Novel antioxidant therapeutic strategies for cardiovascular dysfunction associated with ageing

Ph.D. Doctoral Dissertation

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1. List of abbreviations

ACh - acetylcholine

A/D - analogous/digital

ADP - adenosine diphosphate

AIF - apoptosis inducing factor

ATP - adenosine triphosphate

$CO_3^{-\cdot}$ - carbonate radical

COX-2 - cyclooxygenase-2

DNA - deoxyribonucleic acid

DP - developed pressure

+dP/dt - maximal slope of systolic pressure increment

-dP/dt - maximal slope of diastolic pressure decrement

dUTP - deoxyuridin triphosphate

EDV - end diastolic volume

E_{max} - slope of ESPVR

eNOS - endothelial nitric oxide synthase

ESPVR - end-systolic pressure-volume relationship

FP15 - iron chloride tetrakis-2-(triethylene glycol monomethyl ether) pyridyl porphyrin

H_2O_2 - hydrogen peroxide

ICAM-1 - interstitial cell adhesion molecule

INO-1001 - the potent indeno-isoquinolinone-based PARP inhibitor of Inotek Inc.

iNOS - inducible nitric oxide synthase

LVEDP - left ventricular end diastolic pressure

LVSP - maximal left ventricular systolic pressure

MAP - mean arterial pressure

MDP - left ventricular mean diastolic pressure

MHC-II - major histocompatibility complex class II

MMP-2 - type-2 matrix metalloproteinase

MSP - left ventricular mean systolic pressure

NAD(P)H - nicotinamide adenine dinucleotide (phosphate)

NAD^+ - deprotonated form of nicotinamide adenine dinucleotide

$NO^{\cdot}$ - nitric oxide

$NO_2^{\cdot}$ - nitrogen dioxide radical

NT - nitrotyrosine

$O_2^{-\cdot}$ - superoxide anion

OCl^- - hypochlorite

$OH^{\cdot}$ - hydroxyl radical

$ONOO^-$ - peroxynitrite

ONOOH - peroxynitrous acid

PAR - poly(ADP-ribose)

PARG - poly(ADP-ribose) glycohydrolase

PARP - poly(ADP-ribose) polymerase

PE - phenylephrine

PJ-34 - [11C]2-(dimethylamino)-N-(5,6-dihydro-6-oxophenanthridin-2-yl)acetamide (PARP-inhibitor)

PRSW - preload recruitable stroke work

ROS - reactive oxygen species

RNS - reactive nitrogen species

S.E.M. - standard error of the mean

SNP - sodium nitroprusside

Tau - time constant of left ventricular pressure decay

TGF-β - transforming growth factor beta

TUNEL - terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling

WW85 - a potent peroxynitrite decomposition catalyst (confidential structure)

2. Introduction

The populations in the industrial countries in Europe and Northern America are duratively ageing. 16.7 % of the population of the European Union were 65 years of age or older in 2000, 2.4 % more than in 1990 (Gabányi and Vértes, 2005). In our days there are 35 million people in the United States older than 65 years. This number will double by the year 2030 (Lakatta and Levy, 2003a). In Hungary, the rate of old people is continuously increasing; from 5 % of the population in the year 1837, the proportion of people 60 years of age or older reached 20.41 % in 2001. Whithin this group, the increase of the proportion of people above 80 years will dominate in the next ten years, and this will reach 10 % of the whole population of the industrial countries. According to epidemiological studies, nutrition factors, smoking, diabetes or sedentary lifestyle act as main risk factors of the most important cardiovascular diseases in the population, nevertheless, advanced age unequivocally confers the major risk. The incidence and prevalence of hypertension, coronary heart disease, heart failure or stroke increase continuously with advancing age (Lakatta and Levy, 2003a). Not only the clinically manifest cardiovascular diseases appear more often with age, but so do subclinical or occult diseases such as silent coronary atherosclerosis. Because of a physiological ageing process, cardiovascular structure and functions change with time, and this process alters the substrate on which pathophysiological disease mechanisms can become superimposed. Thus, ageing-associated changes of cardiovascular structure and functions become “partners” with pathophysiological mechanisms of diseases to determine the treshold, severity and progression of cardiovascular diseases in older patients (Lakatta and Levy, 2003a).

2.1. Ageing-associated changes in cardiovascular structure and function in apparent health

2.1.1. The ageing vascular system

2.1.1.1 Structural changes at old age

The most important structural changes associated with advanced ageing in the human vascular system are wall thickening, luminal dilatation, reduction in compliance and elasticity with an increase in stiffness in the large elastic arteries.

Comparative morphologic studies report that the ageing-related aortic wall thickening consists mainly of intimal thickening, and this phenomenon in different populations is shown to be independent of the incidence of atherosclerosis (Virmani et al., 1991). Several epidemiological studies indicate that the carotid wall intimal medial thickness measured by noninvasive methods increase 2-3-fold between 20 and 90 years of age, from approximately 0.3mm to 0.8mm on the average, nevertheless, there are a marked heterogenity in the intimal medial thickness in individuals at a given age (Nagai et al., 1998). Although the arterial remodeling with age has been object of intensive investigations, the exact molecular factors and mechanisms underlying the age-related thickening of large elastic arteries in humans are presently not understood in detail (Lakatta and Levy, 2003a). According to results of experimental studies, the thickened intima at old age is composed of extracellular matrix molecules (collagen, proteoglycanes, fibronectin), and vascular smooth muscle cells derived from the media, and contains markedly higher levels of transforming growth factor beta (TGF-β), type-2 matrix metalloproteinase (MMP-2) and interstitial cell adhesion molecule (ICAM-1) (Lakatta and Levy, 2003c; Li et al., 1999). Based upon these characteristic changes similar to that seen in the development of atherosclerosis, it has been argued that the ageing-associated intimal thickening represents an early stage of atherosclerosis. Age-related thickening of the intima is known to be occur in animals and humans also in the absence of atherosclerosis, thus, it is rather supposed to be correlated with intrinsic arterial

ageing and may act as a risk factor for development of artherosclerosis (Lakatta and Levy, 2003a).

Reduced distensibility and increased stiffness, the other main ageing-associated changes in the vascular system are conventionally assessed by noninvasive measurement of the pulse wave velocity. An increase of pulse wave velocity from approximately 400 cm/s to 800-900 cm/s has been reported in humans between the age of 20 and 80 years both in men and women (Vaitkevicius et al., 1993), and also in populations with low incidence of atherosclerosis, which clearly indicates that ageing-associated vascular stiffening is a phenomenon independent from atherosclerosis (Avolio, 1995). The main molecular mechanisms responsible for the age-related increase in vascular stiffening are structural alterations in the media: elastin fractures/fragmentation and reduced elastin content due to enhanced elastase activity; increased collagen production, cross-linking of collagen by non-enzymatic glycation; calcification and impaired regulation of growth factors and tissue repair mechanisms. Moreover, the role of altered endothelial regulation of the smooth muscle tone at old age has also been recently implicated in the development of vascular stiffening (Lakatta and Levy, 2003a; 2003c; Monos et al., 2005).

2.1.1.2. Ageing-associated changes of the endothelium

Ageing is also associated with impaired structure and function of the vascular endothelium. Several studies describe enhanced expression of adhesion molecules and thus, increased adherence of leukocytes on the endothelial surface at old age (Li et al., 1999; Orlandi et al., 2000). Possibly due to accumulated glucosaminoglycanes in the thickened intima of ageing vessels, the vascular permeability increases (Belmin, et al., 1993).

To our knowledge, the most important functional change associated with advanced ageing in blood vessels is the altered local regulation of vascular tone caused by enhanced calcium-dependent vasoconstriction responses, altered density of potassium channels on smooth muscle cells, and dominantly, by impaired endothelium-dependent vasorelaxation (Lakatta and Levy, 2003c; Monos et al., 2005; Hongo et al., 1988; Asai et al., 2000). Several experimental

and clinical studies have shown that endothelium-dependent vasorelaxation in different vessel types (coronary, brachial, basilar arteries and also resistance vessels) progressively declines with age. This functional impairment occurs earlier in men than in women and has been shown to be independent from structural changes in the vessel wall (Brandes et al., 2005). The impaired endothelium-dependent vasorelaxation affects three major endothelium-derived vasodilators: nitric oxide, prostacyclin and the endothelium-derived hyperpolarizing factor. At advanced age, the nitric oxide-mediated pathways are mostly affected. Accumulating evidence (e.g. increased expression and activity of endothelial nitric oxide synthase in the ageing endothelium) supports the view that possibly not the production, but the bioavailability of endothelial nitric oxide is reduced at old age, which has been interpreted to reflect ageing-associated intensive superoxide-production coupled to rapid removal of endothelial nitric oxide in the reaction of peroxynitrite formation ($O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^-$), as evidenced also by increased protein-nitrosylation (Lakatta and Levy, 2003c; van der Loo et al., 2000). Previous data suggest that also the prostacyclin-mediated vasorelaxation is impaired in ageing humans, in contrast, recent works suggest the upregulation of prostacyclin-synthesis as a possible compensatory mechanism for the ageing-associated lack of nitric oxide-mediated relaxation (Heymes et al., 2000; Brandes et al., 2005).

Under normal conditions, endothelial cells rarely divide and have a turnover rate of 3 years on the average. Important physiological processes occuring during the whole life span, such as angiogenesis, endothelial injury or wound healing trigger the division and proliferation of endothelial cells. Repetition of these processes leads finally to endothelial cellular ageing (endothelial senescence) that has been characterized by expression of senescence-associated β-galactosidase, suppressed telomerase activity and telomere shortening (Sherr and DePinho, 2000; Chang and Harley, 1995). Endothelial senescence has been implicated in impaired angiogenesis and delayed wound healing process observed in the ageing organism, moreover, telomere shortening has been shown to play a role in the development of endothelial dysfunction (Minamino et al., 2002).

2.1.1.3. Vascular system and arterial pressure at old age

As the walls of the aorta and large elastic arteries stiffen with age, the elasticity (distensibility) decreases, and the aortic impedance increases. Arterial pressure is determined by stroke volume, peripheral resistance and central artery stiffness. The primary increase in central artery stiffness at old age lead to a marked elevation of central systolic arterial pressure. Several studies have reported an age-dependent increase in systolic blood pressure during the whole adulthood (from approximately 110-120 mmHg at 20 to 150 mmHg at 80 years of age). In contrast, average diastolic blood pressure has been shown to increase until approximately 50-60 years of age, and remain at this level, or slightly decrease thereafter (Franklin et al, 1997). The net result of these characteristic changes of systolic and diastolic arterial blood pressure at advanced age is widened pulse pressure. These age-dependent blood pressure characteristics are in line with the notion that in young people, arterial pressure is determined mainly by peripheral vascular resistance, while at advanced age by central arterial stiffness (Lakatta and Levy, 2003a).

The ageing-associated physiological changes: widened pulse pressure, elevated systolic pressure and increased arterial stiffness all act as potential risk factors for cardiovascular diseases, over 60 years of age the pulse pressure is found to be the most relevant blood pressure index as a predictor of cardiovascular events. Moreover, isolated systolic hypertension, the most common form of hypertension at old age - even in a mild form - is associated with increased cardiovascular morbidity (Franklin et al., 2001; Sesso, 2000).

In fact, there are conflicting data about arterial blood pressure levels in aged animals of experimental rodent models. The dramatic increase in arterial systolic blood pressure observed in human beings at old age could not been confirmed in rats. Arterial blood pressure tends rather to decrease (Pacher et al., 2004a; 2004b; Gaballa et al., 1998), or stay stable with ageing (Imaoka et al, 1999; Cantini et al., 2001).

2.1.2. The ageing heart

2.1.2.1 Structural changes at old age

The most important ageing-associated physiological structural change in the human heart is the increase of the left ventricular wall thickness. This process occurs in both sexes and has found to be progressive with age. The posterior wall thickness measured by M-mode echocardiography show a continuous increase from approximately 0.4 cm/m^2 body surface area to 0.55 cm/m^2 body surface area between the age of 20 and 80 years (Gerstenblith et al., 1977). As a background of this phenomenon on the tissue level, enlargement of cardiomyocyte size was observed accompanied by a decrease in cardiomyocyte number that was more pronounced in men than in women. The mechanisms underlying the reduction of cardiomyocyte number are necrosis and apoptosis, with the former dominating (Olivetti et al., 1995; Anversa et al., 1990). A focal accumulation of collagen and fibronectin has been descibed in the myocardium at advanced age, moreover, the biophysical properties of collagen become unfavourable due to non-enzymatic cross-linking. The myocyte-to-collagen ratio, however, remains constant or increases, due to cardiomyocyte size enlargement (Lakatta and Levy, 2003b). At old age, progressive fibrosis and calcification have been reported in the annulus fibrosus, the latter also on the basis of the aortic valve leaflets (Monos et al., 2005).

2.1.2.2 Ageing-related changes in the diastolic and systolic cardiac function

The above mentioned structural fibrous changes in the myocardium along with the prolonged calcium transient and diastolic residual calcium activation from the systole lead to reduced distensibility and prolonged relaxation of the ageing left ventricle. The left ventricular early diastolic filling rate progressively decreases after the age of 20 years, reaching a reduction of up to 50 % by 80 years (Schulman et al., 1992). As a compensatory mechanism for that, the late diastolic filling rate increases, mainly due to more powerful atrial contractions. The augmented atrial contractions result in atrial hypertrophy and enlargement,

leading to the appearance of the fourth heart sound (atrial gallop) on the auscultation. These typical age-associated changes in the diastolic filling characteristics produce an exaggerated A wave and a decreased E:A ratio in the Doppler echocardiography (Lakatta, 2002). In humans, the left ventricular end-diastolic volume index (end diastolic volume normalized for body surface area) in the supine position has been found unaltered in ageing individuals when compared with young adults, however, after postural maneuvers or during exercise the end diastolic volume index is higher at old than at young age (Lakatta and Levy, 2003b).

A prolonged ventricular contraction can also be observed at old age, that is caused by prolonged action potential and prolonged cytosolic calcium transient due to reduced calcium sequestration by the sarcoplasmic reticulum (Orchard and Lakatta, 1985; Froehlich et al., 1978). The left ventricular ejection fraction, as a classical measure of systolic function is not influenced by physiological ageing. In contrast, the maximal ejection fraction achieved during exhaustive exercise shows a marked decrease at old age. In healthy young adults, the exercise-induced augmentation of the ejection fraction is associated with a progressive reduction of the end systolic volume index (end systolic volume normalized for body surface area) upon demand. The ageing heart, in turn, shows a remarkable inability to appropriately reduce end systolic volume during exercise and to augment ejection fraction on demand. According to recent studies, the end systolic volume reserv (reduction of end systolic volume index during exhaustive exercise) at the age of 85 years has been found reduced by approximately 80% when compared to that of 20-year-old adults (Fleg et al., 1995). As a result of the described age-related changes of end diastolic and end systolic volumes at rest and during exercise, the stroke volume index is relatively preserved in aged individuals over a wide range of performance demand (Lakatta, 2002).

A marked reduction (50-75 %) in the number of pacemaker cells of the sinus node has been reported, that occurs after the age of 50 years. This phenomenon is supposed to be induced by apoptosis, and may lead to a moderate decrease in resting heart rate. Nevertheless, no significant age-associated change has been observed in heart rate in the supine position at rest. The maximal heart rate during

exhaustive exercise, in turn, progressively decreases with age from approximately 180/min at 20 to 130/min at 80 years of age (Fleg et al., 1995).

The resting supine cardiac index (cardiac output normalized for body surface area) is not significantly influenced by ageing, however, during exhaustive exercise, 30 % reduction of acute cardiac output reserve (exercise-induced increase in cardiac output) has been reported in ageing individuals between the ages of 20 and 80 years. This reduction is entirely caused by the reduction in heart rate reserve as described above (Lakatta, 2002).

A notable ageing-associated deficit in left ventricular intrinsic contractility has been described in experimental animal models and may be expected in humans as well. Recent studies performing invasive hemodynamic measurements in ageing rats report about a progressive decrease in cardiac contractility during ageing (Pacher et al., 2004a; 2004b). The most reliable, loading-independent estimate of myocardial contractility is the slope of the end systolic pressure volume relationship (ESPVR), that is calculated from pressure-volume loops obtained at a range of end diastolic volumes. This index indicates a marked age-related decrease in myocardial contractility in animals, however it has not been measured in a large study population of a broad age range in humans. A single value of end systolic pressure – end systolic volume ratio (as a crude contractility index) during exercise shows an age-associated decline in myocardial contractile reserve in humans as well (Lakatta, 2002; Lakatta and Levy, 2003b).

2.1.2.3 Ageing-related changes in the vegetative cardiovascular regulation

With advancing age, the attenuation of baroreceptor reflexes has been observed that is caused by changes in receptor sensitivity, and modulation of the sympathetic regulation. During any perturbations from the supine resting state, apparent deficits in sympathetic regulation of cardiovascular functions occur accompanied by elevated plasma levels of noradrenalin and adrenalin due to increased production and to reduced clearance of these transmitters (Lakatta, 1993; Monos et al., 2005). In spite of elevated levels of adrenerg transmitters, markedly impaired responses to β-adrenergic stimulation can be observed on the

cellular level. The greater cellular receptor occupancy by adrenalin and noradrenalin leads to desensitization of post-receptor signaling. The importance of the sympathetic dysregulation at old age is reflected by the observation that acute blockade of β-adrenergic receptors changes the exercise hemodynamic profile of young healthy adults to resemble that of ageing individuals (Lakatta, 1993).

Ageing is associated with decreased cardiovagal baroreflex sensitivity. Mechanisms underlying this decrease may involve factors such as increased levels of oxidative stress, vascular stiffening, and decreased cardiac cholinergic responsiveness with age (Monahan, 2007). The impaired vestibulosympathetic reflex is reported to be responsible for the enhanced prevalence of orthostatic hypotension at old age (Monos et al, 2005).

2.2. Ageing and nitro-oxidative stress

2.2.1. The oxidative stress hypothesis of ageing

The oxidative stress hypothesis of ageing (or free radical theory) is one of the most popular explanations of how ageing occurs at the molecular level. In 1956, Denham Harman with his "free radical theory of ageing" was the first to propose that the age-related decline is due to an accumulation of damage to cellular macromolecules by the by-products of oxidative respiration, namely reactive oxygen species (Harman, 1956).

Free radicals are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. They are considered to be the most instable and most reactive atoms or molecules. In biological systems, oxygen and nitrogen radicals, particularly superoxide anions ($O_2^{\cdot-}$), hydroxil radicals ($OH^{\cdot}$) and nitric oxide ($NO^{\cdot}$) play an important role in physiological and pathophysiological processes. Further non-radical reactive oxygen and nitrogen species, such as hydrogen peroxide (H_2O_2), hypochlorite (OCl^-) or peroxynitrite ($ONOO^-$) are other important participants of oxidative and nitrosative stress in living organisms. Reactive oxygen and nitrogen species (ROS, RNS) can be produced as a by-product of oxygen metabolism (during incomplete terminal oxidation) in

mitochondria or from the activity of various enzymes including NAD(P)H-oxidases, xantine oxidase, glucose oxidase, the cytochrome P450s or the endothelial nitric oxide synthase to name a few (Lelbach and Székács, 2005; Cai, 2005). Oxidative and nitrosative stress (or nitro-oxidative stress) may occur as a result of increased production of reactive oxygen and nitrogen species or by deterioration of endogen antioxidant mechanisms, or both. Whatever the reason, nitro-oxidative stress leads in many cases to an increase in the cellular level of oxidatively modified macromolecules. Reactive oxygen and nitrogen species rapidly and agressively attack various biomolecules and cellular structures including lipids (e.g. self-amplifying chain-reactions of lipid peroxidation in membranes), proteins (thereby inactivating important enzymes) and particularly DNA. Damage to DNA is particularly harmful since it may be fixed into mutation, if not repaired in a proper time, and passed onto daughter cells. Moreover, damaged DNA can trigger the activation of several pathways in the cell inducing further important cellular changes related to oxidative injury. Although background levels of oxidatively damaged DNA always exist, nitro-oxidative stress can lead to an increase in the damage, and it is this increase which has been linked to various pathophysiological conditions, such as carcinogenesis, neurodegenerative and cardiovascular diseases, or ageing (Cooke et al., 2006).

