

# Possibilities of experimental and clinical antioxidant protection in cardiovascular diseases

PhD thesis

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2012

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## 1. Abbreviations

AIF	Apoptosis-inducing factor
Ang II	Angiotensin II
AP-1	Activating protein-1
ASI	Aortic stiffness index
ERK1/2	Extracellular signal regulated kinase
IMT	intima media thickness
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JNK	c-jun N-terminal kinase
MAPK	mitogen activated protein kinase
MKP-1	MAP-kinase phosphatase-1
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
PARP	Poly(ADP-ribose)polymerase
PGI	Prostacyclin
PKC	Protein kinase C
RES	Resveratrol
RIP1	Receptor-interacting protein 1
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SHR	Spontaneous hypertensive rat
SNP	Sodium nitroprusside
WKY	Wistar-Kyoto rat

## 2. Background

### *2.1. PARP inhibition in the prevention of vascular remodeling*

Hypertension is a major public health problem mainly in middle-aged and in elderly people. It is both a complex disease and an important risk factor for other cardiovascular outcomes, such as sudden cardiac death, stroke, myocardial infarction, heart failure, renal diseases and peripheral vascular diseases. Unfortunately, the control of arterial hypertension is far from optimal and only minimally improved over the last decades. Side effects of antihypertensive drugs and complaints due to their blood pressure lowering effect are the key factors in the background of inadequate hypertension control (1-5). Hypertension alters the structure and function of blood vessels. These structural alterations which affect every layer of vascular wall involve changes in cell growth, cell death, cell migration, and production or degradation of extracellular matrix. This active process is also known as vascular remodeling. Remodeling is an adaptive process that occurs in response to long-term changes in hemodynamic conditions, but it may also subsequently contribute to the pathophysiology of vascular diseases and circulatory disorders (7-8).

#### *2.1.1. Oxidative stress and consequent activation of poly(ADP-ribose)polymerase enzyme in hypertension*

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have an important pathological role in the development of cardiovascular diseases. ROS generation occurs in every layer of the vascular wall caused by mechanical forces such as shear stress and vasoactive agents like Angiotensin II (Ang II). This is a consequence of excess production of oxidant agents, decreased antioxidant capacity and decreased bioavailability of nitric oxide (NO) which has a prominent part in the maintenance of elevated peripheral resistance. The major sources of ROS are NADPH oxidase, nitric oxide synthase, lipoxygenases, cyclooxygenases, xanthine oxidase and cytochrome P450 enzymes and the mitochondrial respiratory chain (9-10). For instance  $H_2O_2$  at low concentration plays an important role in intracellular signaling in the vasculature such as regulating vascular tone, cell growth and proliferation, cytoskeletal reorganisation, barrier functions, inflammatory responses and vascular remodeling. When it is produced in higher concentrations it causes oxidative injury in tissues by oxidating proteins and lipids and inducing DNA strand breaks. Another important oxidative agent is peroxynitrite ( $ONOO^-$ ) which is generated in the reaction of

superoxide anions ( $O_2^-$ ) and nitric oxide (NO) when the level of these molecules are very high in tissues. In this case the reaction of these two molecules is favoured instead of superoxide with SOD and NO with heme (11). Peroxynitrite can also induce pathophysiological alterations like protein modifications from which tyrosine nitration is the most known, DNA modifications, alterations in signal transduction pathways, changes in inflammatory responses, endothelial glycocalyx disruption, up-regulation of adhesion molecules in endothelial cells and leading to cell death through apoptosis and necrosis in endothelial and vascular smooth muscle cells. Products of oxidative injury, such as 3-nitrotyrosine, are used as biomarkers of oxidative stress in tissues (12).

Furthermore, ROS-induced injury of the ion channels and the decreased amount of high energy phosphates can lead to profound alteration in smooth muscle cell calcium homeostasis and therefore to increased smooth muscle cell proliferation and contractility (13-16).

ROS and especially  $ONOO^-$  are responsible for single-strand DNA breakage and consequent poly(ADP-ribose) polymerase 1 (PARP) activation. PARP is a highly conserved 116 kDa weight nuclear enzyme. It has several physiological cellular functions like DNA repair, gene transcription, cell cycle progression. However, severe DNA damage like single and double strand breaks leads to the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) which uses the energy sources of cells by transferring ADP-ribose units from  $NAD^+$  to nuclear proteins such as histones and PARP-1 itself. This process results in rapid depletion of  $NAD^+$  and intracellular ATP pools and impaired mitochondrial respiration, leading to cellular dysfunction, apoptosis or necrosis (17-18).

### *2.1.2 Cell signaling mechanism in hypertension*

The increased vascular tensile stress, the oxidative stress and neurohumoral factors in hypertension equally influence the activity of downstream signaling molecules (19). The most important signaling pathways are the Jak/STAT system, several PKC isoenzymes and MAP kinases (19-21). MAPKs are protein-serine/threonine kinases that are involved in cell differentiation, cell growth and apoptosis.

One of the consequences of oxidative stress-induced cellular injury is the significant increase in the formation of single stranded DNA breaks which activates poly(ADP-ribose)polymerase-1 (PARP-1) (22-23) leading to the depletion of cellular  $NAD^+$  and ATP pools slowing the rate of glycolysis and mitochondrial respiration, so eventually PARP activation leads to cellular dysfunction and death (24-26) .

PARP activation can induce ROS production, calcium elevation, and activates JNK, p38 MAP kinase and RIP1 which can destabilize mitochondrial membrane system leading to the release pro-apoptotic proteins from the mitochondrial inner membrane space, like Cytochrome C, AIF and endonuclease G (27-29). In addition, PARP activation can activate NF-kappaB and AP-1 transcription factors (30-31) which can contribute to cardiovascular remodeling (32-33).

### 2.1.3 Experimental model of chronic hypertension

The spontaneously hypertensive rat model (SHR) is a widely used model for studying the development and the consequences of hypertension. Several similar features between human primary hypertension and hypertension in SHR were showed both in pathophysiology and the clinical course of this disease. Okamoto and Aoki developed originally SHR strain during the 1960s by selective breeding Wistar Kyoto rats with high blood pressure. SHRs start to develop persistent hypertension after approximately 6 weeks of age but the cause of the rising blood pressure remains unknown. Their systolic blood pressure reaches 200-220 mmHg by adulthood. Between the age of 40 and 50 weeks they start to develop vascular and myocardial hypertrophy. These rats are also characterized by altered vascular tone, increased vascular contractility and by the reduction of distensibility and vascular compliance resulting from wall media hypertrophy and endothelial dysfunction also develops (34).

In our research, as PARP-inhibitor L-2286 was used. L-2286 is derived from 2-mercapto-4(3H)-quinazoline by alkylation with 1-(2-chloroethyl)piperidine (35). L-2286 was chosen, because in an vitro PARP assay it exhibited significantly better PARP inhibitory activity than basic quinazolines such as 4-hydroxyquinazoline or 2-merkapto-4(3H)-quinazolinone (36) (Figure 1.)

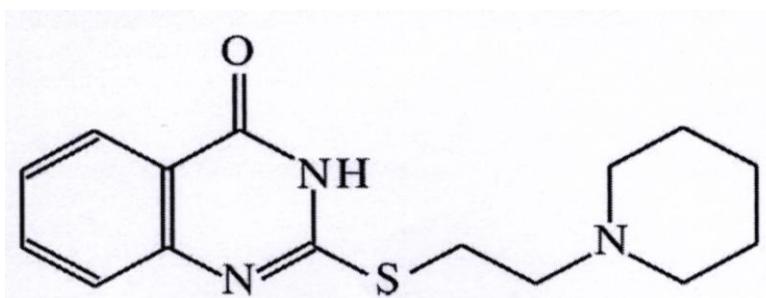


Figure 1. Chemical structure of L-2286 (2-[(2-piperidin-1-yl)ethyl]thio]quinazolin-4(3H)-one.

## *2.2. Biological effects of resveratrol*

French people tend to have a lower incidence of cardiovascular diseases despite having similar coronary risk factors as people in other industrialized countries, which phenomenon is known as the French paradox and attributed to the higher red wine intake by the French (37). Red wine contains high amount of polyphenolic compounds like resveratrol (trans-3,4,5-trihydroxystilbene), epicatechin, catechin, gallic acid, quercetin. Primarily resveratrol is thought to be responsible for the cardioprotective effect of red wine. Several studies supported its antioxidant activity, its ability to decrease low-density lipoprotein (LDL) oxidation (38), and function as a direct free radical scavenger (38). Resveratrol improves endothelial function, and has numerous beneficial effects on vascular tone and vessels in human and animal models. It has been shown that resveratrol improves the release of nitric oxide (NO) and prostacyclin (PGI) which both play a prominent role in the maintenance of endothelial function (39). In endothelial cells obtained from human umbilical vein, resveratrol enhanced the activity of endothelial nitric oxide synthase (eNOS) promoter. (40). In other in vitro human investigations resveratrol resulted in NO depending relaxation of vascular rings of saphenous vein and internal mammary artery (39). Furthermore resveratrol plays an important role in the mitigation of platelet aggregation (41). The protective effect of resveratrol against thrombosis can be explained by the regulation of prostaglandin synthesis with reversible cyclooxygenase 2 (COX2) and irreversible cyclooxygenase 1 (COX1) inhibition. On thromboxane A2 (TXA2), which is produced by COX1 in platelets and enhances aggregation, resveratrol also has an inhibitory effect (42).

It has already been shown that resveratrol reduces serum cholesterol and triglyceride levels in rats (38). It has also been observed that the size, and the density of atherosclerotic lesions in the thoracic aorta as well as the thickness of intima were decreased and flow mediated

dilatation (FMD) was improved by resveratrol (43). Several experiments have been carried out on FMD of the brachial artery, showing the ability of endothelium dependent dilatation of a vessel, which was further increased by red grape polyphenol extract (44).

### **3. Aims of the study**

- 3.1. We tried to reveal the potential role of PARP activation in the pathogenesis of chronic hypertension-induced vascular remodeling.
  - 3.1.1. The aim of this work was to provide evidence for a new molecular mechanism of the vasoprotective effect of PARP inhibition.
  - 3.1.2. We tried to provide evidence whether long-term treatment with L-2286 could beneficially influence intima-media thickness of carotid arteries and aortic stiffness index
  - 3.1.3. We tried to prove the protective effect of L-2286 on vasomotor dysfunction
  - 3.1.4. We estimated the fibrosis decreasing effect
  - 3.1.5. We tested whether PARP inhibition had beneficial effect on signal transduction pathways taking part in vascular remodeling
  
- 3.2. We investigated if resveratrol had a clinically measurable cardioprotective effect in patients after myocardial infarction receiving proper secondary preventive drug treatment.
  - 3.2.1. We tried to examine the effect of resveratrol treatment on echocardiographic parameters.
  - 3.2.2. We hypothesised that resveratrol could enhance endothelial function.
  - 3.2.3. We tested whether resveratrol influenced different hemorheological and laboratory parameters.

## 4. Materials and methods

### 4.1. Animal model and noninvasive blood pressure measurement

Ten-week-old male SHR rats obtained from Charles River Laboratories (Budapest, Hungary) were randomly divided into two groups. One group received no treatment (SHR-C, n = 10), whereas the other group (SHR-L, n = 10) received 5 mg/b.w. in kg/day L-2286 (2-[(2-Piperidine-1-ylethyl)thio]quinazolin- 4(3H)-one), a water-soluble PARP inhibitor for 32 weeks. The third group was an age-matched normotensive control group (WKY-C, n=10, Charles River Laboratories, Budapest, Hungary). The fourth was a normotensive age-matched group receiving 5 mg/b.w. in kg/day L-2286 treatment (WKY-L, n=10). The dose of L-2286 was based on our previous results with this PARP inhibitor (45,46). According to these data, L-2286 can exert protective effects against oxidative cell damage in concentration of 10  $\mu$ M. The serum concentration of L-2286 in the applied dose (5 mg/kg/day) with an estimated average bioavailability is approximately 10  $\mu$ M in rats. L-2286 was dissolved in drinking water on the basis of preliminary data about the volume of daily consumption, but the water was provided ad libitum throughout the experiment. Before the administration of L-2286 and at the end of the 32-week treatment period, ultrasound measurements were performed. Noninvasive blood pressure measurement was carried out in every fourth week from the beginning of the study using tail-cuff method (Hatteras SC 1000 Single Channel System) (47). At the end of the study animals were euthanized with an overdose of ketamine hydrochloride intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p., Biochemie GmbH, Kundl, Austria). Carotid arteries and aortas were removed under an Olympus operation microscope and were freeze-clamped and stored at -70°C or fixed in 10% formalin. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs Medical School.

