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ORIGINAL CONTRIBUTION

Zhi-Dong Ge
Pin-Lan Li
Ya-Fei Chen
Garrett J. Gross
Ai-Ping Zou

Myocardial ischemia and reperfusion reduce the levels of cyclic ADP-ribose in rat myocardium

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Zhi-Dong Ge · Pin-Lan Li · Ya-Fei Chen
Garrett J. Gross, Ph.D. (✉) · Ai-Ping Zou
Department of Pharmacology
and Toxicology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, Wisconsin 53226, USA
Tel.: (414) 456-8627
Fax: (414) 456-6545
E-Mail: ggross@mcw.edu

Abstract Cyclic ADP-ribose (cADPR) is a novel Ca^{2+}-mobilizing second messenger in mammalian cells including cardiomyocytes. It is unknown whether myocardial ischemia and reperfusion affect the metabolism of cADPR in the myocardium. The present study therefore examined the effects of myocardial ischemia and reperfusion on the concentrations of myocardial cADPR using high-performance liquid chromatography. Basal levels of cADPR in rat myocardium were 5.3 ± 1.8 nmol·mg^{-1} protein. Myocardial ischemia for 30 min significantly decreased cADPR concentrations to 2.1 ± 0.4 nmol·mg^{-1} protein. During reperfusion, cADPR was maintained at ischemic levels. The activity of ADP-ribosyl cyclase was expressed as the conversion rate of nicotinamide guanine dinucleotide (NGD^+) to cyclic GDP-ribose. Myocardial ischemia and reperfusion did not alter the activity of ADP-ribosyl cyclase. However, cADPR hydrolase activity, as measured by the conversion rate of cADPR to ADP-ribose, was significantly elevated by ischemia and reperfusion. To determine the mechanism resulting in the enhancement of cADPR hydrolase activity, we examined the effects of changes in ADP, ATP, pH, and PO_2 on the conversion rate of cADPR to ADPR. Alterations of ADP, ATP, or pH in myocardial tissue had no effect on the degradation of cADPR, whereas a decrease in tissue PO_2 markedly increased the hydrolysis of cADPR. These results suggest that myocardial ischemia and reperfusion decrease cADPR in the myocardium by increasing its hydrolysis. Tissue hypoxia may be one of the important mechanisms to activate cADPR hydrolase.

Key words Cyclic ADP – ribose – ischemia – reperfusion – heart – rats

Introduction

Cyclic ADP-ribose (cADPR) is synthesized from β-nicotinamide adenine dinucleotide (NAD^+), an abundant intracellular substrate, by ADP-ribosyl cyclase and hydrolyzed to adenosine diphosphoribose (ADPR) by cADPR hydrolase (14, 26). In a wide range of mammalian cells, cADPR has been found to initiate Ca^{2+} release from intracellular stores (1, 6, 10, 16, 19, 23, 26, 30). The effect of cADPR to mobilize Ca^{2+} is as effective as inositol 1,4,5-triphosphate (IP_3) but completely independent of the IP_3 signaling mechanism (6,10). Therefore, cADPR is widely accepted as a novel second messenger mediating Ca^{2+} mobilization (5, 10, 18). cADPR-mediated Ca^{2+} signaling has been found to participate in the regulation of a variety of cell functions, including insulin secretion, egg fertilization, cell proliferation, coronary vascular tone, the effects of nitric oxide, and mediation of agonist responses (8, 10, 20, 23, 24, 30, 32). In cardiomyocytes, cADPR has been recently reported to potentiate Ca^{2+} transients and Ca^{2+} sparks (2, 11, 21) and mediate or

modulate Ca^{2+} release during excitation-contraction coupling (12, 25). Thus, cADPR might play an important role in the regulation of myocardial contractility.

Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) is an important trigger and regulator of cardiac contractility and metabolism. It is well known that after acute global and regional ischemia, a common event is a progressive rise in $[Ca^{2+}]_i$ of cardiomyocytes that persists during the early stages of reperfusion (1, 22). However, myocardial contractility decreases during ischemia and reperfusion. The mechanism producing this Ca^{2+} paradox has not been fully understood. Since cADPR in cardiomyocytes modulates Ca^{2+} release during excitation-contraction coupling (12, 25), we hypothesized that cADPR decreases during ischemia and reperfusion. To test this hypothesis, the present study was designed to examine the effects of myocardial ischemia and reperfusion on the concentrations of cADPR and to explore the mechanisms by which cADPR is altered under these circumstances. Using high-performance liquid chromatography (HPLC), cADPR concentrations were first determined in rat myocardium subjected to ischemia or post-ischemic reperfusion. Subsequently, the activities of ADP-ribosyl cyclase and cADPR hydrolase in these myocardial tissues were examined to define the role of cADPR formation or degradation in altered cADPR levels in ischemic or reperfused myocardium. Finally, to explore the mechanisms producing changes in cADPR concentrations during ischemia and reperfusion, we further examined the effects of changes in ADP, ATP, pH, and PO_2 in myocardial tissue on the activities of ADP-ribosyl cyclase and cADPR hydrolase.

Methods

In vivo myocardial ischemia and reperfusion and assessment of infarct size (IS)

All the procedures in the present study conformed to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Wistar rats weighing 350 – 450 g were anesthetized via i.p. administration of Inactin (100 $mg \cdot kg^{-1}$). A tracheotomy was performed, and the trachea was intubated with a cannula connected to a rodent ventilator. The rats were ventilated with room air supplemented with O_2 at 60 – 65 $breaths \cdot min^{-1}$. Body temperature was maintained at 37 ± 1 °C using a heating pad. A left thoractomy was performed approximately 10 mm from the sternum to expose the heart at the fifth intercostal space. The pericardium was removed, and the left arterial appendage was moved to reveal the location of the left coronary artery. A ligature (6-0 prolene) was passed below the left vein and coronary artery from the area immediately below the left arterial appendage to the right portion of the left ventricle. The ends of the suture were threaded through a propylene tube to form a snare. Pulling the ends of the suture taut and clamping the snare onto the epicardial surface with a hemostat elicited occlusion of the coronary artery and resulted in regional left ventricular ischemia. Reperfusion of ischemic myocardium was initiated through unclamping the hemostat and loosening the snare and was confirmed by visualizing an epicardial hyperemic response.

Upon completion of 30-min ischemia or postischemic reperfusion for 2 h, the left coronary artery was reoccluded, and Patent blue dye was injected via the jugular vein to effectively stain the nonischemic area of the left ventricle. The heart was excised, and the left ventricle was removed from the remaining tissue and subsequently sliced into five cross-sectional pieces. This allowed for the delineation of the normal area (stained blue) versus the area at risk (AAR) that subsequently remained pink. The AAR was cut from the nonischemic area and the tissues were placed in separate vials and incubated with a 1% triphenyltetrazolium chloride staining in 100 mM phosphate buffer (pH = 7.4) at 37 °C. Triphenyltetrazolium chloride is an indicator of viable and nonviable tissues. Tissues were stored in vials of 1% formaldehyde overnight and the infarcted myocardium was dissected from the AAR under the illumination of a dissecting microscope. Infarct area and AAR were determined by gravimetric analysis. IS is expressed as a percentage of the AAR (4).

Extraction and quantitation of myocardial cADPR

Rats were randomly assigned to the following three groups: group I with no ischemia (control), group II with 30-min coronary artery occlusion without reperfusion, group III with 30-min coronary artery occlusion followed by 2-h reperfusion. The heart was rapidly cut into very small pieces and powdered under liquid N_2. Tissue powder of 100 mg with 3 nanomole internal standard NGD^+ was homogenized with 1 ml of 0.6 $mol \cdot L^{-1}$ perchloric acid and sonicated for 20 s at 45 W with use of a microsonicator. The homogenate was centrifuged at 13,800 × g for 10 min at 4 °C. The supernatant containing nucleotide was neutralized with 7 $mol \cdot L^{-1}$ NH_4OH to pH 7.2 – 7.4, and the ionic contaminants present in the perchloric acid extract were removed by solid-phase extraction on affinity chromatography gel (Affi-Gel 601, BIO-RAD) by using disposable columns which were filled with Affi-Gel 601 preswollen with 0.1 $mol \cdot L^{-1}$ formic acid ammonium. The samples were loaded on the column. The column was washed with 20 ml of 0.1 $mol \cdot L^{-1}$ formic acid ammonium

at a flow rate of 1.5 ml·min^{-1}, and the sample elute collected and lyophilized (3).

