

**THE ROLE OF INTRACELLULAR SIGNALING IN THE  
CARDIOPROTECTIVE EFFECT OF POLY(ADP-RIBOSE)  
POLYMERASE INHIBITION**

PhD thesis

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## 1. ABBREVIATIONS

AP-1	activator protein-1
BNP	B-type natriuretic peptide
BW	body weight
CK	creatine kinase
CrP	creatine phosphate
ERK1/2	extracellular signal-regulated kinase
GSK-3 $\beta$	glycogen synthase kinase-3 $\beta$
HE	haematoxylin and eosin
HQ	4-hydroxyquinazoline
IR	ischemia-reperfusion
ISO	isoproterenol hydrochloride
JNK	c-jun N-terminal kinase
LDH	lactate dehydrogenase
MAPK	mitogen activated protein kinase
MQ	2-merkapto-4(3 <i>H</i> )-quinazolinone
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NIH	National Institutes of Health
NMR	nuclear magnetic resonance
PARP	poly(ADP-ribose) polymerase
PDC-1 $\alpha$	pyruvate dehydrogenase complex-1 $\alpha$
Pi	inorganic phosphate
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
ROS	reactive oxygen species
SEM	standard error of mean
TBARS	thiobarbituric acid reactive substances
TBS	TRIS-buffered saline
TEF-1	transcriptional enhancer factor-1
TL	length of right tibia
TTC	triphenyltetrazolium-chloride
WV	weight of ventricles

## 2. BACKGROUND

### 2.1 MECHANISM OF CARDIOPROTECTION AFFORDED BY POLY(ADP-RIBOSE) POLYMERASE (PARP) ENZYME INHIBITION

Enhanced activation of poly(ADP-ribose) polymerase (PARP) enzyme is a major contributor to oxidative stress-induced cell dysfunction and tissue injury.<sup>1,2</sup> Reactive oxygen species (ROS) and peroxynitrite formation expedites the ischemia-reperfusion-induced cardiac injury, and causes lipid peroxidation, protein oxidation and single-strand DNA brakes.<sup>3-5</sup> Single-strand DNA brakes can activate the nuclear PARP, which ADP-ribosylates different nuclear proteins on the expense of cleaving NAD<sup>+</sup>. If PARP activation exceeds a certain limit, it can lead to cellular NAD<sup>+</sup> and ATP depletion, ultimately resulting in cell death.<sup>1-4</sup> We and other investigators have already shown that PARP inhibitors can efficiently reduce oxidative myocardial damage during ischemia-reperfusion both in isolated heart perfusion and in in vivo myocardial infarction models, furthermore PARP inhibition provided significant protection against postinfarction heart failure.<sup>3,6-9</sup>

Recent studies have however challenged the original conception that protection by PARP inhibitors exclusively rely on the preservation of NAD<sup>+</sup> and ATP stores. In vitro data in Langendorff perfused heart model system indicated for the first time that PARP inhibitors decreased the ischemia-reperfusion-induced increase of mitochondrial ROS production and so all consequent oxidative damages. According to one possible argument, PARP inhibition by moderating cytoplasmic NAD<sup>+</sup> loss can help to retain mitochondrial NAD<sup>+</sup>, and may prevent the decrease of the mitochondrial NAD<sup>+</sup>-linked substrate oxidation and ROS formation. As the nuclear PARP enzyme can probably not play any role in isolated mitochondria, it was also assumed, that PARP inhibitors bound to mitochondrial proteins such as mono-ADP-ribose transferase or NAD<sup>+</sup>-glycohydrolase that play role in the oxidative damage of mitochondria.<sup>3</sup>

Several studies have also suggested that PARP inhibitors may modify the activation state of signaling routes and gene expression.<sup>2,10-12</sup> Importantly, there are strong evidences that PARP enzyme alters the function of a variety of transcription factors, and interfere with the expression of several proinflammatory genes by direct protein-protein interaction or by poly(ADP-ribosylation).<sup>2,10,11</sup> On one hand, *protein-protein interaction* is recognized as a mechanism for PARP to function as a specific transcriptional coactivator of nuclear factor (NF)- $\kappa$ B.<sup>13</sup> It was also proven that PARP modulates transcription by direct interaction with Yin-Yang (YY)-1, activator protein (AP)-2, Oct-1 and transcriptional enhancer factor (TEF)-

1.<sup>10</sup> On the other hand, Amstad et al. have demonstrated that *poly(ADP-ribosylation)* is also an important event for the elongation and activation of protooncogenes.<sup>14</sup> Several transcription factors such as TEF-1, TATA-binding protein and recombinant p50 and p65 NF- $\kappa$ B subunits can also be poly(ADP-ribosyl)ated by PARP in vitro.<sup>10</sup> The dual regulatory role of PARP, i.e., dependent or independent of the poly(ADP-ribosyl)ative activity, was further supported by studies demonstrating that genetic deletion of PARP, but not pharmacological inhibition of its activity, was able to reduce NF- $\kappa$ B binding in glial cells. However, pharmacological inhibition of the catalytic activity of PARP was able to reduce gene expression of inducible nitric oxide synthase.<sup>10</sup>

Nevertheless, to date, limited information is available on how PARP inhibition influences the signaling pathways during myocardial ischemia-reperfusion, or how PARP inhibition might modulate intracellular signaling during the progression of postinfarction remodeling and heart failure. There are data indicating that during reperfusion absence of PARP leads to the reduction of myocardial apoptosis, which is associated with reduced NF- $\kappa$ B activation and altered gene expression of apoptotic regulators.<sup>15</sup> Zingarelli et al. demonstrated for the first time that PARP-1 was requisite for the complete activation of c-jun N-terminal kinase (JNK) during reperfusion injury in vivo, while ischemia-reperfusion-induced elevated JNK activity was reduced in PARP<sup>-/-</sup> mice.<sup>10</sup> It was also demonstrated that mice with genetic ablation of PARP-1 exhibited a significant cardioprotection, which was associated with downregulation of the proinflammatory pathway of AP-1, whereas the anti-inflammatory pathway of heat-shock factor (HSF)-1 was enhanced with consequent modification of gene expression of several inflammatory mediators such as heat shock protein (HSP)-70.<sup>10</sup> Genetic or pharmacological inhibition of PARP-1 also reduced the adhesion molecule expression of P-selectin and intercellular adhesion molecule (ICAM)-1 and neutrophil infiltration in in vivo myocardial reperfusion injury and reduced the plasma release of inflammatory cytokines.<sup>16,17</sup> Furthermore the loss of the PARP gene was associated with a significant reduction of the AP-1-dependent matrix metalloproteinase enzyme, which well correlated with the architectural damage and maintenance of myocyte alignment of the reperfused heart. Finally it was also demonstrated in an ex vivo ischemia-reperfusion model that the ischemia-reperfusion-induced activation of the cardioprotective Akt pathway could be further enhanced by the pharmacological inhibition of PARP.<sup>12</sup>

## 2.2 PROTEIN KINASE CASCADES AND OXIDATIVE STRESS

Hypoxia-reoxygenation as well as other oxidative insults influence tissue survival partially via differential regulation of protein kinase cascades and inflammatory reactions.<sup>18,19</sup> Phosphatidylinositol-3-kinase (PI3K)-Akt and mitogen-activated protein kinase (MAPK) (including extracellular signal-regulated kinase (ERK1/2), JNK and p38-MAPK) signaling networks have all been shown to alter their activation state in response to oxidant injury and therefore could potentially participate in cell fate decisions.<sup>20,21</sup> Signaling through Akt and ERK appears to be prosurvival in nature associated with growth factor receptor stimulation.<sup>22</sup> On the other hand, JNK and p38-MAPK activation was linked to apoptosis, but depending on the context and duration of activation, they can exert opposite effects, as well.<sup>23,24</sup>

Cardiac remodeling, ultimately culminating in heart failure, is also associated with alterations in intracellular signaling including the inhibition of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) via phosphorylation by Akt, protein kinase C (PKC), p70-S6 kinase, p90-RSK and protein kinase A.<sup>25</sup> These pathways and the MAP kinases have all been demonstrated to alter their activation state in response to hypertrophic stimuli and therefore may be responsible for myocardial hypertrophy.<sup>26-28</sup>

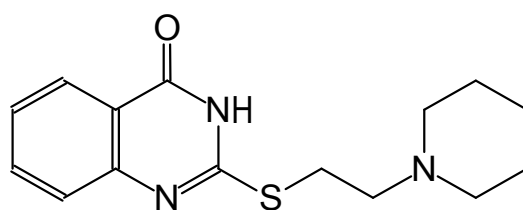
As recent findings in a rodent septic shock model showed that PARP inhibition increased the phosphorylation and activation of Akt and attenuated the lipopolysaccharide-induced phosphorylation of ERK1/2, p90RSK and p38 MAP kinases, it suggested, that PARP inhibitors could modulate these signaling pathways which are also responsible for the cell-fate decisions in myocardial ischemia-reperfusion or in the development of cardiac hypertrophy.<sup>11</sup>

## 2.3 EXPERIMENTAL MODELS OF CARDIAC OXIDATIVE INJURY

To elucidate the role of protein kinase signaling in the mechanism of cardioprotection afforded by PARP inhibitors, we utilized three experimental models. First, we investigated the effect of PARP inhibition on the recovery of energy metabolism in vitro in Langendorff perfused hearts during ischemia-reperfusion cycle, then the PARP inhibitor agent was tested in vivo in isoproterenol-induced myocardial infarction model.<sup>19,29</sup> Third the PARP inhibitor compound was administered in a rat model of chronic heart failure after isoproterenol-induced myocardial infarction. As known, subcutaneous administration of the beta-adrenoceptor agonist isoproterenol induces extensive amount of cardiomyocyte necrosis, ranging from patchy subendocardial necrosis to transmural infarction, while maintaining patent coronary vasculature.<sup>29,30</sup> The exact mechanism of isoproterenol-induced myocardial damage has not

been clarified, but a mismatch of oxygen supply versus demand following coronary hypotension and myocardial hyperactivity may offer the best explanation.<sup>30,31</sup> It has been also demonstrated that ISO administration produces free radicals via  $\beta$ -adrenoceptor mechanism that affects the cell metabolism to such a degree that cytotoxic free radicals are formed producing myocardial necrosis.<sup>30</sup> This effect results in both acute and chronic a deterioration of hemodynamic variables.<sup>29,31,32</sup> The significant cell loss after the experimentally induced myocardial infarction is the initial insult that triggers the development of heart failure. The alteration in work load leads to consequent myocardial hypertrophy and finally ventricular enlargement and dilatation. Furthermore administration of high dosages of catecholamines is also followed by myocardial fibrosis, alterations in the collagen fibers or changes in the neurohormonal system. Finally injection of isoproterenol induces a syndrome in the rat that displays numerous typical characteristics of heart failure.<sup>30</sup>

In this study PARP inhibition was achieved by a novel compound, L-2286, which was derived from 2-mercapto-4(3*H*)-quinazolinone by alkylation with 1-(2-chloroethyl)piperidine (Fig 1.). L-2286 was chosen, because in *in vitro* PARP assay it exhibited significantly better PARP inhibitory activity than basic quinazolines such as 4-hydroxyquinazoline or 2-merkapto-4(3*H*)-quinazolinone.<sup>33</sup>



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

### 3. AIMS OF THE STUDY

The aim of this work was to provide evidence for a new molecular mechanism of the cardioprotective effect of PARP inhibition.

