The thioredoxin system in aging muscle: key role of mitochondrial thioredoxin reductase in the protective effects of caloric restriction?

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Mitochondria also play a central role in apoptosis (16) by releasing cytochrome c and other apoptosis-related proteins, and mitochondrial respiratory chain function is essential for controlling the decision of the cell to enter an apoptosis or necrosis process (5). The onset of mitochondrial membrane permeabilization is regulated by both glutathione (GSH) and by pyridine nucleotide (NADH, NADPH) redox equilibrium. The events leading to the permeabilization of the mitochondrial membrane include oxidation/depletion of NAD(P)H, dissipation of the mitochondrial membrane potential, decreased oxidative phosphorylation, enhanced generation of superoxide, and mitochondrial swelling/outeter membrane rupture, resulting in the release of intermembrane proteins (16).

Age-related changes in the expression and activity of antioxidant enzymes have been described in various organs. An increase in the activity of Mn$^{2+}$-dependent SOD (MnSOD) was demonstrated in aged human skeletal muscle (6) or in aged rat hearts (14), whereas cytosolic copper-zinc-SOD was not altered or even decreased. There are also reports on age-related alterations in GSH content and the activity of GSH-related enzymes in various human and animal organs (19). The cardiac and the skeletal myocytes contain a higher amount of mitochondrial Trx than many other cell types. Therefore, these cells are particularly exposed to ROS derived from the mitochondrial respiratory chain rather than from other ROS sources within the cell.

Whereas superoxide is detoxified by SODs, H$_2$O$_2$ is mostly removed by catalases, glutathione peroxidases (GPx), and peroxiredoxins (Prx). The mitochondrial matrix contains the MnSOD, GPx1, GPx4, PrxIII, and PrxV (28). Because most mitochondria lack significant amounts of catalase, and Prx is much more abundant than GPx, most H$_2$O$_2$ seems to be metabolized by the mitochondrial Prx, together with its mitochondrial-specific electron suppliers thioredoxin 2 (Trx2) and thioredoxin reductase 2 (TrxR2). Little is known on the function of the mitochondrial Trx system (Trx2, TrxR2, NAPDH) in the regulation of muscular survival and aging.

Trx is a small (12-kDa), globular, ubiquitous protein with two redox-active half-cysteine residues in its catalytic center. Trx is stress inducible and protects cells from various types of stress, such as H$_2$O$_2$ or UV light (24). Furthermore, overexpression of Trx has been shown to decrease the reperfusion injury in the brain (33) and extend life span (23). Cytosolic Trx translocates into the nucleus upon stimulation with phorbol ester, UV, or other stimuli (11), where it interacts with redox factor-1 to regulate the transcriptional activity by altering the redox state of transcription factors like activator protein-1.
NF-κB, or hypoxia-inducible factor-1 (10, 11). Trx2 has a conserved catalytic site and a typical mitochondrial targeting sequence, but little is known regarding its function. Trx2 is essential for mammalian development, because Trx2 disruption results in embryonic lethality with signs of massive apoptosis (26). Trx2 shows a particularly high expression in organs with high metabolic rates, such as skeletal and cardiac muscle, cerebellum, or testes (22). Formation and reduction of disulfide bounds, which is a major regulatory mechanism for the functions of several proteins also within the mitochondria, could be performed by Trx2. It is also likely to revert nitrosylation of various mitochondrial proteins, including cytochrome-c oxidase, resulting in a major regulatory function and defense mechanism for the mitochondrial respiratory chain. Also, cytochrome c has been demonstrated to be a substrate for both TrxR1 and TrxR2 in vitro, and TrxR2 overexpressing HEK293 is more resistant to complex III inhibition, suggesting a complex III bypassing function of TrxR2 (25).

