Cardioprotective effect of Poly(ADP-ribose) polymerase inhibition

PhD thesis

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2010.

1. Abbrevations

AIF	apoptosis-inducing factor
BNP	B-type natriuretic peptide
BW	body weight
CFY	CFY Sprague-Dawley rat
DAP	diastolic arterial blood pressure
EF	ejection fraction
ERK 1/2	extracellular signal-regulated kinase
FS	fractional shortening
GSK-3β	glycogen synthase kinase-3β
HF	heart failure
IR	ischemia-reperfusion
ISO	isoproterenol hydrochloride
IVS (d)	thickness of interventricular septum in diastole
IVS (s)	thickness of interventricular septum in systole
JNK	c-jun N-terminal kinase
LVEDV	left ventricular end-diastolic volume
LVESV	left ventricular end-systolic volume
LVID (d)	left ventricular end-diastolic diameter
LVID (s)	left ventricular end-systolic diameter
MAP	mean arterial blood pressure
MAPK	mitogen activated protein kinase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAD^+	nicotinamide adenine dinucleotide
NIH	National Institute of Health
NOS	nitric oxide synthase
PAR	poly(ADP-ribose)polymers
PARP	poly(ADP-ribose)polymerase
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PW (d)	thickness of left ventricular posterior wall in diastole
PW (s)	thickness of left ventricular posterior wall in systole
PTP	permeability transition pore
ROS	reactive oxygen species
RWT	relative wall thickness
SAP	systolic arterial pressure
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
TBS	TRIS-buffered saline
TL	lenght of right tibia
WV	weight of ventricles

1. Introduction

Accumulating evidence suggest that the reactive oxygen and nitrogen species are generated in cardiomyocytes and endothelial cells during myocardial infarction, various forms of HF or cardiomyopathies, circulatory shock, cardiovascular aging, diabetic complications, myocardial hypertrophy, atherosclerosis, and vascular remodelling following injury.

ROS are produced in the ischemic myocardium especially during and after reperfusion when electron transport resumes in the mitochondria after suppression by ischemia.

These reactive species induce oxidative DNA damage and consequent activation of the PARP. 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 long branches of ADP-ribose polymers.

Reactive oxygen and nitrogen species (e. g., peroxynitrite)-dependent citotoxicity in various cardiovascular diseases is mediated by a multitude of effects including lipid peroxidation, protein nitration and oxidation, DNA oxidative damage, activation of matrix metalloproteinases, and inactivation of a series of enzymes. In the heart, ROS can evoke cytotoxicity, myocardial stunning, arrhythmia, reduction of the calcium transient and contractility, elevated diastolic calcium levels and intracellular ATP depletion.

A number of physiological, pharmacological and pathological stimuli initiate cardiac hypertrophy. In addition, cardiac hypertrophy is associated with alterations in intracellular signaling transduction pathways, including alterations of G-protein-coupled receptors, small G protein, MAPK, PKC, calcineurin and calmodulin and so on. Various signaling pathways are involved in the complicated interactions that finally promote cardiac hypertrophy and HF.

Several studies suggest that PARP inhibitors can modulate these intracellular signaling pathways beneficially in various forms of HF or cardiomyopathies, circulatory shock, cardiovascular aging, diabetic complications, myocardial hypertrophy, atherosclerosis, vascular remodeling following injury and during myocardial IR.

To reveal the effect of PARP inhibitors on intracellular signaling pathways and echocardiographic parameters in rats, two experimental models were used. First, we investigated

the action of PARP inhibition in vivo in a postinfarction heart failure model, then PARP inhibitor agent was tested on non-compensated phase of SHRs.

In these studies, as PARP inhibitor L-2286 was used. L-2286 is derived from 2-mercapto-4(*3H*)-quinazolinone by alkylation with 1-(2-chloroethyl)piperidine. L-2286 was choosen, because in vitro PARP assay it exhibited significantly better PARP inhibitory activity than basic quinazolines such as 4-hydroxyquinazoline or 2-merkapto-4(*3H*)-quinazolinone (17), (Fig. 1).

