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Original Article

Long-term strength training causes a downregulation of nephrin in the slit diaphragm of kidneys in rats

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Abstract: Objective: Excessive exercise causes an abnormality of the structure and function of the renal filtration barrier, which produces proteinuria. Our objective was to perform a study examining the molecular mechanism of sustaining exercise-induced proteinuria with cut point, combined with long-term strength training and nephrin in the slit diaphragm (SD) of the kidney. Methods: Male Sprague Dawley rats, aged 8 weeks, were divided into three groups. These groups included a control group (group A), an overload group drawn immediately (group C), and an overload group drawn after 24 hours (group D). A rat model of exercise-induced proteinuria was produced by long-term strength training, then we observed the glomerular structure, tested the nephrin protein expression of the kidney, and the intrarenal and circulating blood renin-angiotensin system (RAS). Results: The urine total protein (TP, $P<0.01$), microalbumin (mALB, $P<0.01$), and microalbumin/creatinine (mALB/CRE, $P<0.01$) of groups C and D are higher than that of group A. The abnormality of the glomerular structure of group C was obvious, and that of group D was slight. The nephrin expression of group C ($P<0.01$) and group D ($P<0.01$) were lower than in group A. The intrarenal renin activity (Ra, $P<0.01$) and angiotension II (AngII, $P<0.01$) of group C were lower than that of group A, but the two groups had no differences in the circulating blood. The intrarenal Ra and AngII of group D were considerably higher than in group A and were the same in circulating blood. Conclusion: The intrarenal and circulating blood RAS were continuously active and induced by long-term strength training, which downregulated nephrin protein expression. This led to abnormalities of the structure and function of SD and resulted in proteinuria.

Keywords: Strength training, slit diaphragm, nephrin, RAS

Introduction

Exercise-induced proteinuria is a normal physiological phenomenon. Excessive training makes the kidney experience repeated stress causing irreversible renal injury. This situation is common in competitive sports and military training and is manifested as proteinuria, hematuria, electrolyte disorders and by other clinical symptoms [1-3]. Excessive exercise makes the structure of the renal filtration barrier abnormal. This leads to declining function and protein exudation exceeding the renal tubular reabsorption threshold (or accompanied by renal tubule damage), which produces proteinuria [4, 5]. SD is the most important and biologically active part of the renal filtration barrier [6] and nephrin is the main structural protein of SD, participating in signal transduction of the podocyte [7]. Overtraining could result in abnormalities of the renal microstructure. It is unclear whether the protein expression of nephrin is affected. If the protein expression is affected, the mechanism is not known.

Renal ischemia is caused by a redistribution of blood throughout the body during strenuous exercise. It is principally regulated by RAS and further affects the function of kidneys [8]. It has been suggested that excessive activity of RAS could lead to irreversible renal damage involving the development of proteinuria, inflammatory response, and glomerular sclerosis, etc [9]. AngII has a hemodynamic effect by regulating the tension of renal vessels and the reabsorption of water and sodium. The overexpression of AngII could damage the integrity of the structure and function of SD [10]. Is the overexcitation of RAS, induced by strenuous exercise, inhibiting expression of nephrin, damaging the SD structure, and therefore causing proteinuria? Do both intrarenal and circulating blood

Table 1. Specific training protocol

Stage	Speed, m.min^{-1}	Duration, min	Grade, degree
1	10	10	10
2	15	30	10
3	25	60	10
4	30	90	10
5	35	120	10
6	40	180	10

Table 2. The change of urine index induced by strength training

Urine index	Control	Overtrained
TP (μg/ml)	0.963±0.369	1.899±0.368**
mALB (μg/l)	13.33±2.38	19.15±3.23**
CRE (umol/l)	7598.8±1000.7	4123±634.6**
mALB/CRE	0.001789±0.000427	0.004755±0.001249**

Control: Group A; overtrained: Group C and D. **P<0.01 vs. group A.

RAS have an effect on the expression of nephrin? Long-term intensive training might cause RAS to remain in a sustainable state of excitement, while the resulting renal injury is not effectively restored. Maximal rates of proteinuria occur approximately 30 minutes after exercise, with a resolution toward resting levels within approximately 24 hours [5]. Whether the continuous activity of RAS would affect the expression of nephrin at 24 hours after exercise will determine the persistent adverse effects of long-term strength exercise on renal structure and function. It is helpful to explore the pathogenesis of exercise-induced renal injury in attempting to understand the causes of proteinuria induced by long-term intensive training.