1. To assess cardioprotection afforded by PARP inhibition
  - a) We tested whether the novel quinazolinone derivate PARP inhibitor, L-2286 facilitated the recovery of myocardial energy metabolism and prevented the oxidative injury during ischemia-reperfusion in a Langendorff-heart perfusion system.
  - b) We tested whether L-2286 prevented oxidative myocardial damage during isoproterenol-induced myocardial infarction by determining serum necroenzyme levels and infarct size.
  - c) We tested whether PARP inhibition protected against postinfarction heart failure and ventricular remodeling by analyzing histological changes and metabolic alterations.
  
2. To provide evidence for a new additional cardioprotective mechanism, it was assessed, how PARP inhibition influenced the activation state of the intracellular signaling pathways responsible for myocyte cell survival or hypertrophic response. Therefore the phosphorylation states of the
  - a) PI3-kinase/Akt pathway,
  - b) protein kinase C
  - c) and mitogen activated protein kinase cascades were examined by Western blotting.



## 4. MATERIALS AND METHODS

### 4.1 HEART PERFUSION

Adult male CFY-strain Sprague-Dawley rats weighing 300-380 g were used for the Langendorff heart perfusion experiments and the myocardial infarction model. The animals were housed in solid-bottomed polypropylene cages and received commercial rat diet and water ad libitum. The investigation conformed 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. Rats were anesthetized with 200 mg/kg ketamine intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p.). Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 mmHg, at 37°C as described previously.<sup>3</sup> The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 11 mM glucose and 0.6 mM octanoic acid and, in the treated group, L-2286 in 10 and 20 µM concentrations. The perfusate was adjusted to pH 7.40 and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> through a glass oxygenator. After a washout, non-recirculating period of 10 min, hearts were either perfused under normoxic conditions for 10 min, or were subjected to a 30-min global ischemia by closing the aortic influx and reperfused for 15 min. The experimental compound was administered into the perfusion medium at the beginning of normoxic perfusion. During ischemia hearts were submerged into perfusion buffer at 37°C. Hearts were freeze-clamped at the end of each perfusion.

### 4.2 NMR SPECTROSCOPY

NMR spectra were recorded with a Varian <sup>UNITY</sup>INOVA 400 WB instrument. <sup>31</sup>P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z•SPEC in a 20-mm broadband probe (Nalorac Co., Martinez, CA, USA), applying GARP-1 proton decoupling ( $\gamma$ B2=1.2 kHz) during acquisition. Field homogeneity was adjusted by following the <sup>1</sup>H signal ( $w_{1/2}$ =10-15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free induction decay (FID). 45° flip angle pulses were employed after a 1.25 s recycle delay and transients were acquired over a 10 kHz spectral within 0.25 s, and the acquired data points (5000) were zero filled to 16 K. Under the above circumstances the

relative concentrations of the species can be taken proportional to the peak areas, because interpulse delays exceeded 4-5xT1 values of the metabolites to be analyzed in <sup>31</sup>P experiments.

#### 4.3 LIPID PEROXIDATION AND PROTEIN CARBONYL CONTENT

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of a described method.<sup>34</sup> Cardiac tissue was homogenized in 6.5% trichloroacetic acid (TCA) and a reagent containing 15% TCA, 0.375% thiobarbituric acid (TBA) and 0.25% HCl was added, mixed thoroughly, heated for 15 min in a boiling water bath, cooled, centrifuged and the absorbance of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using malondialdehyde standard, TBARS were calculated as nmol/g wet tissue.

To measure protein carbonyl content, 50 mg of frozen heart tissue were homogenized with 1 ml 4% perchloric acid and the protein content was collected by centrifugation. The protein carbonyl content was determined by means of the 2,4-dinitrophenylhydrazine-method.<sup>35</sup>

#### 4.4 MYOCARDIAL INFARCTION MODEL

Myocardial infarct was induced by subcutaneous injection of 80 mg/kg isoproterenol hydrochloride (ISO) (Sigma-Aldrich Co, Budapest, Hungary), while physiological saline (1 ml/kg) was given to control rats intraperitoneally. ISO solutions were prepared with sterile distilled water immediately before injection. ISO-treated animals were divided into two groups: while the ISO group received repeated injections of saline, the ISO+L-2286 group received L-2286 10 min before (10 mg/kg) and every hour for 5 hours (3 mg/kg) after ISO administration. Electrocardiogram was made before and hourly (for 5 hours) after ISO administration (Schiller AG electrocardiograph, Switzerland).

#### 4.5 INFARCT SIZE MEASUREMENT

24 h after the ISO administration, animals were sacrificed, hearts were removed and kept overnight at -20°C. Frozen ventricles were sliced into 2-3 mm thick sections and then incubated in 1% triphenyltetrazolium-chloride (TTC) at 37°C in 0.2 M Tris buffer (pH 7.4)

for 30 min. While the normal myocardium was stained brick red, the infarcted areas remained unstained. Size of the infarcted area was estimated by the volume and weight method.<sup>36</sup>

#### 4.6 SERUM NECROENZYME DETERMINATION

Serum lactate dehydrogenase (LDH) and creatine kinase (CK) levels were determined from blood samples collected 24 h after ISO administration. Myocardial enzyme activities were measured by standard methods as described earlier.<sup>37,38</sup>

#### 4.7 CHRONIC HEART FAILURE MODEL

350-380 g male CFY rats received two subcutaneous injections (separated by a 24-hour interval) of 80 mg/kg isoproterenol.<sup>31</sup> Twenty-four hours after the second injection the surviving animals were randomly assigned to receive either 5 mg/kg L-2286, or water daily. The PARP inhibitor treatment was delayed 24 h to avoid the decrease of infarct size by the PARP inhibition.<sup>9</sup> L-2286 was given for 8 weeks. At the end of 8 weeks body weights were measured and standard ECG was recorded to determine the R wave amplitude and J point depression (lead II). Animals were subsequently sacrificed, their hearts were removed, the atria and great vessels were trimmed from the ventricles and the weight of the ventricles was measured. It was then normalized to the body weight and the length of right tibia. Hearts were then freeze-clamped or fixed in 10% formalin.

#### 4.8 DETERMINATION OF PLASMA B-TYPE NATRIURETIC PEPTIDE

Blood samples were collected into the Lavender Vacutaner tubes containing EDTA and aprotinin (0.6 TIU/ml of blood), centrifuged at 1600 g for 15 minutes at 4°C. Plasma were collected and kept at -70°C. Plasma B-type natriuretic peptide-45 (BNP-45) levels were determined by enzyme immunoassay method (BNP-45, Rat EIA Kit, Phoenix Pharmaceuticals Inc., CA, USA).

#### 4.9 MEASUREMENT OF MITOCHONDRIAL ENZYME ACTIVITY

NADH:cytochrome-c oxidoreductase was measured as described previously.<sup>3</sup> Enzyme activity was determined by measuring the rate of cytochrome c reduction at 550 nm in a medium containing 50 mM sodium-phosphate, 1 mM sodium-azide, 1.5 mM NADH and 50-

75 µg mitochondrial protein/ml, pH 7.5. The reaction was started by addition of 40 µl cytochrome c.

#### 4.10 HISTOLOGY

Ventricles fixed in formalin were sliced, and embedded in paraffin. 5 µm thick sections were cut serially from base to apex. 10 to 12 slices at 1-mm intervals were stained with haematoxylin and eosin (HE). The sections were mounted on slides and projected at a magnification of 40x and photomicrographs were taken. Mean myocyte diameters on HE stained sections were calculated by measuring 100 cells per specimen in the region of the cell nucleus using the two-point distance function of the TelPath analyzer system (bollman.com, 2000). Type III collagen was stained as a marker of interstitial fibrosis on frozen sections, 5 µm thick by the Vector M.O.M.<sup>TM</sup> Kit (Vector Laboratories Inc., Burlingame, CA, USA) staining procedure. After fixing and dehydrating, sections were washed in TRIS-buffered saline (TBS) containing 0.5% Tween 20, pH 7.6. To block the endogenous peroxidase activity, sections were incubated in 3% H<sub>2</sub>O<sub>2</sub> then washed in TBS. After 1 h incubation with M.O.M.<sup>TM</sup> mouse IgG blocking reagent (containing 20% normal rat serum) sections were washed in TBS, and incubated in the working solution of M.O.M.<sup>TM</sup> diluent for 5 min. Primary mouse antisera against type III collagen (1:1000, Monoclonal Anti-Collagen, Type III, Sigma-Aldrich Co, Budapest, Hungary) diluted in M.O.M.<sup>TM</sup> diluent reacted at room temperature for 30 min, followed by two 2-minute rinses in TBS. Biotinylated Anti-Mouse IgG Reagent was applied for 10 min and sections were washed twice for 2 min in TBS. VECTASTAIN ABC Reagent was applied for 5 min that was followed by two 2-minute rinses in TBS. Sections were then stained with Vector NovaRED Substrate (Vector Laboratories Inc., Burlingame, CA, USA) for five min, washed in distilled water, dehydrated, mounted on slides and projected at a magnification of 10x. The sections were quantified with the NIH ImageJ analyzer system. All histologic and immunohistological samples were examined by an investigator in a blinded fashion.

#### 4.11 WESTERN BLOT ANALYSIS

Fifty milligrams of heart samples were homogenized in ice-cold Tris buffer (50 mM, pH=8.0) and harvested in 2x concentrated SDS-polyacrilamide gel electrophoretic sample buffer. Proteins were separated on 12% SDS-polyacrilamide gel and transferred to nitrocellulose

membranes. After blocking (2 h with 3% nonfat milk in Tris-buffered saline), membranes were probed overnight at 4°C with antibodies recognizing the following antigens: phospho-specific Akt-1/protein kinase B- $\alpha$  Ser<sup>473</sup> (1:1000), nonphosphorylated Akt/PKB (1:1000), phospho-specific glycogen synthase kinase (GSK)-3 $\beta$  Ser<sup>9</sup> (1:1000), phospho-specific extracellular signal-regulated kinase (ERK1/2) Thr<sup>183</sup>-Tyr<sup>185</sup> (1:1000), phospho-specific p38 mitogen-activated protein kinase (p38-MAPK) Thr<sup>180</sup>-Gly-Tyr<sup>182</sup> (1:1000), phospho-specific c-Jun N-terminal kinase (JNK) (1:1000), phospho-specific protein kinase C (PKC) (pan)  $\beta$ II Ser<sup>660</sup> (1:1000), phospho-specific protein kinase C  $\alpha$ / $\beta$ II (PKC $\alpha$ / $\beta$ II) Thr<sup>638/641</sup> (1:1000), phospho-specific protein kinase C  $\delta$  Thr<sup>505</sup> (1:1000), phospho-specific protein kinase C  $\delta$  Ser<sup>643</sup> (1:1000; Cell Signaling Technology, Beverly, MA, USA), nonphosphorylated PKC (1:1000), N-terminal domain of actin (1:10000; Sigma-Aldrich Co, Budapest, Hungary). Membranes were washed six times for 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween (TBST) before addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution; Bio-Rad, Budapest, Hungary). Membranes were washed six times for 5 min in TBST and the antibody-antigen complexes were visualized by means of enhanced chemiluminescence. The results of Western blots were quantified by NIH ImageJ program.