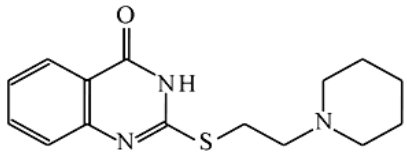

Figure 1. Chemical structure of L-2286 (2-[(2-Piperidine-1-ylethyl)thio]quinazoline-4(3H)-one).

2. Aims

3. Aims of the study

The aim of this work was provide evidence for beneficial in vivo effects of PARP inhibition.

1. To assess cardioprotection afforded by PARP inhibition

a) We tested whether the PARP inhibitor, L-2286 can attenuate the isoproterenol-induced myocardial damage

b) We tested whether the long-term administration of L-2286 can diminish the signs of hypertension induced-HF

c) We compared the protective effect of PARP-inhibition to that of ACE-inhibition against the postinfarction myocardial remodelling.

2. To provide evidence for cardioprotective effects of L-2286, the following parameters were examined:

a) interstitial fibrosis in histological samples

b) phosphorylation state of PI3K/Akt-1^{Ser473}/GSK-3β^{Ser9}, MAPK, PKC cascades by Western blotting

c) echocardiographic parameters with high-resolution imaging system

3. Material and Methods

Postinfarction heart failure model

Male CFY Sprague-Dawley rats were involved into this study. MI was induced by subcutaneous injection of 120 mg/kg ISO, while physiological saline (1 ml/kg) was given to control rats subcutaneously, two times. 24 hours after the second injection the surviving animals were randomly assigned to receive either 5 mg/kg/day L-2286 (a gift of Prof. Dr. Kalman Hideg), a water-soluble PARP inhibitor (ISO+L) or 10 mg/kg/day enalapril maleate (ISO+E), or water (ISO). The fourth group was an age-matched control group (C).

Hypertensive heart failure model

Male 30-week-old SHR rats a compensatory hypertrophic stage were divided randomly into two groups. One group received no treatment (SHR-C), while the other group recieved L-2286 (a water-soluble PARP inhibitor) 5 mg/bw in kg/d for 46 weeks (SHR-L). The third group was an age-matched normotensive control group (CFY).

The investigations conforms with to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Animal Research Review Committee of the University of Pecs Medical School.

Gravimetric parameters

Animals were euthanized with an overdose of ketamine hydrochloride intraperitoneally and heparinized with sodium heparin, sacrified, their hearts were removed, the atria and great vessels were trimmed from the ventricles and weight of the ventricles was measured, which was then normalized to the body mass and to the length of the right tibia (indices of cardiac hypertrophy). The lung wet weight-to-dry weight ratio (an index of pulmonary congestion) were also measured in experimental animals.

Invasive blood pressure measurements

Five rats from each group in the heart failure model were anaesthetized with ketamine hydrochloride intraperitoneally and a polyethylen catheter was inserted into their left arteria femoralis. Blood pressure was measured by CardioMed System (CM-2005).

Determination of Plasma B-type natriuretic peptide

Blood samples were collected into the Lavender Vacutainer tubes containing EDTA. Plasma B-type natriuretic peptide-45 levels (BNP-45) were determined by enzyme immunoassay method.

Histology

Formalin-fixed ventricles were sliced and embedded in paraffin. 5 μm thick sections were cut serially from base to apex. Slices at 1 mm intervals were stained. Slices were stained with Picrosirius Red or Masson's trichrome staining to detect the interstitial fibrosis. Sections were quantified with the NIH ImageJ analyzer system.

