THE ROLE OF REACTIVE OXYGEN SPECIES AND ADP-RIBOSYLATION IN THE OXIDATIVE MYOCARDIAL CELL INJURY

(Ph.D. thesis)

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1. INTRODUCTION

1. 1. THE OXIDATIVE STRESS AND THE HEART

The past decade has seen an explosion of knowledge regarding the role of oxidative stress in the pathogenesis of atherosclerosis, ischemia, reperfusion injury, cancer, chronic inflammation, autoimmun diseases and aging (1,2). Our work will be centered on the changes of intracellular signalling caused by oxidative stress which is generated either by ischemia-reperfusion or by a pharmacological compounds.

Sources of ROS in living cells are represented by physiological enzymatic mechanisms. They are generated from aerobic metabolism that utilises life-sustaining oxygen to oxidise fuels. Besides reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide and hydroxyl radicals, there are also other sources of oxidative stress like singlet oxygen, peroxides, reactive aldehydes, nitric oxide, and other reactive species. 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. Being major producers of ROS, mitochondrial structures are exposed to high concentrations of ROS and therefore be particularly susceptible to their attack. Damage by oxidative stress to mitochondrial components includes lipid peroxidation, protein oxidation and mitochondrial DNA (mtDNA) damage. Of special interest is damage to mtDNA, since this small molecule is extremely susceptible to oxidative damage: being located in the matrix, it is close to the major source of ROS; moreover, lacking introns and being devoid of histones and other DNA-associated proteins, the probability of oxidative modification of a coding region of

mtDNA is very high. Mitochondrial structural genes encode for 13 polypeptide chains belonging to those complexes of the inner mitochondrial membrane which are involved in the transmembrane movement of protons. Thus, the phenotypic consequence of a mtDNA mutation must be a defect in the oxidative phosphorylation machinery of mitochondria and as a consequence can be resulted in decreased ATP synthesis (3).

Assessments of overall oxidative stress must also include other sources of free radicals existing in the cytosolic compartment. Various cytosolic oxidases and dehydrogenases (xanthine oxidase, lipoxygenase e. g.) are known to produce free radicals. Newly added to the list of widely distributed cytosolic enzymes is either constitutive or inducible nitric oxide synthase which produces NO. A major consequence of oxidative stress is the lipid peroxidation and their by-products, the reactive aldehydes which show unusual and diverse reactions. They are known to react with proteins by attacking histidine residues and other amino acids, and 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 be major contributing players in oxidative stress (4).

1. 2. PROTECTION AGAINST OXIDATIVE STRESS IN THE MYOCARDIAL CELL

Cells have developed various enzymatic and nonenzymatic systems to protect them from oxidative damages. Superoxide anion can be scavenged by superoxide dismutase, an enzyme present in all aerobic organisms, which catalyses the conversion of two of these radicals into hydrogen peroxide and molecular oxygen:

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

The hydrogen peroxide formed by superoxide dismutase and by the uncatalysed reaction of hydroperoxy radicals is scavenged by catalase, a ubiquitous hem protein that catalyses the conversion of hydrogen peroxide into water and molecular oxygen:

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

Peroxidases also hem enzymes, catalyse 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 also plays a key role in detoxification by reacting with hydrogen peroxide and organic peroxides. Glutathione, present at high levels (5 mM) in animal cells, serves as a sulfhydryl buffer. It cycles between a reduced thiol form (GSH) and an oxidised form (GSSG) in which two tripeptides are linked by a disulphide bond. GSSG is reduced to GSH by glutathion reductase, a flavoprotein utilising NADPH as the electron source. The ratio of GSH to GSSG in most cells is greater than 500 (5). In addition to GSH, other biological thiols could, 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 glutathion are water-soluble antioxidants which regenerate alfa-tocopherol. However, the protective effect of ascorbate and GSH against oxidative attack is attenuated or even reversed in the absence of vitamin E in the membranes (6).

Ubiquinone (coenzyme Q), in addition to its function as an electron and proton carrier in

mitochondrial and bacterial electron transport coupled to ATP synthesis, acts in its reduced form (ubiquinol) as an antioxidant, inhibiting of 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. Physiological compounds, such as urate, bilirubin, 5-hydroxytriptophan, 5-hydroxyindole and DOPA, can also protect against oxidative attack (7).

1. 3. THE REPERFUSION INJURY OF THE HEART

There are certain circumstances when the oxidative threats exceed the capacity of the defence system causing an oxidative stress to the cell. It has been clearly established that myocardial cells cannot survive under prolonged conditions of severe ischemia. Coronary reperfusion therefore appears to be the only appropriate therapeutic strategy. However experimental studies have clearly demonstrated that, although early reperfusion may prevent or lessen the development of necrosis, it is accompanied by various characteristic disturbances, generally referred to as reperfusion injury or reperfusion syndrome. Among the manifestations of reperfusion injury is cardiac stunning, which may persist for several days after reperfusion. Reperfusion of the ischemic myocardium is also frequently associated with the development of arrhythmia. In both cases the pathophysiological mechanisms involved have not yet been fully elucidated. A role for cytosolic free calcium overload and/or an overproduction of reactive oxygen species has been proposed (8). ROS can initiate lipid peroxidation (9), protein oxidation (10) and DNA damages, most frequently resulted in the formation of single-stranded DNA-breaks (DNA-SSB) (11-14).

4. THE ROLE OF POLY-ADP-RIBOSE POLYMERASE IN THE MYOCARDIAL CELL DURING REPERFUSION INJURY

If ROS concentrations are permanently high they can 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 the enzyme and the extent and number of DNA strand breaks in most tissue (15). PARP is a chromatin-bound, monomeric 113 kDa Zn2+-finger protein, present abundantly in the nucleus in an inactive form (16,17). The exact range of functions of PARP has not been established, although the enzyme is thought to play a role in different DNA related processes, such as replication, gene expression, and repair (18,19). PARP utilises NAD as a substrate to transfer up to 100 ADP-ribose groups to a variety of nuclear proteins, including histones as well as PARP itself. The normal function of the protein is restored after catabolism of the poly(ADP-ribose) polymer by another nuclear enzyme, poly(ADP-ribose) glycohydrolase. When DNA damage is extensive the net effect of this metabolism is the rapid lowering of the cellular NAD^+ pool. In efforts to resynthesize NAD, ATP may also be depleted leading to cell death due to energy depletion (20,21). Therefore, inhibition of PARP can, partially, prevent ROS toxicity (22,23) and the ischemia-reperfusion induced cell death (22). Therefore PARP inhibitors are widely used to protect cells from oxidative damages (24,25) and the disruption of PARP gene increases the tolerance against oxidative damages (13,19).

Mitochondria play a significant role in oxidative cell damage because mitochondrial respiratory complexes are the major source of ROS in postischemic hearts (26,27). ROS can induce mitochondrial permeability transition leading to apoptotic and necrotic cell death

(28,29). In addition, ROS and peroxynitrite can suppress the mitochondrial respiration causing impaired energy metabolism and cell death, but this process can be partially reverted by PARP inhibitors indicating a connection between oxidative mitochondrial damage and PARP activation (30-31).

1.5. THE ROLE OF MONO-ADP RIBOSYLATION IN THE MYOCARDIAL CELL DURING STRESS SITUATIONS

Mono-ADP-ribosylations occur mainly outside the nucleus. This may be the mechanism of regulation of certain enzymes and regulatory proteins. It is a reversible process, and both synthetic enzymes, the mono-ADP-ribosyltransferases, and degrading enzymes, the mono-ADP-ribosylhydrolases have been characterised. These enzymes are specific for the bond formed between the amino acid and the ADP-ribose group. Arginine, cysteine and diphtamide specific ADP-ribosyltransferases exist. Various G proteins have been shown to be modified by endogenous ADP-ribosylation, and this alters their activity. In many other cases, though, the exact nature of the acceptors or how the ADP-ribosylation reaction itself is regulated is not known (32). Previous results have demonstrated the presence of a 52 kDa ADP-ribosylation product which is modified at an arginine residue in the adult rat heart plasma membrane (15,33).The mono-ADP-ribosylation of a 39 kDa cytosolic protein in rat heart cytosol increases upon treatment with nitrous oxide-generating agents.(15) It is also known that GRP78 (the 78 kDa glucose-regulated protein also known as BIP, the immunoglobulin heavy chain-binding protein) is post-translationally modified by ADP-ribosylation. GRP78 is a highly conserved member of the stress protein family that resides within the lumen of the ER. GRP78/BIP is believed to bind transiently to nascent proteins as

they are translocated into the ER, facilitating the attainment of the correct conformation of these proteins destined for surface expression or secretion. The ADP-ribosylated and unmodified forms of GRP78 are functionally different. Only the unmodified form of GRP78 is functional and capable of binding to ER proteins (33-36).

The protection of cells from oxidative damage can be based on the reduction of ROS level (by antioxidants (37-41) and by ROS scavenging enzymes, or by the reduction of ROS-induced cell injury or adverse reactions (42-45).

In the first part of my work the protective effect of lipoamide, as an antioxidant against oxidants-induced cell injury during ischemia-reperfusion, in the second part BGP15 as a novel PARP inhibitor and in the third part the harmful side effects of AZT causing an oxidative burden on the cell metabolism will be discussed.

2. ENHANCED ADP- RIBOSYLATION AND ITS DIMINUTION BY LIPOAMIDE FOLLOWING ISCHEMIA-REPERFUSION IN PERFUSED RAT HEART

The ischemia-reperfusion induced oxidative cell damages, and changes in the intracellular signalling mediated by reactive oxygen species (ROS) and peroxynitrite were studied in Langendorff heart perfusion system. Ischemia-reperfusion significantly increased ROS formation *in vitro* and *in situ* in perfused hearts, and activated the nuclear poly-ADP-ribose polymerase which increased the rate of NAD^+ catabolism. Cytoplasmic ADP-ribosylation reactions, such as the mono-ADP-ribosylation of glucose regulated protein (GRP78/BIP) was also activated by ischemia-reperfusion as determined by Western blot analysis with antibody against ADP-ribose. ADP-ribosylation causes the inactivation of GRP78, and so it may affect the protein transport and folding in the lumen of ER in postischemic heart. The presence of lipoamide in the perfusate significantly decreased the ischemia-reperfusion induced ROS production and single-strand DNA breaks, and reduced the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase, the ADP-ribosylation of the GRP78 and the NAD^+ catabolism. In perfused hearts lipoamide diminished the ischemia-reperfusion induced lipid peroxidation as determined by measuring the free malondialdehyde production, and helped to maintain the cell membrane integrity as judged by the release of intracellular enzymes (LDH, CK and GOT). These data show that the reduced form of lipoamide can modulate the ROS-induced signalling through poly- and mono-ADP-ribosylation reactions, the NAD^+ catabolism, and improve the recovery of postischemic myocardium.

Previous works indicated that lipoamide influences the substrate selection of the postischemic heart by promoting the carbohydrate utilisation *versus* fatty acid oxidation, increases the rate of citric acid cycle and improves the recovery of postischemic myocardium (46).

In this work we studied the mechanism by which ischemia-reperfusion regulates signalling through mono- and poly-ADP-ribosylation of cardiac proteins in a Langendorff heart perfusion system. In addition, ischemia-reoxigenation-induced ROS production, lipid peroxidation, DNA breaks, NAD^+ catabolism and changes of the membrane integrity were also studied, addressing the question how lipoamide regulate ROS-mediated signalling in heart perfusion model.

2. 1. MATERIALS AND METHODS

2.1.1. Chemicals

NAD, DL-Lipoamide (DL-6,8-thioctic acid amide), DL-Lipoic acid (DL-6,8-thioctic acid), dihydrorhodamin123 (DHR), and IgG peroxidase were purchased from Sigma Chemical Co. Malondialdehyde-bis-(diethylacetal) was obtained from Merck (Darmstadt, Germany). Anti-ADP-ribose antibody was a kind gift from Alexander Buerkle (Heidelberg, Germany) and Masanao Miwa (Tsukuba, Japan). DnaK antibody was a kind gift from Thomas Langer (Marburg, Germany). LMW Electrophoresis Kit was purchased from Pharmacia. DL-Dihydrolipoic acid and DL-dihydrolipoamide were prepared as described previously (46). All other reagents were of the highest purity commercially available.

2.1.2. Animals

The heart of adult male Wistar rats weighing 300-350g were used for Langendorff heart perfusion experiments. All animal experiments were conducted in conformity with the guiding principles in the care and use of animals.

2.1.3. Heart perfusion

Rats were anaesthetised with ketamine (200 mg/kg i.p.) intraperitoneally and heparinized with sodium heparin (100 IU/rat i.p.). Hearts were perfused via the aorta according to the Langendorff method (47) 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 $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose and 0.6 mM octanoic acid supplemented with 50 mM lipoamide in the case of the treated group. The perfusate was bubbled with a 95% O_2/ 5% CO_2 through a glass oxigenator and adjusted to pH 7.40.

After a stabilisation period of 15 minutes, hearts were either perfused under normoxic conditions for 15 or 30 minutes, or were subjected to a global ischemia of 1 hour 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.

2.1.4. Assay of NAD^+

The concentration of NAD^+ in the neutralised perchloric acid extract of the cardiac muscle was measured by using alcohol dehydrogenase reaction (48). 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 ml of the tissue extract, 650 ml 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.