Ageing organisms are exposed to continuous oxidative injury, due to the higher rate of superoxide and hydrogen peroxide production from the mitochondrial electron-transport chain (Sohal and Sohal, 1991). Accordingly, the amount of nitro-oxidative damage to various macromolecules (such as lipids, proteins, and DNA, as discussed above) has been shown to increase exponentially during ageing in a variety of tissues in different species (Sohal and Weindruch, 1996).

Experimental studies on life-expectancy and oxidative stress showed, that the life expectancy of experimental animals is inversely related to the rate of accrual of oxidative damage, moreover the rates of mitochondrial $O_2^{-\cdot}$ and H_2O_2 production and amounts of oxidized products of lipids, proteins, and DNA were

relatively lower under conditions that extended the life spans of experimental animals (Agarwal and Sohal, 1994).

On the basis of the existing experimental evidence and correlative information, the involvement of nitro-oxidative stress as a causal factor in the ageing process is quite probable, however additional evidence is needed to exactly define the nature of this involvement.

2.2.2. Peroxynitrite

The peroxynitrite ($ONOO^-$) anion is a short-lived oxidant species that is produced by the reaction of nitric oxide ($NO^{\cdot}$) and superoxide ($O_2^{\cdot-}$) radicals at diffusion-controlled rates. The sites of peroxynitrite formation are assumed to be spatially associated with the sources of superoxide (such as the plasma membrane NAD(P)H oxidases or the mitochondrial respiratory complexes) because although $NO^{\cdot}$ is a relatively stable and highly diffusible free radical, superoxide is much shorterlived and has restricted diffusion across biomembranes. Despite the short half-life of peroxynitrite at physiological pH (~10 ms), its ability to cross cell membranes in the protonated form, implies that peroxynitrite generated from a cellular source could influence surrounding target cells within one to two cell diameters (5–20 μm). A fundamental reaction of $ONOO^-$ in biological systems is its fast reaction with carbon dioxide (in equilibrium with physiological levels of bicarbonate anion), which leads to the formation of carbonate ($CO_3^{\cdot-}$) and nitrogen dioxide ($NO_2^{\cdot}$) radicals, which are one-electron oxidants, that can readily oxidize amino acids such as cysteine and tyrosine to yield the corresponding cysteinyl and tyrosyl radicals. In addition, $NO_2^{\cdot}$ can undergo diffusion-controlled termination reactions with biomolecule-derived radicals, resulting in nitrated compounds. Alternatively, the protonated form of peroxynitrite (peroxynitrous acid, ONOOH) can undergo homolytic fission to generate hydroxyl ($OH^{\cdot}$) and $NO_2^{\cdot}$ radicals. Although this reaction is relatively slow, it become relevant in hydrophobic phases to initiate lipid peroxidation and lipid and protein nitration processes. Moreover, ONOOH in the membranes may undergo direct reactions with metal centres such as hemin or membrane-associated thiols (Pacher et al., 2007; Szabo et al., 2007).

Many biomolecules are oxidized and/or nitrated by peroxynitrite-derived radicals, including tyrosine residues, thiols, DNA and phospholipids. Indeed, tyrosine nitration, dimerization and hydroxylation by peroxynitrite to form 3-nitrotyrosine, 3,3′-dityrosine and 3,4′-dihydrophenylalanine, respectively, are entirely dependent on free-radical pathways (Radi, 2004). Thiols can be oxidized by one-electron reactions by peroxynitrite-derived radicals and initiate radical-dependent chain reactions to produce higher oxidation states of sulphur, including sulphinic and sulphonic acid derivatives. In DNA, purine nucleotides are vulnerable to oxidation and to adduct formation, with 8-oxo and 8-nitroguanine being two of the major products. Also, peroxynitrite can cause deoxyribose oxidation and single strand breaks. The reaction of peroxynitrite-derived radicals with lipids leads to peroxidation and the formation of nitrated lipid oxidation adducts (Pacher et al., 2007; Szabo et al., 2007).

In the cardiovascular system peroxynitrite is also known to impair important regulatory functions. Peroxynitrite formation ($O_2^{-\cdot}$ + $NO^{\cdot}$) strongly reduces the bioavailability of nitric oxide by scavengeing this key vasorelaxant, while nitration of tyrosine by peroxynitrite inactivates among others the prostacyclin syntase enzyme, the manganese superoxide dismutase, src-kinases, mitochondrial complex I, sarcoplasmic reticular calcium-ATPase; all resulting in cardiovascular dysregulation (van der Loo et al., 2000).

Although peroxynitrite has generally been considered as directly biotoxic towards cardiomyocytes, few studies suggest its role as a mediator of cell signal transduction. Short episodes of exposure to low concentrations of peroxynitrite has been reported to inhibit NF-κB activation in cultured cardiomyocytes, thereby it might unexpectedly down-regulate pro-inflammatory mediators (Levrand et al., 2005). However, the *in vivo* relevance of this observation remains unclear.

Because of the nature of its formation and decomposition, which result in a short half-life and low steady-state concentration, peroxynitrite cannot be directly measured *in vivo*, and investigators have been relying on secondary markers such as protein-3-nitrotyrosine (NT, “footprint of peroxynitrite”) to document its production. Elevated levels of nitrotirosine has been reported in various tissues of ageing organisms, suggesting ageing-associated overproduction of peroxynitrite

and other reactive nitrogen species (Drew and Leeuwenburgh, 2002; van der Loo et al., 2000).

2.2.2.1. The therapeutic perspective of peroxynitrite decomposition

Although the list of compounds with peroxynitrite scavenging potential *in vitro* is long (e.g. ascorbic acid, thioredoxin, ebselen, nebivolol, penicillamine, simvastatine, deprenyl, rasagiline…), for most compounds the rate constants of their reactions are low and their action may be attributed to reaction with the secondary radicals (for example $OH^{\cdot}$, $NO_2^{\cdot}$, $CO_3^{\cdot-}$). It is unlikely that most classical scavengers would effectively react as peroxynitrite scavengers *in vivo* and so their therapeutic potential as peroxynitrite neutralizing agents is low (Szabo et al., 2007). In turn, various groups of synthetic molecules react directly and catalytically decompose peroxynitrite, including metalloporphyrins of iron and manganese. These metalloporphyrins (e.g. FP15 an N-PEGylated-2-pyridyl iron porphyrin) can effectively attenuate the toxic effects of peroxynitrite *in vitro* and *in vivo*, and are currently being developed for clinical applications. (Pacher et al., 2007; Szabo et al., 2002b; Szabo et al., 2007). Recent studies with pharmacological catalytical decomposition of peroxynitrite (Szabo et al., 2002b; Pacher et al., 2003; Pieper et al., 2005; Bianchi et al, 2002), emerge as novel potent antioxidant therapeutic possibilities in multiple cardiovascular pathophysiological conditions induced by nitro-oxidative stress, such as diabetic vascular complications, doxorubicin-induced cardiotoxicity, heart transplantation and rejection or myocardial infarction.

2.2.3. The peroxynitrite-PARP-pathway

As detailed above, nitro-oxidative stress, accompanied by increased formation of reactive oxygen and nitrogen species - mainly peroxynitrite - are endogenous inducers of DNA single strand breakage that is the obligatory trigger of PARP-activation. The nuclear enzyme poly(ADP-ribose) polymerase 1 (PARP-1), the most abundant isoform of the PARP enzyme family, is a 116-kDa protein, which is highly conserved in eukaryotes. PARP-1 functions as a DNA damage sensor and signaling molecule binding to both single- and double-stranded DNA breaks.

Upon binding to damaged DNA, PARP-1 forms homodimers and catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose to form poly(ADP-ribose), long branches of ADP-ribose polymers on glutamic acid residues of a number of target proteins including histones and PARP-1 itself. Poly(ADP-ribosyl)ation deliberates negative charge to histones leading to electrostatic repulsion among histones and DNA, a process implicated in DNA repair, and transcriptional regulation. Poly(ADP-ribosyl)ation is a fast dynamic process, which is also indicated by the short (<1 min) *in vivo* half-life of the polymer, and determined also by two catabolic enzymes poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase (Virag and Szabo, 2002).

According to the unifying concept (Virag and Szabo, 2002) cells exposed to DNA damaging agents can enter three major pathways based on the intensity of the trigger. Moderate genotoxic stimuli facilitate PARP-1 activation leading to DNA repair by signaling cell cycle arrest and by interacting with other DNA repair enzymes. Consequently, DNA damage is restored and cells survive without the risk of passing on mutated genes in this pathway. More severe DNA damage triggers the second apoptotic cell death pathway during which caspases (the main executor enzymes of the apoptotic machinery) inactivate PARP-1 by cleaving it into two fragments by destroying its ability to respond to DNA strand breaks, thereby preventing the loss of cellular ATP associated with PARP activation and allowing the maintenance of the cellular energy essential for the execution of apoptosis. This route is intended to prevent cells from the pathological consequence of the third pathway mentioned later in which cells die by necrosis, a less controlled mechanism also posing a risk for neighboring cells. As such, PARP cleavage has been proposed to function as a molecular switch between apoptotic and necrotic modes of cell death (Pacher and Szabo, 2007; Virag and Szabo, 2002).

Extensive nitro-oxidative stress triggers the third pathway by inducing extensive DNA breakage, overactivation of PARP, and consequent depletion of the cellular stores of its substrate NAD^+, impairing glycolysis, the Szent-Györgyi-Krebs cycle, and mitochondrial electron transport, and eventually resulting in ATP depletion and consequent cell dysfunction and death by necrosis.

By this route, PARP activation in cardiomyocytes and endothelial cells leads to a cellular energetic crisis, which subsequently causes functional impairment of contractile function at the cellular level and reduced ability of endothelial cells to produce nitric oxide when stimulated by an endothelium-dependent relaxant agonist, such as acetylcholine (Soriano et al., 2001b; Szabo and Bahrle, 2005). Impairment of endothelial function in the coronary arteries may lead to regional or global myocardial ischaemia, which secondarily impairs cardiac performance (Pacher et al., 2004b).

Two additional roles of PARP-1 has been described, the first one is its involvement of regulating the mitochondria-to-nucleus translocation of apoptosis-inducing-factor (AIF), a 67-kDa mitochondrial death-promoting protein, which induces DNA fragmentation by initiating the activation of a yet unidentified nuclease (Susin et al., 1999). PARP-1 activity appears to be essential for AIF to translocate to the nucleus in cells exposed to nitro-oxidative stress, a process most likely mediated by small PAR fragments signaling into the mitochondria. As such, AIF is currently believed to play an important role in PARP-1-dependent cell death, supporting the hypothesis that a nuclear-mitochondrial crosstalk dependent on poly(ADP-ribosylation) is critical in determining the fate of oxidatively injured cells (Andrabi et al., 2006; Yu et al., 2006).

The second additional role of PARP-1 is its involvement in the regulation of the expression of various proteins implicated in the inflammation at the transcriptional level, e.g. inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), or major histocompatibility complex class II (MHC-II) (Pacher and Szabo, 2007).

2.2.3.1. The therapeutic perspective of PARP-inhibition

The inhibition of the PARP-pathway - especially of the third mechanism detailed above - by pharmacological PARP inhibitors may offer tremendous therapeutic benefit; for instance, in various cardiovascular pathophysiological conditions (e.g., during the myocardial ischemia-reperfusion following myocardial infarction, bypass surgery, cardiac transplantation, cardiac arrest, aortic reconstructive surgery, neointima formation, atherosclerosis…) by

preventing acute cell death. Many pharmacological inhibitors of PARP with different molecular structures have been developed over the last two decades (Jagtap and Szabo, 2005; Graziani and Szabo, 2005). Based on their promising effects in various – among others cardiovascular – indications mentioned above, some of them (e.g. INO-1001, an indeno-isoquinolinone-based PARP-inhibitor) have entered the stage of clinical testing (Pacher and Szabo, 2007).

In chronic cardiovascular dysfunction associated with advanced ageing increased formation of reactive oxygen and nitrogen species (increased nitro-oxidative stress) and increased poly(ADP-ribosyl)ation were reported both in cardiomyocytes and endothelial cells in rodent models (Csiszar et al., 2005; Ungvari et al., 2005; Pacher et al., 2007). Pharmacological inhibition of PARP might therefore beneficially affect myocardial and endothelial function in these animal models of diseases.

3. Aim of the work

Based upon the oxidative stress theory of ageing and recent studies supporting it, we hypothesized that the activation of the nitro-oxidative stress - DNA-injury - poly(ADP-ribose) polymerase (PARP) pathway may play an important pathophysiological role in the functional decline of the cardiovascular system at old age. Thus, interrupting this pathway at different steps may beneficially affect the ageing-associated cardiac and vascular dysfunction.

The aims of the present studies were:

1. In the *in vitro* model of vascular oxidative stress induced by hydrogen peroxide on isolated rat aortic rings:

-Investigation of the possible pathophysiological role of the DNA-damage - PARP pathway in the development of vascular dysfunction induced by oxidative stress

-Testing the effects of pharmacological PARP-inhibition with INO-1001 on endothelial dysfunction induced by hydrogen peroxide and underlying cellular and molecular changes in the vessel wall

2. In the *in vivo* rat model of ageing-associated cardiovascular dysfunction

-Investigation of the possible pathophysiological role of endogenous peroxynitrite overproduction and activation of the PARP pathway in the development of myocardial and endothelial dysfunction associated with advanced ageing

- investigation of the effects of single dose acute PARP-inhibition by INO-1001, and rapid catalytic decomposition of peroxynitrite by FP15 on cardiac performance, vascular functions and the underlying cellular and molecular changes in the heart and in the vessel wall

As a summary, our main goal was to establish novel potent antioxidant therapeutic strategies for ameliorating the cardiovascular dysfunction associated with advanced ageing.

4. Methods

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). All procedures and handling of animals during the investigations were reviewed and approved by the local Ethical Committee.

4.1. Experimental models

4.1.1. *In vitro* model of vascular dysfunction induced by oxidative stress

In organ bath experiments for isometric tension with isolated rat thoracic aortic rings we investigated the effects of *in vitro* hydrogen peroxide exposure on vasoconstriction, endothelium-dependent and –independent vasorelaxation as described detailed below. Endothelial injury was induced by exposing the isolated aortic rings of young male rats to H_2O_2 (200 and 400 μM) for 30 minutes.

4.1.2. The rat model of cardiovascular dysfunction associated with advanced ageing

We investigated ageing (20-24 months old) and young (3 months old) male Lewis and dark agouti rats in our experiments as models for ageing-associated cardiovascular dysfunction. Systolic and diastolic cardiac performance were investigated by left ventricular catheterisation, endothelium-dependent and -independent vascular functions were determined by *in vitro* vascular reactivity measurements on isolated thoracic aortic rings of the rats as described detailed below.

4.2. Animals, experimental groups, treatment protocols

During the whole period of the experiments, all animals were housed in a room at a constant temperature of 22 ± 2 ºC with 12 hours light/dark cycles and fed a standard laboratory rat diet and water ad libitum.

4.2.1. Animals for the *in vitro* measurements of endothelial dysfunction induced by hydrogen peroxide

3-month-old male Sprague-Dawley rats (250-350 g; Charles River, Sulzfeld, Germany) were used for these experiments. After excision and preparation of the descending thoracic aorta of the rats (as described below), aortic rings from each animal were placed in Krebs-Henseleit solution at 37 ºC, aerated with 95 % O_2 and 5 % CO_2 and divided into 5 groups (n=5 in each group) as follows: Control group (no treatment), H_2O_2 groups (exposure to 200 or 400 μM H_2O_2 for 30 minutes), H_2O_2 + INO-1001 group (10 minutes preincubation with the potent PARP-inhibitor INO-1001 (1 μM, a concentration found to be effective in previous studies (Xiao et al., 2005)) and subsequent exposure to 400 μM H_2O_2 for 30 minutes), INO-1001 control group (10+30 minutes incubation with 1 μM INO-1001).

4.2.2. Animals of the FP15 experiments

3-month-old young adult (230-250 g) and 24-month-old ageing (320-370 g) male dark agouti (DA) rats (Harlan Winkelmann, Germany) were used in the experimental setup with FP15. Ageing rats were treated with vehicle (ageing control group, n=6), or the peroxynitrite decomposition catalyst FP15 intraperitoneally (i.p.) for 3 weeks (0.1 mg/kg/day, a dose found to be effective in previous studies (Pacher et al., 2003)) (ageing treatment group, n=6). Young rats treated for the same time with vehicle (young control group, n=6), or same dosed FP15 (young treatment group, n=6) were used as controls.

4.2.3. Animals of the INO-1001 experiments

Young adult (3 months old, 200-250 g, n=10) and ageing (20 months old, 450-600 g, n=10) male Lewis rats (Charles River, Sulzfeld, Germany) were used in the series of experiments with INO-1001.

Animals used for the hemodynamic measurements received a single dose iv. injection of the potent PARP-inhibitor INO-1001 (5 mg/kg). Hemodynamic recordings were performed before and 60 minutes after the treatment.

In additional experiments for vascular reactivity measurements, young and ageing rats received a single intraperitoneal injection of vehicle (young control group, ageing control group) or the PARP-inhibitor INO-1001 (5 mg/kg) (young treatment group, ageing treatment group) 120 minutes before anaesthesia and removal of the thoracic aorta.

4.3. Hemodynamic measurements

Rats were anesthetized with thiopentone sodium (60 mg/kg intraperitoneally), tracheotomized, intubated and artificially ventilated. Animals were placed on controlled heating pads and core temperature measured via a rectal probe was maintained at 37 ºC. The thoracic cavity was opened to permit access to the apex of the heart. All incisions were kept to a minimum to avoid major blood loss. The left ventricle was punctured by a 20 G plastic cannula, through which a 2 F microtip pressure-volume catheter (SPR-838, Millar Instruments, Houston, TX, USA) was inserted into the left ventricular cavity. Mean arterial pressure was measured via the right femoral artery. After stabilization for 5 minutes, the signals were continuously recorded using a pressure-volume conductance system coupled to an A/D converter (EMKA Technologies, Paris, France) at a sampling rate of 1000/s, stored and displayed on a computer by the IOX Software System (EMKA Technologies, Paris, France). With the help of a special blood pressure analysis program (EMKA Technologies, Paris, France) mean arterial pressure (MAP), maximal left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), developed pressure (DP), mean left ventricular systolic (MSP) and diastolic pressure (MDP), maximal slope of systolic pressure increment (+dP/dt) and diastolic decrement (-dP/dt), time constant of left ventricular pressure decay (Tau) were computed and calculated. Additionally, in the INO-1001 series, left ventricular pressure-volume relations were measured by transiently compressing the inferior vena cava. The slope (E_{max}) of the left ventricular end-systolic pressure-volume relationships (ESPVR), preload recruitable stroke work (PRSW) and maximal slope of systolic pressure increment – end-diastolic volume relation (+dP/dt-EDV) were calculated as load-independent indexes of left ventricular contractility.