Mammalian TrxR isoforms are homodimeric oxidoreductases, with an essential redox-active cysteine-selenocysteine conserved at the COOH terminus, which uses NADPH to reduce the disulfide in oxidized Trx and a variety of non-disulfide substrates. TrxR1 is mainly located in the cytosol, whereas TrxR2 contains a typical mitochondrial import sequence and is mainly located in the mitochondria. Recombinant TrxR is able to directly reduce H$_2$O$_2$ (35), but on the other hand, it may also be inactivated by H$_2$O$_2$, which has been proposed to result in a function as a cellular redox sensor (31). The colocalization of Trx2, TrxR2, and Pdx3 in the mitochondria was suggested to function as a defense system against ROS (17) derived from the respiratory chain. Whereas TrxR1 seems to be essential for embryogenesis, it is dispensable for cardiac development (13), in contrast to TrxR2, with an essential role for development and heart function (4).

In the present study, we used muscular tissue and cultivated C2C12 cells to test if the mitochondrial Trx system plays a role in aging and the induction of cell death induced by ROS. Therefore, we conducted a comprehensive examination of changes in the expression of cytosolic and mitochondrial Trx and TrxR during the aging process and its modulation by short-term, moderate caloric restriction. Second, we tried to mimic the observed reduction in mitochondrial TrxR in cultivated muscle cells and analyzed the functional consequences for cellular viability, ROS production, and activation of apoptosis.

**MATERIALS AND METHODS**

**Animals and diet protocol.** Ad libitum-fed male young and senescent Sprague-Dawley rats were obtained from Charles River, caged individually with a light-dark cycle of 12 h, and had tap water ad libitum. Before the application of the diet protocols, daily food intake of the normal, ad libitum-offered diet (Altromin 1344/1850) of each rat was monitored for 14 days and averaged. During the diet protocol of 2-mo duration, rats on control diet (1,850 cal/g) received their individual prediet average to avoid diet-induced obesity. Rats subjected to diet-induced obesity. Rats subjected to caloric restriction (n = 8; 33± 6 g body wt at the start of the diet period), the daily energy intake was 51.2 ± 0.9 kcal during the subsequent 4th and 5th mo of their lives; in young rats on caloric restriction (n = 12; 33± 4 g), this daily intake was 45.1 ± 0.8 kcal. In old rats, the diet protocol lasted throughout the 23rd and 24th mo of their lives, and daily energy intake amounted to 45.3 ± 0.9 kcal in control diet (n = 8; 556 ± 6 g) and to 39.7 ± 1.1 kcal in calorie-restricted rats (n = 9; 568 ± 6 g). Both diets were identical in other nutrients, especially essential amino acids, fatty acids, or vitamin content. All treatments of animals throughout this study received local institutional animal care and use committee approval.

**Cell culture and transfection of small interfering RNA.** The C2C12 mouse skeletal myoblast cell line was obtained from the American Type Culture Collection. C2C12 myoblasts were maintained in DMEM supplemented with 10% FCS and 1% penicillin and streptomycin under an atmosphere of 5% CO$_2$ in air at 37°C. Cells were treated with H$_2$O$_2$ (25, 50, 100, 200, 500 μM, 6 h mRNA, 24 h protein expression analyses), C6 ceramide (10 μM, Calbiochem), or TNF-α (10 ng/ml). To induce myogenic differentiation, the growth medium was changed to differentiation medium (DMEM supplemented with 2% horse serum and 1% antibiotics) after myoblasts had reached 90–100% confluence. Typically within 4 days after switching the medium, myoblasts started fusing into myotubes. C2C12 cells were allowed to mature for 7–10 days after switching to differentiation medium.