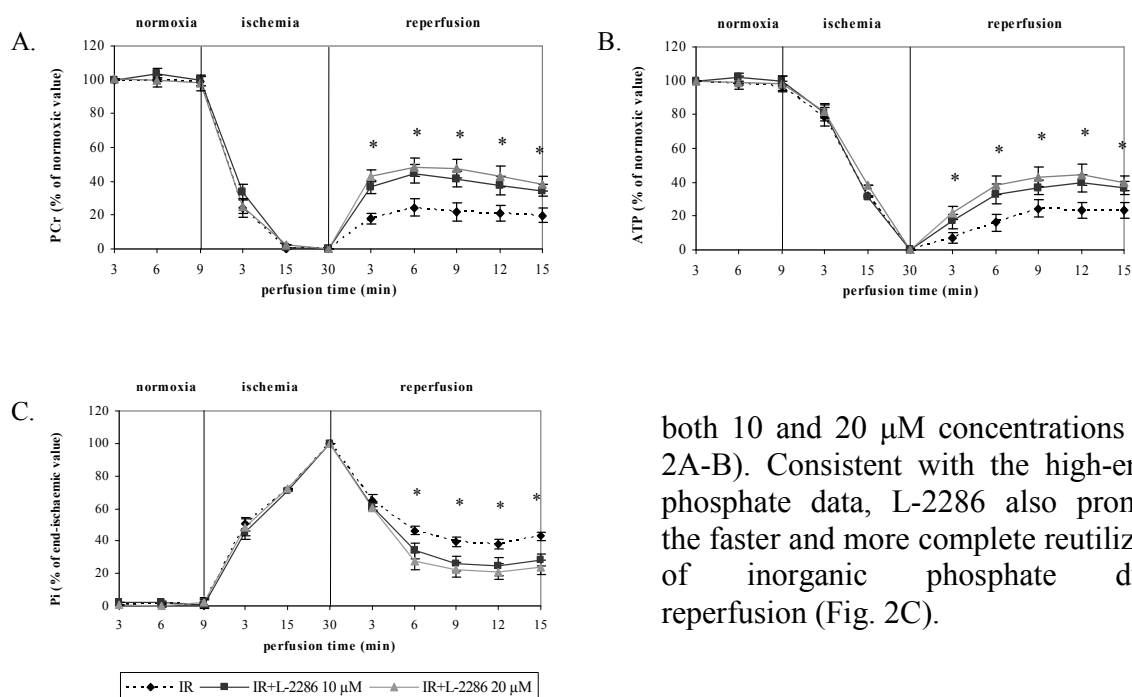
#### 4.12 STATISTICAL ANALYSIS

Statistical analysis was performed by analysis of variance and all of the data were expressed as the mean  $\pm$  SEM. Significant differences were evaluated by use of unpaired Student's *t*-test and *p* values below 0.05 were considered to be significant.

## 5. RESULTS

### 5.1 L-2286 PROMOTED THE POSTISCHEMIC RECOVERY OF MYOCARDIAL ENERGY STORES

Energy metabolism of Langendorff perfused hearts was monitored in the magnet of a  $^{31}\text{P}$  NMR spectroscope enabling the detection of changes in high-energy phosphate intermediates. Ischemia induced a rapid decrease in ATP and creatine phosphate levels and a fast evolution of inorganic phosphate (Fig. 2A-C). In our experimental setting, high-energy phosphate intermediates recovered only partially in untreated hearts during the 15-min reperfusion phase. On the other hand, L-2286 facilitated the recovery of creatine phosphate and ATP in



both 10 and 20  $\mu\text{M}$  concentrations (Fig. 2A-B). Consistent with the high-energy phosphate data, L-2286 also promoted the faster and more complete reutilization of inorganic phosphate during reperfusion (Fig. 2C).

**Figure 2. L-2286 attenuates ischemia-reperfusion-induced injury of isolated hearts.** Time-course of creatine phosphate (A), ATP (B) and inorganic phosphate levels (C) as measured by  $^{31}\text{P}$  NMR spectroscopy. Values are given as the mean $\pm$ SEM for five experiments. IR: ischemia-reperfusion. \*Significant difference from control untreated sample ( $p < 0.01$ ).

### 5.2 L-2286 ATTENUATED THE ISCHEMIA-REPERFUSION-INDUCED LIPID PEROXIDATION AND PROTEIN OXIDATION

In this study, ischemia-reperfusion increased the amount of TBARS compared to the normoxic hearts (from  $24.4 \pm 1.1$  to  $43.2 \pm 1.25$  nmol/g wet tissue,  $p < 0.001$ ). However, 10

$\mu\text{M}$  L-2286 administration significantly reduced the formation of TBARS (to  $30.2 \pm 1.7$  nmol/g wet tissue,  $p < 0.01$ ) compared to untreated hearts, indicating that L-2286 prevented the ischemia-reperfusion-related lipid peroxidation. On the other hand, ischemia-reperfusion markedly elevated the level of protein oxidation (from  $1.2 \pm 0.05$  to  $2.2 \pm 0.1$   $\mu\text{mol/g}$  wet tissue,  $p < 0.001$ ); however, L-2286 significantly attenuated ( $p < 0.01$ ) the increase in the quantity of protein-bound aldehyde groups (to  $1.5 \pm 0.08$   $\mu\text{mol/g}$  wet tissue).

### 5.3 ENHANCED AKT AND MAP KINASE ACTIVATION UPON L-2286 TREATMENT DURING ISCHEMIA-REPERFUSION

The moderate phosphorylation of Akt under normoxic conditions in our study increased after ischemia-reperfusion; nevertheless, L-2286 treatment further enhanced the activation of Akt (Fig. 3A). GSK-3 $\beta$  was not phosphorylated during normoxia, but became moderately phosphorylated after ischemia-reperfusion and strongly phosphorylated upon L-2286 treatment. As GSK-3 $\beta$  is phosphorylated by Akt leading to its inactivation, the marked phosphorylation of GSK-3 $\beta$  in treated hearts is in accordance with enhanced Akt activation in the same tissue samples (Fig. 3A). Interestingly, L-2286 also brought about Akt as well as GSK-3 $\beta$  phosphorylation during the 10-min normoxic perfusion, which is in clear contrast with the untreated normoxic condition where moderate or no phosphorylation was observed.

Additionally, L-2286 promoted the phosphorylation of ERK, p38-MAPK as well as JNK both in normoxic and ischemic-reperfused hearts (Fig. 3B). Ischemia-reperfusion by itself only slightly increased the phosphorylation of ERK, p38-MAPK and JNK.

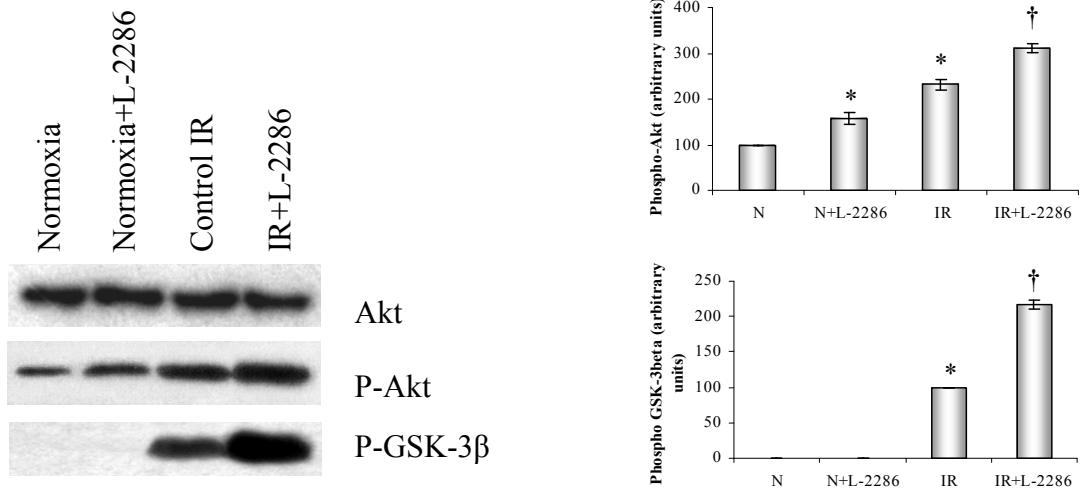
### 5.4 L-2286 INHIBITED THE ISOPROTERENOL-INDUCED MYOCARDIAL CELL LOSS

ISO administration results in compromised cell membrane integrity and causes necroenzyme release from cardiomyocytes. In comparison with the control group, ISO administration significantly increased CK and LDH release from the injured cardiomyocytes ( $p < 0.01$ ). By contrast, L-2286 treatment significantly reduced CK and LDH levels in the serum ( $p < 0.05$ ) (Fig. 4A-B). ECG monitoring of the heart rate revealed that L-2286 did not interfere with the tachycardia elicited by ISO administration (data not shown).

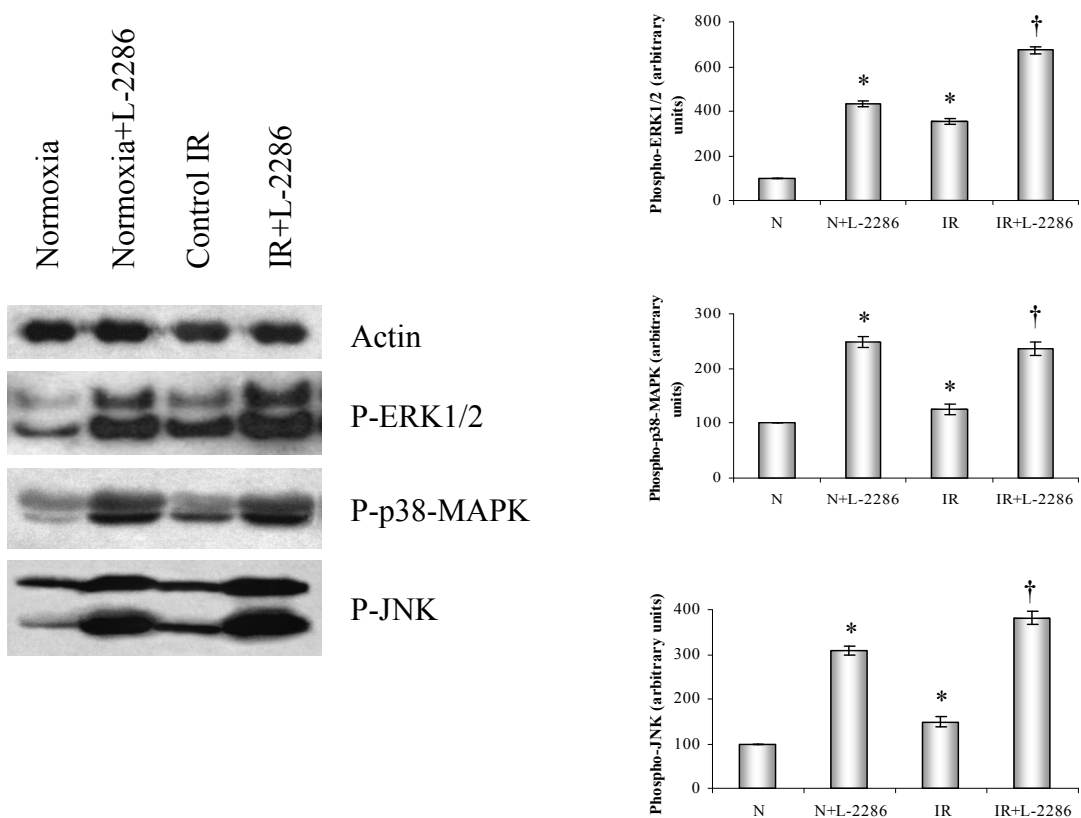
As the TTC staining in five consecutive samples demonstrated, ISO administration caused a large,  $21.1 \pm 2$  % infarct of the ventricles. In the meantime, L-2286 treatment significantly

reduced the infarct size to  $8.9 \pm 1\%$  ( $p < 0.05$ ). TTC staining of control hearts rendered no appreciable infarcted area.

