

## Exercise-induced apoptosis in rat kidney is mediated by both angiotensin II AT1 and AT2 receptors

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**Summary.** Excessive physical exercise may lead to disturbance of the entire homeostasis in the body, including damage not only in skeletal muscles but also in many distant organs. The mechanisms responsible for the exercise-induced changes could include oxidative stress or angiotensin II. We previously showed that acute exercise led to apoptosis in kidney but not as a result of oxidative stress. In this study, we examined the role of angiotensin II and its AT1 and AT2 receptors in mediation of exercise-induced apoptosis in kidney. We clearly demonstrated that acute physical exercise induced apoptosis in renal cells of distal convoluted tubuli and cortical and medullary collecting ducts. Moreover, the cells displayed an increased expression of both AT1 and AT2 angiotensin II receptors and of p53 protein. The results suggest that angiotensin II could upregulate p53 expression in renal distal convoluted tubular cells and in the cells collecting ducts via both AT1 and AT2 receptors, which might be the crucial apoptosis-mediating mechanism in kidneys after excessive exercise.

**Key words:** Apoptosis, Exercise, Angiotensin II, AT1, AT2, Kidney

### Introduction

It is well known that excessive physical exercise may lead to disturbance of the entire body homeostasis, including damage not only in working skeletal muscles but also in many distant organs (Carraro and Franceschi, 1997; Podhorska-Okolow et al., 1998). The mechanisms responsible for the exercise-induced changes could

include oxidative stress or generation of angiotensin II (Liu et al., 2000; Bhaskaran et al., 2003). Intense exercise is often accompanied by an excessive generation of oxygen free radicals. Reactive oxygen intermediates react with cellular components, which may cause extensive damage to cellular structures such as DNA. The DNA damage could finally lead to apoptotic or necrotic processes in many organs, including skeletal muscles, heart, cells of the immune system, lungs and liver (Azenabor and Hoffman-Goetz, 1999; Liu et al., 2000). Oxidative stress is widely known to activate the apoptotic process (Phaneuf and Leeuwenburgh, 2000). However, evidence for tissue oxidative stress and damage due to exercise still remains incomplete. In our previous work we demonstrated that apoptotic changes in kidney observed 6 and 96 hr after exercise were not associated with oxidative stress (Podhorska-Okolow et al., 2004b).

Intense training activates the adrenergic system which is responsible for arterial contraction and blood redistribution, such as decrease of blood flow in kidneys, liver, intestine and non-working skeletal muscles. Moreover, the adrenergic system activation markedly increases the release of renin from renal juxtaglomerular apparatus (Di Bona, 2001). In response to a decreased glomerular filtration and sodium depletion, activation of renin-angiotensin-aldosterone (RAA) system develops. Angiotensin II, an active end product of the system has a variety of physiological actions, including proliferation and apoptosis (Bonnet et al., 2001). Angiotensin II acts through its receptor subtypes, type 1 (AT1) and type 2 (AT2) which involve different molecular mechanisms and lead to different effects (Kaschina and Unger, 2003). The role of both receptors in mediation of the processes remains controversial (Bonnet et al., 2001). It was initially assumed that angiotensin II stimulates cell growth and proliferation via AT1 receptor while apoptosis via AT2 receptor but recently it has been suggested that both AT1 and AT2 receptors are involved

in parallel in the two processes (Dimmeler et al., 1997; Cao et al., 2000).

Apoptosis is a form of a genetically controlled cell death, characterized by specific morphological, biochemical and molecular events (Steller, 1995). This process is essential for many physiologic functions, such as maintenance of tissue homeostasis and deletion of damaged or potentially dangerous cells. In the adult kidney low levels of apoptosis are normally observed but, as has been shown recently, the number of apoptotic cells may increase as a result of disease or injury (Hammerman, 1998). Many authors have demonstrated that apoptosis contributes to the loss of tubular cells and that the loss can be induced by many diverse stimuli (Bonegio and Lieberthal, 2002; Sekhon et al., 2003). Renal tubular cells die by apoptosis as well as by necrosis in experimental models of ischemic and toxic acute renal failure (Bonegio and Lieberthal, 2002). One of the postulated mechanisms of apoptosis induction in the kidney is the activation of angiotensin II which promotes upregulation of p53 and other pro-apoptotic proteins (Pierzchalski et al., 1997; Bonnet et al., 2001).

