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Hindlimb unloading decreases thioredoxin-related antioxidant proteins and increases thioredoxin-binding protein-2 in rat skeletal muscle

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Abstract

To investigate role(s) of thioredoxin-related antioxidant proteins in disuse muscle atrophy, we examined the levels of thioredoxin-1 (Trx-1), peroxiredoxin-3/SP-22 (Prx-3) and thioredoxin-binding protein-2 (TBP-2) in rat soleus muscle subjected to hindlimb unloading (HU) for 2, 4, 7 or 14 days. The muscle weight loss was initially observed on day 4. The increases in aclorein- and malondialdehyde-modified proteins, and the decreases in the levels of Trx-1, Prx-3 and Mn-SOD were observed in the late phase of muscle atrophy, whereas, the increase in mRNA expression of TBP-2, a negative regulator of thioredoxin, preceded muscle atrophy. These findings suggest that the decrease of those antioxidant proteins, particularly a marked decrease of Trx-1, may be responsible for the enhanced oxidative damage during the late phase of disuse muscle atrophy. Furthermore, the increase in TBP-2 preceding the muscle atrophy may suppress the thioredoxin-mediated redox signaling, which can be an initial trigger leading to disuse muscle atrophy.

Keywords: *Oxidative stress, antioxidant defense system, thioredoxin-1, peroxiredoxin-3/SP-22, thioredoxin-binding protein-2/vitamin D3 up-regulated protein 1*

Introduction

Muscle disuse due to a variety of conditions such as prolonged bed rest, orthopedic injury and space flight is known to induce skeletal muscle atrophy [1]. Disuse atrophy of skeletal muscle is considered to be an important problem in the field of rehabilitation medicine [2] and space biology. In general, the atrophy of skeletal muscle is caused by a decrease in protein synthesis and an increase in protein degradation [1,3]. It has been reported that oxidative stress is elevated in atrophied skeletal muscle [4–8] and oxidative damage to proteins accelerates their degradation [9] by proteolytic pathways such as the ubiquitin–proteasome system, Ca^{2+}-dependent calpain system and the lysosomal proteases [1,10,11].

Several animal studies have demonstrated an increase in oxidative stress during disuse muscle atrophy with immobilization [4,6,7] or hindlimb unloading (HU) [5,8,10]. Lawler et al. demonstrated that 28-day HU decreased the activities of Mn-superoxide dismutase (Mn-SOD), catalase and glutathione peroxidase and increased the activity of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) [8]. They also reported that HU led to a decrease in non-enzymatic antioxidant capacity in rat soleus muscle.

Recently, a growing body of evidence has indicated that the molecular events underlying muscle atrophy involve several critical pathways regulated by intracellular signaling molecules such as phosphatidylinositol 3-kinase (PI3K) and Akt [12]; these events are also regulated by transcription factors such as nuclear factor (NF)-κB [13,14] and FOXO forkhead

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transcription factors [15,16]. These signaling pathways eventually result in the enhancement of muscular protein degradation via the ubiquitin–proteasome system, and/or the Ca^{2+}-dependent calpain system, and also lead to the suppression of muscular protein synthesis and vascular formation required for the growth of muscle cells [1].

Thioredoxin-1 (Trx-1) is a cytosolic protein which contains a redox-active dithiol/disulfide in the active site [17] and is involved in antioxidant defence mechanisms such as the elimination of peroxide [18] and the reduction of oxidized proteins [19,20]. Peroxiredoxin-3/SP-22 (Prx-3) is a member of thioredoxin-dependent peroxidase family that is localized in mitochondria where reactive oxygen species (ROS) are continuously generated as by-products of aerobic energy metabolism. Recent studies have shown that Prx-3 constitutes a novel antioxidant defence system together with mitochondrial thioredoxin (thioredoxin-2) and mitochondrial thioredoxin reductase [21,22] and exerts a protective effect against oxidative stress in the mitochondria of vascular endothelial cells [23]. Furthermore, a number of evidence has indicated that thioredoxin plays multiple regulatory roles in various cellular processes [24], some of which are closely associated with the molecular processes underlying disuse muscle atrophy. The involvements of thioredoxin and thioredoxin-related antioxidant proteins in disuse muscle atrophy, however, have not yet been investigated.

