Protective Effect of Captopril on Ischemic Myocardium

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The protective effect and mechanism of action of the angiotensin-converting enzyme inhibitor (ACE-I) captopril was investigated in organelles from ischemic myocardial cells in a canine coronary ligation model. Sarcoplasmic reticulum (SR) and mitochondrial fractions were extracted from ischemic and nonischemic myocardial cells from captopril- and saline-treated (control) hearts. Heart rate, cardiac output, and right ventricular systolic blood pressure were similar in the captopril-treated and control groups. Left ventricular systolic blood pressure (LVSPs) decreased gradually to 89% of the baseline value after captopril administration, and to 78% of the baseline value after ligation. Ca-ATPase activity in the SR, the respiratory control ratio (RCR) in the mitochondria, and dinitrophenol (DNP)-stimulated ATPase activity were significantly higher in ischemic myocardium from the captopril-treated group than from the saline-treated (control) group. The SH group content of both organelles was higher in the captopril-treated group. Our results suggest that, in addition to their hemodynamic effects, ACE-I agents containing SH groups protect the myocardium from ischemic damage by preventing enzyme oxidation.

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Angiotensin-converting enzyme inhibitors (ACE-I) lower blood pressure by 2 mechanisms. Firstly, they inhibit the conversion of angiotensin I to angiotensin II,¹ resulting in inhibition of the effects of angiotensin II on smooth muscle contraction, aldosterone secretion, and the release of epinephrine from sympathetic nerve endings. Secondly, they inhibit inactivation of bradykinin, resulting in an increase in bradykinin.^{2–5}

In clinical mass epidemiological studies, beneficial effects can be achieved in patients with cardiac failure by administering ACE-I agents. Studies of left ventricular dysfunction (SOLVD)^{6,7} have shown that enalapril increases the long-term survival of patients significantly more than hydralazine and isosorbide dinitrate. A SAVE (Survival and Ventricular Enlargement) study⁸ showed that early treatment with captopril for acute myocardial infarction reduced cardiovascular mortality and prevented deterioration of left ventricular function.⁹ In addition, captopril has been found to provide protection from reperfusion injury after myocardial ischemia.¹⁰

However, agents differ in efficacy depending on whether or not they contain SH groups.¹¹ Pretreatment with captopril, which contains SH groups, resulted in better recovery of myocardial contractility after 15 min-ischemia reperfusion in an adult dog model.¹² ACE-I agents containing SH groups have greater in vitro effects on scavenging superoxide anions than those without SH groups.¹² Furthermore, agents containing SH groups, ie captopril, cysteine and glutathione, achieve significantly greater increases in coronary blood flow in the isolated rat perfusion heart model.¹³

ACE-I agents containing SH groups are therefore potentially useful therapeutic agents for the treatment of cardiovascular disease. The SAVE study found, by evaluating both hemodynamic flow and left ventricular wall motility in patients with acute myocardial infarction, that treatment with ACE-I agents improved cardiac function. However, only a few studies have investigated the mechanism of action of ACE-I at the level of cellular organelles. We have demonstrated decreases in the activity of Ca-dependent ATPase,

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the content of the major ATPase proteins of the sarcoplasmic reticulum (SR), and in mitochondrial respiration, all of which lead to irreversible myocardial cell necrosis 20–30 min after cessation of coronary blood flow^{14,15} Furthermore, membrane-bound protein enzymes are irreversibly digested by proteases in ischemia, and the free radicals generated abundantly during reperfusion injury inhibit enzyme activity owing to oxidation of protein SH groups^{16,17}

Thus, to investigate the effect of captopril on ischemic cellular metabolism, we used an experimental model of acute cardiac ischemia to measure Ca-dependent ATPase activity of the SR, mitochondrial respiratory function, electron transport enzyme activities, and SH group content as indicators of oxidative disturbance of membrane proteins.

