of GH,[5] controlled studies in humans have been predominantly negative.[7,8] Nevertheless, ghrelin has been shown to have GH-independent effects, stimulating vasodilation,[10–12] reversing cachexia,[13–15] and inhibiting sympathetic nerve activity[24] and myocyte apoptosis.[22] Thus, ghrelin may have additional therapeutic potential compared with GH supplementation. Ghrelin improved cardiac function and exercise capacity in not only cachectic CHF patients but also noncachectic ones. Nevertheless, the best candidates may be cachectic CHF patients because ghrelin stimulates feeding and improves muscle wasting.

Conclusions

These preliminary results suggest that repeated administration of ghrelin improves LV structure and function, exercise capacity, and muscle wasting in patients with CHF. Thus, administration of ghrelin may be a new therapeutic approach for the treatment of CHF.

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Optimal Windows of Statin Use for Immediate Infarct Limitation

5′-Nucleotidase as Another Downstream Molecule of Phosphatidylinositol 3-Kinase

Shoji Sanada, MD, PhD; Hiroshi Asanuma, MD, PhD; Tetsuo Minamino, MD, PhD; Koichi Node, MD, PhD; Seiji Takashima, MD, PhD; Hiroko Okuda, PhD; Yoshiro Shinozaki, MD, PhD; Akiko Ogai, PhD; Masashi Fujita, MD; Akio Hirata, MD; Jiyoong Kim, MD; Yoshihiro Asano, MD, PhD; Hidezo Mori, MD, PhD; Hitonobu Tomoike, MD, PhD; Soichiro Kitamura, MD, PhD; Masatsugu Hori, MD, PhD; Masafumi Kitakaze, MD, PhD

Background—Although statins are reported to have a cardioprotective effect, their immediate direct influence on ischemia-reperfusion injury and the underlying mechanisms remain obscure. We investigated these issues an in vivo canine model.

Methods and Results—Dogs were subjected to coronary occlusion (90 minutes) and reperfusion (6 hours) immediately after injection of pravastatin (0.2, 2, or 10 mg/kg), pitavastatin (0.01, 0.1, or 0.5 mg/kg), or cerivastatin (0.5, 5, or 50 μg/kg). Then myocardial phosphatidylinositol 3-kinase (PI3-K) and 5′-nucleotidase activities were measured, as well as infarct size. After 15 minutes of reperfusion, pravastatin caused dose-dependent activation of Akt and ecto-5′-nucleotidase in the ischemic zone, and the effect was significant at higher doses. Pitavastatin also significantly increased these activities, and its optimal dose was within the clinical range, whereas cerivastatin caused activation at the lowest dose tested. In all cases, both Akt and ecto-5′-nucleotidase showed activation in parallel, and this activation was completely abolished by wortmannin, a PI3-K inhibitor. The magnitude of the infarct-limiting effect paralleled the increase in Akt and ecto-5′-nucleotidase activity and was blunted by administration of wortmannin, α,β-methyleneadenosine-5′-diphosphate, or 8-sulfophenyltheophylline during reperfusion. Both collateral flow and the area at risk were comparable for all groups.

Conclusions—Activation of ecto-5′-nucleotidase after ischemia by PI3-K activation may be crucial for immediate infarct-size limitation by statins. There seems to be an optimal dose for each statin that is independent of its clinical cholesterol-lowering effect. (***Circulation*. 2004;110:2143-2149.**)

Key Words: statins ■ myocardial infarction ■ adenosine ■ enzymes ■ phosphates

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) block the biosynthesis of cholesterol[1] and are widely used clinically to decrease serum cholesterol levels. Recent studies have focused on the pleiotropic effects of either hydrophilic[2,3] or hydrophobic[4,5] statins, which are independent of their cholesterol-lowering effect.[2,3,5] Protection against ischemia-reperfusion injury is one of them, which is particularly evident after 12 hours.[6,7] In addition, some studies showed that statins activate the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway within 1 hour,[8,9] as well as activating endothelial nitric oxide synthase (eNOS),[9,10] to cause immediate infarct limitation.[9]

On the other hand, other studies revealed that statins also acutely activate ecto-5′-nucleotidase,[11] which produces the endogenous cardioprotective substance adenosine,[12] especially in response to certain stresses.[13] Ecto-5′-nucleotidase can act only when localized on the cell membrane,[13] and the density of this enzyme on the membrane regulates its activity.[11,14] Endocytotic turnover of ecto-5′-nucleotidase (5′-nucleotidase localized on the cell surface) is inhibited by PI3-K activation,[14] which subsequently increases total 5′-nucleotidase activity within a period as short as 10 minutes.[14] Therefore, we hypothesized that an increase of ecto-5′-nucleotidase activity might be critical for early cardioprotec-

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From the Department of Internal Medicine and Therapeutics (S.S., H.A., T.M., S.T., H.O., M.F., A.H., Y.A., M.H.), Osaka University Graduate School of Medicine, Suita; the Department of Cardiovascular and Renal Medicine (K.N.), Saga University Faculty of Medicine, Saga; the Department of Physiological Science (Y.S.), Tokai University School of Medicine, Isehara; and the Cardiovascular Division of Medicine (A.O., J.K., H.M., H.T., S.K., M.K.), National Cardiovascular Center, Suita, Japan.

Correspondence to Masafumi Kitakaze, MD, PhD, Director, Cardiovascular Division of Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, 565-8565 Japan. E-mail kitakaze@zf6.so-net.ne.jp

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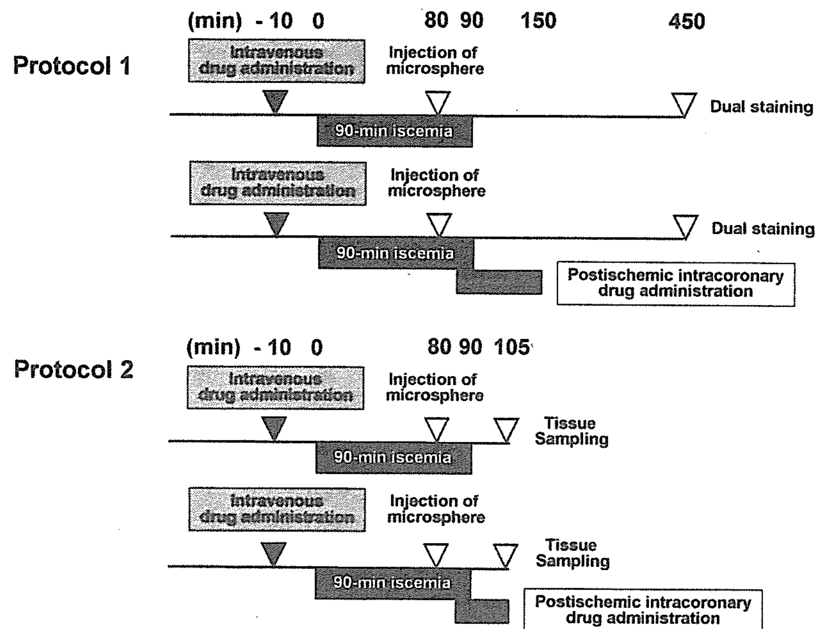

Figure 1. Experimental protocols to measure infarct size (protocol 1; Upper) and kinase activity (protocol 2; Lower).

tion mediated by statins and might be associated with rapid activation of PI3-K.

Here we used a dog model to determine whether 3 statins with different water solubilities (pravastatin, pitavastatin, and cerivastatin) could acutely limit infarct size, as well as whether adenosine and PI3-K were involved in the underlying mechanism.

Methods

All procedures were performed in conformity with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, 1996 revision) and were approved by the Osaka University Committee for Laboratory Animal Use. Pravastatin, pitavastatin, and cerivastatin were obtained from Sankyo, Kowa, and Takeda Pharmaceuticals, respectively. The other drugs were obtained from Sigma.

Instrumentation

Beagle dogs weighing 8 to 13 kg were anesthetized and connected to an extracorporeal bypass tube as described previously.[15,16] In all experiments, the average baseline values of mean aortic blood pressure (ABP), heart rate (HR), and arterial blood Po_2 were 102±2.2 mm Hg, 129±2.5 min^{-1}, and 109±4.1 mm Hg, respectively. Both ABP and HR were measured continuously during the study.

Experimental Protocols

Protocol 1: Measurement of Infarct Size and Myocardial Collateral Blood Flow

After hemodynamic stabilization, we infused pravastatin (0.2, 2, or 10 mg/kg), pitavastatin (0.01, 0.1, or 0.5 mg/kg), cerivastatin (0.5, 5, or 50 μg/kg) or saline intravenously for 10 minutes before 90 minutes of sustained ischemia, which was followed by 6 hours of reperfusion (n=9 to 13 each). Some groups also received intracoronary administration of a selective ecto-5′-nucleotidase inhibitor (α,β-methyleneadenosine-5′-diphosphate [AMP-CP; 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$); a nonselective adenosine receptor antagonist (8-sulfophenyltheophylline [8-SPT; 50 $\mu g \cdot kg^{-1} \cdot min^{-1}$]); or a selective PI3-K inhibitor (wortmannin [1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$]) between 5 minutes before and 60 minutes after reperfusion. We measured infarct size and regional myocardial collateral blood flow during 90 minutes of ischemia as described previously.[15]

We have already confirmed in the same model that the doses of AMP-CP,[17] 8-SPT,[17,18] or wortmannin[19] used in this study were appropriate to block ecto-5′-nucleotidase, the adenosine receptors, or PI3-K, respectively. Figure 1 shows the details of this protocol, and the Table lists all of the groups studied.

Protocol 2: Myocardial Enzyme Assays

Another 54 dogs underwent a procedure identical to that of some groups from protocol 1 and were studied for enzyme assays (n=3 or 4 each). In this protocol, not only wortmannin (1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) but also LY294002 (60 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was used as another selective PI3-K inhibitor. After 15 minutes of reperfusion, a myocardial tissue sample was obtained from the ischemic border zone to ensure evaluation of viable ischemic myocardium and was used for the measurement of PI3-K and ecto-/endo-5′-nucleotidase activity. The myocardial tissue was rapidly frozen in LN_2 and stored at −80°C. Measurement of PI3-K and 5′-nucleotidase activity was done as reported previously[15,19] with minor modifications.

