Original Article

jraas

Journal of the Renin-Angiotensin-Aldosterone System
July-September 2020: 1–11

DOI: 10.1177/1470320320949850
journals.sagepub.com/home/jra
SAGE

Influence of Angiotensin II on cell viability and apoptosis in rat renal proximal tubular epithelial cells in in vitro studies

Aleksandra Piotrowska[1], Magdalena Chmielewska[2], Waldemar Andrzejewski[3], Piotr Dziegiel[1,3] and Marzenna Podhorska-Okolow[4]

Abstract

Introduction: Angiotensin II (Ang II) is multifunctional peptide that plays an important role in blood pressure regulation and maintenance electrolyte homeostasis. It shows biological effects by activating two main receptors: AT_1 and AT_2. The aim of the present work was to investigate the effect of Ang II on NRK-52E cells in in vitro studies. Furthermore, an attempt was made to determine the effectiveness of the AT_1 and AT_2 receptor blocker activity (respectively, losartan and PD123319).

Methods: The study was carried out using adherent NRK-52E cell line. Immunofluorescence and Western Blot method were used to confirm the presence of AT_1 and AT_2 receptors in the cells. The SRB and MTT tests showed decrease in the viability of NRK-52E cells incubated with Ang II in comparison to the control (without Ang II).

Results: The blockade of the AT_1 receptor caused an increase in cell viability in comparison to cells incubated with Ang II only. The blockade of AT_2 receptor also triggered statistically significant increase in cell viability in comparison with cells only exposed to Ang II. Combined administration of blockers for both receptors (losartan and PD123319) decreased Ang II cytotoxicity against NRK-52E cell line. The apoptosis was only observed in cells incubated with Ang II in comparison with control cells. However, simultaneous use of both blockers caused statistically significant decrease in apoptosis.

Conclusions: The result of our study indicates that Ang II causes damaging effect on NRK-52E cells by directing them to programmed cell death. It seems that not only does the AT_2 receptor itself play an important role in the induction of apoptosis, but also its interaction with AT_1 receptor does as well.

Keywords

Angiotensin II, Ang II, AT_1, AT_2, losartan, PD123319, apoptosis

Date received: 5 April 2020; accepted: 5 July 2020

Introduction

The systemic renin-angiotensin system (RAS) is a complex enzymatic-hormonal system that plays an important role in cardiovascular homeostasis. Angiotensin II (Ang II) is the main effector substance composing the classical RAS. It affects a number of organs, such as adrenal gland, kidney, brain, pineal gland, or smooth muscles of blood vessels.[1] Angiotensin is an important factor in pathogenesis of many cardiovascular diseases, such as hypertension, atherosclerosis, cardiac hypertrophy, or cardiac infraction.[2] On a cellular level, Ang II modulates contraction of smooth muscle cells ad regulates life processes,

[1]Division of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, Poland
[2]Amphibian Biology Group, Department of Evolutionary Biology and Conservation of Vertebrates, Faculty of Biological Sciences, University of Wroclaw, Poland
[3]Department of Physiotherapy, Wroclaw University School of Physical Education, Poland
[4]Division of Ultrastructure Research, Wroclaw Medical University, Poland

Corresponding author:
Aleksandra Piotrowska, Division of Histology and Embryology, Department of Human Morphology and Embryology, Wroclaw Medical University, Chalubinskiego 6A, Wroclaw 50-368, Poland.
Email: aleksandra.piotrowska@umed.wroc.pl

such as cell growth, cell division, cell death via apoptosis, or cell differentiation.[3]

Ang II causes multi directional biological effects on target cells via activation of two main types of receptors: AT_1 and AT_2. The receptors are located in the membrane of cells in many organs, but their distribution is uneven.[3] The most physiological effects of Ang II are signaled by AT_1 receptor that belongs to the family of seven-transmembrane domain receptors related to G proteins.[4] Cellular responses to the activation of AT_1 receptor pathway include inter alia: smooth muscle contraction, adrenal steroid hormone production, as well as cell growth, and proliferation.[1] The AT_2 receptor as less known. They show antagonistic effect for AT_1 receptors by exhibiting for example, proliferation inhibitory activity and thus promoting cellular differentiation process.[5]

As Ang II is known to affect the development of some diseases (e.g., hypertension), one of the first angiotensin-inhibiting drugs were Ang II convertase inhibitors (ACEi), for example, captopril. However, attempts to find compound that would be effective in Ras system inhibition were concentrated on discovering blockers of Ang II receptors, mainly AT_1 receptor that is responsible for most of the biological effects caused by Ang II peptide.[6,7] This was due to the fact that in many organs there are alternative pathways of Ang II production (e.g., with other enzymes such as chymase, trypsin, chymotrypsin, cathepsin G, or tonin). Losartan (LOS), which belongs to sartans group, was the first drug effectively blocking AT_1 receptor. LOS is a specific, non-protein AT_1 receptor antagonist. Nowadays, the AT_1 blockers are commonly used as antihypertensive medications.[8] Therefore, declined research interest in discovering novel AT_2 receptor blockers is not surprising. PD123319 is one of the most selective, non-protein angiotensin AT_2 receptor antagonists. Also, the first non-protein agonist of AT_2 receptor– C21 was synthesized.[9]

Already in 2004 r. Zhang et al. showed the effect of Ang II on cell proliferation, differentiation, apoptosis, and regeneration in renal proximal tubules.[10] It was shown that concentration of Ang II in urine of renal proximal tubules is much higher than in blood plasma, which can have a significant effect on the development of local lesions in the cells of renal glomeruli and tubules, as well as in interstitial renal cells. One of the histological features indicating chronic renal damage caused by Ang II is renal tubular atrophy, which can be caused by the damage and apoptosis of renal tubular epithelial cells.[11] This process can be induced by various factors. In their research, Garrido et al. proved that Ang II is NAD(P)H oxidase activator in the cells of renal proximal tubules, which leads to the formation of oxygen free radicals responsible for DNA damage and thus initiation of apoptosis.[12] Also, Podhorska-Okołów et al. showed the effect of oxidative stress on cell apoptosis in renal tubules of rats subjected to intense physical activity.[13] On the other hand, the group of Qin showed that AT_1 receptor blockade decreases cell damage by inhibiting NADPH oxidase pathway.[14]

The aim of the present work was to determine the effect of Ang II on rat renal proximal tubular cells, as well as potential involvement of AT_1 and AT_2 receptors in apoptotic lesions of these cells in in vitro model.