Western blot analysis

Fifty miligrams of heart samples were homogenized in ice-cold 50mM Tris buffer, pH 8.0 (containing protease inhibitor cocktail 1:100, and 50 mM sodium metavanadate, and harvested in 2x concentrated SDS-polyacrylamide gel electrophoresis sample buffer. Sodium metavanadate was used as phosphatase inhibitor. Proteins were separated by 10% or 12% SDS-polyacrylamide gel electrophoresis. After blocking (2 h with 3% nonfat milk in Tris-buffered saline), membranes were probed overnight at 4°C with primary antibodies and the next day with the secondary antibodies. Complexes were visualized by means of enhanced chemiluminescence. After scanning, results were quantified by NIH ImageJ program.

Noninvasive evaluation of cardiac function

At the beginning of the experimets all animals were examined by echocardiography to exclude rats with any heart abnormalities. Transthoracic two-dimensional echocardiography was performed under inhalation anesthesia at the beginning of the experiment and on the day of sacrifice. Rats were lightly anesthetized with a mixture of 1.5% isoflurane and 98.5% oxygen. The chest of animals was shaved, acousting coupling gel was applied and warming pad was used to maintain normothermia. Animals were imaged in the left lateral decubitus position. Cardiac dimensions and functions were measured from short- and long-axis views at the mid-papillary level by a VEVO 770 high resolution ultrasound imaging system (VisualSonics, Toronto, Canada) - equipped with a 25 MHz transducer.

4. Conclusions

4.1 Cardioprotection by PARP inhibition in postinfarction heart failure model

Our study strengthened the previous data of our workgroup that an isoquinoline derivate PARP inhibitor had very prominent protective effect against postinfarction myocardial remodeling in rats. However our recent work demonstrated firstly, that PARP inhibitors can activate the Akt-1/GSK-3β prosurvival signaling pathway, during postinfarction heart failure. We also compared the efficacy of complete PARP inhibition to that of complete ACE inhibition.

Accumulated data suggest that PI3K/Akt signaling tranduces adaptive cardiac hypertrophy and constitutive activation of cardiomyocytes by PI3K/Akt activation did not transit into a maladaptive hypertrophy. ACE inhibitor also influenced the activity of Akt-1. Our recent work showed, that the phosphorylation of Akt-1^{Ser473} was elevated in ISO-treated group, and both PARP-inhibiton and ACE-inhibition caused a further growth of it. The PARP-inhibitor L-2286 caused a significantly greater activation of Akt-1, compared to enalapril. In our experiment the phosphorylation (therefore the inhibition) of GSK-3β^{Ser9} was the highest in the L-2286 treated group. Enalapril exerted a significantly less inhibition of GSK-3β^{Ser9}.

The MAPKs ERK, JNK, and p38 can all be activated by AngII. The exact role of MAPKs is still controversial in chronic HF. The moderate phosphorylation of ERK1/2$^{Thr183\text{-}Tyr185}$ was further attenuated by ISO and became more elevated by other treatments. It was reported that ERK 1/2 activation leads to a concentric form of hypertrophy with enhanced cardiac function and MEK1-ERK2 protects the heart from ischemia induced apoptotic insults in mices. In our study the ISO-treated group p38 MAPK$^{Thr180\text{-}Gly\text{-}Tyr182}$ was slightly phosphorylated, while all other treatments increased the phosphorylation of p38-MAPK$^{Thr180\text{-}Gly\text{-}Tyr182}$ significantly. In case of JNK, ISO significantly decreased its phosphorylation and both L-2286 and enalapril treatment augmented its activation.

The phosphorylation of PKC pan βII^{Ser660} and PKC α/βII$^{Thr638/641}$ increased after ISO-induced MI, however their phosphorylation decreased upon administering ACE or a PARP-inhibitor. The PARP-inhibitor - L-2286 decreased the activity of the prohypertrophic PKC α/β more effectively than the ACE-inhibitor – enalapril. A very similar phosphorylation pattern was revealed in the case of PKCδ Thr^{505} and PKC ζ/λ Thr$^{410/403}$.