Materials and Methods

Animal Experiment

Twenty adult mongrel dogs were anesthetized with iv injections of urethane, α -chloralose, and diazepam (450 mg/kg, 45 mg/kg, 10 mg, respectively), breathing room air under controlled respiration with a Harvard respirator. The left anterior descending artery (LAD) was isolated and ligated just below the first diagonal branch to produce myocardial ischemia. Production of acute myocardial ischemia was confirmed by continuous ST elevation of 0.5 mV or greater on leads V₃ and V₄ on the ECG and by the presence of cyanosis. Simultaneously, a pigtail catheter was inserted through the femoral artery to the left ventricle, and a Swan-Ganz catheter was inserted through the femoral vein to the pulmonary artery.

Left ventricular pressure, cardiac output, and right ventricular pressure were monitored continuously from before occlusion of the LAD until 1 h after coronary occlusion. After the hemodynamic study, captopril (2.5 mg/kg body weight) was intravenously infused from 30 min before coronary occlusion until the end of the occlusion in the animals of the captopril-treated group (n=10), while the same amount of physiological saline was administered as a control in the saline-treated group (n=10). After 1 h ischemia, the heart was removed and immediately washed with cold saline. The ischemic myocardium of the anterior wall in the area supplied by the LAD and the left ventricular posterior wall in the area supplied by the circumflex artery were reserved as nonischemic controls.

Preparation of SR

The modified method of Harigaya and Schwartz¹⁸ was used. Ischemic and nonischemic myocardia were

minced with scissors, homogenized with a Polytron homogenizer in a solution of 0.01 mol/L NaHCO₃, 0.005 mol/L NaN₃, and 0.001 mol/L glycol ethylenediaminetetraacetic acid (GEDTA), and centrifuged twice at 9,000×G for 10 min. The supernatant was ultracentrifuged at 120,000×G for 30 min. The precipitate was solubilized in a solution of 0.6 mol/L KCl and 0.02 mol/L TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid]-Tris (pH 7.0) and ultracentrifuged again. The light microsomal fraction obtained was suspended in a 0.05 mol/L KCl and 0.02 mol/L TES-Tris solution (pH 7.0) and used as the fraction containing SR.

Extraction of Mitochondria

The modified method of Sordahl and Stewart¹⁹ was used. The heart muscle specimen was minced and homogenized with a Polytron homogenizer in a solution containing 0.25 mol/L sucrose, 0.01 mol/L GEDTA, and 0.03 mol/L Tris-HCl (pH 7.2), and centrifuged at 27,000×G for 15 min. The precipitate was treated with 1 mg of alkaline protease per gram of heart tissue, reacted for 8 min in ice, homogenized again in a 0.18 mol/L KCl, 0.01 mol/L GEDTA, 0.5% bovine serum albumin (BSA), 0.03 mol/L Tris-HCl solution (pH 7.2), and centrifuged at 500×G for 10 min. The supernatant was filtered through gauze and centrifuged twice at 12,000×G for 10 min. The precipitate was finally suspended in a solution containing 0.18 mol/L KCl, 0.5% BSA, and 0.03 mol/L Tris-HCl (pH 7.2) as a mitochondrial fraction. An aliquot of mitochondria was fragmented ultrasonically (Branson Sonifier 200, dial 4, duration every other second for 2 min) to make submitochondrial particles. All the above procedures were carried out at 4°C. The protein content was determined by the Biuret reaction. All chemicals used were reagent grade.

Measurement of Ca-ATPase Activity of SR and Electrophoresis of SR Protein

Ca-dependent ATPase activity in SR was measured according to the modified method of Harigaya and Schwartz¹⁸ The SR fraction was incubated with 0.0002 mol/L CaCl₂ or 0.001 mol/L GEDTA at 37°C in a solution of 0.1 mol/L KCl, 0.01 mol/L MgCl₂, 0.005 mol/L NaN₃, 0.003 mol/L ATP, and 0.02 mol/L TES-Tris (pH 7.0) for 10 min, and liberated inorganic phosphorus was measured by the modified method of Fiske and Subbarow²⁰ The activity was expressed as the differences in the presence of Ca²⁺ and GEDTA. An aliquot of SR protein was analyzed by electrophoresis and stained with silver for semiquantitative analysis using the Laemmli method²¹

