Thioredoxin Induction of Peripheral Blood Mononuclear Cells in Mice in Response to a Single Bout of Swimming Exercise

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Abstract. Thioredoxin (TRX) is a stress-inducible protein with diverse intracellular functions, which is expressed under conditions of oxidative stress. Exercise is known to cause oxidative stress by the generation of oxygen radicals from various biological pathways. The purpose of this study was to determine the level of TRX induction of cellular extracts prepared from peripheral blood mononuclear cells after a 30-min swimming exercise in mice. Plasma corticosterone concentration, considered to be a marker for exercise-induced various stress, rose significantly ($p < 0.05$) 0.5 h after exercise and rapidly dropped down following recovery. The carbonyl proteins as a marker of oxidative stress were significantly ($p < 0.05$) higher after 6 and 12 h of recovery in cytosolic extracts. The cytoplasm and nucleus TRX expressions were slightly higher to resting values after 12 and 24 h of recovery. The nucleus TRX expression was significantly ($p < 0.05$) higher after 24 h of recovery. These findings demonstrate that exercise-induced oxidative stress may be associated with increased intracellular TRX expression after 12 and/or 24 h after exercise in peripheral blood mononuclear cells. It is implied that this delayed and prolonged over-expression of TRX may play some roles in response to exercise-induced oxidative stress.

Key words: Thioredoxin — Exercise — Oxidative stress — Redox — Mouse — Carbonyl protein

Introduction

Thioredoxin (TRX) is a redox-regulatory protein with conserved cysteine (Cys) residues at the -Cys-Gly-Pro-Cys- active site (Holmgren 1985). TRX has been

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shown to regulate various intracellular functions, such as cell growth, apoptosis, cytokine expression, and modulation of transcription factors including NF-κB, activator protein 1 (AP-1, Fos/Jun), and nuclear redox factor 1 (Ref-1) (Schenk et al. 1994; Hirota et al. 1997). Furthermore, TRX is a stress-inducible protein, whose expression is enhanced by various oxidative stresses, including hydrogen peroxide (H_2O_2), UV light, irradiation, and ischemic reperfusion (Taniguchi et al. 1996; Nakamura et al. 1997). Although TRX is induced by oxidative stresses, it can also play crucial roles as a scavenger of oxygen radicals (Tanaka et al. 1997; Nishinaka et al. 2001). TRX, as an antioxidant protein, would be induced to provide for cellular protection against oxidative stress (Nordberg and Arner 2001).

It is known that the intracellular and extracellular generation of oxygen radicals increases during exercise (Sjodin et al. 1990; Ji 1999). Exercise causes oxidative stress by increased generation of oxygen radicals, which is associated with oxidative damage to cellular molecules.

Recently, oxygen radicals have been shown to be an important intermediate in regulating a number of signal transduction pathways (Schreck et al. 1991; Sen and Packer 1996; Suzuki and Forman 1997; Adler et al. 1999). Intracellular redox regulation of oxygen radicals and reducing molecules is linked to biological events, such as apoptosis, inflammation, and aging, and is involved in gene expression (Morel and Barouki 1999). TRX is a redox regulator protein induced by intracellular oxidative stress, which plays a crucial role in redox regulation of transcription factors and DNA binding proteins (Schenk et al. 1994; Hirota et al. 1997; Nakamura et al. 1997; Yodoi et al. 2002). The induction of oxidative stress by exercise is thought to regulate the signal pathways through the regulation of TRX induction. However, it is still not clear whether cellular TRX protein induction is enhanced by exercise. The cellular responses to exercise-induced oxidative stress may be involved in the induction of TRX. The present study was undertaken to investigate the induction of TRX after a single bout of swimming exercise in mice.

Materials and Methods

Animals, swimming exercise and blood collection

Male Crj:CD-1 (ICR) mice (8 weeks old, 30–37 g) were obtained from Charles River (Hino, Shiga, Japan). Animal care and animal experiments were carried out in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals published by the US Department of Health and Human Services. Mice were randomly assigned to at rest control or exercise groups. The swimming exercise was performed for 30 min in a tank ($0.55 \times 0.56 \times 0.48$ m) with water kept at 35 ℃. The mice were sacrificed at rest and after 0.5, 3, 6, 12 and 24 h of recovery after the swimming exercise. Mice were killed by decapitation. Trunk blood was rapidly collected, and pooled from five mice in a tube with ∼0.1 ml of heparin (1000 IU/ml). The experiment was repeated twice with the identical procedure.

