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Expression of metallothionein in renal tubules of rats exposed to acute and endurance exercise

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Abstract: The induction of exercise-induced apoptosis in not actively involved in exercise organs, such as kidney could be a result of oxidative stress. Metallothionein (MT) exerts a protective effect in the cell against oxidative stress and apoptosis. We have previously demonstrated an increased incidence of apoptosis in distal tubular cells and collecting ducts in rat kidney after acute exercise. The present study was designed to test the hypothesis that MT may play a protective role in rat renal tubules against exercise-induced apoptosis after the acute exercise and regular training. Male Wistar rats were divided into control, acute exercised and 8-wk regularly trained groups. The kidneys were removed after a rest period of 6 h and 96 h. The ultrastructure of renal tubular cells was examined by electron microscopy. Apoptosis was detected in paraffin sections by the TUNEL technique. Expression of MT was examined by immunohistochemistry. The level of lipid peroxidation (thiobarbituric acid reactive substances - TBARS) was assayed in renal tissue homogenates. After acute exercise, the occurrence of apoptosis was restricted to distal tubules and collecting ducts of rat kidney, whereas the proximal tubules remained unaffected. The 8-wk training did not result in increased apoptosis in tubular cell. MT expression was confined exclusively to proximal tubules in all groups. However, it was significantly increased in acutely exercised animals, as compared to control and trained rats. After the 8-wk training, MT expression remained unaltered as compared to the control group. TBARS levels were significantly increased after acute exercise, while after regular training they remained unchanged. A significant correlation between TBARS level and MT expression was demonstrated. The findings could suggest a protective role of MT against oxidative stress and apoptosis in proximal tubular cells.

Key words: Metallothionein - Kidney - Apoptosis - Exercise - Rat

Introduction

It is widely accepted that physical activity can improve physiological and functional capacity of many organs [11]. However, variety of studies have demonstrated that acute physical exercise can induce a stress response and considerable pathological changes, including apoptosis not only in working skeletal muscles but also in distant organs, such as kidney, liver and intestine [3, 24, 30, 32, 33, 34, 35]. On the other hand a chronic, moderate training is known to develop in many tissues adaptive structural and biochemical mechanisms protecting against exercise-induced damage [21, 31, 44].

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Although the mechanisms leading to induction of apoptosis in distant organs during or after bouts of exercise are not fully understood, they may include such factors as formation of reactive oxygen species (ROS). An increased production of ROS in many tissues might be a result of considerable increase in oxygen utilization during exercise and/or ischemia-reperfusion phenomenon which could occur especially in not working organs, such as liver, kidney and resting muscles. During intense exercise, such organs undergo partial ischemia because of reduced blood supply, followed by tissue reoxygenation, which mimics the ischemia-reperfusion phenomenon, known to cause excessive production of ROS [7, 10]. An increased concentration of ROS could be detrimental to tissue as a result of direct oxidation of cellular components, such as lipids, proteins or DNA or due to changed the redox-sensitive signaling pathways, which

could result in apoptotic death of various cell types [16]. Several defence mechanisms exist to decrease the concentration of ROS, including both antioxidant enzymes, like superoxide dismutases (SOD), glutathione peroxidase (GPx) and catalase (CAT) and non-enzymatic agents, such as metallothionein (MT), reduced glutathione (GSH), vitamin C and vitamin E.

Metallothionein (MT) is an intracellular, low-molecular-weight, cysteine-rich protein, expressed ubiquitously in most mammalian tissues. The main biological function of MT is cytoprotection of organism against heavy metal and free radical induced damage [27]. MT has the capacity to bind and detoxify heavy metal cations and, moreover, it plays an important role in free radical scavenging and thus protects cells against cytotoxic and DNA-damaging effects [16]. Many studies have shown that MT overexpression increases resistance of tissue and cells to oxidative stress [17, 42]. Most recently, the anti-apoptotic function of MT became evident [37], involving not only ROS scavenging and inhibition of ROS formation but also suppression of mitochondrial cytochrome c release [20, 43]. In many studies it has been demonstrated that the enhanced production of free radicals, evoked during exercise, may trigger an increase in the MT level, facilitating effective scavenging of the reactive oxygen species generated during exercise [16, 29].

We have previously demonstrated that acute exercise results in apoptosis of distal tubular cells and collecting ducts of rat kidney and that this process is most likely induced by activation of angiotensin II (Ang II) receptors AT1 and AT2, which are expressed in the same cells in which the apoptotic process occurs [31, 32, 33]. Moreover, we have shown increased levels of lipid peroxidation in rat kidney after the acute exercise, while the antioxidant enzyme activities remained unchanged. However, we have never observed apoptosis in the proximal tubules which are extremely susceptible to ischemic injury.