A.

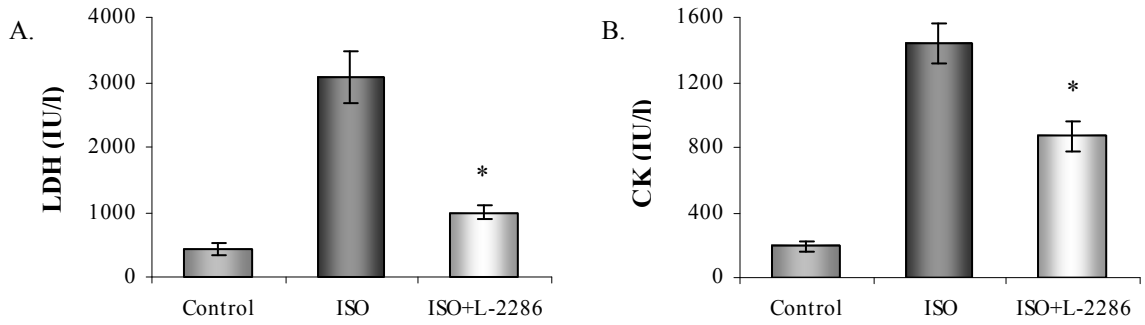


B.



**Figure 3. L-2286 promotes Akt, ERK, p38-MAPK and JNK phosphorylation in ischemic-reperfused hearts.** Western blot analysis of Akt-1 and GSK-3 $\beta$  phosphorylation (**A**) as well as ERK, p38-MAPK and JNK phosphorylation (**B**). Actin is shown as loading control. N: normoxic perfusion; IR: ischemia-reperfusion. Representative immunoblots from five experiments and densitometric evaluation are demonstrated. \*Significant difference from normoxic sample ( $p < 0.01$ ). †Significant difference from IR sample ( $p < 0.01$ ).



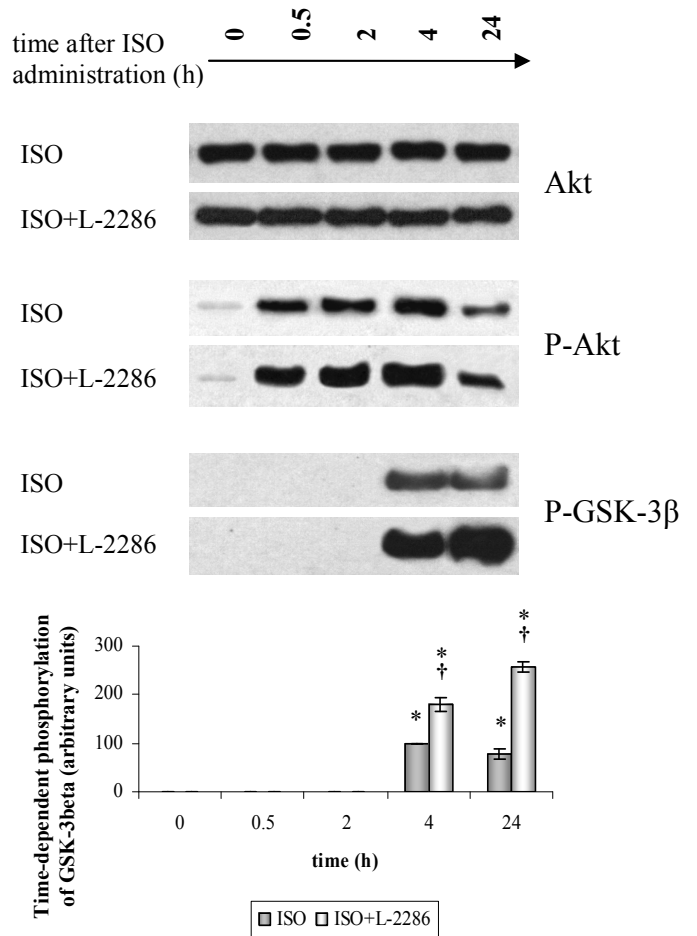


**Figure 4. L-2286 inhibits the isoproterenol-induced in vivo myocardial injury.** Serum levels of lactate dehydrogenase (A) and creatine kinase (B) were measured in the serum 24 hours after ISO administration. Values are given as the mean±SEM for at least five experiments. \*Significant difference from control untreated sample ( $p < 0.01$ ).

### 5.5 L-2286 ENHANCED AKT-1, ERK AND P38-MAPK, WHILE REDUCED JNK PHOSPHORYLATION IN ISOPROTERENOL-INDUCED MYOCARDIAL INJURY

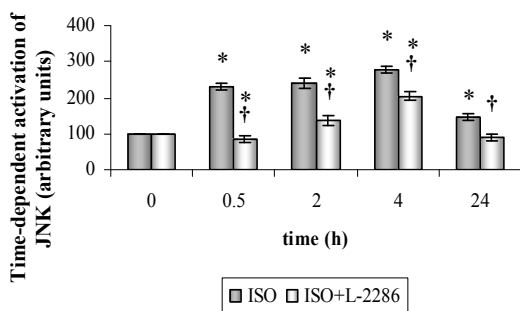
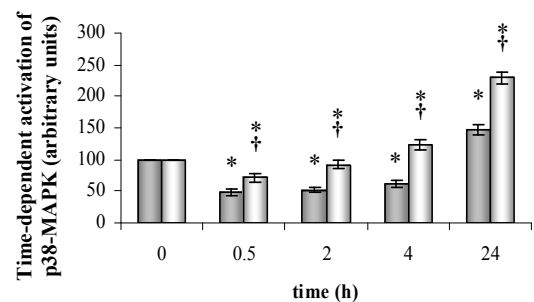
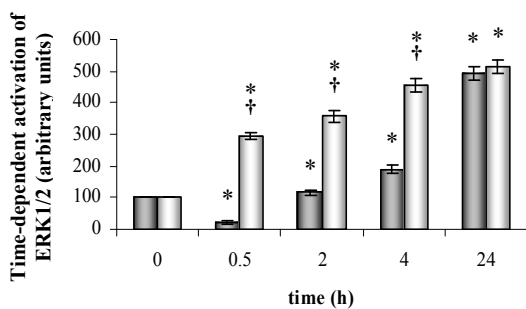
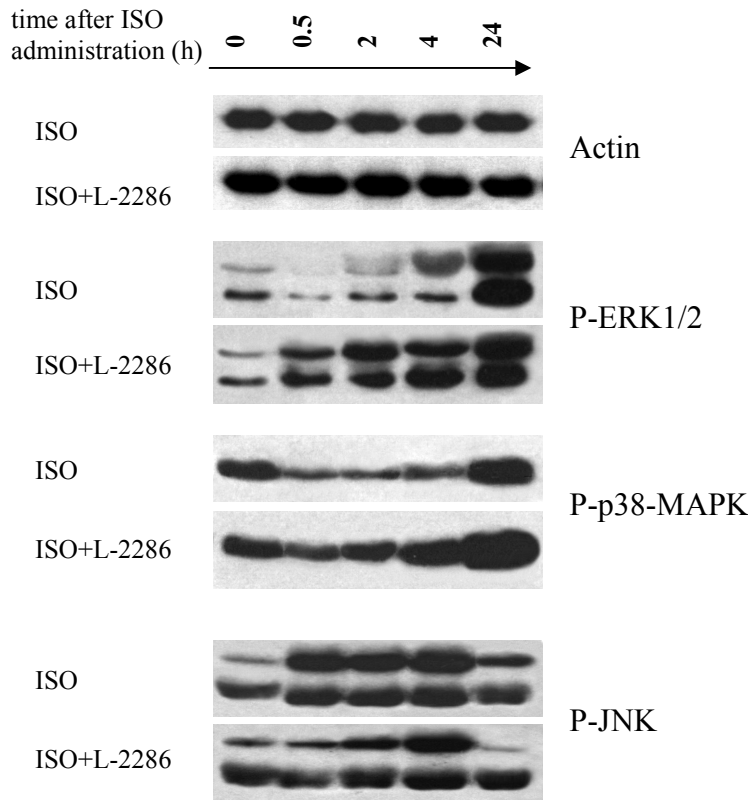
While ISO administration rapidly increased Akt phosphorylation, co-treatment with L-2286 could further enhance the activation of the Akt protein (Fig. 5A). Subsequent elevation in GSK-3 $\beta$  phosphorylation was delayed until 4 hours after ISO administration, but L-2286 triggered a more pronounced phosphorylation than ISO by itself (Fig. 5A).

Figure 5. A.



**Figure 5. L-2286 increases Akt-1, ERK, and p38-MAPK, while suppresses JNK phosphorylation in isoproterenol-induced myocardial injury.** Western blot analysis of Akt-1 and GSK-3 $\beta$  phosphorylation (A) as well as ERK, p38-MAPK and JNK phosphorylation (B) 0, 0.5, 2, 4 and 24 hours after ISO administration. Actin is shown as loading control. Representative immunoblots from five experiments and densitometric evaluation are demonstrated. \*Significant difference from control untreated sample ( $p < 0.01$ ). †Significant difference from L-2286-treated samples ( $p < 0.01$ ).

Figure 5. B.



While ISO administration led to higher ERK phosphorylation only after 24 hours, L-2286 co-treatment accelerated this process immediately after ISO injection (Fig. 5B). Similarly, the initial p38-MAPK phosphorylation level rather diminished following ISO treatment and recovered only after 24 hours (Fig. 5B). By contrast, the ISO-induced decrease in p38-MAPK phosphorylation was prevented by L-2286, therefore the ISO+L-2286 hearts exhibited

higher phosphorylation levels than the ISO hearts at each examined time point (Fig. 5B). Furthermore, JNK became highly phosphorylated within the first four hours following ISO injection to ultimately return to

baseline at 24 hours (Fig. 5B). In contrast to our ischemia-reperfusion data, L-2286 reduced the ISO-induced JNK phosphorylation throughout the entire experiment (Fig. 5B).