Criteria for Exclusion

To ensure that all of the animals included in analysis were healthy and were exposed to a similar extent of ischemia, the exclusion criteria reported previously[16] for hemodynamics, excessive collateral flow, and lethal arrhythmia were adopted.

Statistical Analysis

Results were expressed as mean±SEM, and the number of animals or experiments is shown as n. Statistical analysis was performed by ANOVA with a modified Bonferroni post hoc test, and significance was defined at $P<0.05$.

Results

Mortality and Exclusions in Protocol 1

Among 222 dogs used in protocols 1, 56 dogs met the exclusion criteria of ventricular fibrillation or excessive myocardial collateral blood flow (>15 $mL \cdot 100 g^{-1} \cdot min^{-1}$). Therefore, 166 dogs completed these protocols satisfactorily and were included in the data analysis (Table).

Changes in Hemodynamic Parameters, Risk Area, and Collateral Blood Flow in Protocol 1

The changes in ABP and HR were comparable among all groups throughout the protocol (data not shown), and both the

TABLE 1. Mortality, Exclusion, Area at Risk, and Collateral Flow in Each Group in Protocol 1

		Excluded					
		Lethal Arrhythmia					
Groups	Initial No.	During Ischemia	After Reperfusion	Excessive Collateral Flow	Final No.	Area at Risk, %	Collateral Flow, mL/100 g per minute
Control	13	1	2	1	9	40.1±2.1	8.2±1.0
Prava							
0.2	9	0	1	0	8	38.8±2.0	8.4±1.2
2.0	10	0	0	2	8	39.1±2.2	8.9±1.1
10	10	0	0	2	8	39.6±2.1	8.9±1.4
Pitava							
0.01	9	1	1	0	7	38.7±2.2	8.1±1.3
0.1	11	0	1	2	8	39.3±2.0	9.2±1.5
0.5	10	1	0	2	7	39.9±1.9	8.8±1.5
Ceriva							
0.5	11	0	1	2	8	39.2±1.9	8.5±1.3
5.0	10	1	1	1	7	38.9±2.1	8.7±1.4
50	11	0	1	3	7	39.0±2.0	9.1±1.5
AMP-CP							
+Prava 10	9	0	2	0	7	40.4±2.3	8.6±1.3
+Pitava 0.1	9	0	1	1	7	39.8±2.0	8.4±1.5
+Ceriva 0.5	9	1	1	0	7	40.4±2.3	9.0±1.4
8SPT							
+Prava 10	10	0	1	1	8	38.7±2.2	8.3±1.3
+Pitava 0.1	11	1	2	0	8	39.9±2.1	8.2±1.6
+Ceriva 0.5	11	0	2	1	8	38.4±2.6	8.5±1.5
WTMN							
+Prava 10	10	0	2	1	7	38.6±2.3	9.5±1.5
+Pitava 0.1	10	0	2	0	8	38.9±2.1	9.2±1.6
+Ceriva 0.5	10	0	1	1	8	39.8±2.8	8.8±1.4
AMP-CP	9	0	2	0	7	38.8±2.5	8.5±1.6
8SPT	11	0	3	0	8	39.6±2.5	8.2±1.5
WTMN	9	1	2	0	6	40.5±2.3	8.6±1.6

Data expressed as mean±SEM. Prava indicates pravastatin (mg/kg); Pitava, pitavastatin (mg/kg); Ceriva, cerivastatin (μg/kg); 8SPT, 8-sulfophenyltheophilline; and WTMN, wortmannin.

area at risk and collateral blood flow were also comparable (Table).

Infarct Size

Figure 2 shows infarct size in the groups of protocol 1. Pravastatin (0.2, 2, and 10 mg/kg) dose-dependently reduced the infarct size (29.5±3.5%, 22.5±4.0%, and 18.8±3.4%, respectively) compared with that in the control group (39.8±3.6%), and the difference was significant at 2 mg/kg or more. Pitavastatin (0.01, 0.1, and 0.5 mg/kg) also reduced infarct size (32.9±3.9%, 23.6±3.8%, and 31.4±3.9%, respectively), although the optimal dose was 0.1 mg/kg (the only dose that produced a significant difference). Although cerivastatin (0.5, 5, and 50 μg/kg) caused infarct limitation (26.2±3.2%, 32.1±5.3%, and 37.1±4.4%, respectively), it was significant at the lowest dose only, and the effect was weaker at higher doses. Furthermore, cotreatment with AMP-CP, 8-SPT, or wortmannin between 5 minutes before and 60 minutes after reperfusion abrogated the infarct-limiting effect of pravastatin (39.9±4.0%, 42.6±4.0%, or 38.6±3.6%, respectively), pitavastatin (40.4±3.1%, 39.4±3.6%, or 39.1±3.1%, respectively), and cerivastatin (41.1±3.7%, 42.1±3.9%, or 40.4±4.0%, respectively), although these drugs per se did not affect infarct size (42.7±4.5%, 40.3±3.5%, or 42.7±4.5%, respectively).

5′-Nucleotidase Activity at Reperfusion

Figure 3 shows the activity of ecto-/endo-5′-nucleotidase in protocol 2. Sustained ischemia for 90 minutes and 15 minutes of subsequent reperfusion did not significantly change the activity of ecto-5′-nucleotidase (41.0±5.7 versus 33.2±1.2 nmol · mg protein^{-1} · min^{-1} at baseline). Preischemic treat-

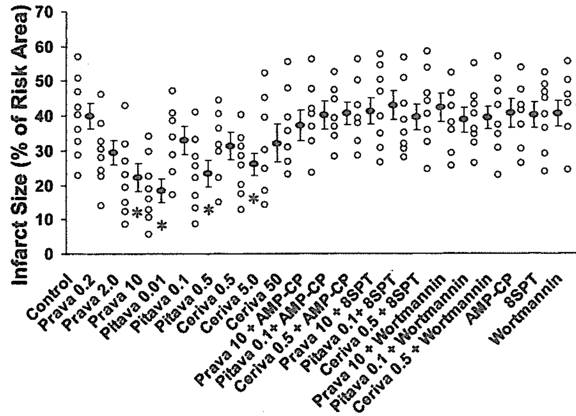

Figure 2. Infarct size in each group in protocol 1. Data are expressed as mean±SEM. *$P<0.05$ vs control. Open circles show infarct size in each individual. Prava indicates pravastatin; Pitava, pitavastatin; and Ceriva; cerivastatin. All other abbreviations are as defined in text.

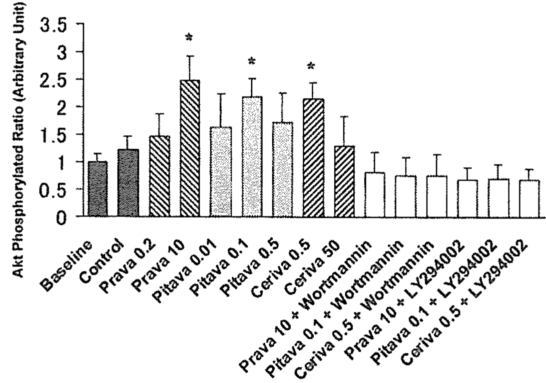

Figure 4. Myocardial PI3-K activity represented by phosphorylated ratio of Akt in each group in protocol 2. Data are expressed as mean±SEM. n=4 each, *$P<0.05$ vs control. Abbreviations are as defined in text and in legend to Figure 2.

ment with pravastatin caused a dose-dependent and acute increase of ecto-5′-nucleotidase activity in the ischemic zone, which became significant at the highest dose (72.6±6.0 nmol · mg protein^{-1} · min^{-1} at 10 mg/kg, $P<0.05$ versus control). Pitavastatin also caused significant activation at its optimal (medium) dose (66.7±6.1 nmol · mg protein^{-1} · min^{-1} at 0.1 mg/kg, $P<0.05$ versus control). Cerivastatin caused activation at the lowest dose (62.5±5.6 nmol · mg protein^{-1} · min^{-1} at 0.5 μg/kg, $P<0.05$ versus control). All of these increases were canceled by the selective PI3-K inhibitors wortmannin (39.5±6.8 nmol · mg protein^{-1} · min^{-1} for pravastatin, 37.0±7.1 nmol · mg protein^{-1} · min^{-1} for pitavastatin, and 38.4±6.5 nmol · mg protein^{-1} · min^{-1} for cerivastatin) or LY294002 (33.5±6.5 nmol · mg protein^{-1} · min^{-1} for pravastatin, 35.0±6.2 nmol · mg protein^{-1} · min^{-1} for pitavastatin, and 37.5±6.7 nmol · mg protein^{-1} · min^{-1} for cerivastatin). The activity of endo-5′-nucleotidase remained unchanged in all cases.