A recent DNA microarray study reported by Stevenson et al. [25] revealed that thioredoxin-binding protein-2 (TBP-2) [26], which is also called thioredoxin-interacting protein (Txnip) [27] or vitamin D3 upregulated protein 1(VDUP-1) [28], was upregulated in the soleus muscle atrophied by HU. TBP-2 has been reported to serve as a negative regulator of the biological function and expression of thioredoxin [26,29].

In the present study, we examined the changes in the protein and mRNA levels of Trx-1 and Prx-3 and the mRNA level of TBP-2 during disuse-induced muscle atrophy. Our results, particularly a marked decrease in the level of Trx-1, suggest a possible involvement of thioredoxin and thioredoxin-related proteins in increased oxidative stress observed during the late phase of disuse muscle atrophy. Furthermore, the mRNA increase of TBP-2, a negative regulator of Trx-1, during the early phase of disuse muscle atrophy also suggests the involvement of Trx-1 in the molecular events committing muscle to disuse atrophy.

Materials and methods

Animals

Male Wistar rats (age: 11 weeks) were obtained and acclimated for a one-week period, and were then randomly assigned to a HU group or to the control group. For the HU groups, the hindlimbs of the animals were suspended for 2 ($n = 5$), 4 ($n = 6$), 7 ($n = 6$) or 14 ($n = 5$) days according to the tail-casting technique, previously described [30]. Since the tail casting procedure for HU of the rats was performed while the animals were under anaesthesia with an intraperitoneal injection of pentobarbital sodium (50 mg/kg), the control rats were also anesthetized in the same procedure, in order to maintain equivalent acclimatized conditions. The HU rats were fed and watered *ad libitum*, and the control rats were pair-fed to the HU rats in order to maintain equal nutritious conditions. The control groups were kept in unrestricted cages for 2 ($n = 5$), 4 ($n = 6$), 7 ($n = 6$) or 14 ($n = 5$) days. Cages for all groups were maintained at 22°C and 50% humidity under a 12:12 h light–dark cycle. After the HU periods, the rats were euthanized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg), and then the soleus muscles which are typical slow-twitch muscles from both hindlimbs were collected, and the wet tissue weight was measured. All experimental procedures were carried out with the approval of the Ethics Committee for Animal Care and Experimentation in accordance with the Guiding Principles for Animal Care and Experimentation of the University of Occupational and Environmental Health, and according to the Japanese Law for Animal Welfare and Care.

Reagents

Polyclonal rabbit antibodies against Trx-1 and Prx-3 were prepared by immunization with *C*-terminal synthetic peptides, as described previously [23,31]. Monoclonal antibody against Mn-SOD was purchased from Chemicon International, Inc. (Temecula, CA). Monoclonal antibodies against aclorein- and malondialdehyde (MDA)-modified proteins were purchased from NOF Co. (Tokyo, Japan). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was purchased from Medical Biological Laboratory (Nagoya, Japan) and HRP-conjugated rabbit anti-mouse IgG was purchased from Zymed Laboratory, Inc. (San Francisco, CA). All other chemicals and biochemicals used were of analytical and molecular biological grade.

Immunohistochemistry of markers of oxidative stress

Isolated soleus muscles were attached to cork boards with tragacanth gum, and then the samples were immersed in isopentane, which was chilled by liquid nitrogen at a temperature of nearly −150°C. Cryopreserved-frozen muscles kept at nearly −80°C were transversely cut into approximately 10 μm-thick serial sections using a cryostat (Microm, Walldorf, Germany). Sections were fixed with acetone for 5 min and then the endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol. These thin sections were

reacted with the monoclonal antibody to the acrolein- or MDA-modified proteins, and the sections were stained using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). When the sections were reacted with non-immune rabbit serum as a negative control, the positive signals were difficult to detect (data not shown).

