Potential sources of oxidative stress that induce postexercise proteinuria in rats

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Exercise-induced proteinuria is a well-known phenomenon in both animals and humans (6, 23, 24, 26). The proposed mechanism of exercise-induced proteinuria involves increased glomerular permeability and exceeding the maximum tubular reabsorption capacity (23, 24). Renal blood flow and glomerular filtration rate decrease during exercise. The decrease in blood flow is more apparent, and the filtration fraction increases during exercise, which facilitates the passage of proteins into the ultrafiltrate (23, 24). Also, the loss of fixed negative charge from the capillary wall of the glomerular tuft may be responsible for exercise-induced proteinuria (23, 41). Still another mechanism suggested for postexercise proteinuria is the maximal tubular reabsorption capacity being exceeded during heavy exercise (23–25, 41). However, the transient effects of exercise on renal function are incapable of explaining the proteinuria that occurs 24–48 h after exercise. In addition to these mechanisms, our previous studies established that exercise-induced oxidative stress also contributes to the occurrence of postexercise proteinuria in both rats (6) and humans (31). However, the sources of oxidative stress that induce urinary protein excretion after exercise are still unknown.

The role of reactive oxygen species (ROS) in various kidney diseases has been well documented. Glomerular disorders (minimal change disease, membranous glomerulonephritis, and neutrophil-induced damage), ischemic or toxic acute renal failure, obstructive nephropathy, and progressive renal failure are kidney diseases in which ROS play role in the pathogenesis (10, 12, 32). It has also been shown that in various kidney diseases accompanied by increased urinary protein excretion, antioxidant intervention reduces proteinuria by oxidative stress suppression (15, 17, 19, 20, 35). Similarly, it is well known that there is a close relationship between oxidative stress and exercise. In addition to the emergence of free radicals from mitochondrial leakage due to enhanced oxygen consumption, the ischemia-reperfusion process and leukocyte activation may also contribute to oxidative stress during and after exercise (11). The latter two mechanisms are particularly responsible for oxidative stress in extramascular organs and tissues after physical exercise (27, 34). The enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is involved in damage induced by leukocyte activation (21), whereas the enzyme xanthine oxidase (XO) plays a role in the ischemia-reperfusion process (11, 37).

The aim of this study was to determine the possible sources of ROS that contribute to the occurrence of postexercise proteinuria. To that end, we focused our study on two enzyme systems. One enzyme, NADPH oxidase, is mainly responsible for leukocyte activation and increasing production of ROS. The other enzyme, XO, can be activated via the ischemia-reperfusion mechanism. We blocked each process using respective inhibitors to clarify the difference between these two pathways of ROS generation.

MATERIALS AND METHODS

Animals

Ninety-six female Wistar rats weighing 200–280 g were used in this study. All rats were given standard rat chow and tap water ad libitum and housed at 23 ± 2°C on a 12:12-h dark-light cycle. All procedures were approved by the Akdeniz University Animal Care and Usage Committee and followed the guidelines established by...
American Physiological Society. The animals were divided into two groups, either NADPH oxidase or XO, and the experiments were performed in two steps.

**NADPH oxidase study.** The effect of NADPH oxidase blockage on exercise-induced proteinuria was evaluated. Control (CNADPH; n = 10), exhaustive exercise (ENADPH; n = 10), NADPH oxidase inhibition (INADPH; n = 10), and exhaustive exercise + NADPH oxidase inhibition (EINADPH; n = 10) groups were created. Diphenyleneiodonium chloride (DPI; 1.6 mg·kg⁻¹·day⁻¹) was used as a NADPH oxidase inhibitor, which was administered intravenously through the tail vein for 4 days before exercise. The last dose of DPI was injected to EI animals 1 h before exhaustive exercise, and the rats were then put in metabolic cages.

**XO study.** The effects of XO blockage on exercise-induced proteinuria were evaluated in this set of experiments. Control (CXO; n = 14), exhaustive exercise (EXO; n = 14), XO inhibition (IXO; n = 14), and exhaustive exercise + XO inhibition (EIXO; n = 14) groups were created. Oxypurinol (40 mg·kg⁻¹·day⁻¹) was used as a XO inhibitor, which was given intraperitoneally for 3 days before exercise. The last dose of oxypurinol was injected to EI animals 1 h before exhaustive exercise, and the animals were then put in metabolic cages as in DPI group.