PI3-K Activity at Reperfusion

Figure 4 shows the activity of PI3-K in protocol 2. Sustained ischemia for 90 minutes and subsequent reperfusion for 15 minutes did not change PI3-K activity significantly (123±23% versus 100±14% at baseline). Preischemic treatment with pravastatin caused dose-dependent and acute activation of ecto-5′-nucleotidase in the ischemic zone, which was significant at the highest dose (249±44% at 10 mg/kg, $P<0.05$ versus control). Pitavastatin also caused significant activation at its medium dose (218±34% at 0.1 mg/kg, $P<0.05$ versus control), whereas cerivastatin caused activation at the lowest dose (214±31% at 0.5 μg/kg, $P<0.05$ versus control). We confirmed that all of these increases were also blocked by wortmannin (81±38% for pravastatin,

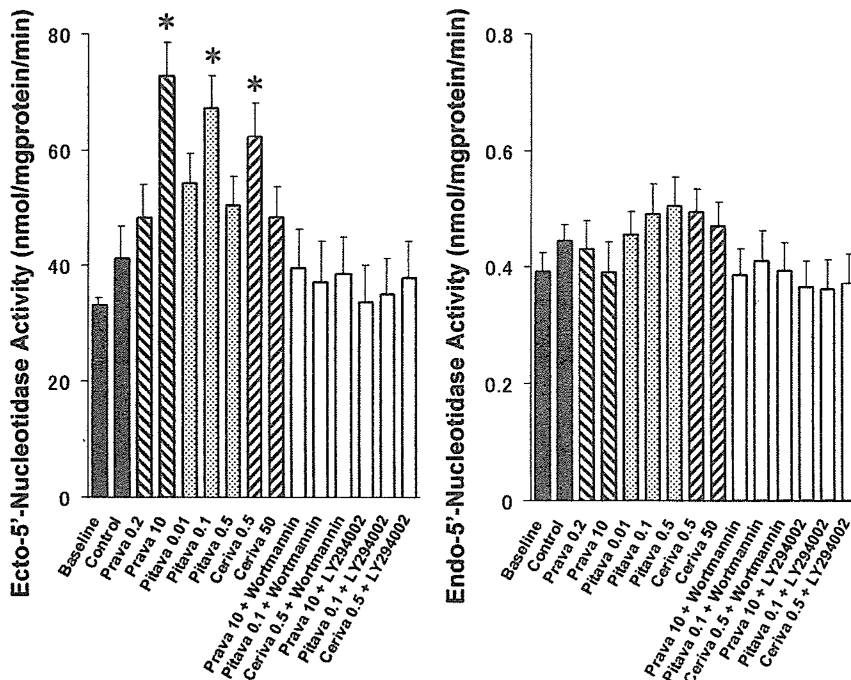

Figure 3. Myocardial ecto-/endo-5′-nucleotidase activity in each group in protocol 2. Data are expressed as mean±SEM. n=4 each, *$P<0.05$ vs control. Abbreviations are as defined in text and in legend to Figure 2.

77±32% for pitavastatin, and 76±39% for cerivastatin) or LY294002 (69±23% for pravastatin, 70±27% for pitavastatin, and 68±21% for cerivastatin).

Discussion

The present study demonstrates that several statins provide immediate infarct limitation of different magnitudes and at different optimal doses. Our results also suggest that activation of ecto-5′-nucleotidase through the activation of PI3-K after ischemia was involved in this cardioprotective mechanism of statins.

Cholesterol-Lowering Effects and Immediate Infarct Limitation of Statins

In this study, we set the doses of statins in line with their clinical cholesterol-lowering properties. In Japan, the standard clinical doses to obtain a 20% to 30% reduction of total plasma cholesterol levels were 10 mg/d for pravastatin, 2 mg/d for pitavastatin, and 0.15 mg/d for cerivastatin. Our preliminary trials in the same dog model revealed that a single intravenous injection of 0.2 mg/kg pravastatin, 0.1 mg/kg pitavastatin, or 5 μg/kg cerivastatin approximated the clinical cholesterol-lowering dose based on the maximal plasma concentration of each statin (data not shown). Because (1) the maximal infarct limitation was achieved by a higher dose of pravastatin than the clinical dose, whereas the dose was similar to the clinical dose for pitavastatin and lower for cerivastatin, and (2) these statins showed early cardioprotection within 2 hours of administration in this model, it is strongly suggested that the magnitude of immediate infarct limitation by each statin is not correlated with its cholesterol-lowering effect.

Existence of Optimal Cardioprotective Doses for Each Statin

In the present report, we have directly shown that pitavastatin has the optimal dose to reduce infarct size. Obviously, there is also an optimal dose for cerivastatin under the lowest dose we tried, because infarct size with far lower doses of cerivastatin near zero will converge with those of control levels. In the case of pravastatin, our additional experiment, within the limitation with regard to the total amount of the drug we could obtain, showed that 100 mg/kg pravastatin administered in the same manner as in protocol 1 exerted similar (but a slightly weaker) magnitude of reducing infarct size (20.9±4.5%, n=5) compared with that achieved with 10 mg/kg of this agent. Although we could not show direct evidence in this case, it would at least not deny the possibility for the existence of an optimal dose of pravastatin. Furthermore, other reports also showed the existence of an optimal dose of atorvastatin for infarct limitation[9] or of simvastatin for PI3-K activation.[8] Taken together, the existence of optimal doses should be ubiquitous among all (or at least all hydrophobic) statins.

Although direct exhibition of the reason for this phenomenon remains unclear in this study, there might be some reasons to regulate the respective optimal windows for each statin, eg, differences in the ability to attenuate inflammatory response[20] or in the potency of direct absorption into cellular membrane to modulate intracellular signaling systems. In addition, our present finding that infarct limitation completely paralleled the activation of PI3-K leads us to hypothesize that the lesser effects by the higher doses of statins should be regulated upstream of PI3-K. One possibility is that all hydrophobic statins can dose-dependently activate apoptosis-related signals,[21] which might also explain the wide range of higher cardioprotective doses for pravastatin specifically. Finally, additional studies will need to be performed to obtain direct evidence.

Cardioprotective Mechanisms

Our observations that (1) activation of PI3-K and ecto-5′-nucleotidase was coincident with a substantial limitation of infarct size, (2) either wortmannin or AMP-CP abolished cardioprotection by all 3 statins, (3) different PI3-K inhibitors at reperfusion actually inhibited PI3-K activity (Figure 4) and subsequently reduced ecto-5′-nucleotidase activity (Figure 3), and (4) our preliminary documentation that PI3-K inhibition by either wortmannin of LY294002 before ischemia did not abolish the infarct limitation by statins in the present study (n=4 or 5, data not shown), together suggest that infarct limitation in this model was linked to the activation of PI3-K during reperfusion, not before ischemia, followed by ecto-5′-nucleotidase activation.

In this study, we did not determine the exact mechanism of how PI3-K activates ecto-5′-nucleotidase. Although we have previously reported that phosphorylation of ecto-5′-nucleotidase might be crucial,[22] other mechanisms may also be involved, such as endocytotic turnover.[17] In addition, although we did not evaluate real-time regional myocardial production of adenosine in each group, treatment with a potent adenosine receptor antagonist (8-SPT) during reperfusion also blunted infarct limitation by statins along with the inhibition of ecto-5′-nucleotidase, further suggesting that cardioprotection against ischemia-reperfusion injury via ecto-5′-nucleotidase activation might be mediated by an increase of adenosine, the main product of ecto-5′-nucleotidase.[11,13,22] However, other implicated mechanism of enhanced activation of the adenosine receptor (eg, increased receptor sensitivity) should be determined by future studies.

Possible Link Between Cardioprotection by Adenosine and NO

Previous studies support our present findings that statins rapidly activate the PI3-K/Akt pathway,[8,9] and we obtained another preliminary finding that the cotreatment with N^{ω}-nitro-L-arginine methyl ester (10 $\mu g \cdot kg^{-1} \cdot min^{-1}$) in the same manner as in protocol 1, which we confirmed did not affect baseline infarct size in the present model,[23] blunted the infarct limitation by pravastatin (36.8±4.1%, n=7), pitavastatin (39.9±3.9%, n=6), and cerivastatin (42.6±4.6%, n=5). Therefore, there is a possibility that ecto-5′-nucleotidase and NO act in series to cause statin-induced cardioprotection.

Although elucidation of a direct effect should be the focus of future studies, there are at least 2 lines of evidence to support the explanation that adenosine and NO synergistically caused infarct limitation in this study. First, NO directly exerts cardioprotection[24]: NO inhibits cell-to-cell adhesion,

such as that between platelets[25] or between neutrophils and endothelial cells,[26,27] by reducing expression of P-selectin,[27] E-selectin, and intercellular adhesion molecule-1,[28] which leads to attenuation of the inflammatory response[22,24,25] or protects against ischemia-reperfusion injury.[25–28] In addition, NO is reported to inhibit caspase-3 activity and to block apoptosis of cardiac myocytes.[29] On the other hand, adenosine also rescues injured myocardium through activating adenosine receptors.[13,30–32] Either administration of adenosine or enhancement of endogenous adenosine release during reperfusion after sustained ischemia limits infarct size.[13,17] We and others have shown that (1) adenosine receptor (A_1 and A_2) activation improves contractile dysfunction after reperfusion,[14] (2) inhibition of norepinephrine release from the presynaptic vesicles and attenuation of calcium influx occur through the A_1 receptor and the coupled inhibitory G protein,[33,34] (3) inhibition of platelet aggregation and leukocyte activation occurs through the A_2 receptor and the coupled stimulatory G protein,[34–36] and (4) activation of extracellular signal–regulated kinase, one of the reperfusion injury survival kinase pathways,[37] takes place during reperfusion through the A_3 receptor.[38] Therefore, either adenosine or NO similarly and potentially protects injured myocardium through multiple pathways.

Second, recent articles have shown that either adenosine[38–40] or NO[41] can reactivate PI3-K downstream. However, increasing the production of both agents is known to negatively regulate further increases of production of these molecules,[42,43] suggesting the requirement of both pathways to confer sufficient cardioprotection in the physiological system. Taking all of these together, it is likely that adenosine and NO synergistically confer the statin-derived immediate cardioprotection shown in this study.

In conclusion, our findings suggest the cellular mechanism by which statins attenuate myocardial injury, which may indicate the possibility of acute protective therapies for ischemia and associated myocardial stresses.

