The Effect of Nitric Oxide on Exercise-Induced Proteinuria in Rats

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Running Head: NO and exercise proteinuria

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ABSTRACT

Temporary proteinuria occurring after exercise is a common finding, and is explained predominantly by alterations in renal hemodynamics. In this study, it was investigated whether nitric oxide (NO), which is known to be effective on renal hemodynamics and to increase during exercise, has a role in post-exercise proteinuria.

At the first step of this study, the effect of acute nitric oxide synthase (NOS) blockage on exercise proteinuria was evaluated. The urinary protein levels in animals, which performed acute exhaustive treadmill running exercise, were considerably elevated compared to the control animals. Significantly elevated urinary protein levels were also detected in animals, which received L-NAME before exhaustion, compared to both control and exhausted groups, and mixed type proteinuria was detected in electrophoresis, as in all exhausted animals. In the second step of the study, an NO donor (isosorbide mononitrate) was given to rats at one hour before exhaustive exercise. Mixed type proteinuria and the elevation in urinary protein levels that occur as a consequence of exhaustive exercise were prevented by NO donor treatment. Finally in the third step of our study, a calcium channel blocker (diltiazem), another vasodilator, was applied to the rats one hour before exhaustive exercise. Urinary protein levels were not different in exhausted rats with or without calcium channel blocker treatment, on the other hand, in both groups, urinary protein levels were higher than control group. The tail-cuff blood pressure alterations caused by vasodilator drug applications prior to exercise were not different for NO donor and calcium channel blocker groups.

These results suggest that, endogenous NO might prevent the post-exercise proteinuria to grow more severe by affecting hemodynamic changes that occur during exercise.

Key words: Nitric oxide, nitric oxide synthase, exercise, proteinuria.
INTRODUCTION

Nitric oxide (NO) has an important role in regulation of kidney functions, as of many other tissues and organs. NO is effective in regulation of glomerular and medullary hemodynamics through vascular resistance, tubuloglomerular feedback response, renin release, and regulation of extracellular fluid volume (16,17). It was demonstrated that acute systemic nitric oxide synthase (NOS) blockage causes a prominent increase in resistance of efferent and afferent arterioles and decreases renal blood flow besides increasing the systemic blood pressure (1). However, glomerular filtration rate either is not affected or is decreased less compared to the renal blood flow, due to the increased filtration fraction after NOS blockade (15,16). In addition, it was shown that NOS blockage affects proximal tubular functions, and decreases total (absolute) and fractional tubular reabsorption (9).

NO also contributes to the blood flow redistribution occurring during exercise. Increased NO production during exercise induces a vasodilatation in heart and skeletal muscles, and prevents excessive vasoconstriction in the renal and splachnic region (18,33). It was demonstrated that the vasoconstriction response in kidney, especially in the renal cortex, increased prominently when NOS blockage was applied prior to exercise (15). On the other hand, there is a lot unknown about the effect of NO on kidney blood flow and functions, which are altered during exercise. The effect of NO on proteinuria occurring after exercise has not been investigated either.

Exercise proteinuria is common in athletes. It is described as a “temporary increase in protein excretion by urine in healthy individuals after exercise”, and was shown in various exercising laboratory animals as well as in humans (5,14,27). The basic mechanisms of postexercise proteinuria are increased glomerular permeability and/or exceeding the maximum tubular reabsorption capacity for proteins (6,28). The permeability of the glomerular capillary membrane, which does not allow the filtration of macromolecules at
normal circumstances, increases due to the renal hemodynamic alterations occurring during exercise. It is known that a considerable decrease in renal blood flow and an increase in filtration fraction occur during exercise and this facilitates the passage of macromolecules/proteins to the ultrafiltrate (13,23). While a lot of factors such as prostaglandin, renin-angiotensin system, sympathetic stimulation, negative charge loss from the glomerular membrane are accounted for the glomerular permeability change occurring during exercise (25,26,30), but the effect of NO on exercise proteinuria is not yet known.

It is possible that NO which is increased during exercise, may have a role on relevance of exercise proteinuria because of either exercise or NO being effective on renal hemodynamic. The aim of this study is to demonstrate whether NO has an effect on occurrence of exercise-induced proteinuria. To test this hypothesis we evaluated exercise proteinuria in rats under conditions of both NOS inhibition and increased NO availability.

