Increase in Content of Glycogen Phosphorylase during Ischemia in Isolated Rabbit Heart

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Summary The amount of a 97-kDa protein in the sarcoplasmic reticulum-enriched microsomal fraction from rabbit heart changed in a manner dependent on the oxygenated condition after global hypothermic ischemia and subsequent normothermic reperfusion. Isolated hearts were immersed in physiologic saline for 0 h (control group) or 1, 2 and 3 h at 4°C (ischemia group) and were subjected to 40-min reperfusion at 37°C after 1 h of hypothermic ischemia (reperfused group). The amount of the 97-kDa protein in the microsomal fraction apparently increased after ischemia, but decreased after reperfusion. The N-terminal amino acid of the protein was blocked and two internal sequences were determined, demonstrating the 97-kDa protein to be glycogen phosphorylase. A polyclonal antibody against rabbit skeletal muscular glycogen phosphorylase (GP) stained only the 97-kDa protein in the microsomal fraction in Western blot analysis. Microsomal fractions isolated from rabbit brain, liver, spleen, and white skeletal muscle were also examined by Western blot analysis using anti-GP. Liver and skeletal muscle contained a 97-kDa protein that reacted with anti-GP in control preparations. Ischemia induced a modest increase in the amount of the 97-kDa protein skeletal muscle microsomes, whereas no change in the amount of the protein occurred in liver microsomes. The results indicate that alteration in the energy metabolism from ischemia to the subsequent reperfusion is associated with the amount of GP.

Key Words: sarcoplasmic reticulum-enriched microsomes, rabbit heart, ischemia, reperfusion, glycogen phosphorylase

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Ischemia-reperfusion injury is inevitable during surgical procedures, and such injuries are often caused by disruptions in blood flow, e.g., myocardial infarct, ulcer, and the crush syndrome. In well-oxygenated, contracting myocardium, the energy for muscular contraction is provided by mitochondrial oxidative phosphorylation. In the ischemic myocardium, disruptions in the supply of oxygen and in fatty acid metabolism result in accelerated glycolysis and the accumulation of lactate [1-3]. Ischemia-reperfusion injuries are believed to be due to abrupt and large increase in flows of Ca\(^{2+}\) and oxygen into cells, known as the 'calcium paradox' [4] and 'oxygen paradox' [5]. Adenosine 5'-triphosphate (ATP) provides energy not only for muscular contraction but also for cellular ionic homeostasis as well. Depletion of ATP thus induces decreased contractility and deterioration of the ionic composition of the cardiac cells. Ionic conditions in cardiac cells are maintained by the action of the Na\(^+/\)Ca\(^{2+}\)-exchanger and Na\(^+\), K\(^+\)-ATPase in the sarcolemma (SL) and by Ca\(^{2+}\), Mg\(^{2+}\)-ATPase in the sarcoplasmic reticulum (SR). It has been postulated that glycogen phosphorylase (GP) forms a complex with glycogen and attaches to SR membranes [6-9]. Furthermore, a series of glycolytic enzymes are also associated with SR membranes [10]. These facts suggest that ATP produced by mitochondrial oxidative phosphorylation is used for muscular contraction and that ATP produced by glycolysis is used for ion transport.

We previously reported that after ischemia-reperfusion injury, Ca\(^{2+}\)-transporting Ca\(^{2+}\), Mg\(^{2+}\)-ATPase in cardiac SR was degraded and that both ATPase and Ca\(^{2+}\)-uptake activities were inhibited [11, 12]. In addition to Ca\(^{2+}\), Mg\(^{2+}\)-ATPase, a variety of protein levels changed during and after ischemia-reperfusion including the level of a 97-kDa protein. In the present study we isolated this 97-kDa protein in the SR-enriched microsomal fraction and analyzed its content during ischemia and after reperfusion. The 97-kDa protein increased after ischemia and decreased after reperfusion. From the internal amino acid sequence and reactivity against a rabbit skeletal muscle GP polyclonal antibody, the 97-kDa protein was identified as GP.

MATERIALS AND METHODS

Animals and hearts. We followed the Guide for Animal Experimentation issued by our college, which was based on the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animals Resources and published by the National Institutes of Health (NIH Publication No.85-23, 1985, revised 1996).

