PREVENTION OF OXIDATIVE CELL INJURY WITH ANTIOXIDANTS AND POLY(ADP-RIBOSE) POLYMERASE INHIBITORS

Ph.D. Thesis

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CONTENTS

1. ABBREVIATIONS

3-AB	3-aminobenzamide
3-ABA	3-aminobenzoic acid
4-HQ	4-hydroxyquinazoline
AST	aspartate aminotransferase
ATP	adenosine triphosphate
β-ATP	β-phosphoryl group of ATP
BGP-15	O-(3-piperidino-2-hydroxy-1-propyl)nicotinic amidoxime
CABG	coronary artery bypass graft
CK	creatine kinase
DHR123	dihydrorhodamine123
DNA	dezoxyribonucleic acid
dP/dt	the rate of pressure development
DTE	dithioerythritol
EDTA	ethylenediamine tetraacetate
FID	free induction decay
GSH	reduced form of glutathione
GSSG	oxidized form of glutathione
H_2O_2	hydrogen peroxide
H-2545	*N*-3-(2,2,5,5-tetramethyl-3-pyrroline-3-carboxamido) propylphthalimide
H-2954	*N*-hydroxyl metabolite of H-2545
HO-2434	1-(2,6-dimethylphenoxy)-[2-*N*-(2,5-dihydro-3-methyl-2,2,5,5-tetramethyl-1*H*-pyrrol)]aminopropane
HO-2433	*N*-hydroxyl metabolite of HO-2434
HR	heart rate
IC_{50}	half-maximal inhibitory concentration
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LVDP	left ventricle developed pressure
MDA	malondialdehyde

MOPS	3-[*N*-morpholino]propanesulfonic acid
MPT pore	mitochondrial permeability transition pore
NA	nicotinamide
NAD^+	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
$NO^{\bullet}$	nitric oxide
$O_2^{\bullet-}$	superoxide anion
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly(ADP-ribose) polymerase
Pi	inorganic phosphate
PCr	creatine phosphate
PTCA	percutaneous transluminal coronary angioplasty
δ	chemical shift between Pi and PCr in ppm
ROS	reactive oxygen species
RPP	rate-pressure product
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TEMPOL	4-hydroxy-[2,2,6,6-tetramethylpiperidine-1-oxyl]
Trolox	water-soluble vitamin E analogue

2. INTRODUCTION

Cardiovascular diseases and especially ischemic heart disease are the major cause of death in adults and among elderly patients in the developed and in many developing countries [1]. The availability of invasive diagnostic and therapeutic procedures (coronary angiography, PTCA, stent implantation, CABG) is limited, mostly because of their excessive cost, thus the development of new agents for the treatment of ischemic heart disease is in the limelight of scientists.

The past decade has seen an explosion of knowledge regarding the role of oxidative stress in the pathogenesis of a wide variety of diseases, such as atherosclerosis, ischemia-reperfusion injury, cancer, chronic inflammation, autoimmune diseases and aging [2-8].

2.1. Sources of free radicals in the cell

Sources of reactive oxygen species (ROS) in living cells are represented by physiological enzymatic mechanisms. They are generated from aerobic metabolism that utilizes life-sustaining oxygen to oxidize fuels [9]. Besides ROS such as the superoxide anion, hydrogen peroxide and hydroxyl radicals, there are also other sources of oxidative stress like singlet oxygen [10], peroxides [11], reactive aldehydes [12], nitric oxide [13], and other reactive species [14]. Mitochondria are well documented as a major source of $O_2^{\cdot -}$ and H_2O_2. It is calculated that 1-4% of oxygen reacting with the respiratory chain is incompletely reduced to ROS [9]. Being major producers of ROS, mitochondrial structures are exposed to high concentrations of ROS and, therefore, are particularly susceptible to their attack [15].

Assessment of overall oxidative stress must also include other sources of free radicals existing in the cytosolic compartment. Various cytosolic and membrane bound oxidases and dehydrogenases (e.g. xanthine oxidase, lipoxygenase and NADH oxidase) are known to produce free radicals. The constitutive and inducible forms of nitric oxide synthase, which produces $NO^{\bullet}$ are also important sources of free radicals [16]. A major

consequence of oxidative stress is lipid peroxidation and its by-products, the reactive aldehydes that show unusual and diverse reactions. They are known to react with proteins by attacking histidine residues and other amino acids, and also to modify DNA. Considering their relatively longer half-life, high affinity, high diffusivity to both hydrophilic and hydrophobic regions, and their proximity to targets within the membrane, these aldehydes could play a major contributory role in oxidative stress [12, 15-16].

2.2. Protection against oxidative stress

Free radicals are generating continuously in the living aerob organism. Nevertheless, cells have developed various enzymatic and nonenzymatic systems to protect themselves from oxidative damages. Superoxide anions can be scavenged by superoxide dismutase, an enzyme, which is present in all aerobic organisms, which catalyzes the conversion of two of these radicals into hydrogen peroxide and molecular oxygen:

$$O_2^{-\cdot} + O_2^{-\cdot} \xrightarrow{superoxide-dismutase+2\,H+} H_2O_2 + O_2$$

Hydrogen peroxide formed by superoxide dismutase and by the uncatalysed reaction of hydroperoxy radicals are scavenged by catalase, an ubiquitous heme protein that catalyzes the conversion of hydrogen peroxide into water and molecular oxygen:

$$H_2O_2 + H_2O_2 \xrightarrow{catalase} 2\,H_2O + O_2$$

Peroxidases are also heme enzymes and catalyze an analogous reaction in which hydrogen peroxide is reduced to water by a reductant (AH_2):

$$H_2O_2 + AH_2 \xrightarrow{peroxidase} 2\,H_2O + A$$

Glutathione has a key role in detoxification by reacting with hydrogen peroxide and organic peroxides. Glutathione, which is present in high concentrations (5 mM) in animal cells, serves as a sulfhydryl buffer [17-18]. Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG), in which two tripeptides are linked by a disulphide bond. GSSG is reduced to GSH by glutathione reductase, a flavoprotein utilizing NADPH as the electron source. The ratio of GSH to GSSG is in most cells greater than 500 [19]. Furthermore, other biological thiols could also

potentially be of importance in specific cellular locations.

Other important physiological antioxidants are vitamin E and reduced coenzyme Q (CoQ). Vitamin E is regarded as the most important lipid-soluble antioxidant. In vitamin E-deficient microsomal fractions, the protection against lipid peroxidation was diminished. Ascorbate and reduced glutathione are water-soluble antioxidants, which regenerate alfa-tocopherol. However, the protective effect of ascorbate and GSH against oxidative attack can attenuate or even reverse in the absence of vitamin E within the membranes [20].

In addition to its function as an electron and proton carrier in mitochondrial and bacterial electron transport coupled to ATP synthesis, ubiquinone (coenzyme Q) acts in the reduced form (ubiquinol) as an antioxidant, inhibiting lipid peroxidation in biological membranes and in serum low-density lipoprotein (LDL). According to recent evidence it can also protect mitochondrial inner-membrane proteins and DNA against oxidative damage [21].

Physiological compounds, such as urate, bilirubin, and ceruloplasmin, can also protect against oxidative attack [22-24].

2.3. The mechanism of myocardial reperfusion injury

Despite of the complexity of the above mentioned antioxidant system, free radicals generated excessively in certain circumstances can break this barrier and cause an oxidative stress to the cell. In various forms of oxidative stress there are different sources of ROS. During ischemia-reperfusion, mitochondrial respiratory complexes, especially complex I, are the main sources of toxic oxygen intermediers [25]. Hydrogen peroxide- or doxorubicine-induced oxidative injury in turn has at least two components. They can cause an enhanced mitochondrial ROS production, but their reaction with transition metals (e.g. Fe^{2+}) inducing a site-specific oxyradical formation is also very important in the mediaton of oxidative cell injury [26-27].

Among the above-mentioned pathological states ischemia-reperfusion has the most pronounced importance. It has been clearly established that myocardial cells cannot survive under severe prolonged ischemia. Coronary reperfusion, therefore, appears to be the only appropriate therapeutic strategy [28]. Early reperfusion may

prevent or lessen the development of necrosis, but experimental studies have demonstrated that, in the meantime, it is accompanied by various characteristic disturbances, generally referred to as reperfusion injury [29]. Although the generation of free radicals is slightly elevated also during ischemia, in the third-fourth minutes of reperfusion cells break into the formation of a huge amount of ROS, which phenomenon is called respiratory burst [14].

The excessive amount of ROS, such as H_2O_2, superoxide radicals and hydroxyl radicals are predominantly derived from the mitochondrial respiratory chain. Nevertheless, NADH oxidase of the neutrophil granulocytes and xanthine oxidase are also important sources of ROS [14, 16].

Reactive oxygen species injure a wide variety of biomolecules and cell compartments. ROS initiate lipid peroxidation [30], protein oxidation [31] and the formation of single-stranded DNA breaks [32-33]. The major product of lipid peroxidation is 4-hydroxy-2-nonenal, which is highly cytotoxic and can readily react with and damage proteins [15]. Intracellular sodium and calcium accumulation is the consequence of the injury of ion channels and the decrease of myocardial high-energy phosphate levels. The high level of intracellular Ca^{2+} activates the proteolytic enzymes and it causes the conversion of xanthine dehydrogenase to xanthine oxidase [34]. High intracellular Ca^{2+} level together with high intracellular inorganic phosphate concentration induces the opening of mitochondrial permeability transition pores (MPT pore). As a consequence of opening of the MPT pores, the mitochondrial membrane potential will collapse and the mitochondrial energy production will be ceased [35]. Mitochondrial respiratory complexes have a central role in the development of postischemic myocardial damage. They are the main sources of ROS during reoxygenation, but themselves are also injured by ROS and by reactive aldehydes, such as 4-hydroxy-2-nonenal, and they exhibit a reduced activity during oxidative stress [15]. The consequences are a decreased energy generation, an increased ROS production and eventually cell death.

ROS and NO-induced single-strand DNA break formation, on the other hand, activate the nuclear poly(ADP-ribose) polymerase (PARP) [36, 37]. Upon activation, PARP cleaves NAD^+ to nicotinamide and ADP-ribose which is coupled to different proteins and protein-bound ADP-ribose residues. Excessive PARP activation leads to cellular NAD^+ and ATP depletion which causes necrosis [38]. The possible roles of

PARP under physiological and pathophysiological circumstances will be discussed in Chapter 2.4.

Under the clinical manifestations of the above-mentioned reperfusion injury is the cardiac stunning, which may persist for several days after reperfusion. Reperfusion of ischemic myocardium is also frequently associated with the development of arrhythmias, especially ventricular tachycardias or ventricular fibrillation [39].

2.4. Physiological and pathophysiological role of the poly(ADP-ribose) polymerase enzyme. Protection against reperfusion injury using PARP inhibitors.

If ROS concentrations are permanently high, they cause significant amount of single-strand DNA breaks, which activate the poly(ADP-ribose) polymerase (PARP). There is a direct correlation between the activity of this enzyme and the extent and number of DNA strand breaks in most tissues [40]. PARP is a chromatin-bound, monomeric 113 kDa Zn^{2+}-finger protein present abundantly in the nucleus in an inactive form [41-42].

The exact range of functions of PARP has not yet been established, although the enzyme is thought to play an essential role in different DNA related processes, such as replication, gene expression, and repair and maintenance of genomic stability [43-45]. PARP utilizes NAD as a substrate to modify proteins. Although this modification is transient, it is very extensive in vivo, since polymer chains reach more than 200 ADP-ribose units on protein acceptors. More than 30 nuclear substrates of PARPs have been identified. Most of them are nuclear proteins involved in the metabolism of nucleic acid, DNA repair, regulation of cell cycle and modulation of chromatin structure (e.g. histones, DNA ligases, polymerases and topoisomerases) as well as PARP itself. Normal function of proteins is restored after catabolism of the poly(ADP-ribose) polymer.

The efficient degradation of the polymer requires two additional nuclear enzymes, poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase. PARG is responsible for the hydrolysis of glycosidic bonds between ADPr units located at the extremity and within the polymer. ADP-ribosyl protein lyase hydrolyses the most proximal unit of ADPr from the acceptor protein [45].

In case DNA damage is extensive, the net effect of this metabolism is the rapid depletion of cellular NAD^+ pool. NAD^+ is an essential cofactor in energy metabolism. Synthesis of ATP and the balance of redox potential directly depend on NAD^+ level in cells. In addition, in efforts to resynthetize NAD^+, ATP may also be depleted leading to cell death due to energy depletion [38, 46]. Therefore, inhibition of PARP can partially prevent ROS toxicity and ischemia-reperfusion-induced cell death [47-48]. As a consequence, PARP inhibitors are widely used experimentally to protect cells from oxidative damages [49-50] and the disruption of PARP gene increases the tolerance against oxidative damages [44, 51].

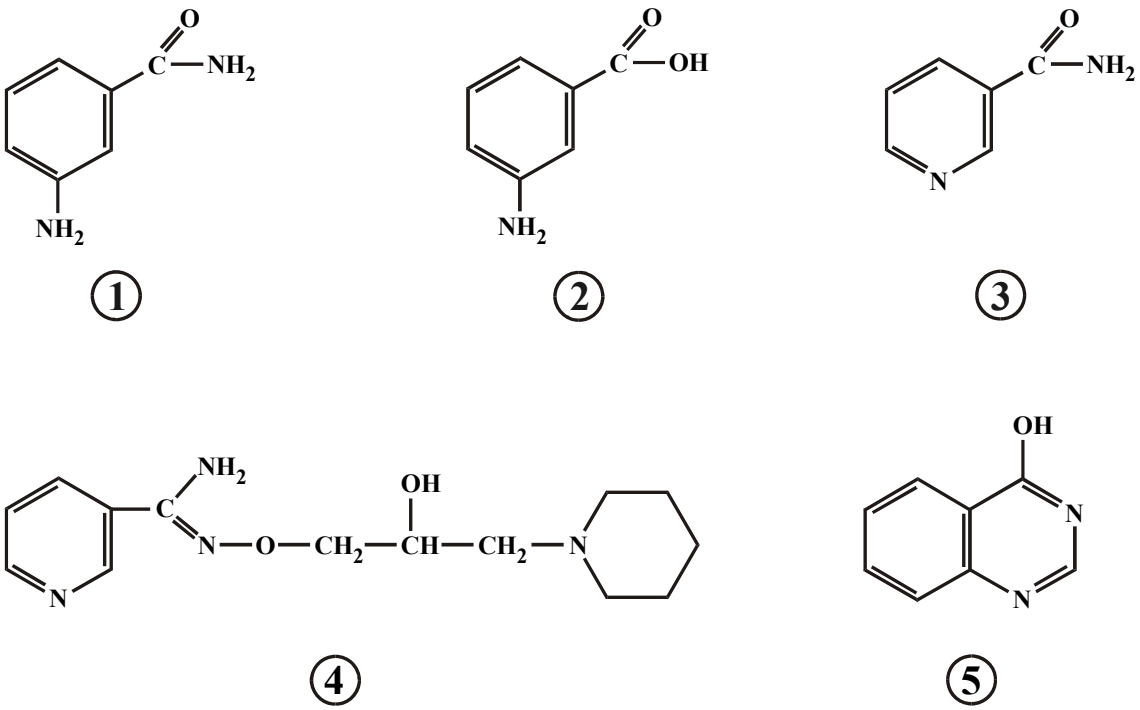


Fig. 2.1. Chemical structure of the studied PARP inhibitors and 3-aminobenzoic acid.
1., 3-aminobenzamide; 2., 3-aminobenzoic acid; 3., nicotinamide; 4., BGP-15; 5., 4-hydroxyquinazoline.

2.5. Protection against reperfusion injury using scavenger molecules.

Oxygen free radical damage has been implicated in a wide range of diseases. Therefore, scavenging these radicals should be considered as a basically important therapeutic approach. Antioxidant molecules and enzymes can potentially limit the oxidative injury but they are not readily internalized within myocardial cells, or they can not reach the right cell compartment to exert their protective effect [52-53].

Consequently, there is a long-standing effort to design small, non-toxic molecules which can reach the right cell compartments and exhibit marked antioxidant properties against various types of oxidative injury [54].

Small molecular weight, stable nitroxides have been shown to have potential therapeutic values in a variety of diseases, including myocardial reperfusion injury and doxorubicine-induced cardiotoxicity. Protective effect of sterically hindered nitroxides have been attributed to antioxidative processes including: 1) superoxide dismutase-mimicking activity, 2) induction of catalase-like activity in hemeproteins, 3) oxidation of reduced transition metal ions, and 4) radical scavenging [55-58]. In addition, nitroxides are cell permeable, making it possible to provide both intracellular and extracellular protection against oxidative stress. It is important because ROS are highly reactive compounds with very short half-life time, so antioxidants must be at the right place at the time of the radical formation. In biological tissues, nitroxides are reduced to hydroxylamine form, and these two forms of nitroxide coexist [59].

Class I antiarrhythmic drugs are accumulating in the membrane of cardiomyocytes providing an ideal transport molecule for sterically hindered nitroxides and can improve their protective effect against myocardial oxidative stress. This group of drugs accumulates in heart membranes and can be characterized by the presence of three structural units: (a) an aromatic ring capable of intercalating between the alkyl chains of phospholipids, (b) an amino group undergoing ionization in the biological system at pH 8-9, and (c) an interconnecting chain (between the aromatic ring and the amino group) [60].

A group of pyrroline-based compounds has been shown to have a class I antiarrhythmic activity [61]. The amino compound *N*-3-(2,2,5,5-tetramethyl-3-pyrroline-3-carboxamido)propylphthalimide (H-2545) is oxidized to the hydroxylamine and nitroxide form in mice [62]. Figure 2.2 shows the metabolism of H-2545 caused by mixed function amine oxidases or ROS. H-2545, an amine compound can be oxidized to form a stable nitroxide radical. This radical can be reduced by ascorbic acid to the *N*-hydroxyl form, which undergoes reversible one-electron oxidation to an *N*-oxyl compound. The *N*-oxyl molecule is sufficiently reactive to scavenge another ROS by transferring its hydrogen atom [62].