4.4. Preparation of isolated aortic rings

Rats were anesthetized with thiopentone sodium (60 mg/kg intraperitoneally), the descending thoracic aorta was carefully removed from the open-chest animals and placed in cold (+4 ºC), oxigenized Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.77 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.4 mM glucose; pH=7.4). The aortae were prepared and cleaned from periadventitial fat and surrounding connective tissue and cut transversely into 4-mm width rings (n=3 or 4 from each animal, mean internal diameter 1700-1800 μm) using an operation microscope. Special attention was paid during the preparation to avoid damaging the endothelium.

4.5. *In vitro* organ bath experiments for vascular reactivity

Isolated aortic rings were mounted on stainless steel hooks in individual organ baths (Radnoti Glass Technology, Monrovia, CA, USA), containing 25 ml of Krebs-Henseleit solution at 37 ºC and aerated with 95 % O_2 and 5 % CO_2.

Isometric contractions were recorded using isometric force transducers (Radnoti Glass Technology, Monrovia, CA, USA), digitized, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France).

The aortic rings were placed under a resting tension of 2 g and equilibrated for 60 minutes. During this period, tension was periodically adjusted to the desired level and the Krebs-Henseleit solution was changed every 30 minutes. Maximal contraction forces to potassium chloride (KCl, 100 mM) were determined and aortic rings were washed until resting tension was again obtained. Phenylephrine (PE, 10^{-6} M) was used to precontract the rings until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of endothelium-dependent dilator acetylcholine (ACh, 10^{-9}-10^{-4} M) and endothelium-independent dilator sodium nitroprusside (SNP, 10^{-10}-10^{-5} M). Contractile responses are expressed as grams of tension, relaxation is expressed as percent of contraction induced by phenylephrine (10^{-6} M).

4.6. Histology

4.6.1. Immunohistochemical analysis

Myocardial sections of the rats used for the hemodynamic measurements in the INO-1001 series were removed for immunohistochemical processing immediately after completing the left ventricular pressure-volume analysis. After the preparation of the descending thoracic aorta in all series, an additional thoracic aortic segment from each animal and from each experimental group was prepared and separated for immunohistochemical processing. All samples were fixed in buffered paraformaldehyde solution (4 %) and embedded in paraffin. Three adjacent sections were processed for each of the following types of immunohistochemical labelling.

According to the methods previously described (Liaudet et al., 2000), we performed immunohistochemical staining for nitrotyrosine (NT, product of the nitrating effect of peroxynitrite; a marker of nitrosative stress in general, and as "footprint of peroxynitrite" obvious evidence for *in vivo* peroxynitrite generation in particular (van der Loo et al., 2000; Halliwell, 1997), and for poly(ADP-ribose) (PAR, the enzymatic product of PARP). Primary antibodies used for the stainings were polyclonal sheep anti-nitrotyrosine antibody (OXIS, Portland, OR, USA) and mouse monoclonal anti-poly(ADP-ribose) antibody (Calbiochem, San Diego, CA, USA). For immunohistochemical detection of apoptosis inducing factor (AIF) we applied the procedure described previously (Xiao et al., 2004) using rabbit polyclonal anti-AIF antibody (Chemicon International, Temecula, CA, USA). To detect endothelial nitric oxide synthase (eNOS), a routine immunohistochemical procedure was applied using the avidin biotin method (Gross et al., 2005). Primary antibody was rabbit polyclonal anti-eNOS antibody (Dinova, Hamburg, Germany).

4.6.2. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction

TUNEL assay was performed for detection of DNA strand breaks. The detection was carried out using a commercial kit following the protocol provided

by the manufacturer (Chemicon International, Temecula, CA, USA). Briefly, aortic segments of all groups were fixed in neutral buffered formalin and embedded in paraffin. 4 μm thick sections were placed on adhesive slides. Rehydrated sections were treated with 20 μg/ml DNase-free Proteinase K (Sigma-Aldrich, Germany) to retrieve antigenic epitopes, followed by 3% H_2O_2 to quench endogenous peroxidase activity. Free 3`-OH termini were labeled with digoxigenin-dUTP for 1 hour at 37 ºC utilizing a terminal deoxynucleotidyl transferase reaction mixture (Chemicon International, Temecula, CA, USA). Incorporated digoxigenin-conjugated nucleotides were detected using a horseradish peroxidase conjugated anti-digoxigenin antibody and 3,3`-diaminobenzidine. Sections were counterstained with Gill's hematoxylin. Dehydrated sections were cleared in xylene, mounted with Permount (Fischer Scientific, Germany) and coverslips were applied.

4.6.3. Quantification of immunohistochemistry and TUNEL

Semiquantitative histomorphological assessment was performed on all of the stained specimens of the H_2O_2 and FP15 projects in a blinded fashion using conventional microscopy and the COLIM software package (Pictron, Budapest, Hungary). The results were expressed with a scoring system. On the base of staining intensity specimens were coupled with intensity score values as follows: 0= no positive staining, 1 to 3= increasing degrees of intermediate staining and 4= extensive staining. According to the amount of positive stained cells, an area score was assigned (1= up to 10 % positive cells, 2= 11 % to 50 % positive cells, 3= 51 % to 80 % positive cells, 4= >80 % positive cells). Finally an average score (0-12) for the whole picture was calculated (intensity score multiplied by area score).

In the case of apoptosis inducing factor staining the total number of positively stained endothelial cell nuclei was obtained in each section, and an average value was calculated for each experimental group.

For assessment of TUNEL-labelled cells, the number of positive cell nuclei/ microscopic examination field (250x magnification) were counted (four fields

characterizing each specimen), and an average value was calculated for each experimental group.

4.7. Statistical analysis

All data are expressed as means ± S.E.M.. Intergroup comparisons were performed by using one-way analysis of variance followed by Student's unpaired t-test with Bonferroni´s correction for multiple comparisons. Differences were considered significant when $P<0.05$.

4.8. Drugs

Hydogen peroxide solution (AppliChem, Darmstadt, Germany) was diluted with distilled water. Phenylephrine, acetylcholine and sodium nitroprusside (Sigma-Aldrich, Germany) were dissolved in normal saline; FP15 (FeCl tetrakis-2-(triethylene glycol monomethyl ether) pyridyl porphyrin) and INO-1001, the potent indeno-isoquinolinone-based poly(ADP)-ribose polymerase inhibitor (Inotek Pharmaceuticals Corporation, Beverly, MA, USA; Jagtap et al., 2005) were dissolved in 5 % glucose solution.

5. Results

5.1. Vascular dysfunction induced by H_2O_2 *in vitro* – effects of PARP-inhibition

5.1.1. TUNEL and immunohistochemical analysis

Using the TUNEL assay we found pronounced DNA-damage in the aortic wall (intima and media) in the H_2O_2 groups as reflected also by the quantitative assessment of TUNEL-positive cells. Pretreatment with INO-1001 tended to decrease H_2O_2-induced DNA strand breaks (Fig. 1., Fig. 2.A). In turn - as expected – blood vessels not exposed to H_2O_2 (i.e. the control and the INO-1001 control group) showed essentially no TUNEL-positivity. Figure 1. (upper panel) shows representative sections for TUNEL in the different groups.

As shown in Figure 1. and Figure 2.B, a marked degree of PARP activation was observed in the aortic wall sections of the H_2O_2-groups - when compared to controls - , as evidenced by higher poly(ADP-ribose) scores. In the case of 200 μM H_2O_2 we found a tendency towards increased poly(ADP-ribose)-immunoreactivity, which reached statistical significance in the 400 μM H_2O_2-group. Pretreatment with the potent PARP-inhibitor INO-1001 resulted in significantly reduced formation of poly(ADP-ribose) in the aortic rings exposed to 400 μM H_2O_2 (Fig. 1., Fig. 2.B), while it had no effect on control rings. Figure 1. (middle panel) shows representative stainings for poly(ADP-ribose) in the different groups.

An altered pattern was found in the localization of apoptosis inducing factor in the intima of the H_2O_2 groups, whereby a diffuse (mitochondrial) localization of apoptosis inducing factor converted into a nuclear localization, consistently with mitochondrial-to-nuclear translocation of this factor. H_2O_2-exposure notably increased the number of apoptosis inducing factor positive aortic endothelial cell nuclei, which was significantly decreased in the H_2O_2-INO-1001 group (Fig. 1., Fig. 2.C). Figure 1. (lower panel) shows representative stainings for apoptosis inducing factor in the different groups.

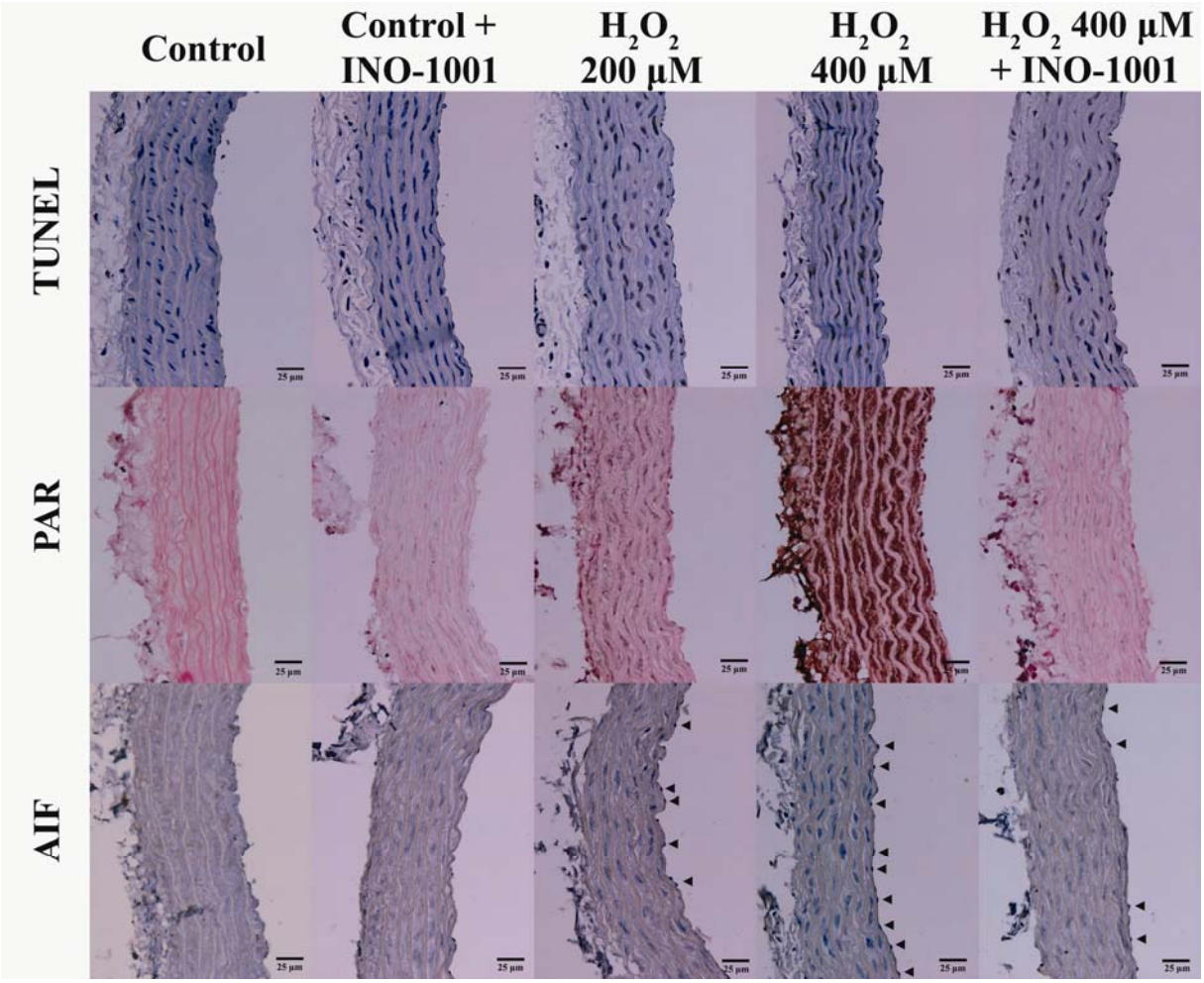


Figure 1. Photomicrographs of TUNEL assay, PAR- and AIF-immunohistochemistry Representative photomicrographs of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction (upper panel; brown staining in the cell nuclei), representative immunohistochemical stainings for poly(ADP-ribose) (PAR; middle panel; brown staining) and for apoptosis-inducing factor (AIF; lower panel; arrows show the positively stained (dark brown, black) endothelial cell nuclei) in the vessel wall of control, INO-1001-pretreated control, hydrogen peroxide-exposed (H_2O_2; 200 and 400 µM), and INO-1001-pretreated H_2O_2-exposed thoracic aortic rings (magnification: 400X, scale bar: 25 µm).

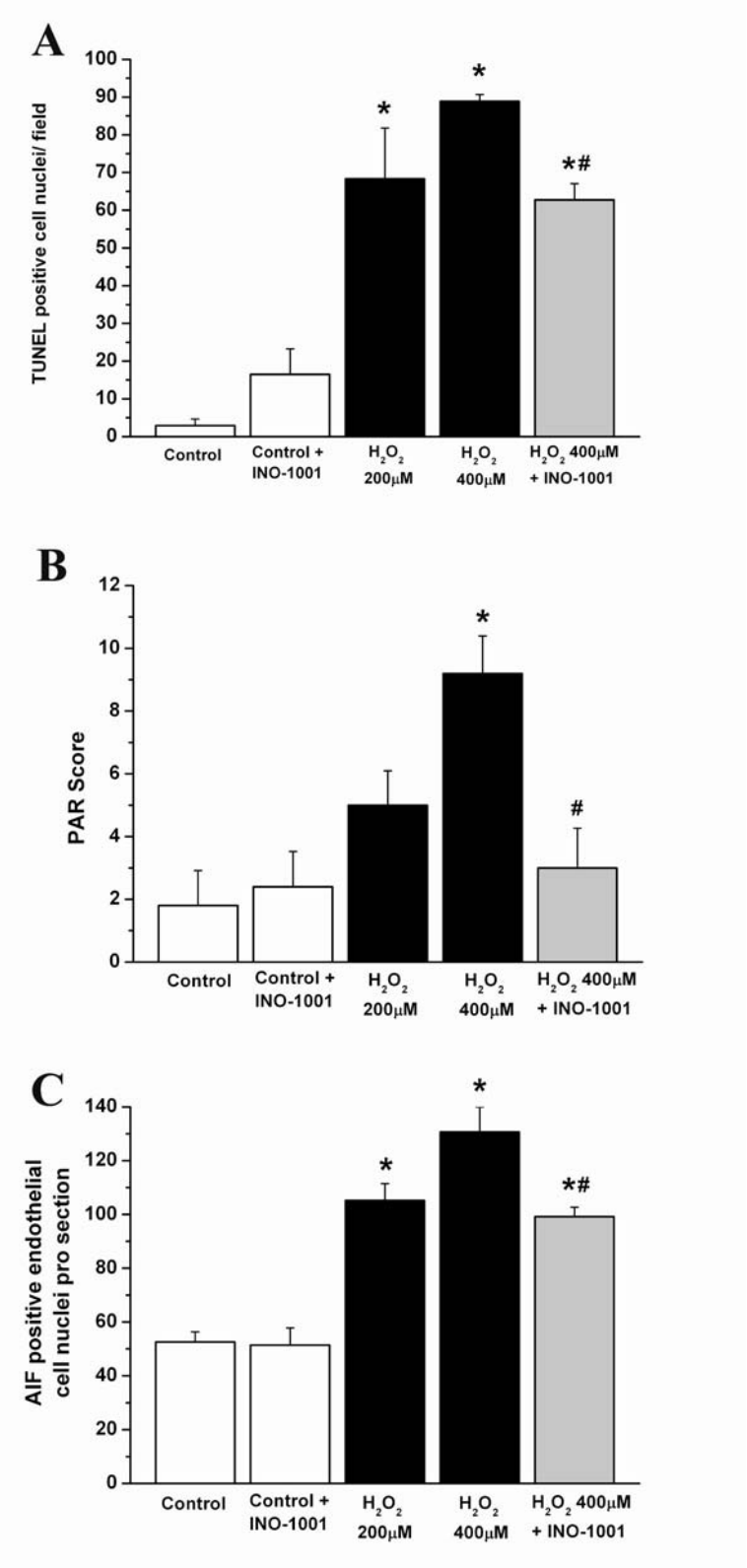


Figure 2. Scoring of TUNEL assay, PAR- and AIF-immunohistochemistry

Average number of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) positive cell nuclei in a microscopic field (magnification 250X) of the aortic wall of

control, INO-1001-pretreated control, hydrogen peroxide-exposed (H_2O_2; 200 and 400 μM), and INO-1001-pretreated H_2O_2-exposed thoracic aortic rings (A). Immunohistochemical scores for poly(ADP-ribose) (PAR) in the aortic wall in the different groups (B). Average number of endothelial cell nuclei stained positively for apoptosis-inducing factor (AIF) in the intima of aortic wall in the different groups (C). Values represents mean ± S.E.M. of scores of all specimens of the different groups *, $P<0.05$ versus control; #, $P<0.05$ versus H_2O_2 400 μM.

5.1.2. Vascular function

Regarding the contractions of aortic rings exposed to phenylephrine (10^{-6} M), only the 400 μM H_2O_2-group showed significantly lower contraction forces, when compared to controls (Fig. 3.A). A dose-dependent impairment of endothelial function caused by H_2O_2 was demonstrated in the *in vitro* organ bath experiments. The endothelial dysfunction induced by the reactive oxidant H_2O_2 was indicated by the reduced maximal relaxation of isolated aortic rings to acetylcholine (86.2 ± 1.6 % control vs. 72.6 ± 2.0 % 200 μM H_2O_2 vs. 66.9 ± 2.0 % 400 μM H_2O_2, $P<0.05$), and the H_2O_2-dose-dependent rightward shift of the dose-response curve as compared to the control group (Fig. 3.B). The endothelium-independent vascular smooth muscle function indicated by the vasorelaxation of aortic rings to sodium nitroprusside was not impaired by H_2O_2 (Fig. 3.C).