#### 5.6 PARP INHIBITION IMPROVES GRAVIMETRIC PARAMETERS IN ISO-INDUCED CHRONIC HEART FAILURE

After myocardial infarction the initial loss of myocardium can initiate progressive ventricular hypertrophy. The surviving myocytes undergo hypertrophy showing increases in both cell length and diameter. In this study gravimetry performed 8 weeks after ISO-induced myocardial infarction showed significantly elevated ventricular weight ( $p<0.05$ ) as well as ventricular weight normalized to body weight ( $p<0.01$ ) and to tibia length ( $p<0.001$ ) (Table 1). However, L-2286 treatment prevented the unfavourable changes in these parameters (Table 1). Additionally, L-2286 administration also attenuated the ISO-induced weight gain (Table 1).

	<b>Control</b>	<b>ISO</b>	<b>ISO+L-2286</b>
<b>BW (g)</b>	403 ± 53.7	431.3 ± 28.6*	397.2 ± 22.3**
<b>WV (g)</b>	1.02 ± 0.078	1.132 ± 0.123*	0.977 ± 0.074**
<b>WV/BW (g/kg)</b>	2.51 ± 0.14	2.62 ± 0.19*	2.53 ± 0.15**
<b>WV/TL (g/mm)</b>	0.022 ± 0.001	0.025 ± 0.0025*	0.023 ± 0.0013**

**Table 1. PARP inhibition improves gravimetric parameters in ISO-induced chronic heart failure.** Conditions for performing gravimetry are detailed in Methods. BW: Body weight, WV: Weight of ventricles, TL: Length of right tibia, WV/BW: Weight of ventricles/body weight ratio, WV/TL: Weight of ventricles/length of tibia, ISO: animals 8 weeks after ISO administration; ISO+L-2286: animals treated with L-2286, 8 weeks after ISO administration. All values are means±SEM, \* $p<0.05$  (vs. Control group), \*\* $p<0.05$  (vs. ISO group).

#### 5.7 L-2286 MODERATED THE ELECTROCARDIOGRAPHIC CHANGES ASSOCIATED WITH CHRONIC HEART FAILURE

Decreased R wave amplitude ( $0.18 \pm 0.02$  vs.  $0.59 \pm 0.03$  mV,  $p<0.01$ ) as well as J point depression ( $-0.04 \pm 0.04$  vs.  $0.12 \pm 0.01$ ) were observed on the electrocardiogram of the ISO group 8 weeks after myocardial infarction. These electrocardiographic changes were

significantly improved ( $0.4 \pm 0.03$  and  $0.05 \pm 0.02$  mV, R wave amplitude and J point, respectively,  $p < 0.05$ ) by L-2286 treatment.

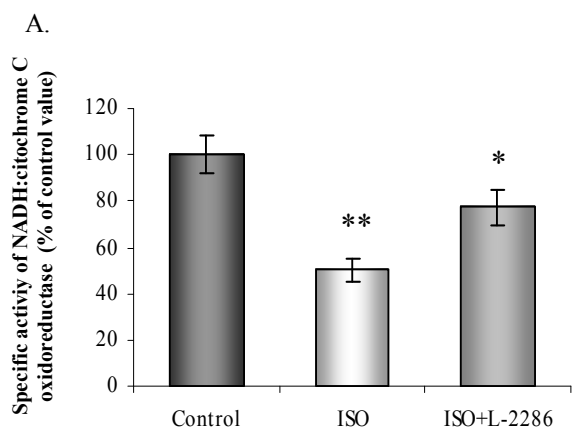
#### 5.8 L-2286 INHIBITED THE HEART FAILURE-INDUCED ELEVATION OF PLASMA BNP LEVEL

Elevation of plasma BNP level is associated with the severity of heart failure, while low plasma BNP concentrations provide strong evidence against heart failure. Plasma BNP level in our study showed a significant increase in the ISO group 8 weeks after myocardial infarction ( $3.5 \pm 0.22$  ng/ml) in comparison with the control group ( $2.2 \pm 0.18$  ng/ml,  $p < 0.01$ ). On the other hand, L-2286 treatment significantly reduced the plasma BNP level ( $2.56 \pm 0.16$ ,  $p < 0.05$ ) suggesting that the PARP inhibition was able to decrease the severity of postinfarction heart failure.

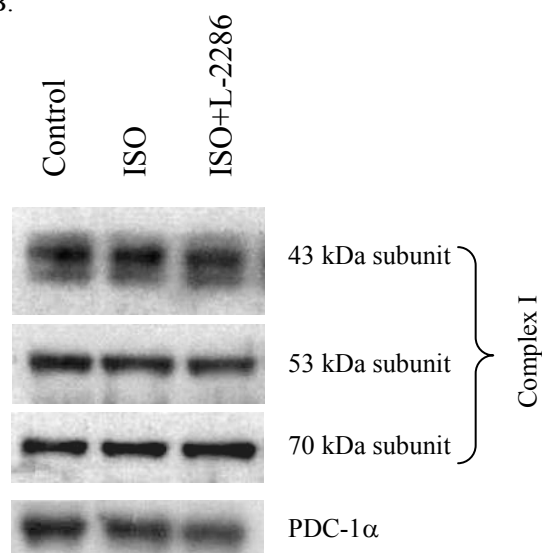
#### 5.9 L-2286 PREVENTED THE OXIDATIVE DAMAGE OF RESPIRATORY COMPLEXES IN CHRONIC HEART FAILURE

Ventricular remodeling is accompanied by altered mitochondrial energy metabolism. Under our experimental conditions, chronic heart failure induced a partial inactivation of respiratory complexes I-III assessed by the measurement of NADH:cytochrome c oxidoreductase activity ( $p < 0.01$ ) (Fig. 6A). As a contrast, L-2286 could partially protect ( $p < 0.05$ ) NADH:cytochrome c oxidoreductase activity in the failing heart mitochondria. Importantly, the expression of mitochondrial enzymes (including NADH dehydrogenase (complex I) 43, 53, 70 subunits and pyruvate dehydrogenase complex-1 $\alpha$ ) showed no changes in either the untreated or L-2286-treated heart samples, therefore the reduced activity of mitochondrial enzymes can be completely attributed to posttranslational oxidative injury (Fig. 6B).