Materials and methods

Cell culture

A cell line of rat renal proximal tubular epithelial cells (ATCC, Washington, CO, USA) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC), supplemented with 5% Calf Bovine Serum (CBS), 4 mM L-glutamine, penicillin (100 I.U./ml) and streptomycin (100 µg/ml) (ATCC). The culture was maintained at 37°C in a humidified atmosphere containing 5% CO_2. At confluence, the cells were washed and maintained in serum free DMEM for 48 h prior to the experiments. Viability was checked using the trypan blue exclusion method. The following reagents were used in the experiments: Angiotensin II (10^{-9} M), PD123319 (10^{-6} M), and losartan potassium (10^{-6} M) (Sigma-Aldrich, Darmstadt, Germany). In some combinations, losartan (10^{-6} M), PD123319 (10^{-6} M) or both blockers simultaneously were added to the cell cultures 1 h prior to the experiment. The experiment was divided into five groups: (1) control, (2) Ang II (10^{-9} M), (3) Ang II (10^{-9} M) + LOS (10^{-6} M), (4) Ang II (10^{-9} M) + PD123319 (10^{-6} M), (5) Ang II (10^{-9} M) + LOS (10^{-6} M) + PD123319 (10^{-6} M). The incubation was stopped at selected time points (6, 12, 24, and 48 h).

Detection of AT_1 and AT_2 receptors

Immunofluorescence. The NRK-52E cells (9×10^4 cells/vessel) were grown in eight Chamber Polystyrene Vessel—Culture Slides (Becton Dickinson, Franklin Lakes, NJ, USA). Following the experiment described above, the cells were washed in PBS and fixed in wells with the use of 4% paraformaldehyde in PBS for 12 min at room temperature. Cell membrane permeabilization was performed with 0.2% Triton-X100 in PBS for 10 min in room temperature. Cells were incubated overnight at 4°C with primary antibodies: rabbit polyclonal anti-AT1R N-10 (dilution 1:50; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and goat polyclonal anti-AT2R C-18 (dilution 1:50; Santa Cruz Biotechnology Inc). Next, the slides were incubated for 1 h with Fluorescein (FITC)-conjugated AffiniPure Donkey Anti-Rabbit IgG (for AT1R antibody) and Rhodamine (TRITC)-conjugated AffiniPure Donkey Anti-Goat IgG (for AT2R antibody) secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:50 in the reagent with background-reducing components.

Preparations were mounted using Vectashield with DAPI Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). The slides were analyzed using BX microscope (Olympus, Tokyo, Japan) coupled with CellF software (Olympus).

Western Blot analysis

After the experiment, the cells were trypsinized, centrifuged, and washed in PBS. The number of cells was estimated using Burker's chamber. For each experimental group, 2×10^6 of NRK-52E cells in exponential phase were used. Whole protein lysates were obtained from cells using CellLytic MT Cell Lysis Reagent (Sigma-Aldrich, Darmstadt, Germany) with the addition of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific Inc., Rockford, IL, USA) and 0.2 mM PMSF (Sigma-Aldrich). To determine the concentration of the protein, Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) and NanoDrop 1000 (Thermo Fisher Scientific Inc.) spectrophotometer were used. Equal amounts of total protein (40 µg) were mixed with sample buffer and dithiothreitol (DTT) and resolved by SDS-PAGE.[15] After electrophoresis, the samples were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore, Bedford, MA, USA) and incubated in 3% BSA solution in TBST buffer (0.2 M Tris; 1.5 M NaCl; 0.1% Tween-20). After blocking, the membranes were incubated for 1 h in room temperature with the primary rabbit polyclonal anti-AT1R N-10 antibody (dilution 1:200; Santa Cruz Biotechnology, Inc.) and overnight at 4°C with the primary rabbit polyclonal anti-AT2R H-143 (dilution 1:500; Santa Cruz Biotechnology, Inc.). Next, the membranes were treated with the secondary HRP-conjugated donkey anti-rabbit antibody (dilution 1:3000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature, rinsed and incubated with the Luminata Classico Western HRP Substrate (Merck; Millipore, Bedford, MA, USA). The reactions were visualized using ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA). For each line, the amount of protein in AT1R and AT2R bands was normalized to β-actin.

Analysis of cell viability

SRB method. Cell viability was measured using the SRB method. For the experiment, NRK-52E cells were seeded at 9000 cells/well in 96-well plates. After experiment, the cells were fixed with 50% trichloroacetic acid and subsequently stained for 30 min using 0.4% SRB dissolved in 1% acetic acid. The unbound dye was removed by rinsing the cells in 1% acetic acid and the cell protein-bound dye was extracted with 10 mM unbuffered Tris solution. The optical density of the solution (OD) was measured using EL× 800 plate reader (Bio-Tek Instruments Inc., Vinooski, VT, USA) at 562 nm wavelength.[16] As a control, the medium alone subjected to the procedure described above was used.

MTT method. Cell viability was also measured using MTT assay.[17] Reduction of yellow MTT salt to a purple formazan crystals by mitochondrial dehydrogenases in viable cells was determined by measuring the light absorbance at 570 nm using EL× 800 plate reader (Bio-Tek Instruments Inc.)

Detection of apoptosis

Terminal transferase dUTP nick end labeling assay. Apoptotic assay was conducted using ApopTag In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA). After the experiment, NRK-52E cells (9×10^4 cells/vessel) cultured on eight-well chambered slides (Becton Dickinson) were washed in PBS, fixed in cold acetone-methanol (1:1) for 15 min at 4°C and air-dried. The sections were washed in PBS and digested using Proteinase K (Dako, Glostrup, Denmark) for 5 min in room temperature (RT). The activity of endogenous peroxidase was blocked by 5 min incubation in 3% H_2O_2 in PBS. In the next step, the sections were incubated first with Equilibration Buffer for 10 min in RT, and then with TdT Enzyme and Reaction Buffer at 37°C for 1 h. The reaction was stopped by the incubation with Stop Buffer for 10 min in RT. Subsequently, the sections were incubated with anti-digoxygenin peroxidase conjugated secondary antibodies and the substrate for peroxidase, diaminobenzidine (DAB), was applied for 10 min at RT. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated in graded ethanol concentrations (70%, 96%, 99.8%) and xylene the preparations were mounted in SUB-X Mounting Medium (Dako). Percentage of apoptotic nuclei was evaluated by scoring the brownish-labeled cell nuclei (positive cells) in selected hot-spots under 400× magnification (Olympus BX 41 light microscope with visual mode Analysis 3.2 software for computer-assisted image analysis).

Measurement of caspase-3 activity

After the experiment, caspase-3 activity was detected by the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, 1.5×10^6 NRK-52E cells were lysed in the lysis buffer to obtain total protein in each sample. Subsequently, 80 µg of protein in a total volume of 50 µl was added to 50 µl of reaction buffer and 5 µl of DEVD-pNA substrate (200 µM final concentration). After incubation (3 h, 37°C), the hydrolysis of DEVD-pNA by caspase-3 released free pNA that produces a yellow color detected at 405 nm using a microplate reader (Tecan, Mannedorf, Switzerland).