For further purifying cADPR, AG MP-1 ion exchange columns were used. Lyophilized extract was dissolved in 1 ml of water and injected into the AGMP-1 column. A gradient from water (eluent A) to 150 mmol·L^{-1} trifluoroacetic acid (eluent B) was used at a flow rate of 8 ml·min^{-1}. The gradient was (in % of eluent B): 0 min, 10%; 6 min, 10%; 11 min, 20%; 16 min, 40%; 18 min, 58%; 20 min, 100%; 25 min, 100%; 26 min, 10%; 31 min, 10%. Absorbance was measured at 254 nm, and the eluting fraction following 12 – 15 min including the internal standard (NGD^+) was collected and lyophilized. The lyophilized sample was subsequently redissolved in 30 µl of H_2O and analyzed by a reverse-phase HPLC with a 1090A photodiode array detector (3).

Preparation of homogenate

On completion of 30-min ischemia or 2-h post-ischemic reperfusion, left ventricular muscle was minced and homogenized in 10 mmol·L^{-1} HEPES buffer (pH7.4) containing (in mmol·L^{-1}) NaCl 148, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.3, and glucose 5.5. The resultant homogenate was centrifuged at 4 °C for 10 min at 2,000 × g to remove unbroken cells and nuclei. The crude supernatant (homogenate) contained membrane protein and cytosolic component. The homogenate was frozen in liquid N_2 and subsequently stored at –80 °C until assayed.

Assay of ADP-ribosyl cyclase activity

Activity of ADP-ribosyl cyclase was determined by HPLC analysis of conversion of NGD^+ into the fluorescent product cyclic GDP-ribose (cGDPR), as described previously (7, 9). Briefly, a reaction mixture containing 10 µg homogenate, 1 mmol·L^{-1} NGD^+, 20 mmol·L^{-1} HEPES, and 1 mmol·L^{-1} $MgCl_2$ was incubated at 37 °C for 30 min, and then rapidly frozen in liquid N_2 to terminate the reaction. Before analysis with HPLC, the reaction mixtures were ultrafiltered using an Amicon filter to remove the protein. The reaction products were analyzed by a HPLC system using a Hewlett Packard 1090L solvent delivery system and a 1046A programmable fluorescence detector (Hewlett Packard). The excitation wavelength was 300 nm, and the emission wavelength was 410 nm. Nucleotides were resolved on a Supelcosil LC-18 column (3 µm; 4.6 × 150 mm, Supelco). The injection volume was 20 µl. The mobile phase consisted of 150 mmol·L^{-1} ammonium acetate (pH 5.5), which contained either 5% methanol (Solvent A) or 50% methanol (Solvent B). The nucleotides were separated in solvent A with a gradient of 0% solvent B to 25% solvent B for 12 min, 25% solvent B to 100% solvent B for 4 min, and then 0% solvent B for 5 min. The flow rate was 0.8 ml·min^{-1}. Peak identities were confirmed by comigration with known standards. Quantitative measurements were performed by comparing known concentrations of standards.

Assay of cADPR hydrolase activity

The activity of cADPR hydrolase in rat myocardium was determined by analysis of conversion rate of cADPR into ADPR using UV HPLC (20). Briefly, the homogenate was incubated with 1 mmol·L^{-1} cADPR at 37 °C for 30 min. The reaction products were chromatographed and analyzed using a Hewlett Packard HPLC system with a 1090A photodiode array detector. The column effluent was monitored at 254 nm. Data were collected and analyzed by a Hewlett Packard chemstation. Nucleotides were resolved on a 3-µm Supelcosil LC-18 column (4.6 × 150 mm, i.d.) with a 5 µm Supelcosil LC-18 guard column (4.6 × 20 mm, i.d.; Supelco, Bellefonte, BA). The injection volume was 20 µl.

Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). The significance of differences in mean values within and between multiple groups was examined using ANOVA for repeated measures followed by a Duncan's multiple range test. Student's t test was used to examine the significance of difference in two groups. $P < 0.05$ was considered significant.

Results

IS and AAR

No infarct was observed in the rat heart without a coronary artery occlusion. Coronary artery occlusion for 30 min created a large infarct (52.4 ± 2.3%, n = 7). The IS expressed as a percentage of the AAR in the rats with 30-min occlusion/2-h reperfusion was 58.7 ± 2.7% (n = 7).

Changes in cADPR concentrations in ischemic and reperfused myocardium

Figures 1a – c are representative chromatographs showing that cADPR (peak at retention time of 3.2 min) levels were markedly lower in the myocardium subjected to ischemia and post-ischemic reperfusion than in control myocardial tissue. The results are summarized in Fig. 1d.

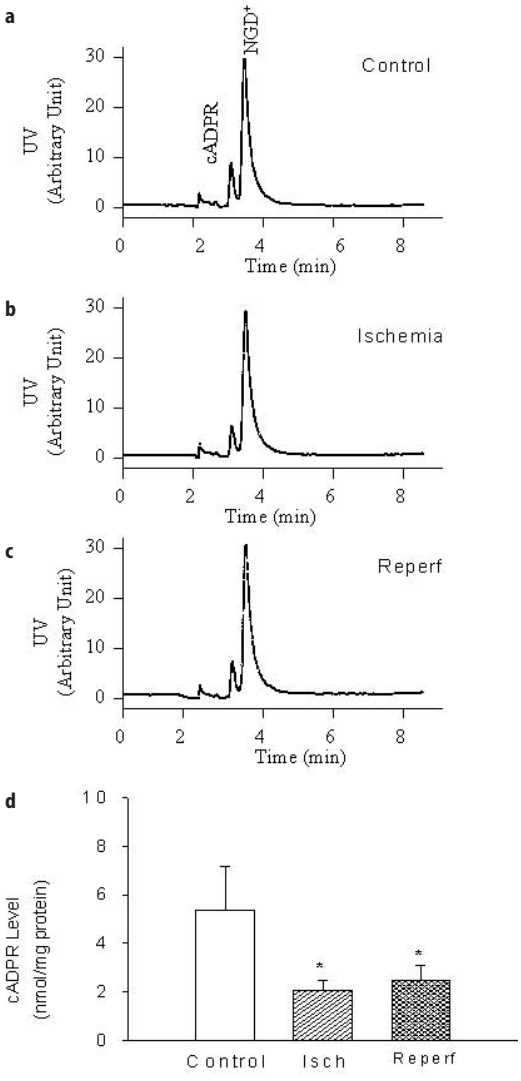

Fig. 1 cADPR levels in the rat myocardium of control, ischemia, and post-ischemic reperfusion. **a, b**, and **c** HPLC chromatograms of cADPR extracted from rat myocardium of control, ischemia and post-ischemic reperfusion, respectively, are shown. NGD^+ was used as an internal standard. Injection volume is 20 μl that contained identical amounts of extracted protein. **d** The data showing the cADPR concentrations in the rat myocardium of control, ischemia, and post-ischemic reperfusion are summarized. Isch indicates 30-min ischemia; and reperf indicates 2-h reperfusion after ischemia. *P < 0.05, compared to the control (n = 7–8).

In the control rat myocardium, the basal level of cADPR was 5.3 ± 1.8 nmol·mg^{-1} protein (n = 8). When the hearts of rats were subjected to ischemia for 30 min, the cADPR concentration was decreased to 2.1 ± 0.4 nmol·mg^{-1} protein (n = 8, P < 0.05) and continuously maintained at a low level (2.5 ± 0.6 nmol·mg^{-1} protein) during reperfusion.