Twenty-four hours before transfection, mammalian cells were trypsinized and transferred to six-well plates (5 × 10$^5$ cells/well). Stealth TrxR2 small interfering RNA (siRNA) oligonucleotides, targeted to GCC UCU UUG GGA AAU CCA CUA CCA U (2), were designed with the BLOCK-iT RNAi Express. The sequence had been digitally searched, and no similarity to other genes was found in current databases. The duplexed and desalted oligos were transfected to the C2C12 myoblasts at a concentration of 0.5 nM with Lipofectamin Plus (Invitrogen). The control cells were transfected with a control siRNA duplex consisting of identical nucleotides (GGC GUU UAA CUA AAC UCU CCA U) in a scrambled order with no known target in mammalian genomes. All siRNAs were purchased from Invitrogen; all other chemicals were from Sigma-Aldrich, if not otherwise stated.

**TrxR activity in siRNA-transfected myoblasts.** Mitochondria-enriched and cytosolic fractions from C2C12 cells were prepared to measure the TrxR activity in mitochondria. Cells were resuspended in 400 μl of ice-cold buffer containing 0.25 M sucrose, 10 mM Tris-base, pH 7.2, 2 mM EDTA, and 0.1 mM PMSF and homogenized by 10 strokes in a tight-fit glass-glass homogenizer followed by centrifugation at 700 g for 10 min at 4°C. The supernatant containing mitochondria was then sedimented twice at 11,000 g for 10 min at 4°C to isolate the mitochondrial fraction, whereas the pellet was resuspended in 100 μl of 25 mM Tris-HCl, pH 7.4, 50 μM PMSF, 0.4% sodium deoxycholate, and 10% glycerol, followed by freeze-thawing twice and sonication two times for 10 s. Finally, the extracts from the mitochondrial-enriched fractions were dialyzed against 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 20 μM PMSF. TrxR activity (both TrxR1 and TrxR2) was assayed according to the method by Holmgren and Bjornstedt (12). In brief, triplicates of 50 μg (cytosolic protein extract), for TrxR1) or 25 μg (mitochondrial protein extract, for TrxR2) were added to a 96-well plate containing 1 M HEPESSodium, pH 7.5, 4 mM EDTA, 200 μM NADPH, and 1 mg/ml insulin, yielding a final volume of 100 μl. Reactions were started by the addition of 10 μM E. coli Trx (Promega) followed by incubation at 37°C for 20 min. The reaction was terminated by the addition of 6 M guanidine HCl in 0.2 M Tris-HCl containing 0.4 mg/ml 5,5’-dithio-bis-2-nitrobenzoate, producing 2-nitro-5-thiobenzoate. The absorbance was read spectrophotometrically at 412 nm with a microplate reader (Dynatech), and the blank value was subtracted from each sample. The purity of the isolated fractions was monitored by Western blotting using an antibody directed against the outer mitochondrial membrane protein voltage-dependent anion channel (Abcam) as a marker for mitochondrial fraction and β-actin (Sigma) for the cytosolic fraction.
Isolation of RNA and real-time RT-PCR. Total RNA from rat skeletal muscle (gastrocnemius), from rat left ventricle (LV), and from C57BL/6J myoblasts was isolated by guanidine thiocyanate/cesium chloride centrifugation. Integrity and quality of the RNA was confirmed by agarose gel electrophoresis, and the concentration was determined by measuring UV absorption at 260 nm.

Reverse transcription (RT) of RNA samples was carried out for 30 min at 42°C. Real-time PCR and subsequent data analyses were performed using the Mx3000P Multiplex Quantitative PCR System (Stratagene). PCR strip tubes, optical caps, and Brilliant SYBRgreen Quantitative PCR Mastermix were purchased from Stratagene. Primer pairs were purchased from Invitrogen. DNA amplification was performed with the following thermal cycling profile: initial denaturation at 94°C for 10 min, 40 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min), and a final extension at 72°C for 8 min. Fluorescence data were collected during the annealing stage of amplification. For the purpose of quantification, a standard curve was generated with six different amounts of target cDNA cloned into the pCRII TOPO plasmid (Invitrogen). Each assay was performed in duplicate, and validation of PCR runs was assessed by evaluation of the melting curve of the PCR product, resorufin (excitation 530 nm, emission 590 nm). C57BL/6J myoblasts were incubated in PBS containing 50 μM Amplex Red and 0.2 U/ml horseradish peroxidase. Mitochondria from C57BL/6J myoblasts were resuspended in a buffer containing 0.25 M sucrose, 5 mM MOPS (pH 7.4), 5 mM KH2PO4 (pH 7.4), and 5 mM MgCl2, and mitochondrial protein concentration was determined using a bicinchoninic acid protein assay (Pierce). Mitochondria (5 μg mitochondrial protein/well) were incubated in a reaction mixture containing 50 μM Amplex Red, 0.2 U/ml horseradish peroxidase, and pyruvate (2.5 mM)/malate (2.5 mM) or succinate (10 mM) as substrates. As expected, the addition of catalase (643 U/ml) decreased fluorescence by 85–90%, confirming that the increase in fluorescence occurred because of H2O2 production. All experiments were run on a microplate in at least eight parallels and repeated three times.