**MATERIAL AND METHODS**

**Animals and Groups**

Eighty, 3 months-old male Wistar rats weighing 200-280 grams were used in this experimental study. All rats were given standard rat chow and tap water *ad libitum* and housed at 23±2 °C on a 12 h dark and 12 h light cycle. All procedures were approved by the Akdeniz University Animal Care and Usage Committee and followed the guidelines established by American Physiological Society. We randomly divided animals into ten groups and we tested our hypothesis in three steps of experiments:

**Step I:** The effect of acute NOS blockage on exercise proteinuria was evaluated. Nω-nitro-L-arginine methyl ester (L-NAME) was used for NOS blockage and it was applied two hours prior to exercise as 10 mg/kg i.p. Control (C; n=8), Exhaustive exercise (Exer; n=8), NOS blockage (L-NAME; n=8) and Exhaustive exercise + NOS blockage (L-NAME-Exer;
(n=8) groups were involved in this step. C and Exer groups received 0.5 ml sterile saline solution (vehicle) via i.p. Blood pressure (BP) measurements were made in all groups. Basal (pre-treatment of L-NAME) and post-treatment (just before exercise or two hours after L-NAME administration) BP were measured by a non-invasive tail-cuff method and data were obtained with the MAY-BPHR200 unit and MP 100A-CE data acquisition system (BIOPAC Systems, Inc., Santa Barbara, CA-USA) connected to a personal computer.

**Step II;** Isosorbide mononitrate (ISMN) was used as the NO donor in this step where the effect of exogenously applied NO on exercise-induced proteinuria was examined. Control (C; n=8) animals received 0.5 ml of sterile saline solution intragastrically. NO donor was administered in a group of animals (ISMN; 2 mg/kg p.o., n=8) while the other was subjected to additional exercise protocol, one hour after ISMN treatment (ISMN-Exer; 2 mg/kg/ p.o., n=8). Basal (pre-treatment of ISMN) and post-treatment (just before exercise or one hour after ISMN administration) BP were measured by a non-invasive tail-cuff method.

**Step III;** Diltiazem (calcium channel blocker), another vasodilator agent was used to reveal whether the effect of NO on exercise proteinuria is specific, and the effect of this drug on exercise proteinuria was tested. For this purpose, one group received calcium channel blocker without exercise (DILTI; n=8), whereas another received it one hour before acute exhaustive exercise (DILTI-Exer; n=8) at the dose of 0.3 mg/kg, i.p. 0.5 ml sterile saline solution was administrated via i.p. injection in control rats (C; n=8). Basal (pre-treatment of DILTI) and post-treatment (just before exercise or one hour after DILTI administration) BP were measured by a non-invasive tail-cuff method.

**Exercise Protocol and Sampling**

Acute exhaustive exercise was performed on a motor driven treadmill (MAY-TME 9805, Commat Ltd., Ankara, Turkey). All the rats performing exercise were familiarized to treadmill running 5 days before actual experiment day. They were put on the treadmill just to
recognize the treadmill in first two days. They walked very slowly on treadmill for 5 minutes during subsequent two days and finally they were rested in the last day. 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 and no incline. The grade was gradually increased and reached a 15% in 20 minutes. Running was continued until exhaustion. The point of exhaustion was determined by animals’ loss of righting reflex when turned on its back.

24-hours urine samples were collected in metabolic cages from all animals and were used for protein and creatinine measurements. All animals in exhausted groups were placed in metabolic cages immediately after treadmill running. The animals which received only drug treatment were placed in metabolic cages after drug administrations (two hours later for L-NAME group and one hour later for ISMN and DILTI groups).

**Proteinuria Assessment**

Total urinary protein levels were measured by spectrophotometric technique as described by Lowry (19) and values are expressed as mg/mg creatinine. Creatinine was measured by kinetic-spectrophotometric methods (BioSystems).