Effects of ischemia and reperfusion on ADP-ribosyl cyclase and cADPR hydrolase

Synthetic standard cGDPR displayed a peak at 2.1 min in fluorescent chromatograph. The conversion rate of NGD^+ to cGDPR in myocardial homogenates of control rats was 14.8 ± 0.5 nmol·min^{-1}·mg^{-1} protein (n = 8, Fig. 2a). Using NGD^+ as substrate, the cyclization reaction catalyzed by the homogenate was kinetically analyzed. The K_m and V_{max} calculated by Lineweaver-Burk plots were 0.1 μmol·L^{-1} and 0.92 μmol·min^{-1}·mg^{-1} protein, respectively. When rat myocardium was subjected to 30-min ischemia and 30-min ischemia followed by 2-h reperfusion, the conversion rates were 14.9 ± 0.5 and 14.2 ± 0.8 nmol·min^{-1}·mg^{-1} protein (n = 8), respectively (Fig. 2a). There was no significant difference among the three groups.

The activity of cADPR hydrolase was quantified by measurement of conversion rate of cADPR to ADPR. In

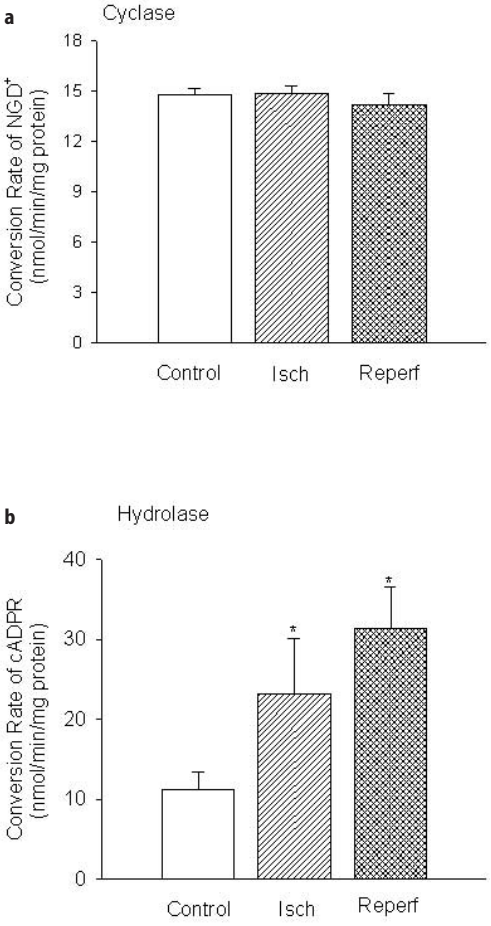

Fig. 2 The activities of ADP-ribosyl cyclase (**a**) and cADPR hydrolase (**b**) in ischemic and perfused rat myocardium. The activities of ADP-ribosyl cyclase and cADPR hydrolase were expressed as the conversion rates of NGD^+ to cGDPR and cADPR to ADPR, respectively. *P < 0.05, compared to the control (n = 8).

UV chromatograph, one peak at a retention time of 3.0 min was identified as cADPR, and the other at 15.6 min was ADPR. Similar to synthetic standards, the incubation of the myocardial homogenate with 1 mmol·L^{-1} cADPR produced cADPR and ADPR peaks. The conversion rate of cADPR to ADPR in the homogenate averaged 11.2 ± 2.1 nmol·min^{-1}·mg^{-1} protein (n = 7, Fig. 2b). The K_m and V_{max} via Lineweaver-Burk plot analysis were 0.7 μmol·L^{-1} and 2.72 μmol·min^{-1}·mg^{-1} protein, respectively. When the rat myocardium was subjected to ischemia, the conversion rate of cADPR to ADPR was significantly increased to 23.1 ± 4.6 nmol·min^{-1}·mg^{-1} protein (n = 8, P < 0.05). At two hours of reperfusion, the conversion rate remained elevated at 31.3 ± 5.2 nmol·min^{-1}·mg^{-1} protein (n = 7, P < 0.01).