### 4.2. Transthoracic echocardiography

At baseline, all animals were examined by ultrasound to exclude rats with any abnormalities. Two-dimensional ultrasound was performed under inhalation anesthesia at the beginning of the experiment and on the day of sacrifice. Rats were lightly anesthetized with a mixture of 1.5% isoflurane and 98.5% oxygen. The necks and the upper part of the chests of the animals were shaved, acoustic coupling gel was applied, and warming pad was used to maintain

normothermia. Aortic stiffness index (ASI) and intima-media thickness of carotid arteries (IMT) were measured by a VEVO 770 high-resolution ultrasound imaging system (VisualSonics, Toronto, Canada) - equipped with a 40 MHz transducer. Aortic elastic property was calculated according to a previously proposed and evaluated formula (48):  $(ASI) = \ln(SBP/DBP) \times DD / (SD - DD)$ .

#### *4.3. Isometric force measurement*

The method was performed according to a standard protocol. The common carotid arterial (CCA) ring isolated from WKY, SHR-C and SHR-L rats. The contractile force was measured isometrically by using standard bath procedures as described previously (49). After ketamin-xylasin anesthesia the carotid arteries were removed, quickly transferred to ice cold (4°C) oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) physiological Krebs solution (PSS), and dissected into 5-mm rings. Each ring was positioned between two stainless steel wires (diameter 0.0394 mm) in a 5-ml organ bath of a Small Vessel Myograph (DMT 610M, Danish Myo Technology, Aarhus, Denmark). The normalization procedure was performed to obtain the basal tone to 1.0 g (13,34 mN), and artery segments were allowed to stabilize for 60 min before measurements. The software Myodaq 2.01 M610+ was used for data acquisition and display. The rings were precontracted and equilibrated for 60 min until a stable resting tension was acquired. Tension is expressed as a percentage of the steady-state tension (100%) obtained with isotonic external 60mM KCl. Cumulative response curves were obtained for Sodium Nitroprussid (SNP), Acetil choline (ACh), and KCl in the presence of endothelium. The bath solution was continuously oxygenated with a gas mixture of 95% O<sub>2</sub> plus 5% CO<sub>2</sub>, and kept at 36.8°C (pH 7.4). In the first series of experiments, the carotid rings were exposed to increasing doses of SNP (10<sup>-9</sup> to 10<sup>-5</sup> mol/L), ACh (10<sup>-9</sup> to 10<sup>-5</sup> mol/L) and in some experiments, arterial rings showing relaxation to ACh of more than 70% were counted as endothelium intact. At the end of the experiments the administration of 60 mM KCl was repeated to examine the viability of the carotid arteries. Each experiment was carried out on rings prepared from different rats. The composition of PSS (in mMol) was 119 NaCl, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 1.2 Mg<sub>2</sub>SO<sub>4</sub>, 11.0 glucose and 1.6 CaCl<sub>2</sub>.

#### 4.4. Immunohistochemistry and confocal laser fluorescence microscopy

Carotid arteries and aortic segments separated for immunohistochemical and immunofluorescence processing were fixed immediately after excision in buffered paraformaldehyde solution (4%) for 1 day. Five micrometer thick sections were cut.

Immunohistochemical staining was performed for nitrotyrosine and for poly(ADP-ribose) (PAR). Primary antibodies used for the stainings were monoclonal mice anti-nitrotyrosine antibody (Upstate, Chicago, USA) and anti-PAR (Alexis Biotechnology, London, UK). Binding was visualized with biotinylated secondary antibody followed by the avidin-biotin-peroxidase detection system (Universal Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). Using 3,3'-diaminobenzidine (DAB) as the chromogen, progress of the immunoreaction was monitored under a light microscope and the reaction was stopped by the removal of the DAB. Some slices were stained with Masson's trichrome staining to detect the interstitial fibrosis as described earlier and quantified with the NIH ImageJ analyzer system. All histological samples were examined by an investigator in a blinded fashion (50).

Fluorescence immunohistochemistry was performed for apoptosis inducing factor (AIF), NF-kappa B (NF-κB) and MAP kinase phosphatase-1 (MKP-1). Primary antibodies used for the staining were polyclonal rabbit antibodies (Cell Signaling Technology, Kvalitex Kft. and Santa Cruz Biotechnology). As secondary antibody donkey-anti-rabbit antibody (R and D Systems, NorthernLights, fluorochrome-labeled antibody; 1:200) was used. The stainings were finished by Hoechst (Sigma) counterstain. Sections were examined using a confocal laser scan microscope (Olympus Fluoview 1000). Recording for RRX (excited with the green laser, Helium-Neon) was followed by recording for Hoechst with a 405 nm laser.

#### 4.5. Western blot analysis

Carotid arteries and aortic segments were homogenized in ice-cold 50 mM Tris-buffer, pH 8.0 (containing protease inhibitor cocktail 1:1000, and 50 mM sodium vanadate, Sigma-Aldrich Co., Budapest) and harvested in 2x concentrated SDS-polyacrylamide gel electrophoresis sample buffer. Proteins were separated on 10 or 12% SDS-polyacrylamide gel electrophoresis sample buffer. After blocking (2 h with 3% non-fat milk in Tris-buffered saline), membranes were probed overnight at 4°C with antibodies recognizing the following antigens: phospho-specific Akt-1/protein kinase B-α<sup>Ser473</sup> (1:1000), anti-actin (1:10000), phospho-specific extracellular signal regulated kinase (ERK 1/2)<sup>Thr183-Tyr185</sup> (1:1000), phosphorylated p38 mitogen-activated protein kinase<sup>Thr180-Gly-Tyr182</sup> (p38 MAPK) (1:1000), phospho-specific c-Jun

N-terminal kinase (JNK) (1:1000), phosphorylated MAP kinase phosphatase-1<sup>Ser359</sup> (MKP-1) anti poly(ADP-ribose) (anti-PAR, 1:5000). Antibodies were purchased from R and D Systems, Biomedika Kft., Hungary, except for anti-actin, which was bought from Sigma-Aldrich Co, Budapest, Hungary and anti-PAR, which was bought from Alexis Biotechnology, London, UK. Membranes were washed six times for 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween (TBST) before the addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution, Bio-Rad, Budapest, Hungary). The antibody–antigen complexes were visualized by means of enhanced chemiluminescence. After scanning, results were quantified by NIH Image J program.

#### *4.6. Electron microscopy*

For electron microscopy blocks were prepared from the same aortic segments that were used for light microscopy. 1-mm long blocks were cut from the aorta and were fixed in 4% buffered formaldehyde mixed with a 2,5% glutaraldehyde solution for 24 hours in refrigerator. After washing in phosphate buffer the samples were fixed in 1% osmium tetroxide in 0.1 M PBS for 35 minutes. After that the samples were washed after that in buffer several times for 10 minutes each and dehydrated in an ascending ethanol series, including a step of uranyl acetate (1%) solution in 70% ethanol to increase the contrast. Dehydrated blocks were transferred to propylene oxide before being placed into aluminum-foil boats containing Durcupan resin (Sigma) and then embedded in gelatin capsules containing Durcupan. The blocks were placed in termostate for 48 hours at 56°C. From the embedded blocks 1 micrometer thick semithin and serial ultrathin sections (70 nanometer) were cut with a Leica ultramicrotome, and mounted either on mesh, or on Collodion-coated (Parlodion, Electron Microscopy Sciences, Fort Washington, PA) single-slot, copper grids. Additional contrast was provided to these sections with uranyl acetate and lead citrate solutions, and they were examined with JEOL 1200EX-II electron microscope (50).

#### *4.7. Statistical analysis*

All data are expressed as mean  $\pm$  SEM. Comparisons among groups were made by using Student's t-test (SPSS for Windows 11.0). To post hoc comparison, Bonferroni test was applied. Values of  $p < 0.05$  were considered statistically significant.

## 5. Results

### 5.1. Effect of L-2286 treatment on systolic blood pressure

During the 32-week-long treatment period systolic blood pressure of hypertensive (SHR-C, SHR-L) rats were significantly higher than that of normotensive (WKY) rats (at the age of 10 week SHR:  $180 \pm 5.6$  Hgmm, WKY:  $130 \pm 5.4$  Hgmm, at the age of 42 weeks SHR-C:  $230 \pm 6.3$ , WKY-C:  $130 \pm 5.4$ , WKY-L:  $135 \pm 5.5$  Hgmm) ( $p < 0.05$  SHR-C vs. WKYs). L-2286 treatment did not alter systolic blood pressure of SHR animals (SHR-C:  $230 \pm 6.3$ , SHR-L:  $225 \pm 2.4$  Hgmm) during the 32-week-long treatment period (Figure 2.).

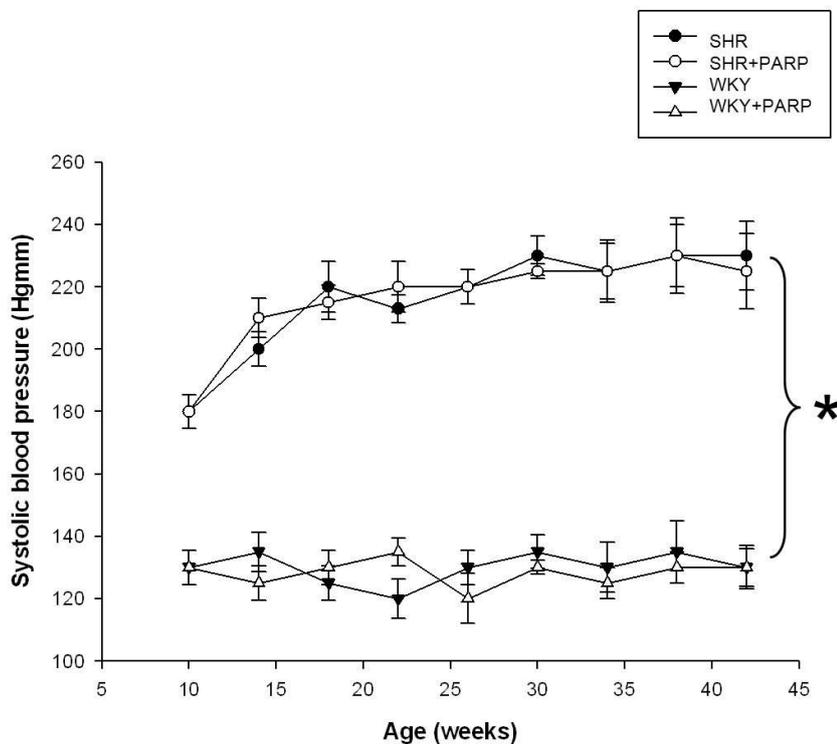


Figure 2. Systolic blood pressure values of normotensive (WKY-C, WKY-L) and hypertensive (SHR-C, SHR-L) rats. Values are means  $\pm$  SEM. \* $p < 0.05$  (WKY vs. SHR).

### 5.2. Effect of L-2286 administration on IMT of carotid arteries and on aortic stiffness

At the beginning of the study there was no significant difference between the normotensive (WKY) and the hypertensive (SHR) animals (IMT: WKY:  $39 \pm 2.5$   $\mu$ m vs. SHR:  $40 \pm 2.3$   $\mu$ m; ASI: CFY:  $3.79 \pm 0.46$ , SHR:  $3.86 \pm 0.41$ ). IMT of normotensive animals did not change during the 32-week-long treatment period. However, in hypertensive rats IMT of carotid arteries almost doubled by the end of the study period (WKY:  $41 \pm 2$   $\mu$ m vs. SHR-C:  $78 \pm 5$   $\mu$ m,

p<0.05). L-2286 treatment decreased significantly the thickness of carotid arteries induced by elevated blood pressure (SHR-L: 63±1 μm, p< 0.05) (Figure 3A).

Arterial stiffness index of the aorta (ASI) changed in parallel with the change of IMT. ASI values increased significantly in hypertensive rats and were significantly reduced by L-2286 treatment (at the age of 10 weeks: WKY: 3.79±0.46, SHR: 3.86±0.41), (at the age of 42 weeks CFY: 4.1±0.1 vs. SHR: 5.8±0.3, p<0.01; SHR-L: 4.3±0.4 p<0.05) (Figure 3B).

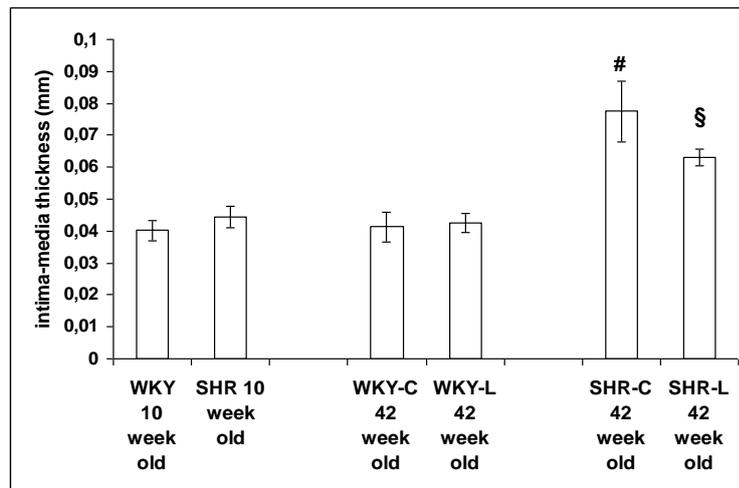


Figure 3A. Intima-media thickness (IMT) of carotid arteries in normotensive (WKY-C, WKY-L) and hypertensive (SHR-C, SHR-L) rats. Values are means±SEM. #p<0.05 (WKY vs. SHR-C 42 week old), § p<0,05 (SHR-C vs. SHR-L 42-week old).