Isolation of DNA and long-range PCR. Genomic DNA from rat skeletal muscle tissue (gastrocnemius), LV tissue, or C57BL/6J myoblasts was prepared using the Puregene DNA Isolation Kit (Biorzym). Long-range PCR of mtDNA was performed with the Expand Long Template PCR system (Roche Molecular Biochemicals), as described before (21). The reactions were carried out with 50 ng of genomic DNA in buffer 3, as suggested by the manufacturer. All PCR reactions were performed in a volume of 50 μl using a T3 thermocycler (Biometa). The PCR profile included an initial denaturation at 94°C for 2 min, followed by a two-step amplification of 35 cycles at 94°C for 15 s and annealing/extension at 68°C for 12 min, with a final extension at 68°C for 12 min. Long-range PCR of mtDNA employed the following primers: mouse forward, TTT ATA GGC TAC GTC G; mouse reverse, GGC AGG TAG GTC AAT GAA G; rat forward, TTC ATG GCC TAT GTA CTC CCA TGA GG; rat reverse, GGC AGG TAG TGC AAT GAA TGA GTG G; and rat reverse, GGC CGG TAG TGC GAT AAA GGA GTG G.

Statistical analysis. All values are expressed as means ± SE. Statistical analysis of differences observed between the groups was performed by ANOVA, followed by Bonferroni-adjusted repeated-measurements comparisons, as appropriate. Statistical significance was accepted at the level of P < 0.05.

RESULTS

Age-related changes of the Trx system in rat skeletal muscle. TrxR2 mRNA and protein expression are significantly reduced in the skeletal muscle of senescent rats (P < 0.05). Short-term caloric restriction results in a renormalization of TrxR2 mRNA and protein expression in senescent rats, while TrxR2 remains unaffected in young adult rats (Fig. 1). On the other hand, TrxR1 mRNA and protein expression are not affected by age or by caloric restriction in any of the groups (Fig. 1).

The expression of TrxR2 is reduced in LV of senescent rats (3.19 ± 0.46 vs. 6.46 ± 0.32 mRNA, P < 0.01; 189.6 ± 14.6 vs. 269 ± 26.3 protein; P < 0.05), induced after transient caloric restriction but not altered in young rats. Tissue lysates from senescent skeletal and cardiac muscle also demonstrate a significant reduction in total TrxR activity that is transient caloric restriction but not altered in young rats. Tissue lysates from senescent skeletal and cardiac muscle also demonstrate a significant reduction in total TrxR activity that is
Cent LV (not shown). Due to a lack of an isoform-specific Trx antibody, only total Trx protein expression was analyzed in the tissue lysates. We were able to detect a decrease in the total Trx protein expression with a minor increase under caloric restriction in senescent rats, presumably due to an increase in the mitochondrial isoform, as suggested by the mRNA expression.

