Prevention of myocardial reperfusion injury by poly(ADP-ribose) synthetase inhibitor, 3-aminobenzamide, in cardioplegic solution: in vitro study of isolated rat heart model

Kazuhiro Yamazaki, Senri Miwa, Kunihiro Ueda, Seigo Tanaka, Shinya Toyokuni, Oriyanhan Unimon, Kazunobu Nishimura, Masashi Komeda

Department of Cardiovascular Surgery, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawahara-machi, Sakyo-ku, 606-8507, Kyoto, Japan
Laboratory of Molecular Clinical Chemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto, Japan
Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Received 15 September 2003; received in revised form 12 March 2004; accepted 6 April 2004

Abstract

Objective: Cardioplegic arrest remains the method of choice for myocardial protection in cardiac surgery. Poly(adenosine 5'-diphosphate-ribose) synthetase (PARS) inhibitor has been suggested to attenuate the ischemia-reperfusion injury in myocardial infarction by preventing energy depletion associated with oxidative stress. We investigated the efficacy of a cardioplegic solution containing a PARS inhibitor, 3-aminobenzamide (3-AB), for myocardial protection against ischemia-reperfusion injury caused by cardioplegic arrest.

Methods: Isolated hearts were set on a Langendorff apparatus and perfused. The hearts were arrested for 90 min with a cardioplegic solution given at 30-min intervals and then reperfused for 20 min. The hearts of rat in the 3-AB(−) group (n = 8) were perfused with a standard cardioplegic solution and terminal warm cardoplegia, whereas the 3-AB(+) group (n = 8) received these solutions supplemented with 3-AB (100 μM). Left ventricular function and release of cardiac enzymes were monitored before and after cardioplegic arrest. After reperfusion, NAD(+) (nicotinamide-adenine dinucleotide) levels were assessed, and the tissues were examined immunohistochemically for oxidative stress and apoptosis.

Results: During reperfusion, the 3-AB(+) group showed significantly higher (P = 0.005) dp/dt and lower creatine phosphokinase (CPK) level and glutamic-oxaloacetic transaminase (GOT) in the effluent (CPK; P = 0.003, GOT; P = 0.001). The cardiomyocytes of the 3-AB(+) group also preserved a higher NAD(+) level (P < 0.001). Immunohistochemical study of oxidative stress revealed a lesser extent (P = 0.007) of nuclear staining and a lower fraction of apoptosis in the 3-AB(+) group.

Conclusion: Cardioplegic solution supplemented with 3-AB provides efficient myocardial protection in cardioplegic ischemic reperfusion by suppressing oxidative stress and overactivation of PARS.

Keywords: Myocardial protection; Cardioplegia; PARS [Poly(ADP-ribose) synthetase] inhibitor; Ischemic reperfusion injury; Heart surgery; Reactive oxygen species (ROS)

1. Introduction

In cardiac surgery, hyperkalemic solutions are conventionally used for rapidly inducing depolarization of the cell membrane and electromechanical arrest, thereby reducing cellular energy expenditure during the ischemic period. However, the cardioplegic arrest and reperfusion generate more or less reactive oxygen species (ROS) and induce myocardial injury [1]. The duration of cardioplegic arrest and reperfusion is known to be positively correlated with the extent of cardiomyocyte damage [2]. Therefore, we need to develop a cardioplegic solution that offers greater cardioprotection.

The generation of ROS associated with ischemia and reperfusion leads to lipid peroxidation, protein oxidation and the formation of DNA single-strand breaks [3]. Poly(adenosine 5'-diphosphate-ribose) synthetase [PARS, also referred to as poly(ADP-ribose) polymerase-1; PARP-1] (EC2.4.2.30) is an enzyme implicated in DNA repair in the nucleus. The enzyme is activated by oxidant-mediated DNA single-strand breaks. Excessive activation of PARS...
results in a fall in the intracellular level of the substrate NAD$^+$, as NAD$^+$ is indispensable for mitochondrial respiration, the depletion of NAD$^+$ leads to a deficiency of ATP. This ‘energy crisis’ hypothesis could explain the molecular mechanism of cell death after oxidative stress [3,4].