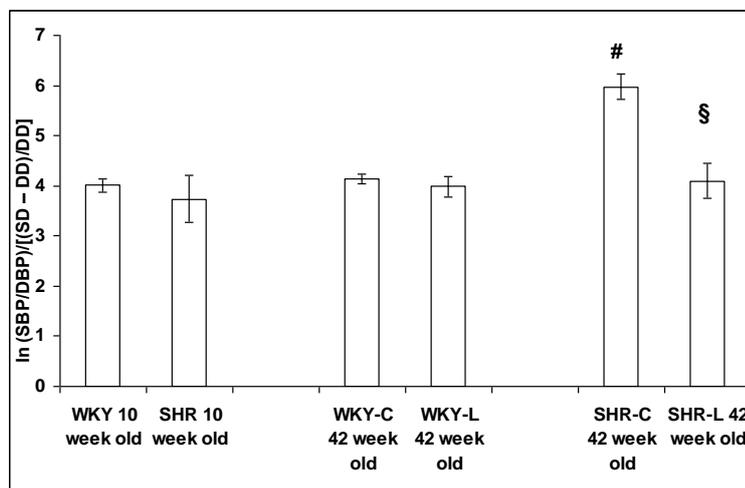


Figure 3B. Arterial stiffness index (ASI) of aorta of normotensive (WKY-C, WKY-L) and hypertensive (SHR-C, SHR-L) rats. Values are means±SEM. #p<0.05 (WKY vs. SHR-C), § p<0.05 (SHR-C vs. SHR-L).

### 5.3. Effect of L-2286 administration on poly(ADP-ribosyl)ations in aortas and carotid arteries

To determine whether the administered PARP-inhibitor treatment was effective, we detected the poly(ADP-ribosyl)ation of proteins in the samples. Western blot analysis of the great vessels revealed that ADP-ribosylation of proteins was the highest in the SHR-C group. This elevation was significantly decreased by L-2286 treatment ( $p < 0.05$ ). (Figure 4A, 4B)

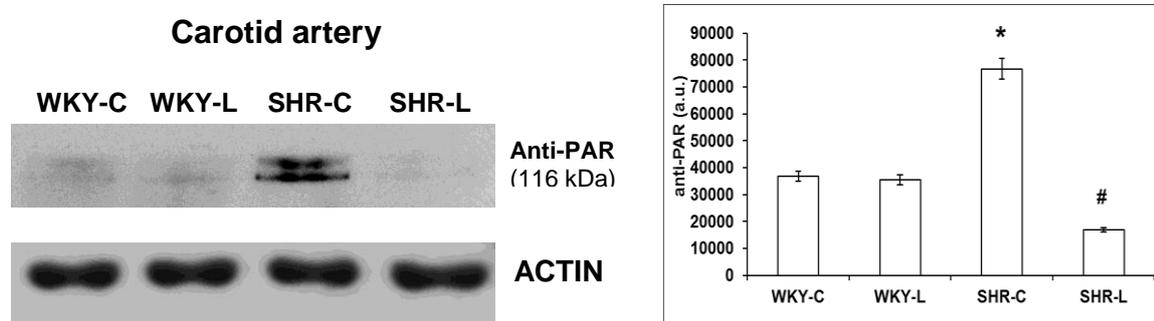


Figure 4A

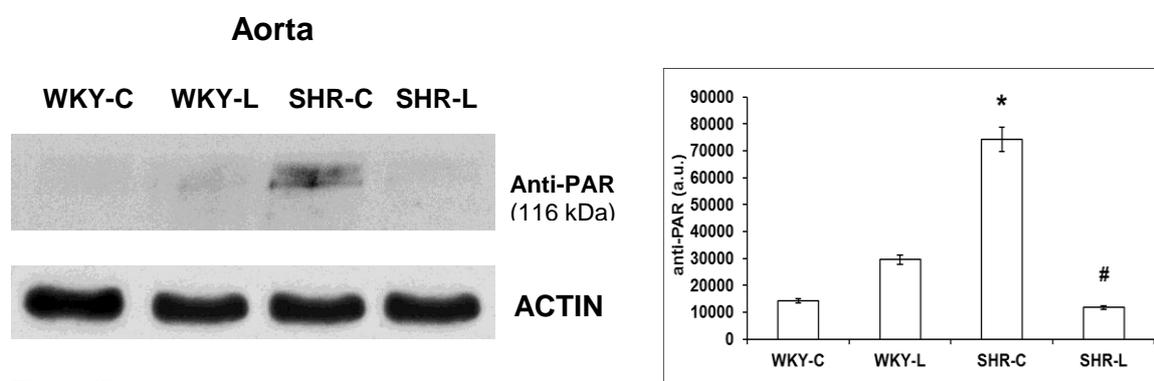


Figure 4B

Effect of L-2286 treatment on PARP activation in carotid arteries and aortas of normotensive (WKY-C, WKY-L) and hypertensive (SHR-C, SHR-L) rats. Representative western blot analysis of, anti-PAR and densitometric evaluation are shown ( $n = 4$ ). Actin is shown as loading control. Values are means  $\pm$  SEM. \* $p < 0.05$  WKY vs. SHR-C, # $p < 0.05$  SHR-C vs. SHR-L.

*5.4. Effect of L-2286 treatment on the phosphorylation state of Akt-1 and MAP kinases in carotid arteries and aortas*

Akt-1<sup>Ser473</sup> was activated in 42-week-old WKY-C rats but its activity was higher in the SHR-C group ( $p < 0.05$ ), which was further increased in SHR-L ( $p < 0.05$ ) animals due to the PARP inhibitor treatment. Phosphorylation of Akt-1<sup>Ser473</sup> was also increased in the WKY-L group compared to WKY-C rats ( $p < 0.05$ ). In case of MAPK kinases, the modest phosphorylation of the examined MAPKs — p38-MAPK<sup>Thr180-Gly-Tyr182</sup>, JNK, and ERK1/2<sup>Thr183-Tyr185</sup> — occurred in WKY rodents ( $p < 0.05$  vs. SHR groups). The phosphorylation of p38-MAPK<sup>Thr180-Gly-Tyr182</sup>, ERK1/2<sup>Thr183-Tyr185</sup> and JNK in SHR-C rats was the highest, and these alterations were significantly attenuated by the L-2286 treatment ( $p < 0.01$ ) (Figure 5).

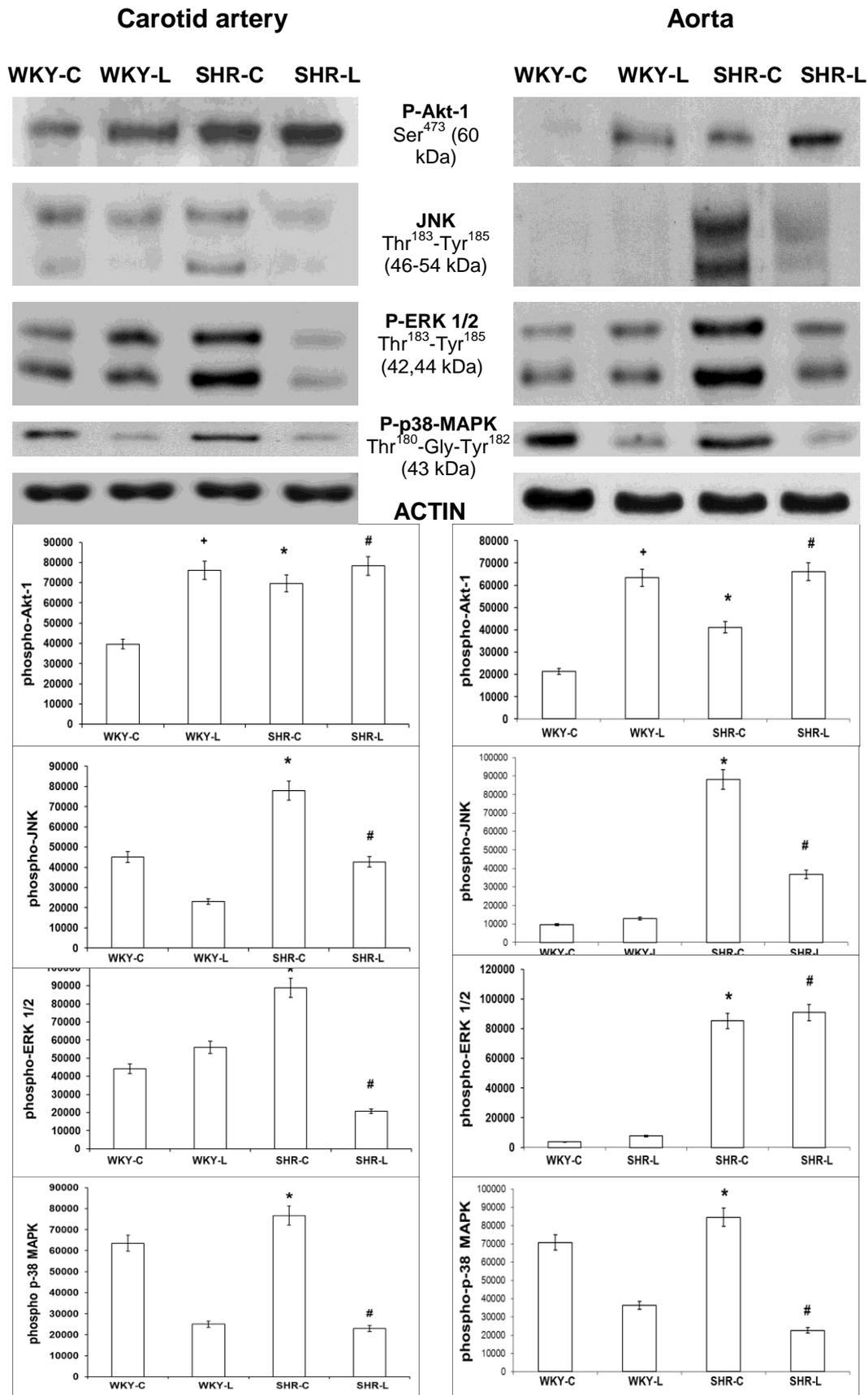


Figure 5. Effect of L-2286 treatment on the phosphorylation state of Akt-1Ser473, JNK<sup>Thr183-Tyr185</sup>, ERK<sup>1/2Thr183-Tyr185</sup> and p38-MAPK<sup>Thr180-Gly-Tyr182</sup> in carotid arteries and aortas of normotensive (WKY-C, WKY-L) and hypertensive (SHR-C, SHR-L) rats. Representative western blot analysis of Akt-1<sup>Ser473</sup>, JNK<sup>Thr183-Tyr185</sup>, ERK<sup>1/2Thr183-Tyr185</sup> and p38-MAPK<sup>Thr180-Gly-Tyr182</sup> phosphorylation, and densitometric evaluation are shown (n = 4). Actin is shown as loading control. Values are means±SEM. \*p< 0.05 WKY vs. SHR-C, #p< 0.05 SHR-C vs. SHR-L, + p< 0.05 WKY-C vs. WKY-L.

### 5.5. Collagen content in vascular wall of aorta and carotid arteries

Masson's trichrome staining was used to evaluate the degree of vascular fibrosis. The highest collagen content was observed in carotid arteries of non-treated hypertensive rats (SHR-C) (WKY: 18.16±0.61%, SHR-C: 26.23±0.68%, SHR-L: 20.86±1.05%,  $p < 0.05$  SHR-C vs. WKY groups). A similar result could be seen in the aortic wall as well (WKY: 19.22±0.8%, SHR-C: 27.62±1.45%, SHR-L: 21.24±0.63%,  $p < 0.05$  vs. WKY groups and vs. SHR-L) and the vascular fibrosis was significantly moderated by the L-2286 treatment ( $p < 0.05$  vs. SHR-C) (Figure 6.).