H-2545 has low toxicity, its therapeutical index is 8.8 in animals [61]. Previous data showed that H-2545 could suppress the coronary-ligation-, digitalis- and

adrenaline-induced arrhythmias due to its effect on ion channels: H-2545 is a strong sodium channel blocker compound with a weak potassium and calcium channel blocking effect [63].

Furthermore, in 50-μM concentration - which is approximately 10 times higher than its maximal therapeutic serum concentration - H-2545 improved efficiently the functional recovery of Langendorff perfused rat hearts [64].

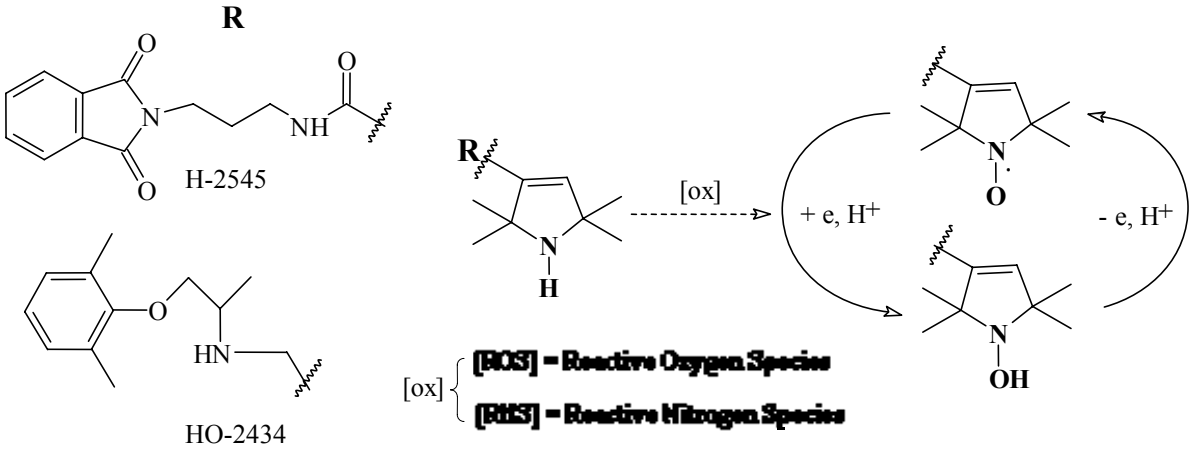


Fig. 2.2. Structure of 2,2,5,5-tetramethylpyrrolin-3-yl drugs. In biological systems sterically hindered amine compounds are oxidized to nitroxides, which undergo reversible one-electron reduction to *N*-hydroxyl compounds. These metabolites behave as antioxidants reducing toxic ROS.

3. OBJECTIVES OF THE STUDY

3.1. Antioxidant compounds

In our work we aimed to clarify what the lowest concentration is at which H-2545 can offer a significant protection against oxidative myocardial injury. Previous data were obtained from experiments using H-2545 in high (50 μM) concentration in animals.

We have supposed that H-2545 is a superior scavenger molecule in myocardium compared to natural antioxidants because it accumulates in heart membranes and during its metabolism yields other molecules with marked antioxidant property. In addition, nitroxides, such as H-2545, have multiple action against oxidative stress. Therefore, we compared the cardioprotective effect of H-2545 to the well-known antioxidant Trolox.

In various forms of oxidative stress the source of ROS is different. During ischemia-reperfusion ROS are dominantly derived from the mitochondrial respiratory chain. In the case of hydrogen peroxide or other externally administered oxidants there are at least two components. They also enhance the mitochondrial ROS formation but their damaging effect is mainly due to their reaction with transition metals (e.g. Fe^{2+}) initiating a site-specific oxyradical formation. The effectiveness of H-2545 and its metabolite was examined both in ischemia-reperfusion and in hydrogen peroxide-induced oxidative stress.

We also strived for clarifying whether the cardioprotective effect of H-2545 can be transferred to another drug accumulating in membranes (mexiletine), substituting it with a sterically hindered amine compound.

3.2. PARP inhibitors

It was reported previously that BGP-15, a new PARP inhibitor decreased the myocardial oxidative stress during ischemia-reperfusion. We wanted to test whether it was the specific effect of BGP-15 or it was a common feature of PARP inhibitors.

The diminution of oxidative stress using BGP-15 was not due to a free radical scavenging effect. These data raise the possibility that PARP inhibitors may interfere with endogenous mitochondrial ROS formation by a different mechanism than antioxidants. We have compared the protective effect of PARP inhibitors on ischemia-reperfusion and on hydrogen peroxide-induced lipid peroxidation. During reperfusion mitochondrium is the main source of ROS, however, in the case of hydrogen peroxide ROS are produced predominantly in site-specific reactions and just a small proportion of them are derived from the mitochondria.

We aimed to investigate whether the influence of PARP inhibitors on the endogenous mitochondrial ROS formation can be attributed to a direct mitochondrial effect of these compounds, or it is an indirect effect due to altered NAD catabolism and high-energy metabolism. Therefore, protective effect of PARP inhibitors against hydrogen peroxide-induced oxidative injury were studied in isolated mitochondria.

Finally, we intended to examine whether the PARP inhibiting moiety of the compounds, or another common chemical structure is essential for the direct mitochondrial protective effect.

4. MATERIALS AND METHODS

4.1. Chemicals

The synthesis of H-2545 and H-2954 has already been published [61]. These compounds were synthetized at the University of Pecs, Institute of Organic and Medicinal Chemistry. Mexiletine, Trolox, 3-aminobenzamide, 3-aminobenzoic acid, 4-hydroxyquinazoline, nicotinamide, H_2O_2, NAD^+ and dihydrorhodamine123 (DHR) were purchased from Sigma - Aldrich Chemical Co. (Budapest, Hungary). Malondialdehyde-bis (diethylacetal) was obtained from Merck (Darmstadt, Germany). BGP-15 was a gift from N-Gene Research Laboratories, Inc. (Budapest, Hungary). All other reagents were of the highest purity commercially available.

4.2. Animals

The hearts of adult male Wistar rats weighing 300-350 g were used for the Langendorff heart perfusion experiments. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication NO. 85-23, revised 1996), and approved by the Animal Research Review Committee of the University of Pecs Medical School.

4.3. Heart perfusion

Rats were anesthetized with ketamine, 200 mg/kg 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^{o}C. The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 0.6 mM octanoic acid. In treated groups, perfusion medium additionally contained antioxidants (H-2545, H-2954, mexiletine, HO-2434 or HO-2433) in 10 μM concentration or PARP inhibitors (3 mM nicotinamide, 3 mM 3-aminobenzamide, 100 μM 4-hydroxyquinazoline or 40 mg/l = 113,9 μM BGP-15) or 3-aminobenzoic acid (3 mM). The perfusate was adjusted to pH 7.40 and bubbled with 95% O_2/ 5% CO_2 through a glass oxygenator.

4.3.1. Ischemia-reperfusion

After a washout, non-recirculating period of 15 minutes, hearts were either perfused under normoxic conditions for the given time, or were subjected to a global ischemia of 45 or 25 minutes by closing the aortic influx and reperfused for 15 or 30 minutes. During ischemia hearts were submerged into perfusion buffer at 37^{o}C. At the end of perfusion hearts were freeze clamped.

4.3.2. Perfusion with hydrogen peroxide

Hearts were perfused after the washout period with or without 0.5 mM hydrogen peroxide and, in treated groups, in the presence of a scavenger or a PARP inhibitor compounds for 30 min.

4.4. Determination of heart function

A latex balloon was inserted into the left ventricle through the mitral valve and filled to achieve an end-diastolic pressure of 8-12 mmHg. All measurements were performed at the same balloon volume. Hearts were selected on the basis of the stability of high-energy phosphates (assessed by NMR) during a control period of 15 min before the experiment. The length of normoxia, ischemia and reperfusion were 15, 25, 45 min, respectively. Experimental drugs were added to the perfusion medium after the 15 min of control period. Functional data of rat hearts (LVDP - left ventricle developed pressure, RPP - rate-pressure product, HR – heart rate, and dP/dt) were monitored during the perfusion [65].

4.5. Assessment of cell membrane integrity

Release of lactate dehydrogenase EC 1.1.1.27 (LDH), creatine kinase EC 2.7.3.2 (CK) and aspartate aminotransferase EC 2.6.1.1 (AST) enzymes were measured in the perfusate of Langendorff perfused hearts under normoxic and postischemic conditions. Enzyme activities were measured by standard methods as described in [66] for LDH, [67] for AST and [68] for CK.

4.6. Lipid peroxidation

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of a described method [69]. 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 absorbency of the supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. Using MDA standard TBARS were calculated as nmoles/g wet tissue.

4.7. Determination of protein carbonyl content

Fifty mg of freeze-clamped perfused 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 the 2,4-dinitrophenylhydrazine-method [70].

4.8. Determination of DNA single-strand breaks

Single-strand DNA breaks were determined by the alkaline fluorescence analysis of DNA unwinding as described before [71]. DNA samples were prepared from normoxic and from reperfused hearts. To estimate the quantity of undamaged double stranded DNA, samples were divided into 3 sets of tubes. DNA fluorescence was determined under different conditions. To determine F value, DNA was kept at pH 12.4 to permit partial unwinding of DNA. To determine F_{min}, DNA was kept at pH 12.4, but at the beginning of the incubation period DNA sample was sonicated for 60 sec. To determine F_{max}, DNA sample was kept at pH 11.0, which is below the pH needed to induce unwinding. Solutions were incubated for 30 minutes at $0^{o}C$ followed by 15 minutes of incubation at $15^{o}C$. Unwinding was stopped by adjusting the pH to 11.0. Fluorescence was measured after the addition of the dye ethidium bromide 0.67 µg/ml with an excitation wavelength of 520 nm and an emission wavelength of 590 nm on a Perkin Elmer luminescence spectrometer. Results are expressed as D (percent of double stranded DNA) = $(F-F_{min})/(F_{max}-F_{min})$ X 100.

4.9. Assay of NAD^+

The concentration of NAD^+ in the neutralized perchloric acid extract of the cardiac muscle was measured by using alcohol dehydrogenase reaction [72]. The freshly prepared reaction buffer contained 0.1M Tris, pH 8.40, 1 mM EDTA, 4 mM L-cysteine chloride, 2% ethanol. Each cuvette contained 300 µl of the tissue extract, 650 µl of the reaction buffer and 4 units enzyme. The reaction was initiated by the addition of the enzyme and the exact tissue NAD^+ concentrations were determined from a

calibration curve.

4.10. Direct scavenger effect of antioxidant compounds

Reactive oxygen species formation was detected using the oxidation-sensitive non-fluorescent probe dihydrorhodamine 123 which can be oxidized by ROS to fluorescent rhodamine 123 [73]. Rhodamine 123 content was determined in the suspension of mitochondrial inner membrane by a Perkin Elmer fluorescence spectroscope at an excitation wavelength of 496 nm and an emission wavelength of 536 nm. Mitochondrial inner membrane was suspended in 2 ml 20 mM Tris buffer pH 7.40 containing 150 mM KCl, 2 mM succinate, 8 μM antimycine A, 1 μM dihydrorhodamine 123 and, in some experiment, 1 mM sodium nitroprusside. H-2545 and H-2954 were added to this system in the concentrations indicated in Table 4.

Antioxidant effect of H-2545 and H-2954 were also studied in an another system where hydroxyl radicals were generated in Fenton reaction and the hydroxylation of benzoic acid was used to follow the hydroxyl radical reaction fluorimetrically. The reaction took place in 20 mM sodium phosphate, pH 6.8, containing 100 μM benzoic acid, 100 μM H_2O_2, 10 μM Fe^{2+}-EDTA. Fluorescence was detected at an excitation wavelength of 305 nm and an emission wavelength of 407 nm.

4.11. Determination of mitochondrial ROS production

Mitochondria were incubated in a buffer containing 150 mM KCl, 1 mM EDTA, 5 mM MOPS, 1 mM succinate at pH 7.4 and in the presence of PARP inhibitors and 3-amino-benzoic acid (0.2, 1, 3 mM). Mitochondrial suspension was stirred and after the addition of hydrogen peroxide (final concentration 1 mM), ROS production was continuously monitored by following the oxidation of DHR to rhodamine123 in a Perkin Elmer fluorescence spectroscope at an excitation wavelength of 496 nm and an emission wavelength of 536 nm [74].

4.12. Isolation of mitochondria and mitochondrial inner membrane

Mitochondria and inner mitochondrial membrane vesicles were isolated from sacrificed or perfused rat hearts as described before [75] and were stored in 5 mM MOPS pH 7.4, 150 mM KCl and 1 mM EDTA.

4.13. Measurement of mitochondrial enzyme activity

Citrate synthase [76], NADH: cytochrome c oxidoreductase and cytochrome oxidase [77] activities were measured as described previously.

4.14. NMR spectroscopy

NMR spectra were recorded with a Varian UNITY^{INOVA} 400 WB instrument (Varian Inc., Palo Alto, CA, USA).

^{31}P measurements (161.90 MHz) of perfused hearts were run at 37°C in a Z•SPEC® 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 1H signal (w1/2=10-15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each FID. 45° flip angle pulses were employed after a 1.25 s recycle delay, and transients were acquired over a 10 kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16K.

Under the above circumstances, the relative concentrations of the species can be taken proportional to the peak areas, since interpulse delays exceeded 4-5×T1 values of the metabolites to be analyzed in ^{31}P experiments [78].

4.15. Statistical analysis

Statistical analysis was performed by ANOVA and all of the data were expressed as the mean ± S.E.M. Significant differences were evaluated by use of unpaired Student`s t test and *P* values below 0.05 were considered to be significant.

5. 2,2,5,5-TETRAMETHYLPYRROLINE-BASED ANTIARRHYTHMIC COMPOUNDS IN THE PREVENTION OF OXYRADICAL-INDUCED MYOCARDIAL DAMAGE

5.1. RESULTS

5.1.1. Assessment of cell membrane integrity

Membrane damage resulting from ischemia-reperfusion causes enzyme release from cardiomyocytes. The activity of CK in the coronary effluent is extremely low during the normoxic perfusion of rat hearts, indicating that cell membranes are undamaged under these conditions (Fig. 5.1.). Ischemia-reoxygenation caused a significant release of this enzyme into the perfusate.

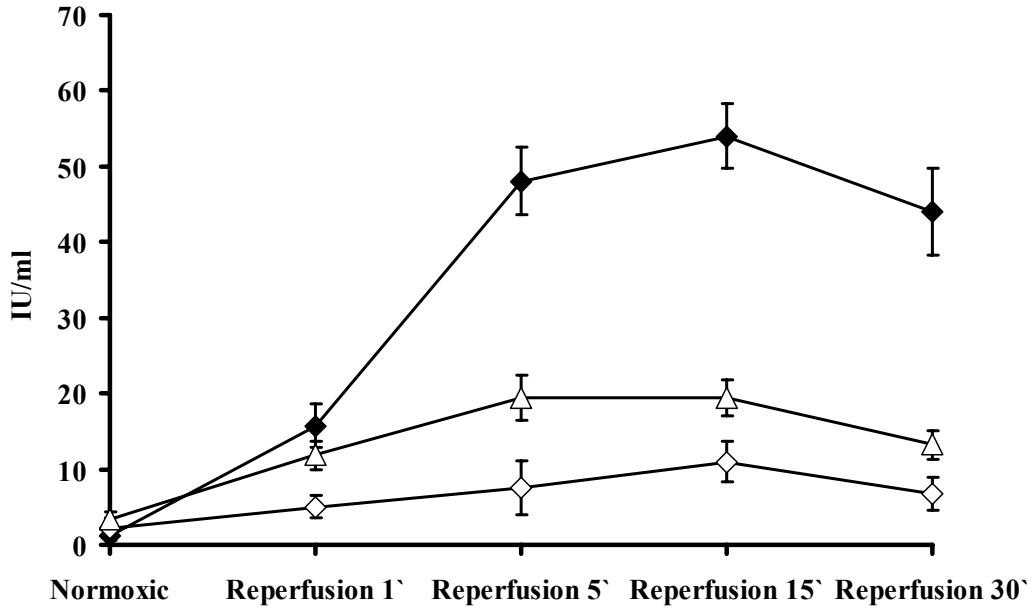


Fig. 5.1. Time-dependence of CK release from myocytes during ischemia-reperfusion. The activity of CK in the coronary effluent was measured as described under „Materials and Methods" at the end of the normoxic perfusion and during 30 min reperfusion. Ischemia-reperfusion (♦); ischemia-reperfusion in the presence of 10 μM H-2545 (◊); ischemia-reperfusion in the presence of 10 μM H-2954 (Δ). Values are mean ± SEM for five experiments.

When the hearts were perfused with 10 μM H-2545 or H-2954 15 minutes prior to ischemia, during the 30 min of reperfusion a significantly reduced ($p<0.001$) release of CK was seen in the perfusate of postischemic rat hearts. Our results showed that H-

2545 provided a significantly better ($p<0.01$) protection against ischemia-reperfusion-induced damage of plasma membranes compared to the metabolite (H-2954) (Fig. 5.1.). A similar effect could be detected measuring the activity of AST and LDH in the coronary effluent (Table 5.1.).

Table 5.1. Effect of H-2545 and H-2954 on intracellular enzyme release during ischemia-reoxygenation in Langendorff perfused rat hearts.
Membrane integrity was followed by enzyme release from cardiomyocytes into the perfusate during heart perfusion. Normoxic values were measured at the end of the normoxic perfusion and postischemic values after 45 minutes ischemia and 15 minutes reperfusion. For details see Materials and Methods. Values are means±SEM for five heart preparations.

	CK	AST	LDH
		IU/ml	
Normoxic	2.4±1.1*	2.7±1.3*	9.7±2.6*
Ischemia-reoxygenation (IR)	52.6±7.3	118.2±7.1	463.5±25.4
IR + 10 μM H-2545	7.6±3.2*	29.3±4.6*	99.6±21.9*
IR + 10 μM H-2954	21.8±4.2*‡	73.8±4.4*‡	187.3±19.6*‡

* Values are different from the respective ischemia-reoxygenation values at the significance of $p<0.01$.
‡ Values are different from the respective IR + H-2545 values at the significance of $p<0.01$.