Protein carbonyl assay

Oxidative damage to proteins was evaluated by measuring the protein carbonyl content using 2,4-dinitrophenylhydrazine (DNPH) according to the method of Reznick and Packer [32]. Briefly, 50–100 mg of soleus muscle tissue was homogenized in 50 mM phosphate buffer, pH 7.4, containing 0.1% digitonin, a cocktail of antiproteases (0.5 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin and 0.5 μg/ml p-amidino-phenylmethylsulfonylfluoride) and 1 mM EDTA. A nucleic acid-depleted protein extract was prepared from this homogenate by treatment with 1% streptomycin, and the extract was reacted with 10 mM DNPH–2.5 M HCl in the dark. The absorbance of the resultant DNPH derivatives (protein hydrazones) was spectrophotometrically measured at 356 nm. The protein carbonyl content was calculated by using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} for the DNPH derivatives.

Western blot analysis of Trx-1, Prx-3 and Mn-SOD

Soleus muscles from all groups were homogenized in lysis buffer (10 mM phosphate buffer, pH 7.5, containing 0.25 M sucrose, 0.5 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin and 0.5 μg/ml p-amidino-phenylmethylsulfonylfluoride) and the lysates were centrifuged for 10 min at 8000*g*. An equal protein amount of the supernatant was separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE), and was probed with an antibody to Trx-1, Prx-3 or Mn-SOD. HRP-conjugated secondary antibodies were used in conjunction with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Little Chalfont, UK) or a POD Immunostain kit (Wako, Osaka, Japan) in order to visualize the immunopositive bands. The intensity of the bands was quantified by densitometric analysis using the public-domain computer program NIH Image (Wayne Rasband, NIH, Research Service Branch, NIMH, Bethesda, MD).

RT-PCR for Trx-1, Prx-3 and TBP-2

RT-PCR was performed to quantitate Trx-1, Prx-3 and TBP-2 mRNAs in the soleus muscles. The total RNA was isolated from the soleus muscles using TRIzol reagent (Life Technologies Inc., Gaithersburg, MD), based on the method reported by Chomczynski and Sacchi [33]. RT reactions were carried out with moloney murine leukemia virus reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) by using the random hexanucleotide primer. The reverse-transcribed cDNA products were amplified with Taq DNA polymerase in a DNA thermal cycler (Perkin-Elmer, Wellesley, MA). The conditions for Trx-1 for each cycle were 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The conditions for Prx-3 for each cycle were 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. The conditions for TBP-2 for each cycle were 94°C for 1 min, 60°C for 1 min and 72°C for 2 min. The following gene-specific primers for Trx-1 were used: 5′-GGTGAAGCTGATCGAGAGCAAG-3′ (bases 3–24, sense) and 5′-CAGCAACATCCTG-GCAGTCATC-3′ (bases 178–199, antisense), which amplified a 197-base pair product. The gene-specific primers for Prx-3 were 5′-GTTGCGGTTTCTGT-GGATTCC-3′ (bases 419–439, sense) and 5′-TGG-GGCTCTCTTTTGGCATG-3′ (bases 840–859, antisense), which amplified a 441-base pair product. The gene-specific primers for TBP-2 were 5′-ACC-CGTTATTTCCGTGTGACTCCT-3′ (sence) and 5′-TGCCTCCATCAGTGTTAGGGCATC-3′ (antisence), which was reported by Saitoh et al. [34]. Each primer set yielded a single PCR product of the predicted size. The identity of the PCR products was confirmed by direct cycle sequencing. RT-PCR was also performed for the housekeeping gene β-actin as a control for the amount of RNA used in the RT reaction. A negative control, in which reverse transcriptase was omitted, was also performed in order to exclude the possibility of amplification of contaminating genomic DNA. Linear relationships were observed between the quantity of RNA subjected to the RT reaction and the amount of amplified PCR product under the PCR conditions used for Trx-1 (23 cycles), Prx-3 (21 cycles), TBP-2 (21 cycles) and β-actin (22 cycles). The quantity of the ethidium bromide-stained PCR products was measured by densitometric analysis using the public-domain computer program NIH Image (Wayne Rasband, NIH, Research Service Branch, NIMH, Bethesda, MD).