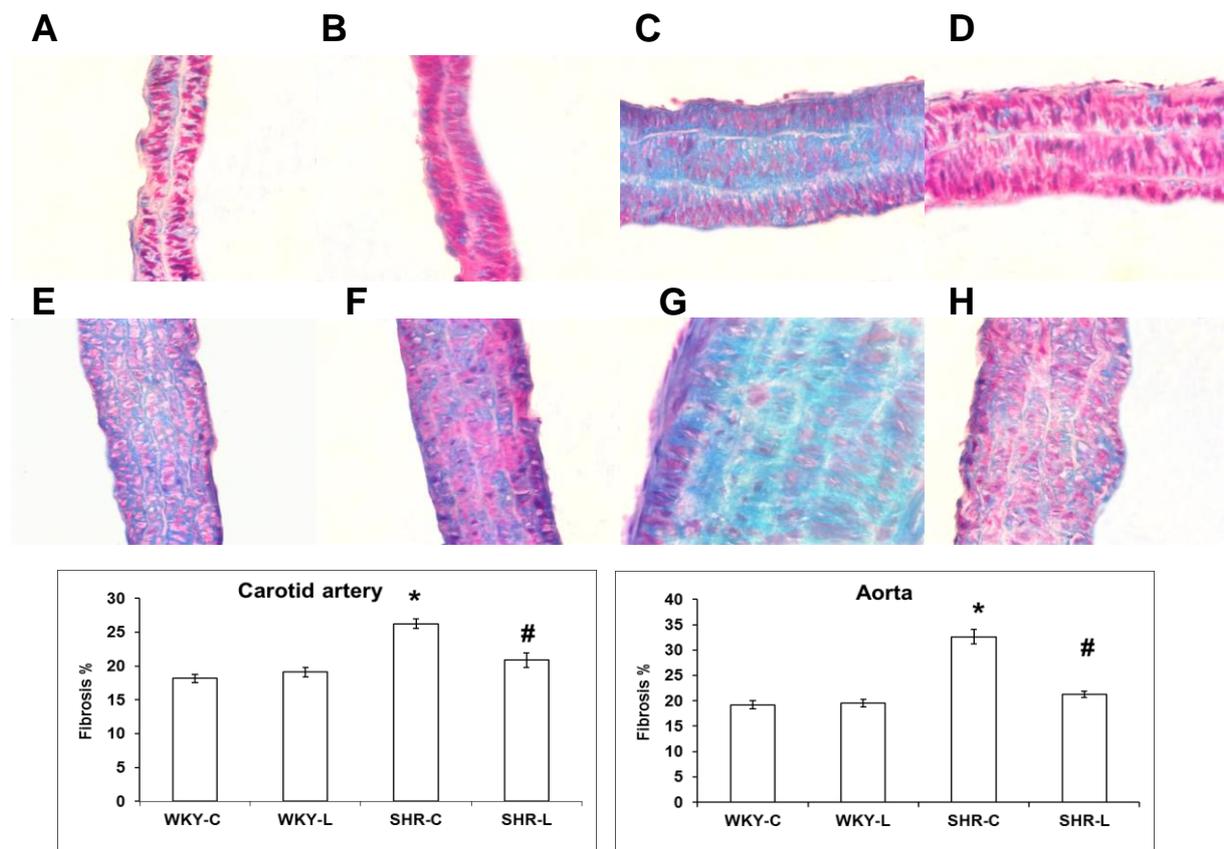


Figure 6. Influence of L-2286 treatment on the deposition of interstitial collagen. Representative histologic sections stained with Masson's trichrome ( $n = 4$ ). Magnifications 40x fold. A: carotid artery of WKY-C, B: carotid artery of WKY-L, C: carotid artery of SHR-C, D: carotid artery of SHR-L, E: aorta of WKY-C, F: aorta of WKY-L, G: caorta of SHR-C, H: aorta of SHR-L. Values are means±SEM. \* $P < 0.05$  CFY vs. SHR, # $P < 0.05$  SHR-C vs. SHR-L.

### 5.6. Immunohistochemical analysis

Immunohistochemical staining showed a highly elevated expression of nitrotyrosine in aortas and in carotid arteries of hypertensive animals (SHR-C) compared to that of the control groups ( $p < 0.05$  vs. WKY-C and WKY-L). The treatment with L-2286 significantly decreased

the degree of nitrosative stress of the vasculature in the SHR-L group ( $p < 0.05$  vs. SHR-C) (Figure 7).

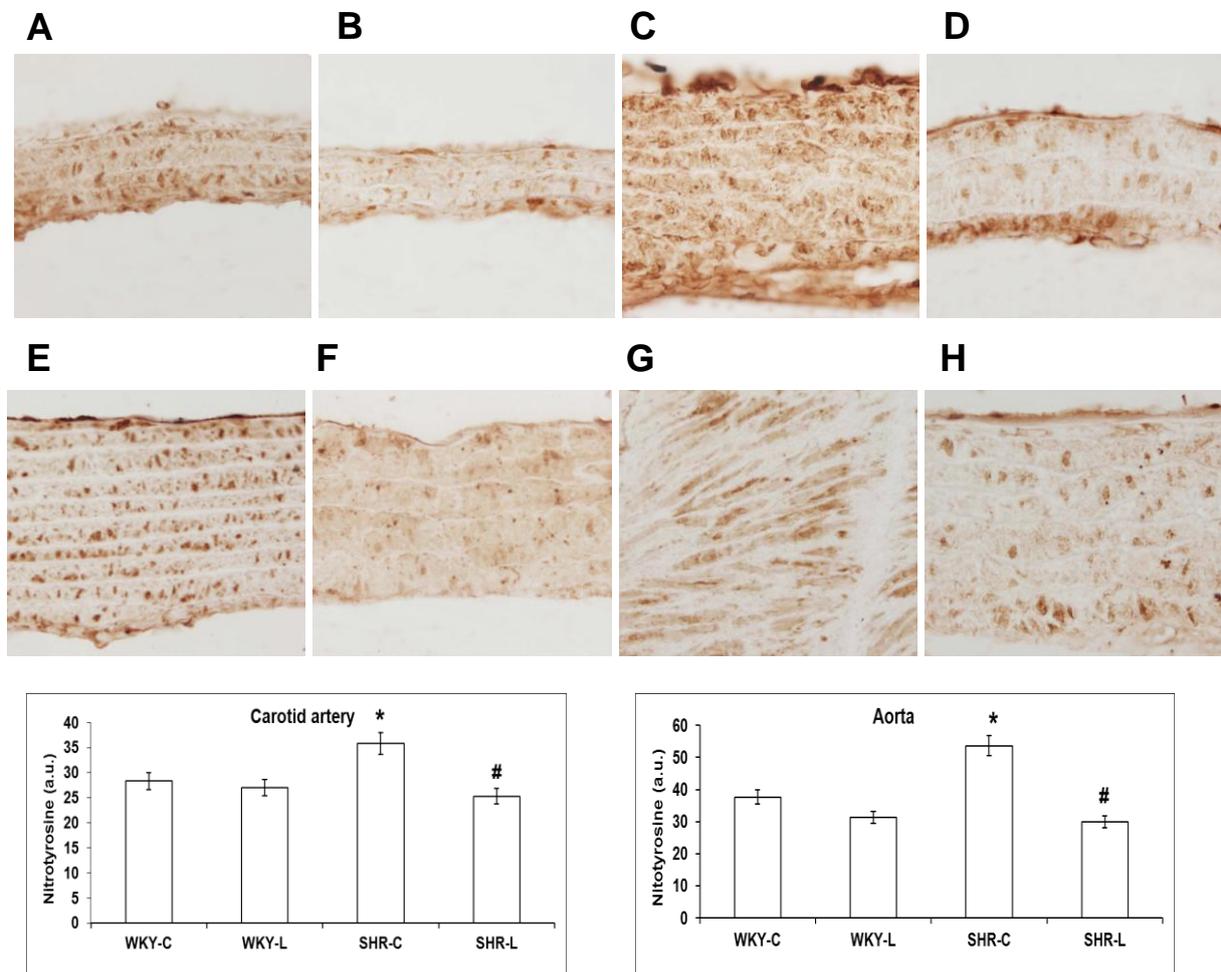


Figure 7. Photomicrographs of immunohistochemistry. Representative immunohistochemical stainings for nitrotyrosine formation (NT, brown staining) in the vasculature of normotensive (WKY-C, WKY-L) and hypertensive (SHR-C, SHR-L) animals. Magnification 40 x fold. A: carotid artery of WKY-C, B: carotid artery of WKY-L, C: carotid artery of SHR-C, D: carotid artery of SHR-L, E: aortic wall of WKY-C, F: aortic wall of WKY-L, G: aortic wall of SHR-C, H: aortic wall of SHR-L. Values are means $\pm$ SEM. \* $P < 0.05$  CFY vs. SHR, # $P < 0.05$  SHR-C vs. SHR-L

In normotensive animals (WKY-C and WKY-L) the apoptosis-inducing factor (AIF) could only be found in the cytoplasm of cells (Figure 6.). In the SHR-C group we could demonstrate the nuclear translocation of AIF, which was significantly decreased in the SHR-L group due to the PARP inhibition by the L-2286 treatment ( $p < 0.05$ ) (Figure 8.).

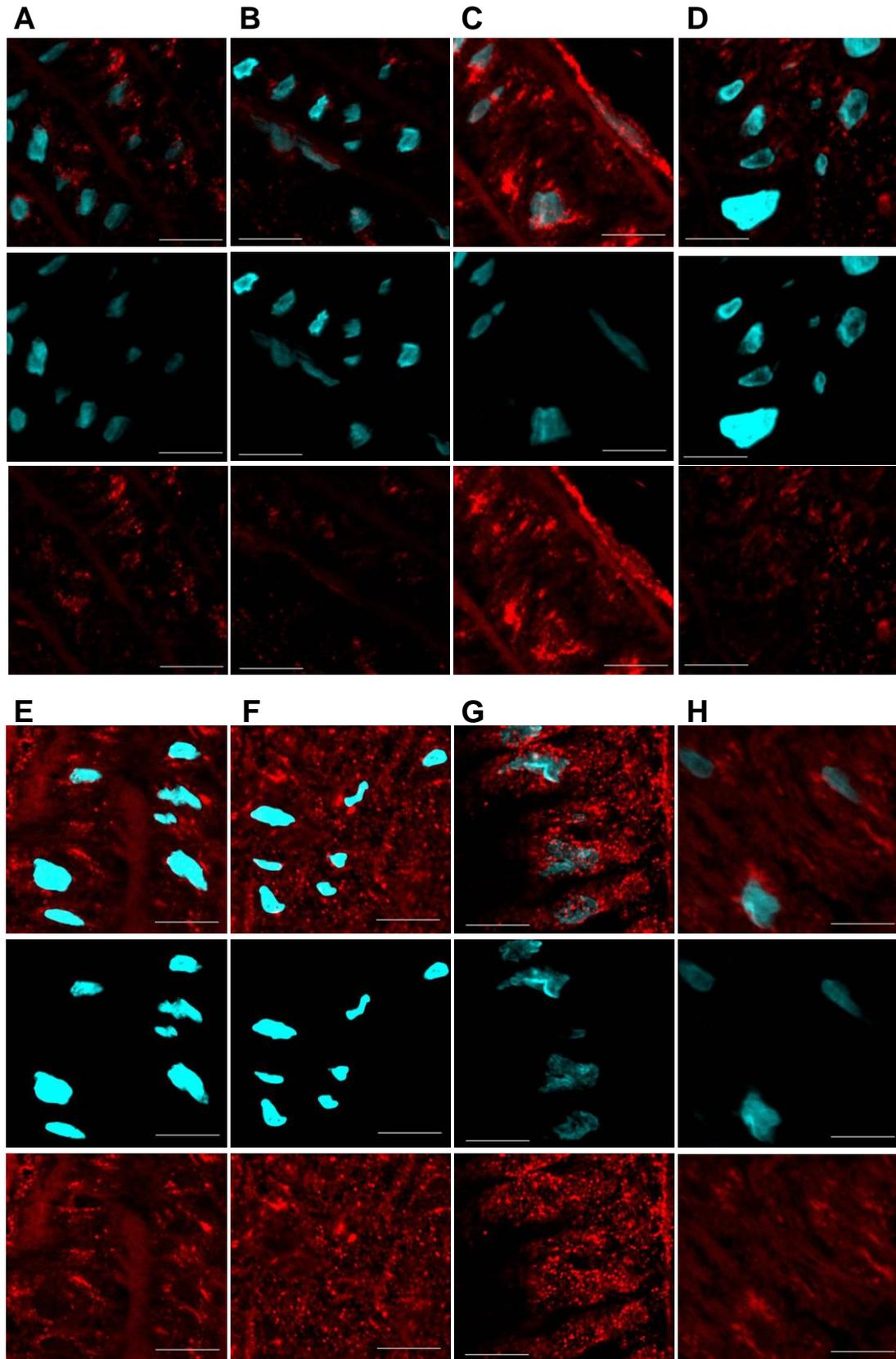


Figure 8. Representative merged confocal images of nuclear localisation of AIF. AIF immunoreactivity (red) and Hoechst nuclear staining (blue) were presented individually and merged (scale bar 50  $\mu$ m) A: carotid artery of WKY-C, B: carotid artery of WKY-L, C: carotid artery of SHR-C, D: carotid artery of SHR-L, E: aortic wall of WKY-C, F: aortic wall of WKY-L, G: aortic wall of SHR-C, H: aortic wall of SHR-L.

Oxidative stress provoked an enhancement in the expression of MKP-1 which is also evident in our samples since MKP-1 activity was more elevated in SHR-C than in WKY animals showed by Western blotting ( $p < 0.05$  vs. WKY-C) and immunofluorescence. Inhibition of PARP enzyme with L-2286 further increased the phosphorylation of MKP-1 in aortas and carotid arteries of hypertensive rats ( $p < 0.05$  vs. SHR-C) (Figure 9,10.).