Ten minutes after rabbits (New Zealand White, male, 2.0–2.5 kg) had received ketamine hydrochloride (40 mg/kg, i.m.), heparin Na (1,000 U) and Na-pentobarbitone (2.5 mg/kg) were injected via the left ear vein. After profound anesthesia had been achieved, the left carotid artery was dissected for blood letting, and 2 ml of ice-cold KCl (15%, w/v) was anterogradely injected into the coronary artery.
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from the aorta. The heart was immediately dissected, and a cannula was set in the aorta through which ice-cold physiologic saline was infused to wash out blood from the coronary artery and cardiac chambers. The ischemia groups were prepared by immersing the isolated hearts in physiologic saline at 4°C for 1, 2, and 3 h. The reperfusion group was subjected to Langendorff perfusion after 1 h of hypothermic ischemia using a modified Krebs-Henseleit bicarbonate solution (in mM, 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 25 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 11.1 glucose, pH 7.4), that had been equilibrated with 95% O₂ and 5% CO₂, at 37°C and 100 cm hydrostatic pressure for 40 min.

Tissue water content and tissue glycogen content. The apices of three hearts from each group were dried at 80°C for 24 h for determination of water content. The tissue water content was estimated as \( \frac{(\text{wet weight}) - (\text{dried weight})}{\text{wet weight}} \).

Four hearts were quickly frozen in liquid nitrogen after each treatment for determination of glycogen content. Glycogen content was estimated after hydrolysis by amyloglucosidase to glucose according to the method described by Keppler and Decker [13].

Isolation of microsomes. SR-enriched microsomes from rabbit heart were isolated by the method of Fukumoto et al. [12], which was based on the procedure of Chamberlain et al. [14]. All procedures were carried out at 4°C. Reperfused hearts were flushed with ice-cold physiologic saline to cool the tissue. Atrium, coronary artery, papillary muscles, and fatty tissue were trimmed off. Ventricular muscle was finely diced with scissors and homogenized (Nissei Excel Auto Homogenizer, Tokyo, Japan) in 5 v/w of 0.29 M sucrose, 3 mM NaN₃, 0.5 mM dithiothreitol, 10 mM 3-morpholinoethanesulfonic acid, pH 6.8 (solution A) by using the following sequence: 16,000 rpm for 15 s, off for 10 s, then 17,000 rpm for 30 s. The homogenate was centrifuged at 3,800 x g for 15 min, and passed through eight layers of medical gauze. The filtrate was centrifuged at 27,900 x g for 15 min, and the subsequent supernatant centrifuged at 119,200 x g for 100 min. The pellet was suspended in the solution A containing 0.65 M KCl and left on ice for 30 min. After centrifugation at 4,400 x g for 10 min, the supernatant was obtained and centrifuged at 251,800 x g for 100 min. The resultant pellet of SR-enriched microsomes was suspended in the solution A, frozen in liquid nitrogen, and stored at −80°C until used.

In a parallel study, crude SR-enriched microsomes from cardiac tissue were centrifuged in the presence of a discontinuous sucrose concentration gradient. Crude microsomes in approximately 10% sucrose were layered on a gradient of 28, 40, and 60% sucrose and centrifuged at 160,000 x g for 180 min (Beckman SW 40Ti). Each fraction was collected, diluted to approximately 10% sucrose, and centrifuged at 251,800 x g for 60 min. The resulting pellets were treated similarly as crude microsomes.

Microsomes from rabbit brain, liver, spleen, and skeletal muscle (psoas) were isolated using a method similar to that for isolation of the cardiac microsomes.

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Tissues were treated with either 0 or 1 h of ischemia by immersing in physiologic saline at 4°C. Microsomes were frozen in liquid nitrogen and stored at −80°C until used.

**SDS-PAGE.** Composition of microsomal fractions was analyzed by SDS-PAGE according to Laemmli [15]. Bands were stained with Coomassie brilliant blue R-250 (CBB), and their relative amount was estimated by densitometric analysis (Densito-Pattern Analyzer, EPA-3000, Cosmo Bio, Tokyo, Japan).

**Isolation and purification of 97-kDa protein.** The 97-kDa protein was electrophoretically isolated (Biophoresis III, ATTO, Osaka, Japan) using a 6% polyacrylamide gel in the presence of 0.1% SDS at constant 20 mA. SR-enriched microsomes (500 mg protein) were subjected to Biophoresis III. Fractions were collected into 20% sucrose, 0.37 M Tris-HCl (pH 8.8) at a flow rate of 1 ml/min. Each fraction was separated by an air bubble so that there was little cross-contamination. Protein composition was analyzed by SDS-PAGE (8% acrylamide), in which 20 μl of each fraction was applied and bands were stained with silver nitrate. Fractions containing the 97-kDa protein were collected, dialyzed exhaustively against water, lyophilized, and stored at −80°C.