5.1.2. Ischemia-reperfusion-induced lipid peroxidation and protein oxidation

Lipid peroxidation induced by ischemia-reperfusion in Langendorff perfused heart was characterized by the formation of TBA reactive substances. Under our experimental conditions, ischemia-reperfusion increased the amount of TBARS compared to the normoxic hearts ($p<0.01$) (Table 5.2.). When ischemia-reperfusion occurred in the presence of 10 μM H-2545 or H-2954, the formation of TBARS was significantly decreased ($p<0.01$) compared to the ischemia-reperfusion hearts (Table 5.2.), indicating that both drugs prevented the ischemia-reperfusion-induced lipid peroxidation.

ROS formation in ischemia-reperfusion cycle can also induce the oxidation of proteins in the cardiomyocytes, which can be characterized by the quantity of protein bound aldehyde groups. Table 5.2. shows that ischemia-reperfusion increased significantly the quantity of protein bound aldehyde groups ($p<0.01$). However, the presence of H-2545 or H-2954 during ischemia-reperfusion cycle attenuated significantly ($p<0.01$) the increase of the quantity of protein bound aldehyde groups.

Table 5.2. Effect of H-2545 and H-2954 on the lipid peroxidation, protein oxidation and single-strand DNA break formation during ischemia-reperfusion cycle in Langendorff perfused rat hearts.
Lipid peroxidation was estimated by the formation of TBARS. Protein oxidation was measured as the protein carbonyl content as described in "Materials and Methods". DNA single strand breaks were determined by the alkali unwinding assay. Values are means±SEM for five heart preparations.

	TBARS	Protein oxidation	Non-damaged DNA
	nmol/g wt.	μmol/g wt.	%
Normoxic	39.78±1.59*	1.47±0.08*	87.2±3.3*
Ischemia-reperfusion (IR)	70.69±2.86	2.47±0.14	29.6±3.7
IR + 10 μM H-2545	46.21±3.31*	1.69±0.05*	83.5±1.9*
IR + 10 μM H-2545	49.53±2.01*	1.85±0.08*‡	74.1±3.8*

* Values are different from the respective ischemia-reperfusion values at the significance of $p<0.01$.
‡ Value different from the respective IR + H-2545 value at the significance of $p<0.05$.

5.1.3. Determination of single-strand DNA break formation and NAD^+ catabolism in the postischemic myocardium

Ischemia-reperfusion increased ROS formation in perfused hearts, which can contribute to the formation of single-strand DNA breaks. Under normoxic conditions, most of the DNA was undamaged but ischemia-reperfusion-induced large amounts of single-strand DNA breaks and the quantity of undamaged DNA decreased to under 30% (Table 5.2.). In the presence of H-2545 or H-2954, ischemia-reperfusion increased only slightly the amount of ssDNA breaks and the amount of undamaged DNA were significantly ($p<0.01$) higher than in postischemic hearts and not significantly different from the normoxic values (Table 5.2.).

It is well known that ssDNA breaks activate PARP, which stimulates intracellular NAD^+ catabolism, therefore, it is expectable that ischemia-reperfusion cycle decreased NAD^+ content of perfused hearts. Figure 5.2. shows that ischemia-reperfusion significantly ($p<0.01$) decreased the NAD^+ content of the hearts. The presence of H-2545 or H-2954 (10 μM) in the perfusate during ischemia-reperfusion cycle significantly ($p<0.01$) protected the loss of NAD^+ in postischemic hearts. H-2545 prevented more effectively the NAD^+ loss in the postischemic myocardium than its metabolite ($p<0.05$) (Fig. 5.2.).

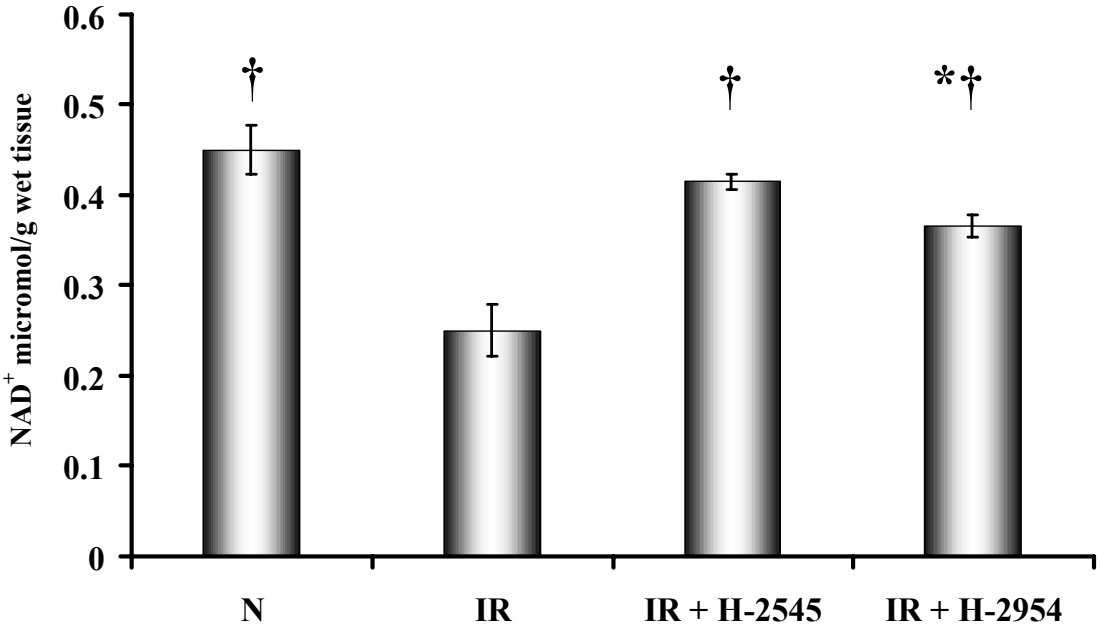


Fig. 5.2. Ischemia-reperfusion-induced NAD^+ loss in Langendorff perfused rat hearts. The NAD^+ content using an alcohol dehydrogenase method was measured as detailed under „Materials and Methods" after 15 min of reperfusion. N, normoxic; IR, ischemia-reperfusion; IR + H-2545, ischemia-reperfusion in the presence of 10 μM H-2545, IR + H-2954, ischemia-reperfusion in the presence of 10 μM H-2954. Values are mean ± SEM for five experiments.
Difference from ischemia-reperfusion: † $p<0.01$. Difference from IR + H-2545: * $p<0.05$.

5.1.4. Effect of H-2545 and H-2954 on the energy metabolism of perfused rat hearts during ischemia-reperfusion

Energy metabolism of Langendorff perfused hearts were monitored in the magnet of NMR spectroscope making possible to detect changes in high-energy phosphorous intermediates (Fig. 5.3.). Ischemia induced a rapid decrease in ATP and creatine phosphate levels and a fast evolution of inorganic phosphate. Under our experimental conditions, high-energy phosphate intermediates recovered only partially in 15 minutes reperfusion phase and H-2545 facilitated the recovery of ATP and creatine phosphate (Fig. 5.3.).

In Figure 5.4, the time dependence of ATP, creatine phosphate and inorganic phosphate levels is shown during ischemia-reperfusion cycle using H-2545 or H-2954 (10 μM). These data showed that both compounds improved significantly ($p<0.01$) the final recovery of high-energy phosphate intermediates and the rate of recovery was increased in the presence of them (Fig. 5.4A and 5.4B). However, H-2545 caused a significantly ($p<0.01$) better recovery than its metabolite. In accord with the high-energy phosphate data, inorganic phosphate levels are decreased in the reperfusion phase (Fig. 5.4C) and

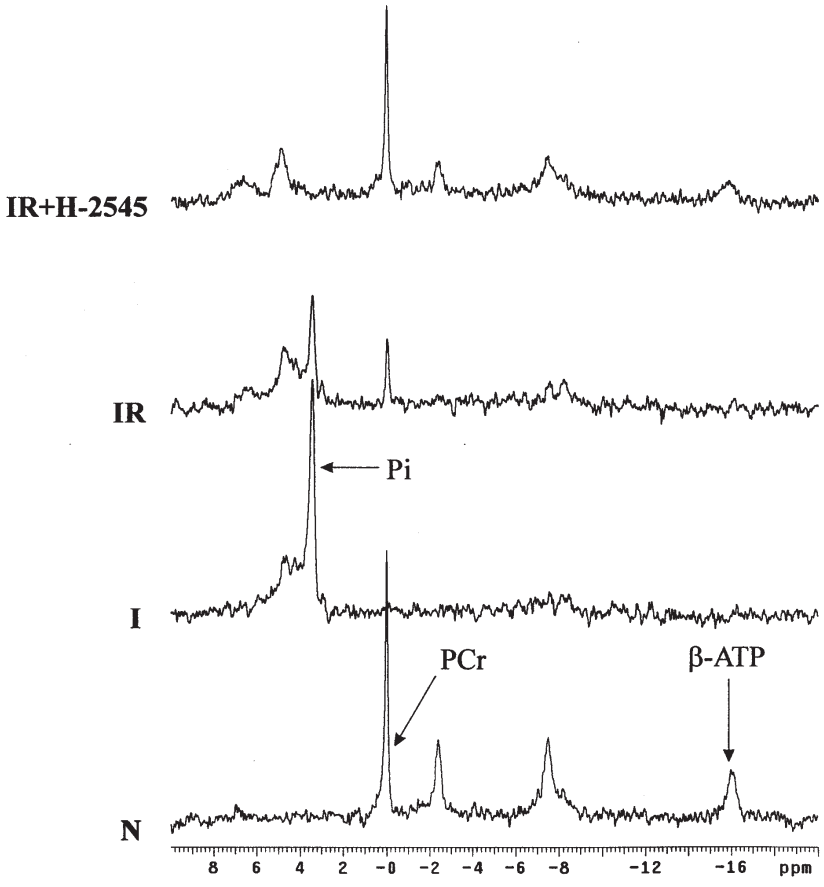
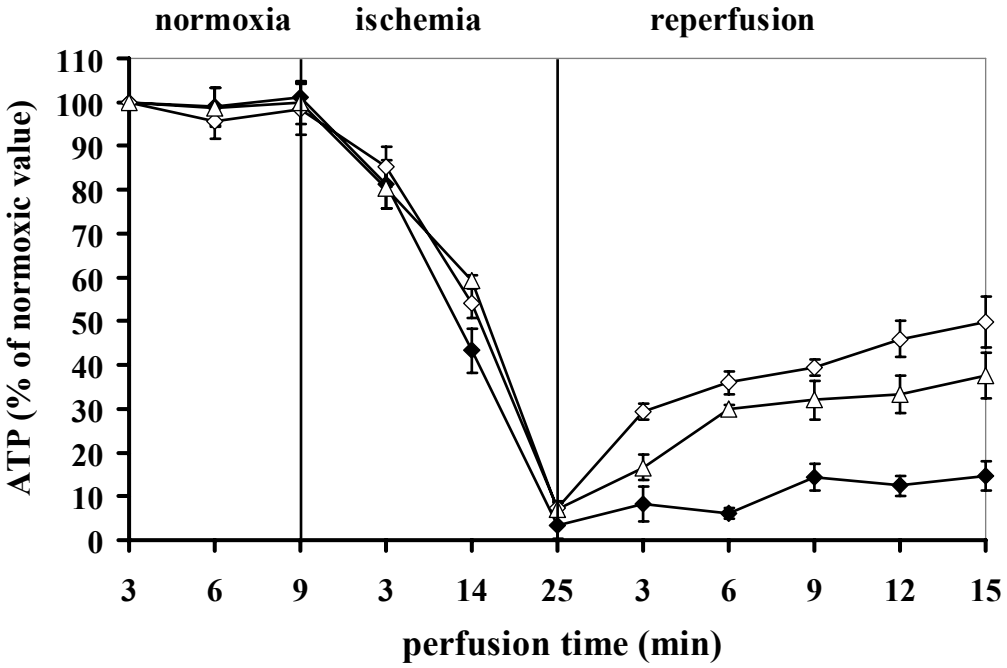


Fig. 5.3. Effect of H-2545 on the recovery of myocardial energy production after ischemia-reperfusion in Langendorff perfused heart. Conditions for heart perfusion and NMR measurements were described under "Materials and Methods". N, normoxic; I, ischemic; IR, ischemia-reperfusion; IR + H-2545, ischemia-reperfusion in the presence of 10 μM H-2545.

both compounds increased the rate of inorganic phosphate utilization.

The intracellular pH fell rapidly during ischemia from 7.41±0.04 to 5.82±0.07. In the control group during reperfusion just a slight, but statistically significant ($p<0.05$) increase could be observed to 5.95±0.07. In the presence of H-2545, pH value in the myocardium increased markedly ($p<0.01$) up to 6.67±0.06. H-2954 caused a significantly less ($p<0.01$) increase in myocardial pH value than H-2545, up to 6.19±0.06. (Fig. 5.4D)

Fig. 5.4A and 5.4B



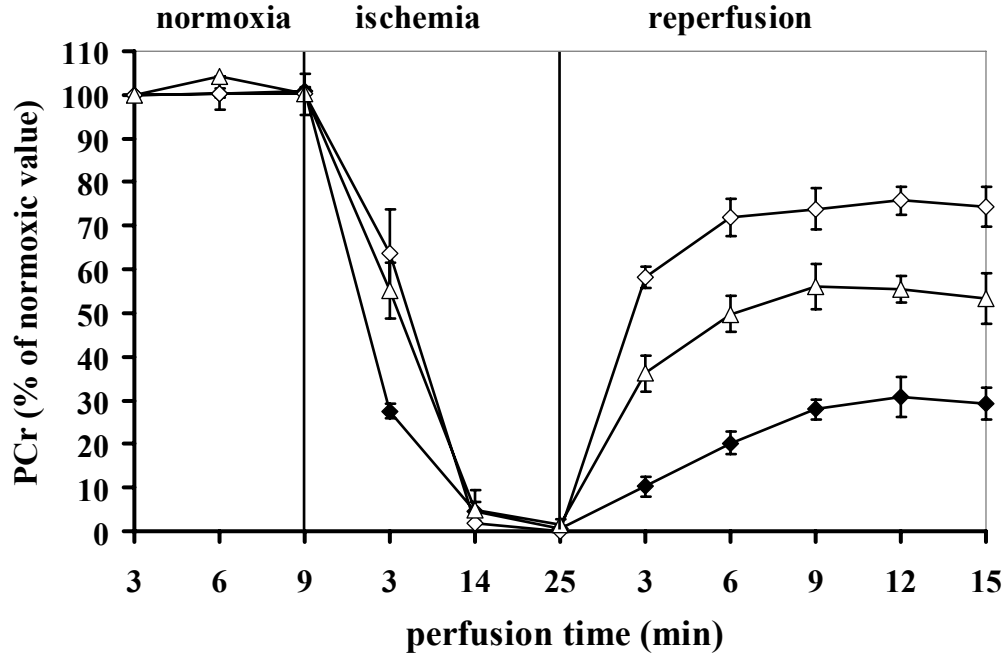


Fig. 5.4C and 5.4D

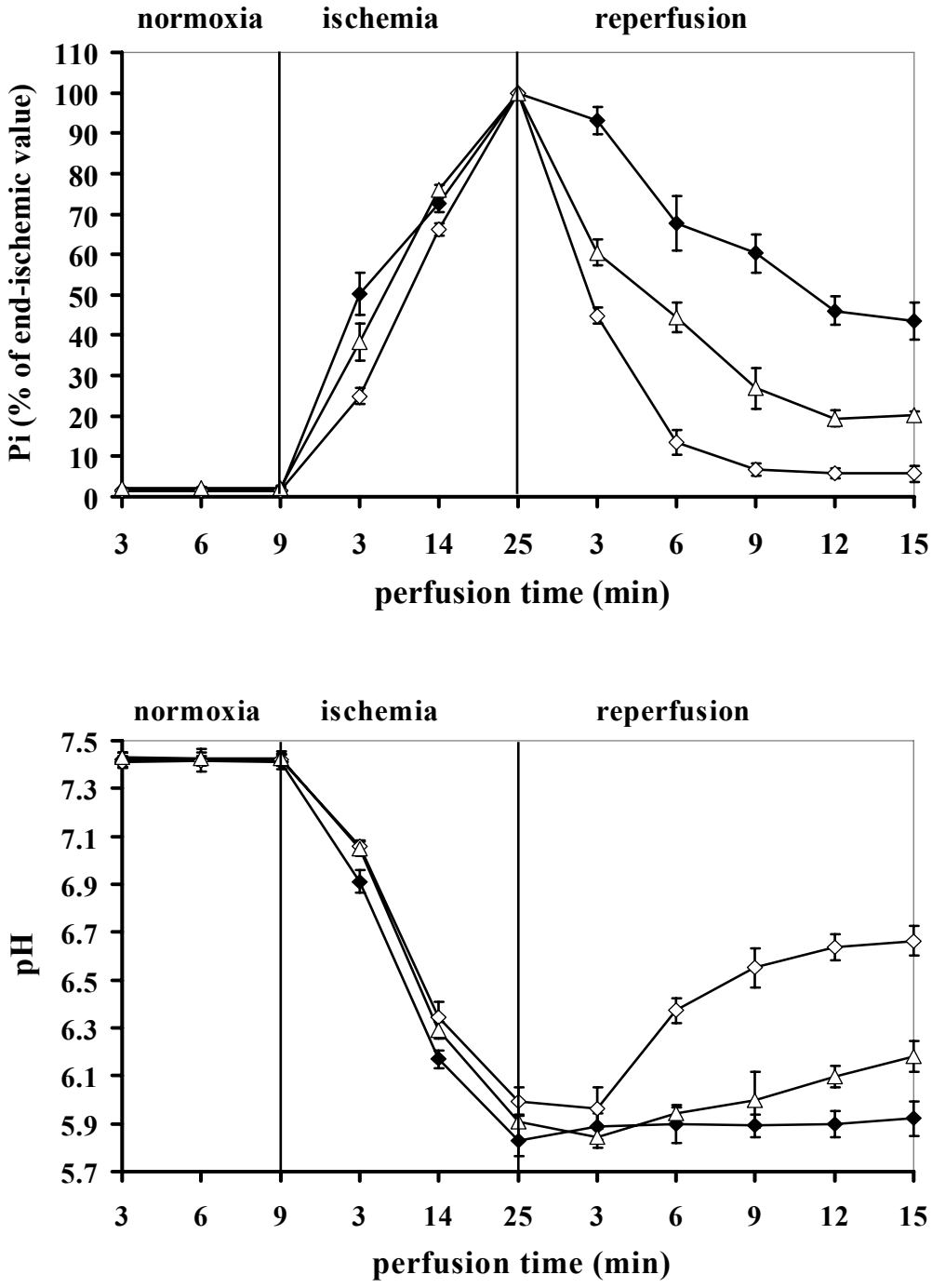


Fig. 5.4. Effect of H-2545 and its metabolite on the recovery of ATP (A), creatine phosphate (B), inorganic phosphate (C) and myocardial pH value (D) in Langendorff perfused hearts. Conditions for heart perfusion and NMR measurements were described under "Materials and Methods". Ischemia-reperfusion (♦); ischemia-reperfusion in the presence of 10 μM H-2545 (◊); ischemia-reperfusion in the presence of 10 μM H-2954 (Δ). Values are given as mean ± SEM for five experiments.