Recent studies have suggested that inhibition of PARS prevents ischemia-reperfusion injury in several organs, such as the brain and intestine [5,6]. In the heart, the inhibition of PARS has been shown to attenuate myocardial infarction by suppressing the energy depletion associated with oxidative stress [7]; neutrophil infiltration into ischemia-reperfused myocardium; and attenuate proinflammatory mediator production [8]. So far, several studies have shown that the administration of PARS inhibitor prior to reperfusion reduces the extent of myocardial infarction after regional or global ischemia in a buffer-perfused heart model [9–11].

Considering the protective effect of PARS inhibitors on myocardial infarction, this study was designed to evaluate the efficacy of cardioplegic solution containing a PARS inhibitor, 3-aminobenzamide (3-AB), in cardiac surgery. We assessed its ability to attenuate myocardial injury in an isolated rat’s heart model of cardioplegic ischemia and reperfusion.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (350–450 g body weight) were used for this study. All animals in this study received humane care in compliance with the ‘Principles of Laboratory Animal Care’ formulated by the National Society for Medical Research and the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health.

2.2. Heart isolation and perfusion

Rats were anesthetized by inhalation of diethyl ether and intraperitoneal injection of sodium pentobarbital (50 mg/kg), and anticoagulated by intravenous injection of heparin (1000 IU/kg). Each heart was rapidly excised and positioned on a nonrecirculating type of Langendorff apparatus. After cannulation of the aorta, coronary circulation was quickly started by retrograde aortic perfusion at a constant pressure of 75 mmHg at 37.5 °C. Krebs–Henseleit solution (NaCl, 118 mM; KCl, 4.7 mM; MgSO4, 1.2 mM; NaHCO3, 25 mM; KH2PO4, 1.2 mM; CaCl2, 2.5 mM; glucose, 11 mM) gassed with a mixture of 95% oxygen and 5% carbon dioxide was used for perfusion.

The experimental protocol is shown in Fig. 1. After a 20-min perfusion, each isolated heart was arrested with warm cardioplegia of Krebs–Henseleit buffer were given with or without 100 μM 3-AB.

St. Thomas’ Hospital solution (NaCl, 110 mM; KCl, 16 mM; MgCl2, 16 mM; CaCl2, 1.2 mM; NaHCO3, 10 mM) (4 °C, 20 ml/kg) as a cold cardioplegic solution. Second and third dose of cardioplegic solution (10 ml/kg) were given at 30-min intervals. After 90 min of cardioplegic arrest, Krebs–Henseleit buffer (10 ml/kg) was administered as a terminal warm cardioplegia, followed by a 20-min reperfusion. Rats were grouped into two groups: 3-AB(−) and 3-AB(+), (n = 8 each). In the 3-AB(+) group, 100 μM 3-AB was added to St. Thomas’ Hospital solution and Krebs-Henseleit buffer. The basal features and functions of the two groups were essentially identical (Table 1).

2.3. Evaluation of left ventricular (LV) function

To measure left ventricular pressure, a latex balloon was inserted through the left atrium into the left ventricular cavity and connected via a fluid-filled polyethylene tube to a pressure transducer. End-diastolic pressure was set at less than 10 mmHg. Heart rate, positive maximum rate of the rise of left ventricular pressure (dP/dt), mmHg/s) and coronary flow were measured and recorded at a 20-min perfusion, and at 5 and 20 min intervals after reperfusion (Fig. 1). Coronary flow was measured by collecting the coronary effluent buffer at the same time points as above.

2.4. Measurement of cardiac enzymes

The extent of release of cardiac enzymes was measured from samples of the coronary effluent buffer. Creatine

<table>
<thead>
<tr>
<th>Variable</th>
<th>3-AB(−) group (n = 8)</th>
<th>3-AB(+) group (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>381.3 ± 39.8</td>
<td>373.8 ± 35.0</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.27 ± 0.12</td>
<td>1.24 ± 0.23</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>293.3 ± 38.4</td>
<td>295.8 ± 33.2</td>
</tr>
<tr>
<td>dp/dt (mmHg/s)</td>
<td>125.1 ± 253.5</td>
<td>1226.6 ± 235.1</td>
</tr>
<tr>
<td>Coronary flow (ml/min)</td>
<td>11.8 ± 2.4</td>
<td>11.8 ± 3.0</td>
</tr>
<tr>
<td>GOT (IU)</td>
<td>0.030 ± 0.016</td>
<td>0.049 ± 0.029</td>
</tr>
<tr>
<td>LDH (IU)</td>
<td>0.09 ± 0.06</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>CPK (IU)</td>
<td>0.29 ± 0.07</td>
<td>0.30 ± 0.10</td>
</tr>
</tbody>
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Data are shown as mean ± SD. There were no significant differences in any variables.
phosphokinase (CPK), glutamic-oxaloacetic transaminase (GOT) and lactate dehydrogenase (LDH) levels were measured using an autoanalyzer AU5200 (Olympus, Tokyo, Japan). Enzyme activities were expressed in units per min (IU).