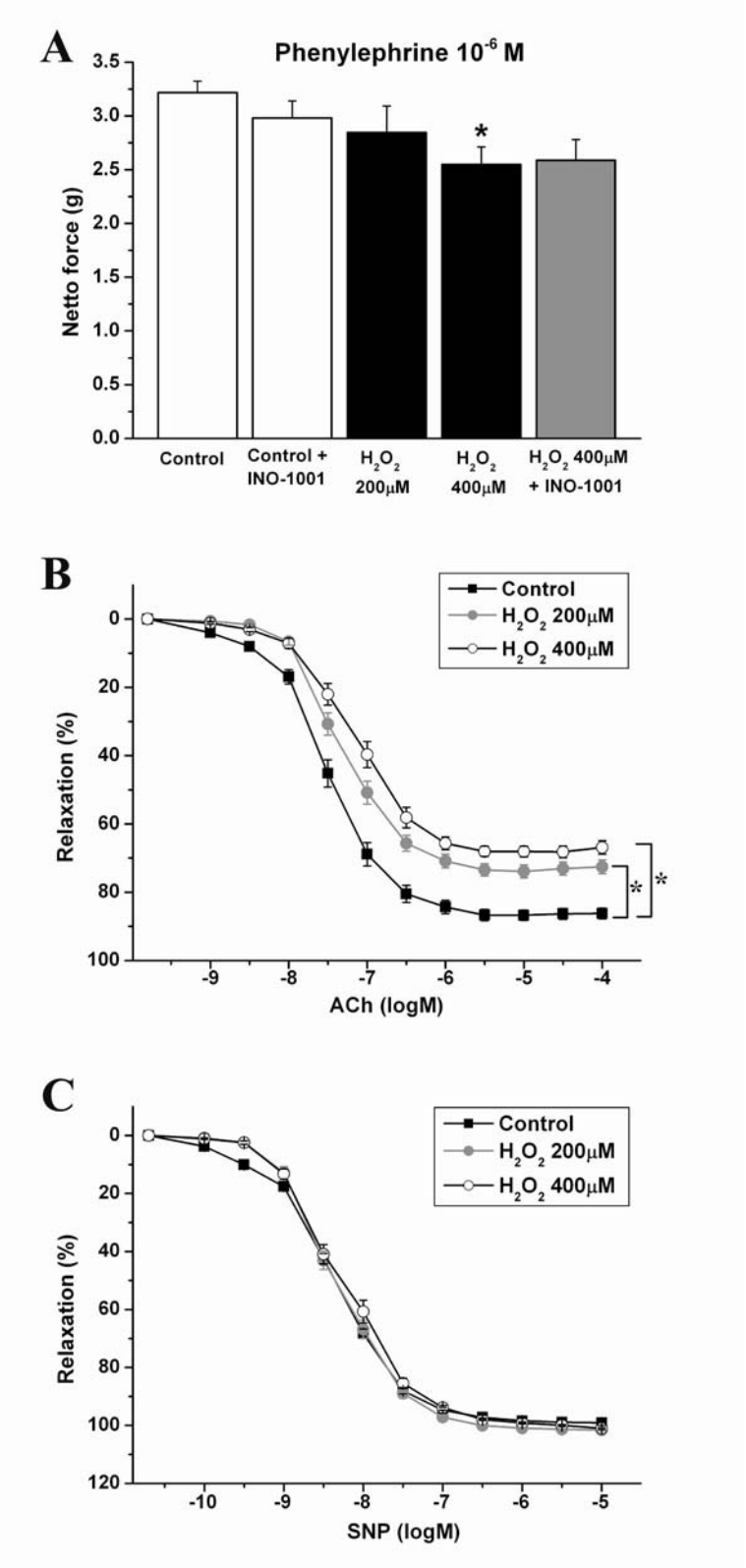


Figure 3. Effects of hydrogen peroxide (H_2O_2) on the vascular function of rat thoracic aortic rings

Contraction forces induced by phenylephrine (10^{-6} M) in the groups of control, INO-1001-pretreated control, hydrogen peroxide-exposed (H_2O_2; 200 and 400 μM), and INO-1001-pretreated

H_2O_2-exposed thoracic aortic rings (A). Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation (B), and sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation (C) in the control and H_2O_2-exposed (200 and 400 μM) groups. Each point of the curves represents mean ± S.E.M. of 18-20 experiments in thoracic aortic rings of the different groups. *, $P<0.05$ versus control.

Inhibition of the PARP-activity by INO-1001 significantly enhanced the acetylcholine-induced, endothelium-dependent, nitric oxide mediated vasorelaxation after exposure with 400 μM H_2O_2 (maximal relaxation: 77.8 ± 3.0 % 400 μM H_2O_2 + INO-1001 vs. 66.9 ± 2.0 % 400 μM H_2O_2, $P<0.05$), indicating improved endothelial function (Fig. 4.A). The same pretreatment had no effect on the endothelium-independent vasorelaxation of aortic rings to sodium nitroprusside (Fig. 4.B).

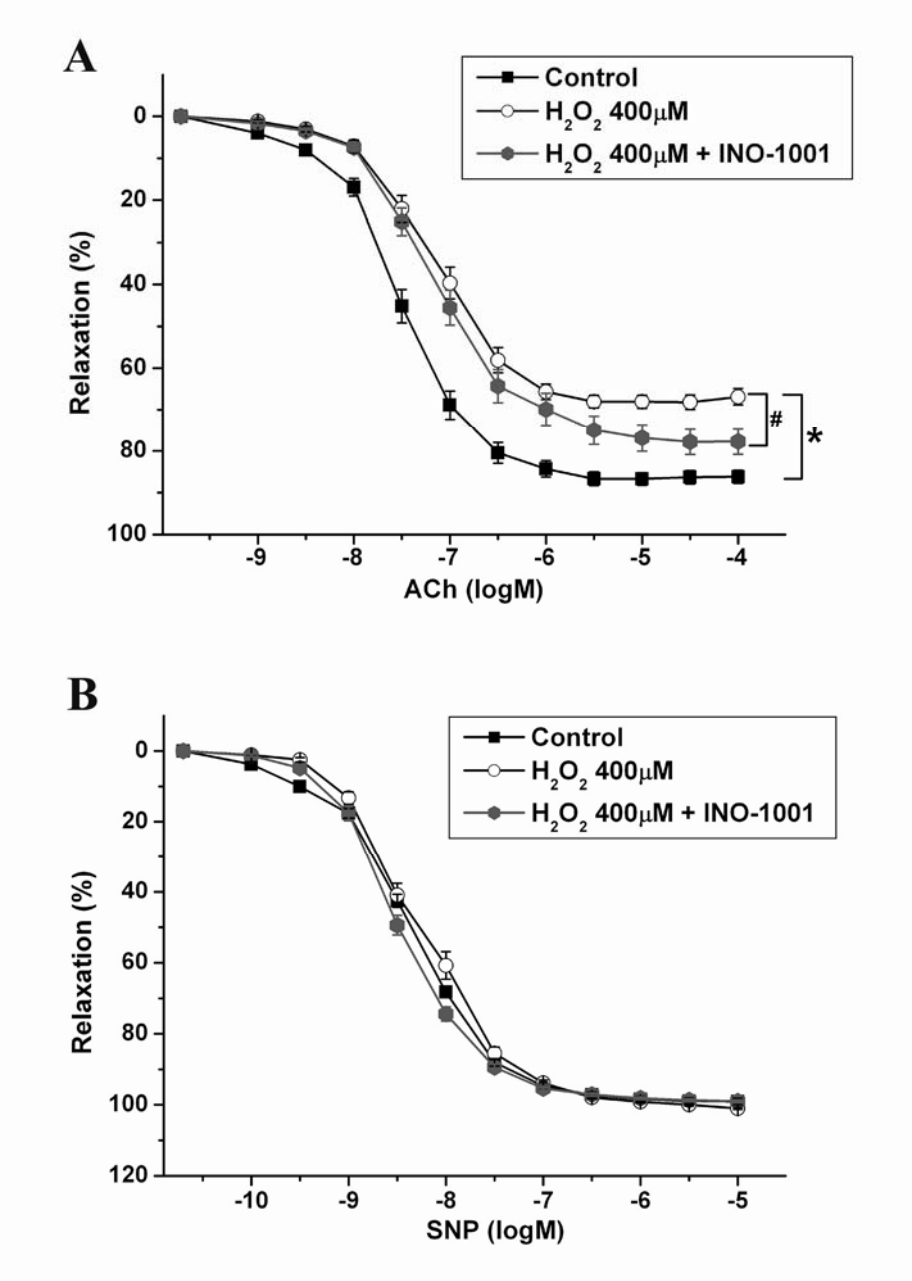


Figure 4. Improvement of hydrogen peroxide-induced endothelial dysfunction by inhibition of poly(ADP-ribose) polymerase with INO-1001

Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation (A), and sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation (B) in the groups of control, 400 μM H_2O_2-exposed, and INO-1001-pretreated H_2O_2 (400 μM) -exposed rings. Each point of the curves represents mean ± S.E.M. of 18-20 experiments in thoracic aortic rings of the different groups. *, $P<0.05$ versus control, #, $P<0.05$ versus H_2O_2 400 μM.

In the INO-1001 control group we found no alterations in both of the acetylcholine- and sodium nitroprusside-induced vasorelaxation when compared to control, INO-1001 did not directly influence the endothelium-dependent and –independent vasorelaxation of aortic rings (Fig. 5.A, B).

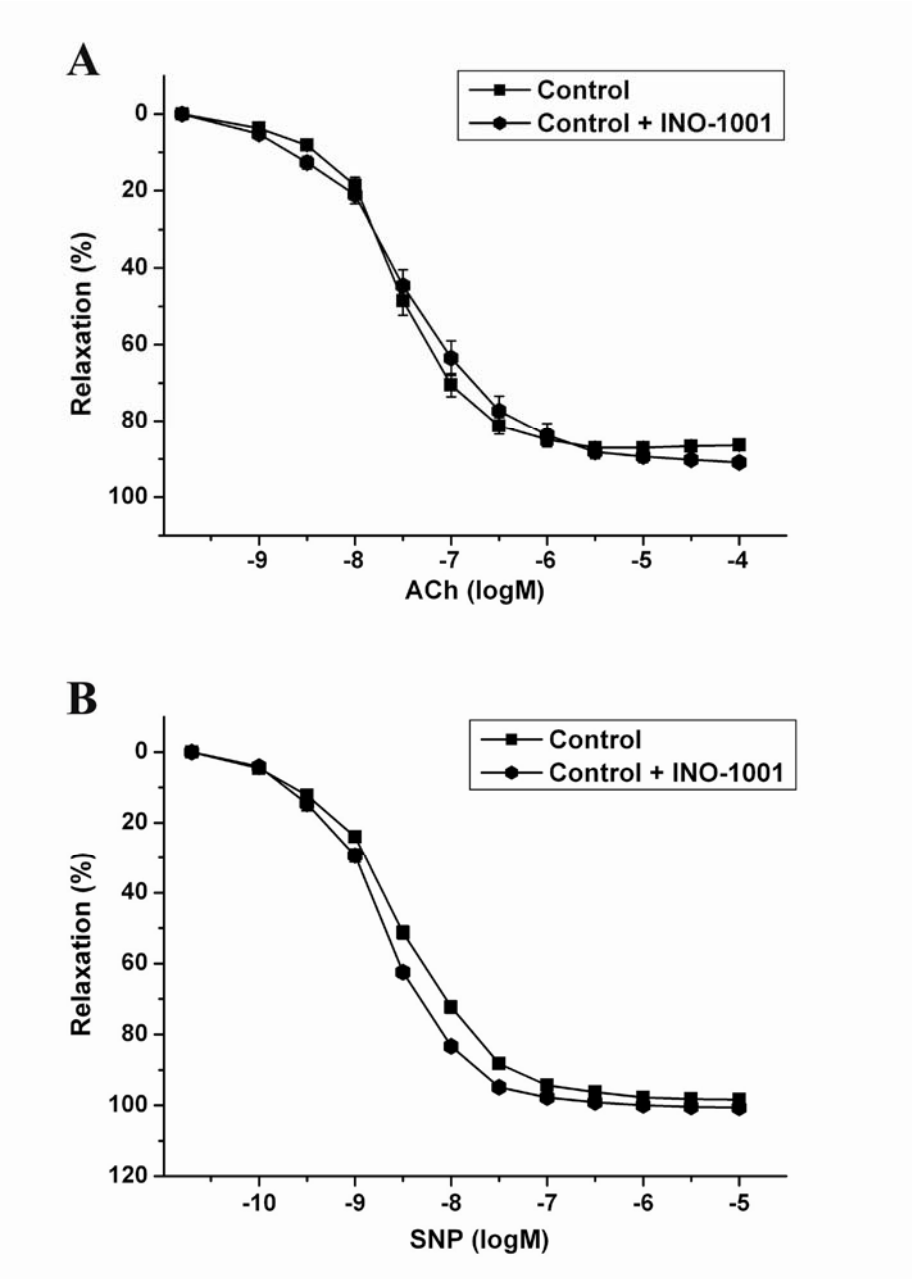


Figure 5. Effects of INO-1001 on the vascular function of control rat thoracic aortic rings
Acetylcholine (ACh)-induced endothelium-dependent vasorelaxation (A), and sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxation (B) in the groups of control and INO-1001-pretreated control aortic rings. Each point of the curves represents mean ± S.E.M. of 21-27 experiments in thoracic aortic rings of the different groups.

5.2. Ageing-associated cardiovascular dysfunction – effects of acute PARP-inhibition

5.2.1. Immunohistochemical analysis

Immunohistochemical staining showed increased immunoreactivity for nitrotyrosine and poly(ADP-ribose) - indicative of nitrosative stress and enhanced activation of PARP - in the left ventricular myocardium and in the aortic wall (mainly in the endothelium) of ageing rats. (Fig. 6., 7.)

Single dose treatment with the potent PARP-inhibitor INO-1001 notably decreased PAR formation both in the myocardium and the aortic wall. Immunoreactivity for nitrotyrosine was not affected by acute PARP-inhibition. Figure 6. and 7. show representative stainings for NT and PAR in the young control, ageing control and INO-1001 treatment groups.

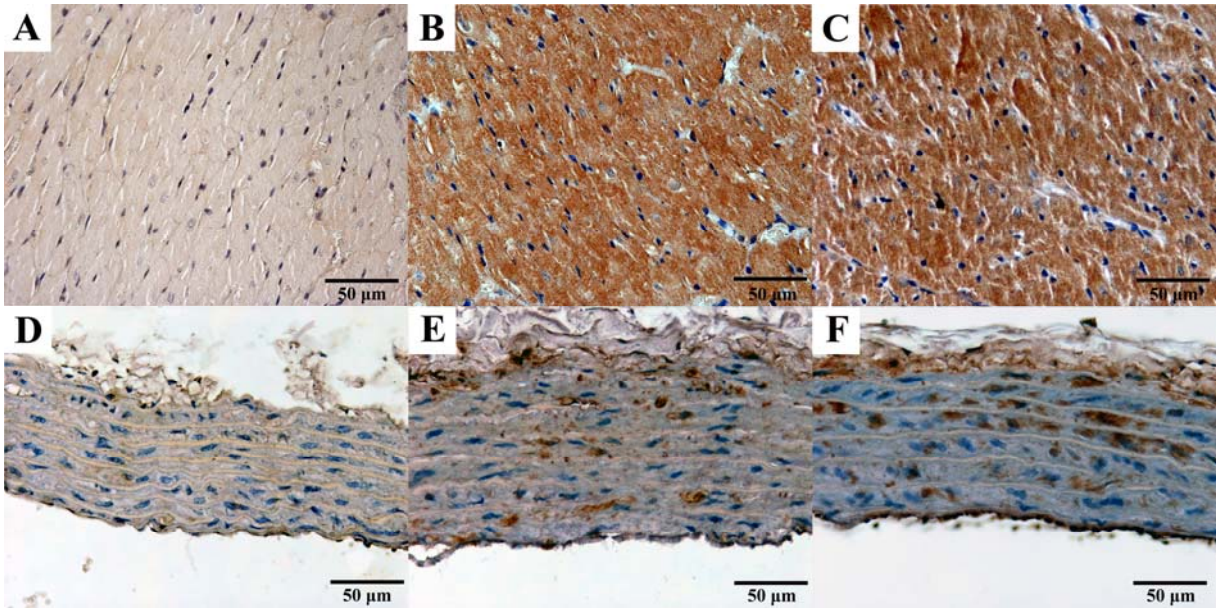


Figure 6. Photomicrographs of nitrotyrosine immunohistochemistry

Representative immunohistochemical stainings for nitrotyrosine (NT, brown staining) in the myocardium (A-C) and aortic wall (D-F). Young control group: A, D; ageing control group: B, E; and ageing INO-1001 treatment groups: C, F (magnification: 400X, scale bar: 50 µm).

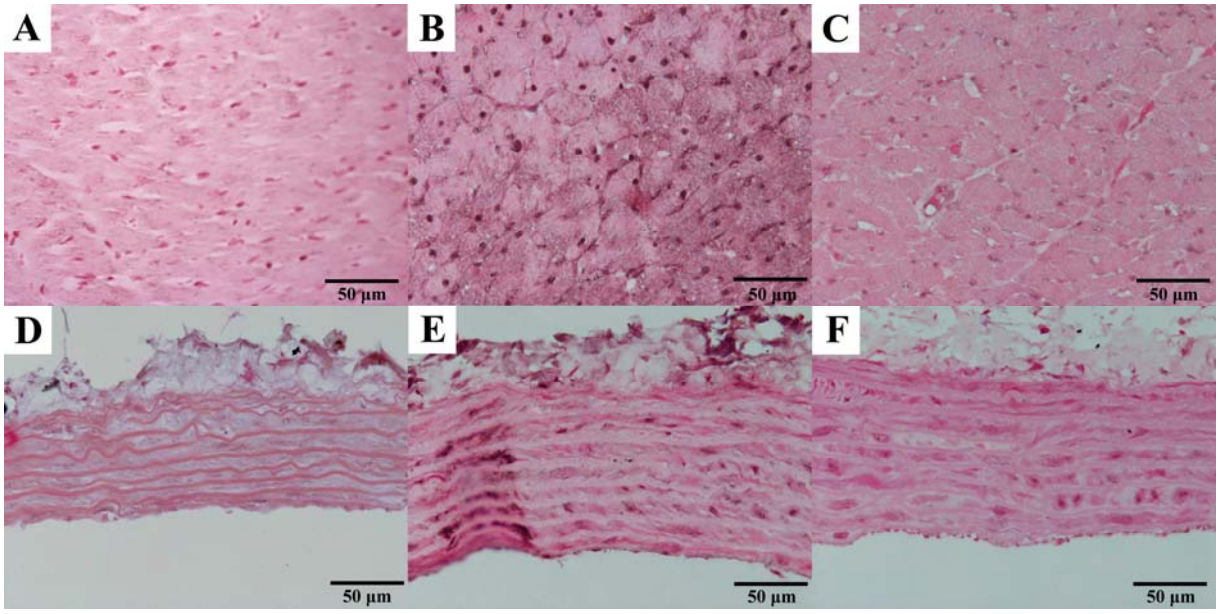


Figure 7. Photomicrographs of poly(ADP-ribose) immunohistochemistry

Representative immunohistochemical stainings for poly(ADP-ribose) (PAR, dark brown/black staining mainly in cell nuclei) in the myocardium (A-C) and aortic wall (D-F). Young control group: A, D; ageing control group: B, E; and ageing INO-1001 treatment groups: C, F (magnification: 400X, scale bar: 50 µm).