Respiratory Activity of Mitochondria

The respiratory activity of mitochondria was measured as described by Hagihara²². The reduction in oxygen concentration was determined polarographically using glutamate and malate or succinate as substrates by adding ADP to a final concentration of 0.000625 mol/L in a solution containing 0.01 mol/L KCl, 0.0001 mol/L EDTA, 0.002 mol/L $MgCl_2$, 0.25 mol/L sucrose and 0.02 mol/L potassium phosphate (pH 7.2). State III and IV respiration and respiratory control ratio (RCR) were calculated according to the method of Chance and Williams.²³

Electron Transfer Enzyme Activities of Mitochondria

NADH-Coenzyme Q Reductase (complex I). Complex I activity was measured by the method of Hatefi and Rieske²⁴. An aliquot of submitochondrial particles was reacted at 37°C in a solution of 0.00003 mol/L NADH, 0.00004 mol/L Co Q₃, 0.003 mol/L NaN_3 , and 0.08 mol/L Tris-HCl (pH 8.0), and reaction in NADH was measured colorimetrically at 340 nm absorbance.

Dinitrophenol (DNP)-Stimulated ATPase (Complex V). DNP-stimulated ATPase activity was measured according to Pullman et al.²⁵. An aliquot of mitochondria was reacted by adding 0.001 mol/L DNP and 0.002 mol/L ATP to a solution of 0.21 mol/L sucrose, 0.02 mol/L KCl, and 0.02 mol/L Tris-malate (pH 7.2), and the activity was calculated by measuring the liberated inorganic phosphorus (Pi) using the method of Fiske and Subbarow.²⁰

Determination of Membrane-bound Sulfhydryl Groups

Sulfhydryl (SH) groups in SR and mitochondrial membrane proteins were measured by the modified method of Ellman²⁶. An aliquot of SR or mitochondrial proteins was reacted with 0.01 mol/L 5,5-dithiobis(2-nitrobenzoic acid) at room temperature in a solution of 0.001 mol/L EDTA, 1% sodium dodecyl sulfate (SDS), and 0.05 mol/L phosphate (pH 8.0), and the 412 nm absorbance was measured colorimetrically to obtain an index for oxidative injury of the membrane protein.

Statistical Evaluation

The results are expressed as means $\pm$ SD. Statistical analysis was carried out using Student's *t* test, with $p < 0.05$ regarded as significant.

Results

Hemodynamics

Hemodynamic changes after captopril or saline treatment and during coronary occlusion were expressed

as ratios (%) of the baseline values. Fig 1a shows that the baseline heart rate was 157 ± 29 beats/min in the captopril-treated group and 160 ± 35 beats/min in the saline-treated group (not significantly different). Neither captopril treatment nor coronary occlusion changed the heart rate significantly over time. The right ventricular systolic pressure (RVP) was 26.4 ± 5.9 mmHg in the captopril-treated group and 27.8 ± 3.9 mmHg in the saline-treated group, and neither captopril treatment nor coronary occlusion changed the values of RVP significantly.

The baseline left ventricular systolic blood pressure (LVPs) was 133 ± 21 mmHg in the captopril-treated group and 134 ± 24 mmHg in the saline-treated group. Intravenous drip infusion of captopril (2.5 mg/kg body weight) starting 30 min before coronary occlusion caused a gradual but significant decrease in LVPs, which reached 89% of the preinfusion value at baseline and 78% 30 min after coronary ligation, but did not fall further thereafter. LVPs in the saline-treated group decreased gradually but nonsignificantly to 93% of baseline minimum at the end of coronary occlusion (Fig 1b). The baseline pressure rate product (PRP) was $20,504 \pm 5126$ mmHg·min in the saline-treated group and $21,616 \pm 7558$ mmHg·min in the captopril-treated group (no significant difference between groups). However, the PRP value decreased significantly in the captopril-treated group to 86% of baseline immediately before coronary occlusion and 77% thereafter, corresponding to the decrease in LVPs, whereas the PRP ratio was well preserved in the saline-treated group during the experiment (Fig 1c). Cardiac output (CO) at baseline was 1.10 ± 0.20 L/min in the saline-treated group and 1.21 ± 0.36 L/min in the captopril-treated group (not significantly different), and the value of CO was not decreased by coronary occlusion in either of the groups (Fig 1d).