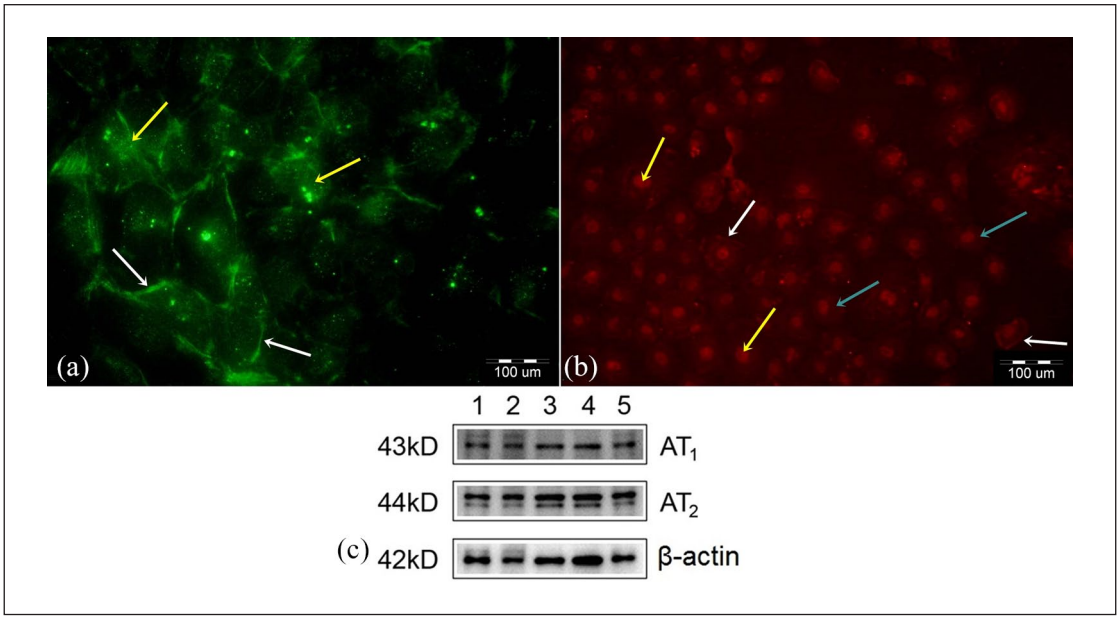

Figure 1. Angiotensin II receptors expression in NRK-52E cells. (a, b) Localization illustrated by immunofluorescence reaction: (a) AT1 receptor—cell membrane (white arrow) and cytoplasm (yellow arrows); (b) AT2 receptor—cell membrane (white arrow), cytoplasm (blue arrow) and nucleus (yellow arrow). Magnification 400×; (c) Representative Western Blot indicated expression of each receptor. Molecular weights of protein bands are denoted on the left side of the images. Lines: 1—control, 2—Ang II 10^{-9} M, 3—Ang II 10^{-9} M + LOS 10^{-6} M, 4—Ang II 10^{-9} M + PD 10^{-6} M, 5—Ang II 10^{-9} M + LOS 10^{-6} M + PD 10^{-6} M.

Transmission electron microscopy

NRK-52E cells were grown in T-75 flasks (Falcon; Fisher Scientific) to reach 95% confluence. Cells were harvested from flasks by gentle scraping, suspended in PBS, spun for 2 min at 60 g and then fixed for 24 h with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C. After fixation the cells were rinsed several times with cacodylate buffer (4 × 15 min), post fixed with 2% osmium tetroxide in cacodylate buffer for 1 h at 4°C, and then dehydrated through a series of graded ethanol concentrations and acetone. The fixed cells were embedded in EPON resin. Ultrathin sections were stained and examined by a JEOL JEM 1011 (Tokyo, Japan) transmission electron microscopy.

Statistical analysis

The statistical significance of differences between individual groups was determined by the Kruskal-Wallis test, as a non-parametric equivalent of the analysis of variance and two-way ANOVA with the Bonferroni multiple comparison test. Statistical significance was defined as $p < 0.05$. All statistical analyses were conducted using Statistica 7.1 software (StatSoft, Krakow, Poland).

Results

The study showed the presence of both Ang II receptors, AT_1 and AT_2, in rat renal proximal tubular cells (NRK-52E). Immunofluorescence method with the use of specific polyclonal antibodies showed that NRK-52E cells express both AT_1 and AT_2 receptors. Following various periods of incubation, all cells subjected to the combinations of the above-mentioned compounds were characterized by the expression of Ang II receptors. The AT_1 receptor was found in the cell membrane and cytoplasm of the cells (Figure 1(a)), whereas AT_2 receptor protein was observed in cell nucleus and cell membrane, as well as in small quantity in cytoplasm (Figure 1(b)).

Western Blot confirmed AT_1 and AT_2 receptor expression in cells incubated with all of the above-mentioned compounds following each incubation period, as well as in control cells. With the use of specific antibodies, bands with molecular mass of 43 kDa, characteristic for AT_1 receptor, as well as bands with molecular mass of 44 kDa for AT_2 receptor (Figure 1(c)) were detected.

The effect of Ang II on NRK-52E cells

In the SRB test, Ang II in 10^{-9} M concentration has a toxic effect on the cells of NRK-52E line. The decrease in the number of cells was shown after each period of exposition to Ang II. The strongest decrease was observed after 6 h—by 68% relative to control. After 12 and 24 h, the OD (optical density) dropped by 8%, whereas after 48 h the OD decreased by 7% in comparison with the control. After 6 and 24 h of incubation with Ang II (in comparison with the control), the differences were statistically significant ($p < 0.05$) (Figure 2(a)).

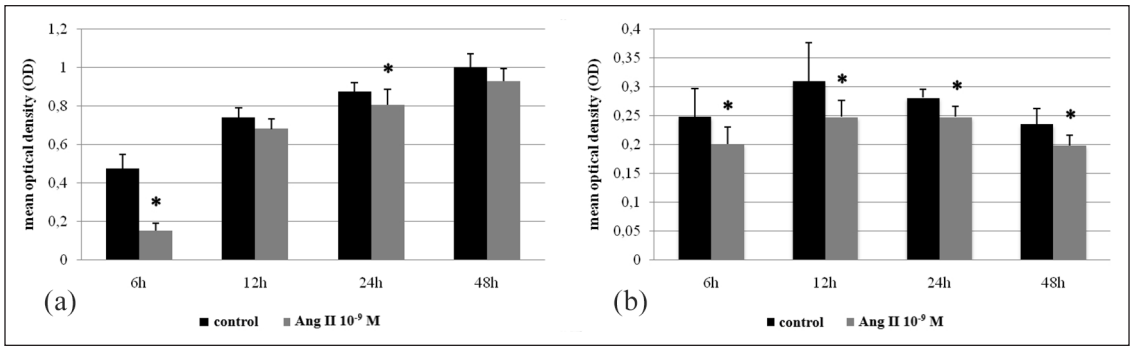

Figure 2. Cell viability of NRK-52E cells incubated with Ang II 10^{-9} M at different times (6, 12, 24, 48 h) (a) SRB method, (b) MTT method. Untreated cells were considered as a control. *$p < 0.05$.

