Changes of thioredoxin, oxidative stress markers, inflammation and muscle/renal damage following intensive endurance exercise

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ABSTRACT

Thioredoxin (TRX) is a 12 kDa protein that is induced by oxidative stress, scavenges reactive oxygen species (ROS) and modulates chemotaxis. Furthermore, it is thought to play a protective role in renal ischemia/reperfusion injury. Complement 5a (C5a) is a chemotactic factor of neutrophils and is produced after ischemia/reperfusion injury in the kidney. Both TRX and C5a increase after endurance exercise. Therefore, it may be possible that TRX has an association with C5a in renal disorders and/or renal protection caused by endurance exercise. Accordingly, the aim of this study was to investigate relationships among the changes of urine levels of TRX, C5a and acute kidney injury (AKI) caused by ischemia/reperfusion injury and oxidative stress following intensive endurance exercise. Also, we applied a newly-developed measurement system of neutrophil migratory activity and ROS-production by use of ex vivo hydrogel methodology with an extracellular matrix to investigate the mechanisms of muscle damage. Fourteen male triathletes participated in a duathlon race consisting of 5 km of running, 40 km of cycling and 5 km of running were recruited to the study. Venous blood and urine samples were collected before, immediately following, 1.5 h and 3 h after the race. Plasma, serum and urine were analyzed using enzyme-linked immunosorbent assays, a free radical analytical system, and the ex vivo neutrophil functional measurement system. These data were analyzed by assigning participants to damaged and minor-damage groups by the presence and absence of renal tubular epithelial cells in the urinary sediments. We found strong associations among urinary TRX, C5a, interleukin (IL)-2, IL-4, IL-8, IL-10, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1. From the data it might be inferred that urinary TRX, MCP-1 and β-N-acetyl-D-glucosaminidase (NAG) were associated with renal tubular injury. Furthermore, TRX may be influenced by levels of IL-10, regulate chemotactic activity of C5a and IL-8, and control inflammatory progress by C5a and IL-8. In the longer duration group (minor-damage group), circulating neutrophil count, plasma concentration of myeloperoxidase (MPO) and serum concentration of myoglobin were markedly increased. In the higher intensity group (damaged group), neutrophil activation and degranulation of MPO might be inhibited, because not only was ROS production observed to be higher, but also antioxidant capacity and anti-inflammatory cytokines were increased. Critically, the newly-developed ex vivo methodology corroborated the neutrophil activation levels in the two groups of participants.

Key words: TRX, C5a, ROS, antioxidant, anti-inflammation, acute kidney injury (AKI)

INTRODUCTION

Endurance exercise not only promotes the generation of reactive oxygen species (ROS), mainly as a result of increased oxygen utilization, ischemia-reperfusion and leukocyte activation, but also consumes endogenous antioxidants (2, 3, 54). This unbalanced state induces oxidative stress and cellular tissue damage in the body. Oxidative stress-induced injury and inflammation are important considerations for athletes.

Recently, it has been reported that aerobic exercise interventions can have a positive effect on chronic renal failure. In the patients with chronic kidney diseases including those undertaking dialysis therapies or in receipt of kidney transplant, it was demonstrated that aerobic exercise reduced oxidative stress and improved quality of life (18, 27). For
these reasons, exercise is recommended for these patients (26). The underlying mechanisms for the direct improvement of renal functions due to aerobic exercise have not yet been identified. However, it has been suggested that moderate exercise may promote nitric oxide production, and inhibit the renin-angiotensin-aldosterone system, or improve renal blood flow by modifying dyslipidemia and intraglomerular pressure (29, 33, 48). However, the acute effects of exercise are different from the chronic effects of exercise training and therefore these exercise modes should be considered separately (33). In acute exercise, it is known that deterioration of glomerular filtration rate and oliguria are induced following endurance exercise (69), and that intensive endurance exercise may cause rhabdomyolysis-induced acute renal failure (10, 52). Rhabdomyolysis may manifest in acute renal failure due to acute tubular necrosis leading to deterioration of glomerular filtration rate. This is induced by glomerulus degeneration and reduced renal blood flow leading to a reduced supply of oxygen and energy, thereby resulting in ischemic vascular endothelial cell damage (6). In animal models of acute renal failure, ROS and lipid peroxides increase, whereas scavengers of ROS such as glutathione and superoxide dismutase (SOD) are decreased in renal tissue (51). It was reported that ROS, such as superoxide anion and hydroxyl radical, contributed to the onset of acute renal failure through reduced renal blood flow and disorder of tubular cells (55). Thus, acute endurance exercise-induced oxidative stress may cause renal failure, and therefore it is important to evaluate oxidative stress in this context.