Ca-Dependent ATPase Activity of SR and SDS Gel Electrophoresis of SR

Among animals in the saline-treated group, Ca-dependent ATPase activity was 8.3 ± 0.9 μ mol Pi/mg protein per h in SR from nonischemic myocardial cells, significantly higher than the average value in ischemic myocardial cells, which was 3.2 μ mol Pi/mg protein per h ($38.7 \pm 13.3\%$). In contrast, in the captopril-treated group, Ca-dependent ATPase activity in SR from ischemic myocardium was well preserved at $59.2 \pm 16.6\%$ of the value in nonischemic myocardial SR, demonstrating a significant protective effect of captopril on the activity of the Ca-dependent ATPase in ischemic myocardium ($p < 0.05$) (Fig 2).

Fig 3 shows the findings of SDS gel electrophoresis

Fig 1. Relative changes in hemodynamic parameters. ○, Saline-treated group; ●, group treated with an iv infusion of captopril (2.5 mg/kg body weight) before and after ischemia. (a) Heart rate (HR). (b) Left ventricular systolic pressure (LVPs). (c) Pressure rate product (PRP) calculated as LVPs×HR. (d) Cardiac output (CO). Data are expressed as means±SE (n=6–8). *p<0.05, **p<0.01.

Fig 2. Ca-stimulated ATPase activities of SR in the captopril-treated and saline-treated groups. The black bar shows the mean relative Ca-stimulated ATPase activity in the captopril-treated group; the striped bar shows the mean relative Ca-stimulated ATPase activity in the saline-treated group. The activity was expressed as the ratio of activity in ischemic to nonischemic myocardium. The Ca-stimulated ATPase activity in nonischemic myocardium was $8.3 \pm 0.9 \mu\text{mol Pi/mg per h}$. Data are expressed means±SE (n=8). *p<0.05. Cap, captopril; Sal, saline.

of SR protein from ischemic and nonischemic myocardium from animals in both groups. In the captopril-treated group the major ATPase band was as well preserved in SR from ischemic myocardium as in SR

from nonischemic myocardium. In contrast, in the saline-treated group, the ATPase band of SR from ischemic myocardium was markedly reduced compared with that from nonischemic myocardium.

Fig 3. SDS gel electrophoresis of SR from ischemic myocardium from animals in the captopril-treated and saline-treated groups and from nonischemic myocardium (control). Arrow shows the major ATPase protein. The major ATPase protein was better preserved in the captopril-treated group than in the saline-treated group. Cap, captopril; Sal, saline.

Fig 4. Respiratory control ratio (RCR) of myocardial mitochondria from animals in the captopril-treated and saline-treated groups. (a) Glutamate plus malate as a substrate ($n=7$). (b) Succinate as a substrate ($n=7$). The black bar shows the mean relative RCR in the captopril-treated group; the striped bar shows mean relative RCR in the saline-treated group. The mean values are expressed as the ratio of RCR from ischemic myocardium to that from nonischemic myocardium. Data are expressed means $\pm$ SE. * $p<0.05$.

Mitochondrial Respiratory Activity

State III respiratory activity in nonischemic myocardium with glutamate and malate as substrates was 154.6 ± 40.6 and 125.0 ± 12.3 n atoms O/mg protein per min in the saline-treated and captopril-treated groups, respectively (not significantly different). The mean ratio of infarcted to noninfarcted myocardium was

64.0% in the saline-treated group and 72.4% in the captopril-treated group. State IV respiration was not affected by ischemia in either group, so that the RCR was reflected mainly by state III respiration. The RCR of infarcted myocardium was expressed as a percentage of that of noninfarcted myocardium. The RCR was 55.2% in the saline-treated group and

Fig 5. Activity of electron transport system enzymes in myocardial mitochondria from animals in the captopril-treated and saline-treated groups. (a) NADH-CoQ reductase (complex I) activity (n=7). (b) DNP-stimulated ATPase (complex V) activity (n=7). The bar shows the ratio of mean relative activity in ischemic myocardium to that from nonischemic myocardium. NADH-CoQ reductase activity in nonischemic myocardium was 0.0562 ± 0.0186 and 0.0660 ± 0.0268 $\mu\text{mol/mg}$ protein per min in the captopril-treated group and saline-treated group, respectively. DNP-stimulated ATPase activity in nonischemic myocardium was 12.51 ± 4.21 and 13.55 ± 0.60 $\mu\text{mol Pi/mg}$ protein per h in the captopril-treated group and saline-treated group, respectively. Data are expressed as means $\pm$ SE. * $p < 0.05$. Cap, captopril; Sal, saline.