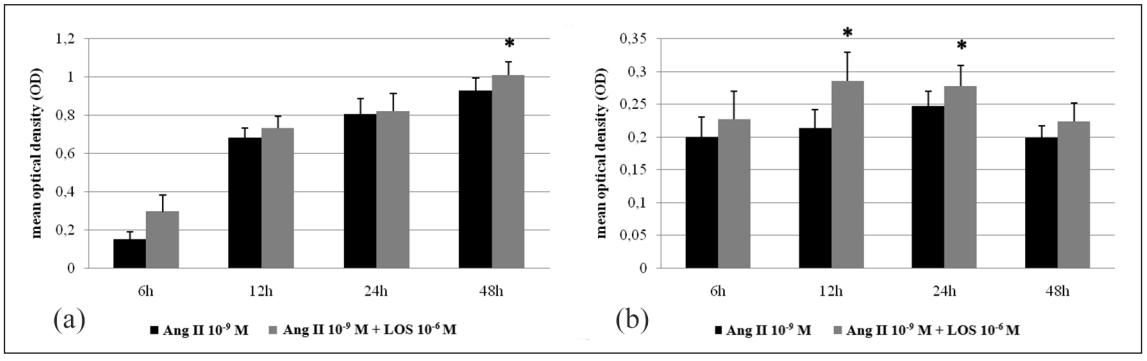

Figure 3. Cell viability of NRK-52E cells preincubated for 1 h with losartan (LOS) 10^{-6} M and then exposed to Ang II 10^{-9} M at different times (6, 12, 24, 48 h) (a) SRB method, (b) MTT method. Cells incubated only with Ang II were considered as a control. *$p < 0.05$.

In the MTT test, the NRK-52E cell line is sensitive to Ang II in 10^{-9} M concentration. Following 6 h of incubation with Ang II, the number of cells decreased by 17%, after 12 h—by 32%, after 24 h—by 12%, and after 48 h—by 15%. All correlations (relative to the control) are statistically significant $p < 0.05$) (Figure 2(b)).

The effect of AT_1 receptor blocker (losartan) on viability of cells incubated with Ang II

The SRB method, for all cell incubations with losartan (LOS) at 10^{-6} M and Ang II at 10^{-9} M, showed an increase in cell viability in comparison with cells treated only with Ang II. However, only the difference after 48 h was statistically significant ($p < 0.05$) (Figure 3(a)).

In the MTT method, LOS used at a concentration of 10^{-6} M together with Ang II (10^{-9} M) increased cell viability in comparison to the incubation with Ang II only. Correlations after 12 and 24 h were statistically significant ($p < 0.05$), (Figure 3(b)).

The effect of AT_2 receptor blocker (PD123319) on viability of cells incubated with Ang II

Following the blockade of AT_2 receptor with PD123319 (PD) compound, in the SRB test, an increase in the viability of cells after each incubation time was observed in comparison with cells exposed only to Ang II. Administration of PD123319 at 10^{-6} M in the presence of Ang II caused almost three times increase in the number of cells after 6 h of incubation. The results obtained after 6, 12, and 48 h of incubation were statistically significant (0.05), (Figure 4(a)).

In the MTT test, PD used at a concentration of 10^{-6} M together with Ang II (10^{-9} M) significantly increased cell viability after 6, 12, and 24 h of incubation relative to the cells treated only with Ang II ($p < 0.05$) (Figure 4(b)).

The effect of concomitant use of LOS and PD on viability of cells incubated with Ang II

In the SRB method, concomitant use of LOS and PD at concentrations of 10^{-6} M together with Ang II (10^{-9} M) causes an increase in call viability in comparison with cells exposed only to Ang II. The most significant, almost threefold increase was observed after 6 h. The values obtained after 6 and 48 h are statistically significant ($p < 0.05$) (Figure 5(a)).

In the MTT test, the addition of LOS (10^{-6} M) and PD (10^{-6} M) to the cells incubated with Ang II (10^{-9} M) significantly decreased Ang II toxicity in comparison to the cells treated only with Ang II after 12, 24, and 48 h of incubation and was statistically significant ($p < 0.05$) (Figure 5(b)).

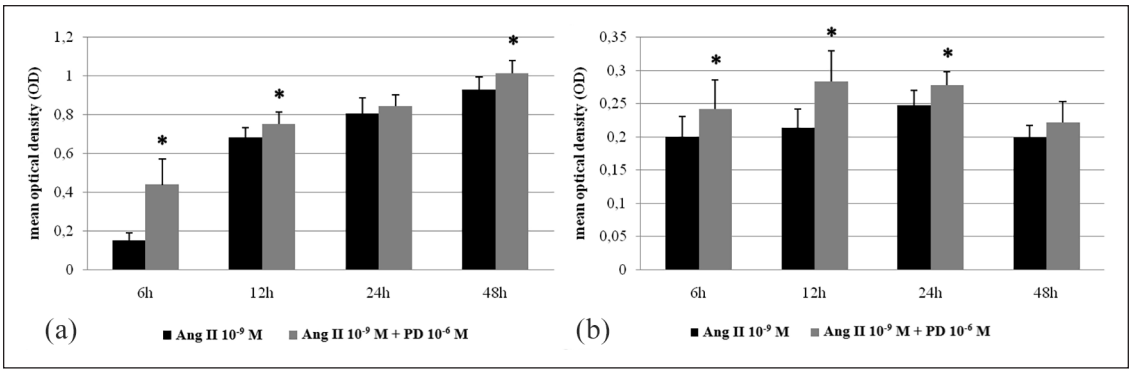

Figure 4. Cell viability of NRK-52E cells preincubated for 1 h with PD123319 (PD) 10^{-6} M and then exposed to Ang II 10^{-9} M at different times (6, 12, 24, 48 h) (a) SRB method, (b) MTT method. Cells incubated only with Ang II were considered as a control. $^{*}p < 0.05$.