**Exercise Protocol and Sampling**

Acute exhaustive exercise was performed on a motor-driven treadmill (MAY-TME 9805, Commat, Ankara, Turkey). All rats performing exercise were familiarized with treadmill running for 3 days before the test day. On the first day, the animals were simply placed on the treadmill. The animals walked very slowly on treadmill for 5 min during subsequent 2 days. The treadmill was equipped with an electric shock grid on the rear barrier to provide exercise motivation to the animals. The protocol was started at a speed of 20 m/min with no incline. The grade was gradually increased and reached 15% in 20 min, and running was continued until exhaustion. The point of exhaustion was determined by loss of righting reflex when animals were turned on their back.

Twenty-four-hour urine samples were collected from all animals while they were in metabolic cages, and the samples were used for protein and creatinine measurements. All animals in the exhausted groups were placed in metabolic cages immediately after treadmill running. At the end of urine collection period, rats were anesthetized with ether, and blood samples were obtained from the abdominal aorta. The kidneys were removed immediately and were stored at −80°C until biochemical analyses.

**Proteinuria Assessment**

Total urinary protein levels were assayed by a commercial colorimetric kit (Randox, Crumlin, UK), and values are expressed as milligrams per milligram of creatinine. Creatinine was measured by a kinetic-spectrophotometric method (18).

**Oxidative Status Parameters**

**Thiobarbituric acid-reactive substances.** Lipid peroxidation of kidney tissue was estimated by measuring thiobarbituric acid reactive substances (TBARS), as described by Stocks and Dromandy (33), using 1,1,3,3-tetraethoxypropane as a standard. TBARS levels were determined by measuring absorbance at 532 nm after reaction with thiobarbituric acid in kidney tissues.

**Carbonyl derivative contents.** Kidney reactive carbonyl derivative contents were measured as a marker of protein oxidation using the method of Levine et al. (16).

**Enzyme Activity**

NADPH oxidase enzyme activity was evaluated in kidney tissues using the fluorometric method, as described by Fang et al. (3), based on reduction of resazurin to resorufin. Results of NADPH oxidase activity are expressed as fluorescent intensity per milligram of protein.

Kidney and plasma XO activity was determined using a commercially available kit (Molecular Probes, Eugene, OR). Results of XO activity measurements are expressed as milliunits per milliliter for plasma and milliunits per milligram protein for kidney.

**Statistical Analyses**

Results are expressed as mean ± SE, and statistical analyses were performed using a one-way ANOVA. The Newman-Keuls post test was used to compare intergroup differences. Results were considered significant for *P* < 0.05.

**RESULTS**

Exhaustion times were not different between exercised groups in both sets of experiments. Latency to exhaustion in exercised groups was found to be 49.1 ± 1.6 min for ENADPH, 52.3 ± 3.0 min for EINADPH, 49.9 ± 2.2 min for EXO, and 51.4 ± 2.1 min for EIXO.

**NADPH Oxidase Study**

Total urinary protein levels are shown in Fig. 1A. A single exposure to acute treadmill running led to an increase in urinary protein excretion in the exercised group (EINADPH) relative to the control animals (*P* < 0.05). Moreover, inhibition of NADPH oxidase activity prevented the exercise-induced proteinuria in EINADPH rats compared with ENADPH group (*P* < 0.01).

Kidney TBARS and protein carbonyl content in the NADPH oxidase groups are shown in Fig. 1, B and C. Exercise induced a significant increase in kidney TBARS level and protein carbonyl content in ENADPH rats relative to control animals (*P* < 0.01). Also, NADPH oxidase inhibitor treatment significantly prevented the increases in kidney TBARS and protein carbonyl derivative levels induced by exhaustive exercise in the ENADPH group (*P* < 0.01).

The enzyme activity results are shown in Table 1. Kidney NADPH oxidase activity increased in the ENADPH group compared with the CNADPH group (*P* < 0.01), and inhibitor treatment prevented the exercise-induced increase in enzyme activity in the EINADPH animals (*P* < 0.01).

