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In vitro susceptibility of thioredoxins and glutathione to redox modification and aging-related changes in skeletal muscle

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ABSTRACT

Thioredoxins (Trx's) regulate redox signaling and are localized to various cellular compartments. Specific redox-regulated pathways for adaptation of skeletal muscle to contractions are attenuated during aging, but little is known about the roles of Trx's in regulating these pathways. This study investigated the susceptibility of Trx1 and Trx2 in skeletal muscle to oxidation and reduction in vitro and the effects of aging and contractions on Trx1, Trx2, and thioredoxin reductase (TrxR) 1 and 2 contents and nuclear and cytosolic Trx1 and mitochondrial Trx2 redox potentials in vivo. The proportions of cytosolic and nuclear Trx1 and mitochondrial Trx2 in the oxidized or reduced forms were analyzed using redox Western blotting. In myotubes, the mean redox potentials were nuclear Trx1, −251 mV; cytosolic Trx1, −242 mV; mitochondrial Trx2, −346 mV, data supporting the occurrence of differing redox potentials between cell compartments. Exogenous treatment of myoblasts and myotubes with hydrogen peroxide or dithiothreitol modified glutathione redox status and nuclear and cytosolic Trx1, but mitochondrial Trx2 was unchanged. Tibialis anterior muscles from young and old mice were exposed to isometric muscle contractions in vivo. Aging increased muscle contents of Trx1, Trx2, and TrxR2, but neither aging nor endogenous ROS generated during contractions modified Trx redox potentials, although oxidation of glutathione and other thiols occurred. We conclude that glutathione redox couples in skeletal muscle are more susceptible to oxidation than Trx and that Trx proteins are upregulated during aging, but do not appear to modulate redox-regulated adaptations to contractions that fail during aging.

The ability of cells and tissues from old mammals to respond to an oxidative stress by increasing the expression and activities of antioxidant defense enzymes seems severely attenuated. Previous data indicate that skeletal muscle from old rodents and humans shows a number of changes in reactive oxygen species (ROS) activities and actions in comparison to that from young rodents and humans [1]. These include increased oxidative damage to many biomolecules and a failure of ROS-stimulated (redox-regulated) signaling systems [1]. In skeletal muscle of old mice and humans there is increasing evidence that adaptations to contractile activity are attenuated [2] and many of these adaptations involve activation of redox-signaling pathways that mediate changes in muscle gene expression [3]. A feasible explanation for the inability of older muscle to respond to oxidative stress may be failed redox signaling through inappropriate oxidation of key regulatory molecules at specific subcellular sites.

Redox-dependent processes can influence the actions of many proteins involved in regulation of vital cell functions such as proliferation, differentiation, apoptosis, etc. [4,5]. Recent attention has focused on proteins that encompass thiol–disulfide regulation for which the redox status depends on a redox-active site that has an amino acid sequence containing one or two active thiols. The major intracellular thiol–disulfide systems include reduced glutathione/oxidized glutathione (GSH/GSSG) and the thioredoxin (Trx) systems. These control diverse cellular events through discrete redox pathways that influence redox signaling [6], are responsive to oxidative stress, and help maintain intracellular redox homeostasis [4]. Trx's are a class of small multifunctional proteins that are present in all prokaryotic and eukaryotic organisms and are characterized by the invariant redox active-site sequence (Trp-Cys-Gly-Pro-Cys-Lys) [5]. The oxidized (inactive) form of Trx ($TrxS_2$) has two cysteines at its active site forming a disulfide bond that is reduced by thioredoxin reductases (TrxR's) in the cytosol (TrxR1) and mitochondria (TrxR2) and NADPH to a dithiol ($Trx(SH)_2$), which can then act as a general protein disulfide reductase [5,7].

The Trx system is present in various cellular compartments; the Trx1 isoform is found in the nucleus and cytosol. Distinct pools are evident, as Trx1 can be imported into the nucleus from the cytoplasm during various forms of oxidative stress [8,9]. Trx2 is localized in mitochondria and is encoded by a nuclear gene and localized to the mitochondrial matrix by a mitochondrial leader sequence [10]. Trx2 is expressed ubiquitously and found at high

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levels in metabolically active tissue such as heart, skeletal muscle, brain, and liver, where it regulates the mitochondrial redox state and may play an important role in cell proliferation [10–12]. Homozygous knockout of either isoform of Trx in mice is embryonically lethal [13,14] and Trx molecules are conserved throughout evolution [15]. Trx1 and Trx2 interact with cysteine residues in many proteins and have been implicated in cellular processes including protein structure/folding, reductive and metabolic enzymes, energy utilization, transcription factors, and immune modulation [16]. Trx's have also been implicated in aging, and overexpression of either Trx1 or Trx2 has been shown to influence life span in experimental models [17,18].

Understanding the redox modifications to proteins within various cellular compartments such as cytosol, mitochondria, and nuclei is of importance because cell signaling events can occur in discrete compartments and antioxidant systems are not distributed uniformly throughout the cell. The development of models to understand the redox potentials in various cell compartments and how they are modified under physiological and pathophysiological conditions could help facilitate the development of novel targeted therapeutic interventions.