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Original Article

Opening of Ca^{2+}-activated K^+ channels is involved in ischemic preconditioning in canine hearts

Yasunori Shintani [a], Koichi Node [b], Hiroshi Asanuma [a], Shoji Sanada [a], Seiji Takashima [a], Yoshihiro Asano [a], Yulin Liao [a], Masashi Fujita [a], Akio Hirata [a], Yoshiro Shinozaki [c], Tomi Fukushima [a], Yoko Nagamachi [a], Hiroko Okuda [a], Jiyoong Kim [d], Hitonobu Tomoike [d], Masatsugu Hori [a], Masafumi Kitakaze [d,*]

[a] *Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Japan*

[b] *Department of Cardiology, Saga University School of Medicine, Saga, Japan*

[c] *Department of Physiological Science, Tokai University School of Medicine, Isehara, Japan*

[d] *Cardiology Division of Medicine, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka Prefecture 565-8565, Japan*

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Abstract

Brief periods of ischemia that precede sustained ischemia can markedly reduce infarct size (IS), a phenomenon that is known as ischemic preconditioning (IP). Several investigators have shown that elevation of the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) during the antecedent brief periods of ischemia triggers the cardioprotective mechanism of IP. Since opening of Ca^{2+} activated K^+ (K_{Ca}) channels is reported to be cardioprotective, we hypothesized that these channels may be involved in the cardioprotective mechanism of IP. In anesthetized open-chest dogs, myocardial ischemia/reperfusion injury was created by occlusion of the left anterior descending coronary artery (LAD) for 90 min followed by 6 h of reperfusion. First, we showed that the treatment with NS1619, a K_{Ca} channel opener, reduced IS (IS in NS1619 group and control group, 19.8 ± 5.5% vs. 45.4 ± 3.5% of the area at risk, $P < 0.05$). Next, four cycles coronary occlusion for 5 min and reperfusion (IP) were performed before the 90-min occlusion with or without the infusion of potent K_{Ca} channel inhibitors, iberiotoxin (IbTX) and charybdotoxin (ChTX). IP markedly reduced IS (IS in the IP group was 8.2 ± 1.8%, $P < 0.01$ vs. control group). Infusion of either of K_{Ca} channel blockers during IP blunted the IS-limiting effect of IP (IS in the IP + IbTX and IP + ChTX groups was 30.7 ± 7.0% and 35.5 ± 3.7%, respectively, $P < 0.05$, vs. IP group). However, the cardioprotective effect of IP was not blunted by the treatment with ChTX when treated only during reperfusion (14.0 ± 4.1%). Thus, we conclude that the opening of K_{Ca} channel is involved in early trigger phase of the molecular mechanism of IP.

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Keywords: Ischemic preconditioning; Ischemia; Reperfusion; Myocardial infarction; Ca^{2+}-activated K^+ channel

1. Introduction

Brief periods of ischemia that precede sustained ischemia can markedly decrease infarct size (IS), a phenomenon which is known as ischemic preconditioning (IP) [1–3]. This endogenous self-defense mechanism is one of the most powerful cardioprotective defenses against ischemia/reperfusion injury that has been demonstrated so far. Several investigators have previously revealed that an increase of the intracellular Ca^{2+} level ($[Ca^{2+}]_i$) during the antecedent brief periods of ischemia triggers or mediates the cardioprotective mechanism of IP [4,5]. Among the intracellular sequelae of Ca^{2+} overload, opening of Ca^{2+}-activated K^+ channels (K_{Ca} channels) is known to occur. Indeed, we have reported that opening of K_{Ca} channels is involved in the limitation of IS by 17β-estradiol or raloxifene [6,7]. A recent report also suggested that K_{Ca} channel opening mediates cardioprotection [8], but it has not been elucidated whether cardioprotection

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} level; ChTX, charybdotoxin; IbTX, iberiotoxin; IP, ischemic preconditioning; K_{Ca} channel, Ca^{2+}-activated K^+ channel; LAD, left anterior descending coronary artery.

* Corresponding author. Tel.: +81-6-6833-5012; fax: +81-6-6836-1120.
E-mail address: kitakaze@zf6.so-net.ne.jp (M. Kitakaze).

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due to IP is mediated through the K_{Ca} channels as well as ATP-sensitive K^+ channels.

We hypothesized that opening of the K_{Ca} channel in response to elevation of $[Ca^{2+}]_i$ may be involved in the cardioprotective mechanism of IP. We found that potent K_{Ca} channel inhibitors, charybdotoxin (ChTX) and iberiotoxin (IbTX), could abolish IP-induced cardioprotection in a canine ischemia/reperfusion model. We also found that this channel contributed to early phase of IP-induced cardioprotective machinery rather than late phase.

2. Materials and methods

All procedures were performed in accordance with 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).

2.1. Instrumentation

Beagle dogs weighing 9–14 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg), intubated with a cuffed endotracheal tube, and ventilated using room air mixed with oxygen (1.5 l/min), as described previously [9]. Thoracotomy was performed through the left fifth intercostal space, and the heart was suspended in a pericardial cradle. After an intravenous dose of heparin (500 U/kg), the proximal left anterior descending coronary artery (LAD) was cannulated and perfused with blood via an extracorporeal tube from the left carotid artery. Further heparin (100 U/kg) was administered intravenously every 3 h throughout the protocol. An occluder was attached to the bypass tube of the carotid-to-LAD shunt, and manual clamping of the tube was performed to produce myocardial ischemia. The pressure-resistant tube from the proximal portion of the cannula was connected to a multichannel recorder (Rm-6000; Nihon Kohden) to monitor arterial pressure. In addition, the left atrium was cannulated for the injection of microspheres.

2.2. Experimental protocols

2.2.1. Protocol 1: Effect of an intracoronary K_{Ca} channel opener (NS1619) on infarct size

Fig. 1 shows the details of this protocol. After hemodynamic stabilization, we injected NS1619 (11 µg/kg per min, Sigma, St. Louis, MO, USA: NS1619 group; n = 6) or NS1619 plus ChTX (0.3 µg/kg per min, Peptide Institute, Minoh, Osaka, Japan: NS1619 + ChTX group; n = 6) into the LAD through the bypass tube from 10 min before coronary occlusion until 1 h after reperfusion without a period of occlusion. We chose these doses of NS1619 and ChTX because these doses are maximal each that does not alter either systemic blood pressure or heart rate (HR), and sufficient to open and block the K_{Ca} channels, respectively. The ED_{50} of NS1619 is about 10 µM [10] and the calculated concentration

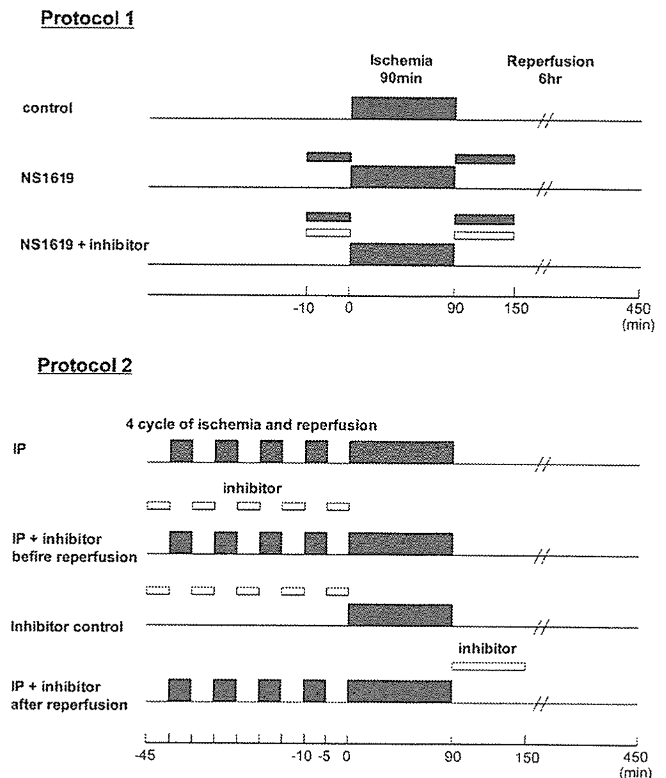

Fig. 1. All experimental protocols in this study are shown. IP: ischemic preconditioning.

of NS1619 used in this model was 40 µM. On the other hand, since K_d of ChTX is 2.1 nM [11], we need to use 10–100 times higher dose of K_d to fully block the channels. The calculated concentration of ChTX used in the present study was 100 nM, indicating that the dose of ChTX used in this protocol are sufficient to block the K_{Ca} channels.

Hemodynamic parameters were measured before the initiation of each protocol, 60 min after the onset of ischemia, and 1, 3 and 6 h after the onset of reperfusion.

2.2.2. Protocol 2: Effect of K_{Ca} channel inhibitors on IP

Fig. 1 displays the details of this protocol. After hemodynamic stabilization, four cycles of coronary occlusion for 5 min and subsequent reperfusion for 5 min (IP) were performed using the occluder with or without infusion of a K_{Ca} channel inhibitor throughout the IP procedure except during coronary occlusion. We used two different K_{Ca} channel inhibitors, IbTX (0.6 µg/kg per min, Peptide Institute) and ChTX. The following six groups were studied: Control group (n = 6), IP group (n = 6), IP + IbTX before reperfusion group (n = 6), IP + ChTX before reperfusion group (n = 6), IbTX group (n = 6), and ChTX group (n = 6). We chose the dose of IbTX because this dose is maximal and does not alter either systemic blood pressure or HR, and is sufficient to block the K_{Ca} channels. Since K_d of IbTX is 1 nM [12], we need to use 10–100 times higher dose of K_d to fully block the channels. The calculated concentrations of IbTX in this study is 200 nM, indicating that the dose of IbTX was also sufficient to block the K_{Ca} channels.

To distinguish the role of K_{Ca} channel in IP during ischemia or reperfusion period, we infused ChTX for 60 min after reperfusion in a group subjected to IP (IP + ChTX after reperfusion group, (n = 8)).

Hemodynamic parameters were measured at the same five times as in protocol 1.

2.3. Exclusion criteria

To ensure that all of the animals included in the analysis of IS were healthy and exposed to a similar extent of ischemia, we adopted the following criteria for exclusion of unsatisfactory dogs: (1) subendocardial collateral flow >15 ml/100 g per min, and (2) more than two consecutive attempts required to correct ventricular fibrillation with a low-energy counter pulse applied directly to the heart.

2.4. Measurement of infarct size and regional myocardial blood flow

We measured IS and regional myocardial blood flow as described previously [9]. For randomization, all measurements were done at completion of the protocol by persons without the knowledge of the treatment given to each heart.

2.5. Statistical analysis

Data are expressed as the mean ± S.E. Statistical significance was assessed with ANOVA, and if differences were found among groups, they were evaluated by Bonferroni's post-hoc test with $P < 0.05$ being considered as significant. The effect of collateral blood flow on IS was analyzed by ANCOVA, with regional collateral flow in the inner half of the left ventricular wall as covariant.

3. Results

3.1. Mortality and exclusions

We excluded 11 dogs from analysis because subendocardial collateral blood flow was greater than 15 ml/100 g per min. Ventricular fibrillation that matched the exclusion criterion occurred in seven animals during the 6-h reperfusion period and six of the seven died of ventricular fibrillation (Table 1). There were no significant differences in the number of exclusions among the groups.