2.1.5. ADP-ribosylation assay

50 mg cardiac muscle was homogenised with Ultra-Turrax in 500 ml 50 mM Tris pH 7.80, and 500 ml 2X Laemmli sample buffer was then added, homogenised with Potter and cleared by centrifugation for 5 minutes at 10.000 rpm. In some experiments the extraction buffer contained 8 M Urea, 20 mM Tris 4 mM EDTA. Samples were subjected to SDS-polyacrylamid gel electrophoresis using a 10 % gel and blotted to nitro-cellulose membrane for Western blot analysis. ADP-ribosylated proteins were detected by anti-ADP-ribose monoclonal antibody and anti-mouse IgG peroxidase complex and visualised by enhanced chemiluminescence (ECL) method.

2.1.6. Isolation of nuclei

The isolation of nuclei from cardiac tissue was carried out by using standard extraction procedure (49). The purified nuclei were prepared for dot blotting using extraction buffer containing 8 M Urea, 20 mM Tris, 4 mM EDTA and 2X Laemmli sample buffer. The immune reaction was carried out as it is described in the ADP-ribosylation assay.

2.1.7. Immunoaffinity purification of GRP78

DnaK antibody was coupled to protein A sepharose beads and incubated with detergent solubilized endoplasmic reticulum proteins (50) at room temperature for 30 minutes. The gel was washed 3 times with 20 mM Tris/HCl buffer pH 7.4 containing 150 mM NaCl to remove unbound proteins. The antigen-antibody complex was removed with equal volume of 2X Laemmli sample buffer, and used for SDS-polyacrylamide gel electrophoresis and followed by Western blot with anti-ADP-ribose antibody.

2.1.8. Detection of ROS

Hydrogen peroxide in the presence of peroxidase, iron or cytochrome C, and hydroxyl radical as well as peroxynitrite oxidise the nonfluorescent dihydrorhodamine123 (DHR) to form fluorescent rhodamine123 (51). In normoxic hearts after 15 minutes washout, DHR (5mM) was added to the perfusate and perfusion was continued for an additional 15 minutes. In the case of hearts subjected to 60 minutes ischemia and 15 minutes reperfusion, DHR (5mM) was added to the perfusate just before reperfusion. In all cases, hearts were perfused with DHR for 15 minutes, and freeze clamped at the end of perfusion. For the extraction of rhodamine123, 90 mg of heart tissue were homogenised in 2 ml deareated ice cold 20 mM Tris buffer pH 7.40 twice for 20 seconds with Ultraturrax homogenizator and an equal amount of ice cold 70 % ethanol containing 0.1 M HCl was then added. The precipitated proteins were removed by centrifugation at 3000 g for 15 minutes. The precipitate was extracted once again, and the unified supernatants aliquots were neutralised with $NaHCO_3$ and centrifuged at 6000 g. The rhodamine123 content in the clear supernatant was determined using a Perkin Elmer fluorescence spectroscope at an excitation wavelength

of 500 nm and an emission wavelength of 536 nm. To control the extraction procedure, 90 mg of heart tissue (which were perfused without DHR) were homogenised in 2 ml 20 mM Tris buffer pH 7.40 containing 5mM DHR and extracted under the conditions described above. The amount of fluorescent rhodamine123 formed under these condition was in the range of the 3-7 % of the rhodamine123 formed in normoxic heart, and these values were substracted from the values obtained in the different hearts having perfused with DHR. To control the effectiveness of extraction, 90 mg of heart tissue (which were perfused without DHR) were homogenised in 2 ml 20 mM Tris buffer pH 7.40 containing 1mM rhodamine123 and extracted under the conditions described above, and the quantity of rhodamine123 fluorescence was compared to the fluorescence of rhodamine just diluted and neutralised in the above buffer. These data showed the 91-96 % of the rhodamine was extracted from the heart homogenate with the procedure described above.

ROS were also detected under *in vitro* conditions in heart tissues following normoxic perfusion (30 minutes), and in heart tissues deriving from hearts subjected to one hour ischemia. In both cases heart tissue (50 mg) was homogenised in 3 ml well oxygenated buffer containing 150 mM KCl, 20 mM Tris, 0.5 mM EDTA, 1 mM $MgCl_2$, 5 mM glucose and 0,5 mM octanoic acid pH 7.4 at 4° C, and incubated in the presence of 5 mM DHR for 30 minutes at 37 °C. The reaction was stopped by the addition of equal amount of ice cold 70 % ethanol which contained 0.1 M HCl and the formed rhodamine123 was extracted as described in the case of in situ assays. To correct background fluorescence, samples were incubated under the same conditions but without DHR, and the 5 mM DHR was given to tissue only at the end of the incubation period.

2.1.9. Determination of mitochondrial ROS production

Rat heart mitochondria were isolated as described before (52). Mitochondria were incubated in 20 mM Tris/HCl buffer, pH 7.4 containing 250 mM sucrose, 1 mM EGTA, 1 mM $MgCl_2$, 30 mM palmitoyl-CoA, 1 mM carnitine, 1 mg albumin/ml, 0.3 mM ADP and variable amount of lipoamide or lipoic acid. The mitochondrial suspension was stirred and the ROS production was continuously monitored by following the oxidation of DHR to rhodamine123 in a Perkin Elmer fluorescence spectroscope at an excitation wavelength of 500 nm and an emission wavelength of 536 nm.

2.1.10. Determination of DNA single-strand breaks

Single-strand DNA breaks were determined by the alkaline fluorescence analysis of DNA unwinding as described by Birnboim and Jevcak (53). DNA samples were prepared from normoxic and ischemic 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. At the determination of F value, DNA was kept at pH 12.4 to permit partial unwinding of DNA. At the determination of F_{min}, DNA was kept at pH 12.4 but at the beginning of the incubation period DNA sample was sonicated for 60 sec. At the determination of 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° C followed by 15 minutes at 15° C. Unwinding was stopped by adjusting the pH to pH 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 (15).

2.1.11. Lipid peroxidation

Lipid peroxidation was estimated from the direct determination of free malondialdehyde (MDA) by high performance liquid chromatography in the neutralised acid-extract of the heart tissue and in the perfusate after acetonitrile addition as described by Lazzarino (54, 55), using a Tosoh, TSK-6011 HPLC system and a C-18, reverse-phase, 25 cm x 4.6 mm column. The absorption was measured at 266 nm.

2.1.12. Assessment of cell membrane integrity

The release of lactate dehydrogenase EC 1.1.1.27 (LDH), creatine kinase EC 2.7.3.2 (CK) and glutamate oxaloacetate transaminase EC 2.6.1.1 (GOT) enzymes were measured in the perfusate of Langendorff perfused hearts under normoxic and ischemic conditions. Enzyme activities were measured by standard methods as described in (56) for LDH, (57) for GOT and (58) for CK.

2.1.13. Dihydrolipoamide determination

Samples were collected from the efflux of perfused hearts and were treated with monochloroacetic acid in 50% methanol as described in (59). HPLC determination of lipoamide and dihydrolipoamide (its monochloroacetic acid adduct) were carried out on a Supelcosil LC-18 5mm column, 25x0.46 cm (Supelco Bellefonte, PA) exactly as described in (59), except we used a 50 ml injection loop and light absorbance detection at 210 nm.

2.1.14. Statistical Analysis

Significant differences ($P<0.05$) between means were determined with the paired t test. Unless otherwise stated, values are presented as mean ± SEM.

2. 2. RESULTS

2.2.1. ROS determination in perfused heart

Hydrogen peroxide in the presence of peroxidase, iron or cytochrome C, and hydroxyl radical oxidise DHR to form fluorescent rhodamine123 (51). Since DHR is cell permeable and the oxidised rhodamine123 is preferentially retained by the mitochondria, the above system seems to be appropriate to detect ROS *in situ* in perfused heart. The oxidation of DHR to rhodamine123 is detectable in normoxic perfused hearts, and the oxidation of DHR to rhodamine123 by ROS is significantly increased as a consequence of ischemia-reperfusion (Table 2.1.).

DHR can be oxidised to rhodamine123 not only by ROS but also by peroxynitrite which can be formed from superoxide and NO (51). To determine the contribution of peroxynitrite to rhodamine123 formation, we perfused hearts with MMArg a well known NO-synthase inhibitor (60) which caused only very small, statistically non-significant decrease in the rhodamine123 production indicating that NO-related compounds like peroxynitrite has only a very small contribution to the oxidation of DHR to rhodamine123. That is, under our experimental conditions, the DHR-rhodamine123 system detects ROS levels. Perfusion of hearts with lipoamide significantly decreased the ischemia-reperfusion induced rhodamine123 formation (Table 2.1.) showing that the intracellularly formed dihydrolipoamide is an excellent antioxidant, directly decreasing the steady-state level of ROS in postischemic hearts.

The dihydrolipoamide concentrations were determined in the perfusate with the method described in (38), and found that the dihydrolipoamide concentration is gradually increasing

with the perfusion time in the efflux of perfused hearts (7.1±1.8μM at 10 minutes perfusion, 11.5±2.3μM at 20minutes perfusion and 15.3 ± 1.8 μM at 30 minutes perfusion). ROS levels were also determined under *in vitro* conditions by the same method from normoxic hearts, from reperfused hearts and reperfused hearts in the presence of lipoamide (Table 2.1.). The same conclusion can be drawn from these data as from *in situ* data.

Table 2.1.
Effect of lipoamide on free radical formation and single-strand DNA breaks during ischemia and reoxigenation cycle in Langendorff perfused rat heart system. ROS formation was followed by the oxidation of DHR to rhodamine123. Under *in vitro* conditions hearts were freeze clamped after 1 hour ischemia and handled as detailed in "Materials and Methods". Under *in situ* conditions the perfusate contained 5 μM DHR during reperfusion period and then treated as described in "Materials and Methods". DNA single-strand breaks were determined by the alkali unwinding assay. Values are given in the percent of non-damaged DNA. Values are mean ± SEM for five heart preparations.

	Rhodamine 123 fluorescence in arbitrary units		Non-damaged DNA
	in situ	*in vitro*	%
Normoxic	16.2±1.2	30.5±2	71±7
Ischemia- reoxigenation	21.1±1.3*	54.2±3*	20±6*
Ischemia-reoxigenation + 1 mM MMArg	20.3±1.2	23.1±5	
Ischemia-reoxigenation + 50 μM lipoamide	2.8±1.7**	5.7±2**	91±6**

* Values are different from the corresponding normoxic values at the significance of $p<0.01$.
**Values are different from the corresponding ischemia-reoxigenation values at the significance of $p<0.001$.

2.2.2. Single-strand DNA breaks in postischemic hearts

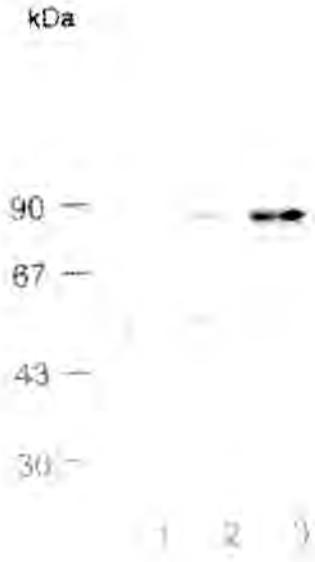
ROS formed under ischemia-reperfusion cycle can damage DNA, resulting most frequently in DNA single-strand breaks. In normoxic perfused hearts most of the DNA is undamaged (Table 2.1.). Ischemia-reperfusion induced large amount of single-strand DNA breaks and the percentage of undamaged DNA was under 20 % in our Langendorff heart perfusion system (Table 2.1.). Perfusing the hearts with MMArg a known NO-synthase inhibitor (60)

did not have significant effect on the ischemia-reperfusion induced DNA damage (Table 2.1.). The perfusion of rat hearts with lipoamide significantly decreased the amount of ischemia-reperfusion induced single-strand DNA breaks, and the percentage of undamaged DNA rose up to 91 % (Table 2.1.).

2.2.3. Effect of ischemia-reperfusion on the mono-ADP-ribosylation of cardiac proteins

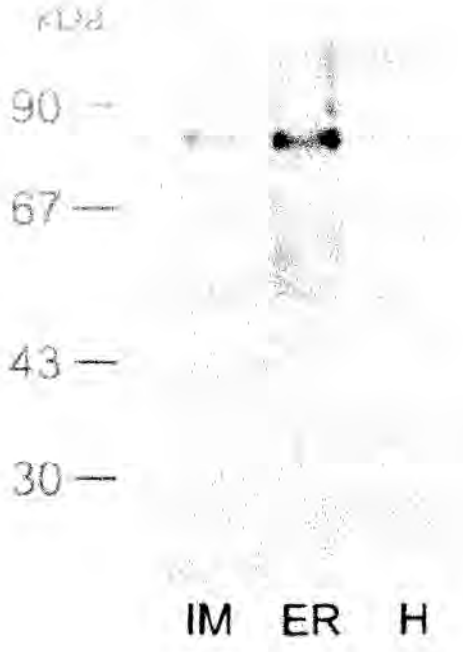
Previous reports indicated the occurrence of mono-ADP-ribosylated proteins (Mw. 78 and 52 kD) in rat heart (32, 61). These ADP-ribosylated proteins were detectable under our experimental conditions using anti-ADP-ribose antibodies in Western blotting (Fig.2.1.A).

Fig. 2. 1. A. Effect of lipoamide on the ischemia-reperfusion induced ADP-ribosylation of cytoplasmic proteins in cardiomyocytes. Western blot analysis of ADP-ribosylation of cytoplasmic proteins in cardiomyocytes with anti-ADP-ribose antibody; 20 μg protein from normoxic heart Lane 1, 20 μg protein from ischemic heart Lane 2, 20 μg protein from postischemic heart (for 30 min) in the absence of lipoamide Lane 3, and 20 μg protein from postischemic heart in the presence of 50 μM lipoamide Lane 4. Protein was extracted as described under "Materials and Methods".