Statistical analysis

Student's unpaired *t*-test was used to compare differences between the control groups and the HU groups. Values of $p < 0.05$ were considered significant.

Results

Muscle weight

Soleus muscle weights were measured as an indicator of the muscle atrophy. The weight loss of the soleus muscle initially became evident on day 4 after the HU. The weights of the soleus muscle were decreased to 75.4, 72.9 and 50.9% of the control by exposure to the 4-, 7- and 14-day HU, respectively (Figure 1).

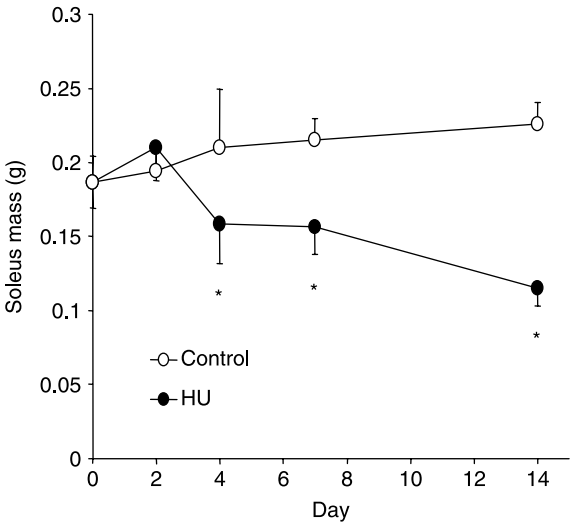

Figure 1. Changes of the wet weight of the soleus muscle of the 2, 4, 7 or 14-day HU rats and those of the control rats. Values are means ± SD ($n = 5$ or 6). *$p < 0.05$ compared with the control for the same period.

Oxidative damage

The accumulation of acrolein- and MDA-modified proteins of soleus muscles subjected to the HU was examined in order to evaluate the degree of lipid peroxidation using antibodies specific to each of the modified proteins. The immunohistochemical analysis showed that the accumulation of acrolein- and MDA-modified proteins were increased in both intercellular and extracellular spaces of the soleus muscles of the 14-day HU rats (Figure 2), whereas, there was no apparent increase in those modified proteins on day 2, 4 and 7 (data not shown).

Oxidative damage to proteins by HU was also evaluated by measuring protein carbonyl content using 2,4-DNPH as a reagent. The specific content of protein carbonyl in the soleus muscles was significantly increased (132%, $p < 0.05$) on day 14 after the HU, compared with that of the control rats (25.7 nmol/mg protein vs 19.4 nmol/mg protein).

The changes of Trx-1, Prx-3 and Mn-SOD

To examine the changes of antioxidant proteins during the HU, we measured the protein levels of Trx-1, Prx-3 and Mn-SOD in the soleus muscle by Western blot analysis. The protein levels of Trx-1 in the soleus muscle started to decrease on day 7 (71.0% of the control; $p < 0.01$) and was markedly decreased on day 14 (18.2% of the control; $p < 0.005$) after the HU, whereas, no significant changes were observed on day 2 and 4 (Figure 3). The RT-PCR results indicated that the mRNA level of Trx-1 started to decrease on day 7 and reached 68.6% of the control ($p < 0.05$) on day 14 (Figure 4).