5.1.5. Functional recovery of postischemic rat hearts during reperfusion

To evaluate the effect of H-2545 and its oxidation metabolite on the postischemic functional recovery of the myocardium, isolated hearts were perfused with 10 μM concentration of both compounds. At the end of the equilibration period, left ventricle developed pressure (LVDP) was 149 ± 22 mmHg, rate-pressure product (RPP) was 3.4 ± 0.1 x 10^4 mmHg/min, dP/dt was 1258 ± 213 mmHg/s and the average heart rate was 227 ± 12 beats/min. Figure 6 shows the recovery of LVDP, RPP and dP/dt during reperfusion. Percentage recovery of LVDP in hearts treated with H-2545 and H-2954 was significantly higher ($p<0.01$) than that of controls. At the end of the 45-min reperfusion, LVDP of hearts perfused with H-2954 showed a 43.9 ± 2.4 % recovery, compared to 26.5 % of controls. The recovery of H-2545 treated hearts was even higher (58.5 ± 3.3 %; $p<0.01$) than that of its oxidative metabolite. Similar recoveries were seen with respect to RPP values. The RPP values at the end of reperfusion were for controls, H-2545 and H-2954 treated hearts: 12.4 ± 3.2, 44.8 ± 3.1, 22.4 ± 2.1 %, respectively. Recovery of dP/dt, expressed as a percentage of the corresponding preischemic value were 14.7 ± 2.9, 53.5 ± 3.4, and 35.4 ± 3.1 % for controls, H-2545 and H-2954, respectively (Fig. 5.5).

Fig. 5.5A

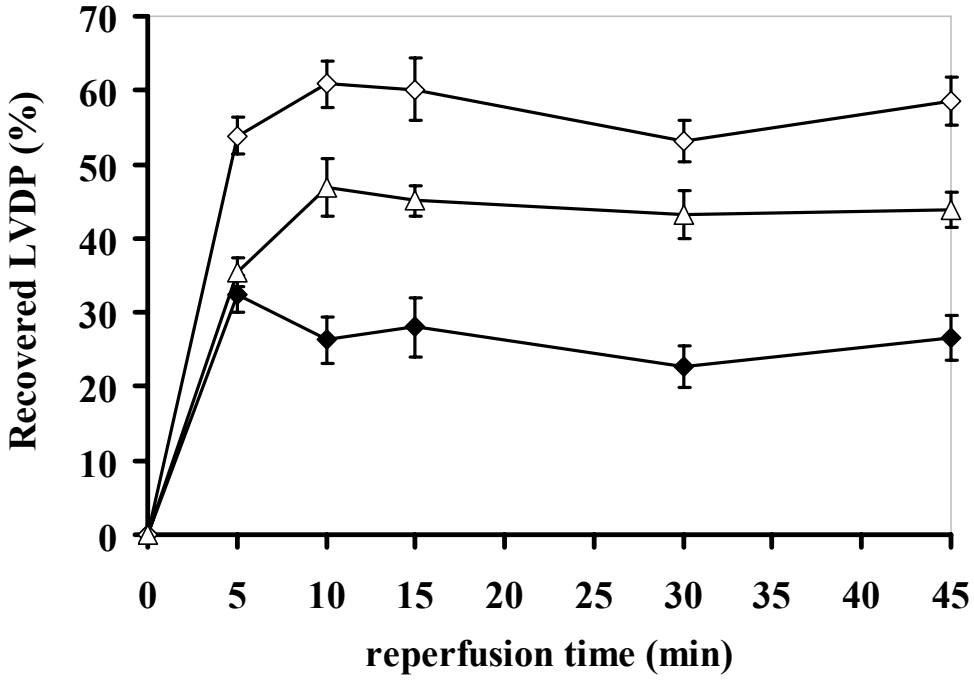


Fig. 5.5B and 5.5C

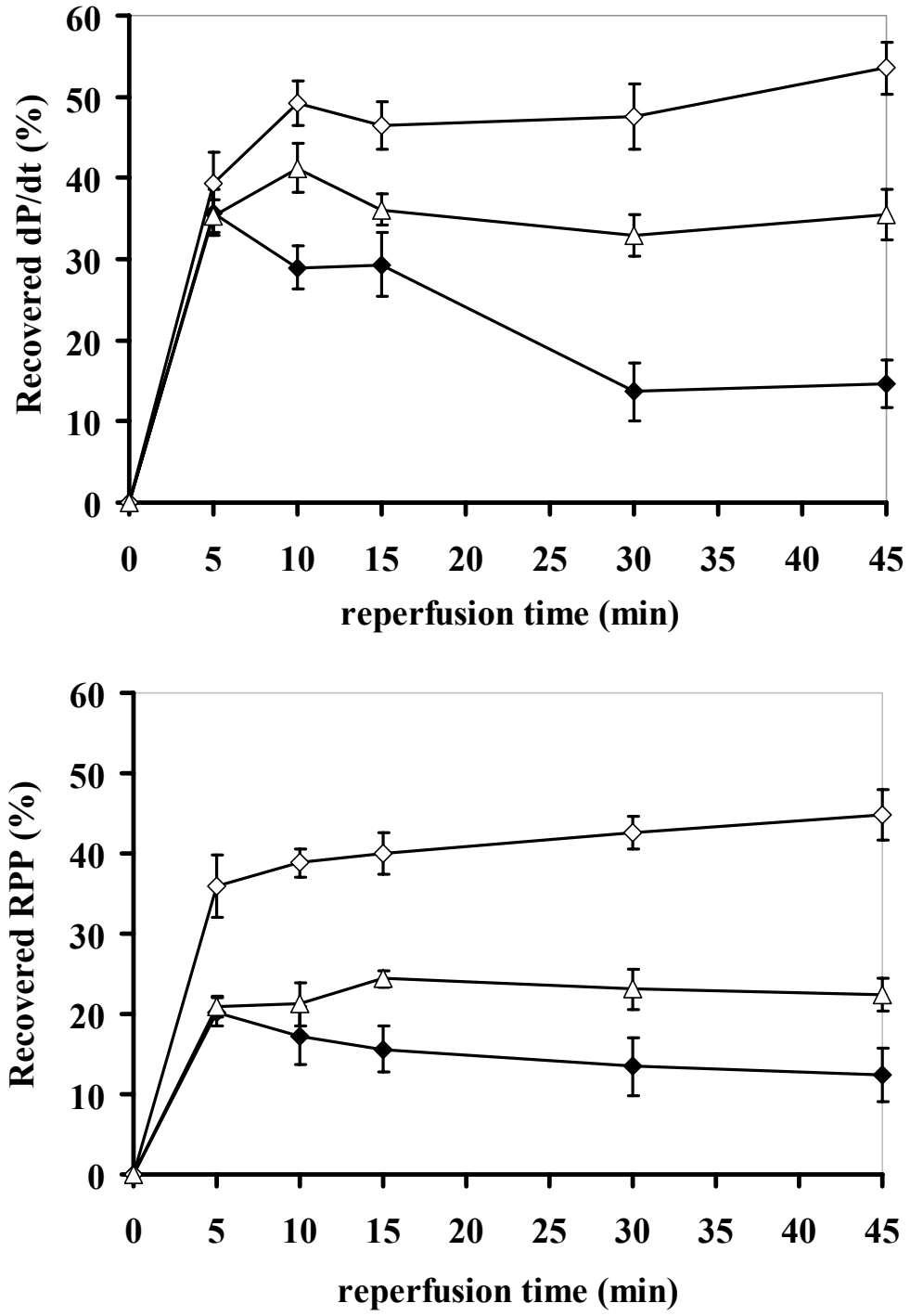


Fig. 5.5. Functional recovery of Langendorff perfused rat hearts during reperfusion. Perfused rat hearts were subjected to 25 min of global no-flow ischemia, followed by a 45-min reperfusion. Treated hearts were perfused with 10 μM H-2545, H-2954 prior to ischemia. During reperfusion LVDP (Panel A), dP/dt (Panel B) and RPP (Panel C) were monitored and expressed as a percentage of the corresponding baseline values. Ischemia-reperfusion (♦); ischemia-reperfusion in the presence of 10 μM H-2545 (◊); ischemia-reperfusion in the presence of 10 μM H-2954 (Δ). Values are mean ± SEM for five experiments.

Fig. 5.6A and 5.6B

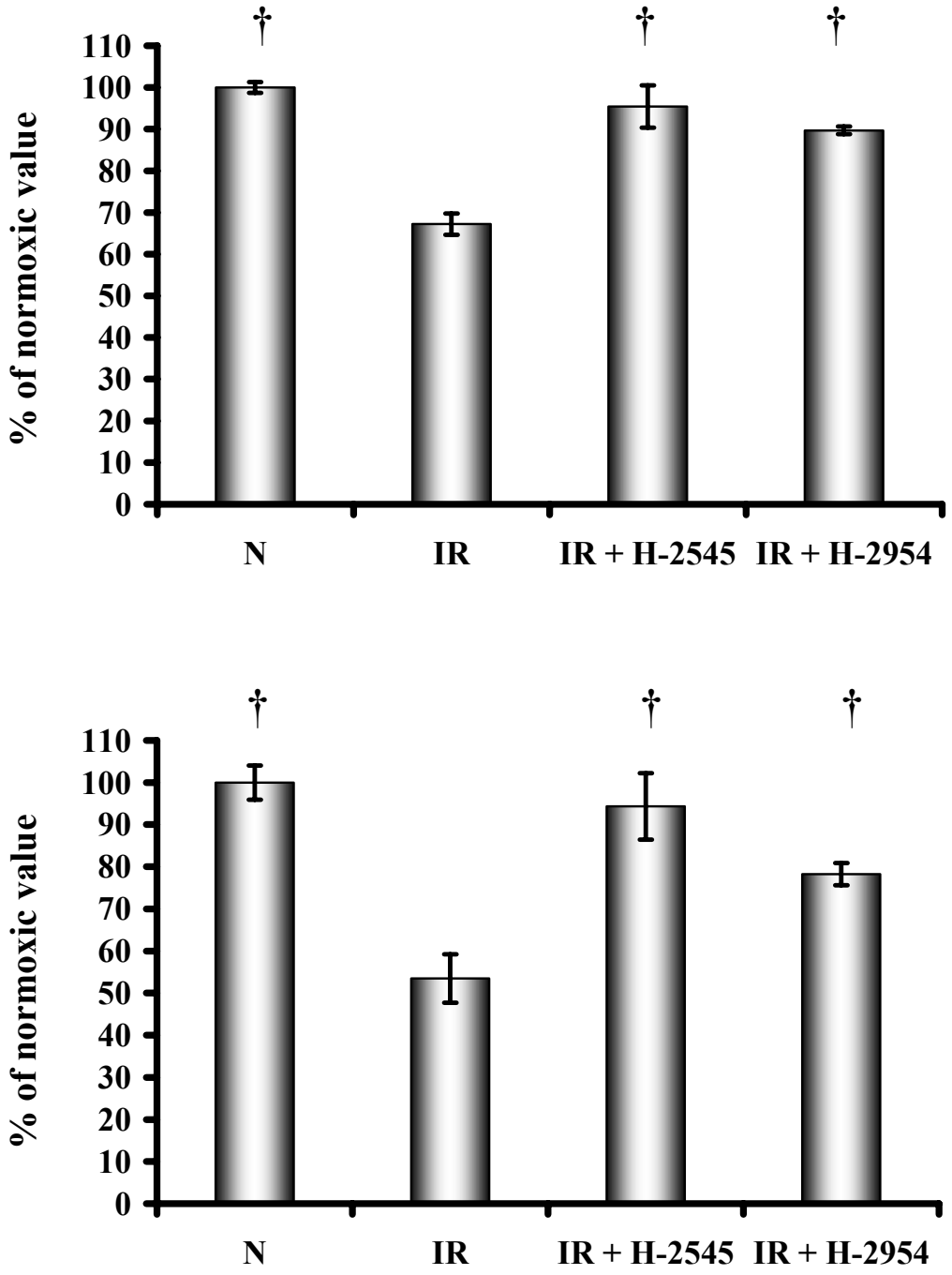


Fig. 5.6. Effect of H-2545 and H-2954 on the ischemia-reperfusion-induced inactivation of respiratory complexes. Conditions for heart perfusion and the measurement of mitochondrial enzyme activities were described under "Materials and Methods". N, normoxic; IR, ischemia-reperfusion; IR + H-2545, ischemia-reperfusion in the presence of 10 μM H-2545, IR + H-2954, ischemia-reperfusion in the presence of 10 μM H-2954. Panel A: activity of cytochrome oxidase. Panel B: activity of NADH:cytochrome c oxidoreductase. Values are given as mean ± SEM for five experiments. Difference from ischemia-reperfusion: † $p<0.01$.

5.1.6. Protecting effect of H-2545 and H-2954 against the ischemia-reperfusion-induced damage of respiratory complexes

Under our experimental conditions, ischemia-reperfusion caused a partial inactivation of respiratory complexes ($p<0.01$) (Fig. 5.6.) which could be the consequence of mitochondrial ROS formation during reperfusion [20]. Our previous data (Fig. 5.1-5.5. and Table 5.1-5.2.) indicated that both H-2545 and its metabolite decreased the oxidative damage for different components of heart during ischemia-reperfusion cycle, therefore, it is reasonable to assume that these drugs may moderate the inactivation of respiratory complexes. We found that cytochrome oxidase activity was almost completely protected ($p<0.01$) from the ischemia-reperfusion-induced partial inactivation using H-2545 and H-2954 (Fig. 5.6A). Due to ischemia-reperfusion the NADH:cytochrome c oxidoreductase activity (Complex I-III) was also partially inactivated (from the normoxic value of 100±5.2 to 53.47±.7.5 %) and both H-2545 and its metabolite could partially protect ($p<0.01$) NADH:cytochrome c oxidoreductase activity in postischemic heart mitochondria (Fig. 5.6B).

5.1.7. Dose response of H-2545, H-2954 and Trolox on the high-energy phosphate levels in the postischemic myocardium

To compare the protective effect of H-2545 against postischemic myocardial oxidative injury to that of Trolox – a water-soluble vitamin E analogue, dose response of H-2545, H-2954 and Trolox was determined on the high energy phosphate recovery in Langendorff perfused rat hearts (Fig. 8) at the end of the reperfusion period (after 15 minutes). These data showed that both drugs promoted the recovery of creatine phosphate in perfused hearts in a concentration dependent way. The IC_{50} values were 4.5, 38 and 29 μM for H-2545, H-2954 and Trolox, respectively. Recovery of the ATP concentration during reperfusion showed a similar dose response as creatine phosphate (data not shown), and IC_{50} values were 6, 41 and 28 μM for H-2545, H-2954 and Trolox, respectively. Our results indicated that comparing to Trolox, a significantly ($p<0.01$) better high-energy phosphate recovery could be observed in hearts perfused with H-2545. H-2954 and Trolox, in turn, have a similar effectivity in promoting recovery of myocardial energy metabolism in postischemic rat hearts (Fig. 5.7.).

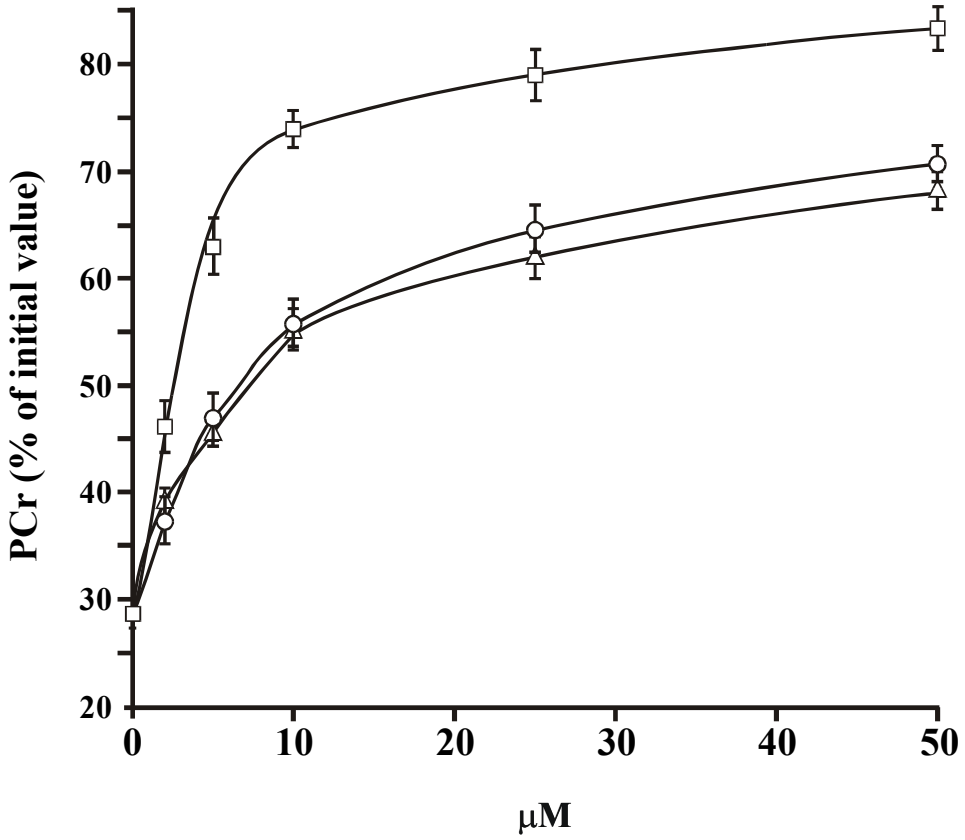


Fig. 5.7. Dose response of H-2545, H-2954 and Trolox on the postischemic creatine phosphate recovery in Langendorff perfused rat hearts.
Conditions for heart perfusion and NMR measurements were described under "Materials and Methods". Hearts treated with H-2545 (□), with H-2954 (Δ) or with Trolox (○). The cardioprotective effect of drugs was tested at 2, 5, 10, 25, 50 μM concentrations. Values are mean ± SEM for five experiments. A significant increase was seen in creatine phosphate level: H-2954 and Trolox, at 2 μM $p<0.05$ and at higher concentration $p<0.01$; H-2545, in all examined concentrations $p<0.01$.