Western blot analysis of ADP-ribosylated heart proteins revealed that ischemia-reperfusion increased the ADP-ribosylation of the 78 kD protein. However, the ADP-ribosylation of the 52 kD protein did not changed significantly. On the basis of previous works (15, 33), we supposed that the 78 kD ADP-ribosylated protein may be the glucose-regulated protein called GRP78 or BIP (33) and belongs to the HSP70 family.

Fig. 2.1.B. Identification of the 78 kDa ADP-ribosylated protein by immunoprecipitation followed by Western blot using antibodies against ADP-ribose. Lane (IM) immunoprecipitated protein from endoplasmic reticulum with anti-DNA-K antibody. Lane (ER) endoplasmic reticular proteins (10 μgram). Lane (H) total heart tissue proteins (10 μgram).



To prove this, we immunoprecipitated members of the HSP70 family from isolated endoplasmic reticulum with DnaK antibody, precipitating the GRP78.

The ADP-ribosylation of the immunoprecipitated protein was detected with

anti-ADP-ribose antibodies by Western blotting (Fig. 2.1.B). These data show that the 78 kD ADP-ribosylated protein indeed belong to the HSP70 protein family, and it is likely identical to GRP78.

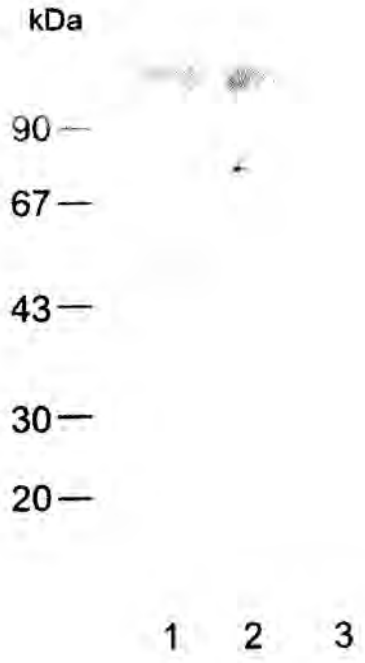
The ischemia-reperfusion significantly increased the ADP-ribosylation of GRP78 in our Langendorff heart perfusion system (Fig. 2.1.A), but the perfusion of hearts with 50 μM lipoamide prevented the ischemia-reperfusion induced ADP-ribosylation of GRP78. In fact, the ADP-ribosylation of GRP78 was under the normoxic values (Fig. 2.1.A).

2.2.4. Activation of poly-ADP-ribose polymerase by ischemia-reperfusion

The nuclear poly-ADP-ribose synthetase could not be extracted by standard extraction procedure from rat heart tissues. However, if the extraction buffer contained 8 M urea (see in Materials and Methods), then the ADP-ribosylation of the high molecular weight nuclear poly-ADP-ribose synthetase become readily detectable with Western blot analysis (Fig. 2.2.). The self ADP-ribosylation of poly-ADP-ribose polymerase (Mw. ~116 kD) was increased by ischemia-reperfusion (Fig. 2.2.) which is in accord to the increased single-strand DNA breaks seen in postischemic hearts. The presence of lipoamide in the perfusate significantly decreased ROS level and the single-strand DNA breaks in the reperfused hearts, and decreased the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase (Fig. 2.2.).

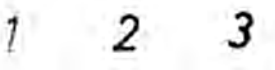
It is possible that a fraction of poly-ADP-ribose polymerase is modified with very large amount of ADP-ribose and it is not able to penetrate into the polyacrylamide gel, and so undetectable with Western blot analysis. To overcome this problem, nuclei were isolated from rat hearts and the poly-ADP-ribose content of nuclei was determined by dot-blot

Fig. 2. 2. Ischemia-reperfusion induced self poly-ADP-ribosylation of nuclear poly-ADP-ribose synthetase. (A). Proteins were separated with SDS polyacrilamide (10 %) gel electrophoresis, and the level of ADP-ribosylation were determined by Western blotting using antibodies developed against ADP-ribose. Extraction of heart proteins were performed in the presence of 8 M Urea, see "Materials and Methods". Lane 1, Normoxic heart (10 μgram protein). Lane 2, Postischemic heart (1 hour ischemia and 30 min. reperfusion) (10 μgram protein). Lane 3, Postischemic heart (1 hour ischemia and 30 min. reperfusion) in the presence of 50 μM lipoamide (10 μgram protein).



analysis (Fig. 2.2.B). These data led to the same conclusion that was drawn from the results of Western blotting (Fig. 2.2.A).

Fig. 2. 2. Ischemia-reperfusion induced self poly-ADP-ribosylation of nuclear poly-ADP-ribose synthetase. (B) ADP-ribosylation of nuclear poly-ADP-ribose-polymerase were also detected by dot blot analysis. Experimental conditions are described under "Materials and Methods". Protein concentrations were 8, 4, 2, 1 μgram in each lanes. Nuclei derived from: Control heart muscle. Lane 1. Heart muscle after ischemia reoxigenation. Lane 2. Heart muscle after ischemia-reoxigenation in the presence of 50 μM lipoamide. Lane 3.



2.2.5. Ischemia-reperfusion induced NAD^+ catabolism in perfused rat hearts

The activation of poly-ADP-ribose polymerase can cause a significant loss of the intracellular NAD^+. Therefore, we determined the NAD^+ content of heart tissues in normoxic hearts, in ischemic hearts and in the hearts subjected to ischemia-reperfusion (Table 2.2.). It was found that 1 hour ischemia caused only a slight decrease in the NAD^+ + NADH content (Table 2.2.), but a significant decrease was seen in the NAD^+/NADH ratio, as expected (data not shown). One hour ischemia followed by 5, 30 and 60 minutes reperfusion induced a significant loss of the intracellular NAD^+ (Table 2.2.). The perfusion of hearts with lipoamide provided a partial protection against the ischemia-reperfusion induced NAD^+ loss (Table 2.2.).

Table 2.2.
Effect of lipoamide on the ischemia-reoxigenation induced NAD^+ loss in Langendorff perfused rat hearts at different reperfusion times. Experimental conditions and NAD^+ determination were performed as described under "Materials and Methods". Values are mean ± SEM for five heart preparations.

Conditions	Reperfusion			
	0 min	5 min	30 min	60 min
	μmoles (NAD^+)/gram wet tissue			
Normoxic	0.45±0.02			
Ischemia	0.38±0.03***			
Ischemia-reoxigenation		0.29±0.02*	0.26±0.01*	0.24±0.02*
Ischemia-reoxigenation + lipoamide 50 μM		0.33±0.03**	0.32±0.02**	0.37±0.02**

* Values are different from the corresponding normoxic values at the significance of $p<0.001$.
** Values are different from the corresponding normoxic values at the significance of $p<0.001$. Values at 60 min reperfusion are different from the corresponding ischemia-reperfusion values at the significance of $p<0.02$.
*** Because a significant fraction of NAD^+ was reduced to NADH, under ischemic condition the sum of NAD^+ and NADH was determined.

2.2.6. Lipid peroxidation in postischemic hearts

It is well accepted that ischemia-reperfusion induced lipid peroxidation (62) and the extent of lipid peroxidation can be characterised by the amount of malondialdehyde production (63). Under our experimental conditions, free malondialdehyde was not detectable by HPLC method in the heart tissue neither in normoxic nor in postischemic hearts, probably because of the good membrane permeability and the high reactivity of free malondialdehyde. However, significant quantity of free malondialdehyde was detected in the reperfusion phase, generally in the first couple of minutes (Table 2.3.). Perfusion of the hearts with lipoamide markedly decreased the amount of free malondialdehyde in the perfusate of postischemic hearts (Table 2.3.). The concentration of free malondialdehyde in the perfusate of normoxic hearts was under the detection limit.

Table 2.3.
Effect of lipoamide on the lipid peroxidation and intracellular enzyme release during ischemia and reoxigenation cycle in Langendorff perfused rat heart system.
Lipid peroxidation was estimated by the MDA production, measured in the effluent in the first 5 minutes by HPLC system. Membrane integrity was followed by the determination of intracellular enzyme release into the perfusate after 15 minutes reperfusion. Values are mean ± SEM for five heart preparations.

	GOT	LDH	CK	MDA
		mU/ml		nmol/ml
Normoxic	2 ± 1	1 ± 1	4 ± 2	<1
Ischemia-reperfusion	96 ± 8*	419 ± 36*	148 ± 17*	23 ± 2.7*
Ischemia-reperfusion + 50 μM lipoamide	47 ± 9**	102 ± 14**	80 ± 7**	5.5 ± 2.4**

* Values are different from the corresponding normoxic values at the significance of $p<0.001$.
** Values are different from the corresponding normoxic values at the significance of $p<0.001$.

2.2.7. Release of cytoplasmic enzymes

Membrane damages due to ischemia-reperfusion cause enzyme release from cardiomyocytes. The release of CK, LDH and GOT were determined in normoxic hearts, postischemic hearts and postischemic hearts in the presence of 50 μM lipoamide (Table 2.3.). The release of these enzymes were very low in normoxic perfusion, but ischemia-reperfusion caused a significant release of CK, LDH and GOT into the perfusate (Table 2.3.). When the hearts were perfused with lipoamide, a significantly reduced release of CK, LDH and GOT were seen in the perfusate of postischemic rat hearts (Table 2.3.). The formation of rhodamine123 from DHR probably did not cause significant damages in heart because perfusion of heart with 2 μM rhodamine123 did not cause significant increase in the release of the enzymes comparing to the normoxic values (data not shown).

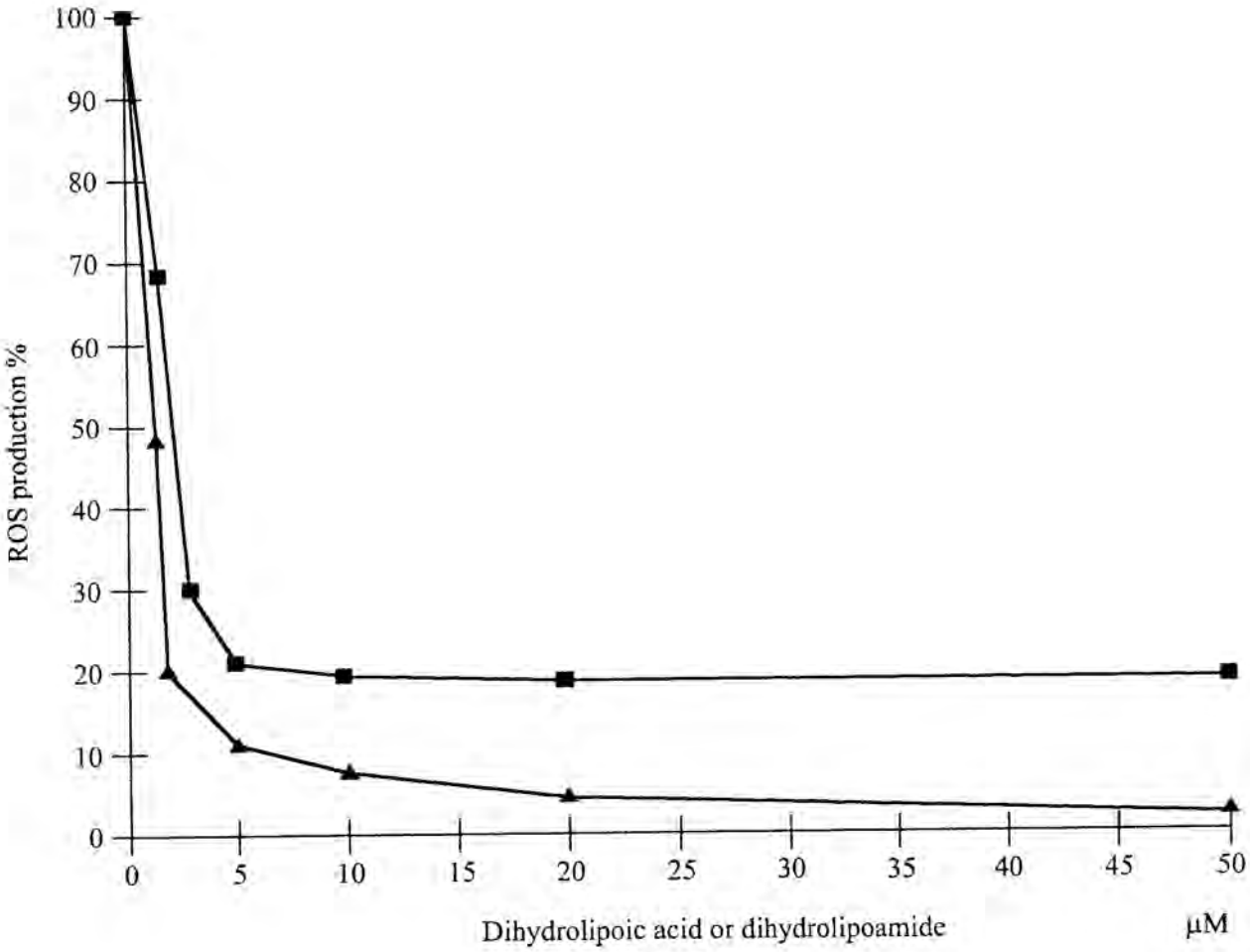
Under our experimental conditions, 4 hearts recovered out of 8 (50%) after ischemia-reperfusion, as compared to 6 out of 7 (85%) when the hearts were reperfused in

the presence of 50 μM lipoamide. These values also indicate the protecting effect of lipoamide against postischemic heart damages.