To determine the type of proteinuria we used the “Sebia Hydragel Proteinurie” kit. After diluting the urine sample with saline to obtain approximately 0.2 g/dl proteins we applied a 5 µl urine sample in each well of the gel and let the samples diffused into the gel for 10 minutes. The gel plate was placed in a Sebia K20 chamber at 8-10 mA, -60 V for 60 minutes during migration. At the end of the migration time, the gel plate was stained with acid violet for 30 minutes and destained for 45 minutes with destaining solution. The gel was dried in a hot 80°C air stream for at least 15 mins and was used to determine proteinuria type. 16, 32, 48, and 64 kDa molecular weight markers were used for determining the protein fraction. The band for 64 kDa indicated albumin.
**Statistical Analyses**

Data are presented as means ± SE. Statistical analyses were performed with two-way analysis of variance test and the Newman-Keuls post hoc test was used to compare inter-group differences when significant F ratio was found. A \( P \) value of 0.05 or less was considered as statistically significant.

**RESULTS**

Blood pressure (BP) values of all groups are shown in Table 1. No difference for basal BP values was detected between the groups. BP levels after two hours in L-NAME groups were considerably higher compared to the basal levels (Table 1A). Significantly decreased BP levels were obtained in groups, which received ISMN (Table 1B) or diltiazem (Table 1C). No statistically significant difference between the BP lowering effects of diltiazem and ISMN was detected. Exhaustion times were not different between all groups (data not shown).

Urinary protein levels are presented in Figure 1. While urinary protein levels increased after acute exhaustive exercise (Exer) compared to the control group, the urinary protein levels of animals which performed acute exhaustive exercise two hours after L-NAME injection (L-NAME-Exer) were found to be significantly higher compared to both C and Exer group (Figure 1A). The urinary protein levels of the rats that received L-NAME treatment without exercise was not different from those of the control group. The urinary protein levels of the rats, which received NO donor without exercise (ISMN) were not different from those of the control group while treatment with NO donor prevented exercise-induced proteinuria in ISMN-Exer group (Figure 1B). There was no difference in urinary protein levels between the group which received diltiazem treatment only (DILTI) and the control group, the urinary protein levels in the group which performed exercise one hour after diltiazem treatment
(DILTI-Exer) was found to be significantly higher compared to the other two groups (Figure 1C).

The results obtained from urinary protein electrophoresis are shown in Figure 2. Acute exhaustive exercise (Exer) caused mixed type proteinuria characterized with an increase in both light and heavy protein chain bands. While band pattern of L-NAME group was similar to controls, mixed type proteinuria was detected in L-NAME-Exer group (Figure 2A). The urinary protein electrophoresis patterns of ISMN and ISMN-Exer groups were similar to that of the control group (Figure 2B). Mixed type proteinuria was also detected in DILTI-Exer group while the results were similar in DILTI and control groups (Figure 2C).

**DISCUSSION**

The renal vasculature is under continuous and strong influence of NO (15,16,17). Although it has been demonstrated that NO plays an important role in blood redistribution during exercise, the effect of NO on exercise-induced proteinuria has not been investigated yet. In this study, it was shown that NOS inhibition increases exercise proteinuria in rats, and that the urinary protein levels return to control values with use of an NO donor. The results suggest that NO prevents a further increase in the proteinuria arising during exercise.

The factors mostly accused for induction of exercise proteinuria are changes in renal hemodynamics during exercise, besides the loss of negative charges in glomerular barrier, exceeding the proximal tubulus reabsorption capacity and exercise-induced oxidative stress (2,11,28). The increase in glomerular permeability during exercise and right after exercise is also explained generally by the increase in filtration fraction (29). Renal blood flow decreases as a consequence of the increase in the renal sympatetic nerve activity and elevated level of catecholamines in circulation during exercise (2,26,31). Furthermore, an increase in resistance of afferent and efferent arterioles and in renin release are achieved by sympatetic stimulation.
during exercise. Angiotensin II, another possible effective vasokonstrictor agent in renal haemodynamics, was investigated extensively. Angiotensin II production following renin release causes an increase in transglomerular pressure and filtration fraction via a more prominent constriction of efferent arteriol (3,6). On the other hand some authors did not observed an involvement of renin-angiotensin system in postexercise proteinuria (24). In addition, it was indicated that sympathetic neurotransmitters as dopamine and neuropeptid Y could increase and these may contribute to the splachnic and renal vasoconstriction during exercise (22). Involvement of vasoconstrictor agents in induction of post-exercise proteinuria were well studied compared to vasodilators except prostaglandin (2,25).