**Figure 6. L-2286 prevented the oxidative damage of respiratory complexes in chronic heart failure. A.**



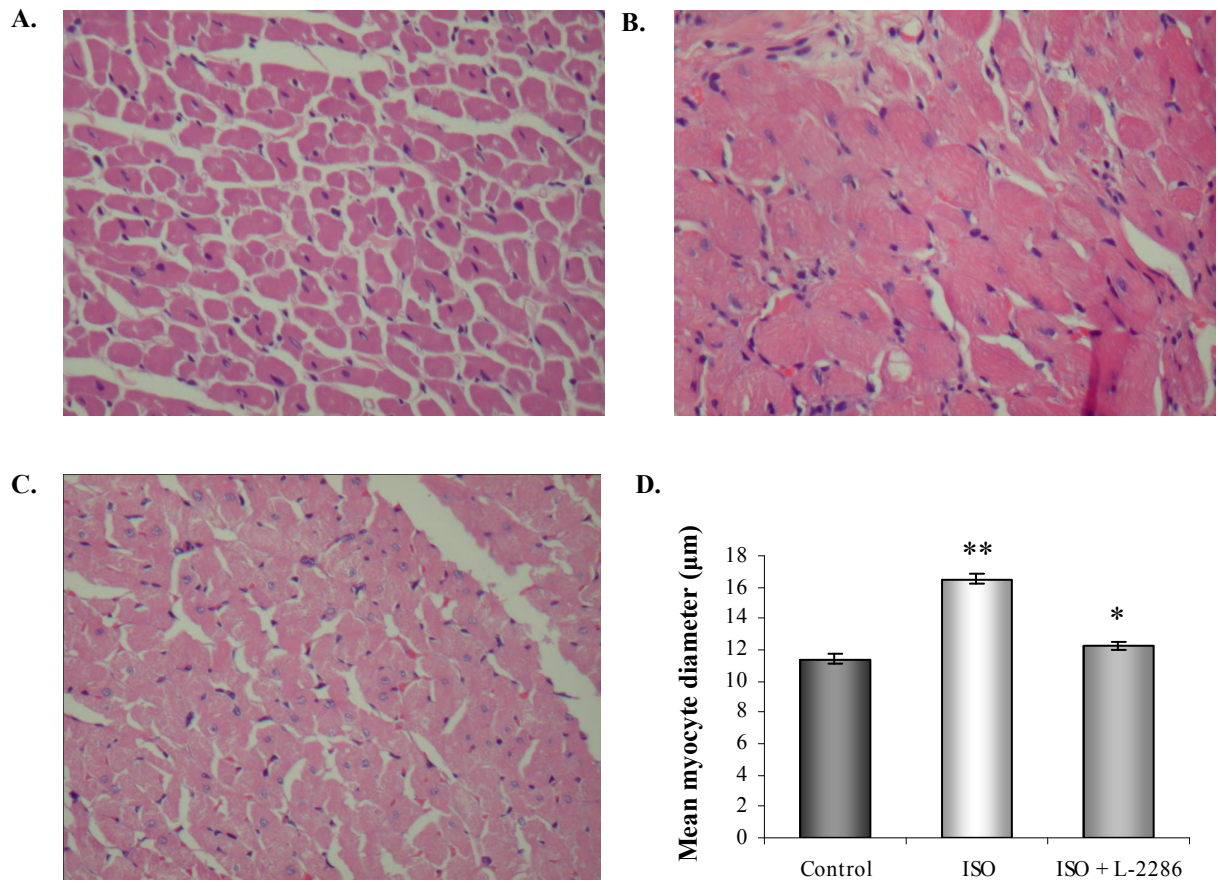
**B.**



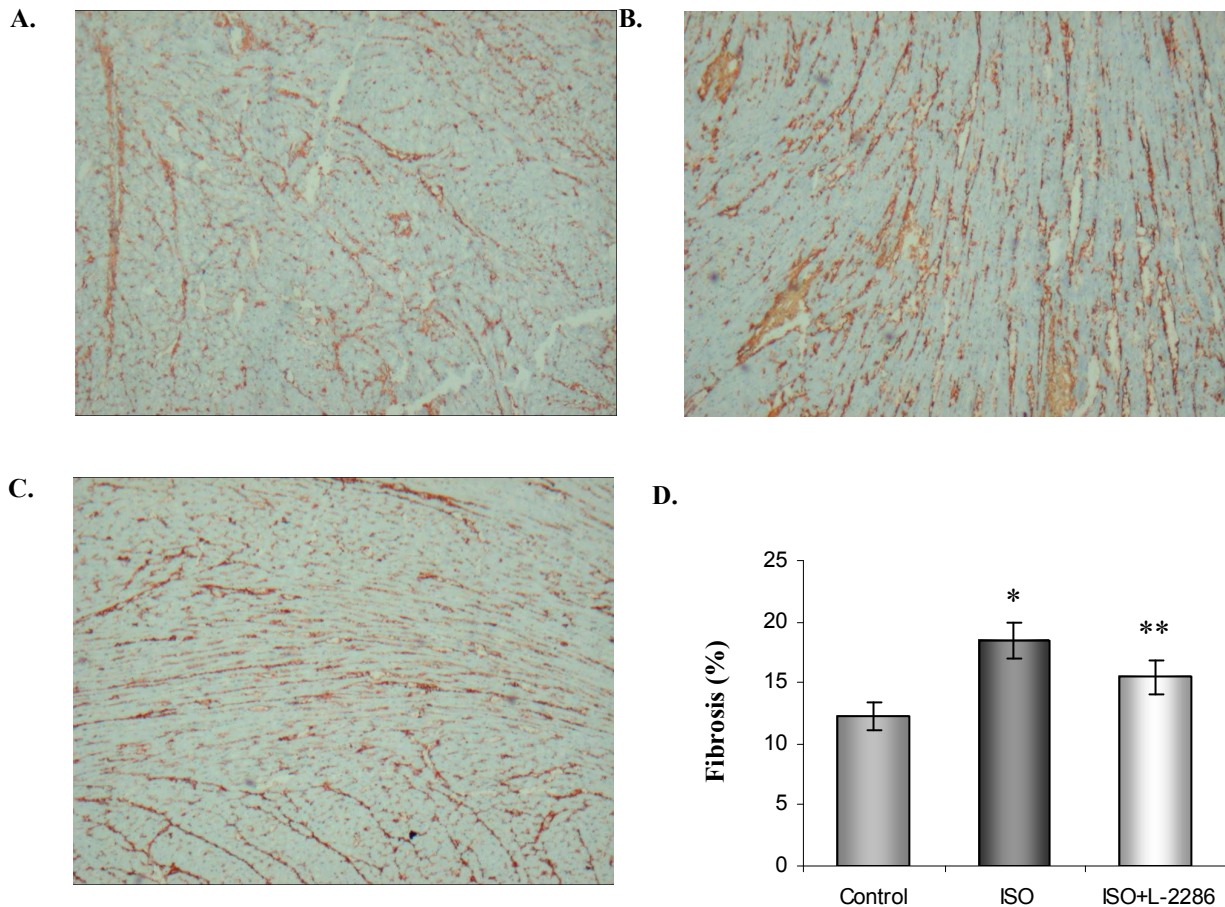
Activity of NADH:cytochrome c oxidoreductase in isolated mitochondria from failing rat hearts. Mitochondrial enzyme activities were measured as described in Methods. ISO: animals 8 weeks after ISO administration; ISO+L-2286: animals treated with L-2286, 8 weeks after ISO administration. Data are expressed as the percent of control values (means±SEM), \* $p < 0.05$  (vs. ISO group), \*\* $p < 0.01$  (vs. Control group). **B.** Expression of mitochondrial enzymes (NADH dehydrogenase complex (complex I) - 43, 53, 70 subunit, pyruvate dehydrogenase complex-1 $\alpha$ ) in heart failure rats. Conditions for Western blot analysis were detailed in Methods. Representative immunoblots from five experiments with similar results and densitometric evaluation are shown. PDC-1 $\alpha$ : pyruvate dehydrogenase complex-1 $\alpha$ ; ISO: animals 8 weeks after ISO administration; ISO+L-2286: animals treated with L-2286, 8 weeks after ISO administration.

#### 5.10 L-2286 ATTENUATED THE MYOCARDIAL HYPERTROPHY AND INTERSTITIAL COLLAGEN DEPOSITION

Histological analysis revealed marked myocyte hypertrophy in failing rat hearts compared to the control group ( $p < 0.001$ ) (Fig. 7A-B). In addition, interstitial type III collagen deposition increased from  $12.2 \pm 1.2$  % in control hearts to  $18.4 \pm 1.4$  % in ISO-treated animals ( $p < 0.001$ ) (Fig. 8A-B). Nevertheless, L-2286 treatment significantly decreased the mean myocyte diameter ( $p < 0.005$ ) (Fig. 7C-D) and prevented the interstitial fibrosis (type III collagen deposition  $15.4 \pm 1.4$  %) ( $p < 0.01$ ) (Fig. 8C-D).



**Figure 7. L-2286 attenuated the ISO-induced myocardial hypertrophy.** Sections obtained from hearts of control (*A*), ISO-treated (*B*), and ISO+L-2286-treated animals (*C*) were stained with hematoxylin and eosin and mean myocyte diameters were calculated (*D*). Conditions for measurement of mean myocyte diameter were detailed in Methods. ISO: animals 8 weeks after ISO administration; ISO+L-2286: animals treated with L-2286, 8 weeks after ISO administration. Values are given as means±SEM, \* $p < 0.005$  (vs. ISO group), \*\* $p < 0.001$  (vs. Control group).



**Figure 8. L-2286 reduced the ISO-induced interstitial collagen deposition.** Sections obtained from hearts of control (A), ISO-treated (B), and ISO+L-2286-treated animals (C) were immunostained with an antibody against type III collagen; additionally, densitometric evaluation is shown (D). Conditions for immunohistology were detailed in Methods. ISO: animals 8 weeks after ISO administration; ISO+L-2286: animals treated with L-2286, 8 weeks after ISO administration. Values are given as means±SEM, \*p<0.001 (vs. Control group), \*\*p<0.01 (vs. ISO group).

#### 5.11 L-2286 REDUCED GSK-3 $\beta$ AND PKC PHOSPHORYLATION IN FAILING MYOCARDIUM

GSK-3 $\beta$  was slightly phosphorylated in control animals, but became strongly phosphorylated 8 weeks after myocardial infarction in the ISO group (Fig. 9A). However, this strong phosphorylation was diminished by L-2286 administration (Fig. 9A). The moderate Akt phosphorylation present in the control samples slightly increased in the ISO group, but the L-2286 treatment did not influence the phosphorylation state of Akt (Fig. 9A). The level of GSK-3 $\beta$  phosphorylation was not in accordance with that of Akt, one of the upstream kinases of GSK-3 $\beta$  causing its inhibition, therefore the L-2286-induced decrease in GSK-3 $\beta$  phosphorylation was probably independent of Akt.

The overall (pan) phosphorylation state of protein kinase C was moderate in control animals, but became strongly enhanced 8 weeks after myocardial infarction without any change in the expression of total PKC (Fig. 9B). As far as the PKC isoforms are concerned, PKC  $\alpha/\beta$ II and PKC  $\delta$  (Ser<sup>643</sup>) seemed to be responsible for the ISO treatment-associated increased pan PKC phosphorylation (Fig. 9B). Most importantly and in concert with the GSK-3 $\beta$  phosphorylation state, the enhanced phosphorylation of pan PKC, PKC  $\alpha/\beta$ II and PKC  $\delta$  (Ser<sup>643</sup>) seen in the postinfarcted samples was significantly attenuated by the PARP inhibitor treatment. Since GSK-3 $\beta$  can be phosphorylated by PKC leading to its inactivation, we propose that the PARP inhibition-caused reduced GSK-3 $\beta$  phosphorylation was probably mediated by the PKC pathway. The phosphorylation state of PKC  $\delta$  (Thr<sup>505</sup>) however remained unchanged in response to any treatment.

#### 5.12 L-2286 HAD NO INFLUENCE ON THE PHOSPHORYLATION OF MAP KINASES

Extracellular signal-regulated kinase (ERK) was moderately phosphorylated in control samples and remained so in ISO-treated as well as ISO+L-2286-treated hearts (Fig. 9C). In contrast, the moderate phosphorylation of both p38-MAPK and c-jun N-terminal kinase (JNK) seen in control hearts was elevated in heart failure animals (ISO group), but L-2286 could not mitigate the phosphorylation of these kinases (Fig. 9C).



Figure 9. A.

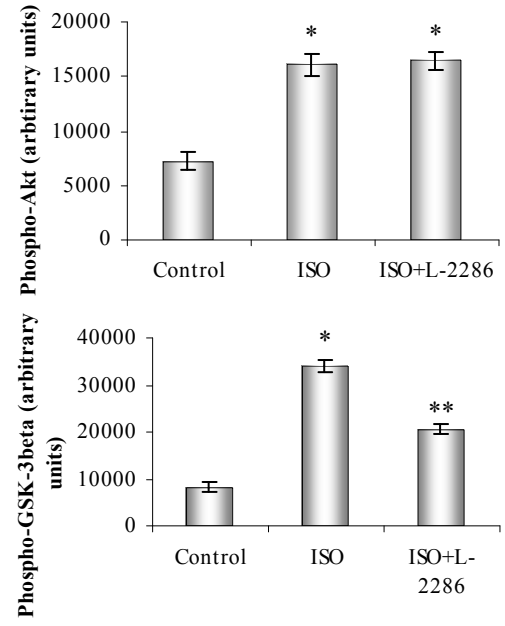
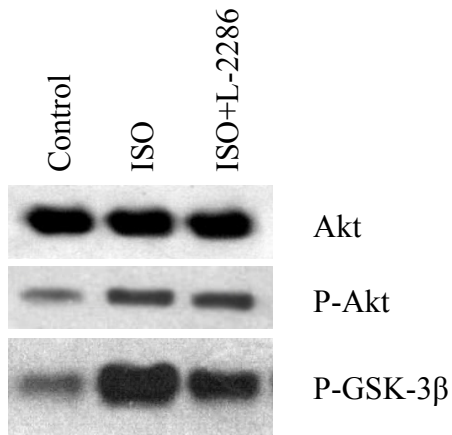


Figure 9. B.