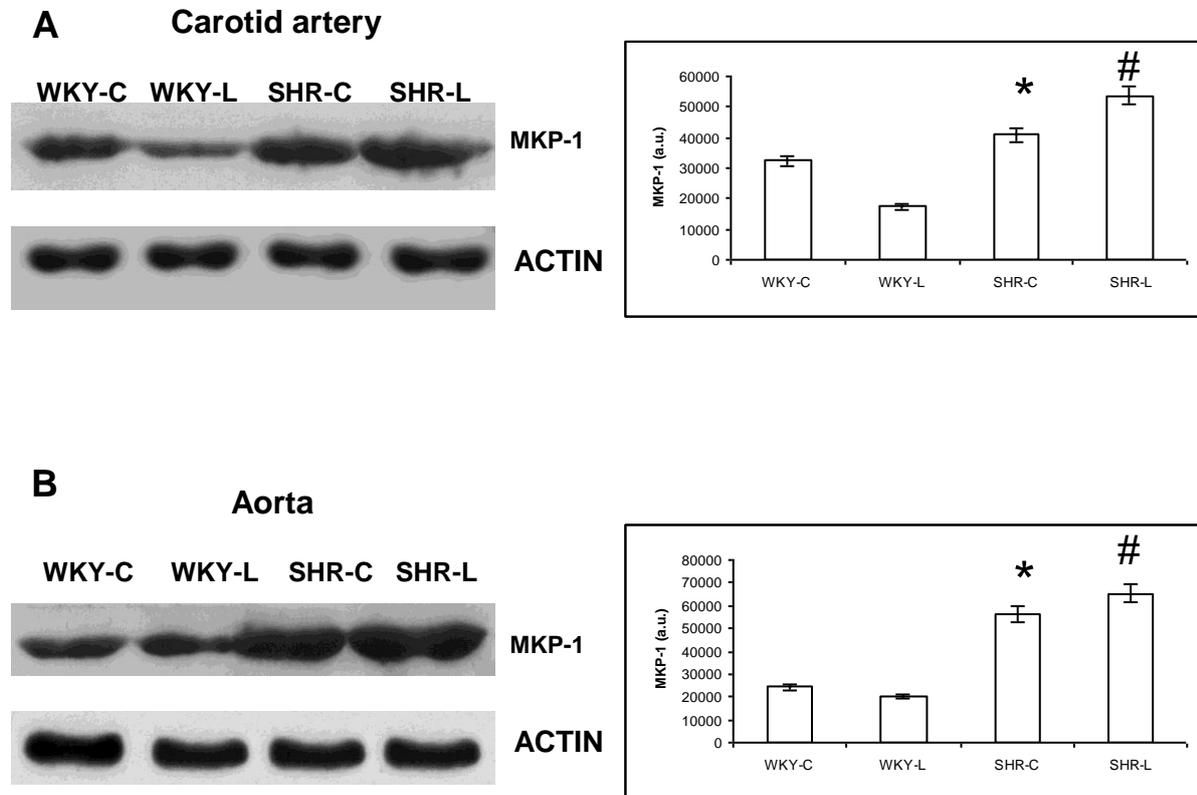


Figure 9 A, B Effect of L-2286 treatment on MKP-1 activation, densitometric evaluation are shown (n = 4). Actin is shown as loading control. Values are means $\pm$ SEM. \* $p < 0.05$  WKY vs. SHR-C, # $p < 0.05$  SHR-C vs. SHR-L.

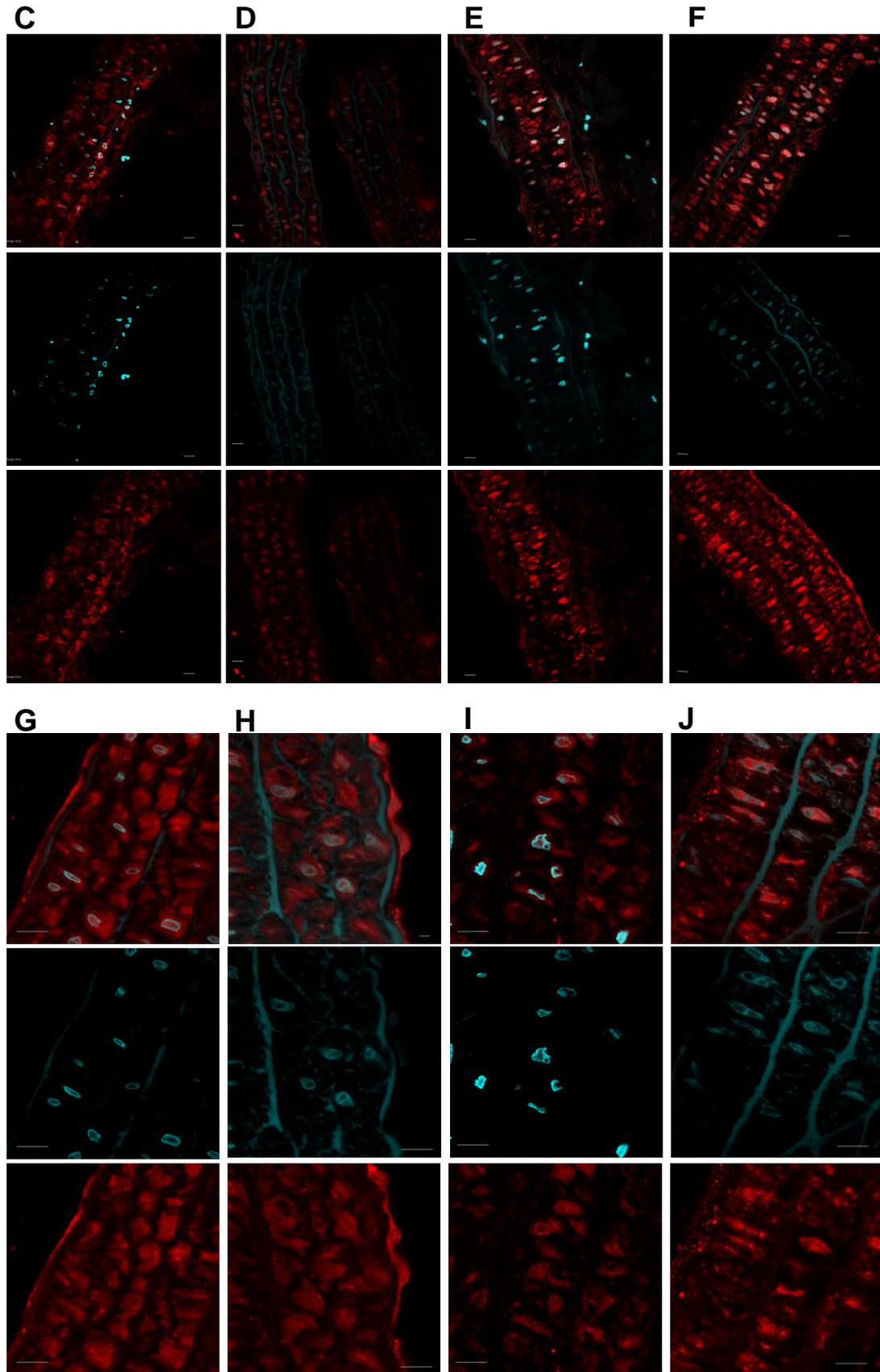
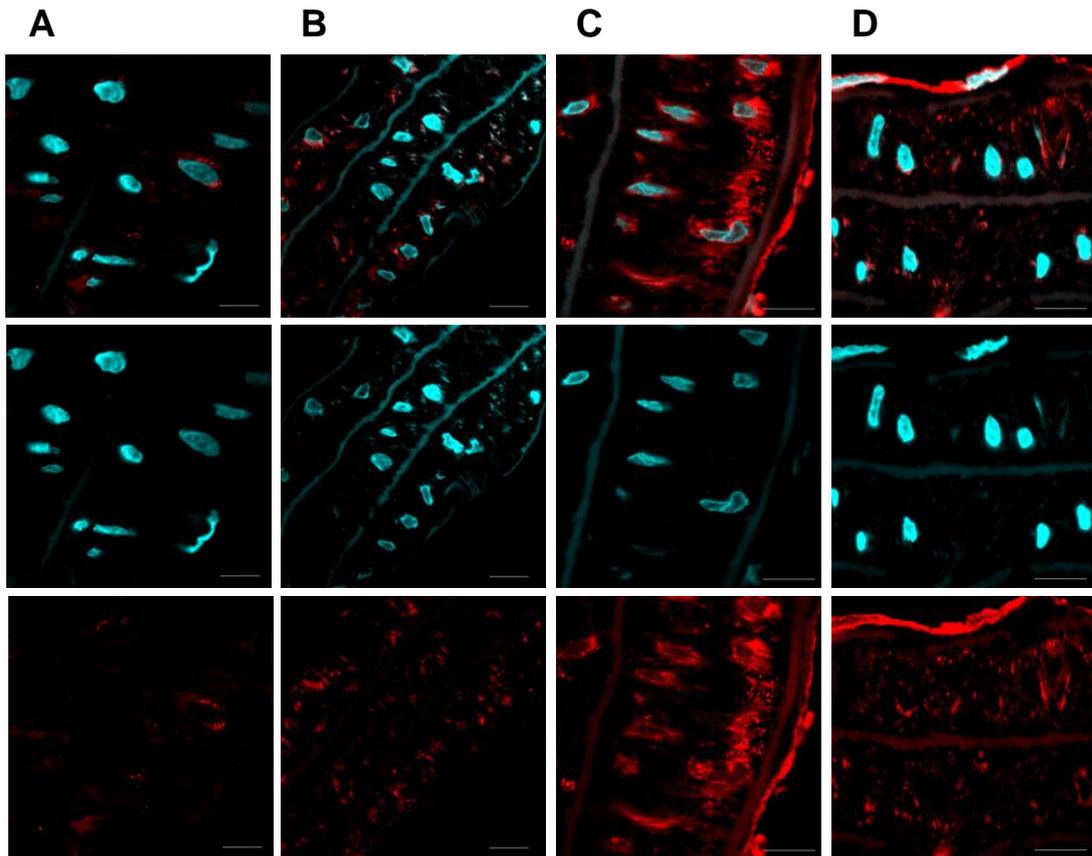


Figure 10. Representative merged confocal images of nuclear localisation of MKP-1. MKP-1 immunoreactivity (red) and Hoechst nuclear staining (blue) were presented individually and merged (scale bar 50  $\mu$ m). C: carotid artery of WKY-C, D: carotid artery of WKY-L, E: carotid artery of SHR-C, F: carotid artery of SHR-L, G: aortic wall of WKY-C, H: aortic wall of WKY-L, I: aortic wall of SHR-C, J: aortic wall of SHR-L.

In control animals NF- $\kappa$ B activity can be seen only in the cytoplasm of cells. Our results showed that hypertension induced the nuclear transport and the activation of NF- $\kappa$ B ( $p < 0.05$  vs. WKY-C). The treatment with L-2286 significantly diminished the nuclear transport and the activity of NF- $\kappa$ B ( $p < 0.05$  vs. SHR-C) (Figure 11.).