**XO Study**

Total urinary protein levels are shown in Fig. 2A. Total urinary protein excretion increased after acute exhaustive exercise in EXO animals (*P* < 0.05). However, we found no significant decrease in urinary protein levels after XO inhibitor treatment in EIXO animals compared with the EXO group.

Kidney TBARS and protein carbonyl content in the XO groups are shown in Fig. 2, B and C. Kidney TBARS level and protein carbonyl content were significantly increased in exercised animals (EXO) compared with the CXO animals (*P* < 0.01 and *P* < 0.01, respectively). The exercise-induced increases in both kidney TBARS and protein carbonyl levels were prevented by oxypurinol treatment in the EIXO group (*P* < 0.01 and *P* < 0.01, respectively).

The XO enzyme activity results are presented in Table 2. We found no significant increase in either plasma or kidney XO activity in EIXO animals relative to CXO animals. However, oxypurinol treatment significantly decreased plasma and kid-
ney XO activity in both the IXO and EXO groups compared with CXO and EXO groups, respectively (P < 0.01).

DISCUSSION

The purpose of this study was to determine the possible sources of ROS that contribute to postexercise proteinuria. Previously, our laboratory found that exercise-induced oxidative stress induces postexercise proteinuria in both rats (6) and humans (28). The results of present study indicate that exercise-induced proteinuria could be prevented by NADPH oxidase inhibition, thereby supporting the suggestion that oxidative stress is involved in mechanisms of postexercise proteinuria.

Table 1. Kidney NADPH oxidase enzyme activity for each treatment group of animals

<table>
<thead>
<tr>
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<th>C_NADPH</th>
<th>E_NADPH</th>
<th>I_NADPH</th>
<th>EINADH</th>
</tr>
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<tbody>
<tr>
<td>Values</td>
<td>67.58±1.80</td>
<td>92.21±5.89*</td>
<td>52.13±3.73</td>
<td>57.8±2.96†</td>
</tr>
</tbody>
</table>

Values are means ± SE given in fluorescent intensity/mg protein. NADPH, nicotinamide adenine dinucleotide phosphate; C_NADPH, control; E_NADPH, exhaustive exercise; I_NADPH, NADPH oxidase inhibition; EINADH exhaustive exercise + NADPH oxidase inhibition. *P < 0.01, difference from control group. †P < 0.01, difference from exercise group.

Proteinuria has been described as a marker of renal disease, and many authors have suggested that exercise-induced proteinuria is a transient, reversible, and benign process that does not lead to pathologies in normal kidney (26). Aside from the well-documented effects of exercise on renal function in normal humans, some contradictory reports indicate that exercise...
could be injurious to impaired renal function and renal histology (14). Regular physical activity is recommended to elderly, diabetic, and hypertensive people when the risk for renal disease rises. The effect of exercise on renal function as well as exercise-induced proteinuria has been insufficiently studied and little is known on the subject.

It is well established that exercise results in increased production of ROS. Mitochondria have long been recognized to be a major cellular site of free radical generation during skeletal muscle contractions (11). Exercise-induced oxidative stress may primarily cause muscle damage, but it also affects several tissues, including heart, kidney, liver, cerebrum, and erythrocytes (11, 27, 30, 39). The precise sources of increased ROS induced by exercise in extramuscular tissues are not yet clear because metabolic rate and oxygen consumption do not increase during exercise in tissues like the kidney. Leukocyte activation (NADPH oxidase enzyme system) and the ischemia-reperfusion process (xanthine oxidoreductase enzyme system) are proposed sources of ROS produced by extramuscular tissues during exercise (21, 27, 28, 37).

It is well documented that oxidative stress leads to proteinuria in several conditions. Minimal-change disease, membranous glomerulonephritis, neutrophil-induced damage, ischemic or toxic acute renal failure, obstructive nephropathy, and progressive renal failure are kidney diseases where ROS play a role in pathogenesis (10, 12). In addition, cobra venom factor (25), daunomycin-induced nephropathy (20), immune complex nephritis (29), Masugi nephritis (38), and adriamycin nephropathy (19) are some examples for oxidative stress-induced proteinuria found in experimental kidney disease models. Antioxidant interventions could be effective in reducing proteinuria with superoxide dismutase (19), dimethyl thiourea (20), catalase (35), probucol (17), and vitamins C and E (15) being utilized in such processes. Also, it has been shown that suppression of activated leukocytes or inhibition of XO activity reduces proteinuria in some experimental kidney diseases (10, 28). The main sources of ROS in these kidney pathologies are NADPH oxidase and XO enzyme systems, similar to in exercise. We hypothesized that the sources of postexercise oxidative stress acting on the kidney might be based on these two enzyme systems. It was shown that acute exercise leads to leukocyte activation and increased production of ROS through mechanisms involving the NADPH oxidase system (21). On the other hand, the XO system is activated through an ischemia-reperfusion mechanism because kidney blood flow decreases during exercise (11, 37).