Materials and methods

Animals and protocol

Male Sprague-Dawley rats, aged 8 weeks and weighing 223.76±6.23 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The 34 rats were randomly divided into three groups, including a control group (group A, 10), an overload group drawn immediately (group C, 12) and an overload group drawn after 24 hours (group D, 12). All rats were maintained in a conventional environment with a regular 12 hour light/dark cycle, 20-24°C temperature, 50-70% humidity, and were provided a standard commercial diet.

The rats of group A did not train; groups C and D ran on a motor-driven treadmill 6 days per week (see **Table 1**). In the first week, groups C and D were familiarized on a treadmill at 10 m/min × 10 min (running time × speed). The second week, they were trained at the beginning of 10 m/min each time, the speed was gradually increased until the target intensity of this week continued to the specified duration. If the rats were unable to maintain their strength in the last week, they would run to exhaustion.

Experimental animal materials

At beginning of the sixth week, group A and the overtraining groups (groups C and D) were randomly selected to have 8 rats. The time of urine collection of group A was random, but the time of urine collection for the overtraining group was 30 minutes after running cessation. The rats were placed in cages with plastic film, and urine was collected into the centrifuge tube after urination. The tube was centrifuged for 20 minutes (2000-3000 RPM) to collect the supernatant for analysis.

The rats of group C were sacrificed using sodium pentobarbital (2% of weight, 2 mL) at 30 minutes after the last training. Blood was taken from the abdominal aorta into the heparin anticoagulant tube, and centrifuged for 10 minutes (3000 rpm) to collect the plasma, and stored in the refrigerator (-20°C) in preparation for the test. We removed both of the kidneys. The three left kidneys were stored in polyformaldehyde fixed solution, and all of the right kidneys were frozen in liquid nitrogen and stored at -80°C for further analysis. Group D rats were operated on 24 hours after the last training while group A were operated on randomly.

Urine indicators

TP - The urine sample was diluted 20 times, then the TP was tested using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™) through ELISA (Bio-Rad xMark).

mALB - The mALB was tested using an enzyme-linked immunoassay kit (Beijing Jin Hai Ke Yu Biological Technology Development Co., Ltd.) through the ELISA (Bio-Rad xMark).

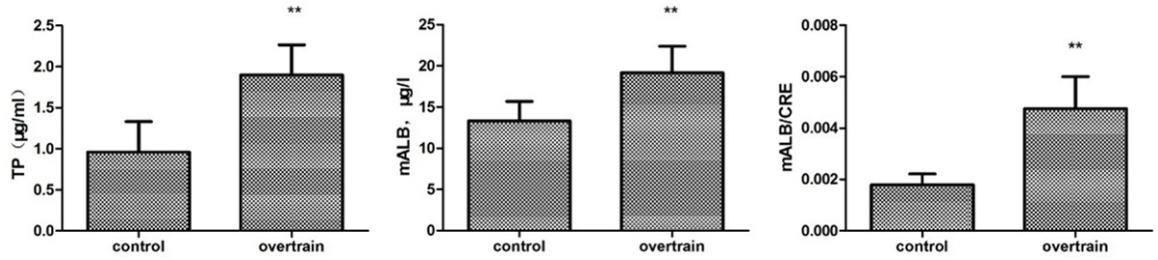

Figure 1. Comparison of urine index of rats with strength training.

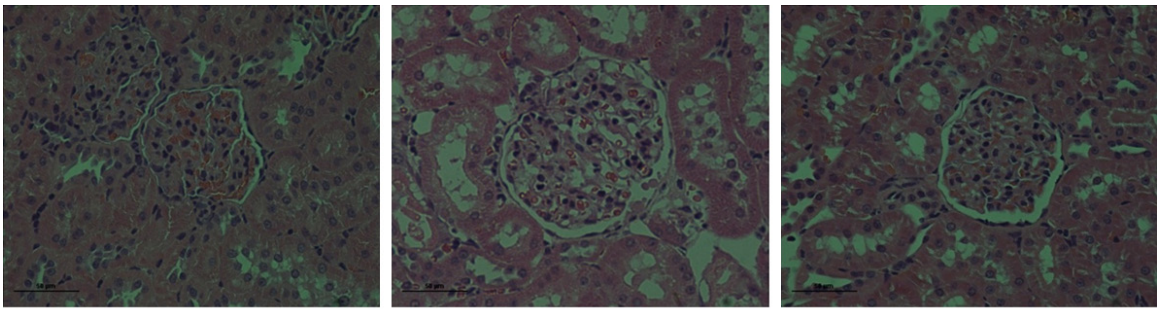

Figure 2. Glomerulus morphology of the three groups.