Currently, oxidative stress is evaluated using metabolic oxidation end products, because the real time evaluation of this phenomenon is difficult. Recently, it was reported that thioredoxin (TRX) is secreted by renal tubular cells due to ischemia and oxidative stress (30). Furthermore, its usefulness as a specific biomarker of acute kidney injury (AKI) caused by ischemia and oxidative stress is being established (30). TRX is a small (12 kDa) multifunctional protein that contains a redox-active dithiol/disulfide in the conserved active site sequence: -Cys-Gly-Pro-Cys- (20). TRX is stress-inducible, and it protects cells from various types of stress (45, 46). TRX functions as an antioxidant, as an inhibitor of chemotaxis, and as a redox-regulating protein in signal transduction (12, 21, 22, 57). TRX eliminates hydrogen peroxide and acts as a radical scavenger, as demonstrated where recombinant TRX has a protective activity against hydrogen peroxide cytotoxicity (23, 38, 45). It is reported that elevated serum TRX in various diseases is associated with increased oxidative stress (25, 32, 40, 46, 47, 60, 61, 83). Moreover, serum levels and expression levels of TRX increase following endurance exercise (36, 70, 85). Recently, it was reported that the urine level of TRX is a specific marker for ischemia and oxidative stress-induced acute renal failure, because it is secreted from renal tubular epithelial cells in response to ischemia/reperfusion injury in renal tissue (30).

Another protein associated with AKI is complement 5a (C5a) (1). C5a is a multifunctional proinflammatory mediator, and a chemotactic factor, which increases the permeability of blood vessels and promotes the migration of leukocytes towards inflammatory sites and their generation of ROS (14, 35). It is reported that C5a increases and causes inflammation following marathon race (8). C5a is an important pathogenic factor in renal ischemia/reperfusion injury (1). The role of C5a in the tubulointerstitial component is demonstrated in an experimental model of progressive glomerulonephritis (80). Indeed, C5a receptor activation in glomerular mesangial cells has been shown to induce proliferation, produce cytokines and growth factors, as well as upregulate certain transcription factors and early response genes (81). The terminal complement complex in plasma and urine, and the anaphylatoxin C5a in plasma and urine, might have potential as an early and reliable marker for acute renal allograft rejection (44). In this regard, urinary C5a level is positively correlated with the severity of renal injury, which highlights the important role of C5a in renal damage of human anti-glomerular basement membrane disease (14, 35).

Given the stresses experienced by endurance athletes, the first aim of this study was to investigate relationships among urine levels of TRX, C5a and AKI caused by ischemia/reperfusion and oxidative stress following intensive endurance exercise. Furthermore, in our previous study, we reported urinary excretion of interleukin (IL)-2, IL-4, IL-8, IL-10, interferon (IFN)-γ and monocyte chemotactic protein (MCP)-1 in stressed athletes suffering from renal tubular epithelial damage. The damaged kidney might be responsible, at least in part, for the kinetics of some cytokines after endurance exercise (59). Therefore, the second aim was to clarify associations between urine levels of TRX or C5a and those of IL-2, IL-4, IL-8, IL-10, IFN-γ, MCP-1 as well as urine albumin (ALB) and serum creatinine (Cr) as renal function markers. The final aim was to determine oxidative stress responses in the circulation after exercise. Here, we applied a newly-developed system of measurement for neutrophil migratory activity and ROS-production. This system uses ex vivo hydrogel methodology with an extracellular matrix as a means to investigate the mechanisms of tissue damage (28).

### METHODS

#### Subjects

Fourteen male triathletes [age 28.7 ± 7.9 (mean ± SD) yr and body mass 63.2 ± 6.0 kg], volunteered to participate in this study. The subjects were seven professional triathletes and seven amateur triathletes. They completed a medical questionnaire and gave written informed consent prior to the study. None of the athletes had been ill in the previous month. The experimental procedure was approved by the institutional ethics committee of Waseda University.

Renal tubular epithelial cells and renal tubular epithelial casts were observed in the urinary sediments of seven subjects, among the fastest eight subjects for race time (59). In this study, according to the values of serum Cr in the AKI diagnosis criteria such as “Risk, Injury, Failure, Loss, End Stage Kidney Disease (ESKD): RIFLE criteria” (4) and “acute kidney injury network: AKIN” (34, 37), AKI following endurance exercise showed “Risk” or “StageI” at 0 h and 1.5 h after the race in the seven subjects with the existence of renal tubular epithelial cells in the urinary sediments. Immediately after exercise in the other seven subjects, there was no evidence of renal tubular epithelial cells in the urinary sediments. After this, the athletes were analysed as two subgroups that were divided according to the existence (damaged group,
n=7) or non-existence (minor-damage group, n=7) based on the levels of renal tubular epithelial cells in the urinary sediments (59).

**Duathlon race**

The present investigation was conducted in an official duathlon race held on the road course of Miyako Island, Okinawa, Japan as described previously (59). Briefly, the race consisted of 5 km of running, 40 km of cycling, and 5 km of running, and began at 14:00. The weather was fair, and the ambient temperature was 24.6 °C.

**Research design**

All participants agreed to avoid the use of vitamin/mineral supplements, herbs and medications from the previous day until after the last sampling point. All participants ate an identical breakfast at 08:30. The breakfast contained 574 kcal, with 22.1 g protein, 13.7 g fat and 88.8 g carbohydrate. The pre-race blood and urine samples (Pre) were collected at 10:30 while the participants were at rest. The athletes did not exercise for approximately 18 h before the pre-race blood and urine sampling. The post-race blood and urine samples were collected immediately (0 h), 1.5 h and 3 h after the race. Peripheral blood samples were drawn by antecubital venipuncture with the participants in the sitting position. Urine samples were collected into designated vessels. They ate lunch at 11:00. The lunch contained 211 kcal, with 9.3 g protein, 2.4 g fat and 38.6 g carbohydrate. All participants drank the same quantity of fluid during exercise. After a warm-up, they each drank 600 ml of fluid before the race. During the race, they each drank 1400 ml of fluid. Therefore, the total fluid intake for each individual was 2000 ml. They each drank 1500 ml of water until 3 h after the race.