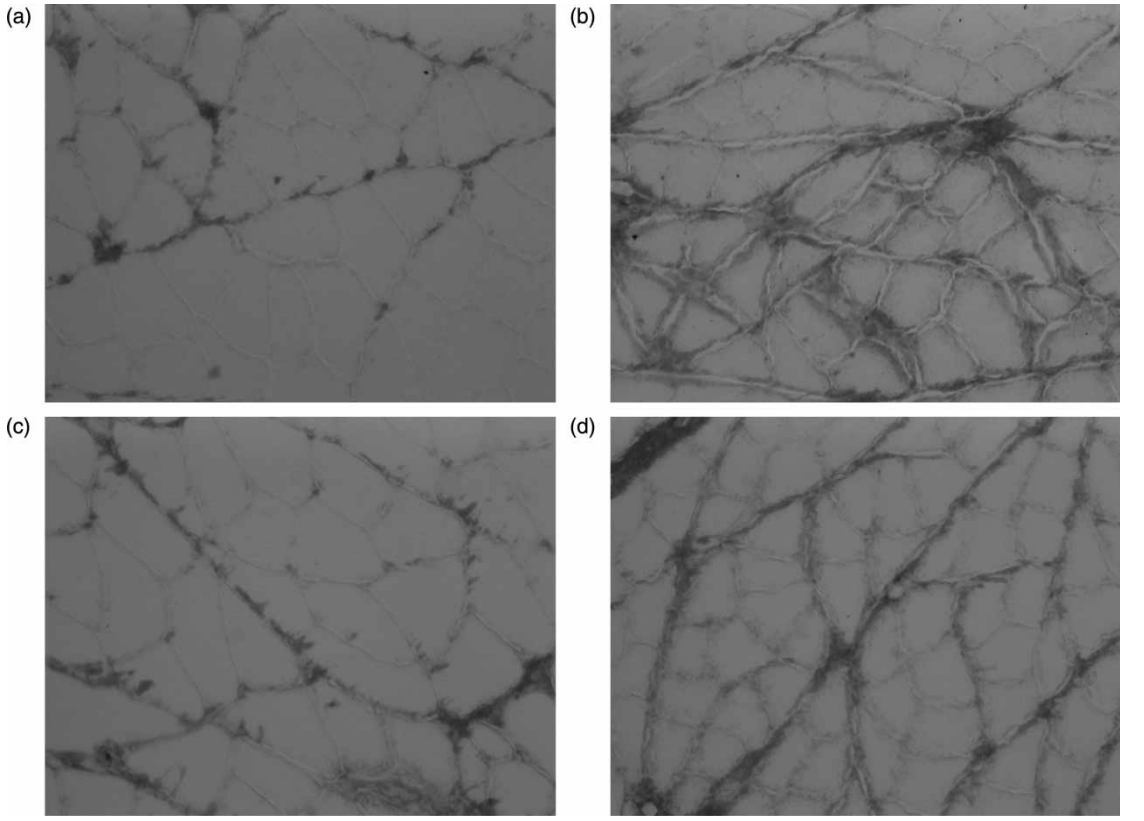

Figure 2. Immunohistochemical analysis of acrolein-(a,b) and malondialdehyde-(c,d) modified proteins in soleus muscle cross-sections from the control (a,c) and the 14-day HU (b,d) rats (× 200).

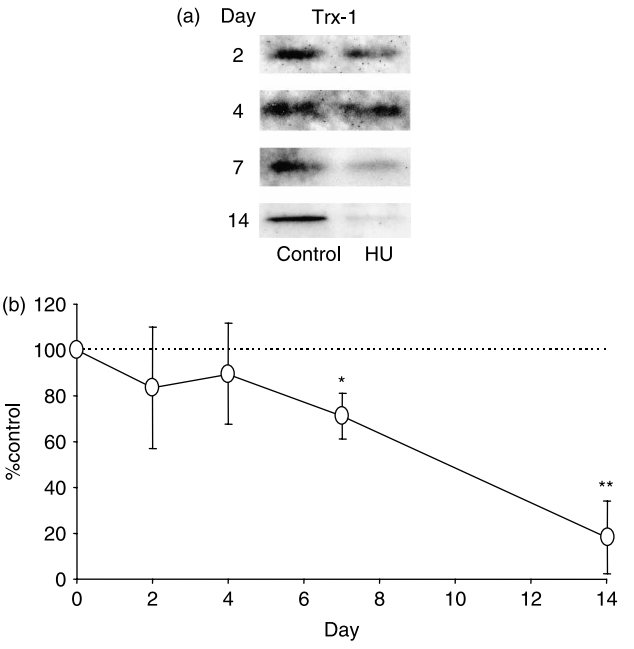

Figure 3. Changes of the protein levels of Trx-1 in the soleus muscles of the 2, 4, 7 or 14-day HU rats. (a) A typical immunoblot result representative of five or six independent experiments is shown. (b) Changes of the protein levels of Trx-1 determined by densitometry of Western blot analysis. The value is expressed as a percentage of the control for the same periods. $\star p < 0.01$ and $\star\star p < 0.005$ compared with the control for the same period.