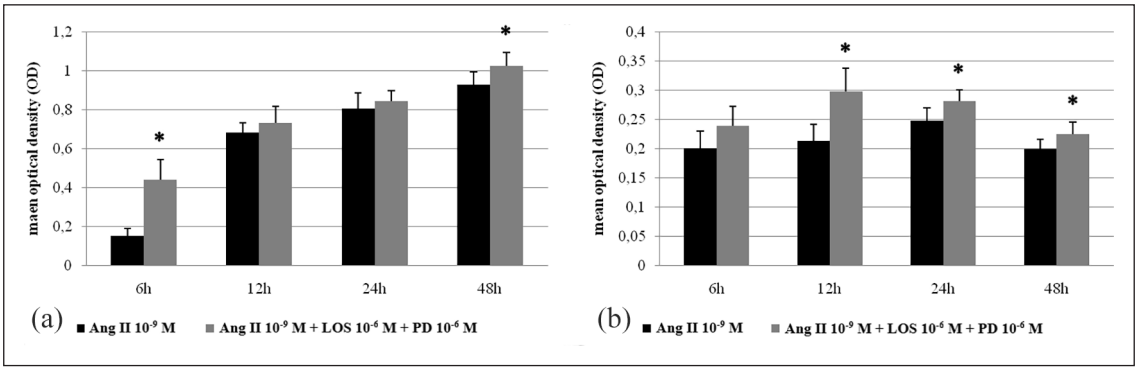

Figure 5. Cell viability of NRK-52E cells preincubated for 1 h with losartan (LOS) 10^{-6} M and PD123319 (PD) 10^{-6} M, simultaneously and then exposed to Ang II 10^{-9} M at different times (6, 12, 24, 48 h) (a) SRB method, (b) MTT method. Cells incubated only with Ang II were considered as a control. $^{*}p < 0.05$.

Terminal transferase dUTP nick end labeling

The effect of Ang II on NRK-52E cells. The experiments performed by the Terminal transferase dUTP nick end labeling (TUNEL) method showed that incubation of NRK-52E cells with Ang II at 10^{-9} M affects the incidence of apoptotic lesions within these cells (Figure 6(c)). An increase in the number of apoptotic cells was observed in comparison to the control after each of the incubation periods. All values (in comparison with the control) were statistically significant, ($p < 0.05$) (Figure 6(a)).

The effect of LOS on the cells incubated with Ang II. Following the use of LOS at a concentration of 10^{-6} M together with Ang II (10^{-9} M) the tendency was observed for the decrease in the number of apoptotic cells for all studied incubation times in comparison to the cells treated only with Ang II. The results were statistically insignificant, ($p < 0.05$) (Figure 6(b)).

The effect of PD on the cells incubated with Ang II. The use of 10^{-6} M PD with Ang II (10^{-9} M) significantly ($p < 0.05$) lowered the number of apoptotic cells in comparison to the cells treated only with Ang II (Figure 6(b)).

The effect of concomitant use of LOS and PD on the cells incubated with Ang II. Simultaneous blockade of both Ang II receptors caused significant decrease in the number of apoptotic cells after each of the exposition times in comparison with the controls ($p < 0.05$) (Figure 6(b)).

Determination of caspase-3 activity

The effect of Ang II on NRK-52E cells. The addition of 10^{-9} M Ang II to the cell culture caused an increase of caspase-3 activity for each of the analyzed incubation times ($p < 0.05$) (Figure 7(a)).

The effect of LOS on the cells incubated with Ang II. The tendency was observed for the decrease in the activity of caspase-3 in comparison to the cells treated only with Ang II when LOS at a concentration of 10^{-6} M was used together with Ang II (10^{-9} M). The differences were statistically significant ($p < 0.05$) (Figure 7(b)).

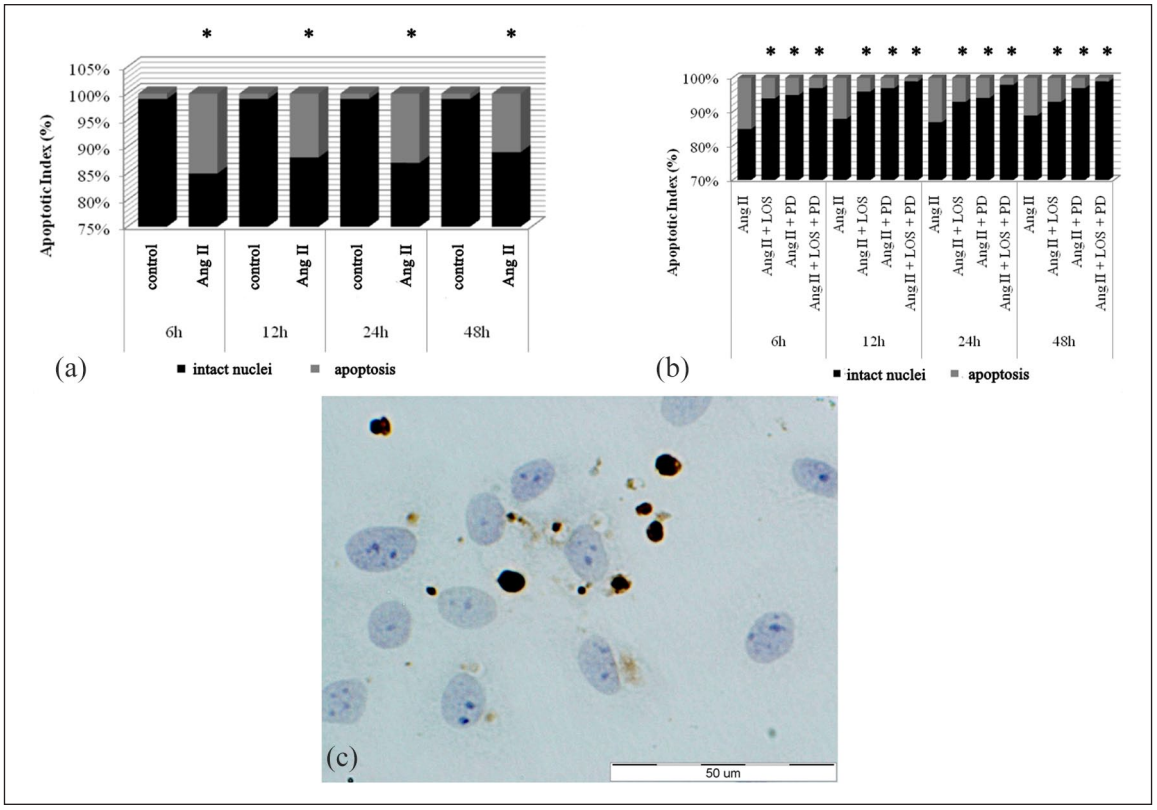

Figure 6. Detection of apoptosis in NRK-52E cells by TUNEL technique (a) Effect of Ang II 10^{-9} M on proportion of apoptotic cell nuclei (%) in NRK-52E cell line at different times (6, 12, 24, 48 h) (*$p < 0.05$); (b) Effect of blockade Ang II (AT1 and AT2) receptors in Ang II-treated NRK-52E cells on proportion of apoptotic nuclei at different times (6, 12, 24, 48 h). Cells incubated only with Ang II were considered as a control. *$p < 0.05$; (c) TUNEL technique detected apoptotic nuclei (brown) in NRK-52E cells after incubation with Ang II 10^{-9} M. Magnification 600×.