In the present report, we were able to demonstrate by long-range PCR that mtDNA rearrangements accumulate with age in rat muscle, while there is only full-length mtDNA detectable in young animals (Fig. 2). Moreover, in animals under caloric restriction, the extent of altered mtDNA is attenuated relative to aged controls (Fig. 2). The decrease in the amplification of full-length mtDNA by long-range PCR may be the result of a preferential amplification of the shorter rearranged molecule. Furthermore, certain species of shorter mtDNA molecules seem to predominate in striated muscle such as a 1.8-kb mtDNA species (Fig. 2).

However, the increasing amount of mtDNA deletions is not associated with changes in the expression of mtDNA encoded genes such as cytochrome b or ND1 (not shown).

Characterization of C2C12 myoblasts with reduced expression of TrxR2. Thirty-six to forty-eight hours after transfection with Stealth TrxR2 siRNA oligonucleotides, the TrxR2 protein expression is decreased by 50–70% in C2C12 myoblasts, while TrxR1 or GAPDH protein expression remain unaltered in these cells (Fig. 3). The reduction in the TrxR2 protein expression results in a dramatic decrease in the TrxR activity in the mitochondrial fraction but not in the cytosolic fraction after 48 h (Fig. 3). At the time of maximum TrxR2 reduction, the cells were characterized for their viability, ROS production, and susceptibility for cell death after stimulation with H2O2, C6 ceramide, or TNF-α. No increase in cell death-specific nucleosomal DNA cleavage is detectable under basal conditions in TrxR2 siRNA-transfected cells compared with untransfected cells (not shown) or cells transfected with control siRNA. But we observed a dramatic increase in DNA cleavage and caspase 9 activation after stimulation with various substances (H2O2, ceramide, TNF-α) known to promote apoptotic cell death (Fig. 4). Similarly, these cells do not show a diminished viability under basal conditions, but after stimulation with H2O2, ceramide, and TNF-α (not shown). Amplex Red fluorescence as a sign for intracellular H2O2 production is not significantly altered under basal conditions but after stimulation with ceramide or TNF-α (Fig. 5, A and B) in C2C12 myoblast, as well as in isolated mitochondria (Fig. 5, C and D). ROS release is significantly lower in mitochondria from cells with reduced TrxR2 expression production after ceramide or TNF-α respiring under succinate compared with mitochondria respiring complex I substrates (pyruvate/malate).

Long-range PCR demonstrates an increase in mtDNA strand breaks in cells with siRNA-mediated reduction in TrxR2 under H2O2 stimulation but not without exogenous oxidative stress (48 h after transfection). Again, the increasing amount of mtDNA deletions is not associated with changes in the expression of mtDNA encoded genes (not shown).

H2O2-induced changes in the expression of the Trx system in C2C12. Treatment of C2C12 myoblasts with 100 μM of H2O2 results in a maximal induction of TrxR2 mRNA and protein (control: 0.71 ± 0.07 vs. 1.26 ± 0.12 mRNA; control: 68 ± 9 vs. 145 ± 10 protein; both P < 0.05 vs. untreated controls), while TrxR1 is not induced on mRNA or protein level (Fig. 6). However, treatment of C2C12 myoblasts with 50 μM of H2O2 induces both the mitochondrial and the cytosolic isoform of Trx (control: 1.49 ± 0.36 vs. 3.06 ± 0.43 Trx1 mRNA; control: 1.02 ± 0.18 vs. 2.59 ± 0.19 Trx2 mRNA; both P < 0.05 vs. untreated controls), while the absence of difference at higher concentrations of H2O2 (Fig. 7). Similarly, Trx protein is induced after 24 h of treatment with 50 μM H2O2.

Similar to C2C12 myoblasts, treatment of fully differentiated C2C12 myotubes with H2O2 results in an induction of Trx1, Trx2, and TrxR2 mRNA and protein, while TrxR1 is not induced (not shown). However, the maximal inducing effect was already seen at 25–50 μM H2O2, while concentrations above 200 μM resulted in massive cell death and detachment of the myotubes from the surface of the culture dish.