Our previous observations revealed after excessive exercise the presence of apoptotic changes not only in skeletal muscles but also in kidneys (Podhorska-Okolow et al., 2004a). Moreover, we demonstrated that induction of apoptosis in rat renal tubular cells did not seem to result from oxidative stress but rather could be associated with the stimulation of angiotensin II receptors.

Since recent studies underline the important role of angiotensin II in the progression of renal injury, including the ischemia/reperfusion mechanism of acute renal failure, the present investigations have been designed to examine the localization of apoptotic process occurring in kidney after physical exercise. Moreover, because of the limited and controversial information regarding the role of both AT1 and AT2 receptors in mediating the apoptosis in rat kidneys we have studied the possible role of activation of angiotensin AT1 and AT2 receptors in induction of apoptosis of renal tubular cells in response to intense physical exercise.

## **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. Forty 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 formed groups of exercised (n=30) and control animals (n=10). Animals from the exercised group were subjected to running on the treadmill at 1.0 km/h until exhaustion. The mean time to exhaustion was

90 min (range: 60-115 min). After the exercise, animals returned to their cages and were randomly grouped into animals killed 2 hrs (n=10), 6 hrs (n=10), or 96 hrs (n=10) after cessation of the exercise. Control animals (n=10) remained in their cages throughout the experiment. All animals were anaesthetized and decapitated. The 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.

### *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). Percentage of apoptotic nuclei was evaluated by scoring the brownish-labelled cell nuclei (positive cells) detected after screening of all tubular cell nuclei under x400 magnification (Olympus BX 41 light microscope with the visual mode AnalySis 3.2 software for computer-assisted image analysis). For each kidney ten transversal sections were done (five for cortex and five for medulla). In each section, the evaluation was performed in five representative fields. Cortex and medulla of each kidney were appraised separately.

### *Immunohistochemistry analysis*

All the immunocytochemical reactions were performed in paraffin sections. Expression of both AT1 and AT2 receptor was demonstrated using rabbit polyclonal antibodies (sc-1173 and sc-7420, respectively, diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Expression of p53 protein was demonstrated using mouse monoclonal antibodies (Clone DO-7; diluted: 1:50, DakoCytomation, Denmark). All the reactions were accompanied by negative controls in which specific antibodies were substituted by the Primary Negative Control reagent (DakoCytomation, Denmark). The investigated antigens were visualised using biotinylated antibodies and streptavidin-biotinylated peroxidase and diaminobenzidine (LSAB2 kit and DAB, DakoCytomation, Denmark).

### *Western blotting analysis*

AT1 as well as AT2 receptor protein expression was assessed by Western blot. The block of the frozen one-half left kidney from each rat was homogenized in a Tris-EDTA buffer. The protein concentration was determined using a BCA protein assay kit (Sigma, Germany). Proteins were separated by SDS-PAGE and were electrotransferred to the supporting nitrocellulose. Both AT1 and AT2 receptor protein bands were detected

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using primary antibodies (1:500 dilutions) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). In order to develop Western blots we used the chromogenic substrate, BCIP/NBT. Quantitative densitometry was performed on the identified bands by using computer-based measurements system Scion Image for Windows version Beta 4.02 (by 2000 Scion Corporation, USA).

### Statistical analysis

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

### Results

#### *Effect of intense exercise on apoptosis of renal tubular cells*

Apoptosis was detected in the paraffin sections by the TUNEL method. Apoptotic cell nuclei were present only in the distal convoluted tubular cells and in cells of the collecting ducts in the renal cortex and medulla of all exercised animals (Fig. 1A). Apoptotic cell nuclei were never observed in proximal convoluted tubuli.

Electron microscopy was employed to examine the ultrastructure of renal tubular cells. The presence of cells with typical apoptotic features was noted, including cell shrinkage, chromatin condensation and apoptotic bodies. The changes were detected only in the distal convoluted tubuli and in the collecting ducts of the cortex and medulla in kidneys of animals subjected to exercise. Moreover, the electron microscopy observations documented shedding of apoptotic bodies directly into the lumen of the renal tubule (Fig. 2).