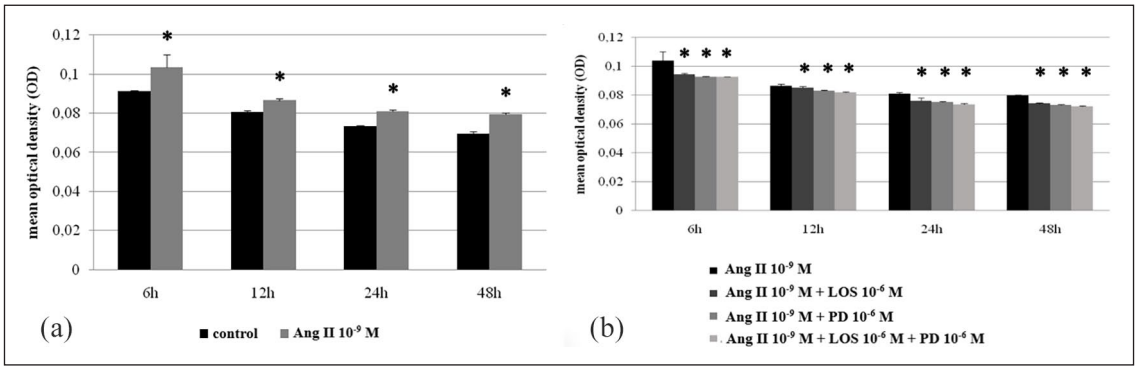

Figure 7. Caspase-3 activity in NRK-52E cells: (a) after incubation with Ang II 10^{-9} M at different times (6, 12, 24, 48 h); (b) in control (Ang II 10^{-9} M) and experimental groups (Ang II 10^{-9} M + LOS 10^{-6} M, Ang II 10^{-9} M + PD 10^{-6} M, Ang II 10^{-9} M + LOS 10^{-6} M + PD 10^{-6} M) at different times (6, 12, 24, 48 h). *$p < 0.05$.

The effect of PD on the cells incubated with Ang II. The use of PD at a concentration of 10^{-6} M together with 10^{-9} M Ang II showed the tendency for the decrease in the activity of caspase-3 for all studied incubation times in comparison to the cells treated only with Ang II. The results were statistically significant ($p < 0.05$) (Figure 7(b)).

The effect of concomitant use of LOS and PD on the cells incubated with Ang II. In the case of simultaneous use of LOS (10^{-6} M) and PD (10^{-6} M) with Ang II (10^{-9} M), for all incubation periods the values for caspase-3 activity decreased significantly in comparison with the cells incubated only with Ang II ($p < 0.05$) (Figure 7(b)).

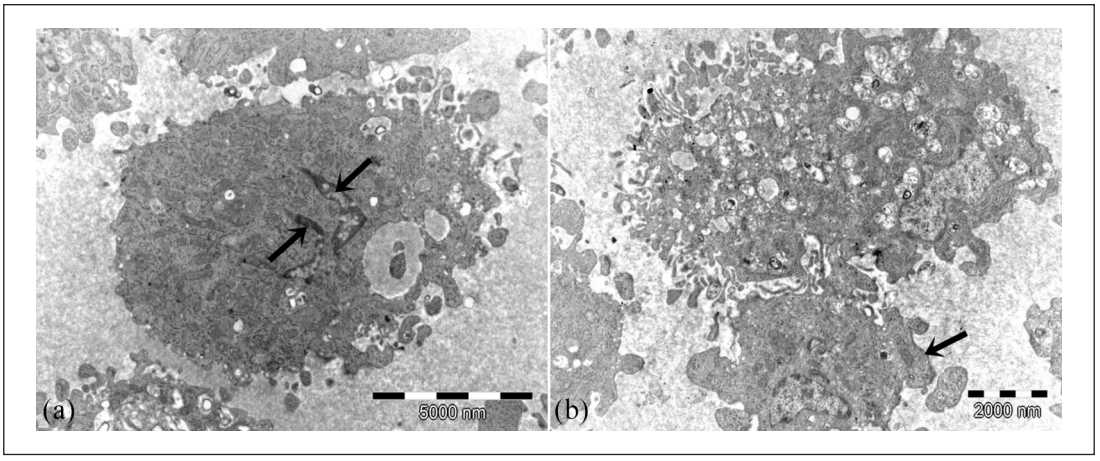

Figure 8. Morphological alterations of NRK-52E cells showed in TEM after incubation with Ang II (10^{-9} M). (a) fragmentation of nucleus and nuclear chromatin condensation (arrow); (b) cell fragmentation and forming apoptotic bodies (arrow).

Transmission electron microscopy

Transmission electron microscopy (TEM) observations of cells treated with Ang II at a concentration of 10^{-9} M showed the presence of features characteristic for apoptosis: peripheral condensation of nuclear chromatin, fragmentation of cell nucleus (Figure 8(a)) and formation of apoptotic bodies (Figure 8(b)). The described images were visible after each period of cell incubation with Ang II.

Discussion

It was already shown 30 years ago that Ang II effects proliferation and differentiation processes of inter alia smooth muscle cells of blood vessels.[18] On the other hand, studies conducted by other authors proved that administration of Ang II to rats causes an increase in the number of renal proximal tubular cells expressing PCNA proliferation marker and apoptotic cells. This, in turn, suggests that Ang II may affect both opposite processes, that is, cell apoptosis and proliferation. However, the use of blockers of both receptors weakened this action.[19] Similarly, Aizawa et al. showed that administering Ang II to rats affects at the same time both proliferation and apoptosis of rat renal proximal tubular cells. However, in this study AT_1 receptor blocker (LOS) alone was sufficient to weaken pro-apoptotic and pro-proliferative activity of Ang II.[20]

Many authors showed the presence of AT_1 receptor at the cells of various renal structures in adults as well as in cultured renal cells, such as interstitial renal fibroblasts. However, the data on AT_2 receptor localization in renal cells are still controversial. Immunohistochemical studies conducted on rat kidneys by the group of Miyata showed the presence of AT_1 receptor in all renal cells, and in particular in the outer layer of renal cortex. Moreover, very strong AT_1 expression in proximal and distal tubules was observed. On the other hand, the same authors found high expression of AT_2 receptor in proximal and distal cortex tubules, as well as in collecting tubules.[21] Most studies are based on Ang II receptor (AT_1 and AT_2) detection in tissues, however, the reports confirming expression of the above-mentioned receptors in NRK-52E cell line are missing. Only Ortega et al. showed the presence of AT_1 receptor in NRK-52E cells using immunofluorescence method.[22] The results of our immunofluorescence studies confirmed the presence of AT_1 receptor in cell membranes. Additionally, the presence of AT_2 receptor was confirmed on NRK-52E cells in cell membranes and nuclei. Similarly, Gwathmey showed this receptor in the nuclei of renal proximal tubular cells in sheep,[23] whereas the group of Tadevosyan reported nuclear localization of AT_2 receptor in canine cardiac fibroblasts.[24]