**Serum, plasma, urine sampling, urinary sediments and biochemical parameters**

Approximately 7 ml of blood was drawn by a standard venipuncture technique from the antecubital vein using vacutainers containing no additive or sodium heparin and disodium EDTA as an anticoagulant to obtain serum and plasma samples, respectively. Collected blood samples containing no additives were allowed to clot at room temperature for 1 h before centrifugation at 1000 × g for 10 min for serum preparation, whereas blood samples containing disodium EDTA were centrifuged immediately for plasma preparation. Plasma was stored at -80 °C until the day of analysis. Serum concentrations of Cr, myoglobin (Mb), uric acid (UA), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and plasma concentration of lactate were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan) (59).

Ulirie sample volume was measured and then approximately 8 ml was stored at 4 °C without centrifugation until analysis of sediments. Remaining urine samples were centrifuged immediately at 1000 × g for 10 min to remove sediments, and the supernatants were stored at -80 °C until the day of analysis. Urinary concentrations of Cr, ALB, UA, Mb and β-N-acetyl-D-glucosaminidase (NAG) activity were measured using an automated analyzer (Model 747-400, Hitachi, Tokyo, Japan) (59). The urinary data are reported as the gross amount per minute (urinary excretion rate) as described previously (59).

**MPO, TRX, C5a, cytokines and chemokines**

Myeloperoxidase (MPO), IL-1 receptor antagonist (IL-1ra), IL-6, IL-8 and IL-10 were measured in plasma, and TRX, C5a, IL-2, IL-4, IL-8, IL-10, IFN-γ and MCP-1 were measured in urine samples with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions (59). The following kits were used for all measurements: MPO (Hbt ELISA test, Hyclut biotechnology, Uden, The Netherlands), TRX (TRX ELISA Kit, Redox Bio Science, Kyoto, Japan), IL-6 (Quantikine HS, R&D Systems, Inc., Minneapolis, MN), IL-1ra and MCP-1 (Quantikine, R&D Systems, Inc.), C5a, IL-2, IL-4, IL-8, IL-10 and IFN-γ (OptEIA, Beckton Dickinson Biosciences, San Diego, CA, USA) (59). For all assays, the absorbance was measured spectrophotometrically on a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) and the concentration of each cytokine was calculated by comparison with a standard curve established in the same measurement. The urinary data are reported as the gross amount per minute (urinary excretion rate).

**Neutrophil function**

Neutrophil function was measured using modified Mebiol (scaffold-thermoreversible galation polymer: S-TGP) gel (Mebiol Co., Hiratsuka, Kanagawa, Japan) and luminal as described previously (17, 28, 68). Peripheral blood samples were drawn in a 2 ml sodium-heparin tubes (Venoject II, Terumo Co., Tokyo, Japan). The blood samples were mixed with 2.5 mM luminol (5-amino-2, 3-dihydro-1, 4-phtha-lazinedione; Sigma Aldrich, MO, USA) at a ratio of 1:1. Then, 150 μl luminol-blood samples were layered on 50 μl S-TGP gel prepared in a tube at 37°C, and was promptly measured by relative light unit (RLU) using a luminometer (Gene Light 55; Microtec Co., Ltd, Funabashi, Chiba, Japan). The samples were incubated at 37°C, and the production of ROS from neutrophils was monitored in a kinetic mode for 60 min. After measurement of luminol-dependent chemiluminencescence (LmCL) for 60 min, luminol-blood samples were removed. The tubes with 50 μl S-TGP gel in which neutrophils migrated were washed three times with PBS at 37°C. Then, the tubes with gel were cooled on ice and mixed well following addition of 50 μl Turk solution (Wako, Osaka, Japan). The suspension obtained in this way was set on the C-Chip (Disposable haemocytometer, Neubauer improved, DHC-No.1, Digital Bio, Seoul, Korea), and the migratory cell number was counted microscopically. Migrating neutrophils were calculated by a 20 times multiplication of the counted cell number (28).