Percentage of apoptotic nuclei was evaluated by scoring the number of brownish-labelled cell nuclei in the paraffin sections using the TUNEL technique. Apoptotic nuclei were present in both parts of the kidney, in cortex (Fig. 3A) and medulla (Fig. 3B). The intense exercise in all exercised animals resulted in a significant increase in the number of apoptotic cell nuclei in renal tubular cells in comparison to the control animals ( $p < 0.05$ ). There was no difference in appearance of apoptosis between the three exercised groups, i. e., 2, 6, or 96 hr after running (Fig. 3A and 3B). The significant correlation was demonstrated ( $r = 0.79$ ;  $p < 0.05$ ) between the number of apoptotic nuclei in the cortex and that in the medulla (Fig. 4).

#### *Effect of intense exercise on expression of angiotensin II AT1 and AT2 receptors*

The role of angiotensin II AT1 and AT2 receptors in mediation of apoptotic changes in renal tubular cells after excessive exercise was documented using the immunohistochemical method, performed in paraffin

sections. In all exercised groups the distal convoluted tubules and collecting ducts displayed a strong expression of both angiotensin AT1 and AT2 receptors 2, 6 and 96 hrs after cessation of the exercise (Fig. 1C, D). The expression of AT1 and AT2 receptors was not observed or very weak in proximal convoluted tubules. In sedentary animals (control group) there was very weak expression of AT1 receptor observed in proximal and distal convoluted tubular cells, as well as in glomeruli (Fig. 1E) while expression of AT2 receptor was not observed of any segments of nephron but only in blood vessels (Fig. 1F).

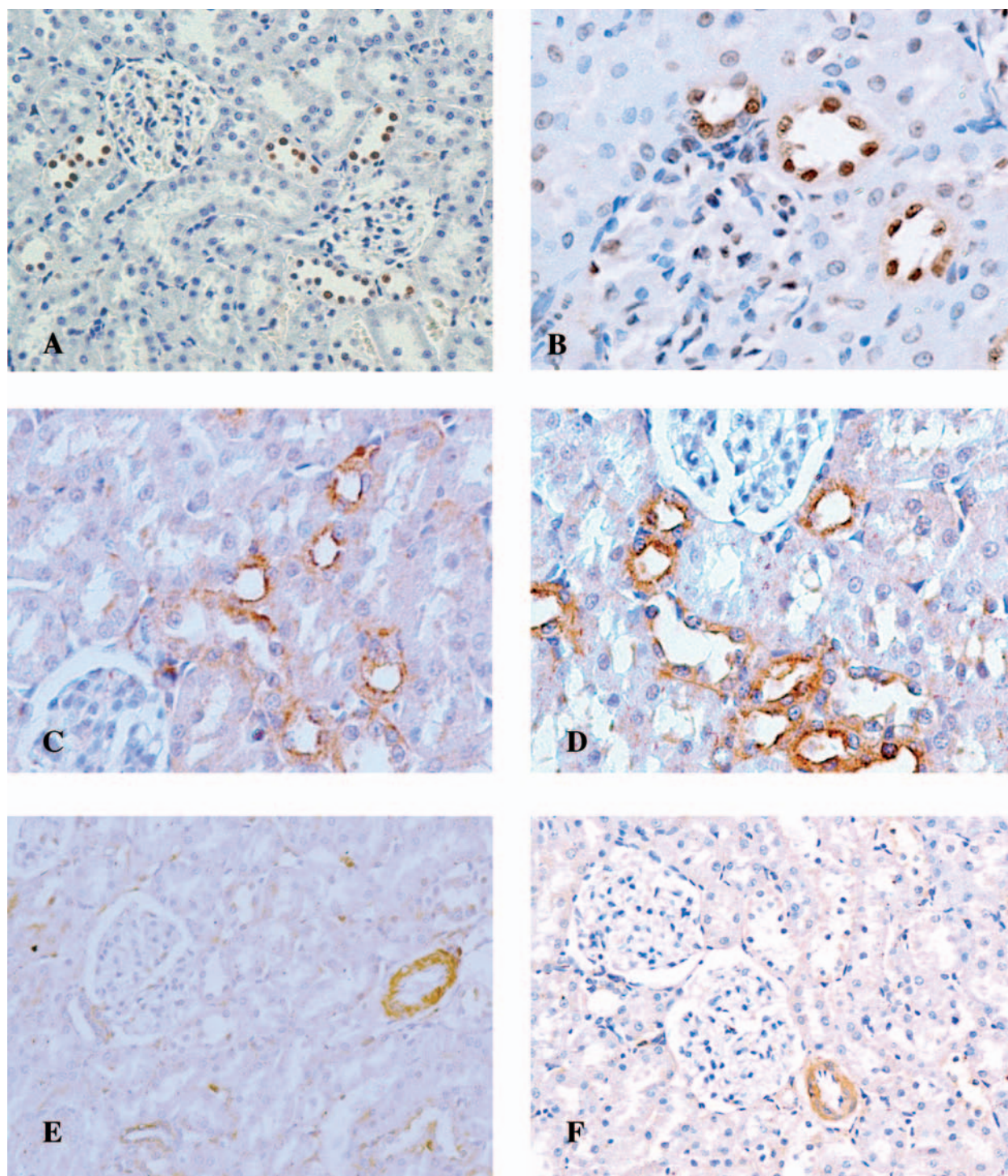
In order to confirm the overexpression of both AT1 and AT2 receptors, Western blot analysis was employed. It revealed an increased expression of AT1 and AT2 receptor proteins ( $p < 0.05$ ) compared with control in kidneys of all exercised animals (Fig. 5B, D, respectively). Representative gels showing the effect of exercise on AT1 and AT2 expression are shown in Fig. 5A and 5C, respectively.