2.2.8. Inhibition of mitochondrial ROS production by DL-dihydrolipoamide and DL-dihydrolipoic acid

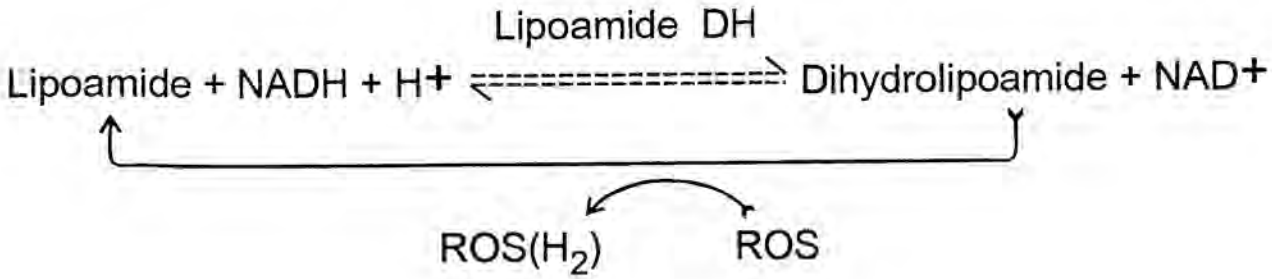
It is known that lipoamide and lipoic acid is reduced in heart tissue and the effective antioxidants are the reduced forms. Therefore, we studied the effect of DL-dihydrolipoamide and DL-dihydrolipoic acid on mitochondrial ROS formation in the 1-50 μM concentration range (Fig. 2.3.). Isolated mitochondria using palmitoyl-CoA plus carnitine as substrate produce ROS which can be determined by the oxidation of DHR to rhodamine123. In this experimental system both DL-dihydrolipoamide and DL-dihydrolipoic acid inhibited the mitochondrial ROS formation, and under our experimental conditions, DL-dihydrolipoamide was found to be a more effective inhibitor of mitochondrial ROS production than DL-dihydrolipoic acid (Fig. 2.3.).

Fig. 2.3. Effect of dihydrolipoamide and dihydrolipoic acid on the mitochondrial ROS formation detected by the oxidation of DHR to rhodamine123. Rat heart mitochondria were incubated in a 20 mM Tris/HCl buffer, pH 7.4 containing 250 mM sucrose, 1 mM EGTA, 1 mM $MgCl_2$, 0.3 mM ADP, 0.03 mM Palmitoyl-CoA and variable amount of dihydrolipoamide or dihydrolipoic acid. The mitochondrial suspension was stirred 30° C and ROS production was continuously monitored by following the oxidation of DHR to rhodamine123 by Perkin Elmer fluorescence spectroscope at an excitation wavelength of 500 nm and an emission wavelength of 536 nm. Effect of dihydrolipoamide, or dihydrolipoic acid on ROS production.



2. 3. DISCUSSION

In a Langendorff heart perfusion model, ischemia-reperfusion increased the formation of reactive oxygen species determined by the oxidation of the nonfluorescent DHR to fluorescent rhodamine123 (Table 2.1.). However, the presence of lipoamide (50μM) in the perfusate diminished the ischemia-reoxigenation induced ROS and peroxynitrite formation - even ROS level was under the normoxic value - probably by inactivating them as suggested in Scheme 2.1.



Lipoamide is converted *in vivo* to dihydrolipoamide which is a very effective antioxidant (Table 2.1.). Even if ROS deoxidise dihydrolipoamide to lipoamide it can be reduced again by the mitochondrial lipoamide dehydrogenase which is in the vicinity of respiratory complexes (64, 65), at the very place where most of the ROS form in postischemic hearts (66). Fig. 2.3. also shows that lipoamide is a better compound to entrap mitochondrially formed ROS than lipoic acid, and that lipoamide has a very low 0.6 μM $I_{0.5}$ for the inhibition of mitochondrial ROS production.

The permanently high level of predominantly mitochondrially formed ROS can affect also other compartments in the cell (like nucleus and ER), and can cause DNA damages in

several forms including single-strand DNA breaks. Under our experimental conditions, we found significant increase in the amount of single-strand DNA breaks as a consequence of ischemia-reoxigenation (Table 2.1.), and a parallel activation of the nuclear poly-ADP-ribose polymerase (Fig. 2.2.). The activation of the nuclear poly-ADP-ribose polymerase (Fig. 2.1.) can affect the whole cellular metabolism by decreasing the intracellular NAD^+ pool (Table 2.2.). The ischemia-reperfusion induced decrease in the nicotinamide adenine dinucleotide pool (Table 2.2.) is probably mainly the consequence of the activation of poly-ADP-ribose polymerase, because the other NAD^+ catabolysing enzymes (such as the NAD^+-glycohydrolase) are not activated under the conditions of ischemia-reperfusion (16, 67). The lack of activation of the nuclear poly-ADP-ribose polymerase in rat heart subjected to ischemia-reperfusion in the presence of lipoamide is probably the consequence of the decrease in the steady-state concentration of ROS (hydroxyl radical and peroxynitrite) (Table 2.1.) and the decreased amount of single-strand DNA breaks (Table 2.1.). The decreased ROS level and low activity of poly-ADP-ribose polymerase in the presence of lipoamide can also explain the smaller damage of cardiomyocytes (Table 2.3.) and the better recovery of postischemic heart (16). These observations are in accord to recent data showing that inhibition of poly-ADP-ribose polymerase significantly decreased the infarct size in hearts subjected to ischemia-reperfusion (25).

Under our experimental conditions, ischemia-reperfusion induces the ADP-ribosylation of GRP78 (Fig. 2.1.). The presence of lipoamide in the perfusate diminished the ischemia-reperfusion-induced ADP-ribosylation of GRP78 so that the level of ADP-ribosylation was under the normoxic values (Fig. 2.1.). It is known that GRP78 (a stress protein) is involved in the binding of transiently nascent proteins as they are translocated into

the ER (36), and facilitates the attainment of correct conformation of these proteins destined for surface expression or secretion (34). The ADP-ribosylation inactivates the GRP78 (35) and so inhibits the protein transport in the ER. The data presented here indicate that reactive oxygen species can regulate the ADP-ribosylation of GRP78 in the perfused heart system which is in accord to the observation in AZT treated rat hearts (68). The high level of reactive oxygen species activated the ADP-ribosylation of GRP78 and lipoamide by decreasing the ROS level diminished the ADP-ribosylation of GRP78 and so can restore the basic function of GRP78, the folding and transport of proteins in the ER.

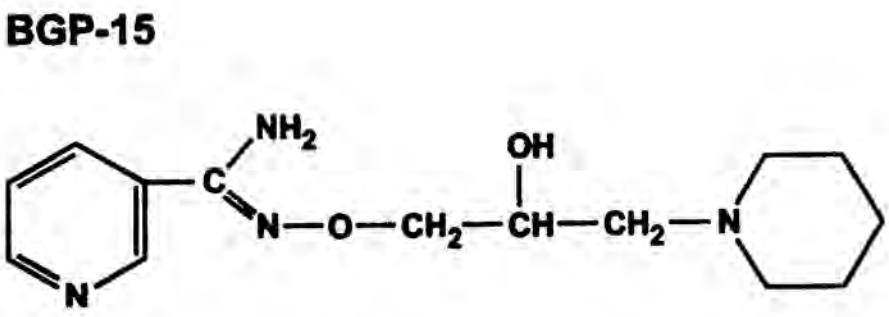
Under our experimental conditions, ischemia-reperfusion induced large increase in malondialdehyde production in perfused hearts which was diminished considerably by lipoamide treatment (Table 2.3.) indicating that the intracellularly formed dihydrolipoamide protected intracellular lipids from peroxidation. Parallel with the lipid peroxidation data, ischemia-reperfusion increased the release of cytoplasmic enzymes in perfused heart (Table 2.3.) which was also markedly decreased by lipoamide treatment, showing that lipoamide protected the membrane integrity of cardiomyocytes from ischemia-reperfusion induced damages. All of these effects of lipoamide treatment can be the consequence of the good antioxidant character of the in vivo formed dihydrolipoamide (Table 2.1.). There are reports in the literature showing that dihydrolipoic acid protects tissues from oxidative damages (69-72) assuming that dihydrolipoic acid reacts with ROS (70, 72). However, it is also known that lipoic acid causes metabolic abnormalities (73) because it can be activated intracellularly to lipoil-CoA depleting this way the free CoA pool. Lipoamide and dihydrolipoamide can not form CoA ester and so they are a non-toxic derivative of lipoic acid with preferential biological application. In addition, dihydrolipoamide can donate, or take up acyl groups

from the mitochondrial α-ketoacid dehydrogenase complexes (46). These properties of lipoamide was utilised in our previous work when it was demonstrated that lipoamide alters the substrate selection of postischemic myocardium by activating the pyruvate dehydrogenase complex and increases the rate of citric acid cycle (46). In spite of these advantageous properties of lipoamide there are almost no available data in the literature about the protecting effect of lipoamide against ROS induced cell damages.

3. BGP-15, A NOVEL POLY(ADP-RIBOSE) POLYMERASE INHIBITOR, PROTECTS HEART FROM ISCHEMIA-REPERFUSION INJURY

The protecting effect of BGP-15, an amidoxime derivative (Fig. 3.1.), against ischemia-reperfusion induced injury was studied in Langendorff heart perfusion system.

Fig. 3. 1. Chemical structure of BGP-15 [O-(2-Hydroxy-3-piperidinepropyl)-pyridine-carbonic acid-amidoxime dihydrochloride].



To understand the molecular mechanism of the cardioprotection, the effect of BGP-15 on the ischemic-reperfusion-induced ROS formation, lipid peroxidation single-strand DNA breaks formation, NAD^+ -catabolism and endogenous ADP-ribosylation reactions were investigated. These studies showed that BGP-15 decreased the reactive oxygen species (ROS) level, lipid peroxidation, membrane damages, single-strand DNA breaks and the rate of NAD^+ catabolism in postischemic hearts. In addition, BGP-15 dramatically decreased the ischemia-reperfusion induced the self ADP-ribosylation of nuclear poly-ADP-ribose polymerase (PARP) and the mono-ADP-ribosylation of ER chaperone GRP78. These data raise the possibility that BGP-15 may have a direct inhibitory effect on PARP which was

verified using isolated enzyme, and the $I_{0.5}$ value for BGP-15 was found to be 0.12 mM.

From these studies we concluded the BGP-15 by reducing the ischemia-reperfusion induced PARP activation decreased the endogenous ROS formation and protected the cell constituents from oxidative damages.

3. 1. MATERIALS AND METHODS

3.1.1. Chemicals

See in chapter 1.1.

3.1.2. Animals

See in chapter 1.1.

3.1.3. Heart perfusion

See in chapter 1.1.

3.1.4. In vitro PARP inhibition on isolated enzyme

The poly-ADP-ribose polymerase was isolated from rat liver based on the method described before (74). The PARP activity was determined in 130 µl reaction mixture contained 100 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$,10 % glycerol, 1.5 mM DTT, [^{3}H], NAD^+ , 10 µg activated DNA and 10 µg histones. The incubation time was 10 minutes, and the reaction was stopped by the addition of trichloroacetic acid (8 %). After addition of 0.5 mg albumin, precipitation was allowed for at least 20 minutes on ice, and the protein was

precipitated with centrifugation (10 min, 10.000xg). The precipitate washed three times with 8 % trichloroacetic acid, and the protein-bound radioactivity was determined with Beckman scintillation counter.

3.1.5. Lipid peroxidation

Lipid peroxidation was estimated from the formation of thiobarbituric acid reactive substances (TBARS). TBARS were determined using a modification of the method described in (75). Cardiac tissue was homogenised in 6.5 % TCA and a reagent containing 15% TCA, 0.375 % 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.

3.1.6. Assay of NAD^+

See in chapter 1.1.

3.1.7. Detection of ROS

See in chapter 1.1.

3.1.8. Determination of DNA single-strand breaks

See in chapter 1.1.

3.1.9. ADP-ribosylation assay

See in chapter 1.1.

3.1.10. Myocardial enzyme leakage

See in chapter 1.1.

3.1.11. Statistical Analysis

Significant differences ($P<0.05$) between means were determined with the paired t test. Unless otherwise stated, values are presented as mean ± SEM.

3. 2. RESULTS

3.2.1. Release of cytoplasmic enzymes

The release of CK, LDH and GOT into the perfusate is extremely low from normoxic perfused rat heart showing that the cell membranes are undamaged under conditions of normoxic perfusion (Table 3.1.). Ischemia-reoxigenation caused serious damages in plasma membranes of cardiomyocytes (76), and induced a significant release of CK, LDH and GOT (Table 3.1.). When hearts were pre-perfused for 10 minutes with 40 mg/l BGP-15 under normoxic condition followed by 45 minutes ischemia and 30 minutes reperfusion, a significant reduction was seen in enzyme release (Table 3.1.) showing that BGP-15 partially protected plasma membranes of cardiomyocytes from ischemia-reoxigenation related damages. BGP-15 has a low toxicity (LD_{50} =1203.4 mg/kg intravenously added to rats) therefore it can be used safely in the biologically effective concentration range.

Table 3. 1.
Effect of BGP-15 on intracellular enzyme release during ischemia and reoxigenation cycle in Langendorff perfused rat heart system. Membrane integrity was followed by the determination of intracellular enzyme release into the perfusate after 30 minutes reperfusion. The details of experimental protocols see "under Materials and Methods". Values are mean ±SEM for five heart preparations.