The aim of this study was to examine if MT could protect rat renal tubular cells against exercise-induced apoptosis after acute exercise and regular, 8-wk training.

Materials and methods

Animals. The study was approved by the Animal Care Ethical Committee of the Clinical Research of the Wroclaw Medical University and was performed according to the guidelines of the Polish Animal Care and Use Committee. Fifty male Wistar rats, 10-12 weeks of age (200-250 g body weight), were obtained from the Department of Pathological Anatomy, Wroclaw Medical University. Rats were randomly divided into three groups (acute exercised N=20; trained N=20; control N=10).

Acute and endurance exercise. Animals from the exercising group were subjected to running on a treadmill, at the speed of 1.0 $km \cdot h^{-1}$, until exhaustion. The mean time to exhaustion was 90 min (range: 60 to 115 min). After the exercise, the animals returned to their cages and were killed 6 h (N=10) or 96 h (N=10) after cessation of the exercise.

Table 1. Evaluation of MT expression by using the semiquantitative IRS scale (0-12 points), according to Remmele and Stegner [36]. The score takes into account the intensity of the color reaction and the percentage of positive cells ($\Sigma = A \times B$).

A - percentage of positive cells	B - intensity of the color reaction
0: no cells with positive reaction	0: no color reaction
1: to 10% cells with positive reaction	1: low intensity of color reaction
2: 11 to 50% cells with positive reaction	2: average intensity of color reaction
3: 51 to 80% cells with positive reaction	3: intense color reaction
4: > 80% cells with positive reaction	

Animals from the adaptive training (endurance exercise) group were trained by running on a treadmill 5 days weekly for 8 wks, and the speed of the treadmill and duration of the training sessions were gradually increased from 0.6 $km \cdot h^{-1}$ for 10 min to 1.68 $km \cdot h^{-1}$ for 65 min by the end of the 8th week. After the last training session, the animals returned to their cages and were sacrificed 6 h (N=10) or 96 h (N=10) after cessation of the training.

Control animals (N=10) remained in their cages throughout the experiment.

Tissue preparation. All animals were anaesthetized and decapitated. Two kidneys of each rat were excised. The right kidney was fixed in 4% buffered formaldehyde solution for 24 hrs and embedded in paraffin. The left kidney was divided into two parts: one half was fixed according to Karnovsky and prepared for electron microscopy, the other half was frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Apoptosis assay. In paraffin sections apoptosis was detected by the TUNEL technique, using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Intergen, Norcross, USA).

Immunohistochemistry. The immunohistochemical reactions were performed in paraffin sections. Expression of metallothionein was demonstrated using mouse monoclonal antibodies against MT-1 and MT-2 (Clone E9; diluted: 1:100, Dako, Denmark). The reactions were accompanied by negative controls in which specific antibodies were substituted by the Primary Negative Control reagent (Dako, Denmark). The investigated antigens were visualised using biotinylated antibodies, streptavidin-biotinylated peroxidase and diaminobenzidine (LSAB2 kit and DAB, Dako, Denmark). MT expression was evaluated using the semiquantitative IRS scale, according to Remmele and Stegner [36] (0-12 points), which took into account the intensity of the color reaction and the percentage of positive cells (Table 1).

Biochemical analysis. Lipid peroxidation was evaluated by thiobarbituric acid color reaction (TBA-test), as previously described by Morel [26]. Tissues were homogenized in 1 ml of 50 mM Tris-HCl (pH 7.4). Trichloroacetic acid (1 ml) was added to the homogenate to stop the lipid peroxidation reaction. After centrifugation, 1 ml of 0.67% thiobarbituric acid was added to the supernatant, followed by 15-min incubation in a boiling water bath. The color produced was read at 532 nm by spectrophotometry, and lipid peroxidation activity was expressed in nmoles TBARS produced/mg protein in tissue. Tissue protein was estimated according to Lowry [25], employing the respective Protein Assay Kit (Sigma Diagnostics, Poznan, Poland).

Statistical analysis. The results were subjected to statistical analysis using Statistica 5.1 PL software (StatSoft, Cracow, Poland). Mann-Whitney, F-Cox, Chi-square, and Spearman's correlation tests were performed. The differences were considered significant at $p<0.05$.