**Amino acid sequence of the 97-kDa protein.** Since the N-terminus of the 97-kDa protein was blocked, the lyophilized protein was hydrolyzed with lysyl endopeptidase (Achromobacter protease I, Wako Pure Chemicals, Osaka, Japan) by the method of Rüegg and Rudinger [16] after reductive pyridylethylation of cysteinyl residues [17]. The hydrolysate was separated by HPLC (μ bondasphere, Waters, IL, USA), and two distinct fragments were analyzed for determination of their amino acid sequence (Protein Sequencer PSQ-I, Shimadzu, Kyoto, Japan).

**Western blot analysis.** The microsomal fraction was separated by SDS-PAGE (7.5% PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford MA, USA) in 18.4% methanol, 0.005% SDS, 25 mM Tris, and 192 mM glycine at constant 15 V for 90 min. Transferred proteins were visualized with gold colloids (AuroDye Forte, Amersham, Buckinghamshire, UK). Sheep anti-rabbit GP-a (Biogenesis, England, UK) was used as the primary antibody, and anti-sheep IgG as the secondary antibody, along with a Vectastain Elite ABC kit (Vector Lab. Inc., CA, USA).

**Statistical treatment.** Values are expressed in average ± SD. Statistical significance of differences in tissue water content and glycogen content was analyzed by analysis of variance followed by Scheffe’s F-test. Differences were considered significant when p was less than 0.05.

**Others.** Protein was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard. All reagents were of analytical grade and purchased from either Wako Pure Chemicals or Nacalai Tesque (Kyoto, Japan).

**RESULTS**

**Tissue water content**

The extent of ischemia-reperfusion injury in the isolated heart is likely to
correlate with that of tissue edema [19, 20]. In the present study, no statistically significant difference in tissue water content was present between the control (0.830±0.047), ischemic (0.764±0.083), and reperfused (0.769±0.051) groups, indicating that edematous alteration was not caused by ischemia-reperfusion treatments.

**Tissue glycogen content**

Tissue glycogen content was estimated as glucose equivalents after enzymatic hydrolysis of glycogen by amyloglucosidase. Tissue glycogen (in μmol glucosyl units/g wet tissue) in the control heart (25.8±13.6) was decreased by ischemia (16.5±12.9) and further decreased by reperfusion (8.2±7.3). Although there was a decrease between groups, the difference was not statistically significant due to scattered values between specimens.

**Protein composition in SR-enriched microsomal fraction**

Figure 1 shows SDS-PAGE profiles of SR-enriched microsomal fractions. The amount of many proteins varied after ischemia and after reperfusion. The 97-kDa protein apparently increased after 2 to 3 h of ischemia, though additional ischemic treatment for 6 h resulted in no further increase (data not shown). Reperfusion for 40 min after 1 h of ischemia apparently decreased the content of the 97-kDa protein.

![Fig. 1. Six percent SDS-PAGE profiles of proteins in SR-enriched microsomes. SR-enriched microsomes (30 μg protein) were applied to each lane. Samples were obtained after ischemia for 0 (I-0), 1 h (I-60), 2 h (I-120), and 3 h (I-180) and also after reperfusion for 40 min following 1-h ischemia (I/R). Four samples having a similar amount of protein were mixed and applied to SDS-PAGE for analysis of protein composition. The bar indicates the position of the 97-kDa protein. Proteins were stained with CBB. Leftmost lane contained molecular weight standards.](image-url)

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Figure 2 compares SDS-PAGE profiles of microsomal fractions centrifuged in a discontinuous sucrose concentration gradient. The 97-kDa protein was located between the 10 and 28% sucrose layers, though the 10% sucrose layer did not contain the protein (data not shown). A Ca$^{2+}$, Mg$^{2+}$-ATPase of approximately 100 kDa was also located mainly in the 10/28% fraction.

Isolation and amino acid sequence analysis of 97-kDa protein

Proteins in the SR-enriched microsomes were electrophoretically fractionated (Fig. 3). The 97-kDa protein was eluted in fractions 8 through 12. Because the N-terminal residue was protected in the 97-kDa protein, the conventional Edman analysis was not effective. We therefore analyzed the internal peptide sequences of two fragments and found them to be T-C-A-Y-T-N-H-T-V-L-P-E and F-G-C-R-D-P-V-R. After comparing these sequences to those in a data bank, we deduced the 97-kDa polypeptide to be GP [21].