#### *Effect of intense exercise on p53 expression*

In order to examine the supposed role of p53 in exercise-induced apoptosis, p53 protein expression was examined using the immunohistochemical method, performed in the paraffin sections. Cell nuclei of renal distal convoluted tubular cells of exercised animals displayed a strong expression of p53 protein, while sedentary animals showed very weak or no expression of p53 (Fig. 1B).

### Discussion

Excessive physical exercise disturbs homeostasis in the body and leads to haemodynamic, metabolic and structural alterations, including apoptosis or necrosis not only in skeletal muscles but also in many distant organs (Podhorska-Okolow et al., 1998, 2004b; Azenabor and Hoffman-Goetz, 1999; Liu et al., 2000; Phaneuf and Leeuwenburgh, 2000). In our experiment the acute exercise resulted in a significant increase in the number of apoptotic cell nuclei in kidneys 2, 6 and 96 hrs after cessation of running, in comparison to sedentary animals. Under normal conditions, regional differences in blood flow are observed in the kidney. The outer-medullary region is marginally oxygenated and segments of tubuli which traverse this region are more sensitive to ischaemic conditions (Vetterlein et al., 1994). Nevertheless, in our experiment we noticed no differences in the number of apoptotic cells between cortex and medulla of the kidneys. Moreover, a significant correlation has been noted in occurrence of apoptotic cells in renal cortex and in medulla after the intensive exercise. Apoptosis was present only in the distal convoluted tubules and in the cortical and medullary collecting ducts of all exercised animals. We have never observed the presence of apoptotic cell nuclei in proximal tubular cells. Likewise, Oberbauer et al.