3.2. Hemodynamic parameters, area at risk, and collateral blood flow

During protocols 1 and 2, the HR and mean arterial blood pressure (MAP) remained stable throughout the study (Table 2). The area at risk and the collateral blood flow were also similar among all of the groups, and there was no statistical difference (Table 3).

3.3. Infarct size

Fig. 2 shows IS in the nine groups from protocols 1 and 2. Treatment with NS1619 before and after ischemia reduced IS (19.8 ± 5.5% vs. 45.4 ± 3.5% of the area at risk, compared with the control group), while ChTX completely blocked this IS-limiting effect (42.2 ± 5.8%). IP markedly reduced IS compared with the control group (8.2 ± 1.8%). Treatment with either K_{Ca} channel inhibitor during IP blunted the IS-limiting effect (IP + IbTX group and IP + ChTX group: 30.7 ± 7.0%* and 35.5 ± 3.7%*, respectively, * $P < 0.05$ vs. the IP group). Either inhibitor alone, had no influence on IS (IbTX group and ChTX group: 37.1 ± 4.5% and 40.7 ± 6.1%, respectively). However, cardioprotective effect of IP was not influenced by treatment after reperfusion with ChTX (14.0 ± 4.1%).

ANCOVA test showed that these effects of K_{Ca} channel opener and inhibitor were independent from that of collateral blood flow (Fig. 3, and Table 4).

4. Discussion

We showed that an intracoronary administration of a K_{Ca} channel opener (NS1619) mimicked the IS-limiting effect in

Table 1
Number of dogs assigned to and excluded from each group for measurement of IS

Group	Number of dogs originally assigned	Number of dogs used for data analysis	Reason for exclusion		
			Vf (>2) during 6 h of reperfusion	Death due to Vf	High collateral flow (>15 ml/100 g per min)
Protocols 1, 2					
Control	7	6	0	1	0
NS1619	10	6	0	1	3
NS1619 + ChTX	8	6	0	0	2
IP	7	6	0	0	1
IP + IbTX before reperfusion	11	6	0	2	3
IP + ChTX before reperfusion	7	6	1	0	0
IbTX	7	6	0	1	0
ChTX	6	6	0	0	0
IP + ChTX after reperfusion	11	8	0	1	2

Vf: ventricular fibrillation, IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin.

Table 2
Hemodynamic parameters during protocol

Group	Baseline		60 min of ischemia		1 h after reperfusion		3 h after reperfusion		6 h after reperfusion	
	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)	MAP (mmHg)	HR (bpm)
Protocols 1, 2										
Control	99 ± 5	141 ± 7	95 ± 5	139 ± 12	98 ± 5	139 ± 9	95 ± 4	132 ± 12	92 ± 4	133 ± 9
NS1619	104 ± 4	135 ± 7	92 ± 7	134 ± 6	92 ± 5	138 ± 7	91 ± 4	146 ± 7	92 ± 4	141 ± 6
NS1619 + ChTX	106 ± 5	142 ± 3	103 ± 6	134 ± 8	100 ± 9	137 ± 9	97 ± 6	136 ± 8	93 ± 6	142 ± 8
IP	106 ± 6	134 ± 7	100 ± 7	133 ± 7	104 ± 7	134 ± 7	102 ± 6	134 ± 7	101 ± 8	132 ± 7
IP + IbTX before reperfusion	104 ± 4	135 ± 9	96 ± 7	134 ± 5	91 ± 3	134 ± 9	92 ± 7	128 ± 14	90 ± 7	133 ± 14
IP + ChTX before reperfusion	105 ± 9	143 ± 8	90 ± 10	146 ± 8	92 ± 9	129 ± 8	96 ± 7	136 ± 9	94 ± 9	138 ± 9
IbTX	101 ± 8	139 ± 8	102 ± 8	137 ± 6	104 ± 7	134 ± 7	98 ± 6	130 ± 6	92 ± 6	133 ± 6
ChTX	99 ± 3	133 ± 9	98 ± 8	136 ± 8	100 ± 7	133 ± 7	104 ± 7	130 ± 7	102 ± 8	134 ± 8
IP + ChTX after reperfusion	104 ± 2	143 ± 6	101 ± 3	135 ± 8	101 ± 3	151 ± 7	99 ± 4	142 ± 9	106 ± 4	137 ± 6

MAP: mean arterial pressure, IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin. Data were shown by mean ± S.E. There was no significant difference among each protocol.

Table 3
Collateral blood flow and area at risk among experimental groups

Group	Collateral blood flow (ml/100 g per min)	Area at risk (%)
Protocols 1, 2		
Control	7.8 ± 1.6	46 ± 4
NS1619	6.5 ± 1.3	49 ± 6
NS1619 + ChTX	7.3 ± 1.5	39 ± 2
IP	9.0 ± 1.4	41 ± 3
IP + IbTX before reperfusion	6.8 ± 1.3	51 ± 5
IP + ChTX before reperfusion	7.9 ± 1.1	42 ± 2
IbTX	7.3 ± 1.4	44 ± 4
ChTX	8.5 ± 1.1	39 ± 2
IP + ChTX after reperfusion	8.7 ± 1.0	41 ± 2

IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin. Data were shown by mean ± S.E. There was no significant difference among each protocol.

Table 4
Linear regression model test in each group

Group	Formula
Control	$y = 50.032 - 0.586x$
NS1619	$y = 30.649 - 1.667x$ *
NS1619 + ChTX	$y = 61.806 - 2.694x$
IP	$y = 15.228 - 0.786x$ *
IP + IbTX before reperfusion	$y = 49.482 - 2.792x$ *, †
IP + ChTX before reperfusion	$y = 42.162 - 0.852x$ †
IbTX	$y = 47.653 - 1.439x$
ChTX	$y = 56.948 - 1.921x$
IP + ChTX after reperfusion	$y = 37.367 - 2.703x$ *

IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin.
* $P < 0.05$ vs. control group. † $P < 0.05$ vs. IP group.

a canine ischemia/reperfusion model potent, and K_{Ca} channel inhibitors (ChTX and IbTX) blocked the cardioprotective effect of IP. We also showed that the cardioprotective effect of IP was not blunted by the treatment with ChTX only during the reperfusion period. These data suggest that opening of K_{Ca} channel is involved in early phase of the molecular mechanism of IP.

Since Murry et al. [1] first demonstrated the intriguing phenomenon known as IP, numerous studies have been done to elucidate the cellular mechanisms responsible. There is considerable evidence that $[Ca^{2+}]_i$ increases transiently during ischemic episodes that produce IP and it may be the key factor in IP [4,5,13–15]. Direct measurement of $[Ca^{2+}]_i$ has shown that a brief period of ischemia increases it two to fourfold [16,17]. On the other hand, exogenous calcium triggers the IS-limiting effect, and our previous study showed that a calcium chelator abolished the cardioprotective effect of IP [5]. While $[Ca^{2+}]_i$ is increased during antecedent ischemia, it is paradoxically reduced during subsequent sustained ischemia [18,19]. Indeed, it is well recognized that ischemia/reperfusion causes intracellular Ca^{2+} overload and thus leads to the death of cardiomyocytes [20]. Generally, the K_{Ca} channel opens after elevation of $[Ca^{2+}]_i$ and causes membrane hyperpolarization, which reduces voltage-dependent Ca^{2+} influx by increasing K^+ efflux and thus prevents Ca^{2+} overload. This sequence suggests that K_{Ca} channel opening is a candidate for mediating IP.

The outward K^+ channels comprise the voltage-dependent channel (K_V channel) and the calcium-dependent channel (K_{Ca} channel). The K_{Ca} channel is separated into three subclasses (BK, IK, and SK) according to its conductance. It has been demonstrated that the K_{Ca} channel (generally BK) is present in various muscular and non-muscular tissues. In addition to its distribution in a variety of cell types, Kawakubo et al. [21] demonstrated the existence of BK channels on ventricular cardiomyocytes by using the patch-clamp technique. In addition, we have previously shown that opening of the K_{Ca} channels has a cardioprotective effect without affecting coronary blood flow. Indeed, it has been reported that the K_{Ca} channel is located on vascular smooth muscle cells (SMC), and that endothelium-derived hyperpolarizing factor causes vasodilation by activating this channel [22,23]. To exclude the possibility of an effect on coronary and collateral flow, we determined the dose of NS1619 (11 μg/kg per min) that did not increase coronary flow. In fact, there was no significant difference of collateral flow (Table 3), which implies that opening of K_{Ca} channels in cardiac tissues other than SMC may also be important.

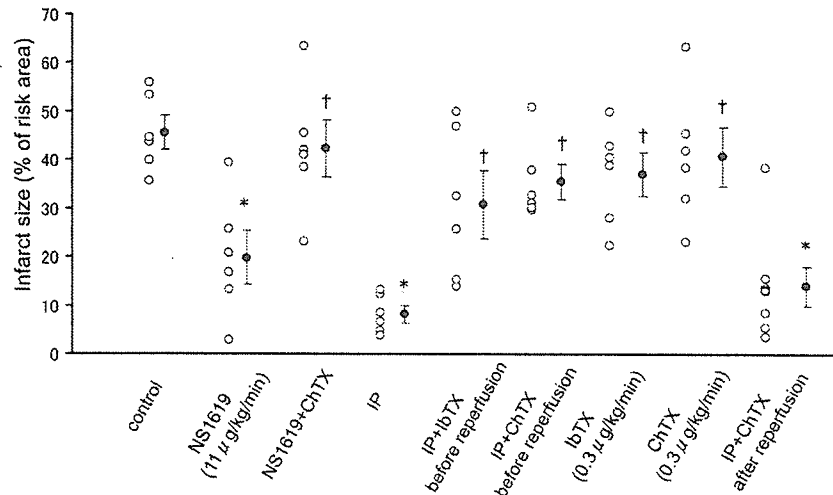

Fig. 2. IS as a percentage of the area at risk in all nine experimental groups in protocols 1 and 2. Data from individual animals and mean ± S.E. are shown. * P <0.05 vs. control group, [+] P <0.05 vs. IP group. IP: ischemic preconditioning, IbTX: iberiotoxin, ChTX: charybdotoxin.