Western blot analysis

Figure 4 shows that only the 97-kDa protein was stained with anti-rabbit skeletal muscle GP-a in the Western blot analysis. The stained area of the 97-kDa band increased after 1 h of ischemia and decreased following reperfusion.

GP in other organs

Since ischemia may modulate the level of GP in other organs, we also
examined the effect of ischemia on rabbit brain, liver, spleen, and psoas skeletal muscle (Fig. 5). Microsomal samples were prepared by a procedure similar to that for the isolation of the heart microsomes. Brain and spleen microsomes did not show any band that could be stained by muscle anti-GP-a. Liver microsomes contained a band that reacted with muscle anti-GP-a, but ischemic treatment did not change the amount of this band. In contrast, skeletal muscle showed an apparent increase in the amount of the 97-kDa protein after ischemia, although the extent of the increase was not as pronounced as in the case of the heart microsomes.
Many aspects of ischemia-reperfusion injury in the heart such as decreased contractility, deteriorating energy balance, and malfunctioning intracellular organelles have been extensively studied. Reperfusion injuries are often attributed to elevated oxygen tension, i.e., the 'oxygen paradox' [5] and elevated intracellular Ca\(^{2+}\), i.e., the 'calcium paradox' [4]. The intracellular Ca\(^{2+}\) concentration is controlled by ATP-dependent Ca\(^{2+}\) pumps in the SL and SR. When the intracellular Ca\(^{2+}\) concentration is extremely elevated, Ca\(^{2+}\) precipitates in the mitochondrial matrices, leading to mitochondrial dysfunction. Homeostasis of intracellular ions, especially Ca\(^{2+}\), is thus essential to ensure myocardial viability. We reported that Ca\(^{2+}\), Mg\(^{2+}\)-ATPase in SR was degraded after hypothermic global ischemia with subsequent normothermic reperfusion [11, 12]. We furthermore observed that the level of a variety of proteins associated with the SR, including that of a 97-kDa protein, changed following ischemia-reperfusion. The goal of the present study was to identify this 97-kDa protein in the SR-enriched microsomal fraction and to examine the effect of ischemia-reperfusion treatment on the level of this protein. Various models have been used to study ischemia-reperfusion injury in the heart, e.g., a model of partial ischemia induced by ligation of the coronary artery in situ and a model of global ischemia using isolated hearts. Ischemia has been classified into two types: anoxic ischemia by perfusion of deoxygenated solution and hypoxic low-flow ischemia [22]. The present model reproduces hypoxic global
Ischemia, since the ischemic period used for these studies may not have been long enough to completely exhaust the oxygen content.

Many studies of energy metabolism during ischemia-reperfusion have shown that glycogen is rapidly depleted and that the glycolytic pathway is activated by ischemia. Glycogen starts to decrease at the onset of ischemia [1, 3, 9, 23], and anaerobic metabolism is accelerated [24-26]. Sakai et al. [27] reported that the glycogen content decreased to 2/3 of the pre-ischemic level in all regions of the heart. The present study corroborated their findings: the glycogen content decreased to 2/3 of control levels after 1 h of ischemia. It has been suggested that glucose transporters in myocardial cells are synthesized in response to hypoxic ischemia [28] and anoxia [29]. Furthermore, it is feasible to postulate that the decrease in glycogen content during ischemia might be attributed to an increased GP content. In the present study, gluconeogenesis was expected to occur during reperfusion with glucose-containing medium. However, the subsequent 40 min of reperfusion decreased the glycogen content by an additional 1/3, whereas the GP content apparently decreased. The decrease in glycogen content was not statistically significant due to scattered values: the content in one heart after reperfusion was similar to the control level, whereas the other three showed a markedly reduced content. Since Cornblath et al. [30] indicated that the glycogen content might vary among individuals and the time of measurement, we examined four hearts to determine their glycogen content. The opposite change in content of glycogen and GP after reperfusion was thus less likely due to inaccurate observation. The results could be explained if gluconeogenesis was inhibited by ischemia-reperfusion, the absence of fatty acids in the perfusate demanding glycolysis for the energy supply [31], and/or if 40 min of reperfusion was not long enough to accumulate glycogen. This question will be the subject of future studies.

