supplemented with 20% (v/v) fetal bovine serum. Cultures were performed in a humidified atmosphere with 5% CO_2 at 37 °C until subconfluence. Hydrogen peroxide-induced oxidative stress was performed as previously described [20]. Cell treatment was carried out with 500 μM H_2O_2 in serum-free proliferation medium. Cells used as controls were cultured in serum-free proliferation medium but without H_2O_2. After 1 h, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS), detached with trypsin (0.05% w/v), centrifuged at 300*g* for 10 min at 4 °C, and washed again with DPBS. Viability of the cells after 1 or 24 h of H_2O_2 treatment was performed on cells cultivated in 96-well microplates (Greiner Bio One, France) using the Cell Proliferation Kit II (XTT), according to the manufacturer's instructions.

Proteasome peptidase activities

Cell pellets were homogenized in an extraction buffer (50 mM Tris–HCl, pH 7, 1 mM dithiothreitol). The homogenates were placed 5 min on ice and centrifuged for 15 min at 20,000*g*. Protein concentration determination by the Bradford method (Bio-Rad protein assay), using bovine serum albumin as standard was performed. Peptidase activities of the proteasome were assayed using the fluorescent peptide substrates succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin, for the chymotrypsin-like activity; Ac-Arg-Leu-Arg-aminomethylcoumarin, for the trypsin-like activity; and *N*-benzyloxycarbonyl-Leu-Leu-Glu-β-naphthylamide, for the peptidylglutamyl peptide hydrolyzing or caspase-like activity; as previously described [22]. Proteasome peptidase activities were determined as the difference between total activity and remaining activity of the crude lysate in the presence of the proteasome inhibitor MG132 (20 μM). All measurements were repeated a minimum of three times and results were expressed as means ± standard deviation. Data were tested for normality, and statistical significance for the comparison of the two groups was determined using Student's *t* test, with $P<0.05$ being considered significant.

Protein sample preparation for OxyBlot and 2D-DIGE

Cellular pellets derived from hydrogen peroxide-stressed and control cells (four independent experiments) were homogenized using a lysis buffer (10 mM Tris–HCl, pH 7.4, 8 M urea, 2 M thiourea, 4% (w/v) Chaps) and clarified by centrifugation. Proteins in the supernatant were precipitated using the 2-D Clean-Up kit, following the manufacturer's instructions, and resuspended in lysis buffer. Protein quantification was performed as described above.

Protein carbonyl immunodetection after 1D or 2D gel electrophoresis (1D- or 2D-OxyBlot)

Immunoblot experiments for carbonyl detection were performed using OxyBlot according to the manufacturer's instructions. For 1D-OxyBlot, 15 μg LHCN-M2 protein lysates was derivatized with 2,4-dinitrophenylhydrazine (DNPH) followed by SDS–PAGE (12% (v/v) polyacrylamide) separation and electroblotting onto nitrocellulose membranes. After being blocked, the membranes were incubated with anti-DNP antibodies, washed, and incubated with peroxidase-conjugated anti-rabbit IgG antibodies.

2D-OxyBlot was performed on protein extracts derived from the same samples used for 1D-OxyBlot, after 2D PAGE separation as described previously [23,24]. For each sample two gels were performed in parallel, one for colloidal blue staining of total proteins and the other for electroblotting onto nitrocellulose membranes. Membranes were then incubated for 2 h in the blocking solution and carbonyl detection was performed using the OxyBlot kit, as described above. Membranes were developed using the Amersham ECL Plus Western blotting detection system. Films were digitized with a UMAX UTA-100 scanner (GE Healthcare) and densitometry analyses were performed using NIH ImageJ or Image Master 2D Platinum 7 software (GE Healthcare).