Original Article

Effects of an HMG-CoA Reductase Inhibitor in Combination with an ACE Inhibitor or Angiotensin II Type 1 Receptor Antagonist on Myocardial Metabolism in Ischemic Rabbit Hearts

Hitoshi KAWABATA, Kizuku NAKAGAWA, and Kinji ISHIKAWA

We investigated the effects of a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, pravastatin, an angiotensin converting enzyme (ACE) inhibitor, temocaprilat, and an angiotensin II type 1 (AT1) receptor antagonist, CV-11974, on myocardial metabolism during ischemia in isolated rabbit hearts using phosphorus 31-nuclear magnetic resonance (^{31}P -NMR) imaging. Forty-five minutes of continuous normothermic global ischemia was carried out. Pravastatin, temocaprilat, CV-11974 or a nitric oxide synthase inhibitor, L-NAME was administered from 60 min prior to the global ischemia. Japanese white rabbits were divided into the following experimental groups, a control group ($n=7$), a group treated with pravastatin (P group; $n=7$), a group treated with pravastatin and temocaprilat (P + T group; $n=7$), a group treated with pravastatin and CV-11974 (P + CV group; $n=7$), and a group treated with pravastatin and L-NAME (P + L-NAME group; $n=7$). During ischemia, P group, as well as either P + T group or P + CV group, showed a significant inhibition of the decreases in adenosine triphosphate (ATP) and intracellular pH (pHi) ($p<0.01$, respectively, at the end of ischemia compared to the control group as well as P + L-NAME group), and a significant inhibition of the increase in inorganic phosphate (Pi) ($p<0.01$, respectively, compared with the control group as well as P + L-NAME group). These results suggest that pravastatin significantly improved myocardial energy metabolism during myocardial ischemia. This beneficial effect was dependent on NO synthase. However, this beneficial effect was not enhanced by either temocaprilat or CV-11974.

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Key Words: HMG-CoA reductase inhibitor, angiotensin converting enzyme inhibitor, angiotensin II receptor antagonist, cardioprotection, ischemia

Introduction

Clinical trials have shown that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors such as pravastatin reduce myocardial infarction and ischemic stroke (1–3). Recent studies have suggested that HMG-CoA reductase inhibitors activate endothelial nitric oxide synthase (eNOS) independently of its effects on lipids (4, 5). The renin-angiotensin system (RAS) is activated upon acute myo-

cardial infarction in both humans (6) and experimental animals (7, 8). In dogs, localized angiotensin II (Ang II) has been shown to exert some deleterious effects on the myocardium of the ischemia-reperfused heart (9). Angiotensin converting enzyme (ACE) inhibitors protect the myocardium from ischemia and reperfusion injury (10, 11). Ang II type 1 (AT1) receptor antagonists also exert cardioprotective effects against ischemia-reperfusion injury (12). We have also shown that the combination of an ACE inhibitor and AT1 receptor antagonist can help protect against ischemia-reperfu-

From the First Department of Internal Medicine, Kinki University School of Medicine, Osakasayama, Japan.

Address for Reprints: Hitoshi Kawabata, M.D., First Department of Internal Medicine, Kinki University School of Medicine, 377–2 Ohno-Higashi, Osakasayama 589–8511, Japan. E-mail: int1@med.kindai.ac.jp

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sion injury in rabbit hearts (13). While ACE inhibitors have been shown to activate bradykinin, bradykinin enhances accumulation of endothelial nitric oxide (NO)/cyclic GMP (14). In addition, although the actions of Ang II are mainly mediated via two types of receptors (15), known as AT1 and AT2 receptors, increased AT2 receptor expression in myocardial infarction has been observed (16). Thus the functions of the AT2 receptor in ischemia-reperfusion injury have been shown to include stimulation of NO production (17). Additional mechanisms that may play a role in ischemia-reperfusion injury include the reduction in myocardial damage by an HMG-CoA reductase inhibitor, reduced formation of Ang II, or activation of eNOS. However, it has not been clearly determined whether or not HMG-CoA reductase inhibitors administered in combination with an ACE inhibitor or AT1 receptor antagonist exert cardioprotective effects against myocardial ischemia-reperfusion injury. The purpose of the present study was to determine whether combination of a HMG-CoA reductase inhibitor and ACE inhibitor or AT1 receptor antagonist serves as a potentiation or not of the cardioprotective effect in the ischemic heart and to search for possible action of these agents, which suggests future clinical applications. Accordingly, we here used phosphorus 31-nuclear magnetic resonance (^{31}P -NMR spectroscopy) imaging of myocardial energy metabolism to investigate whether the above combination treatments would potentiate the cardioprotective effect in ischemic, isolated rabbit hearts, and if so, what their possible mechanisms might be. The administration of HMG-CoA reductase inhibitor alone, as well as in combination with either an ACE inhibitor or AT1 receptor antagonist, was found to improve abnormal myocardial energy metabolism. These data suggest that this beneficial effect was not dependent on either the production of NO synthase or the reduced formation of Ang II induced by either the ACE inhibitor or AT1 receptor antagonist.