In this study, we evaluated whether these two enzyme systems participate in exercise-induced proteinuria via generation of ROS. Because we designed this study as two separate experiments, the results are also discussed as such.

### Table 2. Plasma and kidney XO enzyme activity

<table>
<thead>
<tr>
<th></th>
<th>Cso</th>
<th>Eso</th>
<th>Ixo</th>
<th>EIso</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.18±0.03</td>
<td>1.20±0.02</td>
<td>0.66±0.04*</td>
<td>0.66±0.03†</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.45±0.05</td>
<td>1.65±0.13</td>
<td>0.86±0.04*</td>
<td>0.88±0.07†</td>
</tr>
</tbody>
</table>

Values are mean ± SE given as mU/ml in plasma and as mU/mg protein in kidney. XO, xanthine oxidase; Cso, control; Eso, exhaustive exercise; Ixo, XO inhibition; EIso, exhaustive exercise + XO inhibition. *P < 0.01, difference from control group. †P < 0.01, difference from exercise group.

### NADPH Oxidase Study

As shown by numerous studies (23–25) including ours (6–8, 31), total urinary protein level was significantly increased in exercised animals (E_{NADPH}) relative to control animals (C_{NADPH}). Meanwhile, elevated protein level in urine, TBARS level (as an index for lipid peroxidation), and carbonyl derivative content (as a marker of oxidized protein) in kidney were significantly increased after a single bout of exhaustive exercise (E_{NADPH}). Although TBARS and carbonyl derivative contents are not specific to NADPH oxidase activity, these results suggest that exhaustive exercise induces the oxidative stress and promotes postexercise proteinuria.

Another parameter that was elevated by exercise was kidney NADPH oxidase activity. Consistent with our hypothesis, it has been reported that NADPH oxidase is an important source of ROS that has been associated with mechanisms related to postexercise tissue inflammatory response during and after exercise (21). It has been shown that circulating neutrophil concentration can increase by severalfold within hours of exercise termination, and neutrophil accumulation in tissues remains elevated for several days (36). In addition to neutrophils, many other cells (monocytes/macrophages, podocytes, fibroblasts, endothelial, mesangial, tubular cells, and vascular smooth muscle cells) can also produce ROS via NADPH oxidase in kidneys (1); however, in this study we did not focus on reasons for activation and localization of NADPH oxidase. Inflammatory mediators, angiotensin II, and high-salt diet may also lead to increased NADPH activity in kidney tissue (4). Although the function of constitutively active renal NADPH oxidase is unclear, it was supposed that the enzyme may act as an oxygen sensor in the cortical region (13).

We used DPI as an NADPH oxidase enzyme inhibitor to determine the possible role of this enzyme in exercise-induced proteinuria. The animals that received DPI before exercise (E_{NADPH}) exhibited suppressed NADPH oxidase activity. These results support the idea that NADPH oxidase may be a source of ROS generation that contributes to increased urinary protein excretion following exercise. The increased kidney NADPH oxidase activity may result from infiltration of neutrophils/macrophages to the kidney tissue induced by exhaustive exercise. Another possibility is that increased free radical generation in kidney tissue during exercise may activate NADPH oxidase enzyme in glomerular cells. In rats that received DPI before exercise (E_{NADPH}), urinary protein levels and all oxidative stress parameters were not different from control animals. In other words, NADPH oxidase inhibition completely prevented the increase in urinary protein excretion as well as oxidative stress induced by exhaustive exercise. These results suggest that one of the sources of increased ROS generation is the NADPH oxidase system and that it contributes to postexercise proteinuria. It is possible that NADPH-derived ROS may cause glomerular injury and thus an increase in urinary protein excretion after exhaustive exercise. Several studies have suggested that ROS may play a role in proteinuria by altering glomerular vascular permeability, causing glomerular basement membrane degradation and impairing the electrostatic barrier in various kidney diseases (10, 12).
XO Study