In the first step of this study we investigated whether NO, which is one of the major vasodilator agent in kidney, has an effect on exercise proteinuria. We aimed to demonstrate the effect of acute NOS inhibition on proteinuria arising after exercise. Low dose (10 mg/kg) L-NAME was used for NOS blockage. Previously used high doses in a wide range of 30-300mg/kg, are known to cause reluctance for running in rats (33). In addition, low dose L-NAME treatment would not influence the systemic hemodynamic parameters so much. In this study, blood pressure exhibited an increase of approximately 15% as a result of L-NAME treatment. Exercise-induced proteinuria was significantly higher in L-NAME treated group before exhaustion (L-NAME-Exer) compared to control and only exhausted groups. Elevated NO production due to increased shear stress during exercise increases blood flow in heart and skeletal muscles (10,21). However NO also prevents excessive decrease in the renal and splanchic blood flow that occurs in renal-splanchnic region during exercise (33). As stated before, either sympathetic activation or increased Ag II production decrease renal blood flow during exercise (26,30). As particularly NO plays the balancing role against these vasoconstrictor agents, therefore, inhibition of NO production by NOS blockage might have
induced an excessive proteinuria following exercise as a result of the increase in effectiveness of Ag II on efferent arteriole resistance.

We planned the second step of our study to investigate whether the exogenously given NO (isosorbide mononitrate) has an effect on exercise proteinuria. In addition, we investigated whether this effect is specific to NO by giving non-specific vasodilator agent (calcium channel blocker, diltiazem) to another group in the third step of our study. By preliminary studies, doses of both drugs that caused similar hypotensive effects were determined. Agents that were given one hour before exercise caused approximately 7-13% decrease in blood pressure. These agents did not affect urinary protein concentration and electrophoretic pattern when applied on their own. The level of proteinuria after exercise in the group which received NO donor prior to exercise (ISMN-Exer) was not different from that of the control group. Furthermore, the urinary protein electrophoresis was similar to that of the control group. However, the effect of the calcium channel blocker was different from that of the NO donor. The amount of protein in DILTI-Exer group rats’ urine after exercise was significantly higher compared to the control group, and mixed type proteinuria was detected in electrophoresis. Because NO plays a major role in maintanace of renal blood flow (17), the decrease in renal blood flow that occur during exercise might have been prevented by addition of exogenous NO to endogenously produced NO, thus the occurrence of post-exercise proteinuria might be reduced.

Proteinuria induced by exercise is more prominent in diabetic and hypertensive patients (12,34). Particularly, physical exercise can be used as a provocative test to detect the silent stage of the kidney disorder in both types of diabetes (7). Renin-angiotensin system was focused on mostly in the studies performed to explain the severe proteinuria occurring after exercise in diabetic patients (12,32). The results of our study may indicate that one of the reasons for exercise-induced proteinuria in diabetic and hypertensive patients could be
decreased NO production, since decreased NO production due to endothelial damage was reported in diabetes and hypertension (20). These findings may lead to suggestion that, one of the reasons for severe proteinuria after exercise in diabetic and hypertensive patients is NO deficiency.

In conclusion, proteinuria occurring after exercise becomes more prominent by NOS blockage and is prevented with exogenous NO treatment. Besides sympathetic activation and increased Ag II levels which are suggested to be the most investigated factors in pathogenesis of exercise-induced proteinuria, the effect of NO on renal hemodynamics should be considered to be one of the effective reason for post-exercise proteinuria.
REFERENCES


Table 1. Basal and post-treatment blood pressure measurements (mean arterial pressures, mmHg) of all groups before treadmill running.

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<td>87.5±1.5*</td>
<td>96.0±5.3*</td>
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Values are means±SE. *P<0.05; difference from basal blood pressure.
**Figure 1.** Urinary protein levels of all groups (mg/mg creatinine).

* P<0.05; ** P<0.01; difference from Control group
† P<0.05; difference from Exercise group
‡ P<0.05; difference from DILTI group
Figure 2. Urinary protein electrophoresis of all groups. MWM, molecular weight markers.