**Oxidative stress markers**

To analyze the plasma levels of reactive oxygen metabolites, and antioxidant capacity, diacron reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), and total antioxidant capacity (OXY-absorbent test) were performed respectively using the Free Radical Analytical System (Diacron, Grosseto, Italy) according to the manufacturer’s instructions. The d-ROMs test provides a measure for the oxidative stress of blood samples by evaluating the level of
reactive oxygen metabolites particular to hydroperoxides. This assay is based on the capability of N,N-dimethyl-paraphenylendiamine (DMPD) to give a stable, colored solution when it is transformed into its radical cation (DMPD•). The assay was performed in a 5 ml plastic tube by adding 20 μl of DMPD (final concentration 1mM) and 10 μl of plasma sample to 2 ml of 0.1 M acetate buffer, pH 4.8. The formation of the colored DMPD• was monitored by reading the absorbance at 505 nm. The amount of the colored DMPD• is related to the oxidative stress of the plasma and can be expressed in terms of hydrogen peroxide equivalents, with 1 U. CARR (Carraletti unit) corresponding to 0.8 mg/l hydrogen peroxide (11,74). The BAP assay is a photometric test that determines the serum concentration of antioxidants capable of reducing the iron from the ferric to the ferrous form. A plasma aliquot (10 μl) was dissolved in 1 ml of colored solution obtained by mixing 50 μl of ferric ions (FeCl3; ferric chloride) with a chromogenic substrate (a sulfur-derived compound). Following 5 min incubation, the intensity of the color change was assessed spectrophotometrically at 505 nm. The amount of reduced ferric ions was calculated and the BAP unit was expressed as μM (71). The OXY-absorbent test allows assessment of the antioxidant power of plasma by measuring the ability of such barrier to oppose the large oxidant action of hypochlorous acid (HOCl). HOCl is used as an indicator because it is one of the strongest ROS produced by leukocytes. In the OXY-absorbent test, 1 ml of R1 reagent (HOCl solution) was put into an empty cuvette, to which 10 μl of previously diluted sample (plasma or serum) was added and mixed. The solution was incubated at 37°C for 10 min, before addition of 10 μl of reagent R2 (chromogen). The cuvette contents were mixed and the absorbance measured spectrophotometrically at 546 nm. The results were expressed as μM of HOCl adsorbed by 1 ml of sample (μM HOCl/ml).

**Statistical analyses**

Data were presented as mean ± standard deviation (SD). Statistical validation was assessed using Friedman’s test. If significance was detected, the Scheffe method was used for multiple comparisons. Associations among measured variables were determined by Spearman’s rank correlation coefficient analysis. Statistical findings were deemed to be significant where the probability of events occurring at random was less than 5% (p< 0.05).

**RESULTS**

**Markers of renal function in urine and plasma lactate**

The degree of renal damage, as measured by creatinine clearance, urinary excretion rates of ALB and NAG, was higher in the damaged group than those in the minor-damage group (59). As shown in Figure 2, plasma lactate concentrations increased significantly after the race compared with the pre-race values in both groups, but were higher in the damaged group (3.4-fold) than those in the minor-damage group (2.7-fold).

**Oxidative stress and renal function parameters in the circulation and ex vivo**

As shown in Table 1 and Figure 2, many biochemical variables were affected by the exercise intervention and varied between the two groups. Serum OXY increased significantly immediately after exercise compared with pre exercise in the damaged group only (0 h: 1.2-fold).

**Urinary TRX and C5a**

As shown in Figure 1, the excretion rate of TRX in the minor-damage group did not significantly change, while in the damaged group, TRX was significantly increased at 1.5 h (20.1-fold) after the race and then decreased 3 h (4.9-fold) post-exercise. There was no significant change in the excretion rate of C5a for the minor-damage group. The excretion rate of C5a increased significantly 3 h (31.8-fold) after exercise in the damaged group when compared to pre-exercise values.

**Associations between urinary NAG, TRX, ALB and MCP-1**

As shown in Table 2, the urinary excretion rate of NAG, as a marker of renal tubular epithelial cell injury, was positively correlated with that of TRX, ALB and MCP-1 in the damaged group. In the minor-damage group, the urinary excretion rate of NAG 0 h after the race was positively correlated with that of ALB immediately after exercise only.

**Relations among urinary TRX, C5a, renal function makers and cytokines**

As shown in Table 3, the area under the curve (AUC) for pre-, 0 h, 1.5 h and 3 h of urinary excretion rate of TRX was positively correlated with that of ALB in the damaged group. Furthermore, there was a trend for a positive correlation with serum Cr concentrations, whereas there was a trend for TRX to be negatively correlated with AUC of urinary excretion rate of C5a, IL-2 and negatively correlated with AUC of urinary excretion rate of IL-4, IL-8, IL-10 and IFN-γ. There was a trend for the AUC of urinary excretion rate of C5a to be nega-
Pathogenic mechanisms of redox/inflammatory responses to endurance exercise

Table 1. Changes of leukocytes, cytokines and biochemical variables following the duathlon race.