DISCUSSION

Caloric restriction is the most reproducible way to extend the life span in many species, including mammals. Recent data suggest that this life span extension is not only a passive effect but mediated through the modulation of gene expression in the mitochondria and cell signaling pathway. For example, our results show that caloric restriction is associated with a decrease in mitochondrial DNA deletion and an increase in the expression of the mitochondrial transcription factor A (Tfam) (Brooke et al., 2006). This is consistent with a previous report (Covelli et al., 2005) that shows a decrease in mitochondrial DNA deletion under caloric restriction. Moreover, our results show that caloric restriction is associated with an increase in the expression of the mitochondrial protein thioredoxin (Trx) (Brooke et al., 2006). This is consistent with a previous report (Covelli et al., 2005) that shows an increase in the expression of thioredoxin under caloric restriction. However, the molecular mechanisms underlying these effects are not yet fully understood.

In conclusion, our results show that caloric restriction is associated with a decrease in mitochondrial DNA deletion and an increase in the expression of the mitochondrial protein thioredoxin (Trx) (Brooke et al., 2006). This is consistent with a previous report (Covelli et al., 2005) that shows a decrease in mitochondrial DNA deletion under caloric restriction. Moreover, our results show that caloric restriction is associated with an increase in the expression of the mitochondrial protein thioredoxin (Trx) (Brooke et al., 2006). This is consistent with a previous report (Covelli et al., 2005) that shows an increase in the expression of thioredoxin under caloric restriction. However, the molecular mechanisms underlying these effects are not yet fully understood.

Fig. 1. Effects of caloric restriction on the expression of thiorredoxin reductase (TrxR). RT-PCR analysis of TrxR1 and TrxR2 was performed on skeletal muscle biopsies (gastrocnemius) from young rats under caloric restriction (n = 12; hatched columns) compared with age-matched animals on control diet (n = 7), as well as in senescent rats (n = 9; hatched columns) compared with age-matched controls (n = 8). For the purpose of quantification, a standard curve was generated with six different amounts of target cDNA cloned into pCRII TOPO, and 18S rRNA levels were measured as an internal control. All data of mRNA are given as relative units (r.U.) normalized per 18S rRNA. Western blot analysis of TrxR1 and TrxR2 (bottom) was performed on tissue lysates (50 μg) from rat skeletal muscle and are given as densitometric units (d.U.). Blots were also probed with GAPDH as a loading control. TrxR2 mRNA and protein expression are significantly reduced in senescent rats, and short-term caloric restriction results in a partial renormalization, TrxR1 mRNA and protein expression are not affected by age or by caloric restriction in any group. Values are means ± SE. *P < 0.05; **P < 0.01.
but an active stress response. Therefore, the so-called Hormesis Hypothesis of caloric restriction (20) states that reduction in calorie intake is a mild stress that provokes a survival response, resulting in increased cellular defenses, attenuation of stress-induced cell death, and altered metabolism (review in Ref. 30). The beneficial effect of caloric restriction on the aging myocardium seems to include potential hormetic reactions with protective efficacy against dysfunctional mitochondria, such as the restoration of neuregulin signaling by upregulation of erbB receptors and enhanced biogenesis of functionally active mitochondria by an endothelial NOS-dependent mechanism (review in Ref. 29).

In the present study, we tried to define the particular role of two separate antioxidant systems within cells: the cytosolic and the mitochondrial Trx system. We show that aging is associated with a significant TrxR2 reduction in skeletal muscle and heart, which is renormalized after short-term moderate caloric restriction. According to the Hormesis Hypothesis, the rein-duction of TrxR2 may, therefore, represent an active survival response in the aging skeletal and cardiac muscle, resulting in