5.1.8. Hydrogen peroxide-induced myocardial lipid peroxidation, protein oxidation and impaired myocardial energy metabolism

The perfusion of rat hearts with a buffer containing 0.5 mM H_2O_2 for 30 min caused a pronounced myocardial injury, characterized by a significant increase in the extent of lipid peroxidation (TBARS) and protein oxidation ($p<0.01$) compared to the control samples (Table 5.3.). The co-administration of H-2954 with H_2O_2 in isolated rat hearts caused a markedly decreased ($p<0.01$) myocardial TBARS formation and a reduced amount of protein bound aldehyde groups ($p<0.01$). Protective effect of H-2545 against hydroperoxide-induced myocardial oxidative injury was even more significant ($p<0.05$) than that of its oxidative metabolites (Table 5.3.). Energy metabolism of hearts perfused with hydrogen peroxide was monitored during a 30-min long perfusion. Creatine

phosphate and ATP content of control hearts did not change significantly by the end of the perfusion compared to the initial values. Under our experimental conditions, concentration of high-energy phosphate intermediates decreased markedly in hearts perfused with hydrogen peroxide ($p<0.01$) (Table 5.3.).

Table 5.3. Effect of H-2545 and its metabolite on the lipid peroxidation, protein oxidation and myocardial high-energy phosphate levels in rat hearts perfused with hydrogen peroxide.
Lipid peroxidation was estimated by the formation of TBARS and protein oxidation was measured as the protein carbonyl content after 30 min of perfusion. Measurements were performed from the freeze-clamped heart samples. ^{31}P-NMR measurements were performed as described in "Materials and Methods". Values were measured before and after the 30 min perfusion with hydrogen peroxide and results were expressed as the percent of the two values. Values are means±SEM for five heart preparations.

	PCr	ATP	TBARS	Protein oxidation
	% of control value		nmol/g wt.	μM/g wt
Control	100±4.6*	100±6.8*	39.7±0.7*	1.47±0.12*
Hydrogen peroxide 0.5 mM (H_2O_2)	23.2±3.7	16.3±5.6	66.5±2.6	3.14±0.11
H_2O_2 + 10 μM H-2545	67.2±5.9*	48.4±6.1*	44.8±1.7*	1.89±0.08*
H_2O_2 + 10 μM H-2954	42.6±5.7*‡	33.2±4.2*‡	46.3±2.4*	2.02±0.09*‡

* Values are different from the respective hydrogen peroxide values at the significance of $p<0.01$.
‡ Values are different from the respective H-2545 values at the significance of $p<0.05$.

H-2545 and in a significantly ($p<0.05$) less manner H-2954 markedly reduced the hydroperoxide-induced fall in the myocardial PCr and ATP content (Table 5.3.).

5.1.9. Direct antioxidant effect of H-2545 and H-2954

Superoxide radicals were produced by the mitochondrial respiratory complexes at Complex III by antimycine A and the oxidation of dihydrorhodamine to fluorescent rhodamine123 by superoxide were studied fluorimetrically. Table 5.4. shows that H-2954 directly react with superoxide radical, even better than H-2545, having IC_{50} = 80 μM. When sodium nitroprusside - an NO donor - was added to the reaction mixture resulting in peroxinitrite formation from NO and superoxide radicals, the oxidation of DHR was increased. In this system H-2954 inhibited the DHR oxidation even better indicating an effective direct reaction (IC_{50} = 17μM) between peroxinitrite and H-2954 (Table 5.4.). Reaction of H-2545 and H-2954 with hydroxyl radicals generated in the Fenton reaction were studied by the benzoic acid hydroxylation assay, showing, that both H-2545 and

H-2954 react effectively with hydroxyl radicals (Table 5.5.). In this system, both H-2545 and H-2954 show similar effectivity to scavenge hydroxyl radicals.

Table 5.4. Effect of H-2545 and H-2954 on the mitochondrial antimycine A-induced ROS production, determined from the oxidation of DHR123 to rhodamine123.
Isolation of mitochondrial inner membrane and measurement of dihydrorhodamine oxidation were performed as described in "Materials and Methods". Values are means±SEM for five experiments.

	Oxidation rate of DHR123 (in % of control)			
	In the absence of nitroprusside		In the presence of nitroprusside	
	H-2954	H-2545	H-2954	H-2545
None	100±2.7	100±3.1	100±4.3	100±2.7
10 μM	84.1±3.2‡	98.2±4.1	61.2±2.8*	96.3±2.9
20 μM	72.1±3.0*	97.7±3.8	57.5±3.1*	97.6±4.2
50 μM	66.8±3.7*	97.4±4.4	29.3±2.6*	95.4±1.9
100 μM	42.2±2.8*	96.9±3.4	16.7±2.9*	96.2±4.5

‡ Values are different from the respective untreated values at the significance of $p<0.05$.
* Values are different from the respective untreated values at the significance of $p<0.01$.

Table 5.5. Effect of H-2545 and H-2954 on the Fenton reaction induced hydroxylation of benzoic acid.
Hydroxylation of benzoic acid occurred in 20 mM sodium phosphate, pH= 6.8 containing 100 μM benzoic acid, 100 μM H_2O_2, 10 μM Fe^{2+}-EDTA. Formation of hydroxy-benzoates was determined fluorimetrically using an excitation wavelength of 305 nm and an emission wavelength of 407 nm. Values are mean ± SEM for five experiments.

	Oxidation rate of benzoic acid (in % of control)	
	H-2545	H-2954
None	100±2.1*	100±2.3*
10 μM	93.6±1.9	96.1±2.1
20 μM	84.8±1.8*	85.2±1.8*
50 μM	49.6±2.9*	50.4±2.7*
100 μM	36.4±2.5*	39.2±2.4*

* Values are different from the respective untreated values at the significance of $p<0.01$.

5.1.10. 2,2,5,5-Tetramethyl-3-pyrroline substituted mexiletines

We have proposed according to previous data that the 2,2,5,5-tetramethyl-3-pyrroline group is responsible for the antioxidant and partially for the cardioprotective effect of H-2545. To test this hypothesis, mexiletine, which is a well-known class Ib

antiarrhythmic drug with a moderate antioxidant effect [79], was substituted with this pyrroline ring (HO-2434) [80].

Table 5.6. shows, that mexiletine decreased significantly ($p<0.05$) the ischemia-reperfusion-induced lipid peroxidation, protein oxidation and improved the myocardial energy metabolism in Langendorff perfused rat hearts. HO-2434 in turn caused a significantly better ($p<0.05$) protection against the ischemia-reperfusion-induced oxidative myocardial damage than mexiletine (Table 5.6.). The substitution of mexiletine with a pyrroline ring yielded a similarly efficacious excellent antioxidant and cardioprotective agent that H-2545. Similarly to H-2545, the reaction of HO-2434 with ROS results in the formation of an *N*-hydroxyl metabolite (HO-2433) which has a significantly ($p<0.05$) lower antioxidant and cardioprotective effect than HO-2434.

Table 5.6. The effect of the substitution of mexiletine with a pyrroline ring on its antioxidant and cardioprotective effect

Lipid peroxidation was estimated by the formation of TBARS and protein oxidation was measured as the myocardial protein carbonyl content. Measurements were performed from the freeze-clamped heart samples. ^{31}P-NMR measurements were performed as described in "Materials and Methods". Values were measured before and after the 25 min ischemia and results were expressed as the percent of the two values. Values are means±SEM for five heart preparations.

	PCr	ATP	TBARS	Protein oxidation
	% of control value		nmol/g wt.	μM/g wt
Normoxic	100±2.1†	100±3.6†	39.7±0.7†	1.32±0.11†
Ischemia-reoxygenation (IR)	29.3±3.6	14.7±3.3	70.7±0.6	2.59±0.05
IR + 10 μM mexiletine	41.9±5.7*‡	22.9±4.3*‡	61.8±2.1*‡	2.23±0.10*‡
IR + 10 μM HO-2434	82.1±6.6†	54.4±3.2†	48.4±2.5†	1.49±0.08†
IR + 10 μM HO-2433	67.8±5.2†‡	43.2±4.1†‡	54.8±3.7†‡	1.85±0.09†‡

* Values are different from the respective ischemia-reoxygenation values at the significance of $p<0.05$.
† Values are different from the respective ischemia-reoxygenation values at the significance of $p<0.01$.
‡ Values are different from the respective IR + HO-2434 values at the significance of $p<0.05$

5.2. DISCUSSION

Oxygen free radical damage has been implicated in a wide range of diseases such as atherosclerosis and ischemia-reperfusion injury [16, 73, 81]. Therefore scavenging these radicals should be considered as a basically important therapeutic opportunity. There has been a need for using small antioxidant compounds for preventing oxidative cell damage instead of antioxidant enzymes, because they can not penetrate to all areas susceptible to oxidative damage due to their molecular property [54, 61, 82]. In addition, in various forms of oxidative cell damage there are different sources of ROS. During ischemia-reperfusion mitochondrial respiratory complexes, especially Complex I, are the main sources of toxic oxygen intermediers [25]. Hydrogen peroxide-induced oxidative injury has at least two components. H_2O_2 is a direct cytotoxic agent and its reaction with transition metals (e.g. Fe^{2+}) induces a site-specific oxyradical formation. Hydrogen peroxide can also enhance the mitochondrial ROS production [26].

In the present work, we have investigated the effect of H-2545 and its metabolite on the ischemia-reperfusion- and hydrogen peroxide-induced injury of cardiac muscle in Langendorff heart perfusion system. Ischemia-reperfusion caused a large increase in the release of intracellular myocardial enzymes (CK, AST, LDH) into the coronary effluent. Perfusion of rat hearts with H-2545 nearly totally prevented the release of intracellular enzymes from cardiomyocytes, indicating a significant protection against cell membrane damages. Similarly, the extent of lipid peroxidation in postischemic myocardium could also be diminished by H-2545. We have found that the examined drug, besides the protection of membranes against oxidative damage, could protect other important biomolecules in various cell compartments. The permanently high level of predominantly mitochondrially formed ROS can also affect other regions in the cell (like nucleus and endoplasmic reticulum) and can cause protein oxidation and DNA damages in several forms, including single-strand DNA breaks. During reperfusion the amount of protein carbonyl content and ssDNA breaks indeed increased markedly compared to the normoxic values. However, adding H-2545 to the medium prior to ischemia, the two-fold rise in the extent of protein oxidation could be significantly reduced. Similarly, the amount of undamaged DNA was comparable to that of normoxic rat hearts. Despite its accumulation in biological membranes, H-2545 could also

efficiently protect other cell components against oxidative injury. In postischemic hearts in accord with formation of ssDNA breaks, an increased NAD^+ catabolism could be observed because the DNA-injury caused PARP activation. PARP activation is considered as the consequence of oxidative cell damage mediated by single-strand DNA break formation. Upon activation, PARP cleaves NAD^+ to nicotinamide and ADP-ribose which is coupled to different proteins and protein-bound ADP-ribose residues. Thus, the excessive PARP activation leads to cellular NAD^+ and ATP depletion, which eventually causes necrosis [36-38, 83]. H-2545 decreased the postischemic myocardial NAD^+-loss that is likely to be the consequence of decreased PARP activation. H-2545, through the decrease of ROS levels, could decrease oxidative stress-induced single strand DNA break formation and so indirectly moderated the activation of PARP. This is in concordance with our previous observations that antioxidant lipoamide was able to decrease the ROS induced DNA breaks and PARP activation [73]. Thus, their favourable effect on NAD^+ catabolism is due to the antioxidant effect**.**

Mitochondrial respiratory complexes have a central role in the development of postischemic myocardial damage. They are the main source of ROS during reoxygenation but themselves are also injured by ROS and, in addition, they exhibit a declined activity during oxidative stress [15]. Cytochrome c oxidase and NADH:cytochrome c oxidoreductase were partially inactivated during reoxygenation in mitochondria isolated from perfused rat hearts but the inactivation of the respiratory complexes were significantly attenuated in hearts treated with H-2545. Due to the decreased NAD^+ catabolism and improved mitochondrial function H-2545 increased the recovery of high-energy phosphates in postischemic hearts in comparison with untreated hearts. The recoveries of PCr and ATP during reperfusion were two - or threefold higher in hearts treated with H-2545 than in untreated hearts. In accord with these data, recovery of myocardial pH was also much more pronounced in treated hearts. Consumption rate of Pi was increased and was quite complete in postischemic hearts treated with H-2545, compared to 50% reutilization measured in untreated hearts. The fast and considerable phosphate utilization during reperfusion is a very important step in preventing postischemic myocardial dysfunction and cell death because inorganic phosphate can induce mitochondrial permeability transition (MPT) pores causing the collapse of the mitochondrial membrane potential and the decoupling of mitochondria [35]. Therefore, the faster and more complete utilization of inorganic

phosphate can be advantageous for the preservation of metabolically active, coupled mitochondria in postischemic heart.

Functional recovery of rat hearts in line with the more complete reutilization of inorganic phosphate was also improved during reperfusion in H-2545 treated group compared to untreated hearts.

The metabolite H-2954 decreased also efficiently the ischemia-reoxygenation-induced myocardial injury but its antioxidant and cardioprotective effect was significantly weaker in Langendorff heart perfusion system compared to H-2545. This may confirm our previous assumption that H-2545 can scavenge oxyradicals in two steps, first it converts to a nitroxide, and then is reduced to hydroxylamine form of H-2954, which can scavenge another free radical. We have also evaluated the potency of both drugs to react with different ROS. H-2545 and H-2954 are equally effective scavengers of hydroxyl radicals formed in the Fenton reaction but in isolated mitochondria where H-2545 could not transform to the *N*-hydroxyl form, H-2954 had a significantly higher ability to scavenge superoxide anions than H-2545. However, H-2545 is significantly more effective in the Langendorff perfusion system than the metabolite, a phenomenon that can be explained by the ability of H-2545 to transform to H-2954 by means of amine oxidases and oxidizing agents as well as the higher potency to reach the right cell compartment scavenging free radicals. The lacking antiarrhythmic property (blocking sodium channels) of H-2954 could also explain its lower cardioprotective effect compared to H-2545 [84].

The cardioprotective effect of H-2545, its metabolite and Trolox was also compared to each other. The metabolite and Trolox have a similar protective effect against the impairment of postischemic energy metabolism, but H-2545 exerts significantly greater protection against oxidative myocardial injury than the well-known antioxidant water-soluble vitamin E analogue Trolox.

We have also evaluated the protective effect H-2545 drug and its metabolite on the hydrogen peroxide-induced oxidative myocardial injury. It is well known that beyond the importance of the mitochondria, transition metals play a central role in the mediation of cell damage caused by H_2O_2. Therefore, one of the most effective therapeutic possibilities is the use of iron chelators to diminish the hydroperoxide-induced oxidative cell damage [85]. H-2545 and H-2954 protected hearts efficiently against oxidative cell damage caused by hydrogen peroxide. This protection was

remarkable both in decreasing the oxidative damage of cell components and increasing the concentration of myocardial high-energy phosphates. It has also been reported that nitroxides (e.g. TEMPOL) can oxidize redox active transition metals [86]. Supposedly, the effectiveness of H-2545 and H-2954 - both can transform to nitroxide reacting with ROS - is at least in part due to the ability of altering the redox state of transition metals.

It was supposed that the pyrroline-ring is responsible for the excellent antioxidant and cardioprotective effect of H-2545. For that reason mexiletine, a weak antioxidant compound was substituted with a 2,2,5,5-tetramethyl-pyrroline group (HO-2434) [79, 80]. HO-2434 showed in ischemia-reperfusion experiments a significantly better cardioprotective effect than mexiletine. The modified mexiletine was as effective as H-2545 in preventing postischemic myocardial damage. Therefore, these results confirmed our assumption that the pyrroline-ring is responsible for the cardioprotective effect of H-2545 and substitution of membrane-targeted drugs with a pyrroline ring or with other nitroxide precursors can yield effective scavenger molecules.

In conclusion, H-2545, also in a relatively low concentration, protects cardiac tissue against oxidative cell damage derived from various sources. This type of antiarrhythmic-antioxidant therapy can be a conceptionally new, highly effective treatment in preventing postischemic or drug-induced oxidative damages.

6. EFFECT OF POLY(ADP-RIBOSE) POLYMERASE INHIBITORS ON THE ISCHEMIA-REPERFUSION-INDUCED OXIDATIVE CELL DAMAGE AND MITOCHONDRIAL METABOLISM IN LANGENDORFF HEART PERFUSION SYSTEM

6.1. RESULTS

6.1.1. Effect of PARP inhibitors on ischemia-reperfusion and hydrogen peroxide-induced lipid peroxidation

Lipid peroxidation-induced by ischemia-reperfusion in Langendorff perfused heart was characterized by the formation of TBA reactive substances. Under our experimental conditions, ischemia-reperfusion increased the amount of TBA reactive substances compared to the normoxic conditions ($p<0.01$) (Table 6.1.). In normoxic hearts, PARP inhibitors did not have significant effects on TBA reactive substance formation (Table 6.1.). When ischemia-reperfusion occurred in the presence of PARP inhibitors, the formation of TBA reactive substances were significantly lower than in the hearts

Table 6.1. Effect of PARP inhibitors on the ischemia-reperfusion and hydrogen peroxide-induced lipid peroxidation in Langendorff perfused rat hearts. Lipid peroxidation (TBA reactive substances) was measured as detailed under „Materials and Methods" in Langendorff heart perfusion system either in reperfused heart (25 minutes ischemia followed by 15 min of reperfusion) or in normoxic heart perfused with 1 mM H_2O_2 for 30 minutes. Concentrations of the applied chemicals were 3 mM for 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA) and nicotinamide, 0.1139 mM for BGP-15, and 0.1 mM for 4-hydroxyquinazoline (4-HQ).