Blood mononuclear cells processing

Peripheral blood mononuclear cells and plasma were isolated from heparinized blood samples by using Histopaque-1083 (Sigma Diagnostics, Inc., St. Louis, MO, USA). Aliquots of plasma were taken and kept frozen at −80 ℃ until assayed. A modified extraction of cytosolic and nuclear fractions from mononuclear cells was performed as described (Schreiber et al. 1989). Briefly, blood mononuclear cells were washed in ice-cold phosphate-buffered saline (PBS) and placed into 400 μl of ice-cold lysis buffer containing 10 mmol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.8, 10 mmol/l KCl, 2 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, 1 mmol/l dithiothreitol (DTT), 0.5 mmol/l phenylmethyl-sulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin. After incubation on ice for 15 min, 25 μl of 1 % Nonidet P-40 (NP-40) was added and vortexed for 15 s. After centrifugation at 14,000 rpm for 30 s, supernatants were collected as cytosolic extracts and kept frozen at −80 ℃. Nuclear extracts were prepared by resuspending the pellets in 50 μl of ice-cold lysis buffer containing 50 mmol/l HEPES (pH 7.8), 50 mmol/l KCl, 300 mmol/l NaCl, 0.1 mmol/l EDTA, 1 mmol/l DTT, 10 % glycerol, 0.5 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml pepstatin. The resuspended pellets were incubated at 4 ℃ for 30 min and centrifuged at 14,000 rpm for 10 min at 4 ℃. Supernatants were removed and kept at frozen at −80 ℃.

Western blotting

Protein concentrations were measured by the Bradford method using a protein assay kit purchased from Bio-Rad (Bradford 1976). Equal amounts of (10 or 15 μg protein) were loaded on each lane of a 15 % or 12.5 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Osaka, Japan) using a semi-dry blotter. After blocking with 5 % nonfat milk in PBST (8.1 mmol/l Na_2HPO_4, 1.47 mmol/l KH_2PO_4, 137 mmol/l NaCl, 2.68 mmol/l KCl, 0.05 % Tween 20, pH 7.4) at 4 ℃ overnight, the membrane was incubated with the primary antibody in PBST containing 1 % nonfat milk for 1 h at room temperature. The membrane was washed in PBST, and incubated with the horseradish peroxidase conjugated secondary antibody (Amersham Pharmacia Biotech., England or Santa Cruz Biotech., CA, USA) in PBST containing 1 % nonfat milk for 1 h at room temperature. The expression of mouse TRX was determined by using anti-murine TRX polyclonal antibody that was prepared as previously described (Takagi et al. 1998). Chemiluminescence was detected with the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, England) using X-ray film at room temperature. The intensities of immuno-stained bands were determined as optical densities using NIH Image 1.62 software (National Institute of Health, Bethesda, MD, USA) and presented as arbitrary unit.

Detection of oxidative stress

The carbonyl groups of oxidatively modified proteins of cytosolic extracts were detected by using a protein oxidation detection kit (OxyBlot, Intergen, Co., NY,

USA). The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized proteins samples were separated by SDS-PAGE followed by Western blotting.

Biochemical analyzes

Plasma corticosteron concentrations as a marker of exercise stress were determined by using a radioimmunoassay kit (Diagnostic Products Co., CA, USA).

Data analyzes

Data were analyzed by the InStat 2.02 software (GraphPad, CA, USA). The results were expressed as means ± SEM of two experiments. Unpaired *t*-test was applied to detect differences from resting values. Significance was set at the 0.05 level.

Results

Plasma corticosterone

Plasma corticosterone significantly elevated ($p < 0.05$) after 0.5 h of recovery after exercise compared to the resting values (Fig. 1). This higher value rapidly dropped down after 3 h of recovery, and was significantly lower ($p < 0.05$) than resting values. Although plasma corticosterone significantly elevated ($p < 0.05$) after 6 h of recovery, it was significantly lower ($p < 0.05$) after 24 h of recovery.