Fig 6. Content of protein sulfhydryl (SH) groups in sarcoplasmic reticulum and mitochondria. (a) SR. (b) Mitochondria. The black bar shows the relative SH group content calculated as the ratio of SH group content in ischemic heart muscle to that in nonischemic heart muscle. The SH content of SR protein was 37.3 ± 10.3 and 42.1 ± 8.6 nmol SH/mg protein in the captopril-treated group and saline-treated group, respectively. The SH content of mitochondrial protein was 119.4 ± 9.2 and 132.4 ± 17.1 nmol SH/mg protein in the captopril-treated group and saline-treated group, respectively. Data are expressed means $\pm$ SE (n=8). Cap, captopril; Sal, saline.

72.6% in the captopril-treated group (Fig 4a). State III respiration using succinate as substrate from noninfarcted myocardium was 126.9 ± 42.0 and 103.8 ± 33.6 n atoms O/mg protein per min in the saline-treated group and captopril-treated group, respectively (not significantly different). The ratio of state III respiration in infarcted myocardium to that in noninfarcted myocardium was $57.5 \pm 12.1\%$ in the saline-treated group and $73.3 \pm 17.7\%$ in the captopril-treated group. The relative RCR was significantly higher in the captopril-treated group than in the saline-treated group ($77.4 \pm 6.7\%$ vs $55.0 \pm 13.3\%$, $p < 0.05$) (Fig 4b).

Mitochondrial Electron Transport Enzyme Activities

NADH-CoQ reductase activity in the nonischemic myocardium was 0.0660 ± 0.0268 and 0.0562 ± 0.0186 $\mu\text{mol/mg}$ per min in the saline-treated and captopril-treated groups, respectively. The activity in ischemic myocardium was expressed as a percentage of that in nonischemic myocardium. There was no significant difference in the relative activity of NADH-CoQ reductase between the groups, being $54.1 \pm 12.9\%$ in the saline-treated group and $62.2 \pm 20.4\%$ in the captopril-treated group (Fig 5a). The DNP-stimulated ATPase activity in the nonischemic myocardium was 13.55 ± 0.60 $\mu\text{mol Pi/mg}$ protein per h in the saline-treated group and 12.51 ± 4.21 $\mu\text{mol Pi/mg}$ protein per h in the captopril-treated group (not significantly different). The relative DNP-stimulated ATPase activity in ischemic myocardium from the captopril-treated group remained relatively high at $78.1 \pm 8.0\%$ ($p < 0.05$), whereas it decreased to $62.1 \pm 11.6\%$ in the saline-treated group (Fig 5b).

Changes in Membrane Protein

Sulfhydryl Group Content

Membrane protein sulfhydryl (SH) group content, an index of oxidative stress in the membrane proteins of myocardial organelles, was 42.1 ± 8.6 and 37.3 ± 10.3 nmol SH/mg protein in SR from the saline-treated and captopril-treated groups, respectively (not significantly different). The ratio of SH group content from ischemic myocardial SR to that from nonischemic myocardium was $70.9 \pm 17.3\%$ and $77.3 \pm 9.3\%$ in the saline-treated and captopril-treated groups, respectively (Fig 6a). In nonischemic myocardium, mitochondrial SH group content was 134.8 ± 17.1 and 119.4 ± 9.2 nmol SH/mg protein in the saline-treated and captopril-treated groups, respectively (not significantly different). The relative SH group content in ischemic myocardium was $66.9 \pm 6.0\%$ in the saline-treated group and $88.8 \pm 23.6\%$ in the captopril-treated group (Fig 6b). In both organelles membrane-bound SH

groups tended to be well preserved in the captopril-treated group.