GP has been reported to form a complex with glycogen and bind to SR [6-9]. As shown in the present study, cardiac SR contains many protein other than Ca\(^{2+}\), Mg\(^{2+}\)-ATPase (Fig. 1). Xu et al. [10] showed that a rabbit cardiac SR preparation contained a set of glycolytic enzymes from aldolase through pyruvate kinase, when the SR was not treated with 0.6 M KCl. On the other hand, Entman et al. [6, 7] treated cardiac SR with 0.6 M KCl and isolated SR associated with glycogen and GP in the 33% sucrose fraction after centrifugation in the presence of a linear gradient of sucrose concentration. Since we washed our microsomes with 0.6 M KCl, the resultant SR-enriched microsomes could contain glycogenolytic enzymes without glycolytic enzymes. Since many proteins were present in the SR, it was difficult to isolate a targeted protein. The electrophoretic isolation procedure used in the present study was able to successfully isolate the 97-kDa protein, since the SDS-PAGE of the isolated fraction showed no bands other than the 97-kDa protein one. We analyzed the internal peptide sequence of this protein and identified it as GP.

Frederiks et al. pointed out that irreversible changes in GP correlated with cellular deterioration [32]. Studies of the relationship between ischemia and GP
have mainly focused on examining enzymatic activity. Sakai et al. reported that ischemia doubled the GP activity in all regions of the heart [27]. Lavanchy et al. concluded that increased glycogenolysis was due to a 50% increase in GP activity without a change in the ratio of GP-a/(GP-a+GP-b) [1]. This suggests that ischemia activates GP-b and/or increases the amount of GP and the conversion of GP-b to -a. The present study demonstrates that GP associated with SR increases after ischemia and decreases after reperfusion (Figs. 1 and 4). These results indicate that ischemia not only activates GP as previously reported by others, but also may increase the amount of GP, as shown in the present report.

The activity of GP is regulated in two ways, by covalent modification and by allosteric effectors [33]. Ischemia-induced activation of GP is thought to be the result of an increase in GP-a due to elevated cAMP [23, 34], activation of GP-b by cAMP and/or Pi [1, 32, 35] or by glucose 6-phosphate and Pi [31]. On the other hand, Morgan and Parmeggiani concluded that phosphofructokinase activated GP-b in the anaerobically reperfused isolated heart [3]. In contrast, no change in GP activity was reported in the anoxic heart [28, 34], and GP kinase was reported to be activated by Mg$^{2+}$ and Ca$^{2+}$ [34]. Although the present model most closely resembles hypoxic ischemia, GP kinase may have been activated if 1 h of ischemia induced anoxia. Though the present study did not determine whether GP in the microsomal fraction was GP-a or -b, but it is likely that the majority of GP in the present study was GP-b, since hypoxic ischemia increases GP-a [23, 34] that could be liberated from SR [17]. In addition, reperfusion increased GP-b for long periods after ischemia [9].

Mair recently pointed out that hypoxia liberates GP-BB from a complex with glycogen and SR into the cytosol [8]. He also summarized the distribution of GP isoenzymes in various human organs: GP-MM is in heart and skeletal muscles, GP-BB is in heart and brain, and GP-LL occurs predominantly in liver, spleen, and kidney but is absent from heart, skeletal muscle or brain. In the present study, the 97-kDa protein that reacted with anti-GP was apparent in heart, skeletal muscle, and liver. The antibody used in the present study was derived from rabbit skeletal muscle and thus could be anti-GP-MM. If rabbit and human GPs have a common epitope, the present results are consistent with previously reported results. Western blot analysis showed that after ischemia, only cardiac SR had increased GP levels, whereas microsomes from skeletal muscle and liver showed only slight changes in GP content. Weak signals found in liver microsomes may be due to low antibody cross-reactivity, and low amounts in skeletal muscle may be due to the fact that skeletal muscle is tolerant against hypoxic insult.

In summary, the glycogen content was decreased while the content of GP in SR increased in isolated rabbit heart after hypothermic global ischemia. This suggests that myocardial cells respond to ischemic insult by activating the glycolytic pathway to maintain intracellular Ca$^{2+}$ homeostasis. The response in heart was stronger than that in skeletal muscle (Figs. 4 and 5), indicating that aerobic heart is more vulnerable to the hypoxic condition than anaerobic skeletal muscle.

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muscle. Alteration of GP in SR fraction may support the recent hypothesis that cellular energy metabolism is composed of two compartments, glycolytic and oxidative ATP production.

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