Results

Apoptosis

The acute exercise resulted in a significant increase in the number of apoptotic cell nuclei in renal tubular cells in comparison to the control animals (data not shown). Apoptotic cell nuclei were present only in the distal convoluted tubules and collecting ducts at 6 h and 96 h in all exercised animals, while they were never observed in proximal convoluted tubules (Fig. 1). After the 8-wk training, incidence of apoptosis did not differ from that observed in the control, sedentary group of animals.

Electron microscopy was employed to examine the ultrastructure of renal tubular cells. In the distal convoluted tubules and in the collecting ducts of the kidney in each group of animals subjected to acute exercise, typical shrunken apoptotic cells with condensed chromatin were present (Fig. 2A). The proximal tubular cells remained unchanged (Fig. 2B).

Expression of metallothionein (MT) in the renal tubular cells

In all studied groups of rats: control, acutely exercised and 8-wk trained, MT immnunostaining was confined to the proximal tubular cells (Fig. 3A,B). Other nephron segments were negative. MT immnunoreactivity was present in the cytoplasm and cell nuclei of proximal tubular cells. Acute exercise resulted in a significantly increased expression of MT in renal proximal tubular cells (Fig. 3A), as compared to the control, as well as to the 8-wk trained group (Fig. 3B). There were no differences in expression of MT in kidneys removed 6 h or 96 h after the acute exercise. After the 8-wk adaptive training, MT expression remained unaltered as compared to the control group. There were no differences in expression of MT in kidneys removed from trained rats 6 h or 96 h after the last bout of exercise (Fig. 4).

Lipid peroxidation

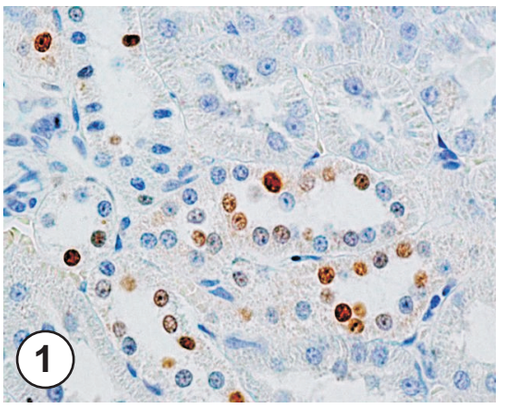
Renal TBARS levels were markedly increased in the acutely exercised animals as compared to the sedentary group. No significant differences were detected in TBARS levels between 6th h and 96th h after the acute exercise. In 8-wk trained group, at 6 h as well as 96 h after the last bout of the exercise no significant changes in the TBARS levels were found, as compared to the control animals. After the adaptive training, the renal TBARS levels were significantly lower than after acute exercise (data not shown).

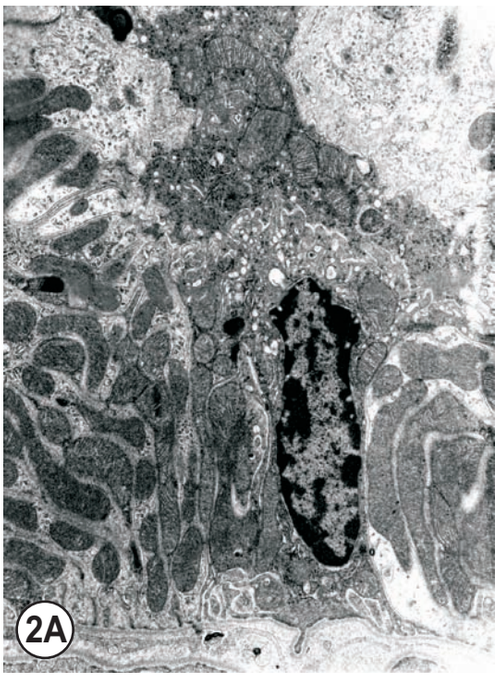
A significant correlation was demonstrated ($r = 0.65$; $p<0.05$) between expression of MT and TBARS peroxidation levels (Fig. 5) . The correlation pertained to both time points after exercise (6 h and 96 h after acute exercise and following 8-wk training).