Table 3. The expression of nephrin in the kidney

	Group A	Group C	Group D
Nephrin	1	0.538±0.209**	0.634±0.293**

**P<0.01 vs. group A.

CRE - The CRE was tested using a creatinine kit (Alkaline Picric Method, Shanghai Kehua Bioengineering Co., Ltd.) via a KHB-1280 automatic biochemical analyzer.

Tissue morphology - We removed the kidneys and put them in paraformaldehyde and cleaned them for 12 hours using water. We then dehydrated the kidneys using gradient alcohol and embedded and immersed them in paraffin, after transparence. Tissue sections (4 μm) were used for HE staining to observe the renal glomerular structure under microscopy at 400 times magnification.

Nephrin

The ground renal samples were stored at -80°C and then lysed. Equal amounts of protein were separated by electrophoresis through 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with BSA, the membranes were treated with primary antibody for one night, washed, and incubated with peroxidase secondary antibody. Antibodies and dilutions included the following: rabbit monoclonal to nephrin antibody (1:2000, Abcam, ab136894) and mice monoclonal to GAPDH antibody (1:20000, Immunoway, YM3029). The blots were visualized with Thermo Pierce ECL reagent and peroxide, followed by exposure to x-ray film, and analyzed by grayscale using Image Lab 5.0.

Ra and AngII in kidney and circulatory system

The Ra and AngII in the ground renal and plasma samples were tested by radioimmunoassay using a XH-6020 full automatic RIA counter (Cnnc Xi, a nuclear instrument factory). The kits included the following: PRL1 (HY-134) riaRIA kit and AngII (HY-10060) RIA kit.

Statistical analysis

The data were presented as the mean ± standard deviation (SD). Statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison test (for comparisons of 3 groups) or Student's t-test (for comparisons of 2 groups). P<0.01 was considered statistically significant.

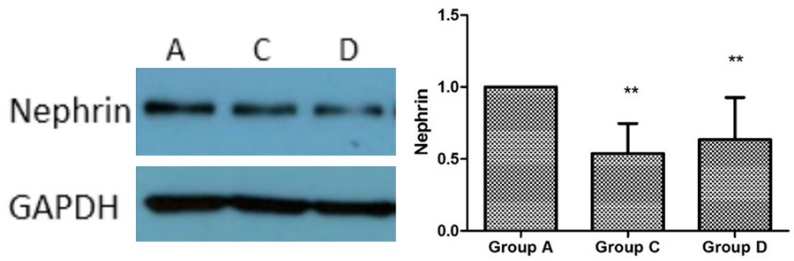

Figure 3. The expression of nephrin and trend chart.

Results

The evaluation of exercise-induced proteinuria

To estimate if strength exercise led to proteinuria in rats via the comparison of urine TP, mALB and mALB/CRE between group C and the overtraining group, we collected the urine of rats 30 minutes after training and tested mALB/CRE to exclude the effect of urine volume on the results (**Table 2**, **Figure 1**).

The six-week intensive exercise used in this study resulted in severe exercise-induced proteinuria in rats. The urine TP, mALB, mALB/CRE level was significantly higher in the exercised rats than in the control rats ($P<0.01$).

Renal morphology

The glomerular morphology changed significantly throughout long-term intensive training. After HE staining, we randomly selected a view of glomerulus to observe the morphological changes of the renal glomerulus in rats. Compared to group A, the glomerular structures of the rats in group C were obviously abnormal, and the rats in group D were still unable to recover after 24 hours (**Figure 2**).

Group A: The intravascular membrane of Bowman's capsule was compact, the glomerulus and the wall of the capsule had obvious boundaries, and the distribution of red blood cells in the blood vessels is regular; Group C: In contrast to group A, Bowman's capsule and the glomerulus of the rats were significantly swollen, the structure of the vascular membrane was destroyed, and the boundary of the glomerulus and the wall of the capsule was blurred; Group D: The glomerular structure was partly restored after 24 hours of recovery, though the vascular membrane remained abnormal.

Nephrin

There was a significant abnormality in the urine index of the overtraining group, and the morphology of the glomerulus was significantly altered by means of histomorphology. The expression of nephrin as the main structural protein of SD also decreased significantly (**Table 3**, **Figure 3**).

Compared with group A, long-term strength training caused renal nephrin protein expression in groups C and D to decrease, and there was a significant difference ($P<0.01$). However, there was no significant difference between group C and group D, indicating that the renal filtration barrier SD remained in the injured state after training, and that 24 hours of rest time was not sufficient for restoration.