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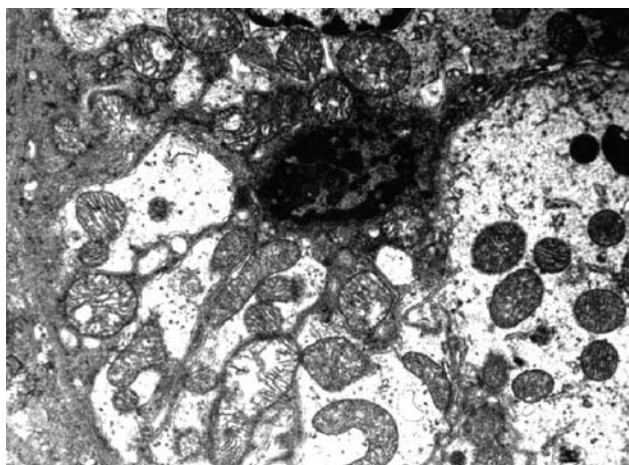
**Fig. 1.** TUNEL and immunohistochemistry showed that physical exercise increased the number of apoptotic nuclei as well as expression of angiotensin II AT1 receptor, AT2 receptor and p53 protein in renal tubular cells. TUNEL technique detected apoptotic cell nuclei (brown cell nuclei) in distal convoluted tubular cells of running animals (6h) (**A**). Immunohistochemistry demonstrated expression of p53 protein in nuclei of distal convoluted tubular cells of running animals (6h) (**B**). Immunohistochemical reaction of expression of AT1 (**C**) and AT2 (**D**) receptor in renal distal convoluted tubular cells and collecting ducts of running animals (6h). Immunohistochemical reaction of expression of AT1 (**E**) and AT2 (**F**) receptor demonstrated a strong staining in vasculature and weak or no reaction in proximal and distal tubular cells of non-running rats (control group). Cortex of kidney counterstained with hematoxylin. A, E, F, x 100; B-D, x 200

(2001) demonstrated that after ischaemic injury in kidneys apoptotic cells were prominent in the distal tubules. The authors suggested that apoptosis in kidneys was regulated at least in part by differential expression of bcl-2 family member genes in distal convoluted tubules, which displayed a strong expression of pro-apoptotic bax protein and suppression of anti-apoptotic bcl-2 protein. In contrast, the proximal tubular epithelial cells displayed up-regulation of bcl-2 protein. This could be one of the protective mechanisms of renal proximal tubular cells which have a vital function for reabsorption of glomerular filtrate. Moreover, the proximal tubules are extremely susceptible to ischaemic injury due to their low glycolytic capacity to generate ATP under ischaemic conditions (Bonventre, 1993). Thus, the acute, severe ATP depletion leads rather to necrotic than to apoptotic cell death in the cells. In contrast, the distal tubules have greater glycolytic capacity to generate ATP under ischaemic conditions, which makes them less vulnerable to necrotic injury and which promotes their eventual apoptotic cell death (Padanilam, 2003). On the other hand, the *in vivo* study of Cao et al. (2000) showed the appearance of apoptosis in rat proximal tubular cells in response to chronic angiotensin II infusion. Similarly Bhaskaran et al. (2003) demonstrated that in response to infusion of angiotensin II the proximal renal cells underwent apoptosis *in vitro*. Unfortunately, no comparative studies have been performed on renal distal tubular cells *in vitro*.

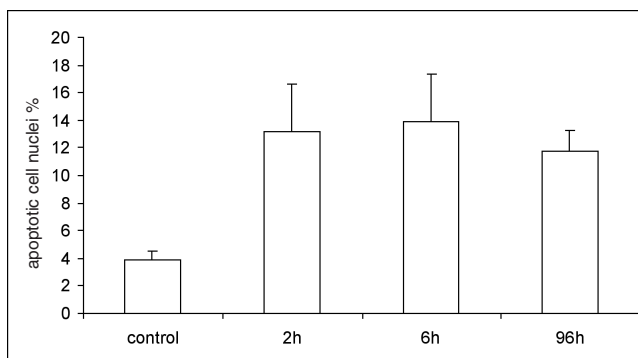
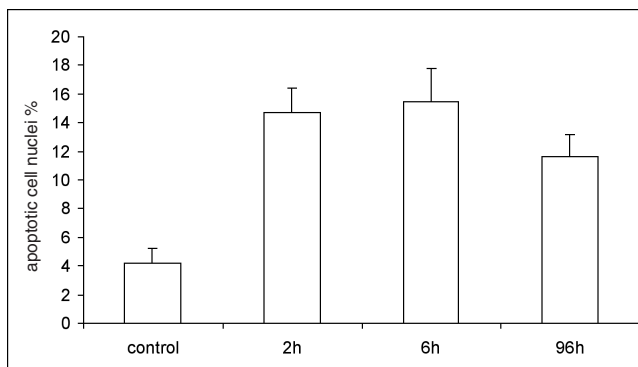
One of the proposed mechanisms, responsible for damage in many tissues after a physical effort, involves an oxidative stress due to manifold augmented oxygen utilization (Liu et al., 2000). The intense exercise is accompanied by the generation of oxygen free radicals, which react with cellular macromolecules and may cause extensive damage to many cellular structures, including DNA during the exercise and even several days later