Materials and Methods

Experimental Protocol

The hearts were divided into the following 7 groups: 1) a control group injected intravenously with vehicle (physiologic saline) at 60 min prior to global ischemia (C group; $n = 7$); 2) a group injected intravenously with pravastatin (Sankyo Co., Tokyo, Japan) (0.025 mg/kg) at 60 min prior to global ischemia (P group; $n = 7$); 3) a group injected intravenously with temocaprilat (Sankyo Co.) (0.027 mg/kg) at 60 min prior to global ischemia (T group; $n = 7$); 4) a group injected intravenously with CV-11974 (Takeda Chemical Industries, Ltd., Osaka, Japan) (0.025 mg/kg) at 60 min prior to global ischemia (CV group; $n = 7$); 5) a group injected intravenously with pravastatin (0.025 mg/kg) coupled with temocaprilat (0.027 mg/kg) at 60 min prior to global ischemia (P + T group; $n = 7$); 6) a group injected intravenously with pravastatin (0.025 mg/kg) coupled with CV-11974

(0.025 mg/kg) at 60 min prior to global ischemia (P + CV group; $n = 7$); and 7) a group injected intravenously with pravastatin (0.025 mg/kg) coupled with N^G -nitro-L-arginine methyl ester (L-NAME) (Sigma Chemical Co., St. Louis, USA) (1.5 mg/kg) at 60 min prior to global ischemia (P + L-NAME group; $n = 7$). This dose of pravastatin, temocaprilat and CV-11974 is necessary to obtain a relevant pharmacological concentration *in situ* such as 10^{-6} M. This high dose of L-NAME is necessary to inhibit cardiovascular NO synthase. After 60 min of treatment, continuous normothermic global ischemia was induced by severing the aorta and removing the heart.

Isolated Heart Preparation

All procedures were in accordance with the guidelines for animal research at our institution. Male Japanese white rabbits weighing 1.6 to 1.7 kg were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg). An endotracheal tube was inserted, and the lungs were ventilated with room air supplemented with 95% oxygen. The chest was opened and heparin sodium (1,000 IU/kg) was injected through the right atrial appendage. The heart was quickly removed and immediately mounted on a ^{31}P -NMR device, and then measurement of ^{31}P -NMR spectra was initiated.

Measurements of High Energy Phosphates and Intracellular pH by ^{31}P -NMR

The heart was placed in an NMR sample tube with a diameter of 25 mm and inserted into a ^{31}P probe. The temperature of the ^{31}P -NMR device was maintained at 37°C. The ^{31}P -NMR spectra were recorded at 161.9 MHz with 45° flip-angle pulses at 2.0 s intervals with a JNM-GX 400 FT NMR spectrometer (JEOL, Tokyo, Japan). Spectra were acquired for 5 min and averaged from 150 transient samples. Quantitative analysis of the whole heart was performed by the relative intensities of the β -ATP (adenosine triphosphate) and Pi (inorganic phosphate) peaks. Areas under each peak were integrated five times with a planimeter. The mean of five such readings was normalized as a percentage of the value given during the initial period. The distance of the intracellular Pi peak relative to the intracellular PCr peak (pH-independent) was measured and the intracellular pH (pHi) was determined using the following equations (18): $\text{pHi} = \text{pk} - \log_{10} [(\delta_0 - \delta_B)/(\delta_A - \delta_0)]$, $\text{pk} = 6.79$, $\delta_A = 3.25$, $\delta_B = 5.75$, where δ_0 is the chemical shift of Pi with respect to PCr.