Thioredoxins seem to be key intracellular regulators of redox signaling and the purpose of this study was to determine the susceptibility of thioredoxins to reduction or oxidation by a physiologically relevant oxidant in various cellular compartments of a well-characterized muscle cell line in culture in comparison with the glutathione system. Additionally, anterior tibialis (AT) muscles from young and old mice were exposed to an isometric muscle contraction protocol to follow the relative changes in the redox status of Trx1 and Trx2 in various cellular compartments in comparison with their glutathione redox status. We reasoned that an increased knowledge of the relative susceptibility to oxidation/reduction of Trx1, Trx2, and GSH in various cell compartments would facilitate a greater understanding of the changes seen in muscle in response to physiological stress (contractions) or aging in vivo. To undertake this, a cell fractionation method for C2C12 myoblasts and myotubes and skeletal muscle tissue from young and old mice was initially developed and fractions were used to analyze the redox status of Trx1 (in nuclear and cytosolic fractions) and Trx2 from the same cells or tissue fraction using redox Western blotting. These cell and tissue samples were also analyzed for the protein content of Trx1, Trx2, TrxR1, and TrxR2; total protein thiol content; and glutathione and oxidized glutathione content. Data indicate that in skeletal muscle, exogenous ROS modulators modified the redox status of the Trx1 system in nuclei and cytosol; in vivo aging influenced the muscle content of Trx1, Trx2, and TrxR2; but neither aging per se nor the physiological generation of endogenous ROS by isometric contractions modified the redox potential of the Trx proteins, although under both of these conditions changes in glutathione and other thiols were seen.

Material and methods

Mice and isometric muscle contraction protocol

Experiments were performed in accordance with UK Home Office guidelines under the UK Animals (Scientific Procedures) Act 1986 and received ethical approval from the University of Liverpool Animal Welfare Committee. A total of 30 male C57Bl6 mice were used in this study, 15 young (mean age: 6 months old) and 15 old (mean age: 28 months old). Animals were maintained in a temperature-controlled environment fed on a standard laboratory chow diet ad libitum and subjected to a 12-h light–dark cycle.

Mice were anesthetized with ketamine hydrochloride (66 mg/kg body wt) and medatomidine hydrochloride (0.55 mg/kg body wt) by intraperitoneal injection, and anesthesia was maintained with additional ketamine (30 mg/kg body wt) as required. Each age cohort was split into three equal groups: (i) control mice, which did not receive the contraction protocol (unstimulated); (ii) mice that received the contraction protocol and were sacrificed immediately at the end of the contractions; and (iii) mice that received the contraction protocol and remained at rest for 15 min before sacrifice. For the mice in groups ii and iii, both hindlimbs were subjected to a 15-min period of electrical stimulation via surface electrodes to generate isometric contractions of the hindlimb muscles. The stimulation protocol used was a square wave pulse of 0.2 ms duration at 60 V and a frequency of 100 Hz contracted every 4 s and repeated 180 times. Mice were sacrificed by administration of an overdose of anesthetic at the times described above and both AT muscles were rapidly removed; half of one muscle was snap-frozen in liquid nitrogen for use in analyses of Trx1, Trx2, TrxR1, TrxR2, glutathione, and total protein thiol contents, and the remaining AT tissue was processed immediately to obtain subcellular fractions for redox Western blotting of Trx1 and Trx2.

Cell culture and treatments

The C2C12 mouse skeletal myoblast line was obtained from the American Type Culture Collection (CRL-1772). C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, Poole, UK) supplemented with 1% L-glutamine (Lonza, Cologne, Germany), 10% fetal bovine serum (FBS; Biosera, Sussex, UK), and 1% penicillin and streptomycin (Sigma) under an atmosphere of 5% CO_2 in humidified air at 37 °C. To induce myogenic differentiation, the growth medium was changed to differentiation medium (DMEM supplemented with 2% horse serum and 1% antibiotics and L-glutamine) after myoblasts had reached 90% confluence, and the cells were allowed to mature to myotubes for 7 days.

Proliferating myoblasts and differentiated myotubes were untreated, treated with hydrogen peroxide (H_2O_2; 300–500 μM), or treated with 5 mM dithiothreitol (DTT); the treatments were applied for 30 min at 37 °C in a tissue culture incubator. Cells were then immediately harvested for cell fractionation and biochemical analyses.

Subcellular fractionation

Cells and freshly harvested AT tissue were homogenized in the presence of STM buffer (250 mM sucrose, 50 mM Tris, 5 mM $MgCl_2$) with protease inhibitors (Sigma–Aldrich). For redox Western blots, 50 mM iodoacetic acid (IAA) was added to the lysis buffer and the pH was adjusted to pH 7.4 using 10 M NaOH (in preliminary experiments, this step was found to prevent artifactual oxidation of the Trx1 redox couple in the nuclear and cytosolic extracts). The suspensions were incubated on ice for 5 min and then centrifuged at 12,000 g for 5 min at 4 °C; the pellet (nuclear fraction) was immediately lysed in G-lysis buffer (6 M guanidine–HCl, 50 mM Tris–HCl, pH 8.3, 3 mM EDTA, 0.5% Triton X-100, 50 mM IAA; Sigma Aldrich). Proteins in the supernatant (cytosolic fraction) were precipitated in cold 100% acetone at −20 °C for 30 min followed by centrifugation at 12,000 g, and the protein pellet was resuspended in G-lysis buffer.

Western blotting

The quality of the cell fractionation was determined for the nuclear and cytosolic fractions (15–30 μg/fraction) by examining marker proteins: histone H3 (nuclei) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cytosol) using monoclonal antibodies for histone H3 (Cell Signaling, Hertfordshire, UK, 1:2000) and GAPDH (Abcam, Cambridge, UK, 1:5000). Whole