	GOT	LDH	CK
		mU/ml	
Normoxic	2 ± 1	1 ± 1	4 ± 2
Ischemia-reperfusion	96 ± 8*	419 ± 36*	148 ± 17*
Ischemia-reperfusion +40 mg/L BGP-15	33 ± 6	125 ± 10	61 ± 5

*Value different from respective normoxic values and ischemia-reperfusion + BGP-15 values at the significance of $p<0.001$

3.2.2. Detection of reactive oxygen species in vitro and in situ

Several reactive oxygen species reoxidise the non-fluorescent dihidrorhodamine123 to a fluorescent rhodamine123 (51). Since DHR is cell permeable and the oxidised rhodamine123 is retained by the mitochondria, this reaction can be used for the detection of ROS in Langendorff perfused heart. The oxidation of DHR to rhodamine123 is well detectable in normoxic perfused hearts (Table 3.2.), but the oxidation rate is significantly increased as a consequence of ischemia-reperfusion (Table 3.2.). The perfusion of hearts with BGP-15 decreased the ischemia-reperfusion induced rhodamine123 formation (Table 3.2.), showing that BGP-15 decreases the steady-state levels of ROS in postischemic hearts.

Similar data were obtained under *in vitro* conditions when ROS were determined by the same method from normoxic hearts, ischemic hearts and ischemic hearts having BGP-15 in the incubation medium (Table 3.2.).

BGP-15 did not inhibit the oxidation of DHR to rhodamine123 by chemically generated ROS (H_2O_2 and Fe^{+2} ion and chemically generated $ONOO^-$) (data not shown) indicating that the observed decrease in ROS level is not the consequence of a direct interaction between ROS and BGP-15.

3.2.3. Effect of BGP-15 on ROS-induced lipid -peroxidation and DNA breaks

Ischemia-reperfusion induced lipid peroxidation in heart and the extent of lipid peroxidation can be characterised by the formation of TBA reactive materials (75). Under our experimental conditions we found that ischemia-reperfusion increased the amount of TBA reactive substances comparing to the normoxic cases (Table 3.2.), and the perfusion of hearts with BGP-15 followed by ischemia-reperfusion under the same conditions reduced

the ischemia-reperfusion induced TBA reactive substance formation (Table 3.2.).

The ischemia-reperfusion increased ROS concentrations which can contribute to the increase of single-strand DNA breaks. In our experimental system most of the DNA is undamaged in normoxic perfused hearts, but the ischemia-reperfusion induces large amounts of single-strand DNA breaks (undamaged DNA < 20 %) (Table 3.2.). The 10 minutes pre-perfusion of hearts with 40 mg/ml BGP-15 decreased the amount of single-strand DNA breaks and increased the quantity of undamaged DNA in postischemic heart (Table 3.2.).

Table 3. 2.
Effect of BGP-15 on free radical formation, single-strand DNA breaks and the lipid peroxidation during ischemia-reoxigenation cycle in Langendorff perfused rat heart system. ROS formation was followed by the oxidation of DHR to rhodamine123. Under *in vitro* conditions hearts were freeze clamped after 1 hour ischemia and handled as detailed in "Materials and Methods". Under *in situ* conditions the perfusate contained 5 μM DHR during reperfusion period and then treated as described in "Materials and Methods". DNA single strand breaks were determined by the alkali unwinding assay. Lipid peroxidation was estimated by the formation of TBARS. Values are given in the percent of non damaged DNA. Values are mean±SEM for five heart preparations.

	Rhodamine-123 fluorescence in arbitrary units		Non-damaged DNA	TBARS
	in situ	*in vitro*	%	nmol/g wt.
normoxic	21.4 ± 1.7	30.5 ± 3	71 ± 7	59,8 ± 3
ischemia-reoxigenation	28.7 ± 1.8*	54.2 ± 4*	20 ± 6*	78.1 ± 2.5**
ischemia-reoxigenation +40 mg/L BGP-15	22.2 ± 2.1	43.4 ± 2	39 ± 5	70.2 ± 2.7

* Values are different from the other respective normoxic values and ischemia-reoxigenation + BGP-15 values at the significance of $p<0.001$.
** Value different from the respective ischemia-reperfusion + BGP-15 value at the significance of $p<0.05$

3.2.4. Ischemia-reperfusion induced NAD^+ catabolism in perfused rat hearts

Ischemia-reperfusion activates intracellular NAD^+ catabolism and can decrease NAD^+

content to such a low level which can compromise the energy metabolism. Under our experimental conditions ischemia caused only a slight decrease in NAD^+ pool (Table 3.3.), but ischemia followed by 5, 30 and 60 minutes reperfusion induced a significant loss of the intracellular NAD^+ (Table 3.3.). The 10 minutes pre-perfusion of hearts with 40 mg/ml BGP-15 could partially protect the heart against ischemia-reperfusion induced NAD^+ loss (Table 3.3.).

Table 3. 3.
Effect of BGP-15 on the ischemia-reoxigenation induced NAD^+ loss in Langendorff perfused rat hearts at different reperfusion times. Experimental conditions and NAD^+ determination were performed as described under "Materials and Methods". Values are mean±SEM for five heart preparations.

Conditions	Reperfusion			
	0 min	5 min	30 min	60 min
	μmoles (NAD^+)/gram wet tissue			
Control	0.45 ± 0.03			
Ischemia	0.38 ± 0.04**			
Ischemia reoxigenation		0.29 ± 0.03	0.26 ± 0.02	0.24 ± 0.02
Ischemia reoxigenation +40 mg/L BGP-15		0.39* ± 0.03	0.31*± 0.01	0.30*± 0.02

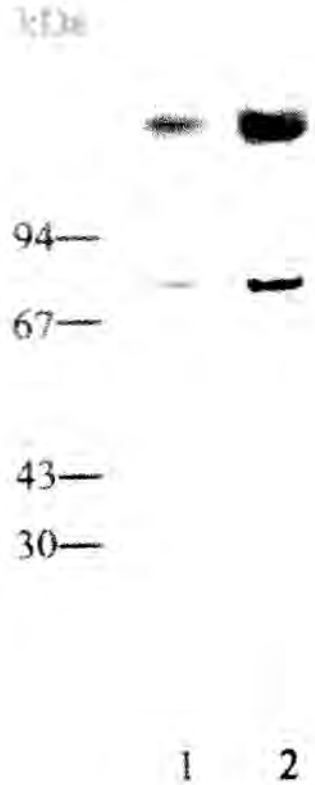
* Value different from values without BGP-15 at the significance of $p<0.02$
** Because a significant fraction of NAD^+ was reduced to NADH, under ischemic condition we determined the sum of NAD^+ and NADH

3.2.5. Ischemia-reperfusion induced ADP-ribosylation of GRP78

Previous reports indicated the occurrence of mono-ADP-ribosylated proteins (Mw. 78 and 52 kD) in rat heart (15, 35). These ADP-ribosylated proteins were detectable under our experimental conditions using anti-ADP-ribose antibodies in Western blotting (Fig. 3.2.). Western blot analysis of ADP-ribosylated heart proteins revealed that ischemia-reperfusion increased the ADP-ribosylation of the 78 kD protein. However, the ADP-ribosylation of the

52 kD protein did not changed significantly. On the basis of previous works (33) we supposed that the 78 kD ADP-ribosylated protein is the glucose-regulated protein called GRP78 (33) and belongs to the HSP70 family. The ischemia-reperfusion significantly increased the ADP-ribosylation of GRP78 in Langendorff heart perfusion system (Fig. 3.2.).

Fig. 3. 2 Effect of BGP-15 on the ischemia-reperfusion induced ADP-ribosylation of cytoplasmic and nuclear proteins in cardiomyocytes. Western blot with antibody against ADP-ribose assay was used to detect ADP-ribosylation in cardiomyocytes in normoxic. Lane 1 (20mg protein), in ischemia and reperfused heart (for 30 min) in the absence of BGP-15, Lane 2 (20mg protein), and in the presence of 40 mg/L BGP-15. Lane 3 (20 mg protein). Extraction of heart proteins occurred in the presence of 8 M Urea, see "Materials and Methods". Proteins of about 116 kDa Mw represent the self ADP-ribosylation of PARP. Proteins of about 78 kDa Mw represent the mono-ADP-ribosylation of the cytoplasmic proteins.



The perfusion of hearts with BGP-15 prevented the ischemia-reperfusion induced ADP-

ribosylation of GRP78. In fact the level of the ADP-ribosylation of GRP78 was under the normoxic values (Fig.3.2.). The GRP78 is involved in the binding of transiently nascent proteins as they are translocated into the ER (33). ADP-ribosylation inactivates GRP78 (49) and inhibits the protein transport into ER, therefore, the inhibition of the mono- ADP-ribosylation of GRP78 can restore the activity of GRP78, as well as the folding and transport of proteins in ER.

3.2.6. Regulation of nuclear poly-ADP-ribose polymerase by BGP-15

The activation of endogenous ADP-ribosylation reactions are the main causes of the ischemia-reperfusion induced NAD^+ catabolism. Therefore, it is worthwhile to study how the ADP-ribosylation of cardiac proteins changes as the consequence of ischemia-reperfusion. The self poly-ADP-ribosylation of PARP can be detected by Western blot as described before. The ischemia-reperfusion in perfused hearts induced the self poly-ADP-ribosylation of PARP (Mw. ~116 kD) comparing to the normoxic hearts (Fig. 3.2.). The pre-perfusion of hearts with BGP-15 showed a clear inhibitory effect on the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase (Fig. 3.2.).

3.2.7. Direct inhibition of nuclear poly-ADP-ribose polymerase

The self ADP-ribosylation of PARP was inhibited by BGP-15 in perfused hearts under *in situ* conditions. (Table 3.2.). This effect of BGP-15 can be either direct or indirect inhibitory effect, and to differentiate between these two alternatives we determine the BGP-15 inhibition on purified PARP under *in vitro* conditions. We found that BGP-15 inhibited the self ADP-ribosylation of PARP under *in vitro* conditions (Table 3.4.), and the $I_{0.5}$ for BGP-

15 was found to be 120 μM on purified PARP.

Table 3.4.
Effect of BGP-15 on the activity of nuclear poly-ADP-ribose polymerase.

BGP-15 or nicotinamide concentration	Enzyme activity
BGP-15	
0	1742 ± 114
10	1260 ± 102
20	703 ± 80
40	219 ± 37
Nicotinamide	
5 mM	138 ± 79

3. 3. DISCUSSION

As it is detailed previously ROS and peroxynitrite generated single-strand DNA breaks induce PARP activation (16, 17) and rapid depletion of intracellular NAD^+ and ATP (21) which are important in the energy metabolism of cells and in the maintenance of healthy mitochondrial function. Therefore PARP inhibitors are widely used experimentally to protect cells from oxidative damages (19, 25, 77, 78) and the disruption of PARP gene increases the tolerance against oxidative damages (77).

In the present work we investigated the effect of BGP-15 (Fig. 3.1.) on ischemia-reperfusion injury of cardiac muscle in Langendorff heart perfusion system. The BGP-15 significantly reduced the release of intracellular enzymes (CK, GOT, LDH) from postischemic cardiomyocytes, indicating a protection against cell membrane damages in postischemic cardiac muscle. Unexpectedly we found that BGP-15 decreased the ischemia-reoxigenation induced ROS formation (Table 3.2.), lipid peroxidation (Table 3.1.) and the amount of single strand DNA breaks (Table 3.2.).

The ischemia-reperfusion increased significantly the self-ADP-ribosylation of PARP and this process was reverted by BGP-15 (Fig. 3.3.) which finding can not be explain with the moderate decrease in single-strand DNA breaks formation (Table 3.2.), but suggesting a direct interaction of BGP-15 with PARP. This idea was further supported by the observation that the 3-pyridinecarboxymimidoil part of BGP-15 show similarity to nicotinamide, a product and an inhibitor of PARP reaction (Fig. 3.1.). The *in vitro* kinetic study on isolated PARP showed that BGP-15 indeed inhibits the PARP catalysed reaction, and $I_{0.5}$ value is equal to 120 μM under our experimental conditions.

Previously it was indicated that externally added oxidants activate PARP and cause mitochondrial damage in thymocytes (79). However, PARP inhibitors significantly reduce oxidative damage of mitochondria and preserve mitochondrial membrane potential (79). Our data show that BGP-15, a novel PARP inhibitor, decreases the ischemia-reperfusion induced endogenous ROS formation, and oxidative damages including lipid peroxidation and the formation of single-strand DNA breaks in cardiac muscle. Since BGP-15 is not a scavenger, the protection of cardiac muscle from oxidative damages can occur by a similar molecular mechanism which was observed by other PARP inhibitors in the case of externally added oxidants in thymocytes (79). Data presented in this paper indicate that PARP inhibitor (BGP-15) besides the inhibition of NAD^+ catabolism and decreasing excess energy consumption can decrease the ischemia-reperfusion-induced endogenous ROS formation providing a novel pathway by which PARP inhibitors protect postischemic cardiac muscle.