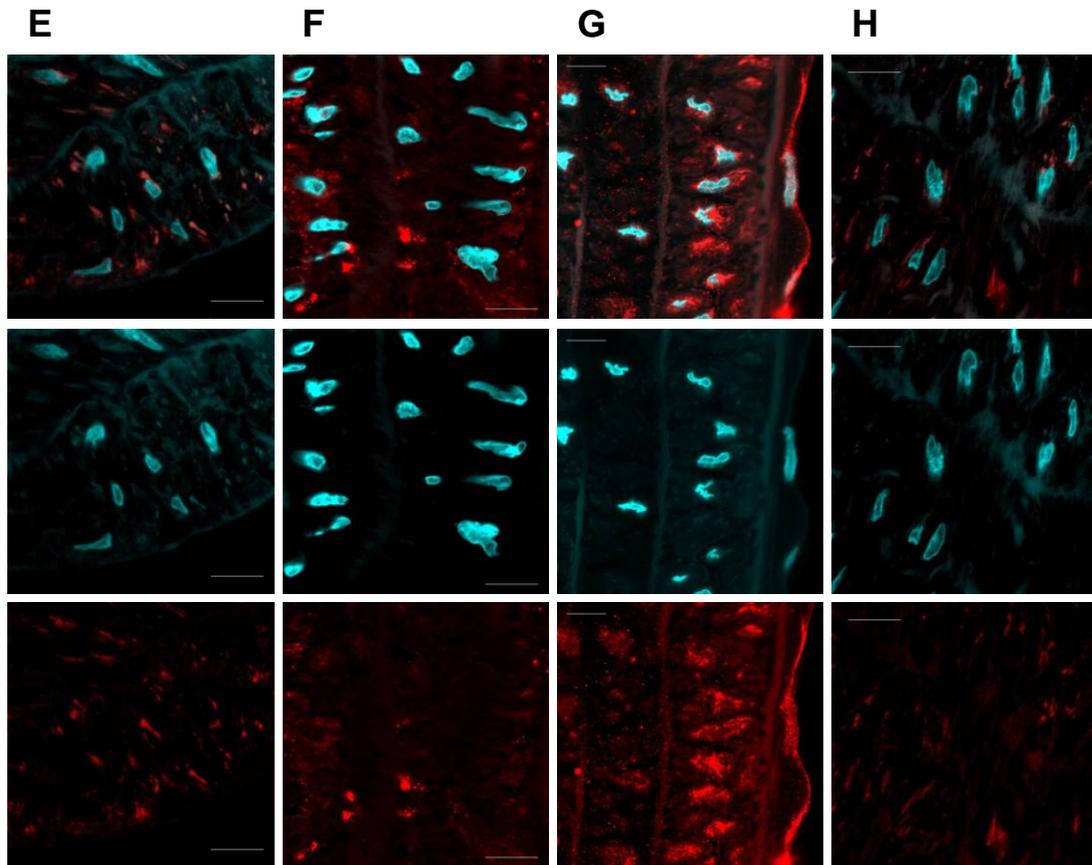


Figure 11. Representative merged confocal images of nuclear localisation of NFκB. NFκB immunoreactivity (red) and Hoechst nuclear staining (blue) were presented individually and merged (scale bar 50 μm). A: carotid artery of WKY-C, B: carotid artery of WKY-L, C: carotid artery of SHR-C, D: carotid artery of SHR-L, E: aortic wall of WKY-C, F: aortic wall of WKY-L, G: aortic wall of SHR-C, H: aortic wall of SHR-L

### 5.7. Electron microscopy

Electron microscopic examinations showed intact architecture of aortic wall in WKY rats. (Figure 9.). However, chronic elevated blood pressure resulted in the alteration of the aortic wall structure in untreated SHR animals (SHR-C).

The endothelial cells were activated, their nuclei were segmented, unfolded and their cytoplasm was extremely widened. The internal elastic lamina (IEL) was periodically interrupted and cytoplasmic processes of medial smooth muscle cells were protruding through the intimal lesions into the subendothelium and into the lumen. The subendothelial space increased and an extreme amount of collagen bundles were produced by activated fibroblasts causing the damage of the basal membrane. “Mushroom-like” formations containing collagen fibers broke through the interendothelial spaces into the lumen. Smooth-muscle cells appeared very frequently producing elastic fibers. These fibers grew in various directions destroying normal layers.

After the treatment with the PARP-inhibitor L-2286, a significantly better situation could be observed. Although we could see activated nuclei in the endothelial layer, the continuity of this layer remained intact. The amount of collagen bundles and elastic fibers was also reduced in the SHR-L group compared to that of the SHR-C animals and therefore the basal membrane was not damaged by collagen fibers and by smooth muscle cells. Thus pharmacological inhibition of PARP enzyme had a significant protective effect on vascular fibroblast and on smooth muscle cells by inhibiting the development of hypertension-induced vascular remodeling (Figure 12.).

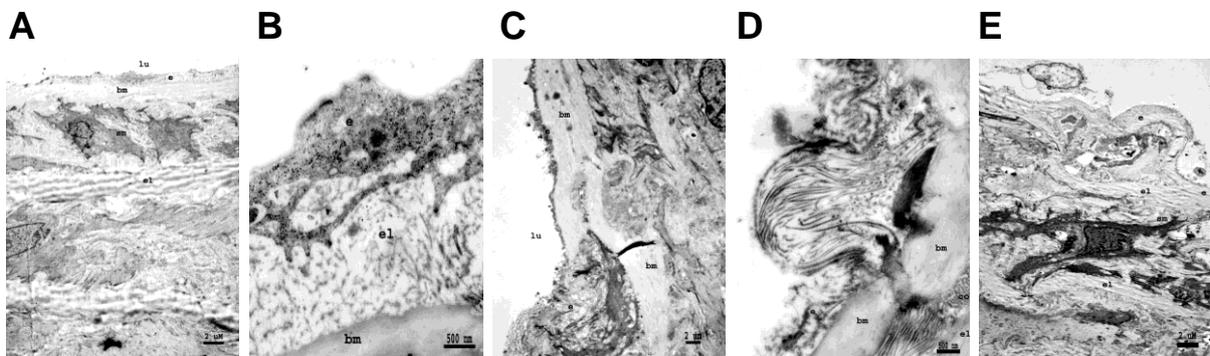


Figure 12. Representative electronmicroscopic sections from aortic wall of 42 week-old animals. Lu: lumen, bm: basal membrane, sm: smooth muscle cell, el: lamina elastica interna, e: endothel. A: aorta of WKY, B: aorta of WKY, C: aorta of SHR-C, D: aorta of SHR-C, E: aorta of SHR-L

### 5.8. *In vitro* isometric vasomotor response of ACh and SNP on rat carotid arteries

Active wall tension (mN/mm) of common carotid arteries (CCA) in WKY rats evoked by KCl (60mmol/L) was primarily mediated by the activation of potassium channels. This pharmacologically evoked contraction was similar to the maximal contractile force in each group of rats. Dose-response curves to SNP and ACh ( $10^{-9}$  to  $10^{-5}$  mmol/L) were determined in CCA rings isolated from normotensive (WKY), or hypertensive (SHR-C and SHR-L) animals. The SNP induced-vasorelaxation (normalized to 60 mM KCL) in the WKY CCA rings was significantly different from that of in the SHR groups, however L-2286 treatment did not change the SNP induced-vasorelaxation (normalized to KCL) (WKY: 100%; 80±5mN, 55±2%, 42±6%, 29±5%, vs. SHR-C: 100%, 57±7%, 40 ±4%, 12±3%, 0% vs. SHR-L: 100%, 42±8%, 20±7%, 18±8%; 0 %; n=6, NS; Figure.13 A.).

The ACh-induced vasorelaxation (normalized to 60 mM KCL) in WKY rats was significantly higher compared to the SHR-C and the SHR-L groups. L-2286 treatment significantly improved the endothelium-dependent vasorelaxation in the SHR-L group compared to the non-treated group (SHR-C) (WKY: 100%; 90±5mN, 60±2%, 0±0%, vs. SHR-C: 100%, 90±7%, 81,7±9%; 60±6%; 59±4% vs. SHR-L: 100%, 90±2%, 77±7%, 20±2%; 22±4%; n=6, p<0.05; (Figure 13 B.).

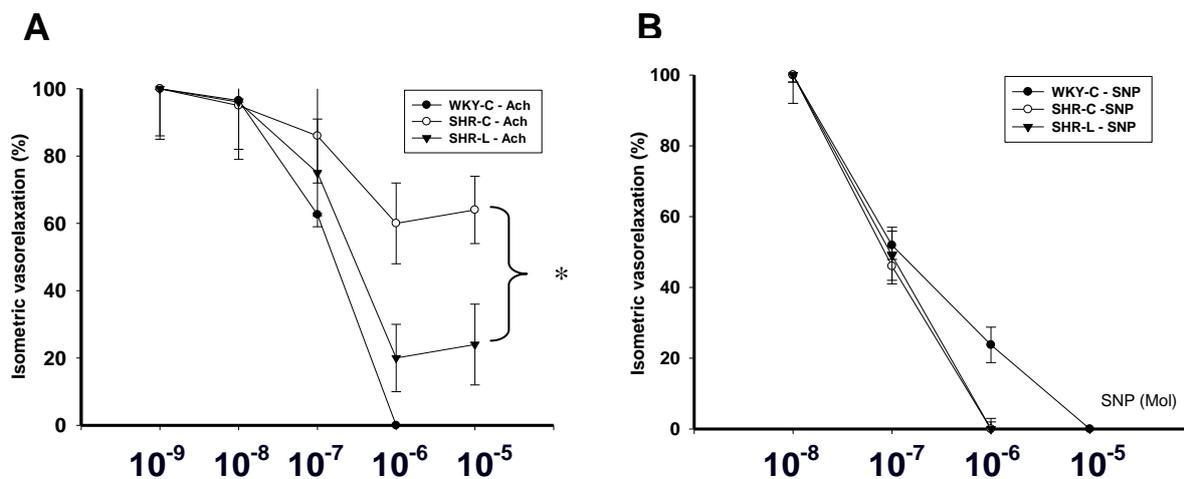


Figure 13. Dose - response isometric vasomotor responses of (WKY, SHR-C, SHR-L) rat carotid arteries to ACh (A) and to SNP (B), all values are normalized to KCL (60mM) responses (100%). Values are means±SEM. (\*p<0,05 WKY-C vs. SHR-C vs. SHR).

## 6. Materials and methods II.

### 6.1. Resveratrol:

Resveratrol (*trans*-3,4,5-trihydroxystilbene) was a kind gift from Admarc Med Diagnostics & Nutraceuticals (Fót, Hungary). 10 mg resveratrol capsule was applied once daily which is in commercial use and possesses official permission for being marketed. Admarc Med Diagnostics & Nutraceuticals has also provided the matching placebo.

### *6.2. Subjects and protocol:*

40 patients were enrolled into our double-blind, placebo-controlled, randomized study (42-80 year old, mean age  $66.3 \pm 8.9$  years, 26 men, 14 women). All patients had a history of myocardial infarction (at least 6 months prior to randomisation) and angiographically verified coronary artery disease. They were randomized into two groups, 13 males and 7 females were in both groups. In one group 10 mg resveratrol, in the other group 10 mg placebo was administered for 3 months. Concomittant medical therapy of the patients remained unchanged for 3 months before randomization and during the study period. Patients received a medical therapy recommended by the current guidelines for secondary prevention of acute myocardial infarction (AMI) including platelet aggregation inhibitors, statins,  $\beta$ -blockers and ACE-inhibitors.

At baseline and after the 3-month treatment period the following examinations were performed: physical examination, blood pressure measurement, clinical chemistry and hemorheological measurements, 12 lead electrocardiography, echocardiography and determination of FMD. The protocol of our study was approved by the Regional Ethics Committee of the University of Pecs and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients to participate in the study.

### *6.3. Clinical chemistry*

From blood samples drawn from the cubital vein after 12-hour fasting white blood cell count, C-reactive protein, TNF- $\alpha$ , glucose, glycosylated hemoglobin-HgA<sub>1c</sub>, fasting lipid levels (total cholesterol, triglyceride, high-density lipoprotein (HDL), LDL-cholesterol) were measured in the Department of Laboratory Medicine, University of Pecs.

### *6.4. Hemorheological parameters*

From blood samples drawn from the cubital vein after 12-hour fasting hematocrit, plasma fibrinogen level, plasma and whole blood viscosity, red blood cell (RBC) deformability, aggregation and platelet aggregation were determined. Hematocrit was measured in a microhematocrit centrifuge (Hemofuge, Heraeus Instr., Germany), plasma fibrinogen concentration was determined by Clauss' method. Plasma and whole blood viscosities were determined in Hevimet 40 capillary viscosimeter (Hemorex Ltd., Hungary). In this viscosimeter the flow of the fluid is detected optoelectronically along a capillary tube and a flow curve is drawn. Shear rate and shear stress are calculated from this curve. Viscosity values are determined as a function of these parameters according to Casson's principle. RBC

aggregation was measured in Myrenne aggregometer (MA-1 Aggregometer, Myrenne Ltd., Germany), applying the light transmission method of Schmid-Schönbein et al. The principle of this technique is based on the increase of light transmission through a red cell suspension. The extent of aggregation is characterized by the aggregation index (AI), calculated from the surface area below the light intensity curve in a 10 s measurement period. Red blood cell filterability was measured in Carat FT-1 filtrometer (Carat Diagnostics Ltd., Hungary) using St. George's technique. In this filtrometer RBC suspension was measured at four pairs of light sources and detectors. The apparatus is interfaced to a computer, which automatically analyzes sequential flow rates and thus distinguishes the relative cell transit time (RCTT) and the pore clogging rate. In our experiments filtration pressure was set for 4 cm of water. All measurements were repeated three times with each sample. Collagen-induced platelet aggregation (2 $\mu$ g/ml collagen) was measured using a Carat TX4 optical platelet aggregometer (Carat Diagnostics Ltd., Hungary) (51).

#### *6.5. Brachial artery flow-mediated dilatation*

Determination of FMD was executed using the modified method described by Celermajer (52). Patients were studied in fasting state, exposure to caffeine and smoking were prohibited for 12 hours before the measurements. FMD was measured on the right brachial artery after 10 minutes resting in a supine position. Images were acquired and saved in digital format using a Technus MPX ultrasound System (ESAOTE, Italy) with a linear vascular transducer. Arterial flow velocity was measured using pulsed wave Doppler signals at a 70° angle to the vessel 5 cm above the antecubital fossa. A pneumatic cuff was then inflated to suprasystolic pressure (250 mmHg) on the forearm for 4 min and a second scan was taken 15 sec. after the cuff deflation and arterial lumen diameter was measured 90 sec. after cuff deflation. FMD was determined as the percentage change in vessel diameter measured at rest and at 90 sec. after cuff release (52,53).