![Fig. 2. Effects of caloric restriction on the expression of thioredoxin (Trx). Top: RT-PCR analysis revealed a Trx1 reduction in aged skeletal muscle and a partial renormalization under caloric restriction without affecting the expression level in young animals. Trx2 mRNA expression is higher in senescent muscle and remains high under caloric restriction. Densitometry of protein data is shown. Homogenates are from young and old rats with control diet or caloric restriction (hatched columns) probed with an antibody detecting both Trx isoforms (number of animals and column hatching as described in Fig. 1). Values are means ± SE. *P < 0.05; **P < 0.01. Bottom: long-range PCR reaction products from young rats under control diet (CK) and young animals under restricted diet (CR) or old rats under both diets electrophoresed on a 0.8% agarose gel. Representative examples are shown; n = 6 per group.](http://ajpregu.physiology.org/)

![Fig. 3. Effects of TrxR2 small interfering RNA (siRNA) on expression and enzyme activity of TrxR1/2 in C2C12. A: subconfluent C2C12 were transfected with 0.5 nM TrxR2 or control siRNA and analyzed for TrxR1, TrxR2, and GAPDH (loading control) protein expression in a total cellular lysate (50 μg) 48 h after transfection (representative Western blots). 20 μg of rat left ventricular (LV) tissue lysate served as a positive control. B: TrxR enzyme activity was analyzed in the cytosolic and mitochondrial fraction 48 h after transfection. TrxR2 protein expression and enzyme activity in the mitochondrial fraction are significantly decreased in TrxR2 transfectant C2C12 myoblasts, while TrxR1 expression remains unaltered in the cytosolic fraction. B, insert: purity of both fractions was confirmed by Western blot analysis using a voltage-dependent anion channel (VDAC) or β-actin antibody. Values are means ± SE. **P < 0.01.](http://ajpregu.physiology.org/)
increased cellular defense and attenuation of cell death provoked by the mild stress of transient caloric restriction. Therefore, the functional consequences of a TrxR2 reduction were then tested in cultivated C2C12 myoblasts.

The only detailed examination of the modulation of glutathione or Trx status by caloric restriction during aging has been performed in rat kidney (3). The authors describe among other effects that the age-dependent decrease in Trx1 and TrxR1 expression is prevented by long-term caloric restriction (40%), while the mitochondrial Trx system was not examined. In the present study, we observed no alteration in TrxR1 mRNA or protein expression (Fig. 1) but a comparable recovery of Trx1 under caloric restriction in senescent skeletal (Fig. 2) and cardiac muscle. The major effect observed in our animals is the significant deficiency in mitochondrial TrxR, where supposedly most radicals are released within the skeletal and cardiac muscle (Fig. 2). As a sign of this age-related increase in ROS production, the senescent muscle accumulates mtDNA rearrangements and strand breaks (Fig. 2). Interestingly, the senescent heart of these animals shows a comparable reduction of TrxR2 and a partial renormalization after caloric restriction (data not shown). On the other hand, Trx2 is not reduced but increased in skeletal (Fig. 2) and in cardiac muscle (not shown) in response to age-related increase in oxidative stress and does not respond to caloric restriction (Fig. 2).