5.2.2. Vascular function

Similar to previous studies, the impairment of endothelial function in ageing rats was demonstrated on the thoracic aorta. The ageing-associated endothelial dysfunction was indicated by the reduced maximal relaxation of isolated aortic rings to ACh (61.2 ± 2.2 % ageing control vs. 80.8 ± 2.0 % young control, $P<0.05$), and the rightward shift of the dose-response curve as compared with the young control group. (Fig. 8.A). Single dose treatment with PARP-inhibitor INO-1001 significantly improved the ACh-induced, endothelium-dependent, nitric oxide mediated vasorelaxation in ageing animals (maximal relaxation: 69.1 ± 2.3 % ageing treatment group vs. 61.2 ± 2.2 % ageing control, $P<0.05$). The same treatment had no effect in young rats. (Fig. 8.A)

The endothelium-independent vascular smooth muscle function indicated by the vasorelaxation of aortic rings to SNP was not impaired in ageing rats and was also unaffected by acute INO-1001 treatment. (Fig. 8.B)

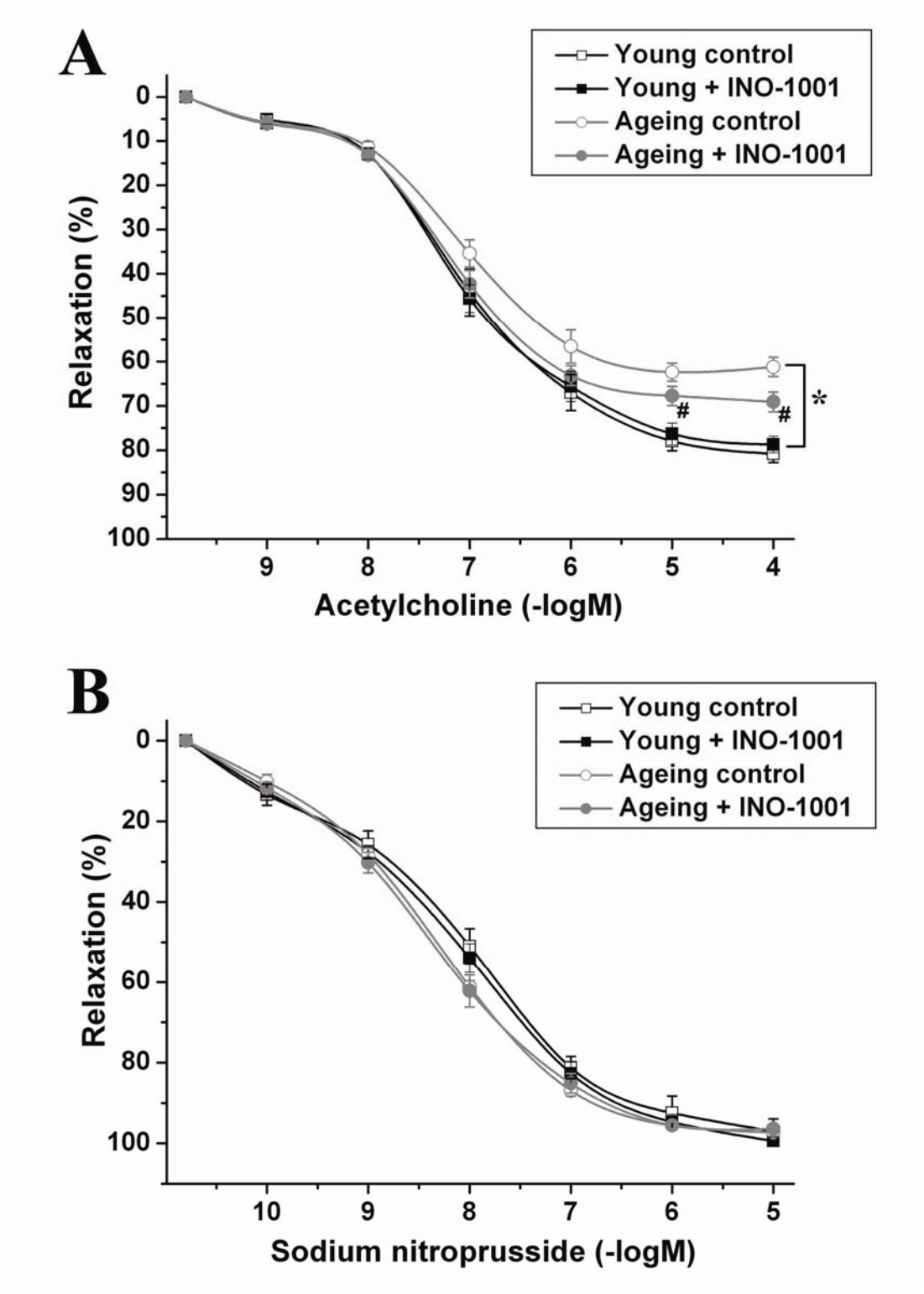


Figure 8. Rapid reversal of ageing-associated vascular dysfunction by treatment with INO-1001 in rat aortic rings

ACh-induced endothelium-dependent relaxation (A), and SNP-induced endothelium-independent relaxation (B). Each point of the curve represents mean ± S.E.M. of 12 experiments with thoracic aortic rings in all groups. *, $P<0.05$ versus young control; #, $P<0.05$ versus ageing control.

Maximal isometric forces produced by the isolated aortic rings precontracted by potassium chloride (100 mM) and phenylephrine (10^{-6} M) were significantly lower in the ageing control group as compared with young animals, which was not influenced by acute PARP-inhibition. (Fig. 9.)

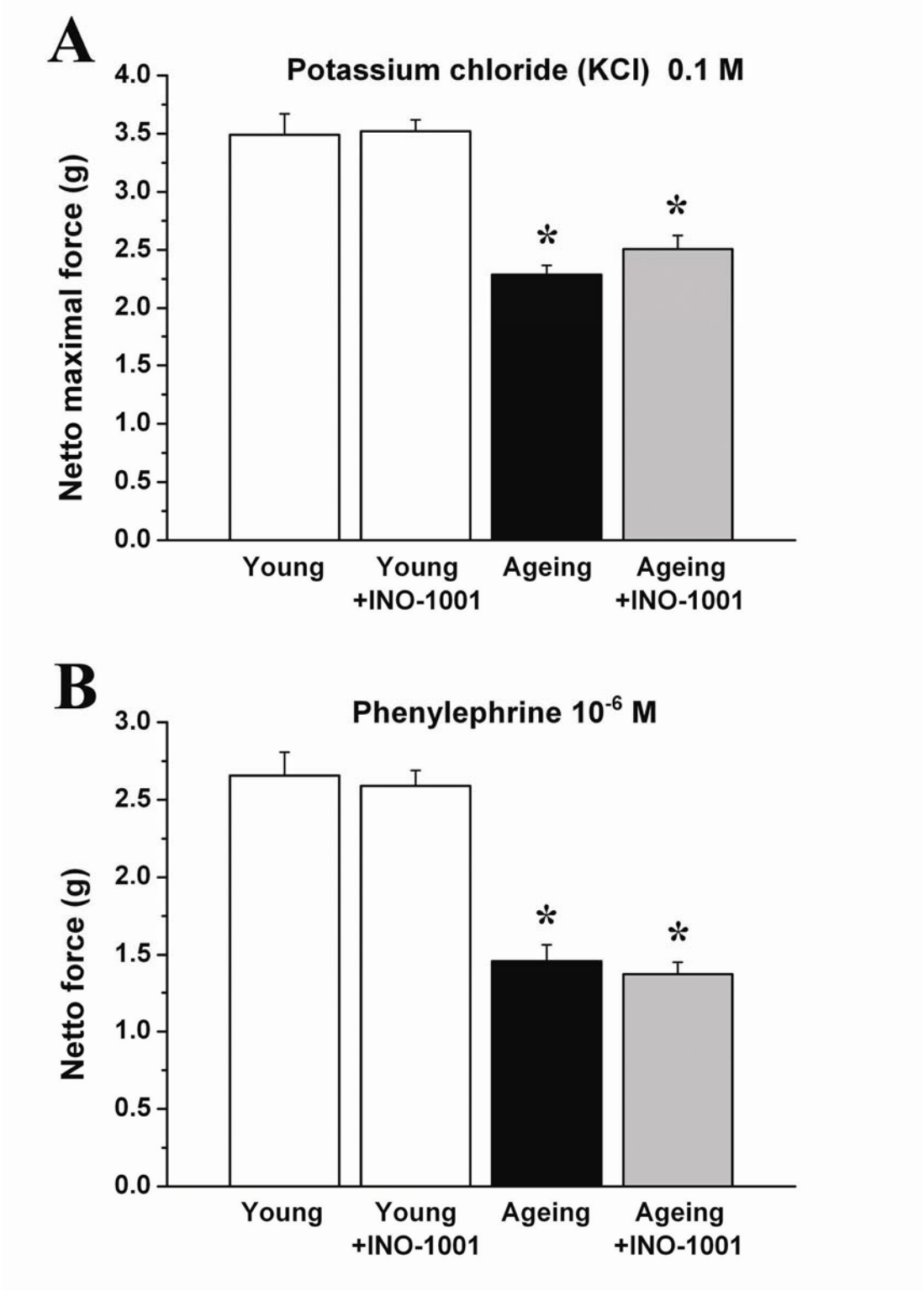


Figure 9. The effect of ageing and acute PARP-inhibition on contraction of rat aortic rings
Contraction forces induced by potassium chloride (KCl; 0.1M) (A) and phenylephrine (PE; 10^{-6} M) (B). Each column represents mean ± S.E.M. of 12 experiments with thoracic aortic rings in all groups. *, $P<0.05$ versus young control.

5.2.3. Cardiac function

In the ageing control group we found significantly decreased mean arterial pressure (MAP), maximal left ventricular systolic pressure (LVSP), developed pressure (DP) and increased left ventricular end-diastolic pressure (LVEDP). Single dose treatment with INO-1001 in ageing rats significantly improved the hemodynamic parameters MAP, LVSP and DP. (Fig. 10.)

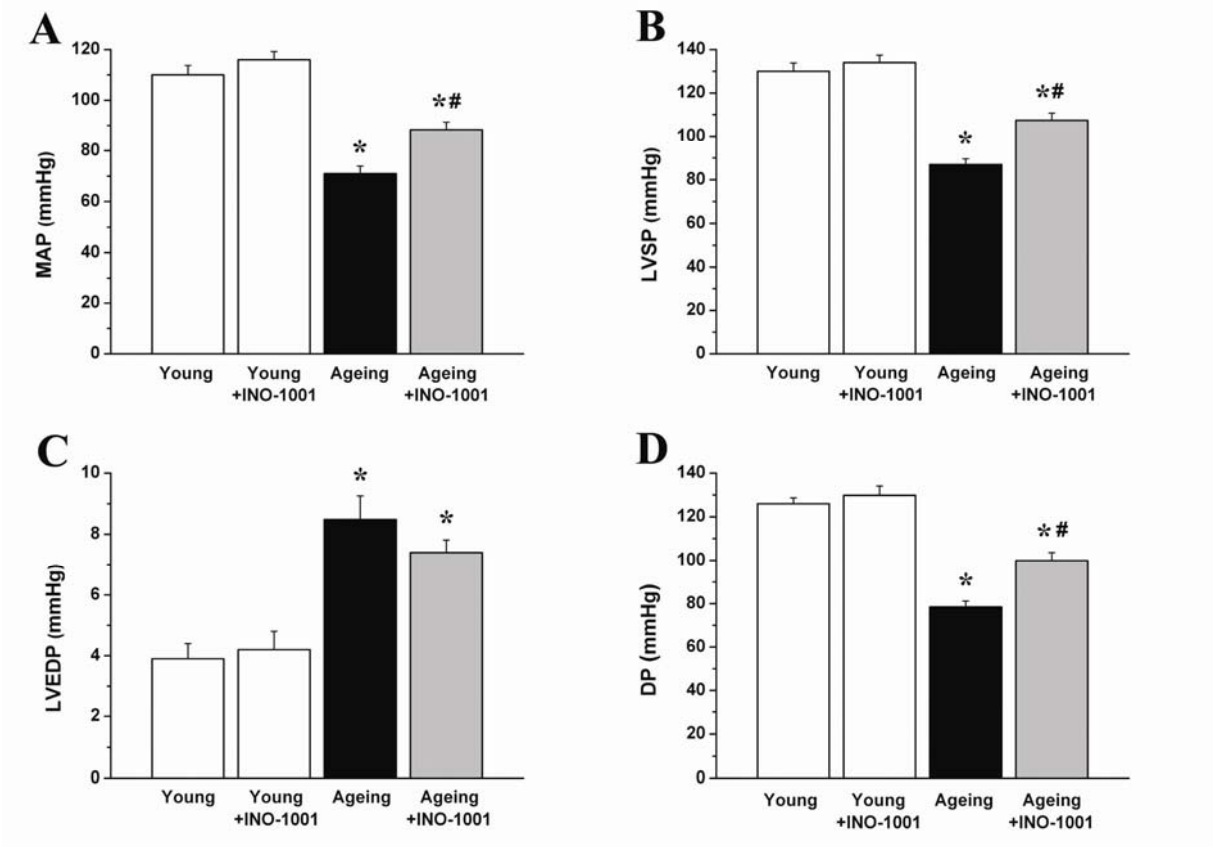
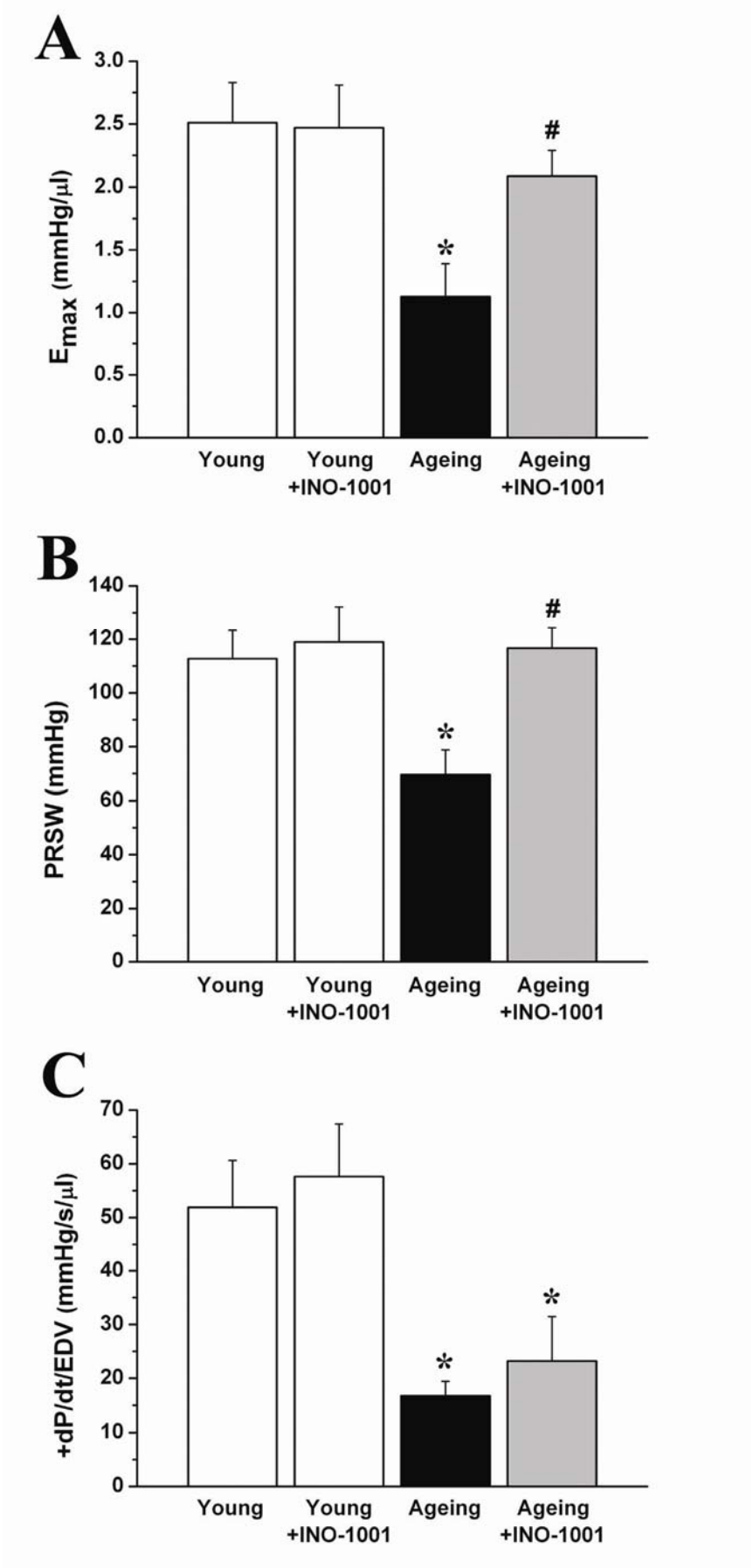


Figure 10. The effect of ageing and acute PARP-inhibition on arterial and left ventricular blood pressure

Mean arterial pressure (MAP), maximal left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and developed pressure (DP) are shown in young adult, young treated with INO-1001, ageing and ageing treated with INO-1001 male rats. Values are mean ± S.E.M. of 7 experiments in each group. *, $P<0.05$ versus young control; #, $P<0.05$ versus ageing control.

When compared to the young group, ageing in rats was associated with significantly decreased left ventricular contractility. The load independent, PV-

loop derived contractility indexes (E_{max}, PRSW, +dP/dt-EDV) showed a marked reduction in ageing animals. After acute PARP-inhibition, we observed significantly increased E_{max} and PRSW, indicating the rapid improvement in left ventricular contractility (Fig. 11.). Treatment with INO-1001 in young rats had no effect on any of the hemodynamic parameters studied (Fig. 10., 11.).


A
3.0
2.5
2.0
1.5
1.0
0.5
0.0
E_{max} (mmHg/μl)
*
#
Young
Young +INO-1001
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Ageing +INO-1001
B
140
120
100
80
60
40
20
0
PRSW (mmHg)
*
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Young
Young +INO-1001
Ageing
Ageing +INO-1001
C
70
60
50
40
30
20
10
0
+dP/dt/EDV (mmHg/s/μl)
*
*
Young
Young +INO-1001
Ageing
Ageing +INO-1001

Figure 11. The effect of ageing and acute PARP-inhibition on cardiac contractility

The slope (E_{es}) of the left ventricular end- systolic pressure-volume relationships (ESPVR), preload recruitable stroke work (PRSW) and maximal slope of systolic pressure increment – end diastolic volume relation (+dP/dt-EDV) are shown in young adult, young treated with INO-1001, ageing and ageing treated with INO-1001 male rats. Values are mean ± S.E.M. of 7 experiments in each group. *, P<0.05 versus young control; #, P<0.05 versus ageing control.

5.3. Ageing-associated cardiovascular dysfunction – effects of catalytic peroxynitrite-decomposition

5.3.1. Immunohistochemical analysis

As shown in Figure 12., significant immunoreactivity for nitrotyrosine and a marked degree of PARP activation were observed in the aortic wall sections of ageing rats, as evidenced by higher NT and PAR scores, when compared with young animals.

Treatment with the potent peroxynitrite decomposition catalyst FP15 in ageing rats significantly reduced nitrotyrosine immunoreactivity and PAR formation in the aortic intima (Fig. 12.). Figure 13. shows representative stainings for NT and PAR in the ageing control and FP15 treatment groups.

Immunohistochemical staining for AIF showed no significant alteration in the localization of this factor in any groups studied (Fig. 12.).