<table>
<thead>
<tr>
<th>Phenomenon</th>
<th>Renal tubular epithelial cells</th>
<th>Unit</th>
<th>Pre</th>
<th>0 h</th>
<th>1.5 h</th>
<th>3 h</th>
<th>Fried test</th>
<th>Scheffe test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood leukocytes</td>
<td>(±) n=7</td>
<td>×10⁶/µl</td>
<td>41.9±8.9</td>
<td>127.1±32.6</td>
<td>128.9±32.5</td>
<td>112.6±25.3</td>
<td>** Pre-0 h**</td>
<td>** Pre-1.5 h**</td>
</tr>
<tr>
<td></td>
<td>(±) n=7</td>
<td></td>
<td>52.9±15.8</td>
<td>168.7±31.2</td>
<td>143.6±39.6</td>
<td>137.3±43.5</td>
<td>** Pre-0 h**</td>
<td>** Pre-1.5 h**</td>
</tr>
<tr>
<td>UA/U</td>
<td>(±) µg/min</td>
<td>11.2±5.2</td>
<td>1.0±0.9</td>
<td>3.4±1.7</td>
<td>4.4±1.3</td>
<td>** Pre-0 h**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(±) µg/min</td>
<td>10.8±5.1</td>
<td>2.1±1.1</td>
<td>5.5±2.4</td>
<td>4.9±1.9</td>
<td>** Pre-0 h**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mb/U</td>
<td>(±) ng/min</td>
<td>20.0±9.6</td>
<td>3.2±2.6</td>
<td>11.0±3.2</td>
<td>15.2±9.2</td>
<td>** Pre-0 h**</td>
<td>** Pre-1.5 h**</td>
<td></td>
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<tr>
<td></td>
<td>(±) 21.0±10.9</td>
<td>6.6±4.5</td>
<td>13.8±9.6</td>
<td>29.7±31.5</td>
<td></td>
<td>* Pre-0 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALB-U</td>
<td>(±) µg/min</td>
<td>5.0±2.0</td>
<td>115.5±60.0</td>
<td>198.1±154.8</td>
<td>41.4±36.8</td>
<td>* Pre-1.5 h</td>
<td></td>
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<tr>
<td></td>
<td>(±) 4.6±2.3</td>
<td>129.7±111.2</td>
<td>44.9±47.4</td>
<td>19.9±27.3</td>
<td>** Pre-0 h**</td>
<td>** Pre-0 h**</td>
<td>** Pre-0 h**</td>
<td></td>
</tr>
<tr>
<td>ALB-P</td>
<td>(±) pg/l</td>
<td>4.5±0.3</td>
<td>5.2±0.4</td>
<td>4.9±0.3</td>
<td>4.8±0.3</td>
<td>** Pre-0 h**</td>
<td></td>
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<tr>
<td></td>
<td>(±) 4.5±0.2</td>
<td>4.9±0.2</td>
<td>4.7±0.3</td>
<td>4.7±0.3</td>
<td>** Pre-0 h**</td>
<td></td>
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<tr>
<td>NAG-U*</td>
<td>(±) mU/min</td>
<td>6.6±2.4</td>
<td>2.5±1.8</td>
<td>8.7±3.6</td>
<td>8.1±2.7</td>
<td>** 0-1.5 h**</td>
<td>** 0-3 h**</td>
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</tr>
<tr>
<td></td>
<td>(±) 5.9±3.6</td>
<td>5.1±1.8</td>
<td>6.8±2.6</td>
<td>4.9±1.2</td>
<td>NS</td>
<td>NS</td>
<td></td>
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<tr>
<td>MCP-1-U*</td>
<td>(±) pg/min</td>
<td>1.7±0.8</td>
<td>0.5±0.3</td>
<td>2.5±1.2</td>
<td>12.2±15.7</td>
<td>** 0-3 h**</td>
<td></td>
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<tr>
<td></td>
<td>(±) 2.7±1.9</td>
<td>2.4±3.2</td>
<td>2.8±2.3</td>
<td>2.6±1.5</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>LDH-S</td>
<td>(±) IU/l</td>
<td>188.0±30.8</td>
<td>266.6±31.9</td>
<td>250.3±35.2</td>
<td>243.1±45.6</td>
<td>** Pre-0 h**</td>
<td>** Pre-1.5 h**</td>
<td></td>
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<tr>
<td></td>
<td>(±) 174.9±38.6</td>
<td>249.7±38.0</td>
<td>235.0±41.5</td>
<td>231.7±45.9</td>
<td>** Pre-0 h**</td>
<td>** Pre-3 h**</td>
<td></td>
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<tr>
<td>AST-S</td>
<td>(±) IU/l</td>
<td>32.3±11.8</td>
<td>41.0±14.9</td>
<td>38.9±13.6</td>
<td>38.9±12.0</td>
<td>** Pre-0 h**</td>
<td>** Pre-3 h**</td>
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</tr>
<tr>
<td></td>
<td>(±) 26.9±8.7</td>
<td>33.4±10.3</td>
<td>33.1±10.1</td>
<td>35.6±11.6</td>
<td>** Pre-0 h**</td>
<td>** Pre-3 h**</td>
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<tr>
<td>AL-S</td>
<td>(±) IU/l</td>
<td>21.9±10.6</td>
<td>26.0±12.3</td>
<td>24.4±11.3</td>
<td>24.1±10.6</td>
<td>** Pre-0 h**</td>
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<tr>
<td></td>
<td>(±) 20.1±4.9</td>
<td>22.6±5.0</td>
<td>21.6±5.1</td>
<td>22.1±5.3</td>
<td>* Pre-0 h</td>
<td>* Pre-3 h</td>
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<tr>
<td>IL-6P*</td>
<td>(±) pg/ml</td>
<td>17.2±11.1</td>
<td>49.8±23.1</td>
<td>35.6±11.3</td>
<td>24.3±11.5</td>
<td>** Pre-0 h**</td>
<td>** Pre-1.5 h**</td>
<td></td>
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<tr>
<td></td>
<td>(±) 16.1±10.4</td>
<td>40.1±17.3</td>
<td>30.1±14.1</td>
<td>19.9±11.3</td>
<td>** Pre-0 h**</td>
<td>** Pre-0 h**</td>
<td>** 0-3 h**</td>
<td></td>
</tr>
<tr>
<td>IL-10P*</td>
<td>(±) pg/ml</td>
<td>5.1±11.6</td>
<td>25.6±42.2</td>
<td>21.8±54.4</td>
<td>20.6±52.3</td>
<td>** Pre-0 h**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(±) 1.0±0.3</td>
<td>6.6±11.6</td>
<td>1.2±0.4</td>
<td>0.8±0.4</td>
<td>** 0-3 h**</td>
<td>** Pre-0 h**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values: means ± SD (n=7). Statistics: ** p < 0.01, * p < 0.05, † p < 0.1 ≤ not significance (NS).
- P: Data are plasma concentrations.
- S: Data are serum concentrations.
- U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).
The preexercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
(±): damaged group: renal tubular epithelial cells existed in the urinary sediments.
(±): minor-damage group: renal tubular epithelial cells did not exist in the urinary sediments.
= Data modified from Figure 1 of the reference No. 59.
Abbreviations: uric acid (UA), myoglobin (Mb), albumin (ALB), β-N-acetyl-D-glucosaminidase (NAG), monocyte chemotactic protein (MCP)-1, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), interleukin (IL).
Pathogenic mechanisms of redox/inflammatory responses to endurance exercise • 135