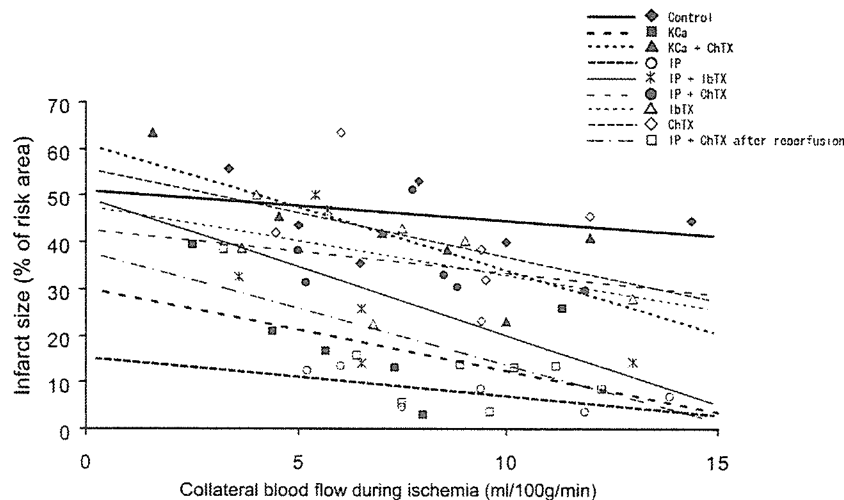

Fig. 3. IS in protocols 1 and 2 as a percentage of the risk area vs. regional collateral blood flow during ischemia. See Fig. 2 legend for explanation of each group. The results of ANCOVA test is indicated in Table 4.

Recently, it was demonstrated that infusion of NS1619 5–10 min before sustained ischemia could reduce IS through mitochondrial K_{Ca} channels in an isolated rabbit heart model [8]. It was suggested that opening of this channel improves mitochondrial ATP production and decreases both the production of reactive oxygen species and Ca^{2+} overload in the mitochondria. This mechanism may also contribute to the cardioprotective effect of the K_{Ca} channel during IP in addition to reduction of voltage-dependent Ca^{2+} influx by promotion of K^+ efflux.

Generally, some care is needed when interpreting experiments that employ pharmacological agents. ChTX mainly acts on BK channels, but it also interacts with IK1 and K_V 1.3 type channels [24]. We tested the influence of IbTX on IP as well as ChTX, since IbTX is a selective blocker of large-conductance BK type K_{Ca} channels. Our results showed that both K_{Ca} channel inhibitors abolished IP-induced cardioprotection, making it likely that this channel is involved in the mechanism of IP rather than the possibility of a nonspecific effect or the contaminating effect of other channels.

Protein kinase C (PKC) is believed to play an important role in triggering IP, and elevation of $[Ca^{2+}]_i$ activates PKC [5,25]. However, activation of PKC was reported to inhibit the K_{Ca} channel [26]. Since we have previously reported that PKA is involved in IP [27] and many authors have shown that PKA activates the K_{Ca} channel, [28–32] it seems that PKA activation following brief ischemia also has a role in opening the K_{Ca} channel as well as the elevation of $[Ca^{2+}]_i$. Adenosine has also been reported to open K_{Ca} channels, and it may be involved in the mechanism of IP [33].

In the present study, we found evidence that K_{Ca} channel opening contributes to IP, as well as the ATP-sensitive K^+ channel which is believed to play a central role in IP. Further investigations will be required to clarify the importance of the K_{Ca} channel to IP.

Acknowledgements

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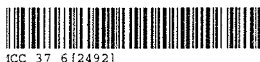

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Original Article

Selective blockade of serotonin 5-HT2A receptor increases coronary blood flow via augmented cardiac nitric oxide release through 5-HT1B receptor in hypoperfused canine hearts

Masashi Fujita [a], Tetsuo Minamino [a,*], Shoji Sanada [a], Hiroshi Asanuma [a], Akio Hirata [a], Hisakazu Ogita [a], Ken-ichiro Okada [a], Osamu Tsukamoto [a], Seiji Takashima [a], Hitonobu Tomoike [b], Koichi Node [c], Masatsugu Hori [a], Masafumi Kitakaze [b]

[a] *Division of Cardiology, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan*

[b] *Cardiovascular Division of Medicine, National Cardiovascular Center of Japan, Suita, Osaka 565-8565, Japan*

[c] *Department of Cardiovascular and Renal Medicine, Faculty of Medicine, Saga University, Saga, Saga 849-8501, Japan*

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Abstract

Serotonin (5-hydroxytryptamine [5-HT]), which induces vasoconstriction via 5-HT2A receptors in smooth muscle cells and vasodilation through activating nitric oxide (NO) synthase (NOS) via 5-HT1B receptors in endothelial cells, possesses divergent effects on regulating vascular tone. These facts lead us to consider that sarpogrelate, a 5-HT2A receptor blocker, may increase coronary blood flow (CBF) via either attenuation of vasoconstriction through 5-HT2A receptor blockade or augmentation of vasodilation by relative stimulation of NOS through 5-HT1B receptor and we tested this hypothesis in ischemic canine hearts. In open chest dogs, coronary perfusion pressure was reduced so that CBF was decreased to 33% of the baseline and kept constant. Thereafter, sarpogrelate was infused selectively into the left anterior descending artery with and without either an inhibitor of NOS (NG-nitro-L-arginine methyl ester (L-NAME)) or a 5-HT1B receptor antagonist (GR55562). An intracoronary administration of sarpogrelate increased CBF (34.0 ± 4.0 to 44.5 ± 4.4 ml/100 g/min, $P < 0.05$), along with the cardiac NOx release (3.2 ± 0.6 to 6.8 ± 1.2 nmol/ml, $P < 0.05$). The increases in both CBF and NOx by sarpogrelate were completely blunted by the co-administration of either L-NAME or GR55562. Interestingly, sarpogrelate increased the cardiac serotonin release (–4.8 ± 3.2 vs. 22.1 ± 1.5 ng/ml, $P < 0.05$, respectively) in the hypoperfused heart. Immunohistochemical analysis showed that sarpogrelate induced serotonin production in ischemic cardiac myocytes. These results suggest that sarpogrelate increases CBF via augmented cardiac NO production through 5-HT1B receptor activation along with the blockade of 5-HT2A receptors. The increase in cardiac release of serotonin may increase NO production in the ischemic heart.

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Keywords: Serotonin (5-hydroxytryptamine, 5HT); Nitric oxide (NO); Ischemia; Coronary circulation; Sarpogrelate

1. Introduction

Serotonin (5-hydroxytryptamine, 5HT) has various subtypes of receptors, and causes both vasoconstriction and vasodilatation [1,2]. Serotonin causes vasoconstriction via activation of 5-HT2A receptors on vascular smooth muscle cells [3], and Vanhoutte [2] reported that serotonin can cause vasodilatation via a nitric oxide (NO)-dependent mechanism via 5-HT1B receptors. However, there is no clear consensus about the effects of serotonin on coronary circulation.

Sarpogrelate is a selective antagonist of 5-HT2A receptor widely used for patients with arteriosclerosis obliterans [4]. Since the blockade of 5-HT2A receptors by sarpogrelate may weaken the vasoconstriction and alternatively stimulates 5-HT1B receptors and therefore NO production, we hypothesized that sarpogrelate may increase coronary blood flow (CBF) in hypoperfused hearts. In the present study, we tested the effects of sarpogrelate on CBF in hypoperfused canine hearts using either NG-nitro-L-arginine methyl ester (L-

* Corresponding author. Tel.: +81-6-6879-3635; fax: +81-6-6879-3473.
E-mail address: minamino@medone.med.osaka-u.ac.jp (T. Minamino).

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NAME), an inhibitor of NO synthase (NOS), or a 5-HT1B receptor antagonist, GR55562. Then we determined whether sarpogrelate increases the differences in the plasma levels of NOx (metabolites of NO) and serotonin between coronary arterial and venous blood. Furthermore, we evaluated a potential mechanism by which sarpogrelate increased NOx release and identified the specific myocardium cell types from which serotonin was released using immunohistochemical technique.

2. Material and methods

All procedures were performed in careful conformance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1996). Experimental protocols were approved by the Osaka University Ethical Committee for Laboratory Animal Use.

3. Instrumentation

Thirty-three hybrid beagle dogs weighing 14–20 kg were anesthetized with sodium pentobarbital (30 mg/kg intravenously). The dogs were prepared as previously described [5]. Briefly, the proximal portion of the left anterior descending (LAD) coronary artery was cannulated and perfused with blood from the left carotid artery through an extracorporeal bypass tube. Either coronary perfusion pressure (CPP) or CBF was monitored at this tube.

A small, short collecting tube (diameter 1 mm, length 7 cm) was inserted into a small coronary vein near the perfused region to sample coronary venous blood in 33 dogs. Arterial samples were collected at the proximal edge of the bypass tube. Sarpogrelate hydrochloride was obtained from Mitsubishi Pharma Co.® (Japan), GR55562 from Tocris® (UK), L-NAME and a primary antibody against serotonin were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4. Experimental protocols

4.1. Effects of sarpogrelate on coronary hemodynamics parameters in the ischemic myocardium (constant low CPP)

After hemodynamic stabilization, CPP was reduced so that CBF was decreased to 33% of baseline, using an occluder attached at the extracorporeal bypass tube. After CPP reduction, the occluder was adjusted to keep CPP constant for 5 min. Saline (n = 7; control group), sarpogrelate (10 μg/kg per min, n = 10; sarpo group), sarpogrelate + L-NAME (10 μg/kg per min, n = 9; sarpo + L-NAME group) or sarpogrelate + GR55562 (1 μg/kg per min, n = 7; sarpo + GR group) was infused selectively into the LAD, and measurements of all hemodynamic parameters were recorded at 5-min intervals for 20 min. Blood was sampled before and 20 min after the onset of hypoperfusion. We chose the dose of 10 μg/kg per min of sarpogrelate for an intracoronary administration so that the concentration of sarpogrelate in coronary circulation became nearly 10 μmol/l. This dose of sarpogrelate is known to abolish serotonin-induced vasoconstriction in an isolated human endothelium-denuded arterial segment of the left internal thoracic arteries [6]. The dose of 1 μg/kg per min of GR55562 was chosen for an intracoronary administration to achieve the concentration of GR55562 to the 1 μmol/l. This level of GR55562 inhibits vasorelaxation by sumatriptan, an agonist of 5-HT1B/1D receptors, in the rat isolated middle cerebral artery [7]. Preliminary experiments confirmed that L-NAME at the dose of the 10 μg/kg per min attenuates the coronary vasodilatory action of bradykinin (20 μg/kg per min, i.c.) by 85% ± 6%.