2.5. Measurement NAD\(^+\) concentration

The concentration of NAD\(^+\) in the perchloric acid extract of the cardiac muscle was measured using an alcohol dehydrogenase reaction. The reaction mixture contained 1000 µl of buffer-substrate [0.1 M Tris acetate (pH 8.80) and 0.5 M ethanol], 100 µl of the tissue extract neutralized and 20 µl of alcohol dehydrogenase. The reaction was initiated by the addition of enzyme, and change in absorbance at 340 nm was recorded by a spectrophotometer [12]. We also assessed the NAD\(^+\) content of normal hearts dissected immediately after anesthesia (untreated control group; UC group).

2.6. Immunohistochemical assay of 8-hydroxy-2’-deoxyguanosine (8-OHdG)

Levels of 8-hydroxy-2’-deoxyguanosine (8-OHdG), one of the major products of oxidative DNA modifications [13], were measured as a marker of oxidative stress [14]. Cardiac tissues were fixed overnight in Bouin’s solution immediately after reperfusion, and then dehydrated sequentially with 50 and 70% ethanol for 24 h each. The specimens were embedded in paraffin, cut into 3.5-µm sections and placed on silane-coated glass slides. For immunohistochemical analysis, the avidin–biotin complex method was carried out as previously described [14]. Briefly, after deparaffinization of the specimens on glass slides, appropriately diluted solutions of normal rabbit serum (Dako Japan Co., Ltd., Kyoto, Japan) (for inhibition of non-specific binding of the secondary antibody), biotin-labeled rabbit anti-mouse IgG serum (Dako) and avidin–biotin complex (Vector Laboratories, Burlingame, CA) were sequentially applied. The substrate for alkaline phosphatase (black) was obtained from a vector. No nuclear counterstaining was performed.

The level of 8-OHdG (8-OHdG index) was measured and quantitated as follows [15]:

\[
8\text{-OHdG index} = \frac{\Sigma (X - \text{threshold}) \times \text{area (pixels)}}{\text{total cell number}}
\]

where \(X\) is the staining density indicated in gray scale, using the NIH image 1.61 [Scion Image Beta 4.02, PC version of NIH Image (Scion Corporation, Frederick, MD) and PHOTOSHOP version 6.0 (Adobe Systems Inc, San Jose, CA)].

2.7. Detection of apoptotic cardiomyocytes

A TUNEL assay was performed for detection of apoptotic cells using a commercial kit (apoptosis in situ detection kit; Wako, Osaka, Japan) according to the manufacturer’s instructions. Digoxigenin-labeled dUTP were catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase, an enzyme that catalyzes a template-independent addition of nucleotide triphosphate to the 3'-OH ends of double- or single-strand DNA.

2.8. Statistical analysis

Statistical analysis was performed with Statview software (ver. 5.0, SAS Institute Inc. CA.). All data were expressed as the mean ± SEM. Two-way repeated-measures analysis of variance (ANOVA) was used to test the effect of cardioplegia group and time on LV function and cardiac enzymes. When analysis of variance indicated a significant effect of cardioplegia group or time (\(P < 0.05\)), the differences were specified with with the Fisher’s test for between-groups comparison. On the concentration of NAD\(^+\) and the 8-OHdG index, differences among the groups were analyzed by one-way ANOVA followed by the Fisher’s test. Statistical significance was determined for \(P\) values less than 0.05.

3. Results

3.1. Time-dependent changes in LV function

Heart rate was slightly decreased at 5 min after reperfusion and partly recovered at 20 min after reperfusion in both the 3-AB(−) and 3-AB(+) groups (Fig. 2).
There were no significant differences between the two groups. However, the 3-AB(+) group exhibited a significantly higher dp/dr value than the 3-AB(−) group at 5 min ($P = 0.003$) and 20 min ($P = 0.026$) after reperfusion. At 20 min of reperfusion, ventricular systolic function was much improved and recovery in the 3-AB(+) group reached a maximum of 120%. Coronary flow was increased at 5 min after reperfusion and recovered at 20 min. However, no significant differences were found in coronary flow between the two groups at any time intervals.