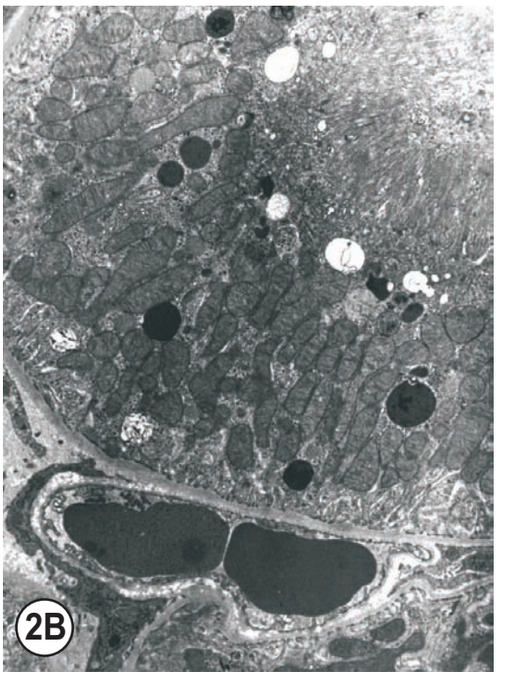
Discussion

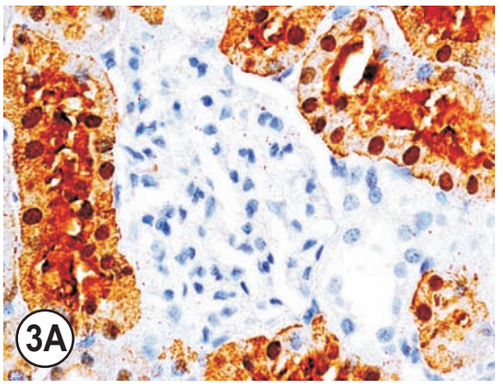
Results obtained in this study confirm our previous reports on the increased incidence of apoptosis in renal distal tubular cells and collecting ducts but not in the proximal tubular cells in response to acute exercise, while after the 8-wk training there were no alterations in the frequency of apoptosis in tubular cells, as compared to sedentary animals [32, 33]. The induction of apoptosis in kidney after physical effort could be associated with oxidative stress and action of Ang II receptors [8, 31, 32]. The exhausting exercise is known to induce an increased free radical generation as a result of increased oxygen consumption rate, especially in working skeletal muscles, but also as a result of ischemia-reperfusion phenomenon, which can occur especially in not working organs, such as kidney [5, 10, 13]. Regardless of the way of their generation, the enhanced production of free radicals evoked during exercise is known to trigger an increase in the enzymatic and non-enzymatic antioxidant system activities [14, 30]. We and the others have shown that acute physical exercise could result in increased lipid peroxidation levels but the antioxidant enzyme activities remain unchanged. On the other hand, the regular physical training has little effect on lipid peroxidation levels and antioxidant enzyme system of not working organs, such as the kidney and the liver [2, 6, 24, 32, 39]. Many studies have demonstrated that MT, one of the non-enzymatic components of the cellular antioxidant system, could have protecting effect against oxidative stress in various tissues [17, 42]. The free radical scavenging capacity of MT was demonstrated in renal as well as in cardiac ischemia/reperfusion injuries, in which oxidative stress is believed to play a major role [18, 19, 40, 43]. Moreover, MT significantly suppressed the cardio- and nephrotoxicity of various anticancer drugs, such as cisplatinum and doxorubicin, the metabolism of which is known to induce ROS generation [4, 12, 22].

In the present experiment, we have demonstrated for the first time an increased expression of MT in proximal tubular cells of the rat kidney at 6 h as well as 96 h after an acute physical effort, while the 8-wk training did not change the MT expression, compared to sedentary animals. MT was expressed in the cytoplasm as well as in the cell nuclei in proximal convoluted tubules, whereas the distal convoluted tubules or collecting ducts displayed no expression of MT. Similarly, overexpression of MT in the proximal tubular cells was demonstrated *in vitro* and *in vivo* studies in response to many stimuli









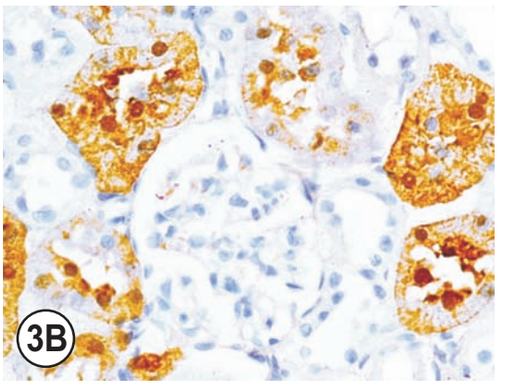


Fig. 1. Apoptotic nuclei (brown) in distal convoluted tubular cells and collecting ducts in kidney of acutely exercised rats, 6 h after the exercise. Cortex of kidney, counterstained with hematoxylin. × 400. **Fig. 2. A:** Electron micrograph of shrunken apoptotic cell, with typical condensed chromatin in distal convoluted tubule. Kidney of rat after acute exercise. × 10 000. **B:** Electron micrograph of normal proximal convoluted tubular cells with prominent microvilli in kidney of rat after acute exercise. × 7000. **Fig. 3. A:** Intense expression of MT in renal proximal tubular cells after acute exercise. **B:** Moderate expression of MT in renal proximal tubular cells after 8-wk training. Cortex of kidney, counterstained with hematoxylin. A, B × 400.