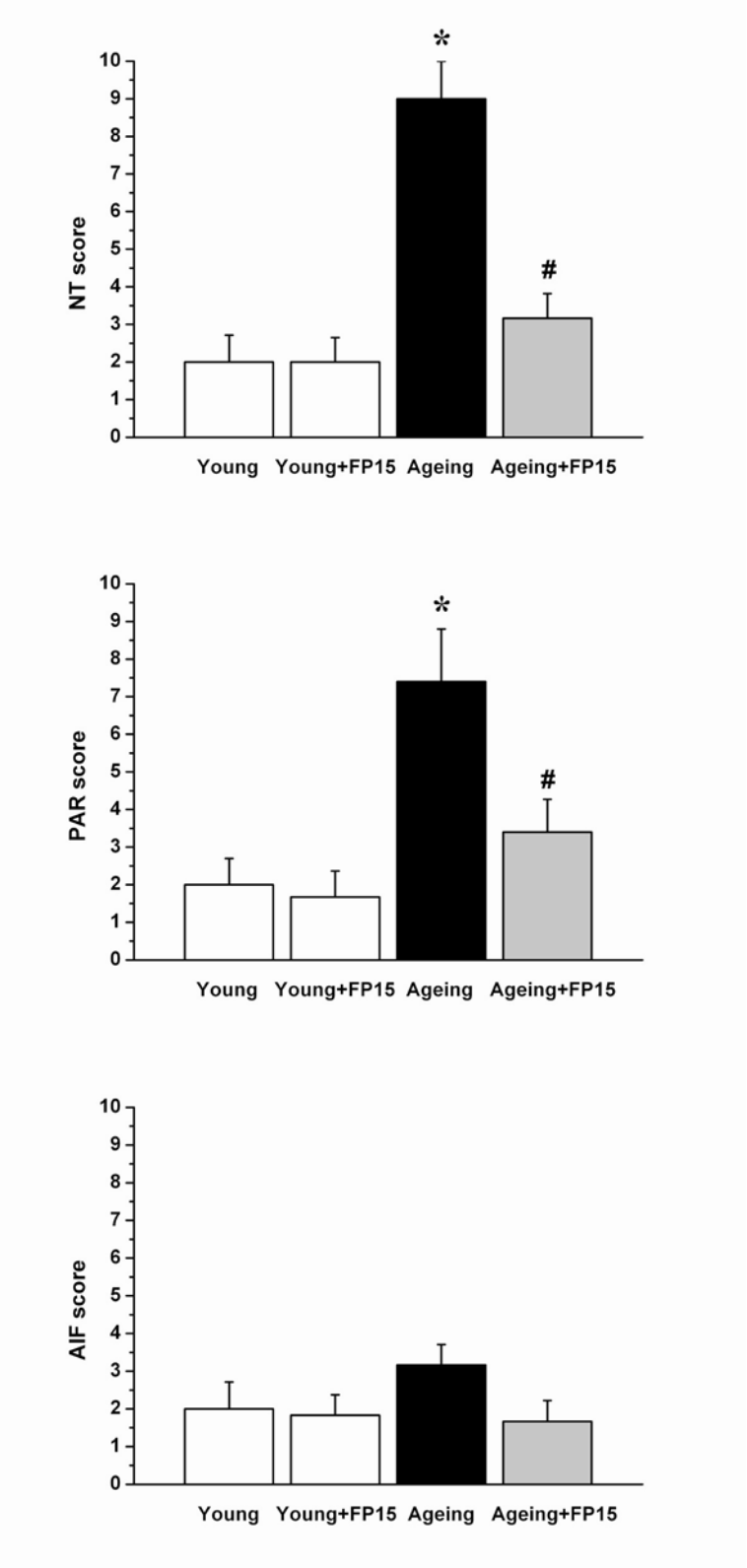

Figure 12. Scoring of NT-, PAR- and AIF-immunohistochemistry

Immunohistochemical scores for nitrotyrosine (NT), poly(ADP-ribose) (PAR), and nuclear apoptosis-inducing factor (AIF) in the intima of aortic wall in young, young treated with FP15,

ageing and ageing treated with FP15 male rats. *, P<0.05 versus young control; #, P<0.05 versus ageing control.

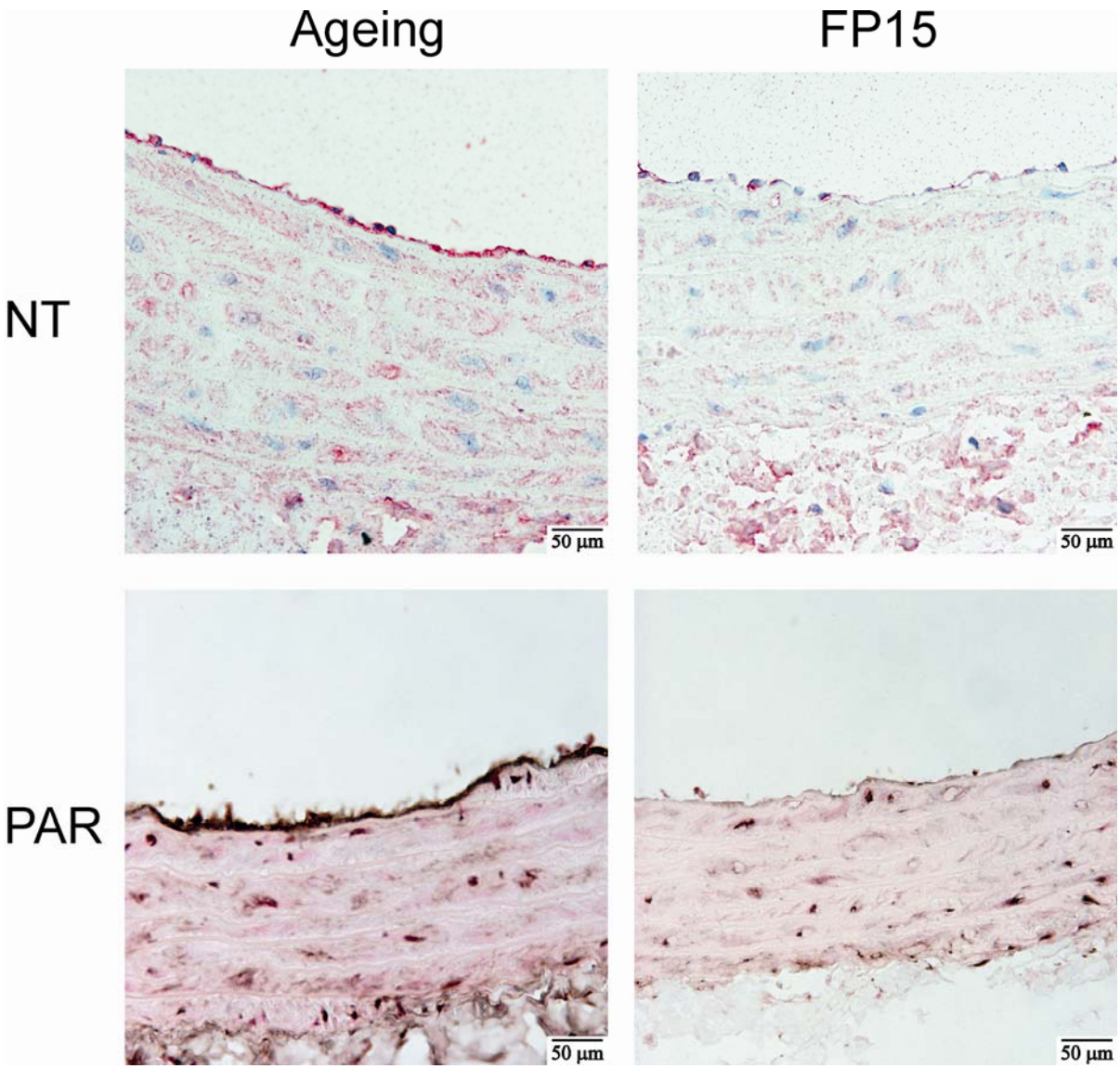

Figure 13. Photomicrographs of NT- and PAR-immunohistochemistry

Representative immunohistochemical stainings for nitrotyrosine (NT) and poly(ADP-ribose) (PAR) in the ageing control, and ageing FP15 treatment groups. (Scale bar: 50 µm)

Immunohistochemical score of eNOS was significantly increased in the aortic endothelium in ageing animals, and was slightly (not significantly) reduced after FP15 treatment (Fig. 14.).

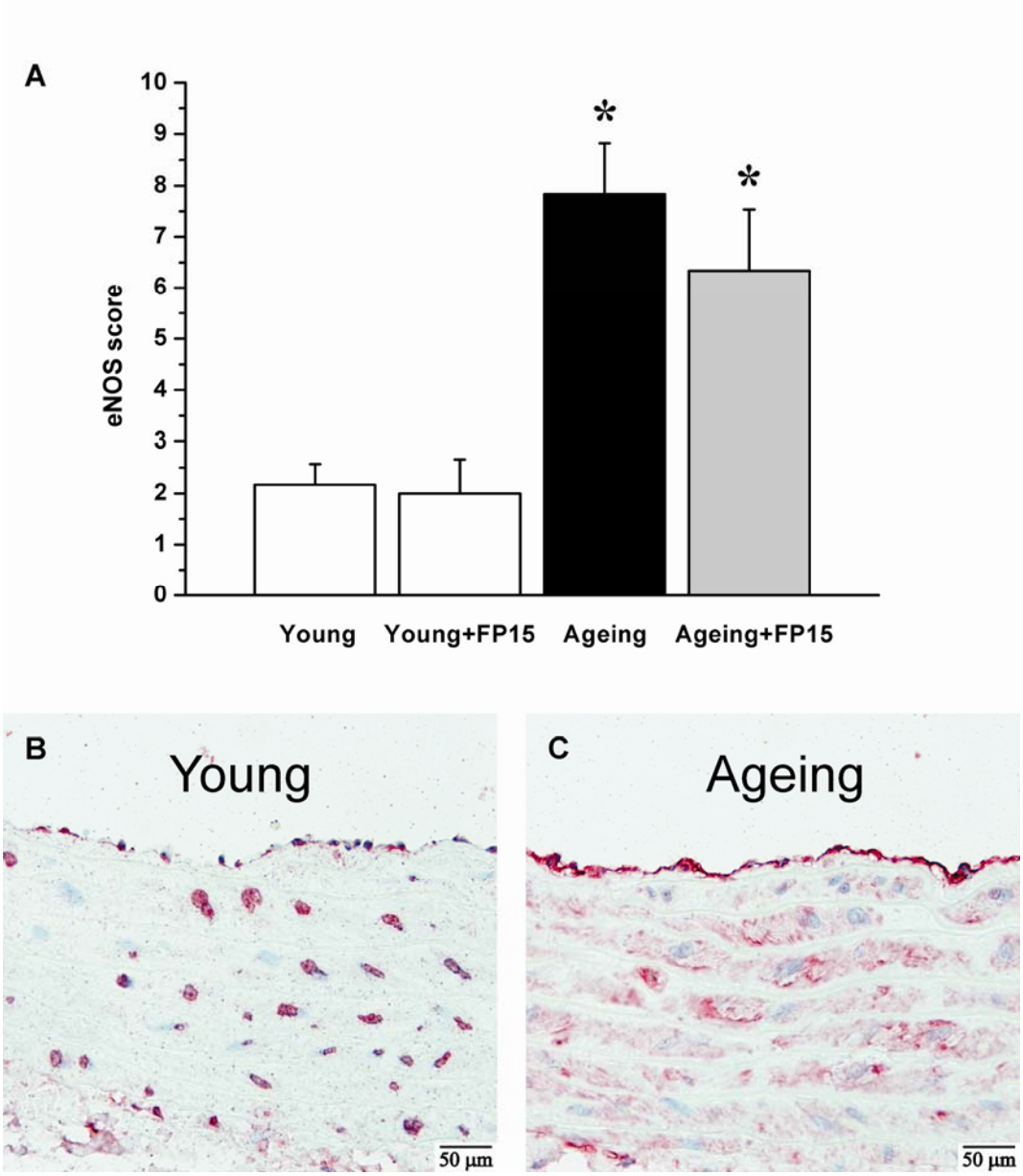

Figure 14. eNOS-immunohistochemistry

Immunohistochemical scores for endothelial nitric oxide synthase (eNOS) in the aortic intima of young, FP15-treated young, ageing and FP15-treated ageing rats (A), and representative immunohistochemical stainings for eNOS in young (B) and ageing control animals (C). *, $P<0.05$ versus young control. (Scale bar: 50 µm)

5.3.2. Vascular function

Similar to previous studies, the impairment of endothelial function in ageing rats was demonstrated in the *in vitro* organ bath experiments. The ageing-associated endothelial dysfunction was indicated by the reduced maximal relaxation of isolated aortic rings to ACh (52.1 ± 1.3 % ageing control vs. 80.8 ± 1.5 % young control,

P<0.05), and the rightward shift of the dose-response curve as compared with the young control group. (Fig. 15.B). Treatment with FP15 for 3 weeks significantly improved the ACh-induced, endothelium-dependent, nitric oxide mediated vasorelaxation in ageing animals (maximal relaxation: 70.3 ± 1.5 % ageing treatment group vs. 52.1 ± 1.3 % ageing control, P<0.05). The same treatment had no effect in young rats. (Fig. 15.B)

The endothelium-independent vascular smooth muscle function indicated by the vasorelaxation of aortic rings to SNP was not impaired in ageing rats and was also unaffected by FP15 treatment. (Fig. 15.C)

Maximal isometric forces produced by the isolated aortic rings precontracted by phenylephrine (10^{-6} M) were significantly lower in the ageing control group as compared with young animals, and there were enhanced maximal contraction forces in the ageing FP15 treatment group. (Fig. 15.A)

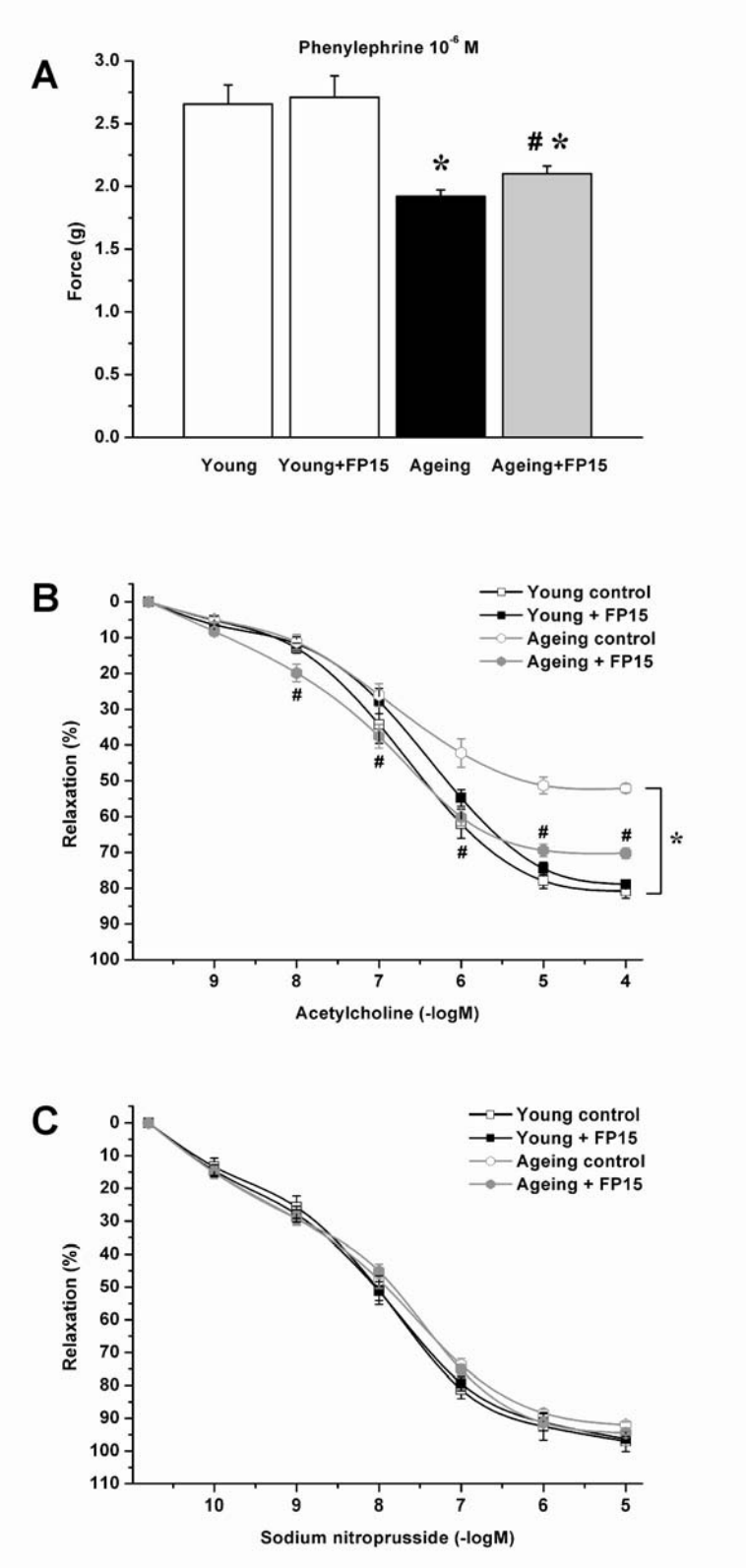

Figure 15. Reversal of ageing-induced vascular dysfunction by treatment with FP15 in rat aortic rings

Contraction forces induced by phenylephrine (10^{-6} M) (A), ACh-induced endothelium-dependent relaxation (B), and SNP-induced endothelium-independent relaxation (C). Each point of the curve represents mean ± S.E.M. of 18-22 experiments in thoracic aortic rings from all 6 animals of all groups. *, $P<0.05$ versus young control; #, $P<0.05$ versus ageing control.

5.3.3. Cardiac function

Ageing in rats was associated with significantly decreased maximal left ventricular systolic pressure (LVSP), developed pressure (DP), mean systolic pressure (MSP), maximal slope of systolic pressure increment (+dP/dt) and diastolic decrement (-dP/dt). In contrast, left ventricular end-diastolic pressure (LVEDP) and the time constant of left ventricular pressure decay (Tau) were increased in ageing animals, indicative of diastolic dysfunction. Mean diastolic pressure (MDP) was not singnificantly altered. (Fig. 16.)

Treatment with the peroxynitrite decomposition catalyst FP15 in ageing rats significantly improved the systolic hemodynamic parameters LVSP, DP, MSP, +dP/dt and the diastolic indexes -dP/dt and Tau. (Fig. 16.)

Mean arterial pressure (MAP) was decreased in ageing animals (77.2 ± 4.7 mmHg vs. 136.1 ± 4.1 mmHg in young control rats), and it was significantly improved after FP15 treatment (146.3 ± 16.6 mmHg, $P<0.05$).

In contrast, in young rats, FP15 had no effect on any of the hemodynamic parameters studied. (Fig. 16.)