Exhaustive exercise may be important in the generation of free radicals in the kidney via activation of XO, similar to the process in ischemia-reperfusion, and thus may be involved in postexercise proteinuria. XO is a cytosolic enzyme that becomes highly active during the ischemia-reperfusion process and can produce ROS (37). This enzyme is often present in a dehydrogenase (XD) form, which does not produce ROS. However, conversion of the XD to the XO form, which is induced during the ischemia-reperfusion process, also occurred during exercise. However, this is not the only process that produces increases in XO activity because elevation of Ca^{2+} and exposure to ROS are also exercise related mechanisms involved in activation of XO (9). It has been indicated that plasma XO activity dramatically increases in rats after exhaustive exercise and that treatment with a superoxide dismutase derivative or an XO blocker attenuates exercise-induced XO activation, glutathione oxidation, and lipid peroxidation (27, 37). However, several studies have demonstrated that renal blood flow is significantly reduced, to as much as 20% of the resting flow rate, depending on the intensity of exercise (23). It is well known that whereas the vascular resistance in skeletal muscles decreases during exercise, the resistance to flow through the kidney increases like in other visceral organs and skin. The decrease in renal blood flow during exercise might lead to XO activation in kidney tissue via ischemia-reperfusion. Thus it is possible that increased XO activity triggered by exhaustive exercise might be a source of ROS that induce postexercise proteinuria. To rule out this possibility, we tested whether inhibition of XO activity could protect against postexercise proteinuria induced by oxidative stress.

The results of present study showed that exhaustive exercise leads to a significant increase in kidney TBARS level and protein carbonyl derivative content, but exercise did not increase plasma and kidney XO activity in EIXO rats. This result may arise from the measurement of XO activity being performed 24 h after the exhaustive exercise in our study. In studies that reported increased plasma and tissue XO activity induced by exercise, the measurements of enzyme activity were performed at a shorter latency from the exercise (27, 34). Therefore, it is likely that we could not detect such increases in plasma and kidney XO enzyme activity because the samples were obtained 24 h after the exhaustion.

Although oxypurinol treatment prevented oxidative stress in kidney tissue, it did not significantly decrease urinary protein excretion in the EIXO group compared with EOXO group. The contribution of the XO enzyme to the occurrence of tubular-type proteinuria may explain this finding. Several investigations have shown that the renal XD/XO enzyme system provides a source of oxygen free radicals in various renal pathologies, such as purumycin aminonucleoside (5), adriamycin nephrosis (2, 5), and ischemic acute renal failure (40). The results of these investigations demonstrated that inhibition of renal XO activity was associated with a marked reduction of proteinuria (2, 5, 22). However, it was also shown that the reduction in proteinuria was transient and that inhibition of XO activity improved the tubulointerstitial, but not glomerular, lesions (22). In this present study, we measured total urinary protein excretion without assessing the glomerular or tubular component. It is well known that during mild to moderate exercise, the proteinuria that occurs is the glomerular type, but when exhaustive exertion is involved, the postexercise proteinuria seems to be of a mixed glomerular-tubular type (24, 26, 31). It is possible that XO enzyme inhibition may improve the tubular component of exercise-induced proteinuria as measured by low-molecular weight proteins such as β2-microglobulin in urine. Therefore, because we determined the total urinary protein levels, it may be said that in present investigation we could not detect the exact effect of XO inhibition on postexercise proteinuria. Although total urinary protein excretion was not decreased by oxypurinol, we cannot exclude the possibility that the XD/XO system may contribute to postexercise proteinuria. For this reason, further investigation specifically investigating each of the tubular and glomerular components of postexercise proteinuria is necessary.

In conclusion, the results of our study suggest that the source of exercise-induced oxidative stress that contributes to postexercise proteinuria was increased by NADPH oxidase enzyme activation during exhaustive exercise. The contribution of the XD/XO enzyme system to postexercise proteinuria needs further evaluations because a likely activation of this enzyme system is also seen in the ischemia-reperfusion process.

GRANTS

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REFERENCES