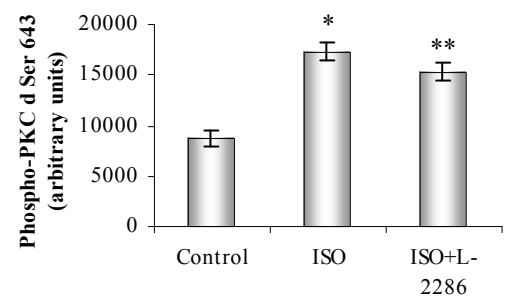
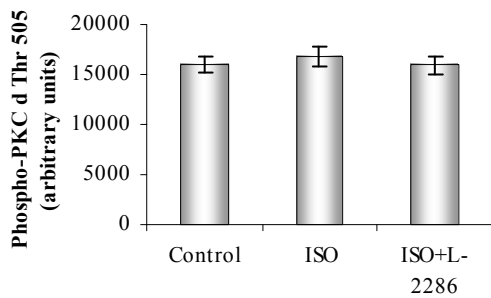
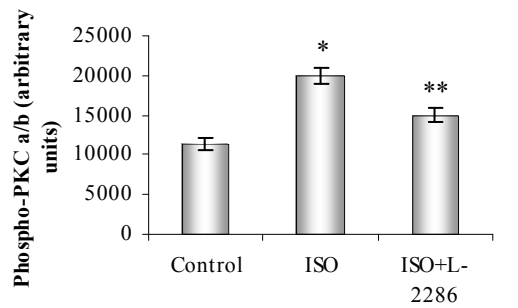
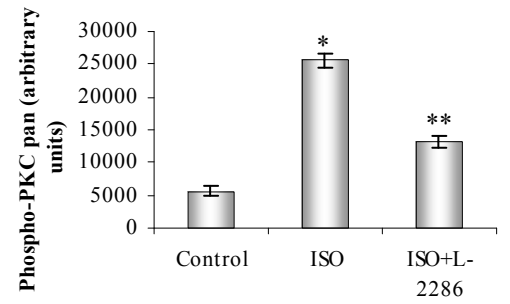
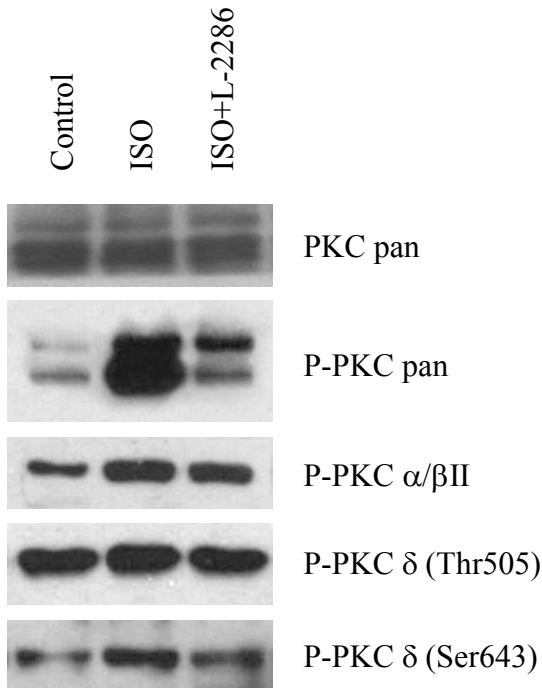
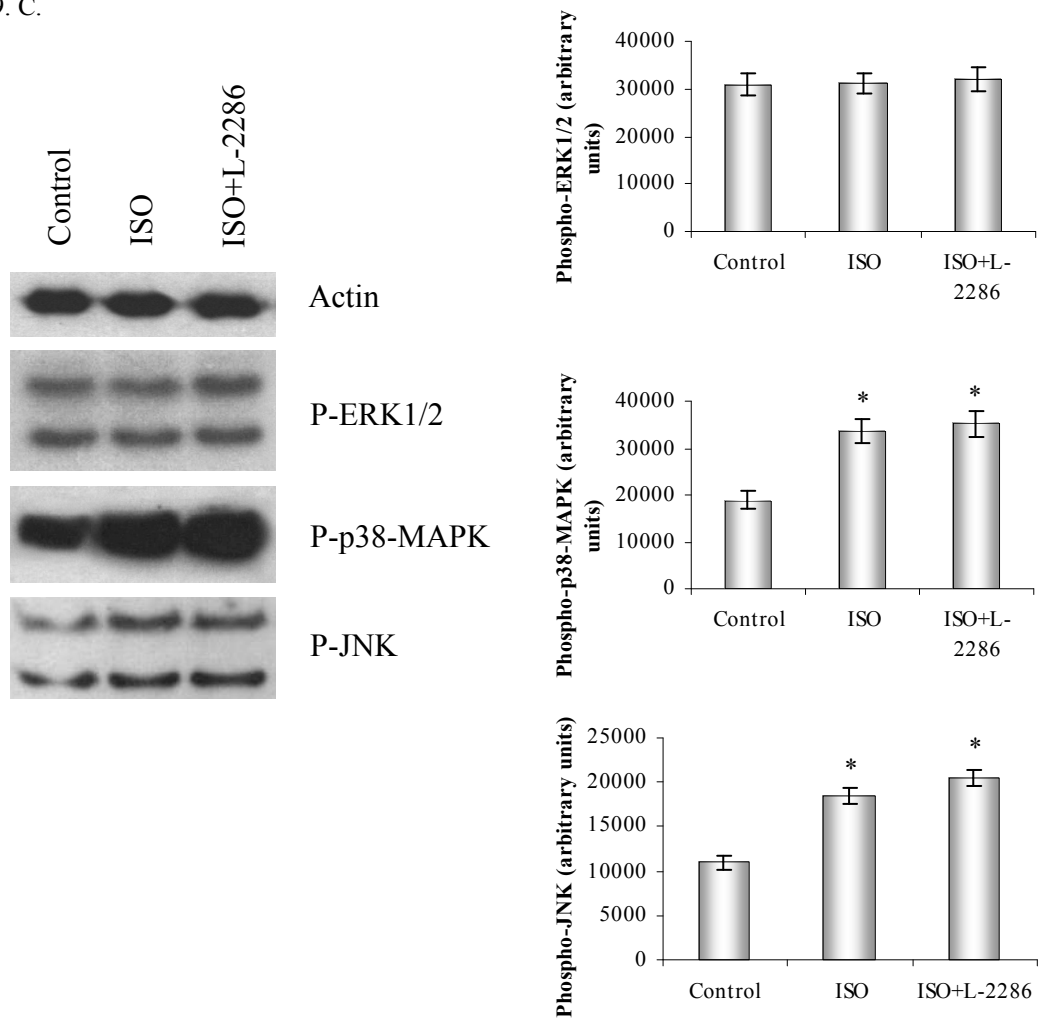


Figure 9. C.



**Figure 9. L-2286 treatment inhibits hypertrophic stimuli-induced GSK-3 $\beta$  phosphorylation via protein kinase C signaling in chronic heart failure.** *A.* Western blot analysis of Akt and GSK-3 $\beta$  phosphorylation and densitometric evaluation are shown. *B.* Western blot analysis of pan PKC, PKC  $\alpha/\beta$ , PKC  $\delta$  (Thr505) and PKC  $\delta$  (Ser643) phosphorylation and densitometric evaluation are shown. *C.* Western blot analysis of ERK, p38-MAPK and JNK phosphorylation and densitometric evaluation are shown. Actin is shown as loading control. ISO: animals 8 weeks after ISO administration; ISO+L-2286: animals treated with L-2286, 8 weeks after ISO administration. Representative immunoblots from five experiments with similar results are shown, \*p<0.001 (vs. Control group), \*\*p<0.001 (vs. ISO group).

## 6. DISCUSSION

This study gave first insight into a novel mechanism of cardioprotection by a novel PARP inhibitor L-2286 in isolated ischemic-reperfused hearts, in *in vivo* isoproterenol-induced myocardial injury and in an experimental model of chronic heart failure. PARP inhibition was first shown to beneficially influence the activation of Akt signaling pathway and the MAPK cascade in two animal models of myocardial ischemia and reperfusion. Furthermore PARP inhibition beneficially influenced the progression of myocardial hypertrophy and remodeling following myocardial infarction eventually by activating the antihypertrophic glycogen synthase kinase-3 $\beta$  via blocking protein kinase C signaling.

### 6.1 CARDIOPROTECTION AFFORDED BY PARP INHIBITION IN MYOCARDIAL ISCHEMIA-REPERFUSION

Massive PARP activation induced by oxidative stress after ischemia-reperfusion contributes to myocardial cell death and the subsequent inflammatory reactions.<sup>2</sup> Consequently, PARP inhibition was shown to exert protection against oxidative injury in cell cultures and *ex vivo* and *in vivo* animal models of regional or global cardiac, brain or renal ischemia-reperfusion.<sup>1-4</sup> Therefore, pharmacological inhibition of PARP or PARP gene ablation improved the functional and metabolic recovery of postischemic myocardium and reduced infarct size in various models.<sup>6,7,10</sup>

Hereby L-2286 was demonstrated to promote the postischemic recovery of myocardial energy metabolism in Langendorff heart perfusion system. L-2286 helped to preserve the high-energy phosphate intermediates during reperfusion as shown by <sup>31</sup>P NMR spectroscopic studies. Moreover, L-2286 facilitated the rapid and more complete consumption of inorganic phosphates during reperfusion. Reutilization of the latter bears crucial importance because excessive amounts of inorganic phosphates, calcium and reactive oxygen species are the most potent triggers of mitochondrial permeability transition, which compromises the integrity of the mitochondrial membranes ultimately leading to apoptosis.<sup>19</sup> The improved metabolic recovery in the presence of L-2286 was accompanied by decreased myocardial oxidative damage, i.e. lipid peroxidation and protein oxidation.

In accordance with Langendorff studies, the isoproterenol-induced myocardial damage *in vivo* was also significantly attenuated by L-2286 treatment, as it was proved by reduced cardiac necroenzyme (CK and LDH) release and smaller infarct size in ISO+L-2286-treated compared to ISO-treated animals. Notably, isoproterenol, a  $\beta$ -adrenergic agonist causes

extensive cardiomyocyte necrosis, which may be induced by a mismatch of oxygen supply versus demand and additionally, by enhanced free radical formation.<sup>29-32</sup>

## 6.2 CARDIOPROTECTION AFFORDED BY PARP INHIBITION IN CHRONIC HEART FAILURE

The experimental PARP inhibitor compound L-2286 was also tested in rats with failing heart following isoproterenol-induced myocardial infarction. As heart failure is one of the leading causes of morbidity and mortality worldwide, the effect of PARP inhibition on the progression of heart failure is worth to be elucidated.<sup>39</sup> PARP inhibition already represents a potential clinical approach to fight against myocardial remodeling: as it has been previously reported, carvedilol, an antiadrenergic drug used, among others, to treat the hypertrophic and failing heart, exhibited antioxidant and PARP-inhibitory properties.<sup>5</sup>

So far PARP inhibition have been reported to moderate cardiac dysfunction and improve vascular relaxation in postinfarcted rats, however, the effects of PARP inhibition on the (sub)cellular and metabolic parameters in the failing heart are still elusive.<sup>9</sup> We therefore examined how L-2286 interferes with the progression of postinfarction heart failure, including changes in cellular mass, interstitial matrix composition, metabolic parameters, gene expression and intracellular signaling.

Cardiac remodeling is characterized by inadequate myocyte hypertrophy and accumulation of extracellular matrix structural proteins (fibrillar collagen, type I and III collagen), which induce tissue stiffness and adversely affect myocardial viscoelasticity, ultimately leading to diastolic and systolic dysfunction.<sup>26,40,41</sup> In addition, perivascular fibrosis and disorganized hypertrophy can exhaust the coronary blood flow reserve and the consequent hypoperfusion may provoke myocardial ischemia.<sup>26,31</sup> In this study L-2286 exerted a beneficial effect on the progression of postinfarction remodeling by attenuating myocardial hypertrophy and interstitial deposition of type III collagen in the failing hearts.

Cardiac hypertrophy and failure can also be characterized by severe dysfunction of mitochondrial energy and substrate metabolism involving impaired mitochondrial function, decline in high-energy phosphates, reduced oxygen consumption and decreased tissue content and activity of complex I through IV of the respiratory chain.<sup>42</sup> Similar to literature data, the activity of complex I to III (NADH:cytochrome c oxidoreductase) has dropped in our heart failure model accompanied with unaltered expression of either complex I (43, 53, 70 subunit) or pyruvate dehydrogenase complex (1 $\alpha$  subunit). Since the dysfunction of these enzymes is

mainly attributed to posttranslational inactivation by reactive oxygen species, this event was efficiently prevented by PARP inhibition.

In response to hypertrophic stimuli such as left ventricular wall stretch and volume overload, cardiac fetal-like gene program is initiated entailing, among others, the expression and secretion of natriuretic factors (BNP, ANP).<sup>26,40,43,44</sup> The increased release of plasma B-type natriuretic peptide from the ventricles, and elevated BNP concentration specifically signals impaired left ventricular function and chronic heart failure.<sup>25,45</sup> In concert with this, plasma BNP level increased in the postinfarcted animals, which was moderated by L-2286 treatment.