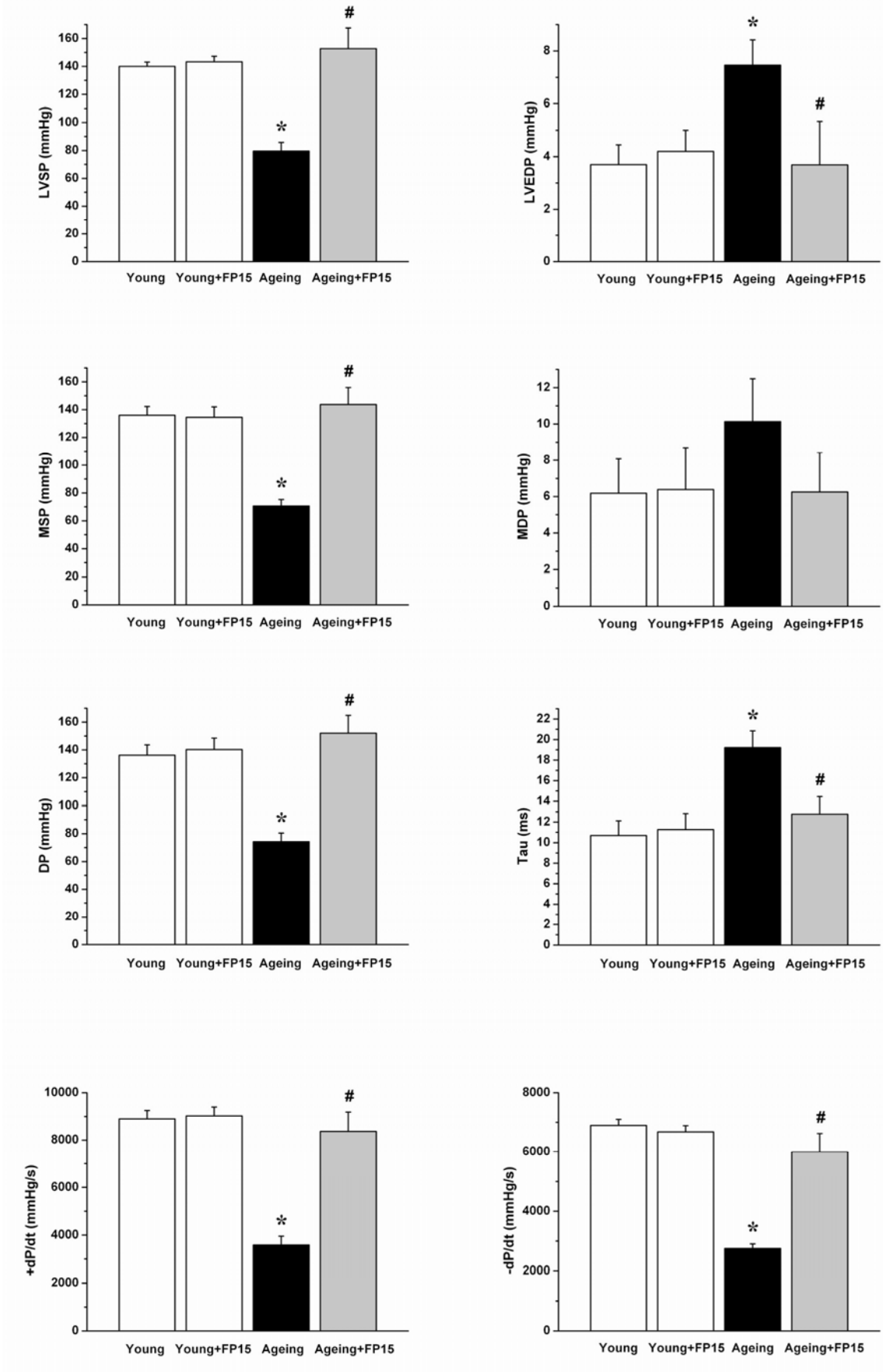
LVSP (mmHg)
LVEDP (mmHg)
MSP (mmHg)
MDP (mmHg)
DP (mmHg)
Tau (ms)
+dP/dt (mmHg/s)
-dP/dt (mmHg/s)
Young
Young+FP15
Ageing
Ageing+FP15
*
#

Figure 16. The effect of ageing and FP15 on cardiac function

Maximal left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), developed pressure (DP), mean left ventricular systolic (MSP) and diastolic pressure (MDP), maximal slope of systolic pressure increment (+dP/dt) and diastolic decrement (-dP/dt) and time constant of left ventricular pressure decay (Tau) are shown in young adult, young treated with FP15, ageing and ageing treated with FP15 male rats. Values are mean ± S.E.M. of 6 experiments in each group. *, $P<0.05$ versus young control; #, $P<0.05$ versus ageing control.

6. Discussion

6.1. *In vitro* model of vascular oxidative stress – novel therapeutic approach by PARP-inhibition

In order to test the possible favourable effects of pharmacological PARP-inhibition on the ageing-associated functional decline of the cardiovascular system as a novel therapy approach against deleterious effects of oxidative stress, we tested as a first step whether this treatment can provide beneficial effects in an *in vitro* model of vascular oxidative damage (i.e. *in vitro* model of endothelial dysfunction induced by hydrogen peroxide).

6.1.1. H_2O_2 and H_2O_2-induced vascular changes

The effects of hydrogen peroxide on the vasculature has been subject of intensive investigations recent years (Dowell et al., 1993; Mian and Martin, 1997; Walia et al., 2000; Thomas et al., 2006; Thengchaisri and Kuo, 2003). There is growing evidence that relatively low concentrations of H_2O_2 (<50 μM) exerts only limited cytotoxicity in many cell types (Halliwell et al., 2000). In this concentration range H_2O_2 participates in vascular signaling and thereby regulates many aspects of vascular endothelial and smooth muscle functions (Thengchaisri and Kuo, 2003; Thengchaisri et al., 2006; Cai, 2005). Under pathophysiological conditions (eg. inflammation or ischaemia/reperfusion) much higher levels of H_2O_2 can be reached in the local circulation of the affected area. At concentrations >100 μM, the direct oxidizing actions of H_2O_2 become dominating causing dysfunction and death of endothelial cells. Recent studies report that *in vitro* exposure of vessels to H_2O_2 at high (200-1000 μM) concentrations but not at relatively low (3-100 μM) concentrations results in impaired function of the endothelium (Dowell et al., 1993; Mian and Martin, 1997; Walia et al., 2000). In accordance with these results we report in the present study impaired endothelium-dependent acetylcholine-induced relaxation of isolated rat aortic rings exposed to 200 and 400 μM H_2O_2. The endothelium-independent relaxation induced by the exogenously administered nitric oxide-donor sodium nitroprusside was unaffected by this concentrations of H_2O_2, indicating normal dilative capacity of the vascular smooth muscle. These functional

data are consistent with the results of Thomas et al. on rabbit aortic rings (Thomas et al., 2006). In contrast, another study (Mian and Martin, 1997) reported inhibition of both endothelium-dependent and –independent vasorelaxation in rat aorta, however at much higher concentrations of H_2O_2 (1 mM). While the phenomenon of H_2O_2-induced endothelial dysfunction is well described, the underlying intracellular pathways and exact molecular mechanisms are still not fully understood.

We demonstrated in the present study, that exposure of rat aortic rings to H_2O_2 resulted in formation of DNA strand breaks in the vessel wall, as evidenced by TUNEL assay. Consistently with these data, previous works on endothelial (Kang et al., 2001) and vascular smooth muscle cells (Li et al., 2000) reported similar results. Furthermore, our immunohistochemical staining for poly(ADP-ribose) clearly demonstrate the subsequent activation of the PARP enzyme in the H_2O_2-groups (Schraufstatter et al., 1986; Junod et al., 1989).

Recent studies report about the possible role of apoptosis-inducing factor in a variety of cell death processes in the cardiovascular system (Xiao et al., 2004; 2005; Ramlawi et al., 2006). Apoptosis-inducing factor is an ubiquitously expressed flavoprotein whose translocation from the mitochondrial inter-membrane space to the nucleus - shown to be regulated by PARP (Xiao et al., 2004; 2005) - has been considered as a critical step in caspase-independent DNA-fragmentation and oxidative cell injury. Consistently with these results we report here enhanced PARP-activation and nuclear translocation of apoptosis-inducing factor in the aortic endothelium after *in vitro* H_2O_2-exposure.

Although we observed intensive PARP-activation by immunohistochemistry also in the vascular smooth muscle in the H_2O_2-groups, our functional results show no impairment (in case of endothelium-independent vasorelaxation to sodium nitroprusside) or only a slight decrease (vasoconstriction induced by phenylephrine) in the vascular smooth muscle functions. Correspondingly, numerous studies on different models of oxidative stress (Szabo et al., 2004; 1997; Pacher et al., 2002e) describe selective impairment of vascular endothelial (but not smoothe muscle) function. Therefore, we assume that the vascular endothelium might be much more vulnerable to oxidative stress injury than smoothe muscle.

6.1.2. Effects of PARP-inhibition on H_2O_2-induced vascular changes

The major finding of these experiments is that pharmacological inhibition of the PARP enzyme (pretreatment of aortic rings with the potent PARP-inhibitor INO-1001) significantly enhanced the endothelium-dependent vasorelaxations (i.e. improved the endothelial function) in aortic rings exposed to 400 μM H_2O_2. Based on this functional improvement we propose that the activation of the PARP pathway play a central role in the pathogenesis of H_2O_2-induced endothelial dysfunction, which is also supported by our immunohistochemical results with PARP-inhibition (prevention of H_2O_2-induced PARP-activation and nuclear translocation of apoptosis-inducing factor, lower amount of DNA-injury and –fragmentation). Previous studies have reported that PARP inhibition also prevents the endothelium-dependent relaxant dysfunction in vascular rings exposed to other reactive species, peroxynitrite (a product of the reaction of superoxide and nitric oxide (Szabo et al., 1997), and hypochlorite (Radovits et al., 2007).

Consistently with our results, previous works with PARP-inhibition showed decreased TUNEL labelling (Yeh et al., 2006), prevention of PARP-activation and nuclear translocation of apoptosis-inducing factor (Xiao et al., 2004; 2005) in several models of oxidative stress-induced diseases. Further data from our group demonstrate translocation of apoptosis-inducing factor in H_2O_2-treated cardiomyocytes *in vitro*, and the prevention of this translocation in the absence of functional PARP enzyme (unpublished observation).

Results of our additional experiments in the INO-1001 control group showed that INO-1001 did not affect vascular function of control aortic rings in the used dose. Thus the improved endothelial function seen in the H_2O_2 + INO-1001 treatment group is a specific phenomenon, ie. preservation of the endothelial responsiveness, rather than the consequence of some nonspecific direct vascular effects of INO-1001.

6.1.3. Proposed underlying molecular pathomechanisms

In spite of the intensive investigations in this field, the exact molecular mechanisms of the pathogenesis of H_2O_2-induced vascular dysfunction still remain unclear, however our current results indicate the important role of the H_2O_2 - DNA-

injury - PARP pathway. As indicated also by our TUNEL labelling in the aortic wall, hydrogen peroxide is known to induce single strand breaks in nuclear DNA in various cell types presumably by indirect mechanisms, since it does not damage purified DNA per se. In the Haber-Weiss reaction catalysed by iron, hydroxyl radicals ($OH^{\cdot}$) are formed from hydrogen-peroxide, which is supposed to be one of the ultimate free radicals to attack DNA (Mello Filho et al., 1984). Another toxic oxidant species, proven to induce DNA single strand breaks is peroxynitrite ($ONOO^{-}$) (Szabo et al., 1996) that is generated in the reaction of superoxide anions ($O_2^{-\cdot}$) and nitric oxide ($NO^{\cdot}$). Considering the observations that H_2O_2 induces production of $O_2^{-\cdot}$ in the vessel wall by activating NADPH oxidase (Coyle et al., 2006), and the abundant NO-production of endothelial cells, this mechanism may be of great importance.

DNA single strand breaks induced by these mechanisms are obligatory triggers of activation of the nuclear enzyme poly(ADP-ribose) polymerase, which mediates the cellular response to DNA injury (Virag and Szabo, 2002). The excessive poly(ADP-ribose) formation (as evidenced in our experiments by poly(ADP-ribose)-immunohistochemistry) results in a cellular energetic crisis (rapid depletion of intracellular NAD^{+} and ATP-pools (Junod et al., 1989; Hu et al., 1998)), which subsequently causes reduced ability of endothelial cells to produce nitric oxide when stimulated by an endothelium-dependent relaxant agonist, such as acetylcholine (Soriano et al., 2001b; Szabo and Bahrle, 2005).

Recent work demonstrated a secondary mechanism by which PARP-activation can lead to impairment of endothelial function. According to these results, certain cellular effectors of DNA fragmentation (such as apoptosis inducing factor) can also be activated by PARP (Xiao et al., 2004). Apoptosis inducing factor appears to be an important factor involved in the regulation of caspase-independent apoptosis in neurons (Cregan et al., 2002) and cardiomyocytes during oxidative stress (Chen et al., 2004). The physiological purpose of this pathway may be that cells with irreparable DNA damage can become safely eliminated.

Based upon the above discussed mechanisms, pharmacological inhibition of PARP can effectively prevent the energy depleting (ADP-ribose)-polymerisation (as verified by decreased poly(ADP-ribose)-formation in our present study), preserves

the energy balance (ATP-pools) of endothelial cells (as evidenced by former studies on cell cultures (Hu et al., 1998) and aortic rings (Soriano et al., 2001a)) thereby protecting endothelial function. Decreasing caspase-independent cell death (as reflected by reduced number of apoptosis inducing factor-positive endothelial nuclei) by INO-1001 pretreatment may also contribute to the showed improvement in endothelial function.

It is frequently assumed that a risk of PARP inhibition in disease conditions is the blockade of DNA repair, and an increase in the number of unrepaired DNA strand breaks. In the current study (similar to studies investigating DNA strand breaks in blood vessels from diabetic rodents with or without PARP inhibition (Garcia Soriano et al., 2001)), we did not see an increase in the number of DNA strand breaks (Fig. 1., 2.). In fact, a tendency towards a reduced number of DNA breaks was noted, which would be consistent with a PARP-dependent, self-amplifying production of mitochondria-derived reactive oxygen species, as a result of the initial oxidant/free radical insult (Virag et al., 1998).

In the present *in vitro* study we investigated the oxidative injury and impairment of vascular responsiveness induced by hydrogen peroxide in the isolated rat aorta. We explored the pathophysiological role of the H_2O_2 - DNA-injury - poly(ADP-ribose) polymerase pathway in this impairment by demonstrating favourable effects of pharmacological PARP-inhibition on H_2O_2-induced endothelial dysfunction. The current *in vitro* data further support the notion that PARP inhibition may represent a potential therapy approach to reduce vascular dysfunction induced by oxidative stress, therefore - based upon the oxidative stress theory of ageing (described above) – it could provide beneficial effects against the functional decline of the cardiovascular system of the ageing organism.

Therefore, further investigations have been performed whether the phenomena observed in the present *in vitro* study have the same significance in an *in vivo* rat model of ageing-associated cardiovascular dysfunction.

6.2. Oxidative stress and the peroxynitrite-PARP pathway in the *in vivo* rat model of ageing-associated cardiovascular dysfunction

Recent studies elucidated numerous cellular and molecular mechanisms responsible for the functional decline of the cardiovascular system at old age (Csiszar et al., 2005). The oxidative stress hypothesis (or free radical theory, as it was originally proposed) (Harman, 1956) is currently one of the most favoured explanations for how ageing leads to progressive cellular damage at the biochemical level. According to this theory, age-related loss of physiological function and ageing is caused by the deleterious effects of progressive and irreversible accumulation of oxidative damage. Several previous studies have demonstrated that ageing organisms have a higher rate of mitochondrial free radical production ($O_2^{-\cdot}$ and H_2O_2) due to the incomplete terminal oxidation at older age (Sohal and Sohal, 1991; Nohl et al., 1978; Kim et al., 1996).

Large amounts of the superoxide anions which are produced in ageing tissues interact with the physiological mediator nitric oxide, forming the potent oxidant peroxynitrite ($O_2^{-\cdot} + NO^{\cdot} \rightarrow ONOO^-$) (Halliwell, 1997). Due to its high diffusibility across lipid membranes in the protonated form, peroxynitrite can easily penetrate cells and tends to attack various biomolecules and cellular structures, thereby inactivating functionally important receptors and enzymes (van der Loo et al., 2000), and causing various forms of DNA-injury (strand breaks and base modifications).
Oxidative and nitrosative stress are endogenous inducers of DNA single strand breakage that is the obligatory trigger of activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which mediates the cellular response to DNA injury (Virag and Szabo, 2002).

Depending on the severity of DNA damage, different cellular pathways can be triggered. In the case of mild DNA damage, PARP facilitates DNA repair and thus cell survival. Severe DNA injury causes excessive PARP activation that initiates an energy-consuming futile repair cycle by transferring ADP-ribose units from NAD^+ to nuclear proteins. The excessive nuclear poly(ADP-ribose) formation results in rapid depletion of intracellular NAD^+ and ATP-pools, slowing the rate of glycolysis and mitochondrial respiration, eventually leading first to cellular energetic crisis and dysfunction, then to cell necrosis (Soriano et al., 2001b). By this route, PARP

activation in cardiomyocytes and endothelial cells leads to a cellular energetic crisis, which subsequently causes functional impairment of contractile function at the cellular level and reduced ability of endothelial cells to produce nitric oxide when stimulated by an endothelium-dependent relaxant agonist, such as acetylcholine (Soriano et al., 2001b; Szabo et al., 1997; Pacher et al., 2002a; 2002b; Szabo et al., 2004a; Pacher et al., 2002d; 2002e; Szabo and Bahrle, 2005). Impairment of endothelial function in the coronary arteries may lead to regional or global myocardial ischaemia, which secondarily impairs cardiac performance (Pacher et al., 2004b).

Recent work demonstrated that certain cellular effectors of DNA fragmentation can also be activated by PARP. According to these results, PARP regulates the mitochondrial-to-nuclear translocation of the apoptosis-inducing factor (AIF) in cardiomyocytes and vascular cells. The physiological purpose of this pathway may be that cells with irreparable DNA damage can become safely eliminated (Xiao et al., 2004).

Enhanced rate of cell death in the ageing myocardium and vessel wall via the necrotic or the apoptotic route by PARP activation results in cardiac and vascular remodeling and impairment of the cardiac and endothelial function (Pacher et al., 2002b; 2002e; Capasso et al., 1990).

The increased peroxynitrite formation and PARP activation together with the above discussed downstream molecular and intracellular mechanisms are considered to play an important role in the pathogenesis of various forms of chronic heart failure (Pacher et al., 2002c; 2005; 2006).

6.2.1. Cardiovascular ageing, oxidative stress and the PARP pathway – immunohistochemical aspects

Similar to other studies (van der Loo et al., 2000; Csiszar et al., 2002; 2005), we demonstrated increased immunoreactivity for nitrotyrosine and activation of PARP in the left ventricular myocardium and aortic wall of ageing rats (Fig. 6., 7., 12., 13.), which confirms the nitro-oxidative stress and the activation of the peroxynitrite-poly(ADP-ribose) polymerase pathway, and are consistent with the above discussed

"free radical theory" of ageing (Pacher et al., 2005; Ungvari et al., 2005). However, our immunohistochemical data for AIF in the aorta showed only a tendency towards more intense nuclear staining (indicative of mitochondrial-to-nuclear translocation of this factor) in ageing rats without reaching the level of significance.

In ageing vessels, the nitro-oxidative damage of the vascular smooth muscle layers were found to be less pronounced, when compared to the endothelium. (Fig. 6., 7., 13.) These immunohistochemical findings are in line with recent reports demonstrating that the vascular superoxide-overproduction at old age occurs mainly in the endothelial cells (van der Loo et al., 2000), which are in addition more vulnerable to oxidative injury.

After treatment with the peroxynitrite decomposition catalyst FP15 in ageing rats we found significantly reduced nitrotyrosine and PAR formation in the aortic intima (Fig. 12., 13.). Similar to other studies with PARP-inhibitors (Xiao et al., 2004; Pacher et al., 2002f), our immunohistochemical data after FP15 treatment indicate the inhibition of the peroxynitrite-PARP pathway, the main downstream mechanism of nitro-oxidative stress. Consistent with our current study, WW85 (another peroxynitrite decomposition catalyst) has also been shown to prevent PARP activation in a different experimental model of cardiovascular injury (heart transplantation and rejection) (Pieper et al., 2005).

Blocking the peroxynitrite-PARP-pathway by direct pharmacological inhibition of the PARP enzyme with INO-1001, effectively decreased PAR-formation in various models of disease, but there are conflicting data about the effect of PARP-inhibitors on tyrosine-nitration (Beller et al., 2006; Farivar et al., 2005; Pacher et al., 2002c). After single dose treatment with INO-1001 in ageing rats we found decreased PAR staining (reflecting decreased PARP activity) both in the myocardium and the aortic intima, however, the immunoreactivity for protein-nitrotyrosine was unaffected. These findings are consistent with the hypothesis that short-term PARP inhibition is sufficient to affect the imbalance between the rapid and reversible polymerization and degradation of ADP-ribose units (by poly(ADP-ribose) polymerase (PARP) and poly(ADP-ribose) glycohydrolase (PARG); (Davidovic et

al., 2001)), but it does not affect upstream processes such as peroxynitrite generation and action (a marker of which is nitrotyrosine), and/or it might exert feedback effects on the generation of oxidants, but this is not reflected in the staining for nitrotyrosine, as this is a rather stable product.