RAS in kidney and circulatory system

The direct effect of exercise on the kidney is to change its blood distribution, and the role of RAS is critical. Intrarenal RAS independently exists, and the abnormal activity of renal local RAS, induced by overtraining resulted in abnormal expression of nephrin. The glomerulus is a clump of capillaries, and the structure and function of SD are also affected by the circulatory system RAS. Long-term intensive training alters the Ra and AngII in the kidney and plasma, which could reflect the effects of exercise stress on RAS excitation.

Long-term exercise has different effects on the activity of the RAS system in the kidney and circulatory system. Compared with group A, the intrarenal Ra and AngII in the rats of group C significantly declined ($P<0.01$), but those of group D were significantly higher than group A ($P<0.01$). RAS in circulatory system, cRa and cAngII were not different between groups A and C, but were higher in group D ($P<0.01$). (**Table 4**, **Figure 4**).

Discussion

This study simulated trainees in competitive sports and military training, that undergo intensive training for a long time. The kidney sus-

Table 4. RAS index of the three groups

RAS		Group A	Group C	Group D
Intrarenal	iRa	0.334±0.024	0.229±0.062**	0.426±0.060**,##
	iAngII	6.442±0.646	5.159±0.485**	7.932±1.103**,##
Circulatory system	cRa	2.832±0.562	2.541±0.460	3.915±0.698**,##
	cAngII	97.475±17.841	85.810±14.281	128.670±19.682**,##

**P<0.01 vs. group A; ##P<0.01 vs. group C. Intrarenal RAS index: iRa (ng/mg/h) and iAngII (pg/mg); RAS in circulatory system: cRa (ng/ml/h) and cAngII (pg/mL).

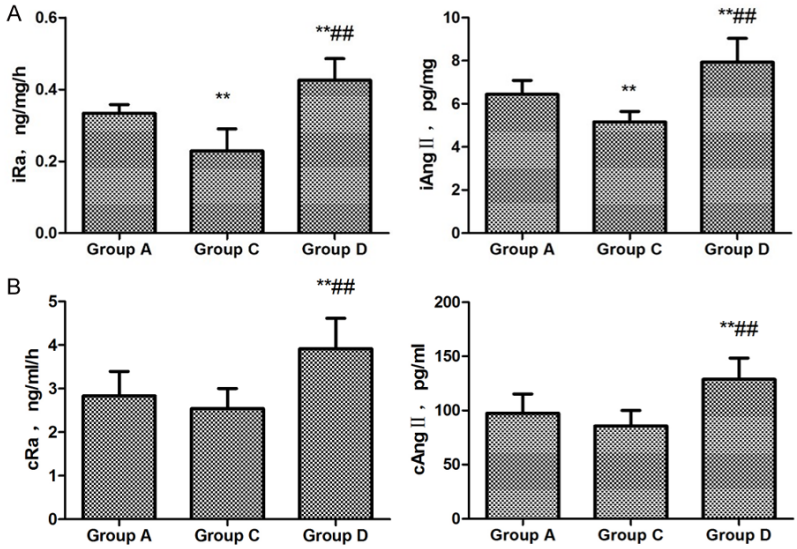

Figure 4. RAS index in kidney (A) and circulatory system (B).

tained injury and could not recover effectively with performance for exercise-induced albuminuria. The sampling time of the urine was fixed at 30 minutes after the training (the most excretion of albuminuria at this time) [5]. The excretion of TP and mALB in the urine of the group of overtraining rats was increased and was higher than in group A ($P<0.01$). Urine TP is the most direct indicator of exercise-induced albuminuria; the exudate of albumin in urine might lead to severe renal injury, and albuminuria could be used as a therapeutic target in clinical medicine [11, 12]. This study collected single urine of rats after training, as the test index of the modeling; mALB/CRE could eliminate the effect of urine volume on the results and was also significantly higher than in group A ($P<0.01$). Therefore, long-term intensive training leads to the decline of renal filtration function and to severe exercise-induced proteinuria.

Excessive exercise stress causes kidney function to decline. Renal tissue would inevitably be accompanied by the corresponding structural changes [13], and six weeks of incremental load training resulted in abnormal morphology of the rat kidney. Compared with group A, the Bowman's capsule and glomerulus of group C were significantly inflated, which was similar to other studies [13]. The damage to the vascular membrane and the space between the glomerulus and the capsule are more serious (similar to the pathological changes of certain diseases) [14]. It is obvious that the long-term excessive exercise caused the buildup of renal structural damage. The renal structure of group D was partly recovered after 24 hours, but it could not return to normal. This study mainly observed abnormalities in the structure of the glomeruli in rats caused by training, therefore expression of nephrin, the main structural protein of SD, could be abnormal.