(Arslan et al., 2001). Oxidative stress is a well known inducer of the apoptotic process (Phaneuf and Leeuwenburgh, 2000). However, in our previous work the induction of apoptosis in renal tubular cells after excessive exercise has not seemed to be associated with the oxidative stress (Podhorska-Okolow et al., 2004b).

Many recent studies underline the role of angiotensin II in the progression of renal injury (Weidekamm et al., 2002; Padanilam, 2003). It is widely known that during intense physical exercise takes place activation of the adrenergic system, which is responsible for decreased glomerular filtration and finally, for the activation of the renin-angiotensin system (Di Bona, 2001). Moreover, there are many tissues, including vascular endothelial and smooth muscle cells, heart, kidney, which have their own local renin-angiotensin (RAS) system capable of producing angiotensin II in response to metabolic changes occurring after physical exercise (Di Bona, 2001). The kidney is a major target organ of angiotensin II, which exerts its regulatory function on both haemodynamic and tubular functions. It has been demonstrated that angiotensin II could also importantly contribute to the pathology via its direct effect on tubular cell growth and proliferation (Kajstura



**Fig. 2.** Electron micrograph of apoptotic tubular cell inside the lumen of collecting duct of outer medulla. x 7,500

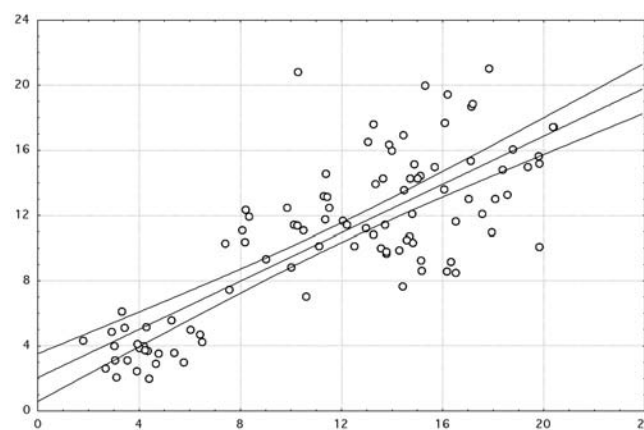


**Fig. 3.** Percentage of apoptotic cell nuclei in tubuli of renal cortex (A) and medulla (B). Control: resting animals. 2h, 6h, 96h groups of exercised animals were killed 2, 6 and 96 hr after physical exercise, respectively. Significant differences: control as compared to 2h, 6h and 96h groups (\*:  $p < 0.05$ ).