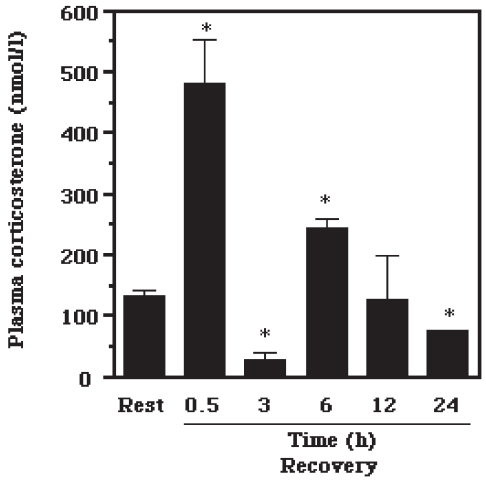

Figure 1. Changes in plasma corticosterone at rest and during recovery after a single bout of swimming exercise in mice. The swimming exercise was performed for 30 min in water at 35 ℃. The results are expressed as means ± SEM. Unpaired *t*-test was performed. * $p < 0.05$, significantly different from rest value.

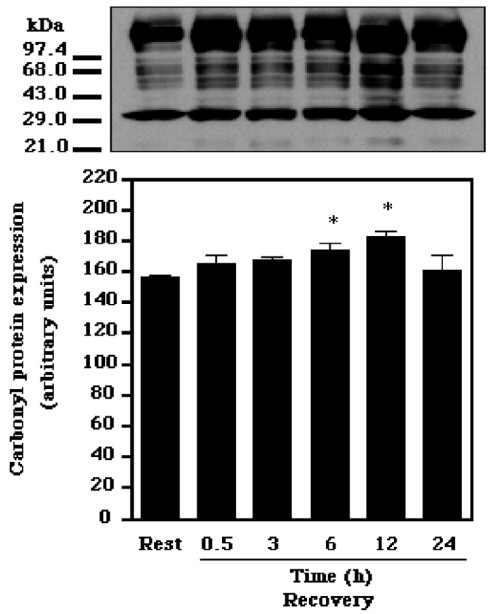

Figure 2. Western blot analysis of protein carbonyl content of cytosolic extracts at rest and during recovery after a single bout of swimming exercise in mice. The swimming exercise was performed for 30 min in water at 35 ℃. The results are expressed as means ± SEM. Unpaired *t*-test was performed. * $p < 0.05$, significantly different from rest value.

Protein carbonyl content

The protein carbonyl content of cytosolic extracts significantly increased ($p < 0.05$) after 6 and 12 h of recovery after exercise (Fig. 2). The carbonyl proteins decreased after 24 h of recover and was similar to the resting values.

Induction of TRX

TRX expression was observed after 12 and 24 h of recovery after exercise. The cytoplasm TRX expression increased slightly after 12 and 24 h of recovery compared to the resting values (Fig. 3A). TRX expression of nuclear extracts was also similar to cytosolic extracts, but was significantly higher ($p < 0.05$) after 24 h of recovery (Fig. 3B).

Discussion

Glucocorticoid secretion is known to be induced by strenuous and prolonged exercise, >60 % maximal O_2 consumption (Howlett 1987; Inder et al. 1998). An increase in plasma glucocorticoid concentration is involved in the exercise-induced ACTH secretion, a marker of stress. The exercise performed in the present study caused