4. ROLE OF REACTIVE OXYGEN SPECIES AND POLY-ADP-RIBOSE POLYMERASE IN THE DEVELOPMENT OF AZT-INDUCED CARDIOMYOPATHY IN RAT

The short term cardiac side-effects of AZT (3'-azido-3'-deoxythimidine, zidovudine) were studied in rats to understand the biochemical events contributing to the development of AZT-induced cardiomyopathy. Developing rats were treated with AZT (50 mg/kg/day) for two weeks and the structural and functional changes were monitored in the cardiac muscle. AZT treatment provoked a surprisingly fast appearance of cardiac malfunctions in developing animals characterised by prolonged RR, PR and QT intervals and J point depression. Electron microscopy showed abnormal mitochondrial structure but the cardiomyocytes had normal myofibers. The AZT treatment of rats significantly increased ROS and peroxynitrite formation in heart tissues as determined by the oxidation of nonfluorescent dihydrorhodamine123 and dichlorodihydro-fluorescein diacetate (H_2DCFDA) to fluorescent dyes, and induced single-strand DNA breaks. Lipid peroxidation and oxidation of cellular proteins determined from protein carbonyl content were increased as a consequence of AZT treatment. Activation of the nuclear poly-ADP-ribose polymerase and the accelerated NAD^+ catabolism were also observed in AZT- treated animals. Western blot analysis showed that mono-ADP-ribosylation of glucose regulated protein (GRP78/BIP) was enhanced by AZT treatment, which process inactivates GRP78. In this way moderate decrease in the activity of respiratory complexes was detected in the heart of AZT- treated animals indicating a damaged mitochondrial energy production. There was a significant decrease in creatine phosphate concentration resulting in a decrease in creatine

phosphate /creatine ratio from 2.08 to 0.58. ATP level remained close to normal but the total extractable ADP increased with 45 %. The calculated free ATP/ADP ratio decreased from 340 to 94 in the heart of AZT-treated rats as a consequence of increased free ADP concentration. It was assumed that the increased free ADP in AZT-treated cardiomyocytes may help cells to compensate the defective ATP production in damaged mitochondria by activating the ATP synthesis in undamaged mitochondria. Southern blot analysis did not show decreased quantity of mtDNA deriving from AZT-treated rat hearts indicating that under our experimental conditions AZT-induced heart abnormalities are not the direct consequence of the mtDNA depletion.

These data show that ROS-mediated oxidative damages, activated ADP-ribosylation reactions and accelerated NAD^+ catabolism play basic roles in the development of AZT-induced cardiomyopathy in our animal model and indicated that these ROS-mediated processes can be important factors in the development of myopathy and cardiomyopathy in zidovudine-treated AIDS patients.

AZT (zidovudine) is widely used for the treatment of AIDS patients as the first drug that inhibits the replication of HIV virus at the point of reverse transcription (80, 81). It has also been well documented that AZT has numerous side effects (82, 83). In mammalian cells AZT is phosphorylated giving AZT-triphosphate which directly inhibits the HIV reverse transcriptase (81). Additionally, AZT-triphosphate inhibits the mitochondrial DNA polymerase (polymerase gamma) (84). The AZT metabolites can incorporate into the host DNA (85) inducing premature chain termination in the cell own DNA, especially in the mitochondrial DNA (mtDNA) lacking of DNA repair processes (86). As a result, significant reduction of mtDNA content was seen in the skeletal muscle of long-term zidovudine

treated HIV patients (87) associated with mitochondrial myopathy, leading to the assumption that the depletion of mitochondrial DNA(mtDNA) can be the primary cause of the myopathy (88, 89). The mtDNA encodes 13 polypeptide that are components of the respiratory complexes (90). Therefore, any damage of mtDNA will cause defective mitochondrial protein synthesis which results in abnormal respiratory complexes and impaired oxidative energy production.

There are additional data suggesting that AZT (or its metabolites) has an inhibitory effect on the mitochondrial oxidative energy production that can not be associated with depletion of mtDNA (91). The inhibitory effect of AZT or its metabolites was localised on NADH:citochrome C oxidoreductase while succinate:citochrome C oxidoreductase was not effected (92). Furthermore, there are data suggesting that the primary intracellular metabolite of AZT (AZT-monophosphate) inhibits glycosphingolipid and ganglioside biosynthesis, and suppresses the incorporation of both sialic acid and galactose into proteins (93). These new data indicate that the molecular mechanism of AZT induced cell damage is more complex than expected.

We observed that AZT treatment significantly increased the production of reactive oxygen intermediates (ROS) in rat hearts raising the possibility that ROS mediated processes may play an important role in the cardiotoxicity of AZT. Therefore, we investigated whether AZT treatment induce *in vivo* lipid peroxidation, protein oxidation and the formation of DNA breaks which are the major changes occur at elevated ROS levels. Since DNA breaks activate the nuclear poly-ADP-ribose polymerase (PARP) an enzyme which play an important role in the NAD^+ catabolism and ROS induced cell death (16, 94), the effect of AZT treatment on the endogenous poly- and mono-ADP-ribosylation reaction were also

investigated. These data may provide new evidence for the role of ROS mediated processes including endogenous ADP-ribosylation reactions in the development of the cardiotoxicity of AZT

4. 1. MATERIALS AND METHODS

4.1.1. Materials

AZT, cytochrome c, CoA-SH, NAD, NADH, were obtained from Sigma. EcoR I was purchased from Amersham. Sst I was purchased from BRL. Anti-ADP-ribose antibody was a kind gift from Alexander Buerkle (Heidelberg, Germany) and Masanao Miwa (Tsukuba, Japan). All other chemicals were of the highest purity commercially available.

4.1.2. General Methods

Approximately 80-100 gram rats were intraperitoneally treated daily with AZT (50 mg/kg) for up to 14 days. Schiller AT-6 ECG was used to monitor cardiac function during and after AZT treatment. ECG was recorded with needle electrodes according to the routine technique (6 limb leads) and RR, PR, QT intervals and J point values were determined by standard methods (95).

The treated rats were sacrificed three days after the completion of AZT treatment. We waited three days to assure that the AZT and its derivatives are released to avoid the direct toxic effect of AZT metabolites. In some cases the treated animals were kept alive for a longer period of time to investigate the possible recovery of heart function.

4.1.3. Electron microscopy

Heart muscle specimens were fixed with 3 % glutaraldehyde in 0.1 mol/l MOPS buffer, pH 7.0 for 1 h at 4 °C. The fixed tissues were washed and mixed with 40 mM OsO_4 in 0.1 M phosphate buffer, pH 7.4, and incubated for 1 h at 4 °C. The samples were washed again and dehydrated in a graded ethanol series to 100 % followed by three changes of propylene oxide. Samples were infiltrated with araldite resin which was polymerised at 56 °C for 48 h. The resultant blocks were sectioned at 80 nm and the sections were stained with lead citrate and uranyl acetate. The transmission electron microscopy was performed by a JEM 1200-EX-II electron microscope operated at an accelerating voltage of 80 kV.

4.1.4. Enzyme assays

Cytochrome oxidase (96), NADH:cytochrome C oxidoreductase (96), citrate synthase (97), carnitine acetyl-transferase (97), lactate dehydrogenase (56) and creatine kinase (46) were measured.

4.1.5. Substrate determinations

For substrate determination, hearts were quickly freeze-clamped, and the freeze-clamped tissues were ground under liquid N_2. The ground tissues were extracted with 4 % perchloric acid containing 2 mM EDTA and neutralised with KOH. The concentration of ATP, ADP, AMP, creatine phosphate, creatine were determined with HPLC (98). The free ADP concentration was calculated by assuming that the cytosolic creatine kinase is in equilibrium, and the apparent equilibrium constant for creatine kinase equal to 166 at pH 7.4.

The concentration of NAD^+ in the neutralised perchloric acid extract of the cardiac muscle was measured by using alcohol dehydrogenase reaction (48). 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 ml of the tissue extract, 650 ml of the reaction buffer and 4 units of enzyme. The reaction was initiated by the addition of enzyme and the exact tissue NAD^+ concentrations were determined from a calibration curve.

4.1.6. Detection of ROS

See in chapter 1.1.

4.1.7. Determination of protein carbonyl content with 2,4-dinitrophenylhydrazine

Fifty mg of freeze-clamped heart tissue from control and AZT-treated rats were homogenised with 1 ml 8 % perchloric acid and the protein content was collected by centrifugation. The protein carbonyl content was determined with the 2,4-dinitrophenylhydrazine method (99, 100).

4.1.8. Determination of single-strand DNA breaks

See in chapter 1.1.

4.1.9. Lipid peroxidation

See in chapter 2.1.

4.1.10. ADP-ribosylation assay

See in chapter 1.1.

4.1.11. Southern blot analysis

The mtDNA was prepared from normal rat liver as described in (101). Radioactively labelled DNA probe (^{32}P) was prepared by random primer method using mtDNA as template. Total DNA was prepared from the heart of control and AZT treated animals. Isolated total DNA was digested with Sst I which linearilized the mtDNA and were electrophoresed through 1.5 % agarose gel and transferred to nylon membrane (Hybound-N, Amersham) as described in (102). Prehybridisation and hybridisation with ^{32}P -labelled rat mtDNA probe were performed as recommended for Hybond-N. The mtDNA was visualised by autoradiography for 24-36 hours at -70 ^{o}C.

4.1.12. Statistical Analysis

Significant differences ($P<0.05$) between means were determined with the paired t test. Unless otherwise stated, values are presented as mean ± SEM.

4. 2. RESULTS

4.2.1. ECG studies

Developing (80-100 gram) rats were treated daily with 50 mg/kg AZT (which is respected as a high human dose) up to two weeks. The rat heart function was monitored by ECG, and after one week treatment we could already detect the development of abnormal heart function. The RR, PR and QT intervals prolonged significantly after one week treatment and the abnormalities further developed by the end of the second week (Table 4.1.). In leads I and aVL which represent the main muscle mass of the left ventricle, significant J point depressions and T wave inversions were found after two weeks of AZT treatment (Table 4.1.).

Table 4. 1.
Effect of AZT treatment on the RR, PR, QT intervals and J point in developing rats. Schiller AT-6 ECG was used to monitor cardiac function. ECG was recorded with needle electrodes according to the routine technique (6 limb leads) and RR, PR, QT intervals and J point values were determined by standard methods.[15] Data represent mean ± SEM for 5 animals.

Treatments	RR	PR	QT(ms)	J(mm)
1 week treatment	213 ± 16*	80 ± 7*	103 ± 3*	-0.8 ± 0.2*
2 weeks treatment	284 ± 16*	82 ± 3*	112 ± 9*	-1.1 ± 0.1*
6 month after treatment	240 ± 56	50 ± 2	95 ± 15	-0.7 ± 0.2**
Age-matched control rats	169 ± 10	55 ± 6	73 ± 5	-0.1 ± 0.1

* Values are different from the corresponding values of age-matched control rats at the significance $p<0.001$
** Values are different from the corresponding values of age-matched control rats at the significance $p<0.01$

After the completion of two weeks AZT treatment the heart function was periodically monitored up to 6 months. We found that suspension of AZT treatment improved but could not restore the normal heart function in AZT-treated animals and ECG detectable heart

abnormalities were observed even 6 month later after the completion of AZT treatment (Table 4.1.).

4.2.2. Electron microscopic studies

Heart samples from 4 AZT-treated rats and from 2 control rats were processed for electronmicroscopy as described under "Materials and Methods". The electronmicroscopic structure of heart muscle did not show gross anatomic changes as a consequence of 2 weeks AZT treatment. The heart myofiber structure in the AZT-treated animals was reasonably good similar to that of control animals (Fig. 4.1.). The most significant changes seen in the heart of AZT-treated animals was the distortion of mitochondrial structure (Fig. 4.1.).

Fig. 4. 1. Transmission electronmicroscopic study of heart muscle in AZT-treated rat. A; Control heart muscle, B; Heart muscle from AZT-treated rat - less damaged area. C; Heart muscle from AZT-treated rat - seriously damaged area.

Fig. 4.1.A.

Fig. 4.1.B.

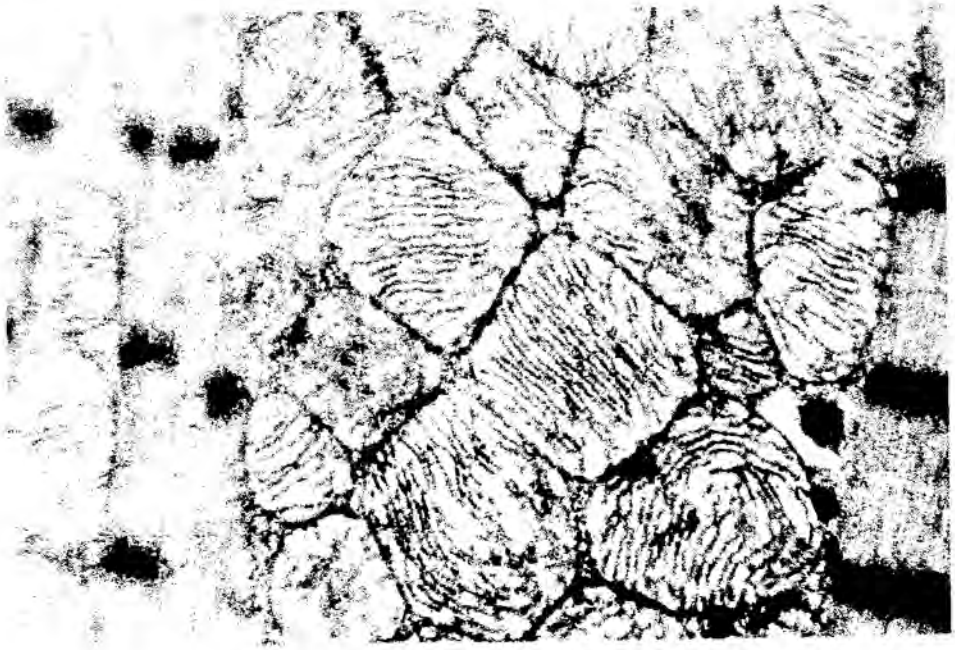
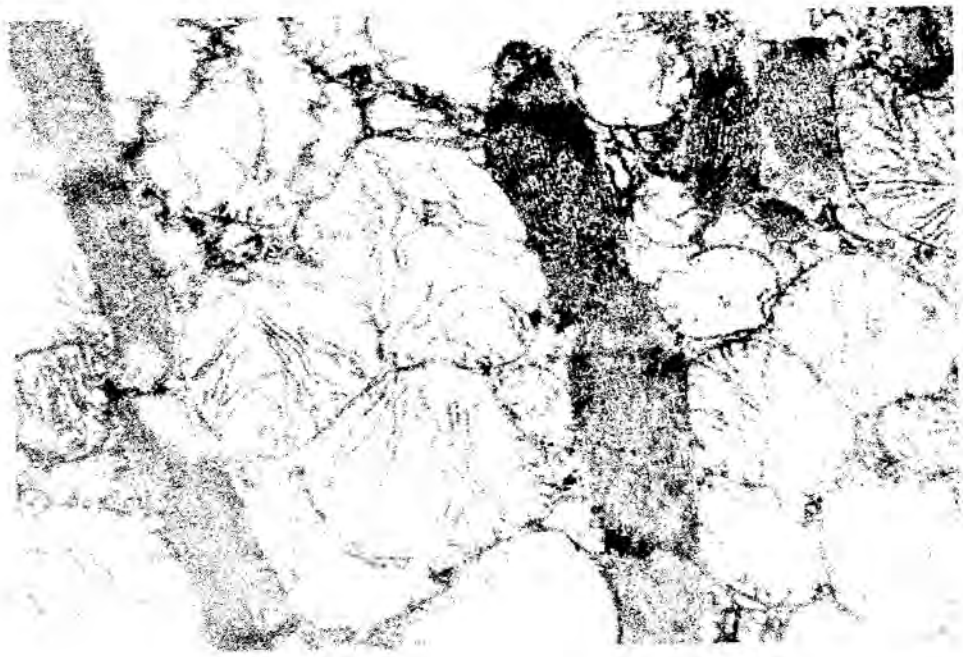


Fig. 4.1.C.