Inhibitors added	Normoxic	Ischemia-reperfusion	No addition	H_2O_2
	Thiobarbituric acid reactive substances in nmols/mg wet tissue			
None	39.78±1.59	70.71±4.66	38.57±2.65	66.53±3.45
3-AB	38.82±2.03	51.05±2.98†	37.19±1.85	56.23±2.67*
3-ABA	41.32±3.42	68.84±3.51	39.54±2.49	67.45±2.14
Nicotinamide	39.25±2.47	42.15±1.76†	38.17±1.71	53.17±1.53*
BGP-15	39.72±3.41	47.39±2.31†	39.08±2.82	55.59±1.34*
4-HQ	38.25±2.23	46.54±1.87†	38.09±1.96	56.12±2.12*

Values are mean ± SEM for five experiments. Difference from ischemia-reperfusion * $p<0.05$, † $p<0.01$. Difference from H_2O_2 treated group * $p<0.05$.

subjected to ischemia-reperfusion without PARP inhibitors (Table 6.1.), indicating that PARP inhibitors prevented the ischemia-reperfusion-induced lipid peroxidation. However, 3-aminobenzoic acid, an inactive structural analogue of the PARP inhibitor 3-aminobenzamide could not prevent the ischemia-reperfusion-induced lipid peroxidation (Table 6.1.).

Dose response of PARP inhibitors on the ischemia-reperfusion-induced lipid peroxidation is showed in Fig. 6.1. indicating that although in different concentration range, PARP inhibitors protect the hearts from lipid peroxidation in a concentration dependent way. The IC_{50} values were 226, 513, 29 and 35 μM for 3-aminobenzamide, nicotinamide, BGP-15 and 4-hydroxyquinazoline, respectively.

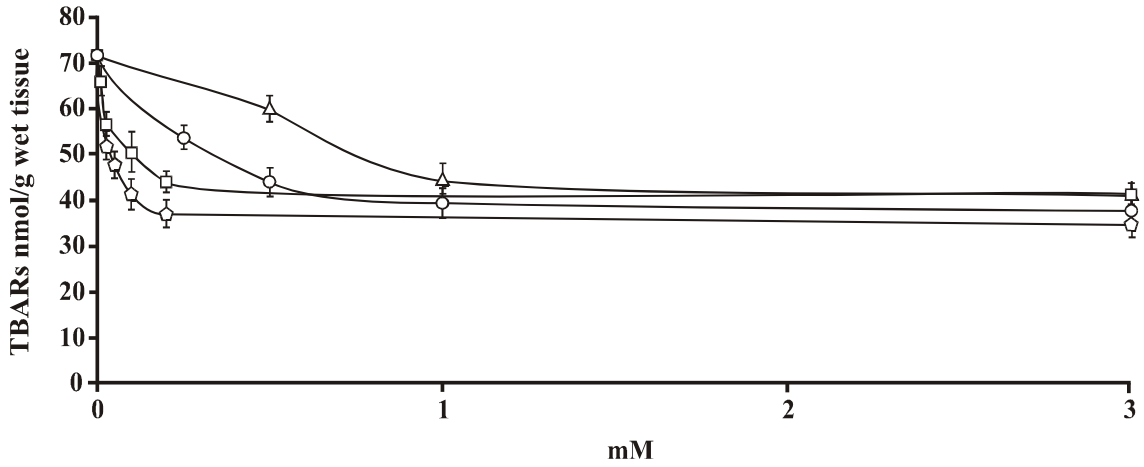


Fig. 6.1. Dose response of PARP inhibitors on the ischemia-reperfusion-induced lipid peroxidation in Langendorff perfused rat hearts. Lipid peroxidation (TBA reactive substances) was measured in Langendorff heart perfusion system in reperfused hearts (25 minutes ischemia followed by 15 min of reperfusion) as detailed under „Materials and Methods". ○ 3-aminobenzamide, □ 4-hydroxyquinazoline, △ nicotinamide, ⬠ BGP-15. Concentrations of the applied chemicals are as indicated in the Figure. Values are mean ± SEM for five experiments. A significant decrease was observed in lipid peroxidation: 3-aminobenzamide, at 0.25 mM $p<0.05$ at higher concentration $p<0.01$; nicotinamide, 1 and 2 mM $p<0.01$; BGP-15 at 0.025 mM $p<0.05$, at higher concentration $p<0.01$; 4-hydroxyquinazoline at 0.01 mM and higher concentration $p<0.01$.

Hydrogen peroxide as an externally added oxidant induced lipid peroxidation in Langendorff perfused hearts, which was determined from the quantity of TBARS formation (Table 6.1.). The presence of PARP inhibitors in the perfusion media could significantly decrease the quantity of TBARS formation in perfused hearts (Table 6.1.) (p values can be seen in the legend). These effects of PARP inhibitors could not be due to their antioxidant property because under our experimental conditions PARP inhibitors did not inhibit the H_2O_2-induced (chemical) oxidation of

dihydrorhodamine123 to rhodamine123 (data not shown).

6.1.2. Effect of PARP inhibitors on ischemia-reperfusion and hydrogen peroxide-induced protein oxidation

ROS formation in ischemia-reperfusion cycle can induce the oxidation of proteins in the cardiomyocytes which can be characterized by the quantity of protein bound aldehyde groups [65]. Table 6.2. shows that ischemia-reperfusion increased significantly ($p<0.01$) the quantity of protein bound aldehyde groups. However, the presence of PARP inhibitors during ischemia-reperfusion cycle prevented the increase in the quantity of protein bound aldehyde groups ($p<0.01$) (Table 6.2.). However, 3-aminobenzoic acid could not prevent the ischemia-reperfusion-induced protein oxidation (Table 6.2.).

Table 6.2. Effect of PARP inhibitors on the ischemia-reperfusion and hydrogen peroxide-induced protein oxidation in Langendorff perfused rat hearts. Protein oxidation (protein carbonyl content with 2,4-dinitrophenylhydrazine) was measured as detailed under „Materials and Methods" in Langendorff heart perfusion system either in reperfused heart (25 minutes ischemia followed by 15 min of reperfusion) or in normoxic heart perfused with 1 mM H_2O_2 for 30 minutes. Concentrations of the applied chemicals were 3 mM for 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA) and nicotinamide, 0.114 for mM BGP-15, and 0.1 mM for 4-hydroxyquinazoline (4-HQ).

Inhibitors added	Normoxic	Ischemia-reperfusion	No addition	H_2O_2
	Protein carbonyl content (nmol carbonyl/mg protein			
None	1.22±0.14	2.47±0.07	1.21±0.13	2.67±0.12
3-AB	1.21±0.07	1.59±0.13*	1.20±0.07	1.75±0.12†
3-ABA	1.22±0.09	2.43±0.09	1.23±0.08	2.71±0.22
Nicotinamide	1.20±0.11	1.45±0.15*	1.20±0.11	1.81±0.21†
BGP-15	1.20±0.12	1.52±0.16*	1.22±0.19	1.82±0.19†
4-HQ	1.21±0.09	1.49±0.11*	1.21±0.07	1.78±0.13†

Values are mean ± SEM for five experiments. Difference from ischemia-reperfusion group * $p<0.01$, and difference from H_2O_2 group † $p<0.01$.

Protective effect of PARP inhibitors was also seen when protein oxidation was induced by externally added H_2O_2 but in these cases PARP inhibitors could only partially protect heart proteins from oxidative damages ($p<0.01$) (Table 6.2.).

Dose response of PARP inhibitors on ischemia-reperfusion-induced protein oxidation is shown in Figure 6.2. indicating that PARP inhibitors, in different concentration range,

protect heart proteins against oxidation in a concentration dependent way. The IC_{50} values were 239, 478, 36 and 22 μM for 3-aminobenzamide, nicotinamide, BGP-15 and 4-hydroxyquinazoline, respectively.

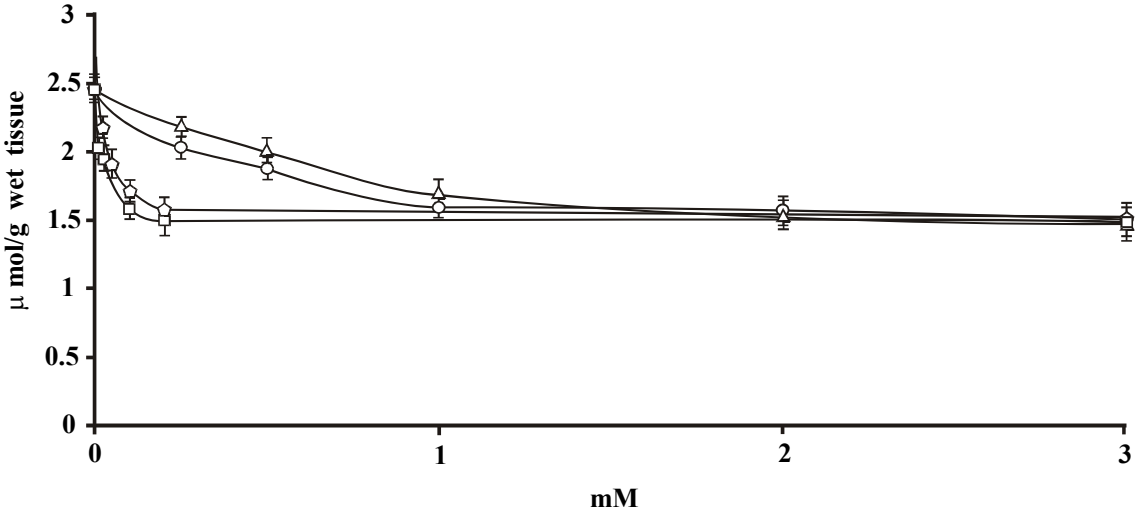


Fig. 6.2. Dose response of PARP inhibitors on the ischemia-reperfusion-induced protein oxidation in Langendorff perfused rat hearts. Protein oxidation (protein carbonyl content with 2,4-dinitrophenylhydrazine) was measured in Langendorff heart perfusion system in reperfused hearts (25 minutes ischemia followed by 15 min of reperfusion) as detailed under „Materials and Methods". ○ 3-aminobenzamide, □ 4-hydroxyquinazoline, △ nicotinamide, ⬠ BGP-15. Concentrations of the applied chemicals are as indicated in the Figure. Values are mean ± SEM for five experiments. Significant decrease was seen in protein oxidation: 3-aminobenzamide, at 0.25 and 0.5 mM $p<0.05$ at higher concentration $p<0.01$; nicotinamide, at 0.5 mM $p<0.05$, at higher concentrations $p<0.01$; BGP-15 at 0.02 mM $p<0.05$, at higher concentration $p<0.01$; 4-hydroxyquinazoline at 0.01 mM and 0.02 mM $p<0.05$, at higher concentration $p<0.01$.

6.1.3. Effect of PARP inhibitors on ischemia-reperfusion-induced single-strand DNA break formation and NAD^+ catabolism

Ischemia-reperfusion increased ROS formation in perfused hearts which can contribute to the formation of single-strand DNA breaks ($p<0.01$). Under normoxic conditions, most of the DNA was undamaged but ischemia-reperfusion-induced large amounts of single-strand DNA breaks and the quantity of undamaged DNA decreased to under 30% (Fig. 6.3.). In the presence of PARP inhibitors, ischemia-reperfusion only slightly increased the amount of ssDNA breaks (Fig. 6.3.) and the amount of undamaged DNA was significantly higher ($p<0.05$ or $p<0.01$) than in postischemic hearts and not significantly lower than the normoxic values (Fig. 6.3.). The inactive analogue of 3-aminobenzamide, 3-aminobenzoic acid, could not decrease the ischemia-reperfusion-induced ssDNA break formation (Fig. 6.3.).

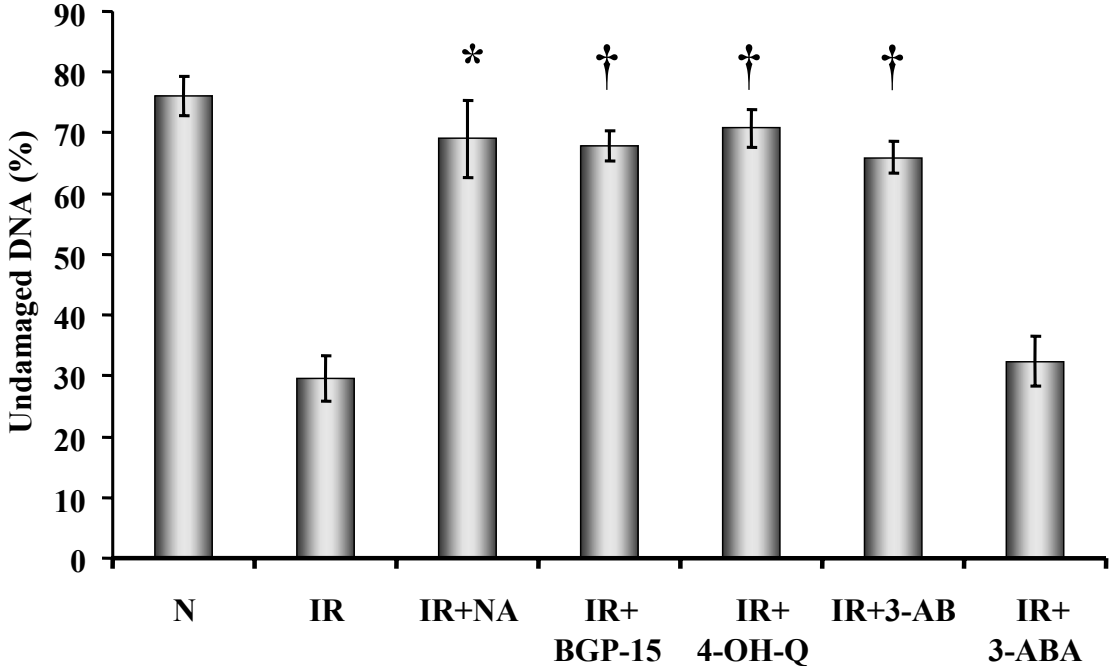


Fig 6.3. Effect of PARP inhibitors on the ischemia-reperfusion-induced single-strand DNA breaks in Langendorff perfused rat hearts. Single-strand DNA breaks (determined by the alkaline fluorescence analysis of DNA unwinding) was measured in Langendorff heart perfusion system in normoxic or in reperfused hearts (25 minutes ischemia followed by 15 min of reperfusion) as detailed under „Materials and Methods". (N) Normoxic; (IR) ischemia-reperfusion; (IR + NA) ischemia-reperfusion in the presence of 3 mM nicotinamide; (IR + 3-AB) ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide; (IR + 3-ABA) ischemia-reperfusion in the presence of 3 mM 3-aminobenzoic acid; (IR + BGP-15) ischemia-reperfusion in the presence of 0.114 mM BGP-15; (IR + 4HQ) ischemia-reperfusion in the presence of 0.1 mM 4-hydroxyquinazoline.
Values are mean ± SEM for five experiments. Difference from ischemia-reperfusion: * $p<0.05$, † $p<0.01$.

It is well known that ssDNA breaks activate PARP which stimulates intracellular NAD^+ catabolism, therefore, it is expectable that ischemia-reperfusion cycle decreases NAD^+ content of perfused hearts. Fig. 6.4. shows that ischemia-reperfusion significantly decreased ($p<0.01$) the NAD^+ content of hearts. The presence of PARP inhibitors in the perfusate during ischemia-reperfusion cycle partially protected ($p<0.01$) the loss of NAD^+ in postischemic hearts (Fig. 6.4.). However, 3-aminobenzoic acid could not prevent the ischemia-reperfusion-induced NAD^+ loss.