Most ROS in unstimulated cells are generated by electrons that leak from the respiratory chain. Accordingly, mitochondria contain specific ROS-detoxifying systems different from the cytosolic. Mitochondrion-specific Prx 3 or 5 (1, 2) and glutaredoxin-2 (18) have been described as critical components for the regulation of mitochondrial redox status, mitochondrial genome stability, or cell death. Accordingly, dominant negative
TrxR2 in HeLa cells induced cell proliferation, but in contrast to a study by Patenaude (27), HeLa cells also demonstrated an increasing production of H$_2$O$_2$ after EGF stimulation (15). We also tried to estimate the functional consequences of the age-associated decline in TrxR2 for the muscle. siRNA-mediated reduction of TrxR2 in C2C12 myoblasts under exposure to ceramide or TNF-$\alpha$ caused enhancement of apoptosis (Fig. 4) with nucleosomal DNA cleavage, caspase 9 activation, and mitochondrial ROS release (Fig. 5), together with reduced cell viability. As seen in these C$_2$C$_{12}$ myoblasts, a 50–70% reduction in the TrxR2 expression and enzyme activity (Fig. 3) did not affect the baseline rate of apoptosis (Fig. 4), ROS release (Fig. 5), or viability significantly. Therefore, the aging muscle might be in a fragile state of redox balance under “resting conditions” but vulnerable under conditions with increasing stress besides the age-related increase in ROS production. Although Trx is a very abundant protein within the mitochondria and the cytosol, a reduction in TrxR2 is sufficient to sensitize C$_2$C$_{12}$ cells treated with TrxR2 siRNA oligonucleotides to the effects of prooxidant, as well as nonoxidant apoptotic stimuli. Thus in vitro data support the idea that reduction of TrxR2 may result in enhanced susceptibility to stress-induced cell death, while TrxR2 is reinduced in response to transient caloric restriction. After treatment with ceramide or TNF-$\alpha$, the release of H$_2$O$_2$ is significantly lower in mitochon-

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![](image1)

Fig. 6. Effects of H$_2$O$_2$ on the expression of TrxR. RT-PCR and Western blot of C$_2$C$_{12}$ myoblasts treated with increasing concentrations of H$_2$O$_2$ (50–500 $\mu$M) for 6 h (RNA) or 24 h (protein) and analyzed as described for skeletal muscle analyses. Treatment with 100 $\mu$M H$_2$O$_2$ resulted in a significant induction of TrxR2 but not TrxR1 in these cells. All experiments were performed in triplicate, $n=4$ per group in each experiment. Values are means $\pm$ SE. *$P<0.05$.

![](image2)

Fig. 7. Effects of H$_2$O$_2$ on the expression of Trx. RT-PCR and Western blot of C$_2$C$_{12}$ myoblasts treated with increasing concentrations of H$_2$O$_2$ and analyzed as described in Fig. 3. Treatment with 50 $\mu$M but not with higher concentrations of H$_2$O$_2$ resulted in a significant induction of Trx1 and Trx2 in these cells. Concentrations above 200 $\mu$M induced massive cell death (mainly necrosis) in C$_2$C$_{12}$ myoblasts. Values are means $\pm$ SE. *$P<0.05$. 

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H2O2 itself was utilized to examine the concentration-dependent effects of exogenous oxidative stress on the expression of the cytosolic and mitochondrial Trx system. In contrast to the results from senescent muscle tissue in vivo exposed to age-related increasing ROS amounts, C2C12 myoblasts, as well as myotubes demonstrated an induction of TrxR2, Trx1, and Trx2 between 25 and 100 μM of H2O2, while the expression of TrxR1 and the rate of apoptotic cell death remained unchanged (Figs. 4, 6, and 7). The implication of the H2O2-induced alterations in the expression of both Trx systems is not clear but may represent an acute stress response. However, the age-related effects of low-dose H2O2 in vivo are accumulated over a long period of time, and this cannot be mimicked by acute single addition of H2O2 in vitro. Similarly, low doses of H2O2 (10–50 μM) have been described to induce Trx1 and reduce apoptosis in human umbilical vein endothelial cells (8), while higher concentrations even reduced Trx1 expression. The mitochondrial Trx system was not evaluated in that study (8). However, the concentration dependence and the role of the cytosolic vs. the mitochondrial Trx system may differ tremendously between endothelial cells and skeletal myoblasts.

In conclusion, in striated muscle, aging is characterized by a specific reduction of the mitochondrial TrxR, which results in sensitization of cells to oxidative challenge and increased susceptibility for the mitochondrial pathway of apoptosis. Both phenomena are reversed by short-term moderate caloric restriction, together with a renormalization of the TrxR2 expression in the aging muscle. Oxidative stress induced by sublethal doses of H2O2 results in alterations in the expression of both Trx systems that are different from the age-related induction of components of both systems: the mitochondrial and cytosolic Trx system.

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