Nephrin is the first transmembrane protein confirmed on SD and plays a key role in maintaining the integrity of the glomerular filtration barrier and its normal function. Some studies have indicated that abnormal expression of nephrin is closely related to innate or acquired proteinuria [15, 16]. Unlike previous research, this is the first study to associate exercise stress with nephrin in SD, and it was observed that expression of renal nephrin of groups C and D was significantly lower than that of group A ($P<0.01$). The results indicate that long-term intensive training reduces expression of nephrin in the kidney and it could not be recovered after a 24 hour rest. Continuation of the intense training could lead to persistent loss of nephrin in the kidney, which might result in a series of kidney

diseases. Although the research result is shows less that exercise causes a decrease in expression of nephrin and leads to severe kidney disease, the reduction of nephrin could still cause a series of abnormalities from a medical perspective. These include changes of SD composition, the molecular connections between podocyte foot processes could be reduced or interrupted, podocytes could be damaged and lost, or breaking the glomerular filtration barrier could increase its permeability, resulting in proteinuria. In addition, nephrin is an important signaling factor for podocytes, and its reduced expression might cause intracellular and extracellular signal transmission. This could lead to further damage to the structure and function of podocytes, forming a vicious circle [16-18]. Excessive exercise stress could also reduce the expression of nephrin, similar to the pathology research of certain specific diseases, and it provides a new idea for the study of the mechanism of exercise-induced renal injury.

Exercise could stimulate RAS excitement. Increasing RAS activity is one of the reasons for chronic renal disease and podocyte injury [19]. AngII, as the main effector molecule of the RAS, could cause glomerular damage through hemodynamics and other approaches [20]. In diabetic nephropathy and multiple glomerular diseases, using Angiotensin II converting enzyme inhibitors (ACEi) or Angiotensin II receptor antagonist (ARB), could reduce podocyte damage, and stabilize the molecular expression of SD structure such as nephrin; the expression of nephrin is closely related to the excited state of RAS [21]. Therefore, the effects of long-term intensive training on RAS activity is an important cause of abnormal expression of nephrin. The training scheme used in this study resulted in different changes in RAS in the local and circulatory system of rats, as follows: the intrarenal Ra (iRa, $P<0.01$) and AngII (iAngII, $P<0.01$) of rats in group C are evidently lower than that of group A and higher in group D than group A ($P<0.01$); the Ra and AngII in plasma was not statistically different between group A and group C, but both are significantly higher ($P<0.01$). Long-term training of the appropriate intensity could reduce the activity of RAS [22], and acute exercise could make the renal RAS more excited [23]. In contrast, the effect of long-term intensive training on the RAS activity of group C is as follows: the activity of intrarenal RAS decreased, and the RAS in the circulation system was not significantly different from the control group. Therefore, long-term overtraining decreased the activity of RAS in the kidney. Contrary to existing research, long-term training did not cause excessive excitement of RAS but resulted in the downregulation of nephrin, which did not completely negate the relationship between RAS and nephrin. This study hypothesized that exercise stress might cause renal RAS excitement; intrarenal Ra and AngII was immediately released into the blood and played a role in the circulation system after a brief increase, and there was no obvious change in the circulatory system of the RAS under the stimulus of long-term training. Both the intrarenal and circulating RAS of group D in rats showed higher arousal states after 24 hours, and the Ra and AngII are significantly higher than in group A and group C. Overtraining resulted in the intrarenal and circulating RAS remaining in a lasting and excessive excited state. Even after 24 hours of rest, the increase of effector molecule AngII led to the expression of renal Nephrin downregulating continuously, severe abnormalities in the structure of SD, the function of kidney filtration obstacle, and exercise-induced albuminuria further damaging the kidney.

Conclusion

This study showed that prolonged intensive training caused severe exercise-induced albuminuria in rats and abnormalities in renal morphology. Excessive exercise stress led to the continuous downregulation of nephrin in SD, which was not able to return to normal in 24 hours. The mechanism of the decreasing expression of nephrin might be related to the excited state of intrarenal and circulating RAS. However, when the active molecules in the renal RAS secrete into the blood immediately after training, they do not show a higher concentration and activity level. Long-term intensive training induces RAS in the kidney, sustains circulatory system excitement, and downregulates expression of nephrin, making the structure and function of SD abnormal. It also exudes a large number of proteins forming proteinuria that may cause kidney damage if there is no effective recovery.

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Disclosure of conflict of interest

None.

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