Statistical Analysis

Statistical analysis was performed by ANOVA with Tukey's post hoc test. All values were expressed as the mean $\pm$ SD, and levels of $p < 0.05$ were considered to indicate statistical significance.

Fig. 1. Changes in Pi in the control group (C), the group treated with 0.025 mg/kg pravastatin (P), the group treated with 0.027 mg/kg temocaprilat (T), and the group treated with 0.025 mg/kg CV-11974 (CV). Data are expressed as the mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C group. § $p < 0.05$, §§ $p < 0.01$: T group significantly different from C group. † $p < 0.05$, †† $p < 0.01$: CV group significantly different from C group.

Results

Metabolic Effect of L-NAME on Isolated Hearts

L-NAME (1.5 mg/kg) alone did not affect the metabolism during ischemia (compared with control, all $p = \text{NS}$, $n = 7$).

Myocardial Metabolism during Ischemia

Pi increased remarkably during ischemia in the C group (Fig. 1). In contrast, the increase in Pi was inhibited significantly in the P group ($p < 0.01$) and in portions of the T and CV groups ($p < 0.05$) in comparison with that in the C group. At 42.5 min after the start of ischemia, the Pi values were 166 ± 14 , 149 ± 6 , 155 ± 14 , and $161 \pm 9\%$ for the C, P, T, and CV groups, respectively. In the P + T and P + CV groups, Pi was significantly ($p < 0.01$) lower than in the C group during ischemia (Fig. 2). At 42.5 min after the start of ischemia, the Pi values were 148 ± 14 and $149 \pm 6\%$ for the P + T and P + CV groups, respectively. However, there were no statistically significant differences in Pi between the P group and either the P + T or P + CV group during ischemia. In the P + L-NAME group, Pi was significantly ($p < 0.01$) higher as well as C group during ischemia than that in P group (Fig. 3). At 42.5 min after the start of ischemia, the value of Pi was $170 \pm 6\%$ in the P + L-NAME group. Pi did not show any statistically significant differences between the C group and P + L-NAME group during ischemia.

During ischemia, ATP gradually declined in the C group (Fig. 4). In contrast, the decrease in ATP was inhibited sig-

Fig. 2. Changes in Pi in the control group (C), the group treated with 0.025 mg/kg pravastatin (P), the group treated with 0.025 mg/kg pravastatin in combination with 0.027 mg/kg temocaprilat (P + T), and the group treated with 0.025 mg/kg pravastatin in combination with 0.025 mg/kg CV-11974 (P + CV). Data are expressed as the mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C group. § $p < 0.05$, §§ $p < 0.01$: P + T group significantly different from C group. †† $p < 0.01$: P + CV group significantly different from C group.

Fig. 3. Changes in Pi in the control (C), the group treated with 0.025 mg/kg pravastatin (P), and the group treated with 0.025 mg/kg pravastatin in combination with 1.5 mg/kg L-NAME (P + L-NAME). Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C and P + L-NAME groups.

nificantly in the P group ($p < 0.01$) and in portions of the T and CV groups ($p < 0.05$) in comparison with that in the C group. Final values at 42.5 min after the start of ischemia were 42 ± 12 , 64 ± 5 , 45 ± 9 , and $53 \pm 5\%$ for the C, P, T, and CV groups, respectively. The P + T and P + CV groups showed a significant ($p < 0.01$) inhibition of the decrease in ATP compared with the C group (Fig. 5). The final values at 42.5 min after the start of ischemia were 64 ± 8 and $65 \pm 8\%$ for the P + T and P + CV groups, respectively. However, there were no differences in ATP between the P group and

Fig. 4. Changes in ATP in the C, P, T, and CV groups. Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C group. † $p < 0.05$: CV group significantly different from C group.