The protein level of Prx-3 was significantly decreased on day 14 (71.2% of the control; $p < 0.05$) after the HU (Figure 5). The mRNA expression of Prx-3 was also decreased (55.4% of the control; $p < 0.005$) on day 14 (Figure 4). The protein level of Mn-SOD was also decreased on day 14 (44.3% of the control; $p < 0.05$) after the HU (Figure 5). There were no significant changes in the levels of Prx-3 and Mn-SOD on day 2-, 4- and 7-day after the HU (Figure 5).

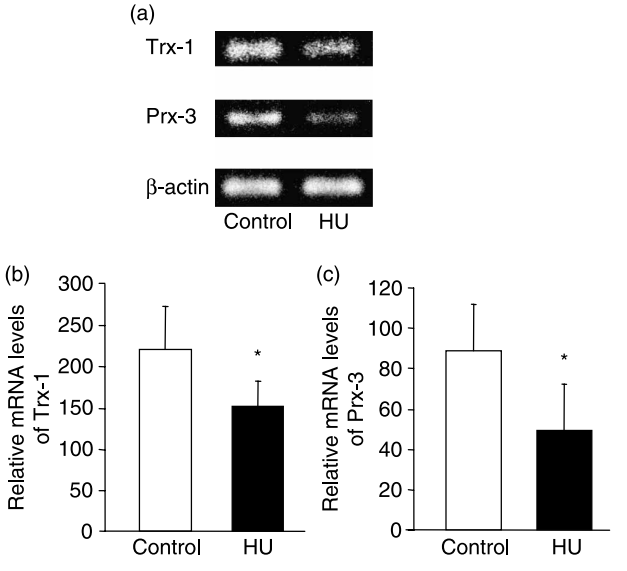

Figure 4. The mRNA levels of Trx-1 and Prx-3 in the soleus muscles of the 14-day HU and the control rats. (a) A typical RT-PCR result representative of five independent experiments is shown. The abundance of mRNA of Trx-1 (b) and Prx-3 (c) was determined by RT-PCR as described in "materials and methods" section. Each value was corrected for the abundance of β-actin mRNA (means ± SD, $n = 5$). $\star p < 0.05$ compared with the control.