The distance between cristae membranes increased significantly and in a fraction of mitochondria the cristae membranes were seriously deteriorated (Fig. 4.1.C). The disease did not affect homogeneously the whole heart. Beside seriously damaged regions less injured areas can also be seen. (Fig. 4.1.B.). The morphological abnormalities were permanent because mitochondrial damages were detectable even 6 months later after the completion of AZT treatment, and only a slight decrease was seen in the quantity of seriously damaged regions (data not shown).

4.2.3. Detection of ROS in AZT-treated rat hearts

Hydroxyl radical, peroxynitrite and hydrogen peroxide in the presence of peroxidase, iron and cytochrome C reoxidize the non-fluorescent dihidrorhodamine123 and dichlorodihydro-fluorescein diacetate (H_2DCFDA) to fluorescent dyes (51,103). Therefore, DHR and

H_2DCFDA can be utilised to detect ROS and peroxynitrite in heart muscle. The oxidation of DHR and H_2DCFDA was monitored in control heart tissues and it was found that under our experimental conditions DHR is about ten times more sensitive in ROS detection than H_2DCFDA (Table 4.2.). The addition of AZT to the reaction mixture did not cause increase in the ROS production indicating that AZT as an organic molecule is not the source of free radicals (Table 4.2). AZT treatment (two weeks, 50 mg/kg body weight/day) induced a large increase in ROS formation, 235 % determined by DHR and 328 % determined by H2DCFDA in the heart muscle tissues (Table 4.2.). Shorter period of AZT treatment also increased ROS production and 3 days treatment was sufficient to get significant increase in ROS production. These data indicate that AZT treatment of rats induced relatively slow unfavourable changes in the heart muscle leading to excess production of ROS.

Table 4. 2.
AZT treatment induced reactive oxygen species and peroxynitrite formation in rat heart studied by the oxidation of dihydrorhodamine123 and dichlorodihydro-fluorescein diacetate (H_2DCFDA). 50 mg heart tissue was homogenised in 3 ml buffer containing 150 mM KCl, 20 mM Tris, 0.5 mM EDTA, 1 mM $MgCl_2$, 5 mM glucose and 0,5 mM octanoic acid pH 7.4 in the presence of 5 μM DHR or 5 μM H2DCFDA and was incubated in 37 °C for 30 minutes. The fluorescent dyes were extracted as described under "Materials and Methods". The fluorescent dye contents in the supernatants were determined using a fluorescence spectroscope at an excitation wavelength of 500nm and an emission wavelength of 536 nm for rhodamine123 and an excitation wavelength of 502nm and an emission wavelength of 523 nm for fluorescein. Values are mean ± SEM for 5 heart preparations.

Experimental conditions	Rhodamine123	Dichloro-fluorescein
	in pmoles/ml	
Untreated hearts	151 ± 8	14 ± 2
AZT added to untreated hearts	132 ± 14*	11 ± 2*
AZT-treated hearts	356 ± 22**	46 ± 5**
AZT added to AZT-treated hearts	347 ± 19***	45 ± 7***

* Values are not significantly different from the corresponding values of untreated hearts.
** Values are different from the corresponding values of untreated hearts at the significance $p<0.001$.
*** Values are not significantly different from the corresponding values of AZT-treated hearts .

4.2.4. Effect of AZT treatment on the endogenous lipid peroxidation, protein oxidation and single-strand DNA breaks

Determination of TBA reactive materials are widely used to estimate the extent of lipid peroxidation. In control rat hearts TBA reactive material values were close to the values reported in the literature (104). Two weeks AZT treatment caused a significant (2.56 fold) increase in TBA reactive materials in the heart tissue comparing to age-matched control (untreated) values (Table 4.3.) indicating a significant increase in the rate of lipid peroxidation.

Free radicals can oxidise proteins forming reactive aldehyde groups which can be stabilised and detected by 2,4-dinitrophenylhydrazine (99). Using age-matched control rats the protein carbonyl group content was similar to data previously reported (99, 100) and two weeks of AZT treatment significantly increased the carbonyl content of the rat heart proteins (Table 4.3.).

Table 4. 3.
Effect of AZT-treatment on the endogenous lipid peroxidation, protein oxidation, NAD^+ content and single-strand DNA breaks in rat heart. Lipid peroxidation (measuring the TBA reactive substances), the protein carbonyl content (determined with the 2,4-dinitrophenylhydrazine method) single-strand DNA breaks (determined by the alkaline fluorescence analysis of DNA unwinding) and the NAD^+ content (using the alcohol dehydrogenase reaction) were measured as detailed under "Materials and Methods". Values are mean ± SEM for
5 heart preparations.

	Control hearts	Hearts from AZT-treated animals
Protein carbonyl content (nmoles/mg protein)	4.9 ± 0.4	10.2 ± 1.1*
TBA reactive substance (nmoles/gram wet tissue)	39.3 ± 2.7	101.2 ± 3.3*
Undamaged DNA %	94.0 ± 3.0	52.0 ± 4.0*
NAD^+ content (moles/gram wet tissue)	0.45 ± 0.03	0.19 ± 0.02*

* Values are different from the corresponding values of control hearts at the significance $p<0.001$.

It is known that reactive oxygen species can induce DNA breaks most frequently DNA single-strand breaks (105). In our experimental system AZT treatment increased significantly the amount of single-strand DNA breaks and the percentage of undamaged DNA decreased to 52%. The quantity of single-strand DNA breaks was very low in the age-matched control rat hearts and the percentage of undamaged DNA was 94 % (Table 4.3.).

4.2.5. AZT-induced poly- and mono-ADP-ribosylation of cardiac proteins and activation of NAD^+ catabolism.

Investigating the mono-ADP-ribosylation of cytoplasmic proteins we found that AZT treatment increased the mono-ADP-ribosylation of the 78 kD protein, but the ADP-ribosylation of 52 and 56 kD proteins did not changed significantly (Fig. 4.2.A).

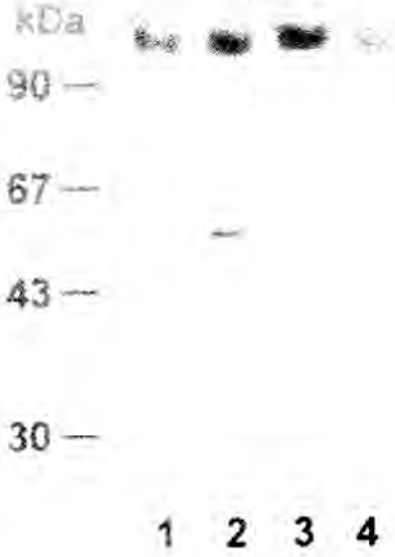
On the basis of previous works (33) we supposed that the 78 kD ADP-ribosylated protein may be the glucose-regulated protein called GRP78 which is an endoplasmic reticular chaperone and the ADP-ribosylation inactivates it.

To further evaluate the significance of free radical production in the development of cardiac side-effect of AZT we investigated the effect of AZT treatment on the self-ADP-ribosylation of nuclear poly-ADP-ribose polymerase, an enzyme which is activated by DNA breaks. The endogenous ADP-ribosylation of cardiac proteins were detected by Western blot analysis (when 8 M urea containing extraction buffer was used) with anti-ADP-ribose antibody. Western blot data indicate that AZT treatment activated the nuclear poly-ADP-ribose polymerase (Mw. ~116 kD) and the steady-state level of self-ADP-ribosylation was significantly higher in the hearts derived from AZT-treated animals than in control heart tissues (Fig. 4.2.B).

Fig. 4. 2. A. Effect of AZT treatment on the endogenous mono-ADP-ribosylation reactions in cardiac muscle. Western blot analysis of ADP-ribosylation of cytoplasmic proteins in cardiomyocytes with anti-ADP-ribose antibody. Lane 1-3, 20 μg protein was subjected to electrophoresis from the heart of AZT-treated animals. Lane 4, 20 μg protein was subjected to electrophoresis from heart muscle of control (untreated) animals. Proteins were extracted as described under "Materials and Methods" in the absence of urea.

It is assumed that the activation of poly-ADP-ribose polymerase (PARP) is the major cause of NAD^+ catabolism in oxidatively damaged heart tissue. Therefore we extended our study to determine how AZT treatment affects NAD^+ level in the AZT-treated heart tissues. We found that two weeks of AZT treatment caused significant decrease in NAD^+ content in heart tissue (Table 4.3.) comparing with age-matched control hearts.

Fig. 4. 2. B. Effect of AZT treatment on the endogenous poly-ADP-ribosylation reactions in cardiac muscle. Extraction of heart proteins occurred in the presence of 8 M Urea and were separated by SDS polyacrilamide (10 %) gel electrophoresis. In this case the nuclear poly-ADP-ribose polymerase was extracted and the level of ADP-ribosylation was determined by Western blotting as before. Lane 1-3. proteins were extracted from the heart of AZT-treated animals (20 μgram protein). Lane 4. proteins were extracted from the heart of control (untreated) animals.



4.2.6. Effect of AZT treatment on mtDNA level in rat hearts

Equal quantity of total DNA from control and AZT-treated heart muscle was digested by Sst I and the linearized DNA was subjected to electrophoresis through 1.5 % agarose gel and the electrophoretic profile of mtDNA was detected by Southern blot (Fig. 4.3.). The Southern blot data did not show depletion of mtDNA deriving from the heart of AZT-treated animals, even some increase was seen in the quantity of mtDNA in the AZT-treated rat heart comparing to control values (Fig. 4.3.). That is, the short-term cardiac side-effect of AZT is not the consequence of the depletion of mtDNA.

Fig. 4. 3. (A) Electrophoretic profile (ethidium bromide stained DNA fluorescence before blotting) of Sst1 digested DNA derived from control animals (Lane 1 - 2) and from AZT-treated animals (Lane 3 -4).

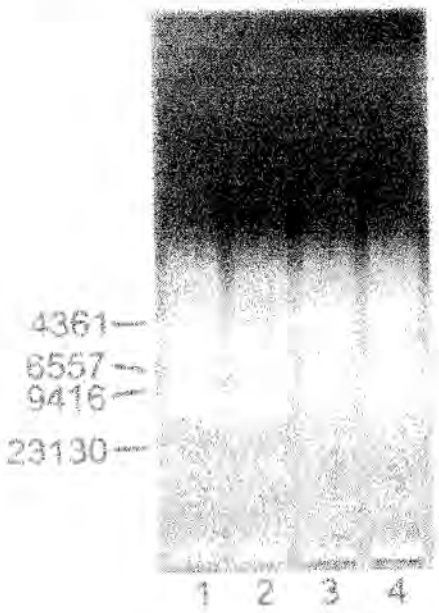
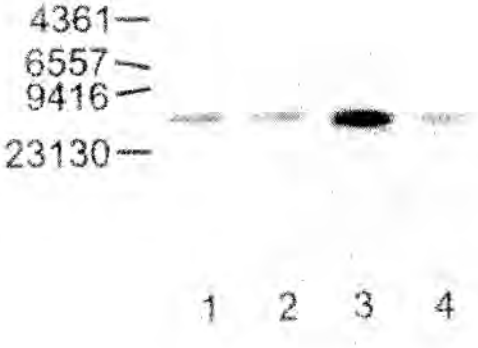


Fig. 4. 3. (B) Southern blot analysis of Sst 1 digested DNA derived from control (Lane 1 -2) and AZT-treated animals (Lane 3 - 4). Four µg DNA was linearized by Sst 1 and electrophoresed through a 1.5 % agarose gel, and transferred onto nylon membrane. Conditions for hybridization and labeling are as described under "Materials and Methods".



4.2.7. Effect of AZT treatment on energy production

Since the AZT-induced abnormalities are concentrated on the mitochondrial membrane systems we determined how AZT treatment influences the activity of respiratory complexes. Statistically significant decrease was found in the activity of NADH:cytochrome C oxidoreductase (from 3.07 ± 0.14 to 1.6 ± 0.21 U/gram tissue) ($p<0.001$) and cytochrome oxidase (from 14.7 ± 1.2 to 9.8 ± 1.1 U/gram tissue) ($p<0.02$) while the activity of malate dehydrogenase, citrate synthase, carnitine acetyl-transferase and lactate dehydrogenase did not change significantly (data not shown).