PKC-ε^{Ser729} is activated by various types of stress. In our study we detected a positive effect (activation) of PARP inhibitor on PKC-ε^{Ser729}, which is responsible for adaptive changes in stress situations, while the levels of other PKC (-α, -β, -ζ, -δ) isoforms were reduced, which are responsible for maladaptive myocardial hypertrophy and remodeling in postinfarction animals.
In our postinfarction model echocardiographic parameters - systolic LV function, wall thickness, LVESV, LVEDV - worsened in ISO-group compared to control animals. This effect can be due to the evolved myocardial fibrosis and cardiomyocyte hypertrophy and partially due to the activation of sevaral protein kinases (e.g. PKC-α/βII$^{Thr638/641}$). Enalapril treatment decreased significantly this worsening, however PARP-inhibitor treatment could nearly completely prevent it. Interestingly, the LVEDV was unchanged despite the ACE-inhibitor, or PARP-inhibitor treatment. The underlying mechanism whereby the PARP-inhibitor L-2286 can exert this favourable effect, is its activator effect on several prosurvival (especially Akt-1-GSK 3β, PKC-ε) and inhibitor effect on prohypertrophic (PKC- α/β, - ζ/λ, -δ) protein kinases.

4.2. *Effect of long-term L-2286 administration on hypertension induced heart failure*

The major findings of this study are that chronic inhibition of nuclear PARP enzyme reduces ADP-ribosylation of nuclear proteins and thus prevents the development of HF from cardiac hypertrophy with inducing reverse remodeling with restoration of cardiac structure and function while changing the altered patterns of signal transducting processes. We used the SHR which provides an animal model of high blood pressure that is similar to essential hypertension in humans. Our study began in the compensated phase of hypertensive cardiopathy in SHR with signs of LVH (at 30-week-old) and after 46 weeks the obvious signs of HF could be detected in SHRs. The development of HF from long-term hypertension can be explained by different mechanism in the literature, but oxidative stress and abnormal signalings are generally respected as the molecular basis of the disease.
In this study, we tested the effect of PARP inhibition in aging SHRs having cardiac hypertrophy and fibrosis related to higher mechanical and oxidative stress and had typical signs of HF (gravimetric parameters, observation daily) and impaired systolic LV function. These conditions have important role in the pathogenesis of diastolic and systolic dysfunctions in hypertensive heart disease.

Both in animal models and in humans, increased blood pressure has been associated with oxidative stress in the vasculature, i.e. with an excessive endothelial production of ROS, which may be both a cause and a consequence of hypertension.

In our experiment the level of plasma-BNP was elevated in both SHR groups. Exalted BNP production and release by cardiocytes occurs in hypertension and has been considered to be a compensatory mechanism against ventricular overload. The Framingham study demonstrated that an increase in BNP predicted the risk of death and cardiovascular events. This alteration could be mitigated by PARP-inhibition and in accordance with this, the survival rate of treated rats was also significantly better. If the heart experiences extended periods of elevated workload, it undergoes a hypertrophic enlargement in response to increased demand. A number of signalling modulators in the vasculature milieu are known to regulate heart muscle mass, including those that influence gene expression, apoptosis, cytokine release and growth factor signaling. One of them is the Akt-1-GSK-3β pathway, which was favorably influenced by PARP inhibitor. In our experiment the down-regulated phosphorylation of Akt-1/GSK-3β in SHR-C samples were increased by PARP inhibitor. Akt-1 is well known to play a central role in the development of physiologic hypertrophy, but also has an important role in cardiac angiogenesis through the activation of mammalian target of rapamycin (mTOR). It is likely that ineffective angiogenesis might contribute to the transition from LVH to HF. The protecting effect of PARP-inhibitors against the development of HF from LVH can be mediated at least partly through the Akt-1/mTOR signaling. MAPKs are ubiquitously expressed, and their specific functions in the heart have been a focus of intensive study. Growing evidence suggests, that modulation of the complex network of MAPKs cascades could be a rewarding approach to the treatment of cardiomyocyte hypertrophy and HF. In our experiment the elevated activation of p38, JNK in the SHR-C groups were decreased, while the activation of ERK was increased by L-2286. While the ERKs are particularly implicated in growth-associated responses, the p38 MAPK and JNKs are generally activated by cytotoxic stress factors.