3.2. Release of cardiac enzymes

Massive levels of all three enzymes tested—GOT, LDH and CPK—were released from ischemia-reperfused hearts in the early phase (5 min after reperfusion) and less abundantly later (20 min). The extent of release of enzymes was in general more marked in the 3-AB(−) group than in the 3-AB(+) group (Fig. 3). Among these changes, a statistical difference was found between the 3-AB(−) and 3-AB(+) groups in GOT at 5 min ($P = 0.016$) and CPK at 5 min ($P = 0.047$) and 20 min ($P = 0.001$).

3.3. Changes of intracellular NAD\(^+\) content

The NAD\(^+\) level (0.21 ± 0.02 μmol/g) in the ischemia-reperfused hearts in the absence of 3-AB (the 3-AB(−) group) was almost a quarter of the level in the untreated control (UC) hearts (0.80 ± 0.02 μmol/g, vs 3-AB(−); $P < 0.001$) (Fig. 4). This marked reduction of NAD\(^+\) was almost completely inhibited by the addition of 3-AB in cardioplegic solution; the level of NAD\(^+\) in the 3-AB(+) group was 0.72 ± 0.04 μmol/g (vs 3-AB(−); $P < 0.001$).

Fig. 3. Release of cardiac enzymes. (A) The 3-AB(+) group released a significantly smaller amount of GOT than the 3-AB(−) group at 5 min of reperfusion (* * $P < 0.05$). (B) There were no significant differences in LDH release between the 3-AB(−) and 3-AB(+) groups. (C) The level of CPK release was significantly lower in the 3-AB(+) group at 5 min (* * * $P < 0.05$) and 20 min (* $P < 0.01$) after reperfusion (Legend: base, before cardioplegic ischemia; 5 and 20 min, 5 and 20 min after reperfusion).

Fig. 4. NAD\(^+\) content after reperfusion. NAD\(^+\) content was decreased in the 3-AB(−) group. Preservation of the NAD\(^+\) content was observed (* $P < 0.001$) in the 3-AB(+) group. There were no significant differences between the 3-AB(+) group and the untreated control group (UC).

However, there was no significant difference between the 3-AB(+) and the UC group.

3.4. Oxidative stress on DNA

We carried out immunohistochemical analysis of 8-OHdG levels to evaluate the oxidative stress on DNA in the rat hearts. In both the 3-AB(+) and 3-AB(−) groups, clear nuclear staining of cardiomyocytes was observed in the anterior, posterior and septal walls. The staining was apparently more prominent in the 3-AB(−) group (Fig. 5). The 8-OHdG index calculated from staining in the 3-AB(−) group (91.2 ± 25.0) was significantly higher than the index in the 3-AB(+) group (36.3 ± 7.2, $P = 0.007$), although the latter was still higher than the index of the untreated control (UC) group (7.6 ± 1.6, vs 3-AB(−); $P = 0.007$) (Fig. 6).

However, no significant differences were found between the 3-AB(+) and the UC group.

Fig. 5. Oxidative stress on DNA. Immunohistochemical analysis of 8-OHdG was carried out using a specific monoclonal antibody (N45.1). Addition in the 3-AB(+) group resulted in a less amount of nuclear oxidative DNA damage than in the 3-AB(−) group. Note that these cells whose nuclei are stained dark suffers from oxidative stress. (Legend: UC, Untreated control group; Anterior, anterior wall; Posterior, posterior wall; Septal, Septal wall). Bar = 50 μm.

Fig. 6. 8-OHdG index in the 3-AB(+) and UC groups.
Under our experimental conditions, the PARS inhibitor the inhibition of PARS activity protects cardiac myocytes. It is conceivable that approximately 30% of PARP activity is required for the most effective myocardial protection. Since the latter group (Fig. 7).

4. Discussion

In this study, the Langendorff heart perfusion model was used to simulate myocardial reperfusion injury during cardiac surgery. A PARS inhibitor, 3-AB, exerted protective effects in this model. The restoration of contractile function and decrease in the release of cardiac enzymes were consistent with amelioration of the myocardial injury.