Effects of ATP and ADP on the activities of ADP-ribosyl cyclase and cADPR hydrolase

The homogenate of rat myocardium was incubated with 1 mmol·L^{-1} NGD$^+$ at 37 °C for 30 min in the absence or presence of ATP or ADP. Neither ATP nor ADP at the concentrations of 1 – 8 nmol·L^{-1} changed the conversion rates of NGD$^+$ to cGDPR (n = 7, Fig. 3a). Similar to the cyclase, the activity of cADPR hydrolase was not changed by either ATP or ADP at 1 – 8 nmol·L^{-1} (n = 5, Fig. 3b).

Effect of pH on the activities of ADP-ribosyl cyclase and cADPR hydrolase

Figure 4a shows the effect of pH on the activity of ADP-ribosyl cyclase in the homogenate of rat myocardium. When pH of the reaction mixture was changed from 6.0 to 7.8, the conversion rates of NGD$^+$ to cGDPR tended to gradually decrease. At pH 7.4, the conversion rate was 11.2 ± 0.3 nmol·min^{-1}·mg^{-1} protein (n = 5). A change in pH from 7.4 to 7.8 did not significantly alter the conversion rates. However, when the pH was changed to 7.2 or lower, the conversion rate of NGD$^+$ to cGDPR significantly increased (n = 5, P < 0.05).

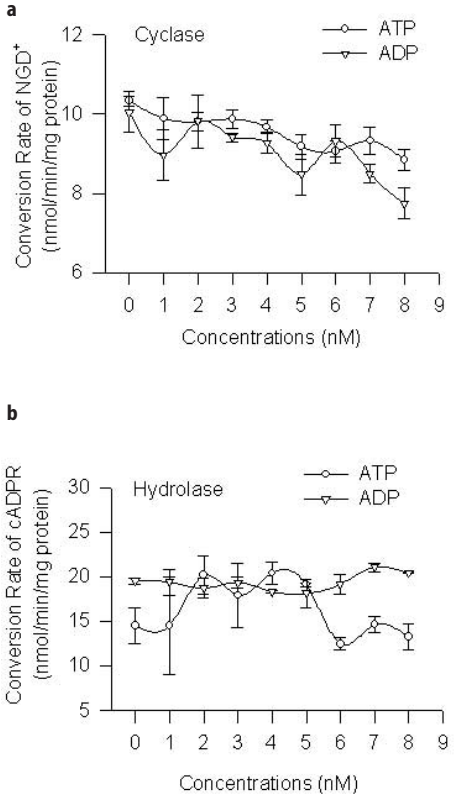

Fig. 3 Effects of ATP and ADP on the activities of ADP-ribosyl cyclase (**a**) and cADPR hydrolase (**b**) in the myocardial homogenate. The activities of ADP-ribosyl cyclase and cADPR hydrolase were expressed as the conversion rates of NGD$^+$ to cGDPR (n = 5) and cADPR to ADPR (n = 7), respectively.

