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

Ph.D. thesis

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1. INTRODUCTION

The ischemic heart diseases and cardiomyopathies are the most important part of cardiology. Reactive oxygen species (ROS) play key role in the pathogenesis of these diseases. Cells have developed various defense systems to protect themselves from oxidative damages. Although there are certain circumstances when the oxidative threats exceed the capacity of the defence system causing an oxidative stress to the cell. Lipid peroxidation, protein oxidation, DNA damages and the increased mono-or poly-ADP-ribosylation of different enzymes are only some of the many important consequences of the excess ROS production, in this elaborate and still not yet fully understood system.

The activation of poly-ADP-ribose polymerase (PARP) seems to play a key role in the pathogenesis of oxidative cell injury. 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 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. 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.

The role of increased mono-ADP ribosylation of different proteins during stress situations are less known. Mono-ADP-ribosylation is the way of regulating certain cytoplasmic enzymes and regulatory proteins. GRP78 (the 78 kDa glucose-regulated protein also known as BIP, the immunoglobulin heavy chain-binding protein) is post-translationally modified by ADP-ribosylation. The unmodified (not ADP-ribosylated) form of GRP78 is functional and believed to bind transiently to nascent proteins as they are translocated into the ER, facilitating the attainment of correct conformation of these proteins destined for surface expression or secretion.

The protection of cells from oxidative damage by different drugs is important in stress situations. It can be based on the reduction of ROS level by antioxidants and by ROS scavenging enzymes, or by the reduction of ROS-induced intracellular signaling, like the excess ADP-ribosylation of different proteins.

2. AIMS OF THE STUDY

The aims of our study were the following:

1. To determine the extent of oxidative cell damages, and changes in the intracellular signalling mediated by reactive oxygen species (ROS) during ischaemia-reperfusion in Langendorff heart perfusion system. The following parameters were studied in perfused hearts: the in vitro and in situ ROS formation, lipid peroxidation, DNA single-strand breaks (DNA SSB), the nuclear poly-ADP-ribose polymerase activation, the NAD^+ catabolism, and the cytoplasmic mono-ADP-ribosylation reactions, such as the mono-ADP-ribosylation of glucose regulated protein (GRP78/BIP).
2. To study the antioxidant character of lipoamide in the above mentioned system. 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 Beside these positive metabolic properties we wanted to know whether lipoamide or the intracellularly formed dihydrolipoamide has also antioxidant character making it an ideal protector against oxidative cell injury.
3. To examine the protective effect of BGP-15, an amidoxime derivative against the oxidative stress, which is not an antioxidant and so would serve an alternative way of cardioprotection.
4. To investigate the role of reactive oxygen species and poly-ADP -ribose polymerase in the development of AZT-induced cardiomyopathy in rat. It is well documented that AZT treatment deplete the mitochondrial DNA (mtDNA). The mtDNA encodes 13 polypeptide that are components of the respiratory complexes. Therefore, any damage of mtDNA will cause defective mitochondrial protein synthesis which results in abnormal respiratory complexes and impaired oxidative energy production. It was also observed that already the short-term AZT-treatment induced cardiomyopathy, when the mtDNA was not yet affected indicating that the molecular mechanism of AZT-induced cell damage is more complex than expected.

3. MATERIALS AND METHODS

Animals: The heart of adult male Wistar rats weighing 300-350g were used for Langendorff heart perfusion experiments.

Heart perfusion: Hearts were perfused via the aorta according to the Langendorff method. The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer. After 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. For AZT traetment 80-100 gram rats were intraperitoneally treated daily with AZT (50 mg/kg) for up to 14 days. ECG was recorded with needle electrodes and RR, PR, QT intervals and J point values were determined.

Assay of NAD$^+$: The concentration of NAD$^+$ in the neutralised perchloric acid extract of the cardiac muscle was measured by using alcohol dehydrogenase reaction.

ADP-ribosylation assay: 50 mg cardiac muscle was prepared for 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.

Isolation of nuclei: The isolation of nuclei from cardiac tissue was carried out by using standard extraction procedure. The purified nuclei were prepared for dot blotting and the immune reaction was carried out as it is described in the ADP-ribosylation assay.

Immunoaffinity purification of GRP78: DnaK antibody was coupled to protein A sepharose beads and incubated with detergent solubilized endoplasmic reticulum proteins. The antigen-antibody complex was removed and used for SDS-polyacrylamide gel electrophoresis followed by Western blot with anti-ADP-ribose antibody.

Detection of ROS: The rhodamine123 content was determined using a Perkin Elmer fluorescence spectroscope at an excitation wavelength of 500 nm and an emission wavelength of 536 nm.

DNA single-strand breaks were determined by the alkaline fluorescence analysis of DNA unwinding as described by Birnboim and Jevcak.

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 using a Tosoh, TSK-6011 HPLC system or from the formation of thiobarbituric acid reactive substances (TBARS).

Assessment of cell membrane integrity: The release of lactate dehydrogenase (LDH), creatine kinase (CK) and glutamate oxaloacetate transaminase (GOT) enzymes were measured in the perfusate of Langendorff perfused hearts under normoxic and ischemic conditions.

Dihydrolipoamide determination: Samples were collected from the efflux of perfused hearts and were treated with monochloroacetic acid in 50% methanol. The lipoamide and dihydrolipoamide content was measured by HPLC.

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.

In vitro PARP inhibition on isolated enzyme: The poly-ADP-ribose polymerase was isolated from rat liver based on the method described before. PARP activity was determined by [^{3}H] NAD incorporation and the radioactivity was determined with Beckman scintillation counter.

Electron microscopy: The transmission electron microscopy was performed by a JEM 1200-EX-II electron microscope operated at an accelerating voltage of 80 kV.

Substrate determinations: The concentration of ATP, ADP, AMP, creatine phosphate, creatine was determined with HPLC.

The protein oxidation: was followed by the determination of protein carbonyl content with 2,4-dinitrophenylhydrazine .

Southern blot analysis: The mtDNA was prepared from normal rat liver. Radioactively labelled DNA probe (^{32}P) was prepared by random primer method. 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. Prehybridisation and hybridisation with ^{32}P -labelled rat mtDNA probe were performed as recommended for Hybond-N.

4. RESULTS AND CONCLUSIONS

1. There are almost no available data in the literature about the protective effect of lipoamide and dihydrolipoamide against oxidative stress. In our study lipoamide significantly decreased the ischemia-reperfusion induced ROS production and single-strand DNA breaks, reduced the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase, the ADP-ribosylation of the GRP78 and the NAD^+ catabolism in perfused rat hearts. Lipoamide diminished the ischemia-reperfusion induced lipid peroxidation and 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, reduce the NAD^+ catabolism, and improve the recovery of postischemic myocardium.
2. BGP-15 - a novel PARP inhibitor, significantly reduced the self ADP-ribosylation of the nuclear poly-ADP-ribose polymerase, the ADP-ribosylation of the GRP78 and the NAD^+ catabolism in perfused rat hearts. BGP15 decreases the ischemia-reperfusion induced endogenous ROS formation, and lipid peroxidation and the formation of single-strand DNA breaks in cardiac muscle. Data presented in this paper 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.
3. The short-term treatment (two weeks) of rats with AZT increased the ROS production in heart, caused single-strand DNA breaks, lipid peroxidation and protein oxidation, activated the nuclear PARP, enhanced the NAD^+ catabolism, increased the ATP consumption. As a part of enhanced ADP-ribosylation GRP78 was also ADP-ribosylated. AZT induced morphological changes in rat heart mitochondria and a significant decrease was seen in the activity of respiratory complexes and energy metabolism. 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.

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