LV systolic function was much improved by the addition of 3-AB. The recovery rate at 20 min after reperfusion was about 120% in the 3-AB(+) group and about 90% in the 3-AB(−) group (Fig. 3). At the early stage, LV function was better preserved in the 3-AB(+) group. The energy crisis theory has been proposed as a possible mechanism by which the inhibition of PARS activity protects cardiac myocytes. Under our experimental conditions, the PARS inhibitor decreased the NAD⁺ depletion induced by ischemia-reperfusion, which is consistent with previous observations in other models [7,8]. The modulation of NAD⁺ levels through this PARS-dependent nuclear process could explain the better recovery of heart functions.

The ischemia-reperfusion sequence results in the generation of free radicals in the myocardium [16]. Ischemia reduces the activity of cellular defense enzymes against free radicals, and reperfusion or introduction of oxygen further disturbs the delicate balance of oxidants/antioxidants and generates a burst of free radicals in the tissue [17]. 3-AB has been shown to neither scavenge superoxide anions nor inhibit the synthesis or action of its precursor [18]. In the previous study, PARS inhibitors abrogated the ischemic-reperfused lipid peroxidation and protein oxidation. Furthermore, PARS inhibitors decreased the ischemic-reperfused mitochondrial ROS formation, which is independent of nuclear PARS activity [7]. 8-OHdG is a major product of oxidative DNA modifications. We recently discovered that the 8-OHdG level assessed using immunohistochemistry is increased in cardiomyocytes after myocardial infarction [19]. In the present study, by calculating the 8-OHdG index, we demonstrated the ability of 3-AB to protect DNA against oxidative stress. These observations suggest that the inhibition of PARS may influence, in an indirect way, the generation of ROS.

It has been reported that apoptosis is evident early after open heart surgery, and the number of apoptotic cells is negatively correlated with cardiac contractility [2,20]. In the present study, 3-AB suppressed apoptotic DNA fragmentation. It has been hypothesized that PARS is implicated in various types of cell death by two mechanisms: (a) PARS could deplete NAD⁺ and ATP of cells, resulting an energy crisis; and (b) PARS serves as a main substrates for caspases [3]. Our results are consistent with the former mechanism, that is, the inhibition of NAD⁺ depletion by PARS inhibitor leads to protection of the cardiomyocyte nuclei from oxidative stress and thus apoptosis.

We used 3-AB at a concentration of 100 µM for the protection of myocyte against reperfusion injury. We also tried a concentration of 1 mM in the same protocol. However, most of the hearts did not start beating again after reperfusion. In a previous study [21], we determined the IC₅₀ of 3-AB to be 23 µM, suggesting that 1 mM and 100 µM 3-AB would results in a 96 and 74% inhibition of the PARP activity, respectively. In their study of protection against myocardial reperfusion injury with PARS inhibitors, Bowes and co-workers also used, a concentration intended too large doses of PARS inhibitor may cause deleterious
effects on other organs by their own toxic actions as well as PARS inhibition. In this sense, the use of 3-AB in a cardioplegic solution at a concentration of 100 μM might be practically safe, even though a small amount of 3-AB would remain in the circulation after cardiac surgery. A lot of studies demonstrated that the blood cardioplegia better preserved myocardial metabolism and cardiac function than the crystalloid cardioplegia [24]. And several experimental studies have shown a cardioprotective effect of leukocyte depleted posts ischemic perfusate [25]. The accumulation of neutrophils is major inflammatory mediators of myocardial injury in the early period of ischemia and reperfusion. A limitation of this study is the lack of influence of neutrophils, proteins and aminoacides. The advantages of the blood-perfused isolated heart Langendorff model have been described previously. However, the use of a support animal can introduce variability related to ionic and hormonal fluctuations. In the present investigation, an isolated heart model with the crystalloid cardioplegic arrest was chosen for two reasons: (1) heart is completely and uniformly affected; (2) there are little influences of neutrophils, immune systems and hormonal fluctuations. However, future studies are required to prove if the concept of 3-AB also improves protection of hearts with the blood cardioplegia or blood perfused models. In conclusion, using a Langendorff perfusion system, we studied, the efficacy of a cardioplegic solution with the addition of PARS inhibitor against cardioplegic ischemia-reperfusion injury. 3-AB effectively preserved LV function and reduced cardiomyocytic injury. We also showed that the nuclear genome against oxidative stress and prevented myocytes from apoptosis. Thus, it can be concluded that the use of cardioplegic solution with 3-AB added in cardiac operations may enhance myocardial protection.

References