Fig. 6. Changes in ATP in the C, P, and P + L-NAME groups. Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C and P + L-NAME groups.

either the P + T or P + CV groups at this final time point. In the P + L-NAME group, ATP was significantly ($p < 0.01$) lower as well as C group during ischemia than that in P group (Fig. 6). At 42.5 min after the start of ischemia, ATP was $39 \pm 3\%$ in the P + L-NAME group. There were no significant differences in ATP between the C group and P + L-NAME group during ischemia.

pHi dropped sharply, but pHi remained significantly ($p < 0.01$) higher in the P group and significantly ($p < 0.05$) higher in portions of the T group than in the C group during ischemia (Fig. 7). Finally, at 42.5 min after the start of ischemia, pHi was significantly ($p < 0.01$) higher in the P group (6.43 ± 0.12), but not in either the T group (6.16 ± 0.21) or CV group (6.11 ± 0.19), compared with that in the C group (6.09 ± 0.11). In addition, pHi remained significantly ($p < 0.01$) higher in the P + T and P + CV groups than in the C group (Fig. 8). At the final time point of 42.5 min after the

Fig. 5. Changes in ATP in the C, P, P + T, and P + CV groups. Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C group. § $p < 0.05$, §§ $p < 0.01$: P + T group significantly different from C group. † $p < 0.05$, †† $p < 0.01$: P + CV group significantly different from C group.

Fig. 7. Changes in pHi in the C, P, T, and CV groups. Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C group. § $p < 0.05$: T group significantly different from C group.

start of ischemia, pHi was significantly ($p < 0.01$) higher in both the P + T (6.36 ± 0.18) and P + CV (6.40 ± 0.10) groups than in the C group. There were no significant differences in pHi between the P group and either the P + T or P + CV groups during ischemia. In the P + L-NAME group, pHi was significantly ($p < 0.01$) lower as well as C group during ischemia than that in P group (Fig. 9). At 42.5 min after the start of ischemia, pHi was 6.13 ± 0.04 in the P + L-NAME group. pHi did not show any statistically significant differences between the C and P + L-NAME groups during ischemia.

Fig. 8. Changes in pH_i in the C, P, P+T, and P+CV groups. Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C group. §§ $p < 0.01$: P+T group significantly different from C group. †† $p < 0.01$: P+CV group significantly different from C group.

Discussion

We examined whether treatment with an HMG-CoA reductase inhibitor and an ACE inhibitor or an AT1 receptor antagonist significantly improved myocardial injury during ischemia compared with either drug alone. In this experiment, ischemia induced changes of myocardial metabolism, such as decreasing ATP and pH_i , and increasing Pi. The decrease of ATP, the fall in pH_i , and the elevation of Pi during ischemia were significantly reduced by pravastatin. However, despite the beneficial effects of either temocaprilat alone or CV-11974 alone, there were no significant differences in myocardial metabolism between the group receiving pravastatin alone and the groups receiving pravastatin in combination with temocaprilat or CV-11974. These findings show that pravastatin had a protective effect on myocardial metabolism during ischemia, and that this effect was not enhanced by either temocaprilat or CV-11974. In addition, there were no significant differences in myocardial metabolism during ischemia between the control group and the group receiving pravastatin in combination with L-NAME, despite the beneficial effect of pravastatin alone. These findings show that pravastatin had a protective effect on myocardial metabolism during ischemia, and this effect may have been caused by NO activation.

The overall events in the transition of ischemia to infarction are very complex and cannot simply be related to depletion of ATP (19). Rather, depletion of ATP should be seen as a marker of 1) the severity of the ischemic process; 2) a depressed rate of anaerobic glycolysis in severely ischemic tissue caused by an accumulation of glycolytic end-products; 3) inhibited lipid metabolism with accumulation of intermediates such as acyl CoA and acyl carnitine, with inhibition of mitochondrial metabolism; and 4) an accumulation of inter-

Fig. 9. Changes in pH_i in the C, P, and P+L-NAME groups. Data are expressed as mean $\pm$ SD. ** $p < 0.01$: P group significantly different from C and P+L-NAME groups.

cellular calcium with ischemic contracture and utilization of ATP. All these processes contribute to damage of the cell membranes (sarcolemma, mitochondria, sarcoplasmic reticulum), which is seen as a critical event in irreversible damage (20). As judged by the rate of fall in ATP, many cells in the severely ischemic zone are destined to die soon, within 30–45 min.

