

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

Ph.D. Thesis

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1. 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. 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.

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. Mitochondria are well documented as a major source of O_2^- and H_2O_2 . Being major producers of ROS, mitochondrial structures are exposed to high concentrations of ROS and, therefore, are particularly susceptible to their attack. However various cytosolic and membrane bound oxidases and dehydrogenases (e.g. xanthine oxidase, lipoxygenase and NADH oxidase) are also known to produce free radicals.

Cells have developed various enzymatic and nonenzymatic systems against continuously generated free radicals to protect themselves from oxidative damages. Superoxide anions can be scavenged by superoxide dismutase, which catalyzes the conversion of two of these radicals into hydrogen peroxide and molecular oxygen: Hydrogen peroxide is scavenged by catalase that catalyzes the conversion of hydrogen peroxide into water and molecular oxygen. Peroxidases catalyze an analogous reaction in which hydrogen peroxide is reduced to water by a reductant (AH_2):

Glutathione, which is present in high concentrations (5 mM) in animal cells, has a key role in detoxification by reacting with hydrogen peroxide and organic peroxides, and serves as a sulfhydryl buffer. Glutathione cycles between a reduced thiol form (GSH) and an oxidized form (GSSG). GSSG is reduced to GSH by glutathione reductase.

Other important physiological antioxidants are vitamin E and reduced coenzyme

Q (CoQ). 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. Physiological compounds, such as urate, bilirubin, and ceruloplasmin, can also protect against oxidative attack.

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. 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.

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. 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.

Reactive oxygen species injure a wide variety of biomolecules and cell compartments. ROS initiate lipid peroxidation, protein oxidation and the formation of single-stranded DNA breaks. The major product of lipid peroxidation is 4-hydroxy-2-nonenal, which is highly cytotoxic and can readily react with and damage proteins. 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. 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. 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 they exhibit a reduced activity during oxidative stress. 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). PARP 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. 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 and inhibition of PARP can partially prevent ROS toxicity and ischemia-reperfusion-induced cell death. As a consequence, PARP inhibitors are widely used experimentally to protect cells from oxidative.

Oxygen free radical damage has been implicated in a wide range of diseases. Therefore, scavenging these radicals should be considered as an other 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 cannot reach the right cell compartment to exert their protective effect. 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.

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. 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.

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.

A group of pyrroline-based compounds has been shown to have a class I antiarrhythmic activity. The amino compound *N*-3-(2,2,5,5-tetramethyl-3-pyrroline-3-carboxamido)propylphthalimide (H-2545) can be oxidized by mixed function amine

oxidases or ROS 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.

H-2545 has low toxicity, its therapeutic index is 8.8 in animals. 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.

2. OBJECTIVES OF THE STUDY

2.1. 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 μ M) 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 actions 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.

2.2. 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.

3. MATERIALS AND METHODS

3.1. Chemicals

The synthesis of H-2545 and H-2954 has already been published. Mexiletine, Trolox, 3-aminobenzamide, 3-aminobenzoic acid, 4-hydroxyquinazoline, nicotinamide were purchased from Sigma - Aldrich Chemical Co. (Budapest, Hungary). BGP-15 was a gift from N-Gene Research Laboratories, Inc. (Budapest, Hungary). All other reagents

were of the highest purity commercially available.

3.2. 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.

3.3. Heart perfusion

Rats were anesthetized with ketamine and heparinized. Hearts were perfused according to the Langendorff method at a constant pressure of 70 mmHg, at 37°C. The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5 mM KCl, 1.25 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, 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% O₂/ 5% CO₂ through a glass oxygenator.

3.3.1. 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°C. At the end of perfusion hearts were freeze clamped.

3.3.2. 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.

3.4. 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. The length of normoxia, ischemia and reperfusion were 15, 25, 45 min, respectively. 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.

3.5. 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 for LDH, for AST and for CK.

3.6. Lipid peroxidation

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). 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.

3.7. 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.

3.8. Determination of DNA single-strand breaks

Single-strand DNA breaks were determined by the alkaline fluorescence analysis of DNA unwinding as described before by Javcek and Birnboim.

3.9. Assay of NAD⁺

The concentration of NAD^+ in the neutralized perchloric acid extract of the cardiac muscle was measured by using alcohol dehydrogenase reaction. The freshly prepared reaction buffer contained 0.1M Tris, pH 8.40, 1 mM EDTA, 4 mM L-cysteine chloride and 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.

3.10. Direct scavenger effect of antioxidant compounds

Reactive oxygen species (ROS) formation was detected using the oxidation-sensitive non-fluorescent probe dihydrorhodamine 123 (DHR) which can be oxidized by ROS to fluorescent rhodamine 123. 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 antimycin A, 1 μM dihydrorhodamine 123 and, in some experiment, 1 mM sodium nitroprusside.

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.

3.11. 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-aminobenzoic 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.

3.12. Isolation of mitochondria and mitochondrial inner membrane

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

3.13. Measurement of mitochondrial enzyme activity

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

3.14. NMR spectroscopy

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

³¹P 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 ($\gamma B_2=1.2$ kHz) during acquisition. Field homogeneity was adjusted by following the ¹H signal ($w_{1/2}=10-15$ 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 ³¹P experiments.

3.15. 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.

4. CONCLUSIONS

4.1. ANTIOXIDANT COMPOUNDS

H-2545 in the concentration of 10 μ M 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 damages.

4.2. PARP INHIBITORS

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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I. Book chapters

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