#### *6.6. Echocardiography*

Echocardiographic measurements were performed with a Vivid 7 Pro (GE, USA) equipment with 3S transducer according to international guidelines. Systolic (54) and diastolic left ventricular function (54, 55) were determined at baseline and at the end of the study period.

### *6.7. Statistical analysis*

All data are expressed as means  $\pm$  SEM. Comparisons among groups were made by using Student's t-test (SPSS for Windows 11.0). To post hoc comparison, Bonferroni test was applied. Values of  $p < 0.05$  were considered statistically significant.

## 7. Results II.

### 7.1. Subjects

There were no significant differences in baseline patient characteristics of the placebo and resveratrol treated group (Table 1).

	<b>Resveratrol-treated group (n=20)</b>	<b>Placebo-treated group (n=20)</b>	<b>P-value</b>
Male gender	13	13	
Age, year (mean±SD)	65.3±9.7	67.4±7.7	ns
<b>Major CV risk factors</b>			
Diabetes	7 (35 %)	8 (40 %)	ns
Hypertension	20 (100 %)	19 (95 %)	ns
Dyslipidemia	13 (65 %)	14 (70 %)	ns
Smoking	3 (15%)	4 (20 %)	ns
Obesity (BMI>30)	8 (40 %)	7 (35 %)	ns
BMI (kg/m <sup>2</sup> )	29.3±2.1	28.1±3.2	ns
<b>Secondary prevention drug treatment</b>			
Antiplatelet drugs	17 (85 %)	18 (90%)	ns
ACEI/ARB	18 (90 %)	18 (90%)	ns
Beta-blockers	18 (90%)	17 (85 %)	ns
Statins	15 (75 %)	16 (80 %)	ns

Table 1. The table shows baseline characteristics of patients. Values are expressed as means ± SEM

### 7.2. The effect of resveratrol on hemorheological, laboratory and blood pressure parameters

In both placebo and resveratrol groups hematocrit, fibrinogen level and whole blood viscosity did not show significant changes during the 3-month follow up. Red blood cell deformability decreased and platelet aggregation increased significantly in the placebo group, which had been prevented by resveratrol treatment ( $p<0.05$ ) (Table 2). Plasma viscosity elevated significantly in both groups during the 3-month follow up period. Routine laboratory and inflammatory parameters (white cell count, platelet count, CRP, HgbA<sub>1c</sub>, TNF $\alpha$ , total cholesterol, triglyceride, HDL-cholesterol) did not show any significant changes after 3

months of treatment with resveratrol (Table 2). The treatment was not associated with any significant changes in blood pressure.

	<b>Placebo baseline</b>	<b>Resveratrol baseline</b>	<b>Placebo 3<sup>rd</sup> Month</b>	<b>Resveratrol 3<sup>rd</sup> Month</b>
<b>Hemorheological parameters</b>				
Hematocrit(%)	43.9±1.22	44.4±0.98	43.47±0.86	44.11±0.91
Fibrinogen(g/l)	3.22±0.12	3.46±0.17	3.38±0.15	3.7±0.21
Red blood cell aggregation (%)	12.8±0.76	11.57±0.33	13.1±0.53	11.32±0.59
Collagen Induced platelet aggregation (%)	43.22±6.57	42.61±6.22	<b>47.95±6.74<sup>*</sup></b>	<b>32.89±4.81<sup>*</sup></b>
Plasma viscosity (mPas)	1.26±0.02	1.26±0.02	1.31±0.02	1.34±0.02
Whole blood viscosity at 90 s <sup>-1</sup> shear rate (mPas)	4.37±0.18	4.5±0.13	4.49±0.17	4.6±0.1
Red blood cell transit time (RCTT)	<b>6.71±0.13<sup>#</sup></b>	7.04±0.14	<b>7.02±0.14<sup>#</sup></b>	7.05±0.1
<b>Laboratory parameters</b>				
White blood cell(x10 <sup>9</sup> /l)	6.11±0.37	6.81±0.49	6.53±0.37	7.08±0.38
CRP (mg/l)	3.27±0.35	3.64±0.57	7.03±3.31	6.51±2.97
HgA <sub>1c</sub> %	6.47±0.26	6.33±0.19	6.18±0.25	6.04±0.23
TNFα pg/ml	5.95±0.59	6.13±0.51	10.12±0.99	10.1±1.01
Platelet count (x10 <sup>9</sup> /l)	195.42±10.9	197.82±8.43	210±11.9	205±8.48
Serum cholesterol level (mmol/l)	4.5±0.25	5.1±0.5	4.5±0.26	4.74±0.26
Serum triglyceride level (mmol/l)	1.94±0.24	1.67±0.21	2.04±0.25	1.84±0.26
Serum LDL-cholesterol level (mmol/l)	2.6±0.24	3.15±0.35 <sup>0</sup>	2.51±0.21	<b>2.7±0.15<sup>0</sup></b>
Serum HDL-cholesterol level (mmol/l)	1.02±0.06	1.2±0.06	1.1±0.08	1.2±0.06

Table 2. Hemorheological and laboratory parameters. Values are represented as means±SEM. \*p<0.05 Resveratrol 3<sup>rd</sup> month versus placebo 3<sup>rd</sup> month, <sup>#</sup> Placebo baseline versus placebo 3<sup>rd</sup> month, <sup>0</sup> resveratrol baseline versus resveratrol 3<sup>rd</sup> month.

### 7.3. The effect of resveratrol treatment on the flow-mediated dilatation of the brachial artery

Flow-mediated dilatation of the brachial artery increased significantly in the resveratrol treated group ( $p < 0.05$ ) (Fig. 14). In contrast, in the placebo group no significant changes could be observed.

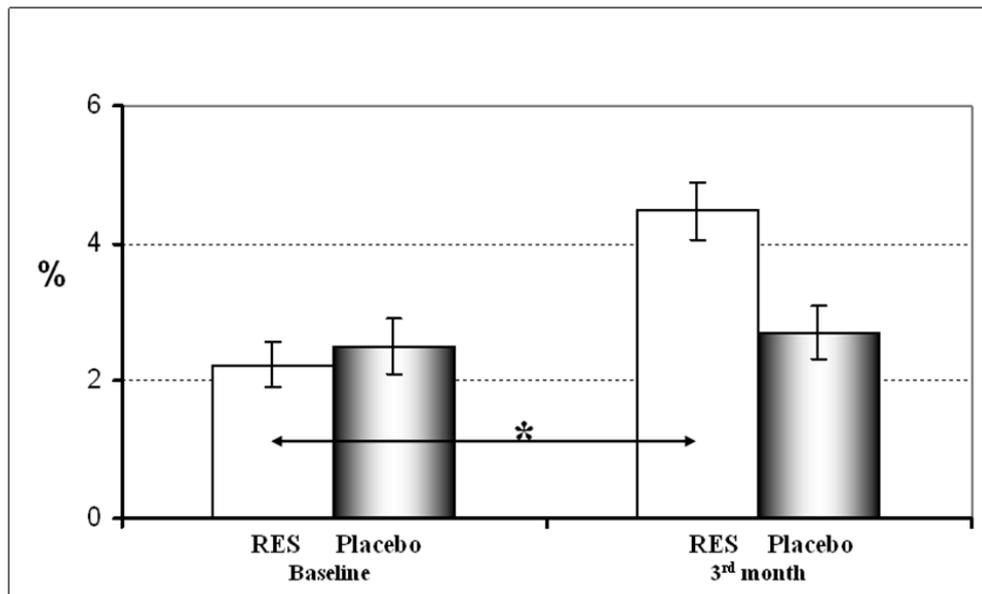


Figure 14. Flow-mediated vasodilatation

Flow-mediated vasodilatation before and after three-months follow-up period. The endothelial function showed a significant improvement in resveratrol-treated group compared to baseline values. In placebo-treated group flow-mediated vasodilatation has not changed. The results were expressed as means  $\pm$  SEM, \* $p < 0.05$ .

%: FMD was defined as the percentage change in vessel diameter measured at rest and 90 sec. after cuff release.

### 7.4. The effect of resveratrol treatment on left ventricular function

After 3 months of resveratrol treatment left ventricular ejection fraction (EF) showed a slight improving tendency compared to placebo group (resveratrol treated group: baseline  $54.77 \pm 13.1\%$ , 3<sup>rd</sup> month  $55.83 \pm 11.5\%$ ; placebo group: baseline  $52.42 \pm 12.3\%$ , 3<sup>rd</sup> month  $51.33 \pm 14.2\%$ ). In LV diastolic function however, significant increase of E/A ratio was observed in the resveratrol group after three months compared to baseline values ( $p < 0.01$ ). In the placebo group diastolic function showed a deteriorating tendency during the 3-month follow up (Figure 15).

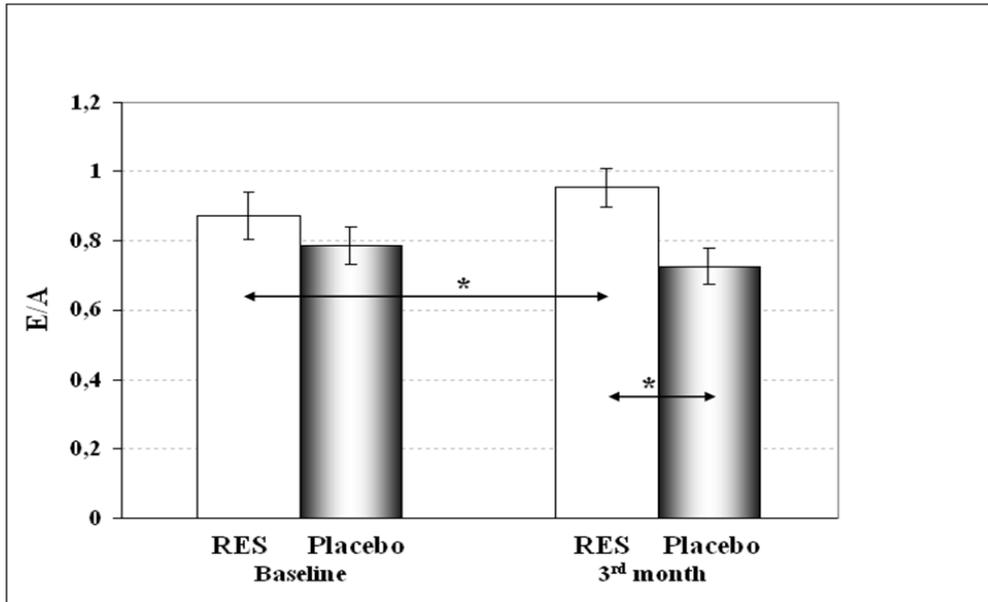


Figure 15. Left ventricular diastolic function

Left ventricular diastolic function (expressed as E/A ratio) of patients at baseline and after three months. The diastolic function in RES-treated group improved significantly. The results were expressed as means $\pm$  SEM. \* p<0.05.

## **8. Discussion**

### *8.1. Vasoprotection by PARP inhibition in a SHR model*

In hypertensive patients, systolic blood pressure and systolic pulse pressure are the dominant prognostic markers. Irrespective of the form of etiology of hypertension, the elevated intraluminal pressure causing cyclic circumferential mechanical strain is the cardinal feature in the development of vascular remodeling. Vascular cells can sense changes in mechanical signal and can transduce these alterations into biological response in a process called mechanotransduction. Therefore the most common protective mechanism against the development of hypertensive vascular remodeling and target organ damage is the lowering of blood pressure. On the other hand side effects of antihypertensive drugs and complaints such as dizziness, headache, fatigue due to the hypoperfusion resulting from lowering the blood pressure are the key factors in the background of inadequate hypertension control (4-5). In order to optimize management of hypertension some recent efforts focus on protecting the vasculature from hypertension induced vascular remodeling with or without lowering the blood pressure. It is well documented that oxidative stress and endothelial dysfunction are consistently observed in hypertension, and recent data suggest that they also have a causal role in the molecular mechanism leading to hypertension. Oxidative stress may directly alter vascular function and induces changes in vascular tone by several mechanisms including altered nitric oxide (NO) bioavailability and altered cellular signaling. NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain and NO synthases can contribute to ROS-production, and are involved in the increased vascular oxidative stress observed during hypertension (56,57). Reactive oxygen species (ROS) coming from different sources are important in regulating endothelial function and vascular tone, and are implicated in endothelial dysfunction, inflammation, hypertrophy, apoptosis, migration, fibrosis and angiogenesis which are important processes involved in vascular remodeling in hypertension (56,57) Angiotensin II, which plays a significant role in hypertension and vascular remodeling, induces oxidative stress, Rho kinase activation and increases intracellular calcium level. It was demonstrated that oxidative stress contributes to VEGF repression and ASK1 activation caused the enhancement of endothelial apoptosis and contributed to a decrease in myocardial capillary density (58). Therefore, oxidative stress induced MAP kinase activation and activation of oxidative stress related transcription factors like NF-kappaB, AP-1 and STAT can contribute to hypertension induced vascular remodeling (59).