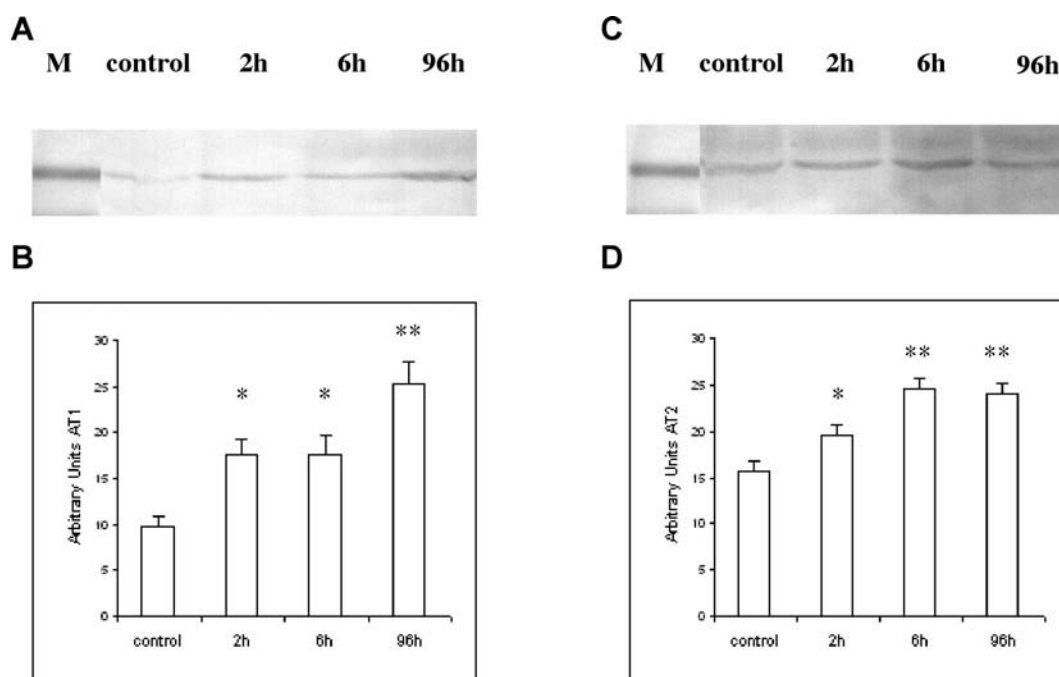
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et al., 1997). Angiotensin II acts through its receptor subtypes, type 1 (AT1) and type 2 (AT2) which involve different molecular mechanisms and lead to different effects (Siragy, 2004). It has been postulated that AT1 and AT2 have opposing actions on proliferation and apoptosis. The proliferative properties of angiotensin II are considered to be associated with the AT1 receptor, whereas the AT2 receptor is viewed to promote apoptosis (Cao et al., 2000). In contrast, many recent studies have demonstrated that AT1 receptor may also activate apoptosis in cultured myocytes or in endothelial cells (Li et al., 1999; Leri et al., 2000). The present study with the use of immunohistochemical techniques, confirmed by Western blot, has demonstrated increased expression of both AT1 and AT2 receptors in distal convoluted renal tubular cells and collecting ducts of all exercised animals. Moreover, in our study correlation has been observed between the occurrence of apoptosis and the increased expression of AT1 and AT2 receptors only in those cells. These results are consistent with our previous preliminary findings, in which it has been demonstrated that the induction of apoptosis in renal tubular cells has been associated with the action of angiotensin II rather than with oxidative stress (Podhorska-Okolow et al., 2004a). Likewise Cao et al. (2000) suggested that proliferative and apoptotic effects of angiotensin II are most likely mediated by both AT1 and AT2 receptors. However, the role of the two angiotensin II receptors still remains controversial. Most of the known physiological and pathologic effects of angiotensin II are mediated through stimulation of the AT1 receptor (Siragy, 2004). Recently, studies have

appeared on involvement of the AT2 receptor in physiological and pathologic processes in adult kidney (Cao et al., 2002). The AT2 receptor has been shown to be expressed mainly in the foetal tissues, which might imply its role in organogenesis (Grady et al., 1991; Mifune et al., 2001). Therefore, its transient reappearance under pathological conditions in the adult organism (for instance after myocardial infarction) could be associated with cell differentiation and regeneration processes (Unger et al., 1996). However, in recent



**Fig. 4.** Correlation between percentages of apoptotic cell nuclei in tubuli of renal cortex and medulla ( $r=0.79$ ;  $p<0.05$ ). The data for correlation was gathered data of all time points after exercise (2, 6 and 96h).



**Fig. 5.** Effect of exercise on AT1 and AT2 expression.

**A.** representative gel showing the effect of exercise on AT1 expression.

**B.** cumulative data showing the effect of exercise on AT1 expression. \*:  $p<0.05$  compared with control. \*\*:  $p<0.05$  compared with control and 2h and 6h after exercise.