5. Biochemical analysis

We measured the metabolites of NO (nitrite and nitrate, NOx), using 2 ml of blood as described previously [8]. Additional blood was immediately placed on ice, and used for measurement for serotonin level as described previously [9]. The cardiac release of NOx and serotonin were defined as the differences in the plasma levels of NOx and serotonin between coronary arterial and venous blood, respectively.

6. Immunohistochemical analysis

After the hearts were perfused with phosphate-buffered saline, we sampled the hypoperfused hearts following a 15 min infusion of sarpogrelate. Immunohistochemical analysis was performed as described previously [10]. Briefly, tissue from the left ventricle of the excised hearts was fixed in 10% formaldehyde for several days and dehydrated with graded concentrations of alcohol for embedding in paraffin. Paraffin slices from each heart were stained with antibody against serotonin. All histopathological sections were scanned with a Olympus light microscope (BX40) equipped with a high resolution digital camera (Fujix HC 2000, Fujifilm).

7. Statistical analysis

The time course of changes in hemodynamic parameters in each group was compared by one-way repeated measures ANOVA. The time course of changes in hemodynamic parameters between groups was compared by repeated measures ANOVA. When ANOVA revealed a significant difference, modified Bonferroni's correction was applied. All values were expressed as mean ± S.E.M. A value of $P < 0.05$ was considered as significant.

8. Results

8.1. Effects of an intracoronary administration of sarpogrelate on coronary hemodynamics

As shown in Fig. 1, both baseline heart rate (HR) and CPP in each group were similar during the experiment. In the sarpo group, baseline HR and CPP averaged 152 ± 10 bpm and 104 ± 8 mmHg, respectively. When CBF was reduced to 33% of baseline, CPP of the LAD coronary artery was decreased to 42 ± 2 mmHg and kept constant thereafter. The intracoronary administration of sarpogrelate increased CBF compared with the control group (33.6 ± 6.1 vs. 44.5 ± 4.4 ml/100 g/min, $P < 0.05$) after 15 min of infusion, and this increase in CBF was completely abrogated by the co-administration of either L-NAME or GR55562 (Fig. 2).

8.2. Cardiac release of NOx in the ischemic canine heart

The infusion of sarpogrelate significantly increased the cardiac release of NOx compared with that of the control group after 20 min of hypoperfusion. Similarly, this increase was abolished by the co-administration of either L-NAME or GR55562 (Fig. 3).

8.3. Cardiac release of serotonin in the ischemic canine heart

Treatment with sarpogrelate for 15 min in a hypoperfused state significantly increased the cardiac release of serotonin (–4.8 ± 3.2 baseline vs. 22.1 ± 1.5 ng/ml, $P < 0.05$), which also reached a significant level compared with the control group after 20 min of hypoperfusion (Fig. 4).

8.4. Serotonin expression in the ischemic canine heart

Immunohistochemical analysis revealed that serotonin was weakly expressed in the non-ischemic heart and potently induced in the ischemic heart after sarpogrelate infusion (Fig. 5).

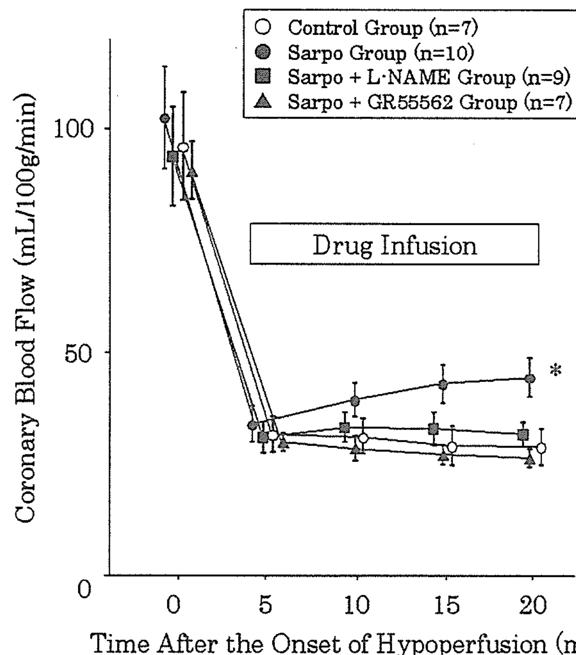
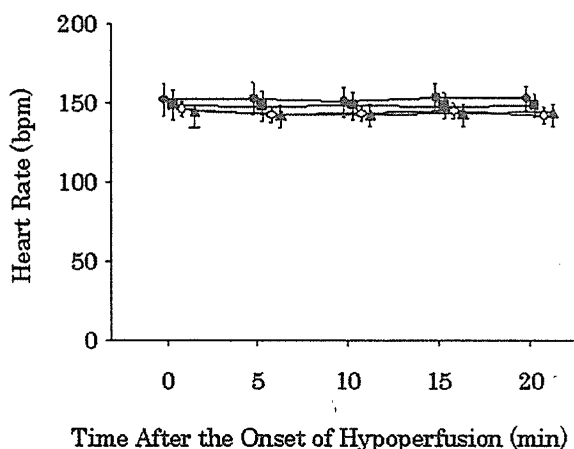


Fig. 2. Changes in CBF after infusion of sarpogrelate with and without L-NAME or GR55562. Although reduced CPP was at a constant low level, sarpogrelate increased CBF, with the effect being blunted by L-NAME or GR55562. * $P < 0.05$ versus the control group.

9. Discussion

We demonstrated here that sarpogrelate, an antagonist of 5-HT2A receptor, increased CBF via a NO-dependent mechanism through 5-HT1B receptor in hypoperfused canine hearts, along with the increase in the cardiac release of serotonin which may be produced in the ischemic myocardium.



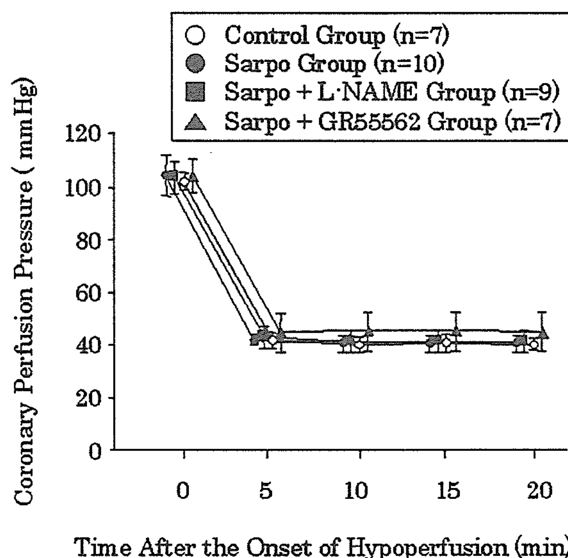


Fig. 1. Changes in HR and CPP among the control, sarpo, sarpo + L-NAME and sarpo + GR55562 groups. Both HR and CPP in each group were similar during the experiment.

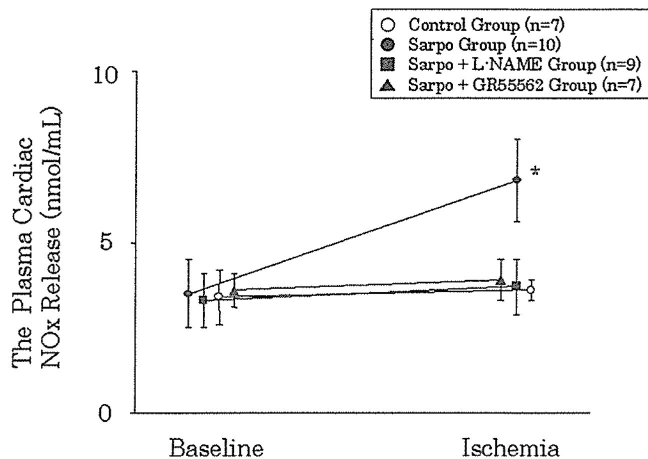


Fig. 3. Changes in the differences in the plasma levels of NOx between coronary arterial and venous blood among the control, sarpo, sarpo + L-NAME and sarpo + GR55562 groups. * $P < 0.05$ versus the control group after 20 min of hypoperfusion (ischemia).

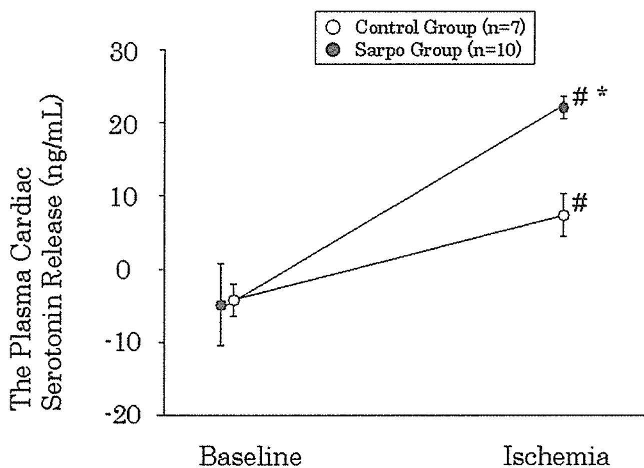


Fig. 4. Changes in the differences in the plasma levels of serotonin between coronary arterial and venous blood among the control and sarpo groups. * $P < 0.05$ versus the control group after 20 min of hypoperfusion (ischemia). # $P < 0.05$ versus before the onset of hypoperfusion (baseline) in each group.