Although it is well-known that oxidative stress induces PARP activation, and PARP activation can contribute to elevated ROS production, inflammation, mitochondrial damages and cell death, there are no data about the role of PARP in the development of vascular remodeling. We presented evidence for the protective role of the PARP inhibitor in the hypertension induced cardiac remodeling, and showed favorable changes in MAP kinases, PKC isozymes and PI-3-kinase-Akt pathway activation (45) although PARP inhibition did not have any antihypertensive effect. Therefore, we raised the possibility that PARP inhibition can also be a new molecular target for the prevention of hypertension induced vascular remodeling.

The major findings of this study are that chronic inhibition of nuclear PARP enzyme reduces ADP-ribosylation of nuclear proteins and thus prevents the development of vascular remodeling with the restoration of vascular structure and function while changing the altered patterns of signal transduction. We used the SHR which provides an animal model of high blood pressure that is similar to essential hypertension in humans (45).

PARP-inhibitors did not have any antihypertensive effect in spontaneously hypertensive rats (45). Despite the lack of blood pressure lowering effect, PARP-inhibitor treatment exerted significant protection against the transition of hypertensive cardiopathy to heart failure in the SHRs (45) and in our present work we also found that chronic inhibition of PARP with L-2286 did not decrease the blood pressure in SHR animals, but it could decrease the hypertension-induced remodeling of the great arteries.

Remodeling is characterized by increased vascular wall thickness, altered vascular tone, increased vascular contractility as well as by reduction of vascular compliance. Chronic elevated blood pressure causes the thickening of the vascular wall in carotid arteries. Increased intima–media thickness (IMT) from ultrasound measurements of the carotid artery is considered as a marker of more generalized atherosclerosis, and has been shown to predict the occurrence of cardiovascular events such as stroke and myocardial infarction (60).

The PARP-inhibitor L-2286 however decreased significantly the hypertension-induced vascular wall thickening in great vessels, measured by a high-resolution ultrasound imaging system. Furthermore L-2286 improved the altered vascular elasticity, expressed as aortic stiffness index, too. The thickness and the elastic properties of vascular wall are determined primarily by the amount of smooth muscle cells and by the amount of collagen content. The vascular collagen content was determined by transmission electron microscopy and by Masson's trichrome staining. Both methods showed a very prominent increase of vascular fibrosis in non-treated SHR animals. Moreover, on electron microscopic pictures the

invasivity of fibrosis could also be seen, because the collagen bundles damaged the basal membrane and broke into the lumen. Pharmacological inhibition of nuclear PARP-1 exerted a significant decrease in the process of fibrotic remodeling and this treatment protected the integrity of vascular structure, too.

Endothelium was thought primarily to be a selective barrier to prevent the diffusion of macromolecules from the blood lumen to the interstitial space, however numerous additional roles have been defined for this tissue, including the regulation of vascular tone, modulation of inflammation, promotion or inhibition of vascular growth and modulation of platelet aggregation and coagulation. Disturbance of these properties is termed endothelial dysfunction, a phenomenon that can easily be demonstrated as an impaired vasorelaxation in response to endothelium-dependent vasodilators such as acetyl-choline (ACh) (61-62). Vasomotor answers of carotid arteries were registered with a wire myograph in the present study. Impaired relaxation of carotid arteries to ACh in hypertensive animals was significantly improved in the L-2286 treated group compared to the SHR-C group.

Oxidative stress and decreased bioavailability of NO are the most important causes of endothelial dysfunction. In accordance with this, the signs of increased oxidative stress were detected in our study in hypertensive animals measuring the amount of nitrotyrosine formation (63,64). Hypertension-induced oxidative stress leads to the activation of PARP-1 enzyme which causes the decrease of high energy phosphate levels thus further aggravating the oxidative cell damage. The PARP-inhibitor treatment did not affect the blood pressure, however it could decrease significantly the formation of ROS/RNS and of course the activity of PARP enzyme. Although L-2286 has a moderated direct scavenger effect too, its main effect on ROS formation is the consequence of its mitochondrial protective effect. PARP-inhibitors can decrease the ROS-induced ROS formation in mitochondria through their protective effect on the mitochondrial respiratory chain complexes (26).

Similarly to our previous results in hypertensive, as well as in other experimental heart failure models, the L-2286 treatment increased the phosphorylation state of Akt-1 in the vascular wall, too. Akt-1 is one of the most important prosurvival intracellular signal transduction factors (45, 46). The activation of Akt-1 yields in numerous beneficial consequences, e.g. its activation can also decrease the ROS production through the inhibition of NADPH oxidases (66-68).

The nuclear translocation of AIF increased significantly in untreated SHR animals, which is a consequence of oxidative stress. AIF is a key factor in apoptosis. It has been demonstrated that AIF regulates cell death through a caspase-independent pathway; mitochondrial AIF

translocates to the nucleus on death stimuli and binds to the DNA that leads to large-scale chromatin fragmentation followed by the cell death (69-70). The L-2286 treatment significantly decreased the nuclear translocation of the pro-apoptotic AIF in vascular smooth muscle cells. Blocking the nuclear translocation has also another beneficial effect, it can influence the vascular remodeling through the modulation of proliferation and migration of vascular smooth muscle cells (71-72) PARP-inhibitor treatment decreased the nuclear translocation of AIF either by decreased formation of PAR, or the modulation of signaling pathways including the activation of Akt-1 which attenuates AIF translocation (73-74).

The importance of MAP kinases was extensively studied in experimental models of various cardiovascular pathologies. Our workgroup previously proved in cell culture, that PARP-1 inhibition by pharmacons, small interfering RNA silencing of PARP-1 expression, or the transdominant expression of enzymatically inactive PARP-1 resulted in the inactivation of these MAPKs through the increased expression and enlarged cytoplasmic localization of MAPK phosphatase-1 (MKP-1) (75). In our recent work this finding was confirmed among much more complicated experimental settings, in a chronic murine model of hypertension. The increased MKP-1 and therefore decreased activity of MAP kinases can be a very important underlying mechanism of the diminished vascular collagen accumulation observed in L-2286 treated SHR. The decreased phosphorylation state of MAP kinases exerts protection against vascular remodeling, because it can reduce the fibrosis through a TGF- $\beta$  independent pathway (76-77). In hypertension the elevated level of AngII via ERK, p38-MAPK and consequent Smad3 activation leads to the upregulation of CTGF, a growth factor, that plays an important role in fibroblast proliferation, migration, adhesion, and extracellular matrix production (78). On the other hand the elevated AngII level via p38-MAPK causes NF- $\kappa$ B activation which in turn can increase the fibrosis via a TGF- $\beta$ 1 dependent pathway (79-80) and can enhance the production of inflammatory proteins that have central role in the development of endothelial dysfunction and atherosclerosis (81-82).

## *8.2. Effect of resveratrol treatment in postinfarction patient*

In this study the possible cardioprotective effects of resveratrol were examined in patients after myocardial infarction. According to previous studies the cardiovascular benefits of resveratrol presumably includes vasorelaxation, antioxidant, antiplatelet and cholesterol lowering effects.

The decreased vasorelaxation response - observed in patients with atherosclerosis – was due to impaired endothelial function. Endothelial dysfunction induces atheromatous plaque formation and it is considered to be an important factor for the development of CAD (84). Several studies have shown the favourable effects of resveratrol on endothelial function (39,40,43,44) but these investigations were carried out on animal models, in vitro human vessels (39) or examined only the acute intake of resveratrol (43). In our study endothelial function was measured by FMD and a significant improvement ( $p < 0.05$ ) was detected in vasorelaxation in resveratrol treated group. According to previous studies these effects are presumably due to an increase in NO level and signalling (39,40,84) and the stimulation of  $Ca^{2+}$  activated  $K^{+}$  channels (44).

The importance of hemorheological processes in the progression of atherosclerosis is well known (51,84). Previous studies have proved the inhibitory effect of resveratrol on platelet aggregation in vitro. The inhibition of platelet aggregation is presumably due to the enhancement of the activity of endogenous antiplatelet substances like prostaglandins (85). In addition, under in vitro circumstances it was shown that resveratrol inhibits type I collagen mRNA expression, and the adhesion of platelets to collagen in a concentration dependent manner (86). RBC deformability has an important role in coronary microcirculation since the average capillary diameter is below the diameter of RBC. Decreased RBC deformability reduces the coronary microcirculation. According to previous studies certain unfavourable changes in hemorheological parameters can be observed in patients after myocardial infarction (87). In our investigation a significant decrease of RBC deformability and an increase of platelet aggregation was also experienced in the placebo group but resveratrol treatment prevented these disadvantageous changes ( $p < 0.05$ ).

High level of LDL-cholesterol is very harmful to endothelial cells and has an important role in the development of atherosclerosis (88). According to the literature the effects of resveratrol on lipid parameters are rather conflicting (38). Some investigations have proved that resveratrol lowered total cholesterol (89), increased HDL level and reduced formation of atherosclerotic plaques (88). In our research a favourable effect of resveratrol was discovered

on plasma LDL level ( $p < 0.05$ ), but no significant effect was detected on other lipid parameters, like total cholesterol, HDL cholesterol and on triglyceride levels.

During our study no significant changes could be measured in white blood cell count, plasma fibrinogen and C-reactive protein levels.

Resveratrol possesses direct protective effect on cardiomyocytes which was demonstrated in several animal studies. In a myocardial remodeling model cardiac fibrosis was inhibited by resveratrol (90), furthermore resveratrol protected cardiomyocytes from ischemia-reperfusion injury through suppression of superoxide levels and activation of potassium channels in animal models (38). In our study resveratrol treatment resulted in a slight but not significant improvement of left ventricular systolic function. On the other hand diastolic function of the left ventricle was significantly improved which might be attributed to the inhibitory effect of resveratrol on myocardial fibrosis with the inhibition of phosphorylation of PKC  $\alpha/\beta$  and activation of Akt pathways described in animal models (91).

In conclusion, our clinical trial provided evidences that resveratrol exerts multiple protective effects on the cardiovascular system in patients after myocardial infarction developing its beneficial effect in addition to routine medical therapy used in the secondary prevention of myocardial infarction. 3-months of resveratrol treatment improved FMD, increased red blood cell deformability, inhibited platelet aggregation, decreased LDL cholesterol level and improved left ventricular diastolic function.

## **9. Summary of new scientific results:**

### *9.1. Investigation of vasoprotective effect of L-2286 in a chronic hypertension model*

- 9.1.1. Pharmacological inhibition of PARP-1 enzyme exerts significant protective effect against hypertensive vascular remodeling in spite of the lack of any antihypertensive effect.
- 9.1.2. Decreased vascular fibrosis as a consequence of elevated MKP-1 activity and thus diminished MAPK phosphorylation are the main underlying mechanisms.
- 9.1.3. PARP-1 inhibition activates Akt which can prevent mitochondrial damages and attenuates cell death.
- 9.1.4. PARP is a promising therapeutical target to prevent hypertensive vascular complications even in those patients, who do not reach the target blood pressure because of complaints or because of side effects caused by antihypertensive drug therapy.

### *9.2. Biological effects of resveratrol in postinfarction patients receiving adequate secondary prevention treatment*

- 9.2.1. Resveratrol administration improves endothelial function measured by FMD
- 9.2.2. Resveratrol has a very important favourable effect on several hemorheological and lipid parameters which play an important part in the progression of atherosclerosis
- 9.2.3. Resveratrol treatment causes improvement of left ventricular diastolic function which is a consequence of resveratrol fibrosis decreasing effect.
- 9.2.4. Resveratrol administration (without alcohol intake) might be recommended in postinfarction patients to slow down remodeling and progression of atherosclerosis.

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## **11. Acknowledgements**

These studies were carried out at the Department of Biochemistry and Medical Chemistry and the 1st Department of Medicine, Medical School of the University of Pecs between 2007 and 2011.

I would like to express my thanks to my teacher and program leader, Professor Kálmán Tóth, who managed my studies and gave a support and useful advises during my work. I am grateful to Professor Balázs Sümegi who taught me a biochemical way of thinking. He directed my work on the field of PARP inhibitors and he ensured the possibility of undisturbed work in his department for me.

I convey my thanks to my project leader Róbert Halmosi for his excellent work and help to perform echocardiographic examinations. I would like to express my gratitude to Professor Ákos Koller, Professor János Hamar, Professor László Seress, Professor Kálmán Hideg who gave me useful advices and help during the experiments.

Dr. Eszter Szabados, Zoltán Vámos, Éva Bartha, Kitti Bruszt, Izabella Solti, Alíz Szabó, László Deres, Krisztián Erős, Eszter Bognár gave also a hand with a part of the experiments.

I am grateful to Istvánne Pásztor, Heléna Halasz, Bertalan Horváth and László Girán, who gave much assistance in the laboratory work.

I express my thanks to my family and friends for their encouraging support during my studies and work.

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