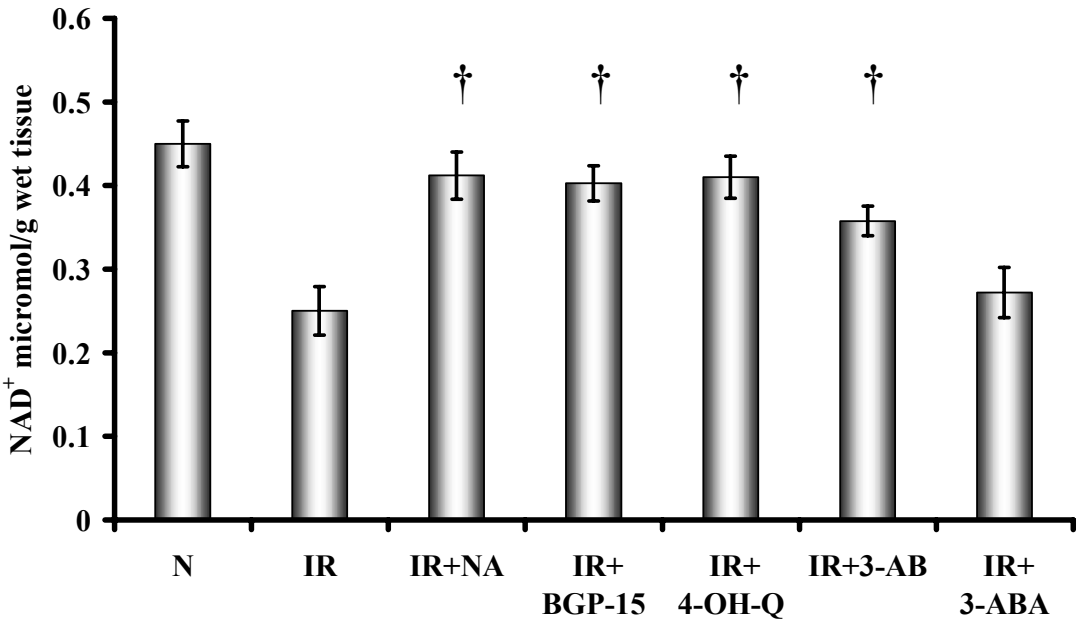
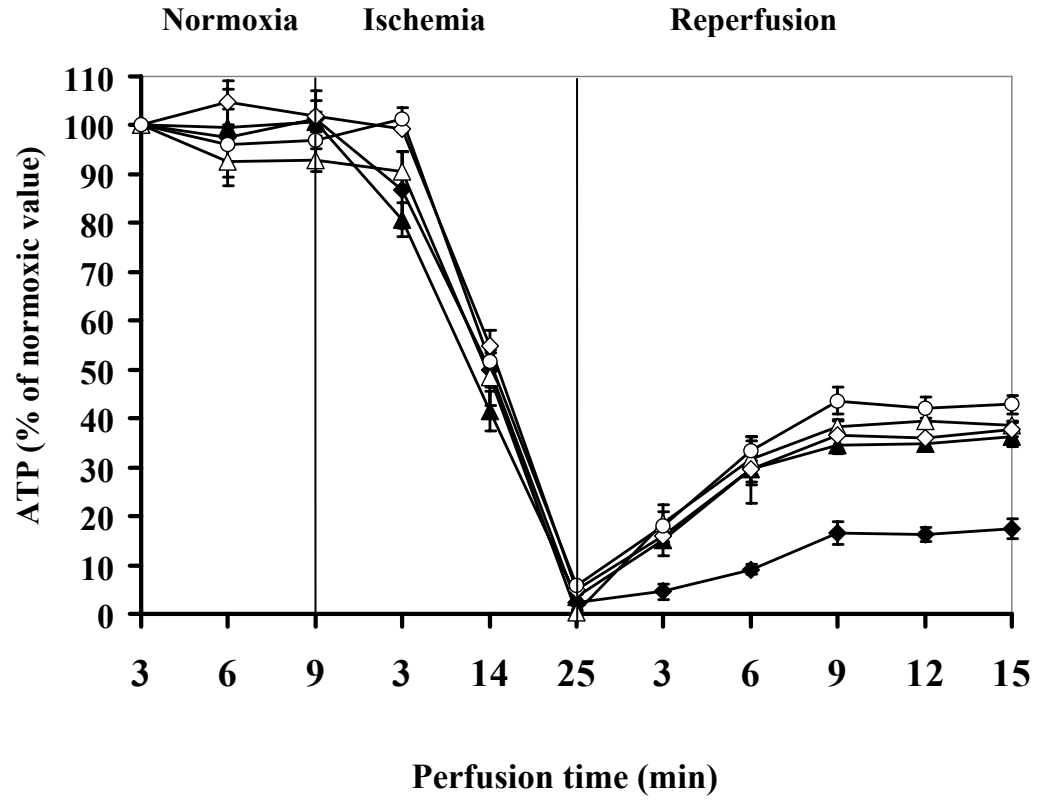


Fig. 6.4. Effect of PARP inhibitors on the ischemia-reperfusion-induced NAD^+ loss in Langendorff perfused rat hearts. The NAD^+ content using an alcohol dehydrogenase method was measured as detailed under „Materials and Methods" after 15 min of reperfusion. (N) Normoxic; (IR) ischemia-reperfusion; (IR + NA) ischemia-reperfusion in the presence of 3 mM nicotinamide; (IR + 3-AB) ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide; (IR + 3-ABA) ischemia-reperfusion in the presence of 3 mM 3-aminobenzoic acid; (IR + BGP-15) ischemia-reperfusion in the presence of 0.114 mM BGP-15; (IR + 4HQ) ischemia-reperfusion in the presence of 0.1 mM 4-hydroxyquinazoline. Values are mean ± SEM for five experiments. Difference from ischemia-reperfusion: † $p < 0.01$.

6.1.4. Effect of PARP inhibitors on the energy metabolism of perfused hearts during ischemia-reperfusion

Energy metabolism of Langendorff perfused hearts was monitored in the magnet of NMR spectroscope making possible to detect changes in high-energy phosphorous intermediates. Ischemia induced a rapid decrease in ATP and creatine phosphate levels and a fast evolution of inorganic phosphate. Under our experimental conditions, high-energy phosphate intermediates recovered only partially in 15-minutes-reperfusion phase and 3-aminobenzamide facilitated the recovery of ATP and creatine phosphate. However, 3-aminobenzoic acid did not improve the recovery of ischemic heart (data not shown) indicating that the inhibition of PARP was responsible for the improved recovery.

Fig. 6.5A and 6.5B



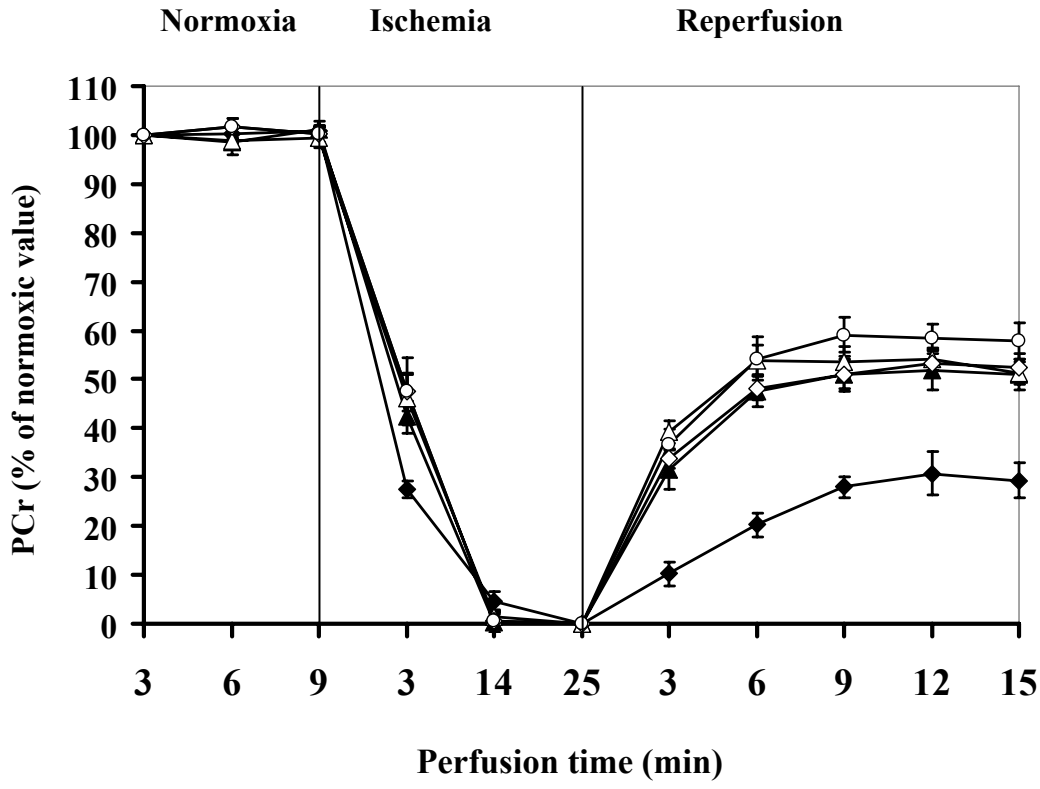


Fig. 6.5C

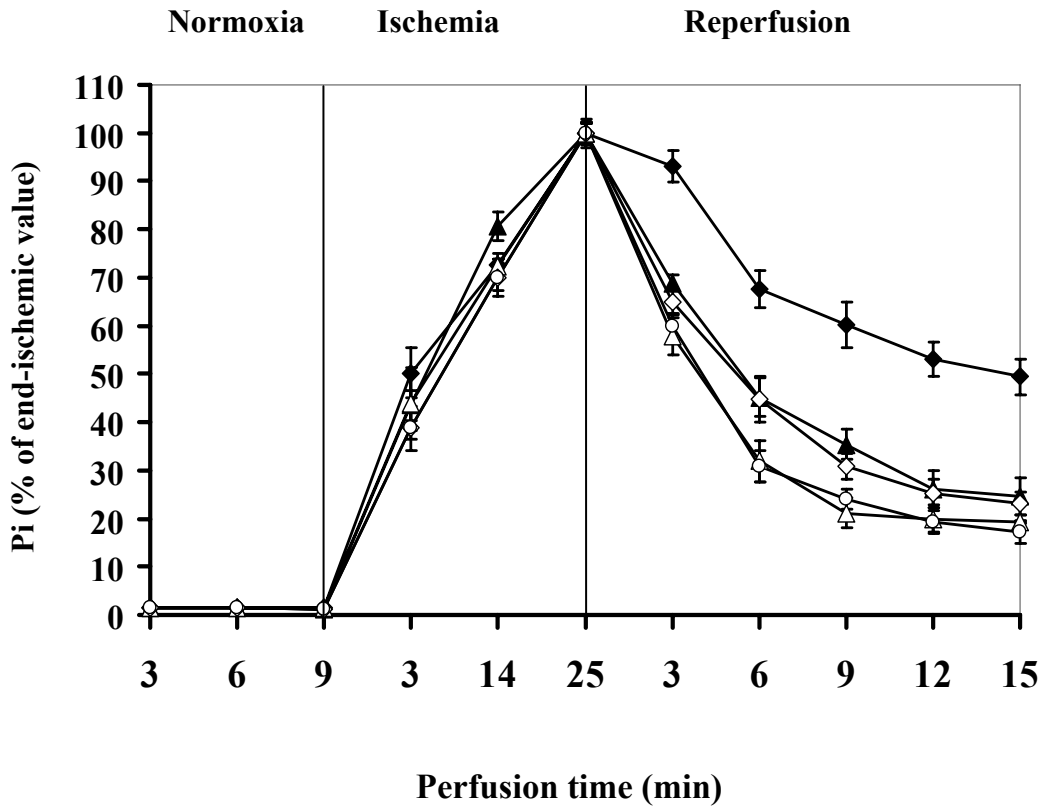


Fig. 6.5. Effect of PARP inhibitors on the recovery of ATP (A), creatine phosphate (B) and inorganic phosphate (C) in Langendorff perfused hearts. Conditions for heart perfusion and NMR measurements were described under "Materials and Methods". (IR) ischemia-reperfusion; (IR + NA) ischemia-reperfusion in the presence of 3 mM nicotinamide; (IR + 3-AB) ischemia-reperfusion in the presence of 3 mM 3-aminobenzamide; (IR + BGP-15) ischemia-reperfusion in the presence of 40 mg/l (0.1139 mM) BGP-15; (IR + 4HQ) ischemia-reperfusion in the presence of 0.1 mM 4-hydroxyquinazoline.
Values are given as mean ± SEM for five experiments. Significant changes were seen:
(A) IR versus IR+ NA, $p<0.05$ at all time point of reperfusion phase, IR versus IR+ 3-AB $p<0.05$ at all time point of reperfusion phase, IR versus IR+ BGP-15, $p<0.05$ at all time point of reperfusion phase, IR versus IR+ 4HQ $p<0.05$ at all time point of reperfusion phase; (B) IR versus IR+ NA, $p<0.01$ at 3, 6, 9, 15 minutes and $p<0.05$ at 12 minutes, IR versus IR+ 3-AB $p<0.01$ at all time point of reperfusion phase, IR versus IR+ BGP-15, $p<0.01$ at all time point of reperfusion phase, IR versus IR+ 4HQ $p<0.01$ at all time point of reperfusion phase; (C) IR versus IR+ NA, $p<0.05$ at all time point of reperfusion phase, IR versus IR+ 3-AB $p<0.05$ at all time point of reperfusion phase, IR versus IR+ BGP-15, $p<0.01$ at all time point of reperfusion phase, IR versus IR+ 4HQ, $p<0.01$ at all time point of reperfusion phase.

In Fig. 6.5., time dependence of ATP, creatine phosphate and inorganic phosphate levels are shown during ischemia-reperfusion cycle using four PARP inhibitors. These data show that each PARP inhibitor improved significantly the final recovery of high-energy phosphates (p values can be seen in the legend).

Dose response of PARP inhibitors on the recovery of creatine phosphate were studied in Langendorff perfused hearts (Fig. 6.6.). These data show that PARP

inhibitors promoted the recovery of creatine phosphate in perfused hearts in a concentration dependent way. The IC_{50} values were 239, 485, 32 and 19 μM for 3-aminobenzamide, nicotinamide, BGP-15 and 4-hydroxyquinazoline, respectively. Recovery of ATP showed similar dose response as creatine phosphate (data not shown), and IC_{50} values were 301, 476, 48 and 26 μM for 3-aminobenzamide, nicotinamide, BGP-15 and 4-hydroxyquinazoline, respectively.

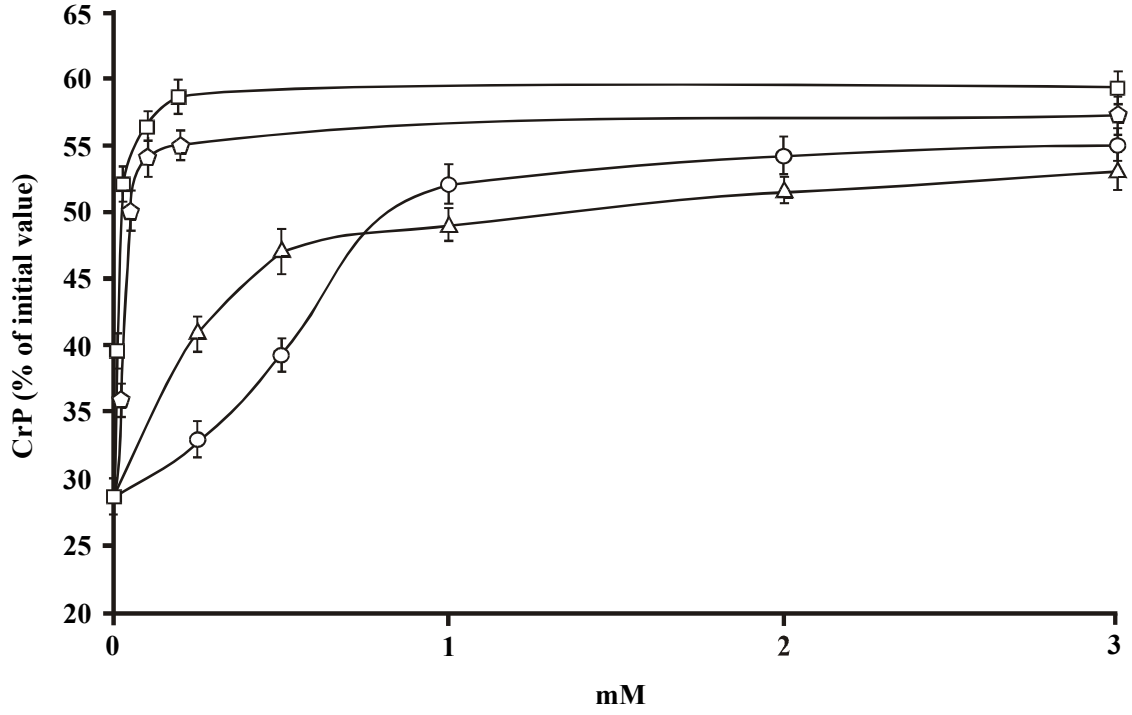


Fig. 6.6. Dose response of PARP inhibitors on the ischemia-reperfusion-induced creatine phosphate recovery in Langendorff perfused rat hearts.
Conditions for heart perfusion and NMR measurements were described under "Materials and Methods". ○ 3-aminobenzamide, □ 4-hydroxyquinazoline, △ nicotinamide, ⬠ BGP-15. Concentrations of PARP inhibitors are as indicated in the Figure. Values are mean ± SEM for five experiments. A significant increase was seen in creatine phosphate level: 3-aminobenzamide, at 0.5 mM $p<0.05$ and at higher concentration $p<0.01$; nicotinamide, 0.25 mM $p<0.05$ and at higher concentrations $p<0.01$; BGP-15 at 0.025 mM $p<0.05$, at higher concentration $p<0.01$; 4-hydroxyquinazoline at 0.01 mM $p<0.05$ and at higher concentration $p<0.01$.

6.1.5. Protecting effect of PARP inhibitors against ischemia-reperfusion-induced damage of respiratory complexes

Under our experimental conditions, ischemia-reperfusion caused a partial inactivation ($p<0.01$) of respiratory complexes (Table 6.3.), which could be the consequence of mitochondrial ROS formation during reperfusion [25, 87-88]. Our

previous data (Table 6.1, 6.2. and Fig. 6.1-6.2.) indicated that PARP inhibitors decreased the oxidative damage for different components of heart during ischemia-reperfusion cycle, therefore, it is reasonable to assume that PARP inhibitors may attenuate the inactivation of respiratory complexes. Using four different PARP inhibitors, we found that cytochrome oxidase activity was almost completely protected ($p<0.01$) from the ischemia-reperfusion-induced partial inactivation (Table 6.3).

Table 6.3. Effect of PARP inhibitors on the ischemia-reperfusion-induced inactivation of respiratory complexes. Conditions for heart perfusion and the measurement of mitochondrial enzyme activities were detailed under „Materials and Methods". Concentrations of the applied chemicals were 3 mM for 3-aminobenzamide (3-AB), 3-aminobenzoic acid (3-ABA) and nicotinamide, 0.114 for mM BGP-15, and 0.1 for mM 4-hydroxyquinazoline (4-HQ).

Treatments	Cytochrome oxidase	NADH:cytochrome C oxidoreductase
	in % of normoxic value	
Normoxic	100±3.2	100±2.9
IR	69.1±2.3	68.3±2.4
IR + 3-AB	99.3±2.8*	93.6±2.9*
IR + 3-ABA	65.7±3.1	67.4±2.1
IR + Nicotinamide	97.5±2.4*	93.5±3.1*
IR + BGP-15	97.8±3.2*	94.4±3.2*
IR + 4-HQ	95.2± 4.1*	97.2±3.8*

Values are given as mean ± SEM for five experiments. Difference from ischemia-reperfusion group:
* $p<0.01$.

Ischemia-reperfusion also partially inactivated ($p<0.01$) the NADH:cytochrome c oxidoreductase activity (Complex I-III) but PARP inhibitors could partially protect ($p<0.01$) NADH:cytochrome c oxidoreductase activity in postischemic heart mitochondria (Table 6.3). Under the same experimental conditions, citrate synthase activity was not affected by H_2O_2 or PARP inhibitors (data not shown).

