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; Jiyong 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 in an 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 and are widely used clinically to decrease serum cholesterol levels. Recent studies have focused on the pleiotropic effects of either hydrophilic or hydrophobic statins, which are independent of their cholesterol-lowering effect. Protection against ischemia-reperfusion injury is one of them, which is particularly evident after 12 hours. In addition, some studies showed that statins activate the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway within 1 hour, as well as activating endothelial nitric oxide synthase (eNOS), to cause immediate infarct limitation.

On the other hand, other studies revealed that statins also acutely activate ecto-5’-nucleotidase, which produces the endogenous cardioprotective substance adenosine, especially in response to certain stresses. Ecto-5’-nucleotidase can act only when localized on the cell membrane, and the density of this enzyme on the membrane regulates its activity. Endocytotic turnover of ecto-5’-nucleotidase is inhibited by PI3-K activation, which subsequently increases total 5’-nucleotidase activity within a period as short as 10 minutes. Therefore, we hypothesized that an increase of ecto-5’-nucleotidase activity might be critical for early cardioprotec-
nary administration of a selective ecto-5'-nucleotidase inhibitor during 90 minutes of ischemia as described previously. In all experiments, the average baseline values of mean aortic blood pressure (ABP), heart rate (HR), and arterial blood PO₂ 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 PI3-K inhibitor (wortmannin 1.5 μg · kg⁻¹ · min⁻¹); a nonselective adenosine receptor antagonist (8-SPT; 50 μg · kg⁻¹ · min⁻¹); or a selective PI3-K inhibitor (wortmannin 1.5 μg · kg⁻¹ · 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.

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. In all experiments, the average baseline values of mean aortic blood pressure (ABP), heart rate (HR), and arterial blood PO₂ 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.

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 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 as 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 · 100 g⁻¹ · 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...
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 ± 4.9, 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 0.5 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 ± 4.5, 42.1 ± 3.9, or 40.4 ± 4.0%, 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-
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, 80±37% for pitavastatin, and 83±35% 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 or of simvastatin for PI3-K activation. 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 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, 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, other mechanisms may also be involved, such as endocytotic turnover. 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. 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 and we obtained another preliminary finding that the cotreatment with Nω-nitro-L-arginine methyl ester (10 μg · kg⁻¹ · min⁻¹) in the same manner as in protocol 1, which we confirmed did not affect baseline infarct size in the present model, 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: NO inhibits cell-to-cell adhesion,
such as that between platelets or between neutrophils and endothelial cells, by reducing expression of P-selectin, which leads to attenuation of the inflammatory response and protects against ischemia-reperfusion injury. In addition, NO is reported to inhibit caspase-3 activity and to block apoptosis of cardiac myocytes. On the other hand, adenosine also rescues injured myocardium through activating adenosine receptors.

Second, recent articles have shown that either adenosine or NO can reactivate PT3-K downstream. However, increasing the production of both agents is known to negatively regulate further increases of production of these molecules, 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.

Acknowledgments
This study was supported by grants on the Human Genome, Tissue Engineering and Food Biotechnology (H13-Genome-11) and grants on Comprehensive Research on Aging and Health (H13-21seikatsu-23) in Health and Labor Sciences Research from the Ministry of Health, Labor and Welfare; a grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and in part by a grant-in-aid for JSPS fellows from the Japan Society for the Promotion of Science and the Japan Heart Foundation.

References


Optimal Windows of Statin Use for Immediate Infarct Limitation: 5′-Nucleotidase as Another Downstream Molecule of Phosphatidylinositol 3-Kinase

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

_Circulation_. 2004;110:2143-2149; originally published online September 27, 2004;
doi: 10.1161/01.CIR.0000143830.59419.73
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/15/2143

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/