The concentration of total extractable ATP, ADP and AMP was determined both in normal and AZT-treated rat hearts (Table 4.4.).

Table 4. 4.
Effect of AZT treatment on the heart metabolism in rats. The concentration of ATP, ADP, AMP, creatine phosphate, creatine in the neutralised perchloric acid extract of the cardiac muscle was determined with HPLC.[19]The free ADP concentration was calculated by assuming that the cytosolic creatine kinase is in equilibrium and the apparent equilibrium constant for creatine kinase equal to 166 at pH 7.4. Values represent mean ± SEM of 5 animals.

Metabolites	Heart muscle	
	Control	AZT treated
	mol/gram protein or (in mM)	
ATP	23.7 ± 2(16.9)	22.8 ± 3(16.2)
ADP	4.9 ± 0.4(3.5)	7.1 ± 0.6(5.1)*
AMP	1.4 ± 0.2(1)	3.2 ± 0.2(2.2)*
Pi	17.4 ± 0.8(12.4)	1.1 ± 1.9(15)
Creatine phosphate	42.4 ± 2.1(30.3)	14.7 ± 1.3(10.5)*
Creatine	20.4 ± 1.2(14.6)	25.3 ± 1.5(18.1)
ADP_f	49.8**	173**
ATP/ADP ratio	340**	94**
log[ATP/ADP.Pi]	4.43**	3.79**

* Values are different from the corresponding control values at the significance of $p<0.001$.
** Calculated parameters using the mean values of metabolic data.

The ATP concentration was close to normal in the heart of AZT-treated animals, but there was a significant increase in the ADP and AMP levels (Table 4.4.). The experimentally determined ATP/ADP ratio decreased from 4.8 in control hearts to 3.2 in the hearts of AZT-treated animals. Since a significant portion of ADP is bound to muscle filament in heart, the changes of free ATP/ADP ratio can be calculated from the creatine phosphate/creatine ratio.

The creatine phosphate concentration decreased significantly in the heart of AZT-treated rats, while the creatine concentration slightly increased as a consequence of AZT treatment (Table 4.4.). The calculated creatine phosphate/creatine ratio decreased from 2.08 control value to 0.58 in the cardiac muscle of AZT-treated animals. The calculated free ATP/ADP ratio decreased with almost 4- fold from 340 (control) to 94 (AZT-treated). The 45 % increase in the experimentally determined ADP concentration and the absence of significant changes in total ATP concentration suggests that the decrease in the free ATP/ADP ratio is the consequence of a significant raise in the free ADP. It is known that the ADP availability is a rate-limiting factor of the mitochondrial ATP production in heart (106), therefore the increased free ADP level (Table 4.4.) can activate the ATP synthesis in the undamaged mitochondria compensating for the damaged mitochondria.

The inorganic phosphate concentration increased in AZT-treated rat hearts (Table 4.4.) and the calculated values for phosphorylation potential (assuming unchanged intracellular pH) decreased from 4.43 to 3.69 in the AZT-treated hearts (Table 4.4.).

4. 3. DISCUSSION

Previous studies indicated that long-term (several months) AZT treatment inhibits the replication of mtDNA and causes mtDNA depletion (88, 89) which was considered as the primary cause of AZT-induced myopathy. However, model studies indicated that a significant reduction of mtDNA level requires several months of AZT treatment. This work showed that a relatively short-term (two weeks) of AZT treatment which did not decrease the quantity of mtDNA (Fig. 4.3.) caused significant functional damages as judged by ECG studies (Table 4.1.) and abnormalities in the morphology of mitochondria. These data raise the possibility of an alternative mechanism for the cardiotoxic effect of AZT.

It was found that the short-term treatment of rats with AZT increased ROS and peroxinitrit production in heart (Table 4.2.), indicating that AZT (or its metabolites) induced unfavourable changes in the mitochondria caused by an excess ROS production. The increased ROS and peroxinitrit production caused single-strand DNA breaks, lipid peroxidation and protein oxidation.

The single-strand DNA breaks activated the nuclear PARP (Fig. 4.2 B), which utilise NAD^+ to form poly-ADP-ribose which binds either to PARP or to other nuclear proteins (Table 4.3.). The poly-ADP-ribose residues are digested to ADP-ribose by poly-ADP-ribose glychohydrolase and as a result of action of these two enzymes intracellular NAD^+ is catabolized to nicotinamide and ADP-ribose (21). ADP-ribose can be converted back to NAD^+ by utilising ATP, therefore an accelerated PARP and glycohydrolase reactions increase the ATP consumption. Additionally, the depletion of the NAD^+ pool negatively affects the energy-producing processes of the cell (41, 53). That is, the activation of PARP

may induce a cycle (increased consumption and decreased synthesis of ATP) which can lead to the collapse of the heart energy metabolism.

As a part of enhanced ADP-ribosylation GRP78 was also ADP-ribosylated under our experimental conditions (Fig. 4.2.) inhibiting its basic function in folding and transport of proteins in ER.

The AZT-induced morphological changes in rat heart mitochondria are similar to the morphological changes seen in chloramphenicol treated cells (swollen mitochondria and defective cristae structure) and in both cases a significant decrease was seen in the activity of respiratory complexes and energy metabolism (Table 4.3.).

5. DISCUSSION FROM A CLINICAL POINT OF VIEW

1. The clinical importance of protecting the heart from oxidative stress has already been recognised. In experimental settings which mimic ROS induced injury, many different agents that posses antioxidant properties have been found to be protective against reperfusion injury. Thus, investigations leading to the development of biologically effective antioxidants or other ptotective compounds against oxidative stress seem to be very important in the clinical practice.

2. Cardiomyopathy is of great importance today, as it is a frequent cause of heart failure. Cardiomyopathy is refractory to conservative medical management, therefore, it is the primary indication for cardiac transplantation world-wide. Despite the more aggressive diagnostic investigation and the introduction of early therapy, the outlook for patients with this disorder remains poor. Our understanding of the aetiology and pathogenesis of this disease has increased significantly over the past years, but several issues need to be clarified before more specific therapeutic strategies can be introduced based on these findings. Investigators from around the world have tried to reveal the mechanisms responsible for the development of cardiomyopathy, but the exact mechanisms are still uncertain. Using AZT for the generation of cardiomyopathy we get closer to the mechanisms by which AZT causes cardiomyopathy , but we also have an easily reproducible, experimentally created model of cardiomyopathy for the better understanding of the mechanisms leading to this disease and a possibility for studying the effects of different drugs on it.

6. CONCLUSIONS

The new results of this study are the following

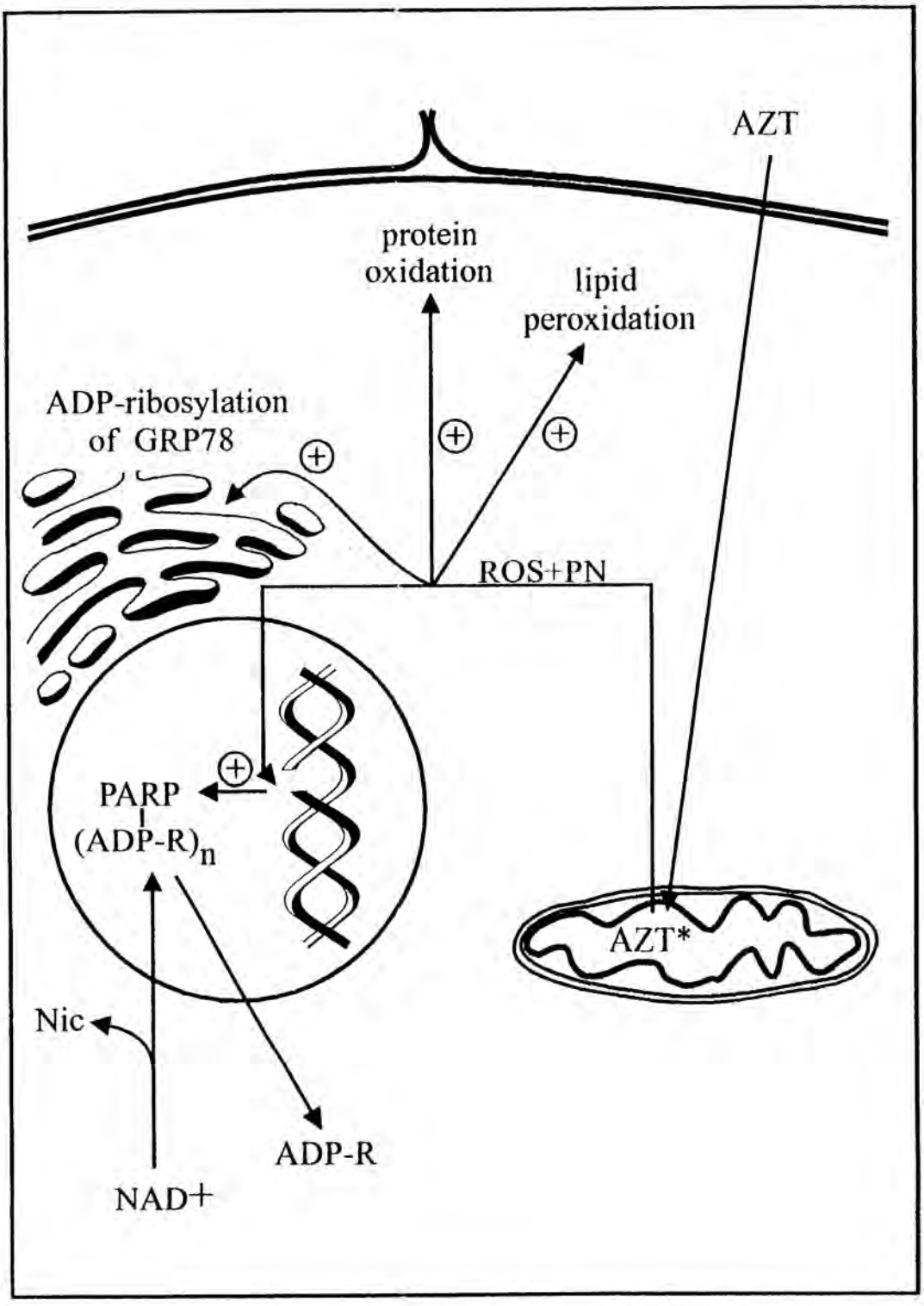
- There are almost no available data in the literature about the protective effect of lipoamide and dihydrolipoamide against oxidative stress. In our study lipoamide (1.) significantly decreased the ischemia-reperfusion induced ROS production and (2.) single-strand DNA breaks, and (3.) reduced the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase, (4.) the ADP-ribosylation of the GRP78 and (5.) the NAD^+ catabolism in perfused rat hearts. Lipoamide (6.) diminished the ischemia-reperfusion induced lipid peroxidation and (7.) helped to maintain the cell membrane integrity . These data show that the reduced form of lipoamide can modulate the ROS-induced signalling through poly- and mono-ADP-ribosylation reactions, the NAD^+ catabolism, and improve the recovery of postischemic myocardium.

- Our data show that (1) BGP-15 is a novel PARP inhibitor, (2) significantly reduced the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase, (3) the ADP-ribosylation of the GRP78 and (4) the NAD^+ catabolism in perfused rat hearts. BGP15 (5) decreases the ischemia-reperfusion induced endogenous ROS formation, and (6) lipid peroxidation and the (7) formation of single-strand DNA breaks in cardiac muscle. Data presented in this paper (8) indicate that PARP inhibitors including BGP-15 by decreasing the ischemia-reperfusion-induced endogenous ROS formation provide a novel pathway by which PARP inhibitors protect postischemic

cardiac muscle.

- The short-term treatment (two weeks) of rats with AZT (1) increased ROS and peroxinitrite production in heart, caused (2) single-strand DNA breaks, (3) lipid peroxidation and (4) protein oxidation, (5) activated the nuclear PARP, (6) enhanced the NAD^+ catabolism, (7) increased the ATP consumption. As a part of enhanced ADP-ribosylation (8) GRP78 was also ADP-ribosylated. AZT induced (9) morphological changes in rat heart mitochondria and a (10) significant decrease was seen in the activity of respiratory complexes and energy metabolism (Table 4.3). Data presented here draw the attention to the fact that a short-term AZT-treatment, instead of depleting mtDNA level, activates the ROS formation in the mitochondria in cardiac muscle (and probably also in other tissues), which triggers a sequence of events unfavourable for cells as outlined in Scheme 4.1.

Scheme 4. 1. AZT-induced ROS mediated signaling in rat heart, ROS, reactive oxygen species; PN peroxynitrite, PARP, poly- ADP-ribose polymerase; Nic, nicotimamide; ADP-R, ADP-ribose; ⊕ , induction or activation; *AZT-triphosphate.



ABBREVIATIONS

ROS	reactive oxygen species
mtDNA	mitochondrial DNA
DNA-SSB	DNA single-strand breaks
PARP	poly-ADP-ribose polymerase
NO	nitric oxide
GRP78	glucose regulated protein
LDH	lactate dehydrogenase
GOT	glutamate-oxaloacetate transaminase
CK	creatin kinase
DHR	dihydrorhodamine123
MMArg	mono-methyl-arginine
HSP70	heat shock protein 70
Mw.	molecular weight
DTT	dithioerithreitol
TBARS	thiobarbituric acid reactive substances
TCA	trichloric acid
MDA	malondialdehyde
ER	endoplasmic retuculum
HIV	human immunodeficiency virus

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