In our studies, the presence of both Ang II receptors was confirmed by Western Blotting in both control cell line as well as in the cells from all experimental groups. Similarly, both Ang II receptors were confirmed in the cells of rat and mouse renal proximal tubules by other researchers.[10,25] Moreover, Shalamanova et al. showed the presence of both receptors in RPTEC (*Renal Proximal Tubular Epithelial Cells*) and HK-2 (*human kidney-2*) cells.[26] Also, some in vivo studies in animal models confirm the presence of AT_1 and AT_2 receptors for example, in renal proximal tubular and mesangial cells.[23]

In our studies, SRB and MTT tests confirmed the decrease in viability of NRK-52E cells caused by Ang II after each incubation time, however, without correlation with incubation time. These observations are in line with Carierre et al., who proved that Ang II cytotoxicity against NRK-52E cells was independent from its concentration and exposition time.[27]

Many researchers showed also that the role of drugs belonging to ACE inhibitors or AT_1 receptor antagonists is associated not only with lowering blood pressure, but also affects blockade of local renal RAS, which in turn lads to

the inhibition of Ang II activity on a cellular level.[25] Our studies on the effect of AT_1 and AT_2 receptor antagonists showed that the use of each one of them separately, as well as their concomitant use with Ang II resulted in an increase in cell viability in comparison to the cells treated only with Ang II. This may prove that cooperation of both receptors is important in signal transduction, which may confirm hypothesis by Cao et al. about so-called cross-talk between AT_1 at AT_2 receptors.[19]

The results of our studies prove that incubation of NRK-52E cells with Ang II affects an increase in the number of apoptotic cells detected with the use of TUNEL method, independently from the incubation time. Bhaskaran et al. also showed that Ang II is associated with the activation of apoptosis in RPTEC (*Renal Proximal Tubular Epithelial Cells*) cell line.[28] Similar results were reported by the group of Zhu with the human renal proximal tubular cells (HK-2 cell line).[29] However, these authors, in the contrary to our results, found pro-apoptotic activity of Ang II correlated with dose and incubation time. In turn, Pang et al. incubated rat neonatal cardiomyocytes with 10^{-7} M Ang II. They observed an increase in the number of apoptotic cells by 14% relative to the control cells (after 48 h), which largely confirms our findings. Our results also confirm the observation by Zimpelmann et al., who reported that 10^{-7} M Ang II causes apoptosis in swine renal proximal tubular cells (LLC-PK1).[30,31] In our research, the apoptosis of NRK-52E cells treated with Ang II was also confirmed by the analysis of the ultrastructure of renal proximal tubular cells with the use of transmission electron microscope (TEM). The presence of typical apoptotic features was observed (nuclear chromatin condensation, fragmentation of nucleus and the whole cell, the presence of apoptotic bodies) in the cells incubated with Ang II, which clearly support the hypothesis of the induction of apoptosis by Ang II in the analyzed cells. Administration of blockers of Ang II receptors, AT_1 and AT_2, as well as simultaneous administration of both their antagonists showed a decrease in the number of apoptotic cells in all combinations and exposition times. Statistically significant results were obtained both in groups with single administration of Ang II receptor blockers and in group with concomitant administration of both blockers. In the study conducted by Ning et al., incubation of cells with Ang II considerably increased the number of apoptotic cells from NRK-52E cell line, whereas the administration of losartan inhibited apoptosis induced by Ang II.[32] Similar anti-apoptotic effect was obtained by the group of Zha with the use of the blocker of AT_1 receptor—telmisartan, and the NRK-52E cell line.[33] Our findings confirm the results obtained by the above authors, however, in our studies the strongest inhibition of Ang II pro-apoptotic activity was observed with concomitant administration of antagonists of both receptors, AT_1 and AT_2.

The strongest anti-apoptotic effect obtained with simultaneous blockade of both Ang II receptors may be a mechanism that protects proximal tubular cells against the damage. These cells have an important function in urine concentration, as they carry out intensive absorption processes. From the point of view of renal physiology, development of protective mechanisms in these exact cells seems justified and understandable.

Ang II is known to have pro-apoptotic properties. Previous studies show that pro-apoptotic activity of Ang II leads to the activation of intrinsic (mitochondrial) apoptosis pathway, for example, by affecting MAPK and ERK kinases, as well as proteins from Bcl family or caspases.[8,31,34] Additionally, Bhaskaran et al. confirmed changes in the expression of apoptosis-regulating proteins: Bax and Bcl-2 in the cells incubated with Ang II.[28] Independently from the type of induction, in the execution phase of apoptosis during apoptotic cell degradation, the most important role is played by caspase-3.[29,35] Our study confirms that the exposition of NRK-52E cells to Ang II resulted in an increased level of active caspase-3 in comparison to the control cells after every incubation time. Similar increase in the level of caspase-3 in cultured cardiomyocytes was previously reported by Pang et al.[30]

Research by Kagawa et al. showed that AT_1 and AT_2 receptor antagonists exert an anti-apoptotic effect by inhibiting caspase-3 activity.[8] The above observations confirm our findings that the strongest inhibition of caspase-3 activity triggered by Ang II results from an interaction between both receptor antagonists.

Our study showed damaging, pro-apoptotic activity of Ang II on renal proximal tubular cells of rat NRK-52E cell line in an in vitro model. The strongest anti-apoptotic effect against NRK-52E cells was observed with the use of antagonists (respectively: losartan and PD123319) of Ang II receptors, AT_1 and AT_2. The exact mechanism of apoptotic death signal transduction by Ang II receptors is not yet fully known and requires further study. Most probably, antagonistic activity commonly attributed to AT_1 and AT_2 receptors at the cellular level is a major simplification. Results of our research showed the most potent apoptosis-inhibitory activity in rat NRK-52E cell line when both receptors were blocked simultaneously. This might be an indirect evidence for the interaction between these receptors in intracellular signaling.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iDs