Figure 2. Changes in neutrophil activity and other variables following the duathlon race.

Statistics: **p < 0.01, *p < 0.05, tp < 0.1.
Box plot: (minimum value)-(mean)-SD-SD=(mean)-SD-(maximum value), N=7
-P: Data is plasma concentration.
-S: Data is serum concentration.
pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.
damaged group: renal tubular epithelial cells existed in the urinary sediments.
minor-damage group: renal tubular epithelial cells did not exist in the urinary sediments.
Abbreviations: Urea acid (UA), diaceon reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), antioxidant capacity (OXY), myeloperoxidase (MPO), reactive oxygen species (ROS), myoglobin (Mb), homainol-dependent chemiluminescence (LncCL), relative light unit (RLU), Carratelli unit (U-CARR).
Table 2. Spearman’s rank correlation coefficient matrix of urinary excretion rates of NAG, TXE, ALB and MCP-1 in the damaged group and the minor-damaged group.

<table>
<thead>
<tr>
<th>Damaged group</th>
<th>NAG</th>
<th>TXE</th>
<th>ALB</th>
<th>MCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>0.950</td>
<td>0.947</td>
<td>0.950</td>
<td>0.938</td>
</tr>
<tr>
<td>NAG</td>
<td>0.407</td>
<td>0.443</td>
<td>0.454</td>
<td>0.498</td>
</tr>
<tr>
<td>TXE</td>
<td>0.363</td>
<td>0.429</td>
<td>0.441</td>
<td>0.448</td>
</tr>
<tr>
<td>ALB</td>
<td>0.350</td>
<td>0.404</td>
<td>0.407</td>
<td>0.353</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.340</td>
<td>0.350</td>
<td>0.340</td>
<td>0.300</td>
</tr>
</tbody>
</table>

Table 3. Spearman’s rank correlation coefficient matrix of urinary excretion rates of TRX, Cls and variables of renal damage following the duallactate race.

<table>
<thead>
<tr>
<th>group</th>
<th>TRX-U</th>
<th>Cls-U</th>
<th>ALB-U</th>
<th>Cr-8</th>
<th>IL-2-U</th>
<th>IL-4-U</th>
<th>IL-10-U</th>
<th>IFN-γ-U</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRX-U</td>
<td>0.993</td>
<td>0.974</td>
<td>0.995</td>
<td>0.974</td>
<td>0.995</td>
<td>0.987</td>
<td>0.994</td>
<td>0.987</td>
</tr>
<tr>
<td>Cls-U</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
<tr>
<td>ALB-U</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
<tr>
<td>Cr-8</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
<tr>
<td>IL-2-U</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
<tr>
<td>IL-4-U</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
<tr>
<td>IL-10-U</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
<tr>
<td>IFN-γ-U</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
<td>0.993</td>
<td>0.987</td>
</tr>
</tbody>
</table>

All data were calculated as area under the curve (AUC).

- AUC total value of pre-, 0 h, 1.5 h and 3 h.
- Values N=27. Statistical ** p<0.01, * p<0.05, p<0.1.
- Data are serum concentration.
- U: Data are the gross amount in the volume of urinary excretion per one minute (urinary excretion rate).
- pre-exercise (pre), immediately post-exercise (0 h), 1.5 hours post-exercise (1.5 h) and 3 hours post-exercise (3 h) are sampling points.

chemokines and tended to be positively associated with oxidative stress markers. AUC of plasma MPO concentrations was positively correlated with antioxidant capacity markers. AUC of neutrophil ROS production ex vivo tended to be positively associated with oxidative stress markers and was associated with IL-1ra, an anti-inflammatory cytokine. Moreover, migratory neutrophil count ex vivo was correlated with IL-1ra. AUC of serum d-ROMs was positively associated with leukocyte count, neutrophil count, IL-1ra and Mb, and tended to be positively correlated with OXY.

In the minor-damage group AUC of plasma concentration of lactate was associated with migratory neutrophil count ex vivo and d-ROMs, and tended to be positively correlated with MPO. Plasma concentration of MPO was correlated with leukocyte count and neutrophil count, and tended to be corre-
labeled with ROS production \textit{ex vivo}. AUC of ROS production \textit{ex vivo} tended to be associated with migratory neutrophil count \textit{ex vivo}. Migratory neutrophil count \textit{ex vivo} was associated with UA. Plasma IL-10 concentrations were correlated with Mb.