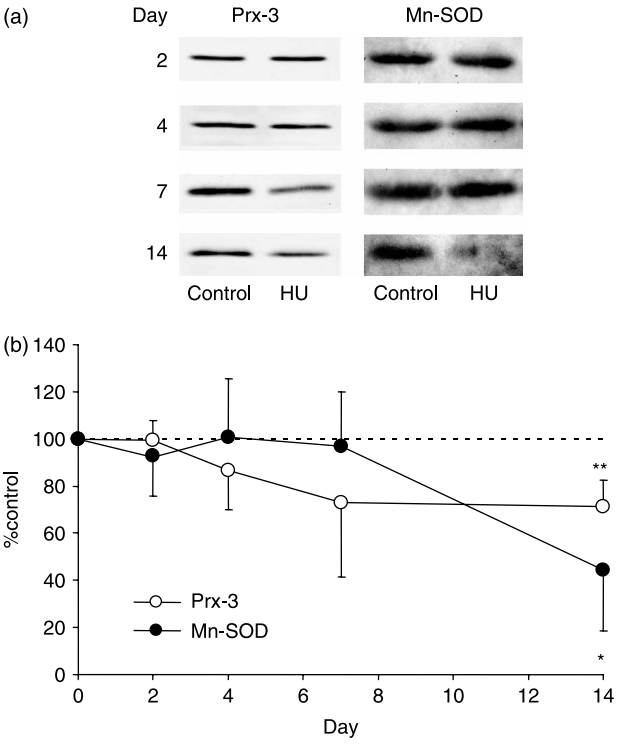

Figure 5. Changes of the protein levels of Prx-3 and Mn-SOD in the soleus muscles of the 2, 4, 7 or 14-day HU rats and those of the control rats. (a) A typical immunoblot result representative of five or six independent experiments is shown. (b) Changes of the protein levels of Prx-3 and Mn-SOD determined by densitometry of Western blot analysis. The value is expressed as a percentage of the control for the same periods. $\star p < 0.01$ and $\star\star p < 0.005$ compared with the control for the same period.

The mRNA expression of TBP-2

We also measured the mRNA expression of TBP-2 which serves as a negative regulator of the function and the expression of thioredoxin by RT-PCR. The mRNA expression of TBP-2 began to increase on day 2 (206% of the control; $p < 0.01$) and reached maximum (224% of the control; $p < 0.05$) on day 4 after the HU (Figure 6), which preceded the onsets of both the muscle weight loss and the decrease in the level of Trx-1 during the HU.

Discussion

Previous reports have demonstrated that various markers of oxidative stress, such as thiobarbituric acid-reactive substances (TBARS), oxidized glutathione, protein carbonyls and 2,3-dihydroxybenzoic acid and dichlorohydrofluorescein diacetate oxidation were increased in atrophied skeletal muscle [5–8,10],

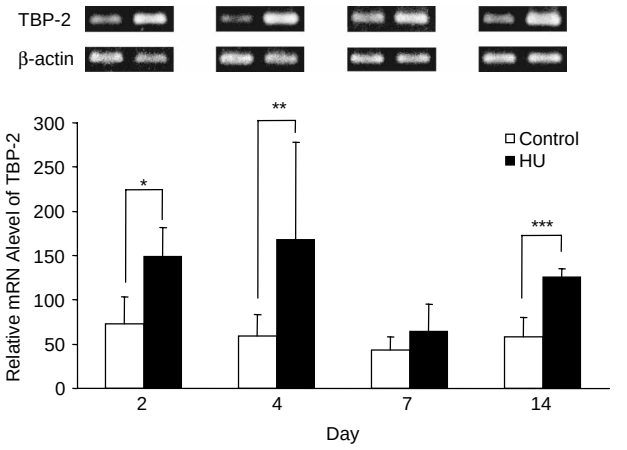

Figure 6. The mRNA levels of TBP-2 in the soleus muscles of the HU and the control rats. Upper panel; a typical RT-PCR result representative of five or six independent experiments is shown. Lower panel; the abundance mRNA of TBP-2 was determined by RT-PCR as described in "materials and methods" section. Each value was corrected for the abundance of β-actin mRNA (means ± SD, $n = 5$ or 6). $*p < 0.01$, $**p < 0.05$ and $***p < 0.001$ compared with the control.

most of which were increased in the late phase of development of muscle atrophy (in most cases, later than 7 days after the unloading or immobilization of hindlimbs) and were preceded by muscle weight loss. We also confirmed the involvement of elevated oxidative stress in the late phase of disuse muscle atrophy by using new indicators of oxidative stress, i.e. the accumulation of acrolein- and MDA-modified proteins. We also indicated that protein carbonyls were significantly increased in the soleus muscle atrophied by 14-day HU.

We demonstrated that both the protein levels of cytosolic Trx-1 and mitochondrial thioredoxin-dependent peroxidase, Prx-3, were decreased in the late phase of disuse muscle atrophy by HU. The protein level of the conventional mitochondrial antioxidant enzyme, Mn-SOD, was also decreased in the late phase. The decreases in the levels of these antioxidant proteins were preceded by the muscle weight loss during the HU. Although, the relative contributions of Trx-1, Prx-3 and Mn-SOD to the suppression of the elevated oxidative stress in the atrophied muscle are not known, the differences in substrate specificities, electron donors and cellular localization suggest that these antioxidant proteins function as redundant defence mechanisms against oxidative stress and compensate each other. The decreased protein levels of both thioredoxin-related antioxidant proteins and the conventional Mn-SOD in the atrophied muscle may result in the impairment of these redundant antioxidant defence systems and lead to the enhancement of oxidative stress.