Aleksandra Piotrowska https://orcid.org/0000-0003-4093-7386

Magdalena Chmielewska https://orcid.org/0000-0002-5480-2530

References

1. de Gasparo M, Catt KJ, Inagami T, et al. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 2000; 52(3): 415–472.
2. Sparks MA, Crowley SD, Gurley SB, et al. Classical renin-angiotensin system in kidney physiology. *Compr Physiol* 2014; 4(3): 1201–1228.
3. Karnik SS, Unal H, Kemp JR, et al. International union of basic and clinical pharmacology. XCIX. Angiotensin receptors: Interpreters of pathophysiological angiotensinergic stimuli [corrected]. *Pharmacol Rev* 2015; 67(4): 754–819.
4. Toth AD, Turu G, Hunyady L, et al. Novel mechanisms of G-protein-coupled receptors functions: AT1 angiotensin receptor acts as a signaling hub and focal point of receptor cross-talk. *Best Pract Res Clin Endocrinol Metab* 2018; 32(2): 69–82.
5. Stoll M and Unger T. Angiotensin and its AT2 receptor: New insights into an old system. *Regul Pept* 2001; 99(2–3): 175–182.
6. Hu WY, Fukuda N, Ikeda Y, et al. Human-derived vascular smooth muscle cells produce angiotensin II by changing to the synthetic phenotype. *J Cell Physiol* 2003; 196(2): 284–292.
7. Wasse H, Naqvi N and Husain A. Impact of mast cell chymase on renal disease progression. *Curr Hypertens Rev* 2012; 8(1): 15–23.
8. Kagawa T, Takao T, Horino T, et al. Angiotensin II receptor blocker inhibits tumour necrosis factor-alpha-induced cell damage in human renal proximal tubular epithelial cells. *Nephrology (Carlton)* 2008; 13(4): 309–315.
9. Sallander J, Wallinder C, Hallberg A, et al. Structural determinants of subtype selectivity and functional activity of angiotensin II receptors. *Bioorg Med Chem Lett* 2016; 26(4): 1355–1359.
10. Zhang SL, Guo J, Moini B, et al. Angiotensin II stimulates Pax-2 in rat kidney proximal tubular cells: impact on proliferation and apoptosis. *Kidney Int* 2004; 66(6): 2181–2192.
11. Weidekamm C, Hauser P, Hansmann C, et al. Effects of AT1 and AT2 receptor blockade on angiotensin II induced apoptosis of human renal proximal tubular epithelial cells. *Wien Klin Wochenschr* 2002; 114(15–16): 725–729.
12. Garrido AM and Griendling KK. NADPH oxidases and angiotensin II receptor signaling. *Mol Cell Endocrinol* 2009; 302(2): 148–158.
13. Podhorska-Okolow M, Dziegiel P, Murawska-Cialowicz E, et al. Effects of adaptive exercise on apoptosis in cells of rat renal tubuli. *Eur J Appl Physiol* 2007; 99(3): 217–226.
14. Qin B, Wang Q, Lu Y, et al. Losartan ameliorates calcium oxalate-induced elevation of stone-related proteins in renal tubular cells by inhibiting NADPH oxidase and oxidative stress. *Oxid Med Cell Longev* 2018; 2018: 1271864.
15. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227(5259): 680–685.
16. Skehan P, Storeng R, Scudiero D, et al. New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990; 82(13): 1107–1112.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65(1–2): 55–63.
18. Paquet JL, Baudouin-Legros M, Brunelle G, et al. Angiotensin II-induced proliferation of aortic myocytes in spontaneously hypertensive rats. *J Hypertens* 1990; 8(6): 565–572.
19. Cao Z, Kelly DJ, Cox A, et al. Angiotensin type 2 receptor is expressed in the adult rat kidney and promotes cellular proliferation and apoptosis. *Kidney Int* 2000; 58(6): 2437–2451.
20. Aizawa T, Ishizaka N, Kurokawa K, et al. Different effects of angiotensin II and catecholamine on renal cell apoptosis and proliferation in rats. *Kidney Int* 2001; 59(2): 645–653.
21. Miyata N, Park F, Li XF, et al. Distribution of angiotensin AT1 and AT2 receptor subtypes in the rat kidney. *Am J Physiol* 1999; 277(3): F437–F446.
22. Ortega A, Ramila D, Izquierdo A, et al. Role of the renin-angiotensin system on the parathyroid hormone-related protein overexpression induced by nephrotoxic acute renal failure in the rat. *J Am Soc Nephrol* 2005; 16(4): 939–949.
23. Gwathmey TM, Shaltout HA, Pendergrass KD, et al. Nuclear angiotensin II type 2 (AT2) receptors are functionally linked to nitric oxide production. *Am J Physiol Renal Physiol* 2009; 296(6): F1484–F1493.
24. Tadevosyan A, Xiao J, Surinkaew S, et al. Intracellular Angiotensin-II Interacts With Nuclear Angiotensin Receptors in Cardiac Fibroblasts and Regulates RNA Synthesis, Cell Proliferation, and Collagen Secretion. *J Am Heart Assoc* 2017; 6(4): e004965.
25. Ruiz-Ortega M, Lorenzo O, Ruperez M, et al. Systemic infusion of angiotensin II into normal rats activates nuclear factor-kappaB and AP-1 in the kidney: Role of AT(1) and AT(2) receptors. *Am J Pathol* 2001; 158(5): 1743–1756.
26. Shalamanova L, Wilkinson MC, McArdle F, et al. Characterisation of the expression of the Renin-Angiotensin system in primary and immortalised human renal proximal tubular cells. *Nephron Exp Nephrol* 2010; 116(3): e53–e61.
27. Carriere M, Avoscan L, Collins R, et al. Influence of uranium speciation on normal rat kidney (NRK-52E) proximal cell cytotoxicity. *Chem Res Toxicol* 2004; 17(3): 446–452.
28. Bhaskaran M, Reddy K, Radhakrishanan N, et al. Angiotensin II induces apoptosis in renal proximal tubular cells. *Am J Physiol Renal Physiol* 2003; 284(5): F955–F965.
29. Zhu Y, Cui H, Lv J, et al. AT1 and AT2 receptors modulate renal tubular cell necroptosis in angiotensin II-infused renal injury mice. *Sci Rep* 2019; 9(1): 19450.
30. Pang JJ, Xu RK, Xu XB, et al. Hexarelin protects rat cardiomyocytes from angiotensin II-induced apoptosis

in vitro. *Am J Physiol Heart Circ Physiol* 2004; 286(3): H1063–H1069.
31. Zimpelmann J and Burns KD. Angiotensin II AT(2) receptors inhibit growth responses in proximal tubule cells. *Am J Physiol Renal Physiol* 2001; 281(2): F300–F308.
32. Ning WB, Hu GY, Peng ZZ, et al. Fluorofenidone inhibits Ang II-induced apoptosis of renal tubular cells through blockage of the Fas/FasL pathway. *Int Immunopharmacol* 2011; 11(9): 1327–1332.
33. Zha D, Yao T, Bao L, et al. Telmisartan attenuates diabetic nephropathy progression by inhibiting the dimerization of angiotensin type-1 receptor and adiponectin receptor-1. *Life Sci* 2019; 221: 109–120.
34. Papp M, Li X, Zhuang J, et al. Angiotensin receptor subtype AT(1) mediates alveolar epithelial cell apoptosis in response to ANG II. *Am J Physiol Lung Cell Mol Physiol* 2002; 282(4): L713–L718.
35. Kanamori H, Takemura G, Li Y, et al. Inhibition of Fas-associated apoptosis in granulation tissue cells accompanies attenuation of postinfarction left ventricular remodeling by olmesartan. *Am J Physiol Heart Circ Physiol* 2007; 292(5): H2184–H2194.