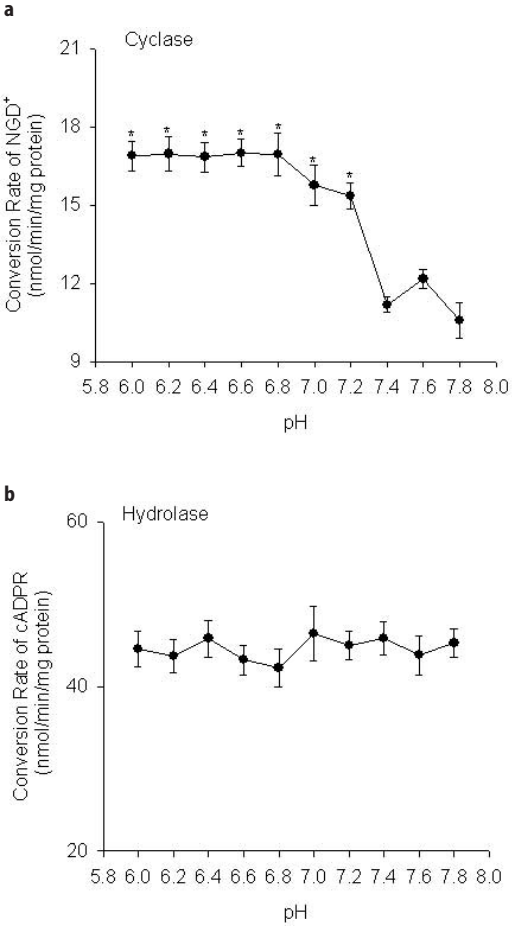

Fig. 4 Effects of pH changes on the activities of ADP-ribosyl cyclase (**a**) and cADPR hydrolase (**b**) in the myocardial homogenate. *P < 0.05, compared to the value obtained at pH 7.4.

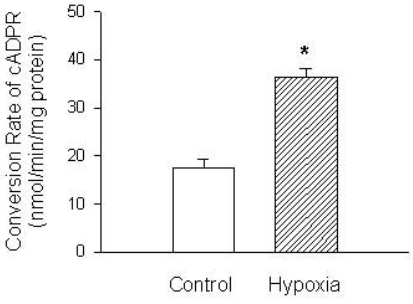

Fig. 5 Hypoxia-induced change in the activities of cADPR hydrolase in the rat myocardial homogenate. Hypoxia was induced via bubbling the reaction mixtures with N_2 during the reaction. *P < 0.05, compared to the control (n = 6).

The effect of pH on the activity of cADPR hydrolase in the homogenate is shown in Fig. 4b. The homogenate of rat myocardium was incubated with 1 mmol·L^{-1} cADPR at 37 °C for 30 min. Change in pH of the reaction mixture from 6.0 to 7.8 did not significantly affect the conversion rates of cADPR to ADPR (n = 5).

Effect of low PO_2 on the activity of cADPR hydrolase

The activity of cADPR hydrolase was expressed as the conversion rate of cADPR to ADPR. In the control group, the myocardial homogenate was incubated with 1 mmol ·L^{-1} cADPR at 37 °C for 60 min under bubbling with room air (PO_2 = 152 ± 8 mmHg). The conversion rate of cADPR to ADPR was 17.9 ± 1.6 nmol·min^{-1}·mg^{-1} protein (n = 6). Low PO_2 was induced and maintained by continuously bubbling the reaction mixture sealed with mineral oil with 99.9 % N_2. After 60 min, the PO_2 in the reaction mixture was 5 ± 1 mmHg. Under such PO_2, the myocardial homogenate was incubated with 1 mmol·L^{-1} cADPR at 37 °C for 60 min. The conversion rate of cADPR to ADPR was significantly increased to 36.2 ± 1.8 nmol·min^{-1}·mg^{-1} protein (n = 6, P < 0.01, Fig. 5).

Discussion

The present study demonstrates that 1) myocardial ischemia decreases cADPR levels, which is associated with an elevated activity of cADPR hydrolase, but without any change in the activity of ADP-ribosyl cyclase, 2) post-ischemic reperfusion does not restore cADPR concentration and the activity of cADPR hydrolase to normal, and 3) exogenous alterations of ATP, ADP, and pH are without effect on the activity of cADPR hydrolase, but a reduction in PO_2 increases the activity of this enzyme. These results indicate that cADPR decreases during myocardial ischemia and that hypoxia-induced enhancement of cADPR hydrolysis may contribute to the change in cADPR.

The values of the risk region and IS obtained in the present study were close to those seen in previous studies in rats for corresponding durations of coronary occlusion (4, 15). In the myocardium subjected to 30-min ischemia or followed by 2-h reperfusion, cADPR levels were found to significantly be lower than those observed in non-ischemic myocardium. Previous studies have shown that cADPR plays an important role in mediating Ca^{2+}-induced Ca^{2+} release, especially in regulating the sensitivity of ryanodine receptors to Ca^{2+} and the speed of Ca^{2+}-induced Ca^{2+} release (17). Therefore, the reduced myocardial contractility during ischemia and reperfusion may be related to a decrease in cADPR.