9.1. Mechanisms by which sarpogrelate increased CBF and the cardiac release of NOx in the ischemic myocardium

In this experiment, we clearly demonstrated that sarpogrelate increased CBF in the hypoperfused hearts which may improve myocardial ischemia. Furthermore, the increase in CBF induced by sarpogrelate was abrogated by either the inhibition of NOS by L-NAME or the blockade of 5-HT1B receptor by GR55562. These results suggested that the increase in CBF induced by sarpogrelate was involved in both NOS and 5-HT1B receptors. Coincident with these findings, we confirmed that sarpogrelate increased NOx release in the coronary circulation, which was blunted by co-administration with GR55562. Thus, sarpogrelate increased CBF via a NO-dependent mechanism through 5-HT1B receptors.

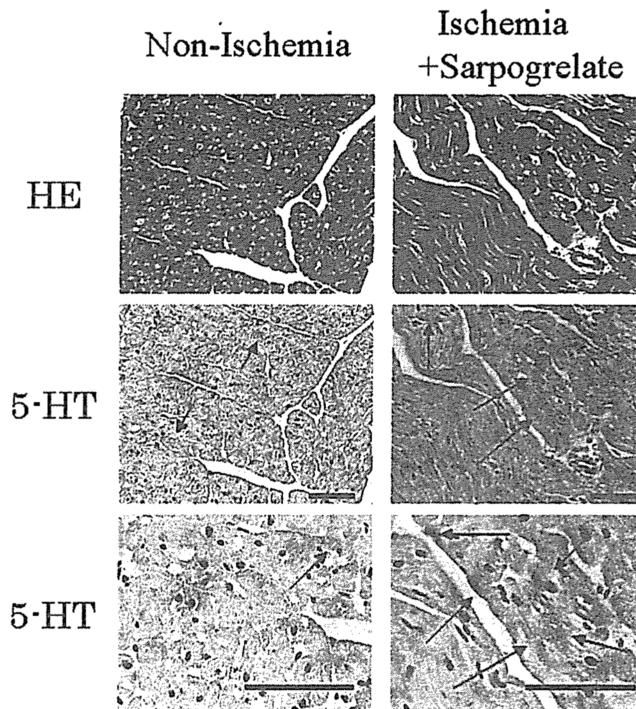


Fig. 5. Immunohistochemical analysis shows staining of serotonin in the non-ischemic myocardium and the ischemic myocardium after 20 min infusion of sarpogrelate. Upper panels show hematoxylin–eosin (HE) staining (×200). Middle (×200) and lower (×400) panels show immunohistochemical staining against 5-HT antibody. Bar indicates 1 μm.

9.2. Mechanisms by which sarpogrelate increased the cardiac release of serotonin in the ischemic myocardium

Vanhoutte [2] reported that serotonin may also enhance NO production via activation of 5-HT1B receptor in the endothelium. Furthermore, there is a report that serotonin evoked NO release in a dose dependent manner in human coronary artery endothelial cells [11]. The resulting production of NO stimulates soluble guanylate cyclase and results in vasodilation [12]. These reports suggest that increased serotonin may increase the cardiac release of NOx via 5-HT1B receptors. Our study confirmed that ischemia induced the cardiac release of serotonin, which was augmented by sarpogrelate. Thus, we suggest that sarpogrelate increased CBF via a NO-dependent pathway through the activation of 5-HT1B receptors induced by enhanced serotonin release. Serotonin is catalyzed by monoamine oxidases (MAO) [13]. A previous report has shown that ischemia decreased MAO activity in an ischemic kidney [14]. Therefore, in ischemic hearts, decreased MAO activity may increase plasma serotonin level by the accumulation of the undegraded serotonin. For the support of this idea, there is a report that the level of serotonin in the coronary effluent was elevated in ischemic isolated rat hearts, suggesting that serotonin was released from the ischemic myocardium [15]. In cardiac tissues, serotonin has been shown to be released from mast cells [16] and ganglia [17]. Although the precise mechanisms by which sarpogrelate augmented an increase in the cardiac release of

serotonin remain unclear, we demonstrated that an infusion of sarpogrelate increased serotonin staining in ischemic cardiomyocytes by immunohistochemistry. This result may partially contribute to an increase in the cardiac release of serotonin in the ischemic myocardium after infusion of sarpogrelate. Importantly, Shimizu et al. [18] showed the contradictory result that interstitial serotonin levels were increased in isolated ischemic rabbit heart that were abrogated by treatment with sarpogrelate. There might be several explanations for this discrepancy. First, we measured the plasma serotonin release in the ischemic canine heart not at the interstitial level as in the infarcted rabbit heart. In our study, we used the dogs with unimpaired endothelium on which serotonin can directly act. Second, as mentioned before, sarpogrelate might have stimulating effects on cardiac myocytes. Although this might be caused by the differences in animal species and models, further investigation is necessary to clarify serotonin released and metabolic mechanisms in the ischemic heart.

10. Clinical implications

In clinical settings, sarpogrelate is a selective 5-HT2A receptor blocker used for patients with arteriosclerotic obliterance because of its vasodilating and antiplatelet action [4]. Furthermore, sarpogrelate has been reported to be protective effects against human angina pectoris through an increase of collateral circulation [19]. We previously reported that an increase of CBF by benidipine attenuated the severity of ischemia gauged by lactate extraction rate and fractional shortening in the hypoperfused canine hearts [20]. Thus, the findings of this study may suggest another cardioprotective effect of sarpogrelate in ischemic heart disease.

11. Study limitation

Since coronary arteries in canine heart are covered with unimpaired endothelium, the beneficial effects of sarpogrelate in patients with arteriosclerosis may differ from our results. Further investigation will be needed to clarify these issues.

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β-Adrenoceptor Blocker Carvedilol Provides Cardioprotection via an Adenosine-Dependent Mechanism in Ischemic Canine Hearts

Hiroshi Asanuma, MD, PhD; Tetsuo Minamino, MD, PhD; Shoji Sanada, MD, PhD; Seiji Takashima, MD, PhD; Hisakazu Ogita, MD, PhD; Akiko Ogai, BS; Masanori Asakura, MD, PhD; Yulin Liao, MD; Yoshihiro Asano, MD, PhD; Yasunori Shintani, MD; Jiyoong Kim, MD; Yoshiro Shinozaki, BS; Hidezo Mori, MD, PhD; Koichi Node, MD, PhD; Soichiro Kitamura, MD, PhD; Hitonobu Tomoike, MD, PhD; Masatsugu Hori, MD, PhD; Masafumi Kitakaze, MD, PhD

Background—Carvedilol is a β-adrenoceptor blocker with a vasodilatory action that is more effective for the treatment of congestive heart failure than other β-blockers. Recently, carvedilol has been reported to reduce oxidative stress, which may consequently reduce the deactivation of adenosine-producing enzymes and increase cardiac adenosine levels. Therefore, carvedilol may also have a protective effect on ischemia and reperfusion injury, because adenosine mediates cardioprotection in ischemic hearts.

Methods and Results—In anesthetized dogs, the left anterior descending coronary artery was occluded for 90 minutes, followed by reperfusion for 6 hours. Carvedilol reduced the infarct size (15.0±2.8% versus 40.9±4.2% in controls), and this effect was completely reversed by the nonselective adenosine receptor antagonist 8-sulfophenyltheophylline (45.2±5.4%) or by an inhibitor of ecto-5′-nucleotidase (44.4±3.6%). There were no differences of either area at risk or collateral flow among the various groups. When the coronary perfusion pressure was reduced in other dogs so that coronary blood flow was decreased to 50% of the nonischemic level, carvedilol increased coronary blood flow (49.4±5.6 to 73.5±7.5 mL · 100 g^{-1} · min^{-1}; $P<0.05$) and adenosine release (112.3±22.2 to 240.6±57.1 nmol/L; $P<0.05$) during coronary hypoperfusion. This increase of coronary blood flow was attenuated by either 8-sulfophenyltheophylline or superoxide dismutase. In human umbilical vein endothelial cells cultured with or without xanthine and xanthine oxidase, carvedilol caused an increase of ecto-5′-nucleotidase activity.

Conclusions—Carvedilol shows a cardioprotective effect against ischemia and/or reperfusion injury via adenosine-dependent mechanisms. (***Circulation*. 2004;109:2773-2779.**)

Key Words: adenosine ■ stress ■ ischemia ■ reperfusion ■ infarction

Beta-adrenoceptor antagonists (β-blockers) are used for the treatment of ischemic heart disease because these drugs reduce adrenergic activity.[1,2] Carvedilol is a β-blocker that has shown efficacy for chronic heart failure in several large-scale trials.[3,4] Carvedilol decreases vascular resistance[3] and improves the pathophysiology of chronic heart failure.[5] This drug dilates both systemic and coronary vessels,[6] which is not a typical characteristic of β-blockers. Although this vasodilatory action may contribute to the beneficial effects of carvedilol in ischemic or nonischemic heart failure,[5] it may not be the primary mechanism of cardioprotection, because vasodilators are not always effective at protecting the heart.[7] Interestingly, carvedilol can also reduce oxidative stress,[8] which causes cellular damage through inactivation of membrane enzymes, pumps, and proteins, such as Na^+/K^+-ATPase,[9] Ca^{2+} channels,[10] and ecto-5′-nucleotidase.[11] Ecto-5′-nucleotidase is the enzyme that produces adenosine, and adenosine is believed to ameliorate chronic heart failure or myocardial ischemia.[12]

To investigate the relationship between the cardioprotective effect of carvedilol and the reduction of oxidative stress on the enhancement of adenosine release, we examined whether carvedilol could reduce infarct size via adenosine- or ecto-5′-nucleotidase–dependent mechanisms in canine hearts. We also investigated whether carvedilol could increase coronary blood flow (CBF) via attenuation of oxidative stress and enhancement of adenosine release in ischemic canine hearts.

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From the Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita (H.A., T.M., S.S., S.T., H.O., M.A., Y.L., Y.A., Y. Shintani, K.N., M.H.); the Cardiovascular Division, National Cardiovascular Center, Suita (A.O., J.K., H.M., S.K., H.T., M.K.); and the Physiology Department, Tokai University School of Medicine, Isehara (Y. Shinozaki), Japan.

Correspondence to Masafumi Kitakaze, MD, Cardiovascular Division, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita City, Osaka Pref. 565-8565, Japan. E-mail kitakaze@zf6.so-net.ne.jp

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