**C.** representative gel showing the effect of exercise on AT2 expression.

**D.** cumulative data showing the effect of exercise on AT2 expression. \*:  $p<0.05$  compared with control. \*\*:  $p<0.05$  compared with control and 2h after exercise.

studies the presence of AT2 receptor has been reported in the adult rat kidney. Ozono et al. (1997) demonstrated expression of AT2 receptor in glomeruli and distal tubules of adult rats. Moreover, functional studies have provided evidence that AT2 receptor could influence renal function, e. g. sodium retention (Miyata et al., 1999). Similarly, in endothelial cells AT2 receptor was shown to mediate inhibition of cell proliferation and to lead in the extremal way to apoptosis of endothelial cells (Unger et al., 1996). In recent studies it has been assumed that both AT1 and AT2 receptors influence the apoptotic process in the kidney (Bonnet et al., 2001; Bhaskaran et al., 2003). The same has been hypothesized by Cao et al. (2000) who have suggested that a "cross-talk" may develop between AT1 and AT2 receptors, which coordinate the biological effect of angiotensin II. It is most likely that both receptors have a similar effect and that the balance between the AT1 and AT2 receptors determines the renal status in health and disease (Siragy, 2004). According to our results, following exercise involvement of both AT1 and AT2 receptors can also be suggested in mediation of the apoptotic process in renal distal convoluted tubular cells and collecting ducts.

Since apoptosis contributes to the deletion of damaged or potentially dangerous tubular cells it is not surprising that it remains under precise genetic control. Therefore, in many cases induction of apoptosis requires many synchronous diverse stimuli, which could employ different pathways like that involving protein kinase C or that progressing via p53 activation (Bonnet et al., 2001). As has been demonstrated by many authors, activation of both AT1 and AT2 receptors could subsequently increase expression of p53, a potent inducer of apoptosis (Miyashita and Reed, 1995; Bedi and Mookerjee, 1998; Horiuchi et al., 1998; Bonnet et al., 2001). Moreover, in *in vitro* studies stimulation of angiotensin receptors AT1 and AT2 in cardiac myocytes has also been reported to be associated with increased expression of p53 (Pierzchalski et al., 1997; De Angelis et al., 2002). Similarly, we have demonstrated an increased expression of p53 in the kidney. p53 expression has been detected only in distal convoluted tubular cells and collecting ducts of exercised animals, which has correlated with an increased expression of AT1 and AT2 receptors and occurrence of apoptotic nuclei also only in those cells. This suggests that angiotensin II could upregulate p53 protein via both AT1 and AT2 receptors. As in cardiac myocytes, development of apoptosis in the kidney after physical exercise could be mediated by angiotensin II, involving the cellular mechanism of p53 activation, activation of the local renin-angiotensin system and of pro-apoptotic genes (Pierzchalski et al., 1997).

This study clearly demonstrates that intense physical exercise may lead to apoptotic damage of renal distal convoluted tubular cells and cortex and medullary collecting ducts but it spares the proximal tubuli. The obtained results have been confirmed by electron microscopy which has revealed the presence of cells

with the typical apoptotic cell nuclei only in the distal convoluted tubuli or in the collecting ducts of cortex and medulla in the kidneys in all groups of animals subjected to exercise. Moreover, we have observed shedding of the apoptotic bodies directly to lumen of the renal tubule or duct, which seems to be the simplest way of getting rid of the apoptotic cells, instead of their phagocytosis by neighbouring epithelial cells. Detection of structural alterations using electron microscopy, as described originally by Kerr et al. (1972), still remains the reliable way of distinguishing between the two modes of cell death.

The induction of apoptosis in kidney tubular cells after physical effort seems to be associated with generation of angiotensin II, which may upregulate p53 and other pro-apoptotic proteins via stimulation of both AT1 and AT2 receptors. Our results suggest that coordinated action of both AT1 and AT2 receptors could play a crucial role in control of apoptosis in kidneys after physical training. Studies should be performed to pinpoint the molecular mechanism involved in angiotensin II-induced apoptosis of renal tubular cells.

We conclude that an intense physical exercise could induce apoptosis in renal distal convoluted tubular cells and collecting ducts. Apoptotic changes were never observed in proximal tubular cells. The induction of apoptosis after physical effort seemed to be associated with the activation of angiotensin II which acts through stimulation of AT1 as well as AT2 receptors. We suggested that angiotensin II receptors upregulated p53 protein in the cells. The findings suggest that activation of both AT1 and AT2 receptors could play a role in p53-mediated apoptosis in kidneys after excessive exercise.

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