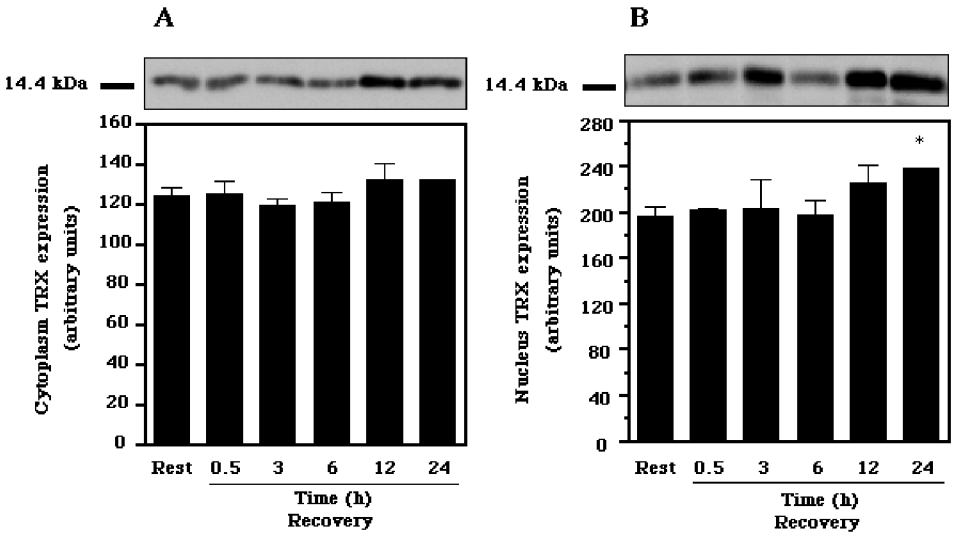

Figure 3. Time course of TRX expressin of blood mononuclear cells after a single bout of swimming exercise in mice. A Western blot for TRX is shown of cytosolic extracts (A) or nuclear extracts (B) of blood mononuclear cells at rest and during recovery after a single bout of swimming exercise. The results are expressed as means ± SEM. Unpaired *t*-test was performed. * $p < 0.05$, significantly different from rest value.

a significant increase in plasma corticosterone concentration after 0.5 h of recovery after exercise. This marked rise in plasma corticosterone was associated with various stresses, including the swimming exercise.

It is well known that strenuous exercise induces oxidative damage to cellular molecules (Davies et al. 1982; Sumida et al. 1989; Ji 1999). Reactive oxygen radicals and other reactive species such as 4-hydroxy-2-nonenal produced by lipid peroxidation modify the side chains of amino acids. The oxidative modification of protein is estimated by measuring the carbonyl content of protein (Davies 1987; Levine et al. 1994). The increase in the carbonyl groups of proteins is caused by the generation of oxygen radicals in biological systems. However, the time-course of recovery of oxidatively modified protein after a single bout of exercise has not been determined. Although it is unclear how reactive oxygen radicals generated by the swimming exercise oxidatively modified proteins of cytosolic extracts in this study, we found that the exercise significantly induced the protein carbonyl content after 6 and 12 h of recovery. Endogenous and/or exogenous oxygen radicals generated by exercise could result in oxidative modification to intracellular protein for 6 and/or 12 h after exercise. The delayed accumulation of intracellular protein carbonyls after exercise may be associated with the balance between the generation of oxygen radicals and protection by antioxidants.

TRX has been shown to play an important role in regulating redox activation of some cellular proteins, including DNA binding proteins (Holmgren 1985; Schenk et al. 1994; Akamatsu et al. 1997; Hirota et al. 1997). In addition, TRX is known not only to scavenge oxygen radicals as an antioxidant protein, but also to be induced by oxidative stress (Sachi et al. 1995; Makino et al. 1996; Nakamura et al. 1997). The induction of TRX has been reported to be higher in the presence of H_2O_2 in a dose-dependent fashion but delayed until 12 h after treatment by H_2O_2 (Sachi et al. 1995; Makino et al. 1996).

In this study, we found maximal TRX expression of cytosolic and nuclear extracts prepared from peripheral blood monocytes at 12 and 24 h after the swimming exercise. This delayed over-expression of TRX was similar to the results of H_2O_2 exposure *in vitro*. It has been reported that TRX gene expression is enhanced through the oxidative stress response element of its promoter (Taniguchi et al. 1996). Numerous studies have demonstrated that exercise-induced oxidative stress of cellular molecules by the production of oxygen radicals (Sjodin et al. 1990; Sen 1995; Ji 1999). A redox balance of exercise-induced oxidative stress and intracellular antioxidants may be an event in the expression of TRX. In this study, although the level of production of oxygen radicals during and after 30 min of swimming exercise is unknown, the delayed secondary oxidative stresses might be associated with the expression of TRX. We found that the protein carbonyl content of cytosolic extracts significantly increased after 6 and 12 h of recovery after the swimming exercise. This result indicate that the cellular oxidative modification of protein was slow in the post-exercise recovery period.

In conclusion, this study was designed to determine if a single bout of exercise would influence the induction of TRX as a stress-inducible protein. Increased levels of TRX in cytosolic and nuclear extracts from peripheral blood mononuclear cells were found at 12 and 24 h after the swimming exercise. Therefore, exercise-induced oxidative stress may affect TRX induction. It is implied that this delayed and prolonged over-expression of TRX may play some roles in the recovery responses after exercise-induced oxidative stress. Effect of exercise on the over-expression of TRX should further be investigated.

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