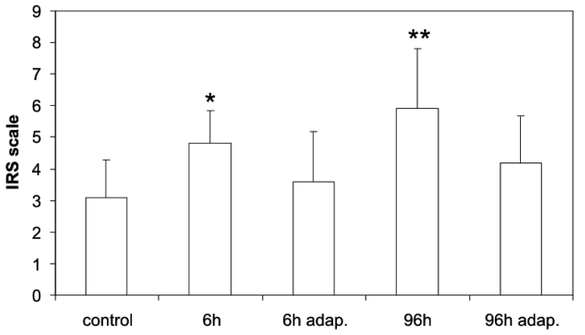


Fig. 4. Intensity of MT expression in proximal tubular cells of rat kidney 6 h and 96 h after acute exercise and adaptive, 8-wk training. *p<0.05 (6 h vs. control and 6 h adaptive training); **p<0.05 (96 h vs. control and 96 h adaptive training).

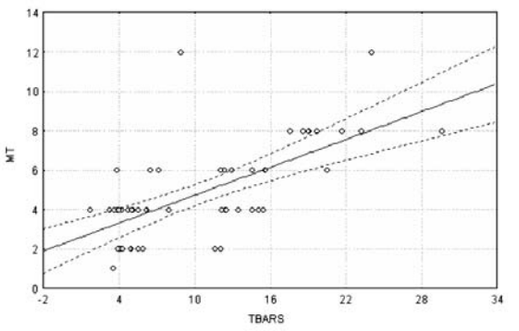


Fig. 5. Correlation between expression of MT and lipid peroxidation level (TBARS) in rat kidney in all studied groups; r=0.65, p<0.05.

known to induce the oxidative stress [1, 40]. In agreement with these findings, the results obtained in the present study strongly suggest that MT could play an important role in protection of renal proximal tubular cells against oxidative stress, known to occur also after the acute physical exercise. Recently, many studies provided supporting evidence for increased levels of MT after acute exercise in skeletal muscles and liver but not in the kidney [15, 23, 29, 38]. However, in these studies the content of MT was estimated in the total kidney homogenate, whereas we have evaluated MT expression immunohistochemically in kidney sections. In the present study, the increased expression of MT was observed exclusively in the renal proximal tubular cells. A significant correlation between the overexpression of MT and the levels of TBARS reflecting lipid peroxidation in response to acute exercise, could suggest a protective role of MT against oxidative stress in proximal convoluted tubules, which are known to be major targets for many toxic agents, like metals and free radicals. Moreover, MT is believed to be associated with resistance of the cells to apoptosis not only by scavenging the apoptosis-inducing free radicals but also by suppressing mitochondrial cytochrome c release [9, 20, 43]. This could, at least partially, elucidate the occurrence of apoptosis after the acute exercise exclusively in the distal tubular cells and collecting ducts, in which MT is not expressed and, therefore, the cells are not protected from apoptosis. The other supposed mechanism of a particular protection of the proximal tubules against apoptosis was proposed earlier by Oberbauer *et al.* [28] who suggested that up-regulation of antiapoptotic protein Bcl-2 in the proximal tubules and suppression of Bcl-2 in the distal tubules in ischemia/reperfusion injury of the kidney could provide the protection of the proximal tubules.

In conclusion, we suppose that apoptosis in renal distal tubular cells and collecting ducts in response to acute physical effort could result from oxidative stress as well as action of Ang II receptors. The increased expression of MT after acute exercise, restricted in turn to the proximal tubular cells, could represent the protective mechanism against oxidative stress and apoptosis in these cells, which are extremely susceptible to ischemic injury whereas they have a vital function in renal reabsorption processes. Regular training seems to prevent occurrence of apoptosis in renal distal tubules and has no effect on MT expression in the proximal tubular cells. Additional studies are needed to elucidate the role of MT in renal tubular cells.

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