Clinical trials with HMG-CoA reductase inhibitors suggest that these inhibitors exert at least part of their cardioprotective action *via* mechanisms other than simply lowering the lipid load of the vessel wall (21). Several recent studies suggest that HMG-CoA reductase inhibitors increase eNOS activity (4, 22–24). And recent studies have provided evidence that endogenous production of NO provides significant myocardial protection from ischemia-reperfusion injury (25, 26). HMG-CoA reductase inhibitors increase eNOS expression through an increase in eNOS mRNA stability (23). The study by Kaesemeyer *et al.* (4) demonstrated that pravastatin stimulated NOS activity and NO release, and that these responses were complete within 10 min. In addition, these authors showed that NO production occurred within the first 30 min after pravastatin administration. The cardioprotective effects of NO have been explained by several factors, such as microvascular effects (27), antineutrophil action (27), induction of stress protein (28), or modulation of cardiac excitability (29). We found that an HMG-CoA reductase inhibitor exerted a cardioprotective effect on ischemic injury by activating the opening of ATP-sensitive K^+ (K_{ATP}) channels *via* an NO-mediated pathway (30).

During ischemia, both the ACE inhibitor and AT1 receptor antagonist had a beneficial effect on myocardial metabolism. This reason is that both agents suppress *via* the Ang II receptor (31). When Ang II binds to the AT1 receptor, which exists in the plasma membrane, cytosolic Ca^{2+} is increased (32–34). Increase of cytosolic Ca^{2+} during ischemia may thus be related to the acceleration of ischemic injury. Fur-

thermore, ACE inhibitors inhibit the degradation of bradykinin, and the increase of bradykinin may have a cardioprotective effect (35, 36). And also, two Ang II receptor subtypes, the AT1 and AT2 receptors, have thus far been identified (37). Blockade of the AT1 receptor has been shown to increase the Ang II concentration (38), which may then activate the AT2 receptor (39, 40). AT2 receptor activation involve the increase of bradykinin and the subsequent action of prostaglandins and/or NO (41). Recently, we have demonstrated that the cardioprotective effect of combination of ACE inhibitor and AT1 receptor antagonist is not dependent on NO synthase in rabbit cardiac tissue (42). In addition, the cardioprotective effects of AT1 receptor antagonist in the stunned myocardium are provided by activation of K_{ATP} channels, which activation is mediated by the bradykinin B_2 receptor *via* AT2 receptor activation (43).

ACE-dependent and -independent (alternate) Ang II-forming pathways also exist in the heart (44, 45). We therefore speculated that Ang II antagonists are more effective at protecting against ischemic injury than ACE inhibitors because ACE inhibitors cannot completely inhibit Ang II production (12). But during ischemia, the effect of the Ang II antagonist on myocardial metabolism was the same as that of the ACE inhibitor in our study. One reason is that in rabbits, Ang II is not produced by chymase (46), and thus ACE may be the main Ang II-forming pathway in rabbit hearts. Another reason is that when the AT1 receptor antagonist binds to the AT1 receptor, ACE activity is increased by feedback regulation (47). Therefore, ACE inhibitor inhibits Ang II production due to the increase of ACE activity.

In the present study, administration of HMG-CoA reductase inhibitor alone was more effective against ischemic injury than administration of either ACE inhibitor or AT1 receptor antagonist alone. The results suggest that the enhancement of NO release by HMG-CoA reductase inhibitor may have played a more important role than reduction of Ang II forms by either ACE inhibitor or AT1 receptor antagonist during ischemia. Furthermore, the protective effect of the combination of HMG-CoA reductase inhibitor with either ACE inhibitor or AT1 receptor antagonist did not result in a significant improvement in myocardial metabolism against ischemic injury compared to that by HMG-CoA reductase inhibitor alone. This may be because the HMG-CoA reductase inhibitor induced a more complete release of NO than either the ACE inhibitor or AT1 receptor antagonist in rabbit cardiac tissue.

In conclusion, dual-therapy with pravastatin and either temocaprilat or CV-11974 did not produce additive reduction in ischemic injury of rabbit hearts compared with HMG-CoA reductase inhibitor alone, and it was suggested that ischemic injury cannot be retarded beyond the level achieved with monotherapy of HMG-CoA reductase inhibitor.

Limitations of the Study

Our studies were performed in rabbit hearts; results may differ in human hearts. The present study did not directly reveal the mechanism by which suppression of Ang II availability and increased bioavailability of NO in tissue inhibits ischemic injury.

Conclusion

We conclude that 1) HMG-CoA reductase inhibitor alone is the cardioprotective effector of ischemic injury with or without either ACE inhibitor or AT1 receptor antagonist; 2) The protective effect caused by HMG-CoA reductase inhibitor alone is sufficient to activate myocardial metabolism against ischemic injury; 3) The effect of HMG-CoA reductase inhibitor is mediated by NO.

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