Activation of ERK causes cardiac hypertrophy and increases survival, while inactivation of ERK contributes to myocyte apoptosis. Cardiac-specific expression of constitutively activated MEK1 promotes cardiac hypertrophy without compromised function or long-term animal survival, suggesting that activation of ERK activity promotes a compensated form of hypertrophy. In our study, the phosphorylation of PKC pan βII^{Ser660}, $\alpha/\beta IIThr^{638/641}$, δ^{Thr505} and $\zeta/\lambda^{Thr410/403}$ were

attenuated in SHR-L compared to SHR-C by PARP inhibitor. Several reports suggest, that PKC α and β are involved in the development of cardiac hypertrophy and HF. The activation of PKC ε was upregulated by L-2286 treatment in SHR-L group. In this experiment, there were no differences in LV systolic functions (EF, FS) at baseline (the age of 30 weeks). These parameters were preserved in the CFY and SHR-L groups, but moderated in the SHR-C group at the end of the study. L-2286 increased EF by reducing end-systolic dimensions. During the development of hypertension, alterations in LV geometry may occur as an adaptation to increasing pressure and volume load. In hypertensive patients, LV geometry can be classified into four patterns on the basis of LV mass index and RWT. In conformity with this classification eccentric hypertrophy was found in SHR-C group (increased LV mass/ BW and normal RWT), while L-2286 administration could preserve concentric hypertrophy (increased LV mass/ BW and increased RWT) state, which could be detected at the beginning of the study in both SHR groups. Therefore, the ineffectiveness of L-2286 on thickness of septum and PW can be considered as a favorable effect because it can add to the maintaining of concentric hypertrophy.

5 Summary

Throughout the last two decades, experimental evidences from in vitro studies and preclinical models of diseases have demonstrated that reactive oxygen and nitrogen species, including reactive oxidant peroxynitrite, are generated in parenchyma, endothelial, and infiltrating inflammatory cells during myocardial and other forms of reperfusion injury, myocardial hypertrophy, heart failure, cardiomyopathies and cardiovascular aging. In related animal models of diseases, pharmacological inhibition of PARP provides significant therapeutic benefits. Therefore, novel antioxidants and PARP inhibitors have entered into the clinical development for the experimental therapy of various cardiovascular and other diseases.

In our experiments, the common feature of the PARP-inhibitor L-2286 treatment was the beneficial action on several intracellular signaling pathways PI-3-kinase-Akt-1^{Ser473} and PKC ε^{Ser729} pathways, it can influence favorably the gravimetric and echocardiographic parameters and cardiac fibrosis. In addition, in our last investigation (HF model), L-2286 treatment could delay the onset of hypertension-induced HF.

6 Acknowledgements

These studies were carried out at the Department of Biochemistry and Medical Chemistry and at the Ist Department of Medicine, Medical School of the University of Pécs between 2005 and 2008.

I would like to express my thanks to my teacher and program leader, Professor Kálmán Tóth, who managed my studies and gave a support and useful advises during my work.

I am grateful to Professor Balázs Sümegi who taught me a biochemical way of thinking. He directed my work on the field of PARP inhibitors and he ensured the possibility of undisturbed work in his department for me.

I am really thankful to Professor Kálmán Hideg who taught me enthusiastic on free radical mediated processes and directed my work on the field of cardioprotective effects of PARP inhibitor compounds.

I convey my thanks to Róbert Halmosi for his excellent work and help to perform echocardiographic examinations.

Tamás Habon, Eszter Szabados, Izabella Solti, Gyöngyi Kiss, Enikő Plózer, Alíz Szabó, László Kereskai and Endre Kálmán gave a hand with a part of the experiments. I am grateful to Istvánné Pásztor, Heléna Halász, Bertalan Horváth and László Girán, who gave much assistance in the laboratory work.

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