**DISCUSSION**

It is known that blood flow is redistributed during endurance exercise. In this study, we provide evidence that the presence of renal epithelial cells in urine may be induced by ischemia/reperfusion caused by a reduction in renal blood flow. We have already investigated AKI caused by endurance exercise and the possible associations between AKI and the increases in urinary levels of IL-2, IL-4, IL-8, IL-10, IFN-γ and MCP-1 (59). This study further analyzed the associations among AKI, cytokines, inflammation and oxidative stress with a special focus on TRX and C5a. In particular, the excretion rate of TRX increased significantly at 1.5 h (20.1-fold) from pre-exercise in the damaged group only (Figure 1), and the excretion rates of NAG (3 h) were positively correlated with those of TRX (3 h) (Table 2). These findings suggested that TRX was related to renal tubular injury. Therefore, it might be possible that the excretion rate of TRX increased in response to oxidative stress as a result of renal tubular injury following intensive endurance exercise.

In a previous study on murine kidney, transgenic hTRX was predominantly observed in the outer medulla after renal ischemia/reperfusion. Thereafter, the immunoreactivity for hTRX was revealed in the intraluminal region of the renal tubule, coinciding with a decrease in TRX protein in the kidneys and an increase in urine. Interestingly, TRX protein concentration did not change in the blood, and expression of TRX mRNA did not reveal localization or change in abundance after renal ischemia/reperfusion. Therefore, it is suggested that urinary TRX protein is derived from proximal tubule cortical region of the kidney (30). In this study, however, it might...
be possible that blood-derived TRX was mixed with TRX from proximal tubule cortical region, because the excretion rates of TRX at the same time points after the race were positively correlated with the excretion rates of ALB at pre, 0 h, 1.5 h h and 3 h after exercise and TRX is small protein of 12 kDa. Whereas urinary excretion rates of ALB at the same time points after the race tended to be positively correlated with the excretion rates of NAG (as a generally accepted marker of renal tubular injury) at pre, 0 h, 1.5 h h and 3 h after the race, the correlations between urinary excretion rates of TRX and NAG at 3 h after exercise were significant (Table 2). Serum NAG protein is not excreted into urine because its molecular weight is 130-140 kDa. Hence, it might be possible that urinary excretion of TRX was derived from both blood and kidney. In this study, renal ischemia/reperfusion of subjects was induced by endurance exercise, but in the previous work, bilateral renal arteries were clipped for 30 min and then released (30). The inconsistency of the results might be derived from the difference of the above induction methods for ischemia and exercise. It is reported that chemokines such as MCP-1 are key modulators in renal ischemia/reperfusion injury, and urinary chemokines are good markers of clinical diseases and AKI (78, 79). In the damaged group, urinary excretion rates of MCP-1 significantly increased (Table 1), and tended to be positively correlated with the excretion rates of TRX and NAG (Table 2). This suggests that TRX, MCP-1 and NAG may be associated with renal tubular injury.

In the present study, the excretion rates of C5a in the damaged group and the minor-damage group significantly increased following intensive endurance exercise (Figure 1). Urinary excretion rate of C5a was positively correlated with that of IL-2, IL-4, IL-8, IL-10 and IFN-γ after intensive endurance exercise (Table 3). It is suggested that the chemotactic factors C5a and IL-8 increased after reperfusion, making inflammatory cells infiltrate into tubular epithelium or glomerular capillary (13, 14, 15, 35, 39, 42, 76, 77 84). On the other hand, it may also be suggested that IL-10 increased to suppress progressive inflammation, and/or to repair damaged tissues (58). Therefore, it might be possible the excretion rates of C5a, IL-8 and IL-10 reflect inflammatory levels in the renal tubular injury. In contrast, in the damaged group, the excretion rates of TRX after intensive exercise were negatively correlated with the excretion rates of C5a, IL-2, IL-4, IL-8, IL-10 and IFN-γ following intensive exercise (Table 3). In the damaged group, it may be possible that levels of TRX were influenced by levels of IL-10 as an anti-inflammatory cytokine, and TRX regulates chemotactic activity C5a and IL-8, or TRX controls inflammatory progress by C5a and IL-8, because TRX functions as an antioxidant, as a chemotaxis inhibitor and as a redox-regulating protein in the signal transduction (12, 21, 22, 57).

We examined systemic oxidative stress and inflammation induced by endurance exercise within two groups (damaged group and minor-damage group) based on urinary measures of AKI. When evaluating the circulating oxidative stress and inflammatory state in these same groups, lactate levels were increased significantly in both (0 h: damaged group 3.4-fold; minor-damage group 2.7-fold). Lactate-related factors such as lactate threshold (LT) and onset of plasma lactate accumulation (OPLA) are critical for setting exercise intensity (16, 72). Since this study was carried out in an actual competition race, we could not examine LT and OPLA, but the athletes’ lactate levels suggested a difference of exercise intensity between the two participant groups.