6.1.6. Effect of PARP inhibitors on the oxidative inactivation of cytochrome oxidase

Hydrogen peroxide (0.5 mM) induced a fast inactivation ($p<0.01$) of cytochrome oxidase in isolated mitochondria (Fig. 6.7.) showing that ROS can indeed inactivate respiratory complexes. In the same system, PARP inhibitors (3-aminobenzamide, nicotinamide, BGP-15 and 4-hydroxyquinazoline) could almost completely protect

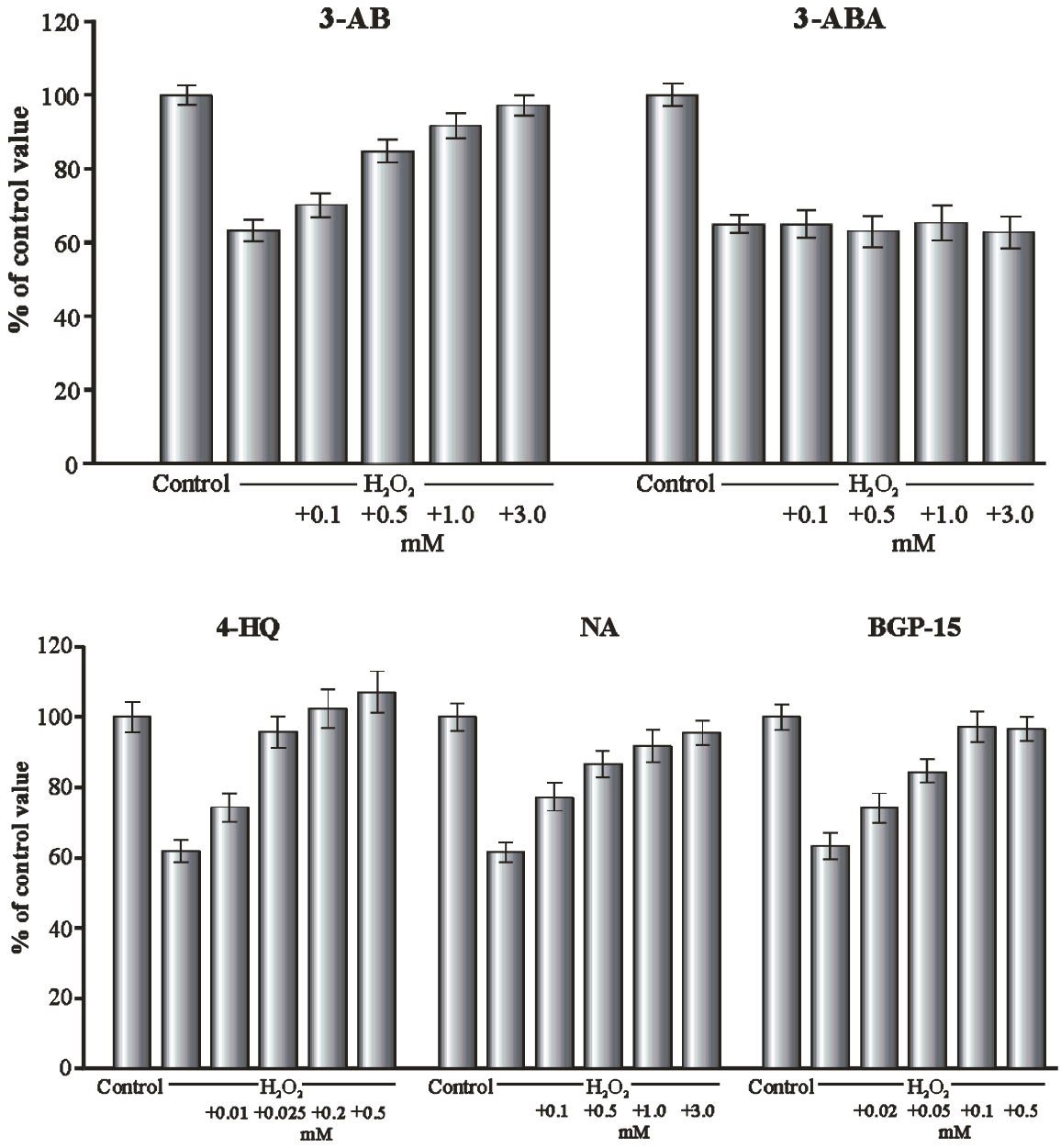


Fig. 6.7. Effect of PARP inhibitors on the H_2O_2–induced inactivation of cytochrome oxidase. Experimental conditions and cytochrome oxidase activity measurement are detailed under „Materials and Methods". Concentrations of the applied chemicals are as indicated in the Figure. (3-AB) 3-aminobenzamide; (3-ABA) 3-aminobenzoic acid; (4-HQ) 4-hyroxyquinazoline; (NA) Nicotinamide; (BGP-15) BGP-15.
Values are mean ± SEM for five experiments. Significant increase was seen: 3-AB at 0.5 mM and higher concentrations $p<0.05$; NA at 0.1 and 0.5 mM $p<0.05$ and at higher concentrations $p<0.01$; BGP-15 at 0.05 mM $p<0.05$ and higher concentrations $p<0.01$; 4-HQ at 0.025 and higher concentrations $p<0.05$.

cytochrome oxidase from H_2O_2-induced inactivation but the chemical analogue of 3-aminobenzamide, 3-aminobenzoic acid, failed to do so (Fig. 6.7.). At the same time, PARP inhibitors did not affect the amount of H_2O_2-induced ROS production as determined by the oxidation of dihydrorhodamine123 to rhodamine 123 (data not

shown), so the protection was not due to the decreased amount of ROS in the presence of PARP inhibitors. Dose response of PARP inhibitors in the protection of respiratory complexes against H_2O_2-induced inactivation is shown on Fig 6.7. The IC_{50} values were 394, 238, 38 and 14 μM for 3-aminobenzamide, nicotinamide, BGP-15 and 4-hydroxyquinazoline, respectively.

6.2. DISCUSSION

The Langendorff heart perfusion system has been successfully used for studying ischemia-reperfusion-induced cardiac damages. In this model, PARP inhibitors were reported to improve the recovery of heart function and ATP level as well as decrease the infarct size [89-90]. Similar protective effects of PARP inhibitors were also observed in local cardiac ischemia in living animals [91-92]. It is known that ROS are mainly produced by mitochondrial respiratory complexes in postischemic heart [25, 88] and that ROS induced oxidative damages represent an important mechanism of the pathological processes in reperfused heart. Our previous data indicated that a new PARP inhibitor (BGP-15) decreased the ischemia-reperfusion-induced oxidative damages in Langendorff perfused heart system without having any obvious antioxidant property [93]. These experiments raise an interesting question whether this is a special property of the compound BGP-15 or other PARP inhibitors have similar characteristics. To answer this question we investigated the effect of well-established PARP inhibitors (3-aminobenzamide, nicotinamide and 4-hydroxyquinazoline) together with BGP-15 on the oxidative cell damage induced by either ischemia-reperfusion or hydrogen peroxide in Langendorff heart perfusion system.

Under our experimental conditions, PARP inhibitors abrogated the ischemia-reperfusion-induced lipid peroxidation (Fig. 6.1., Table 6.1.) as well as protein oxidation (Fig. 6.2., Table 6.2.), and significantly decreased ssDNA break formation (Fig. 6.3.) suggesting that PARP inhibitors somehow reduced the ischemia-reperfusion-induced mitochondrial ROS production and ROS related oxidative damages. When the oxidant was given to the heart externally (1 mM H_2O_2), it induced lipid peroxidation and protein oxidation but PARP inhibitors only partially protected heart tissue from lipid peroxidation and protein oxidation (Table 6.1., 6.2.). These observations could not

be explained by a simple antioxidant effect because the studied compounds could not entrap ROS generated chemically as showed in Chapter 6.1.1. In perfused heart, most of the ROS is produced in the mitochondria during ischemia-reperfusion cycle [25, 88], therefore, our data (Fig. 6.1-6.3.) indicate that PARP inhibitors probably decrease the ischemia-reperfusion-induced elevated mitochondrial ROS production and as so all oxidative damages related to enhanced ROS production. Since the blocking of normal electron flow generally activates the formation of partially reduced reactive oxygen intermediates [94], the observation that PARP inhibitors prevent the ischemia-reperfusion-induced inactivation of respiratory complexes (Fig. 6.7) supports this argument.

In the case of externally added H_2O_2, PARP inhibitors could not prevent the direct oxidative damage caused by H_2O_2 (Table 6.1., 6.2.) but could decrease the H_2O_2-induced inactivation to the components of the mitochondrial respiratory chain (Fig. 6.7.) and as a consequence, the endogenous ROS production and the total amount of oxidative damages seen in Fig. 6.1-6.3., Table 6.1, 6.2. Biochemical effects of H_2O_2 can be different depending on the concentration applied. High concentration (higher than 1 mM) of H_2O_2 induces DNA-breaks, PARP activation, NAD^+ depletion, ATP depletion, cell membrane damage and necrotic cell death which processes can be attenuated by PARP inhibitors [95-97]. H_2O_2 can also induce apoptotic cell death in several cell types, which can also be prevented under certain conditions by PARP inhibitors [98]. In contrast to these, very low concentrations of H_2O_2 can be regarded as a life signal, help proliferation and protect against apoptosis possibly by preventing caspase activation and PARP cleavage [99].

Under our experimental conditions, PARP inhibitors decreased ischemia-reperfusion-induced NAD^+ depletion. This obviously PARP dependent nuclear process through the modulation of NAD^+ level (normoxic 0.45 mg/g wet tissue, ischemia-reperfusion 0.24 mg/g wet tissue and ischemia-reperfusion in the presence of PARP inhibitors 0.35–0.41 mg/g wet tissue) (Fig. 6.4.) could contribute to the better recovery of high-energy phosphate intermediates (Fig. 6.5-6.6.). PARP inhibitors decreased the rate of NAD^+ cleavage and so reduced ATP consumption for the resynthesis of NAD^+ in postischemic hearts. Our data show that PARP inhibitors uniformly promoted the recovery of ATP and creatine phosphate levels and resulted not only in higher percentage of recovery but the rate of recovery was also significantly faster (Fig. 6.5.).

In addition, the consumption rate of inorganic phosphate was enhanced during the recovery period by PARP inhibitors. Since Ca^{2+} and inorganic phosphate can induce mitochondrial permeability transition [100], the faster and more complete utilization of inorganic phosphate can be advantageous for the preservation of metabolically active, coupled mitochondria in postischemic heart. Hearts predominantly produce energy by mitochondrial oxidation, therefore, the faster recovery seen in the presence of PARP inhibitors (Fig. 6.5.) can be, at least in part, the consequence of protected mitochondrial energy metabolism. It is known that ROS can inactivate respiratory complexes (Fig. 6.7.) [94], thus, protection against ischemia-reperfusion-induced inactivation of cytochrome oxidase and NADH:ubiquinone oxidoreductase can also be a factor beyond the inhibition of nuclear PARP in the faster and more complete recovery of high energy phosphate intermediates.

According to one possible argument, PARP inhibition by moderating cytoplasmic NAD^+ loss can help to retain mitochondrial NAD^+ and may prevent the decrease of the mitochondrial NAD^+-linked substrate oxidation and ROS formation between NADH-dehydrogenase and ubiquinone. Therefore, the protective effect of PARP inhibitors against oxidative damages (lipid peroxidation, protein oxidation, single-strand DNA breaks and the inactivation of respiratory complexes) in postischemic heart may not exclusively rely on the inhibition of PARP. It required further confirmation that the inhibition of a nuclear enzyme (PARP) could result in such a good protection against mitochondrial damages. For this reason, we investigated whether PARP inhibitors could have a protective effect against H_2O_2-induced inactivation of cytochrome oxidase in isolated rat heart mitochondria (Fig. 6.7.). Our data showed that the H_2O_2-induced inactivation of cytochrome oxidase was almost completely protected by PARP inhibitors in isolated mitochondria (Fig. 6.7.). In the same experimental system, 3-aminobenzoic acid, an inactive chemical homologue of the PARP inhibitor 3-aminobenzamide, did not protect cytochrome oxidase against H_2O_2-induced inactivation (Fig. 6.7.). In isolated mitochondria, nuclear PARP can not play any possible role (PARP activity was not detectable in the isolated mitochondria with autoradiography, data not shown), therefore, we have to assume that PARP inhibitors bind to another mitochondrial protein which plays a role in the oxidative damage of mitochondria. It is known that several PARP inhibitors can also inhibit mono-ADP-ribose transferase or NAD^+ glycohydrolase which are present in the mitochondria [101-102]. Consequently,

it is possible that PARP inhibitors prevent the inactivation of respiratory complexes by decreasing the activity any of these enzymes.

These data do not conflict with the previous observation using PARP-1 deleted cells or PARP-1 knockout mice [103-106], but they indicate that PARP inhibitors beside their inhibitory effect on nuclear PARP resulting in a significant protection against oxidative damage can have an additional mitochondrial target – a binding site for these molecules - and this direct mitochondrial effect can play a protecting role in oxidative mitochondrial damage.

In conclusion, the studied PARP inhibitors, beside their primary effect of decreasing the activity of nuclear PARP and therefore decreasing NAD^+ depletion and ATP consumption, protect mitochondrial energy metabolism (Fig. 6.4-6.6.), decrease the ischemia-reperfusion-induced mitochondrial ROS formation (Fig. 6.1., 6.2.) and protect the respiratory complexes from ROS induced inactivation (Fig. 6.7.). The mitochondrial protective effect of PARP inhibitors, at least in part, is independent of nuclear PARP activity because it can be observed in isolated mitochondria, as well. Therefore, it represents a novel mechanism for the mitochondrial effects of PARP inhibitors.

7. CONCLUSIONS

New results of our studies

1. H-2545, an antiarrhythmic compound, in the concentration of 10 μM decreased efficiently the ischemia-reperfusion-induced leakage of intracellular enzymes (CK, AST, LDH) into the coronary effluent (1). Similarly lipid peroxidation (2), protein oxidation (3) and ssDNA break formation (4) were also decreased by H-2545 in postischemic myocardium. During reperfusion due to the formation of ssDNA breaks, an increased NAD^+ catabolism could be observed, which can be prevented by using H-2545 (5). Furthermore, H-2545 decreased the partial inactivation of mitochondrial respiratory complexes during reoxygenation (6). Due to the decreased NAD^+ catabolism and improved mitochondrial function H-2545 improved the recovery of high-energy phosphates (PCr, ATP) in postischemic hearts in comparison with untreated hearts (7). Recovery of myocardial pH was also much more pronounced in treated hearts (8). Consumption rate of Pi was increased and quite complete in postischemic hearts treated with H-2545, compared to 50% reutilization measured in untreated hearts (9). In line with the faster and more complete utilization of inorganic phosphate, an improved functional recovery of rat hearts during reperfusion was seen in H-2545 treated group compared to untreated hearts (10). Although, H-2545 and H-2954 are equally effective scavengers of hydroxyl radicals (11), H-2954 had a significantly higher ability to scavenge superoxide anions than H-2545 (12). Despite of this, H-2954 caused a significantly lower protection against the ischemia-reoxygenation induced myocardial injury compared to that of H-2545 (13). Comparing the cardioprotective effect of H-2545, H-2954 and Trolox, H-2954 and Trolox have similar protective effect against the impairment of postischemic energy metabolism, at the same time H-2545 exerts significantly greater protection against oxidative myocardial injury than Trolox (14). H-2545 was effective not only against ischemia-reperfusion-induced myocardial damage but also against hydrogen peroxide-induced cell injury, a phenomenon that derives in part from its scavenging capacity but also from the ability to oxidate redox active metals and as so hindering the Fenton reaction (15). The substitution of mexiletine with a 2,2,5,5-tetramethyl-

pyrroline group improved the cardioprotective effect of the compound and yielded a similarly efficacious drug as H-2545 in preventing postischemic myocardial damage (16). Thus, the sterically hindered pyrroline-ring is responsible for the cardioprotectivity of H-2545 and substitution of drugs accumulating in membranes with a pyrroline ring or with other nitroxide precursors can yield effective scavenger molecules. This type of antiarrhythmic-antioxidant therapy can be a conceptionally new, highly effective treatment in preventing postischemic or drug-induced oxidative myocardial damages.

2. Our data showed that not only BGP-15 but all of the investigated PARP inhibitors decreased the oxidative injury in postischemic rat hearts (1). PARP inhibitors abrogated ischemia-reperfusion induced lipid peroxidation (2) and protein oxidation (3) and significantly decreased ssDNA break formation (4). In case the oxidant was given to the heart externally (H_2O_2), PARP inhibitors only partially protected the heart tissue from lipid peroxidation and protein oxidation (5). Therefore, PARP inhibitors decreased ischemia-reperfusion-induced increase of mitochondrial ROS production and, consequently, all oxidative damages related to enhanced ROS production (6). The observation that PARP inhibitors prevent ischemia-reperfusion-induced inactivation of respiratory complexes (7) supports this argument. PARP inhibitors decreased ischemia-reperfusion induced NAD^+ (8) depletion and uniformly promoted the recovery of ATP and creatine phosphate levels (9) and resulted not only in higher percentage of recovery but the rate of recovery was also significantly faster. In addition, the consumption rate of inorganic phosphate was increased (10) during the recovery period by PARP inhibitors. The studied PARP inhibitors, beside their primary effect of inhibiting the activity of nuclear PARP and, therefore, decreasing NAD^+ and ATP consumption, protect mitochondrial energy metabolism (11), decrease the ischemia-reperfusion induced mitochondrial ROS formation (12) and protect the respiratory complexes from ROS induced inactivation (13). However, 3-aminobenzoic acid, which is a chemical analog of 3-amino-benzamide without any PARP inhibitory effect, was ineffective in the same experimental system (14). The mitochondrial protective effect of PARP inhibitors, at least partially, is independent of nuclear PARP activity because it can be observed in isolated mitochondria (15), as well. Therefore, it represents a novel mechanism for the mitochondrial protective effects of PARP inhibitors.

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10. PUBLICATIONS OF THE AUTHOR

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