### 6.3 EFFECT OF PARP INHIBITION ON CELLULAR SIGNALING IN MYOCARDIAL ISCHEMIA-REPERFUSION

Reports from our and other laboratories have recently challenged the notion that the protection by PARP inhibition is solely attributable to the preservation of cellular NAD<sup>+</sup> and ATP pools.<sup>2,3,10,11,12,15,46,47</sup> These studies suggested that PARP blockade might also modulate a diverse array of signaling cascades and interfere with the expression of several genes in inflammatory processes and cardiac ischemia-reperfusion. In this respect, these findings raised the important prospect that the targeting of PARP might ameliorate the myocardial dysfunction by interrupting the adverse processes at the signal transduction level.

This study demonstrated for the first time that PARP inhibitors could promote Akt activation during cardiac ischemia-reperfusion in *in vitro* and *in vivo* models of myocardial reperfusion injury. We found enhanced L-2286-triggered Akt and GSK-3 $\beta$  phosphorylation not only in isolated hearts, but also in isoproterenol-induced cardiac injury. To our knowledge, this is the first *ex vivo* and *in vivo* report, which attributes a critical role to Akt in the cardioprotection afforded by PARP inhibitors. The PI3-kinase/Akt pathway is one of several prosurvival signaling routes, which is activated as an adaptive response to cellular stress.<sup>22,48</sup> Among others, ischemia-reperfusion itself can expedite Akt signaling in cardiac myocytes, as shown in this study. However, L-2286 administration further increased Akt activation independently of cardiac injury, presumably exerting antiapoptotic and favorable metabolic effects.

Subsequently, this work demonstrated the differential activation of mitogen-activated protein kinases upon ischemia-reperfusion, which was clearly modulated by concomitant L-2286 treatment, strongly depending on the context and timing of the deleterious insult. First, while ERK phosphorylation was enhanced upon ischemia-reperfusion of isolated hearts,

isoproterenol *in vivo* caused a transient fall followed by a delayed increase in ERK activity. This is in accordance with reports showing that ERK activity reduced during ischemia ensued by its recovery during reperfusion in rat hearts.<sup>49-51</sup> Based on ECG recordings, subcutaneous administration of isoproterenol provoked faster heart rate for at least 4 hours, implicating a prolonged “ischemic” period matching the duration of lower ERK activity (data not shown). Most importantly, L-2286 not only promoted ERK phosphorylation in normoxic and ischemic-reperfused isolated hearts, but also accelerated that *in vivo* covering the most vulnerable, first 4 hours immediately after isoproterenol administration. Although ERK is mainly involved in growth factor-induced signaling, it can play protective roles in oxidative stress via blocking apoptosis.<sup>22</sup> Indeed, sustained activation of ERK during reoxygenation was shown to render delayed cytoprotection, probably by downregulating caspase-3 and JNK activity.<sup>52,53</sup> In summary, the ability of L-2286 to elicit robust ERK activation in both *ex vivo* and *in vivo* ischemia-reperfusion may promote cardiac myocyte survival.

Second, phosphorylation of p38-MAPK exhibited a similar dynamics to that of ERK, i.e. a slight activation upon postischemic reperfusion in the Langendorff heart and a protracted activation 24 hours after isoproterenol administration. Our findings are thus consistent with reports where p38-MAPK was not activated by ischemia alone, but was stimulated by reperfusion in rat hearts.<sup>51,54</sup> Remarkably, independently from the model used p38-MAPK phosphorylation was enhanced 2-3-fold in the presence of L-2286. The role of p38-MAPK in myocardial ischemia-reperfusion injury is controversial. Several data suggest that the activation of p38-MAPK/MAPKAPK2/Hsp-27 axis contributes to ischemic preconditioning, however, others concluded that p38-MAPK inactivation might be cardioprotective.<sup>23</sup> This ambiguity might be reconciled knowing that two p38-MAPK isoforms ( $\alpha$  and  $\beta$ ) are expressed in the heart: while p38 $\alpha$  transmits proapoptotic signals, p38 $\beta$  triggers hypertrophic response, i.e. promotes survival.<sup>55</sup> Taken together, L-2286 expedited cardiac p38-MAPK phosphorylation, which might be protective under certain conditions.

Third, JNK was strongly activated by *ex vivo* ischemia-reperfusion and immediately after isoproterenol injection. In the *in vivo* model JNK thus responded differently from ERK and p38-MAPK demonstrating a sharp increase and a slow return (after 24 hours) in phosphorylation. Assuming a prolonged “ischemia” after isoproterenol administration lasting for several hours, our data are consistent with reported ischemia-induced activation of JNK.<sup>49,54</sup> Although JNK was generally regarded as a proapoptotic kinase, recent studies suggested that JNK might only promote apoptosis in cells where the apoptotic program has already been initiated, also depending on the participation of other signaling molecules (such

as NF- $\kappa$ B).<sup>24</sup> While L-2286 strengthened JNK phosphorylation in the Langendorff heart, it blocked that in isoproterenol-induced myocardial injury. The reasons behind these differences remain elusive; however, our in vivo model mimics more closely the pathophysiological events in myocardial infarction. Therefore, L-2286 may inhibit JNK activity during the most critical hours of the failing nutrient and oxygen supply.

#### 6.4 EFFECT OF PARP INHIBITION ON CELLULAR SIGNALING IN CHRONIC HEART FAILURE

Recent years have seen a burst in our understanding of the intracellular signaling mechanisms which mediate extrinsic and intrinsic growth stimuli into coordinated gene expression changes. Hypertrophy is initiated and maintained by vasoactive peptides, peptide growth factors, hormones and neurotransmitters, which all act upon MAP kinases, JAK/STAT, CaMK/calcineurin routes as well as glycogen synthase kinase-3 $\beta$ .<sup>26,27,56-60</sup> Differential activation of MAPKs results in specific cardiac morphologic and functional phenotypes: while ERK1/2 activation leads to a concentric form of hypertrophy with enhanced cardiac function, p38-MAPK and JNK activation gives rise to maladaptive hypertrophy.<sup>26</sup> Interestingly, JNK and p38-MAPK are activated in failing human hearts, while ERK activation remains at a physiologic level.<sup>26,28</sup> Furthermore, several protein kinases including Akt, p70-S6 kinase, p90-RSK, protein kinase C and protein kinase A have been implicated in cardiac hypertrophy, commonly phosphorylating, thereby suppressing the antihypertrophic activity of GSK-3 $\beta$ .<sup>25,28,57,61</sup> The ability of GSK-3 $\beta$  to inhibit hypertrophy in response to  $\beta$ -adrenergic stimulation, pressure overload and calcineurin activation suggests that GSK-3 $\beta$  represents a point of cross-talk and convergence of various hypertrophic signaling pathways.<sup>25,28,57,61</sup> GSK-3 $\beta$  phosphorylation via a phosphatidylinositol-3-kinase dependent pathway can be catalyzed by at least two protein kinases: Akt and insulin like kinase (ILK).<sup>56,61</sup> Growth factors however can regulate GSK-3 $\beta$  not only through the PI3K/Akt pathway, but also via an alternative PKC pathway.<sup>62</sup> Most importantly, several PKC isoforms, i.e. PKC $\alpha$ , PKC $\beta$ , PKC $\gamma$ , PKC $\eta$  and PKC $\delta$ , can phosphorylate GSK-3 $\beta$  at regulatory serine residues.<sup>63</sup> Furthermore, altered gene expression of differential cardiac PKC isozymes including PKC $\delta$ , PKC $\alpha$  and PKC $\beta$ I have been recently reported in myocardial hypertrophy.<sup>64-67</sup>

In this study eight weeks after myocardial infarction no increase in ERK1/2 and JNK activity was found in the heart samples, but a two-fold elevation in Akt and p38-MAPK phosphorylation was seen. Notably, upon L-2286 administration these kinase activities remained unaltered. By contrast, heart failure following ISO injections upregulated GSK-3 $\beta$

phosphorylation, which correlated with the activation of several PKC isoforms rather than that of Akt. Most importantly, PARP inhibition in the infarcted hearts simultaneously mitigated both GSK-3 $\beta$  and PKC phosphorylation. Since GSK-3 $\beta$  can integrate a variety of antihypertrophic signals and transmit them to NF-AT and c-Jun, the L-2286-induced PKC-mediated GSK-3 $\beta$  activation may have contributed to the rescue of failing hearts in our experimental model.<sup>43</sup>

The mechanism by which PARP inhibition diminished the activity of PKC still needs to be elucidated. Nevertheless, oxidative stress in chronic heart failure can increase PKC activation via various pathways. First, oxidative stress can directly or indirectly stimulate PKC via growth factor receptor phosphorylation or lipid secondary messengers (derived from the reactions of other receptor-regulated enzymes such as phospholipases A2, D and C).<sup>68</sup> Second, poly(ADP-ribose) polymerase activation could also contribute to PKC activation, because ROS-induced poly(ADP-ribosyl)ation depletes nicotinamide adenine dinucleotide (NAD<sup>+</sup>) pools or direct poly(ADP-ribosyl)ation blocks glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme.<sup>69,70</sup> The inhibition of GAPDH can divert the glycolytic flux towards the formation of  $\alpha$ -glycerophosphate and diacylglycerol (DAG), the latter being the activator of PKC.<sup>70</sup> On the other hand, reactive oxygen species can also directly oxidize and hinder GAPDH.<sup>71</sup>

Notably, PARP inhibition primarily interferes with the activity of nuclear PARP and, in turn, maintains NAD<sup>+</sup> and ATP pools avoiding NAD<sup>+</sup> depletion-induced inactivation of GAPDH. Furthermore, we have previously reported that PARP inhibitors could prevent the ROS-induced inactivation of mitochondrial respiratory complexes and the consequent mitochondrial ROS formation.<sup>3</sup> We therefore suggest that PARP inhibition can blunt PKC activation eventually by preserving NAD<sup>+</sup> pools and abrogating ROS production, thereby preempting GAPDH inactivation and DAG formation.



## 7. CONCLUSIONS

In conclusion, this study first demonstrates that PARP inhibition can beneficially influence the protein kinase signaling in isolated ischemic-reperfused hearts and isoproterenol-induced myocardial infarction by promoting Akt, ERK and p38-MAPK, but suppressing JNK activity. It is also the first report on changes in intracellular signaling during isoproterenol-induced myocardial infarction. Furthermore this work also provides evidence for the first time that PARP inhibition can halt the progression of cardiac hypertrophy into failure partially by promoting GSK-3 $\beta$  activity via direct or indirect interruption of upstream protein kinase C signaling. Finally PARP inhibition is also reported to protect against cellular changes in the postinfarcted myocardium such as fibrosis, cardiomyocyte hypertrophy, mitochondrial dysfunction and fetal gene expression. The PARP inhibition-induced alterations in intracellular signaling upon myocardial ischemia-reperfusion and postinfarction ventricular remodeling further challenge the original dogma that protection by PARP inhibitors exclusively rely on the preservation of NAD<sup>+</sup> as well as ATP stores.

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## 10. PUBLICATION OF THE AUTHOR

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### **Published abstracts**

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