Endurance exercise increases the circulating number of leukocytes, especially neutrophils, which exhibit the greatest change in cell count and function (7, 41, 49, 50 62-65, 82). Moreover, IL-6, IL-8 and M-CSF responses are positively correlated with the delayed-onset neutrophil mobilization from the bone marrow reserve after exercise (64, 82), particularly when the duration is over 2 h. In this study, leukocytes and neutrophil counts in the minor-damage group (whose race time was over 2 h) tended to be greater than the damaged group (Figure 1 and Table 1). MPO catalyses the conversion of hydrogen peroxide into hypochlorous acid in neutrophils and macrophages (75). MPO is located in the primary (azurophilic) granules (5) and is a marker of neutrophil activation after exercise (7, 50). MPO produces a large amount of ROS and induces oxidative damage to proteins, lipids and DNA (43). MPO increases depending on exercise intensity (49, 53). These findings suggest that the intensity was also higher in the minor-damage group, but MPO increased depending on exercise duration rather than intensity in case of such a long-duration exercise. In the damaged group we found serum concentrations of d-ROMs as an oxidative stress marker, and BAP and OXY as antioxidant capacities tended to be higher than those in the minor-damage group immediately after the race. It was suggested that acute endurance exercise-induced oxygen consumption in many organs in the damaged group was greater and produced ROS, because the intensity of the damaged group was higher compared with that in the minor-damage group. Previous studies showed that scavengers such as enzymatic activities of plasma SOD and catalase (a scavenger for H2O2) and plasma concentration of vitamin C (ascorbate: a scavenger for O2•−, OH, O3• and other oxidants) for toxic ROS might be induced in response to intensive exercise (67). Moreover, it was reported that free radical scavengers prevent not only oxidation of molecules in the body but also adhesion of neutrophils to the endothelial lining and inhibiting neutrophil infiltration (19, 57). It was also reported that anti-inflammatory cytokines prevent inflammatory tissue damage (31, 66, 73). In particular, the anti-inflammatory cytokine IL-10 is an immunosuppressive cytokine that inhibits both proinflammatory cytokine production and ROS production by activated neutrophils (31). Furthermore, anti-inflammatory cytokines and free radical scavengers work to counteract oxidative tissue damage by ROS (9, 24, 56). In this study, the plasma concentration of IL-10 significantly increased only in the damaged group (Table 1). Plasma IL-1ra concentrations increased significantly after exercise in both groups (59) and were significantly correlated with ex vivo neutrophil migratory activity and ROS-production in the damaged group only. Accordingly, increased antioxidant capacity and anti-inflammatory cytokines in the damaged group might inhibit neutrophil activation as compared with those in the minor-damage group. We found also that serum Mb concentrations in the minor-damage group (0 h: 6.2-fold, 1.5 h: 7.0-fold) were higher than those in the damaged group (0 h: 4.8-fold, 1.5 h: 4.2-fold) after the race. These results might suggest that muscle damage increased due to ROS from activated neutrophils in the minor-damage group, whereas in the damaged group, muscle damage was prevent-
ed by elevated antioxidant capacity and anti-inflammatory cytokines.

Kanda et al. reported enhanced neutrophil migration and ROS production after one-leg calf-raise exercise through the use of a newly-developed ex vivo methodology in imitation of tissue damage (28). In this study, we assessed ROS production by neutrophils that migrated into the hydrogel. We found that both neutrophil migration and ROS production increased after exercise in both groups. The ROS production immediately after the race in the damaged group was lower than that in the minor-damage group, but serum concentration of OXY and plasma concentration of IL-10 were higher than those in the minor-damage group. These results suggest that neutrophil activation was suppressed by antioxidant and anti-inflammatory cytokines immediately following intensive endurance exercise.

In conclusion, we infer that the excretion rates of TRX, MCP-1 and NAG were associated with renal tubular injury. It might be possible that the excretion rates of C5a, IL-8 and IL-10 reflect inflammatory levels in renal tubular injury, where the excretion rate of C5a was strongly associated with that of IL-2, IL-4, IL-8, IL-10 and IFN-γ. In the damaged group, the excretion rates of TRX after exercise were negatively correlated with the excretion rates of C5a, IL-2, IL-4, IL-8, IL-10 and IFN-γ following exercise. Therefore, in the damaged group, it could be inferred that levels of TRX were influenced by levels of IL-10 as an anti-inflammatory cytokine, and that TRX regulates chemotactic activity of C5a and IL-8, or that TRX controls inflammatory progress by C5a and IL-8, because TRX functions as an antioxidant, as a chemotaxis inhibitor and as a redox-regulating protein in the signal transduction. Clarification of these pathways might be valuable in the assessment of AKI risk following intensive endurance exercise.

In the circulation of the damaged group ROS production was found to be higher than the minor damage group, while antioxidant capacity and anti-inflammatory cytokines increased immediately after intensive endurance exercise. From these data it might be inferred that neutrophil activation and efflux of MPO were inhibited. Therefore, we suggest that damage to muscle and other tissues are likely to be lower in this group. On the other hand, results from the longer duration group (minor-damage group) showed neutrophil count and efflux of MPO in the circulation were higher when compared to the damaged group. Furthermore, both variables were significantly correlated with neutrophil count and plasma concentration of MPO immediately following intensive endurance exercise. In combination, these results suggest that muscle is likely to be damaged by activated neutrophils to a greater extent than in the damaged group. This inference was further supported by the results from our application of the newly-developed ex vivo method that estimated the functional impact of activated neutrophils.

In the present study, we confirmed that intensive endurance exercise caused “Risk” or “stage I” in the AKI diagnosis criteria such as RIFLE and AKIN, suggesting that not only blood but also urine analyses are important for estimating tissue damage. The relationships among the variables in the urine and circulation, and further delineation of their clinical significance must be revealed in future research.

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