Previous studies found that the concentration of NAD^+ in dog myocardium decreases during ischemia and reperfusion (27, 28). In our preliminary study, NAD^+ levels in rat myocardium subjected to 30-min ischemia or following 2-h reperfusion were also found to be lower than those in the nonischemic myocardium. Theoretically, low concentrations of NAD^+, as the substrate for cADPR synthesis, may result in a lower production rate of cADPR. To explore whether the decreased cADPR observed during ischemia and reperfusion is related to the changes in the activities of its synthase and decrease, we examined the effects of ischemia and reperfusion on activities of ADP-ribosyl cyclase and cADPR hydrolase. It was found that the activity of ADP-ribosyl cyclase was not altered, while the activity of cADPR hydrolase was significantly increased in the ischemic/reperfused myocardium. In the present study, the enzyme activities were measured in extracts. To some extent these assay conditions reflect the ones of well-oxygenated tissue. In our *in vitro* experiments, the activity of cADPR hydrolase was not increased by a rise in oxygen tension. Therefore, the decrease in cADPR during myocardial ischemia and reperfusion may be related to both the decline in NAD^+ levels of myocardium and the enhancement of cADPR hydrolase activity.

Myocardial ischemia and reperfusion lead to the change in the concentrations of ATP and ADP ratio due to the alteration of tissue oxygen supply. In the rat myocardium, the baseline levels of interstitial ATP are approximately 3 – 6 nmol·L^{-1} (15). We therefore examined the effects of exogenous application of ATP or ADP on the activities of the cyclase and hydrolase in myocardial homogenates. In the present study, neither ATP nor ADP at concentrations of 1 – 8 nmol·L^{-1} altered the activities of either enzyme.

In the rat myocardium, baseline pH is around 7.4. At 30-min coronary arterial occlusion, the pH values in the rat myocardium are reduced to about 6.0 (31). We therefore examined the effect of changes in pH on the activities of ADP-ribosyl cyclase and cADPR hydrolase. In the present study, the cADPR hydrolase is not sensitive to the

changes in pH. The activity of ADP-ribosyl cyclase, as the synthase of cADPR, was significantly higher at pH 7.2 to 6.0 than at pH 7.4. Obviously, the increased activity of ADP-ribosyl cyclase by reduced pH during ischemia and reperfusion does not contribute to the decrease in cADPR.

To determine the mechanism of increase in cADPR hydrolase activity during myocardial ischemia and reperfusion, we examined the effect of a decrease in tissue PO_2, since myocardial ischemia often results in a decrease in tissue or cellular PO_2. Low PO_2 was induced by continuously bubbling the reaction mixture with 99.9 % N_2 during a 60-min period. By calculating the conversion rate of cADPR to ADPR, hypoxia was found to increase the activity of cADPR hydrolase. It remains unknown whether low oxygen tension directly increases the activity of cADPR hydrolase. In the rat myocardial homogenates, ATP, ADP, and mitochondria were still present. Low PO_2 can lead to some biochemical changes in the reaction mixture, e.g., low ATP/ADP ratio. ATP at the concentrations of 2 – 6 mmol·L^{-1} was reported to increase the synthesis of cADPR and inhibit the activity of cADPR hydrolase in purified CD38 protein of pancreatic β-cells (13, 29). It is likely that a reduced ATP level in the reaction mixture would eliminate the inhibitory effect of ATP on cADPR hydrolase, which results in the increased activity of the hydrolase, although exogenous addition of ATP at 1 – 8 nmol·L^{-1} in the present study did not produce any effect.

In summary, myocardial ischemia decreased the concentration of cADPR in the myocardium, and the decrease in cADPR appears to be associated with an increased activity of cADPR hydrolase via a decrease in tissue or cellular PO_2. Myocardial ischemia usually leads to contractile dysfunction and cADPR participates in excitation-contraction coupling of myocardium. Therefore, it is likely that the contractile dysfunction of the heart during or after myocardial ischemia is associated with a decrease in cADPR levels.

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