Discussion

Many recent studies^{27,28,29} have reported that ACE-I agents have a beneficial effect on acute myocardial infarction from the standpoint of ventricular remodeling. ACE-I agents are thought to limit the size of the infarction and reduce the collagen content, reducing the asynergy of infarcted left ventricular muscle and the frequency of arrhythmia, however the detailed mechanism of action remains unclear. Thus, we attempted to clarify the mechanism of action ACE-I agents on ischemic myocardium using an ischemic canine model that we have developed. SR Ca-ATPase is very sensitive to ischemic myocardial injury causing irreversible necrosis. We previously reported that the SR Ca-dependent ATPase activity of subendocardial muscle is significantly reduced by necrosis. The major ATPase protein disintegrates around 20 min after the cessation of coronary blood flow, with the effect extending to the subepicardial layer 30–60 min later.¹⁴ Mitochondrial respiratory dysfunction in state III respiration extends from the subendocardium to the subepicardium after 30 min of ischemia, and activity decreases significantly after 60 min. Complex I and DNP-stimulated ATPase activities decrease significantly after 15 min of ischemia in the subendocardium and after 30 min ischemia in the subepicardial muscle.¹⁵ Progression is in accordance with the wavefront phenomenon, and after around 30 min ischemia the membrane phospholipids of the organelle (particularly phosphatidylcholine and phosphatidylethanolamine) are digested by phospholipases.³⁰ Ultrastructural studies of sarcolemma using ionic lanthanum as an indicator show that the permeability is increased in the early stages of ischemia.³¹ These previous reports are compatible with our finding that Ca-dependent ATPase activity, the major ATPase protein band on SDS gel electrophoresis of SR, mitochondrial state III respiration activity, and DNP-stimulated ATPase activity were reduced in infarcted myocardium from animals in the saline-treated group.

Intravenous drip infusion of captopril before coronary occlusion preserved the Ca-ATPase activity, mitochondrial respiratory control ratio (RCR), and DNP-stimulated ATPase activity in mitochondria, confirming that captopril protects against ischemic myocardial injury at the organelle level. However, it is not easy to determine the mechanism of cardiac protection because captopril reduced left ventricular systolic pressure by inhibiting the renin-angiotensin

system and/or by a vasodilatory effect. From the hemodynamic viewpoint, a reduction in cardiac energy metabolism in ischemic areas as a result of a decline in PRP may reduce the risk of myocardial injury in marginally affected areas. In this study, we did not compare the size of myocardial infarction in the captopril-treated and saline-treated groups, as can be done by measuring total release of creatine kinase (CK) or by morphological nitroblue tetrazolium (NBT) staining.

However, vasodilation itself has no direct effect on cardiac metabolism. We have previously reported that coronary dilators such as nitrate do not protect infarcted myocardium.³² According to Longobardi et al,³³ the vasodilatory action of ACE-I agents (captopril, enalapril) does not reduce dipyridamole-induced mechanical dysfunction or ST-segment depression in patients with angiographic evidence of significant coronary artery disease. We confirmed the presence of infarction in our myocardial samples by NBT staining in an acute cardiac infarction model used in our laboratory. We showed that captopril has a direct protective effect on infarcted myocardium, as impaired Ca uptake in isolated SR vesicles caused by oxidation of membrane protein SH groups was restored by addition of dithiothreitol (DTT)³⁴ or a scavenger such as an ACE-I agent containing SH groups.³⁵

Preserving membrane protein SH groups is important for cardiac function. Our study showed that captopril treatment tended to preserve the SH group content more than in the control group in both the SR and mitochondrial membranes, but the difference was not significant. A study³⁶ of cardiac reperfusion injury has suggested that the SH group content of membrane proteins is not significantly reduced even after reperfusion following 90 min of ischemia because the cytosolic sulfhydryl pool, such as glutathione (GSH) is depleted first during reperfusion. Thus, we hypothesize that membrane protein SH groups are only decreased after cytosolic SH sources are exhausted. The tendency for membrane protein SH groups to be preserved may therefore reflect the protective effects of captopril against oxidative injury in ischemia. This study has shown that the myocardial protection afforded by captopril pretreatment may be partly the result of hemodynamic effects and partly due to captopril behaving like DTT or SH-group donors in injured myocardial cells by preserving enzyme activity in the organelles.

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