6.2.2. Ageing-associated endothelial dysfunction

Endothelial dysfunction associated with advanced ageing is a well-known phenomenon and can be explained by the reduced nitric oxide ($NO^{\cdot}$) production of endothelial cells (Soriano et al., 2001b); or by the increased $NO^{\cdot}$-inactivation by superoxide anions (peroxynitrite formation) resulting in altered $NO^{\cdot}$ bioavailibility (van der Loo et al., 2000). The underlying intracellular pathways and molecular mechanisms have been subject of intensive investigations recent years (van der Loo et al., 2000; Pacher et al., 2004b; 2002e; 2002f). In accordance with these studies we report here impaired endothelium-dependent acetylcholine-induced relaxation of isolated aortic rings of ageing rats. The endothelium-independent relaxation induced by the exogenously administered $NO^{\cdot}$-donor SNP was unaffected by ageing, indicating the normal dilative capacity of the vascular smooth muscle. These functional data are consistent with our immunohistochemical findings showing signs of severe nitro-oxidative damage mainly in the endothelium of ageing vessels. In contrast with a previous work using epinephrine for precontraction (Pacher et al., 2002e), we found a significant decrease in contraction forces induced by phenylephrine and potassium chloride in ageing animals which was in line with the results of another study investigating vascular function of diabetic rats (Soriano et al., 2001a) and may be due to alterations in receptor density and/or receptor/effector coupling.

The endothelial dysfunction in ageing can be explained by the reduced ability of endothelial cells to produce nitric oxide when stimulated by an endothelium-dependent relaxant agonist, such as acetylcholine (Soriano et al., 2001b). Interestingly, we found significantly enhanced immunoreactivity for endothelial nitric oxide synthase (eNOS) in the aortic endothelium of ageing rats (Fig. 14.), suggesting intensive $NO^{\cdot}$-generation. Thus, we hypothesize that the reduced

endothelium-dependent relaxation associated with ageing is not due to the downregulation of $NO^{\cdot}$-production of endothelial cells, but rather to the increased $NO^{\cdot}$-inactivation by superoxide anions resulting in altered $NO^{\cdot}$ bioavailibility, as $NO^{\cdot}$ is removed in the reaction of peroxynitrite formation ($O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^{-}$). The paradoxical increase in eNOS expression and activity (demonstrated also by another recent study) may serve as an insufficient compensatory mechanism attempting to counteract the increased inactivation of $NO^{\cdot}$ (van der Loo et al., 2000).

Similarly to the effects of chronic pharmacological PARP inhibitors in experimental models of ageing (Pacher et al., 2004b; 2002e; 2002f), blocking the peroxynitrite-PARP-pathway by rapid catalytic decomposition of peroxynitrite by FP15 treatment in the present work significantly enhanced the endothelium-dependent vasorelaxations (i.e. improved the endothelial function in rats with advanced ageing), but did not affect the normal vasorelaxation to SNP or the vascular function of young rats (Fig. 15.).

Previous studies report acute amelioration of endothelial dysfunction in chronic diseases and pathophysiological conditions: single doses of antioxidants (such as vitamin C), tetrahydrobiopterin and L-arginine were shown to improve endothelial dysfunction associated with hypertension, diabetes, chronic smoking or atherosclerosis (Pieper, 1997; Heitzer et al., 2000; Taddei et al., 1998; Quyyumi, 1998). All of these therapeutical attempts focuses on the restoration of nitric oxide bioavailibility either by directly supplying the endothelial nitric oxide synthase with substrate/co-factor (and reversing eNOS-uncoupling) or by decreasing $NO^{\cdot}$-removal by reducing nitro-oxidative stress. As previously reported, *in vitro* incubation of blood vessels with PARP-inhibitors resulted in rapid reversal of endothelial dysfunction in aortae from diabetic mice (Soriano et al., 2001a) and also in the early stage of atherosclerosis (Benko et al., 2004). Similarly to the results of these studies we report here significantly enhanced endothelium-dependent vasorelaxation (improved endothelial function) in aortic rings of rats with advanced ageing after a single dose injection of PARP-inhibitor INO-1001.

We propose that the ageing-related overproduction of reactive oxygen and nitrogen species represents a continuous trigger of DNA single strand breakage that, in turn keeps PARP in an activated state, which continuously depletes the ATP-pools of endothelial cells, thereby impairing the endothelium-dependent relaxant responsiveness. Under this continuing nitro-oxidative stress the endothelium is likely to exist in a state of chronic energy starvation (metabolic suppression) associated with dysfunction. Due to the reversibility of the rapid polymerization of poly(ADP-ribose) units, pharmacological interruption of this metabolic cycle by acute inhibition of PARP can lead to normalization of the energy balance of endothelial cells by rapid restoration of endothelial ATP-levels to normal levels (Szabo et al., 2002), and according to some reports even to higher levels (Csordas et al., 2006). Considering the fact that - similarly to former studies - our results on young control rats show no direct vasodilatory effects of INO-1001, the found acute effect of PARP-inhibition on ageing-associated endothelial dysfunction can be explained by this intracellular mechanism. However, most of the studies on ATP-levels supporting our hypothesis were conducted on cultured endothelial cells, obvious evidence for the proposed mechanism could be provided by directly measuring *in vivo/ex vivo* tissue ATP-levels, but this was not conducted in the present study, and therefore alternative explanations for the current findings are also theoretically possible.

6.2.3. Ageing-associated myocardial dysfunction

Recent studies performing invasive hemodynamic measurements in ageing rats report decreased cardiac performance and development of progressive heart failure after the age of 20 months (Pacher et al., 2004a; 2004b; Anversa et al., 1989). A recent study provided detailed echocardiographic evidence of a progressive decrement in multiple aspects of systolic and diastolic left ventricular function in ageing rats (Boluyt et al., 2004).

Consistent with these results, we demonstrated that advanced ageing is associated with impaired cardiac relaxation and diastolic dysfunction, as reflected by prolonged time constant of pressure decay (Tau), increased LVEDP and decreased –dP/dt, and a marked depression of systolic pressure development, as indicated by

decreased MAP, LVSP and depressed contractility index +dP/dt (maximal slope of systolic pressure increment).

Furthermore, by measuring left ventricular pressure-volume relations during transiently compressing the inferior vena cava, we calculated sensitive indexes of myocardial contractility. Animals of advanced age showed impaired left ventricular contractility, as reflected by these PV-loop-derived load-independent contractility-indexes E_{max}, PRSW or +dP/dt-EDV. These indexes are widely used as sensitive cardiac contractile parameters, because they are independent from changes in loading conditions and therefore especially informative in assessing cardiac contractility in models, where preload and afterload are altered.

Rapid pharmacological decomposition of peroxynitrite with FP15 ameliorated both systolic and diastolic cardiac function in ageing animals (Fig. 16.), as indicated by the improvement of all hemodynamic parameters measured in our study. These data are also consistent with the results of a recent study investigating the beneficial effects of chronic treatment with the PARP-inhibitor INO-1001 in age-related cardiac dysfunction (Pacher et al., 2004b), blocking the pathway at a stage downstream of peroxynitrite, by directly inhibiting the PARP enzyme. Two other reports on chronic PARP-inhibiton with another compound, PJ34, however, demonstrated improvement in only the diastolic (but not systolic) cardiac function (Pacher et al., 2002e; 2002f).

Our present studies provide the first evidence, that acute inhibition of PARP by a single dose injection of INO-1001 results in rapid improvement of the chronic cardiac and vascular dysfunction associated with advanced ageing. The possible molecular mechanism responsible for these beneficial effects of single dose PARP-inhibition are supposed to be the energy restoration of myocardial cells, similarly to that discussed detailed above. In other words, similar to the situation in blood vessels, it is possible that PARP inhibition acutely affects cardiac contractility via improvement of cardiac high energy phosphate status. An acute improvement in the myocardial blood supply (due to improved endothelial function in the coronary microvasculature) may also be feasible.

In accordance with our previous work with other PARP-inhibitors (Szabo et al., 2002a) we found that INO-1001 did not affect cardiac function of young control rats. Thus the improved cardiac function seen in the ageing treatment group is a specific phenomenon, reflecting a reversal of the ageing-associated suppressed myocardial performance, rather than the consequence of some nonspecific direct cardiac effects of INO-1001.

6.2.4. Inhibition of the peroxynitrite-PARP-pathway as novel therapeutic possibility

This is the first study reporting rapid improvement of the chronic cardiac and vascular dysfunction associated with advanced ageing by acute inhibition of the PARP enzyme and by rapid catalytic decomposition of peroxynitrite. The current findings indicate the importance of the nitro-oxidative stress - PARP - pathway, especially its quickly reversible energy depleting aspects in the pathogenesis of myocardial and endothelial dysfunction at old age. The current work further supports the concept that PARP-inhibition and catalytic peroxynitrite-decomposition may represent novel potential therapy approaches to improve cardiovascular dysfunction associated with ageing. There are many studies and reviews about the clinical treatment perspectives of PARP-inhibitors (Jagtap and Szabo, 2005; Graziani and Szabo, 2005). We probably already know enough about the safety of PARP inhibitors to consider them suitable for short-term treatment of acute situations where the inhibition of PARP may provide considerable benefit, e.g. in myocardial reperfusion injury. However, chronic use of PARP inhibitors may be more challenging because of the unknown potential long-term side effects. Whether or not chronic PARP inhibition is safe, there is no clear answer, as chronic safety studies have not yet been conducted with any of the orally bioavailable PARP inhibitors. Depending on the outcome of such studies, chronic PARP inhibition may or may not turn out to be clinically sustainable. It also remains to be tested as to how long is the period for which a single dose of the PARP inhibitor provides cardiovascular benefit. In chronic heart failure models there is evidence that the beneficial effect of PARP inhibition is sustained after discontinuation of the treatment (Pacher et al., 2002c), therefore the duration of the beneficial effect seen in the current study needs to be delineated.

Regarding the improved cardiac function, rapid pharmacological decomposition of peroxynitrite by FP15 in our model seems to be comparable to or better than the efficacy of blocking the pathway by PARP-inhibition. By eliminating peroxynitrite nitro-oxidative stress can be reduced (as shown by reduced nitrotyrosine formation) and severe damage of DNA can be effectively prevented. Additionally, using this concept we can avoid peroxynitrite-induced modifications of enzymes, receptors and structural proteins and may help to restore the normal bioavailibility of the crucial physiological mediator nitric oxide (as confirmed by the enhanced endothelium-dependent vasorelaxation).

7. Conclusions

In the first *in vitro* study we investigated the oxidative injury and impairment of vascular responsiveness induced by hydrogen peroxide in the isolated rat aorta. We explored the pathophysiological role of the H_2O_2 - DNA-injury - poly(ADP-ribose) polymerase pathway in this impairment and demonstrated favourable effects of pharmacological PARP-inhibition on H_2O_2-induced endothelial dysfunction. The current data further support the notion that PARP inhibition may represent a potential therapy approach to reduce vascular dysfunction induced by oxidative stress in several pathophysiological conditions, e.g. in cardiovascular dysfunction associated with advanced ageing.

This is the first study reporting rapid improvement of myocardial and endothelial functions in the *in vivo* rat model of ageing-associated cardiovascular dysfunction by acute inhibition of the PARP enzyme and by catalytic decomposition of peroxynitrite. The current functional and immunohistochemical findings indicate the importance of the endogenous peroxynitrite overproduction and the activation of the peroxynitrite - PARP - pathway, especially its quickly reversible energy depleting aspects in the pathogenesis of myocardial and endothelial dysfunction at old age.

The current work supports the concept that pharmacological PARP-inhibition and/or rapid catalytic peroxynitrite decomposition may represent novel potential therapy approaches to improve cardiovascular dysfunction associated with advanced ageing.

8. Summary

Overproduction of oxidants and free radicals in ageing tissues induces nitro-oxidative stress, which has recently been implicated in the functional decline of the cardiovascular system at advanced age. Cytotoxic oxidants like hydrogen peroxide or peroxynitrite damage proteins and DNA and activate several pathways causing tissue injury, including the poly(ADP-ribose) polymerase (PARP) pathway.

First, we tested whether the inhibition of the PARP enzyme can improve the endothelial dysfunction induced by hydrogen peroxide, in a simple *in vitro* model of vascular oxidative stress. In turn, our main aim was to investigate the effects of acute PARP inhibition and rapid catalytic decomposition of peroxynitrite on ageing-associated cardiac and endothelial dysfunction.

In vascular reactivity measurements on isolated rat aortic rings we investigated the phenyleprine-induced contraction, and endothelium-dependent and –independent vasorelaxation by using acetylcholine and sodium nitroprusside. Endothelial dysfunction was induced by exposing the rings to H_2O_2. In the treatment group, rings were preincubated with the potent PARP-inhibitor INO-1001. In the *in vivo* rat model of ageing-associated cardiovascular dysfunction, young and ageing rats were treated with vehicle, with a single dose of PARP-inhibitor INO-1001, or with the peroxynitrite decomposition catalyst FP15. Using a pressure-volume conductance catheter, left ventricular pressure-volume analysis of the rats was performed. Endothelium-dependent and -independent vasorelaxation of isolated aortic rings of the rats were investigated by using acetylcholine and sodium nitroprusside.

DNA strand breaks were assessed by the TUNEL method. Immunohistochemical analysis of vessel wall and myocardium was performed for nitrotyrosine (“footprint of peroxynitrite”), for poly(ADP-ribose) (the enzymatic product of PARP) and for apoptosis inducing factor (a pro-apoptotic factor regulated by PARP).

In our *in vitro* model, exposure to H_2O_2 resulted in a dose-dependent impairment of endothelium-dependent vasorelaxation of aortic rings which was significantly improved by PARP-inhibition. The dose-response curves of endothelium-independent

vasorelaxation to sodium nitroprusside did not differ in any groups studied. In the H_2O_2 groups immunohistochemical analysis showed enhanced PARP-activation and nuclear translocation of apoptosis inducing factor, which were prevented by INO-1001. Ageing animals showed a marked reduction of systolic and diastolic cardiac function and loss of endothelium-dependent relaxant responsiveness of aortic rings. Both acute PARP-inhibition and FP15-treatment significantly improved cardiac performance and endothelial function. Immunohistochemistry for nitrotyrosine and poly(ADP-ribose) confirmed enhanced nitro-oxidative stress and PARP-activation in ageing animals, which were reversed in the treatment groups.

Our results demonstrate the importance of endogenous peroxynitrite-overproduction and the activation of the PARP-pathway in the age-related functional decline of the cardiovascular system. Rapid catalytic decomposition of peroxynitrite by FP15 and acute inhibition of PARP may represent a novel therapeutic utility to improve cardiac and vascular dysfunction associated with ageing.

9. Összefoglalás

Az öregedő szövetekben reaktív oxigéntartalmú szabad gyökök és oxidánsok túltermelődése figyelhető meg, mely nitro-oxidatív stresszt indukál. A közelmúlt kutatási eredményei szerint e folyamat fontos szerepet tölt be a szív- és érrendszeri funkciók időskorban megfigyelhető hanyatlásában. E citotoxikus oxidánsok, mint pl. a hidrogén-peroxid vagy a peroxinitrit, fehérjék károsításán túl megtámadják a DNS-t, és több reakcióutat, köztük a poli(ADP-ribóz)-polimeráz (PARP) utat aktiválva szövetkárosodáshoz vezetnek.

Munkánkban elsőként azt vizsgáltuk, hogy a vaszkuláris oxidatív stressz egyszerű *in vitro* modelljében a PARP enzim gátlása képes-e javítani a hidrogén-peroxid által károsított endotélfunkción. Fő célunk viszont az volt, hogy megvizsgáljuk a PARP akut gátlásának, illetve a peroxinitrit katalitikus lebontásának hatásait az időskori szív- és érrendszeri diszfunkcióra, s ezzel új antioxidáns terápiás lehetőségek alapjait fektessük le.

In vitro funkcionális mérésekkel izolált patkány aortagyűrűkön vizsgáltuk az endotélfüggő és nem-endotélfüggő vazorelaxációt acetilkolin ill. nátrium-nitroprusszid adására. Az endotélkárosodást hidrogén-peroxid hozzáadásával váltottuk ki. A kezelt csoportban az érgyűrűket előinkubáltuk a PARP enzim INO-1001 jelű gátlószerével. Az időskori kardiovaszkuláris diszfunkció *in vivo* patkánymodelljében fiatal és öreg patkányokat kezeltünk placebóval, az INO-1001-es PARP-inhibitor egyszeri dózisával, illetve a peroxinitrit lebontásának katalizátorával, FP15-tel. A kezelést követően nyomás- és térfogatmérő konduktancia mikrokatéter segítségével bal kamrai nyomás-térfogat analízist végeztünk patkányainkban a szisztolés és diasztolés szívfunkció megítélésére, valamint izolált aortagyűrűk endotélfüggő és nem-endotélfüggő vazorelaxációját vizsgáltuk acetilkolin, ill. nátrium-nitroprusszid adására. A DNS-lánctöréseket a TUNEL módszerrel detektáltuk. Az aortafal és a balkamrai miokardium szövettani feldolgozása után immunhisztokémiai festést végeztünk nitrotirozinra (a peroxinitrit *in vivo* jelenlétének bizonyítéka), poli(ADP-ribóz)-ra (a PARP enzim terméke) és apoptózis-indukáló faktorra (a PARP által szabályozott pro-apoptotikus faktor).

In vitro modellünkben hidrogén-peroxid hatására az endotélfunkció dózisfüggő károsodását tapasztaltuk, mely szignifikáns javulást mutatott a PARP-inhibitorral előkezelt csoportban. A nem-endotélfüggő vazorelaxáció nem mutatott különbséget a vizsgált csoportokban. A hidrogén-peroxid hatásának kitett érgyűrűkben immunhisztokémiailag intenzív PARP-aktivációt és az apoptózis-indukáló faktor sejtmagi transzlokációját bizonyítottuk, INO-1001 előkezelés hatékonyan védte ki ezeket a változásokat. Öreg patkányainkban a szisztolés és diasztolés szívfunkció jelentős gyengülését figyeltük meg, valamint izolált aortagyűrűikben csökkent endotélfüggő relaxációs választ tapasztaltunk. Az egyszeri, akut PARP-gátlás és az FP15-kezelés is szignifikáns javulást eredményezett a szívműködésben és az endotélfunkcióban egyaránt. Öreg állatok esetében a nitrotirozin- és poli(ADP-ribóz)-imunhisztokémia fokozott nitro-oxidatív stressz és intenzív PARP-aktiváció jeleit mutatta, melyek jelentősen csökkentek a kezelések hatására.

Jelen eredményeink jól mutatják az endogén peroxinitrit-túltermelődés és a PARP-reakcióút jelentőségét a kardiovaszkuláris funkciók időskori hanyatlásában. A peroxinitrit gyors katalitikus lebontása FP15-tel, valamint a PARP enzim akut gátlása új antioxidáns terápiás lehetőséget jelenthet az időskori szív- és érrendszeri diszfunkció kezelésében.

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11. List of publications

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