The time-dependent profile of the changes in the muscle weight loss, oxidative damage and the levels of the antioxidant proteins during the HU suggest that the elevated oxidative stress may not be an initial trigger that causes muscle atrophy. The direct oxidation and/or oxidative modifications of cellular proteins, however, has been reported to render proteins susceptible to degradation by cellular proteolytic pathways [1,9,11], which may possibly facilitate the muscle breakdown in the late phase of muscle atrophy.

It has been reported that acrolein, a reactive aldehyde product of lipid peroxidation, inactivates thioredoxin and thioredoxin reductase, which results in an increase in cellular oxidative damage [35,36]. It has also been reported that acrolein is not merely a footprint of lipid peroxidation but serves as a signaling molecule through the activation of protein kinase cascade and calcium signaling [37], which can be associated with the facilitation of muscle atrophy. Further studies are needed to clarify the role of the reactive aldehydes and aldehyde-modified proteins in cellular signaling pathways underlying skeletal muscle atrophy.

Accumulating evidence indicates that thioredoxin involves in various cellular processes via redox signaling pathways, some of which are closely associated with muscle atrophy. Possible mechanisms by which thioredoxin can exert suppressive effects on the process of muscle atrophy are as follows: (1) thioredoxin suppresses the activation of NF-κB via the inhibition of I-κB breakdown [38], which then inhibits the NF-κB-induced activation of ubiquitin pathway; (2) thioredoxin inhibits the activity of PTEN, an inositol phosphatase specific for the products of PI3K, which subsequently relieves the PTEN-mediated suppression of the PI3K/Akt growth-promoting signal [39], and (3) thiredoxin and Prx-3 eliminate ROS at cytosol and mitochondria, respectively, which in turn suppresses the ROS-mediated activation of NF-κB, FOXO, and the ubiquitin–proteasome system.

In the present study, we demonstrated that the mRNA level of TBP-2, which serves as a negative regulator of the biological function and expression of thioredoxin, was significantly increased by 2- and 4-day HU, which preceded the muscle weight loss and the decrease in the levels of Trx-1. Although, the physiological functions of TBP-2 are not well understood, it has been reported that TBP-2 expression is reduced in many tumor cells and the enforced expression of TBP-2 inhibits cell proliferation by arresting cell cycle progression [40,41]. These natures of TBP-2 may suggest the involvement in muscle atrophy by inhibition of muscle cell growth.

Furthermore, TBP-2 has been reported to interact with thioredoxin through its redox-active cycteines and inhibits the reducing activity [26]. The increase in the level of TBP-2 during early phase of disuse muscle atrophy may suppress the thioredoxin-mediated redox signaling pathways, which could be an initial trigger that incites the muscle atrophy.

Yoshioka et al. demonstrated that TBP-2 controlled cardiac hypertrophy through the regulation of Trx-1 activity [27]. They reported that overexpression of Trx-1 induced protein synthesis, whereas, overexpression of TBP-2 reduced protein synthesis via suppression of Trx-1 in cardiomyocytes. Our results demonstrated the increase in TBP-2 and the decrease in Trx-1 in the atrophied muscle, suggesting that similar to cardiac muscle, the regulation of Trx-1 through TBP-2 is an important mechanism in atrophy of skeletal muscle.

In conclusion, the present results suggest that the decrease in the levels of Trx-1 and Prx-3, particularly a marked decrease in the level of Trx-1, may be one of the critical changes leading to the oxidative damage observed in the late phase of disuse muscle atrophy. Furthermore, taking into consideration the emerging evidence for protective and regulatory roles played by thioredoxin in the antioxidant defence and the redox signaling pathway, the increase in TBP-2 preceding the muscle atrophy may suggest a possible involvement of thioredoxin-mediated redox signaling pathways in the disuse muscle atrophy.

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