

# Simvastatin Restores Ischemic Preconditioning in the Presence of Hyperglycemia through a Nitric Oxide-mediated Mechanism

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**Background:** A growing body of evidence indicates that statins decrease perioperative cardiovascular risk and that these drugs may be particularly efficacious in diabetes. Diabetes and hyperglycemia abolish the cardioprotective effects of ischemic preconditioning (IPC). The authors tested the hypothesis that simvastatin restores the beneficial effects of IPC during hyperglycemia through a nitric oxide-mediated mechanism.

**Methods:** Myocardial infarct size was measured in dogs (n = 76) subjected to coronary artery occlusion and reperfusion in the presence or absence of hyperglycemia (300 mg/dl) with or without IPC in separate groups. Additional dogs received simvastatin (20 mg orally daily for 3 days) in the presence or absence of IPC and hyperglycemia. Other dogs were pretreated with *N*-nitro-L-arginine methyl ester (30 mg intracoronary) with or without IPC, hyperglycemia, and simvastatin.

**Results:** Ischemic preconditioning significantly ( $P < 0.05$ ) reduced infarct size (n = 7,  $7 \pm 2\%$ ) as compared with control (n = 7,  $29 \pm 3\%$ ). Hyperglycemia (n = 7), simvastatin (n = 7), *N*-nitro-L-arginine methyl ester alone (n = 7), and simvastatin with hyperglycemia (n = 6) did not alter infarct size. Hyperglycemia (n = 7,  $24 \pm 2\%$ ), but not *N*-nitro-L-arginine methyl ester (n = 5,  $10 \pm 1\%$ ), blocked the protective effects of IPC. Simvastatin restored the protective effects of IPC in the presence of hyperglycemia (n = 7,  $14 \pm 1\%$ ), and this beneficial action was blocked by *N*-nitro-L-arginine methyl ester (n = 7,  $29 \pm 4\%$ ).

**Conclusions:** The results indicate that simvastatin restored the cardioprotective effects of IPC during hyperglycemia by nitric oxide-mediated signaling. The results also suggest that enhanced cardioprotective signaling could be a mechanism for statin-induced decreases in perioperative cardiovascular risk.

THE enzyme 3-hydroxyl-3-methylglutaryl coenzyme A reductase catalyzes the rate-limiting step in cholesterol synthesis. Inhibitors of this enzyme are collectively called *statins* and prevent the conversion of 3-hydroxyl-3-methylglutaryl coenzyme A to mevalonic acid. Statins are commonly prescribed to reduce low-density lipoprotein cholesterol, a major risk factor for coronary artery disease. The Scandinavian Simvastatin Survival Study demonstrated marked reductions in low-density lipoprotein cholesterol and overall cardiovascular mortality in patients with hypercholesterolemia treated with this statin.<sup>1</sup> More recent clinical trials confirmed these important results and further indicated that statins also protect against atherosclerotic disease in patients with normal cholesterol levels.<sup>2</sup> Notably, statins seem to confer relatively greater cardiovascular benefits in diabetic patients as compared with those without the disease.<sup>3</sup> The results of these and other studies have stimulated an intense interest in mechanisms responsible for the cardioprotective effects of statins that may occur independent of reductions in low-density lipoprotein cholesterol. Increased availability of nitric oxide, preservation of endothelial function, and antithrombotic and antiinflammatory effects are key mechanisms that may contribute to statin-induced protection.<sup>4</sup>

Previous investigations demonstrated that diabetes and hyperglycemia impair nitric oxide availability and abolish the cardioprotective effects of ischemic preconditioning (IPC),<sup>5,6</sup> volatile anesthetic-induced preconditioning,<sup>7,8</sup> and activation of mitochondrial adenosine triphosphate-regulated potassium ( $K_{ATP}$ ) channels.<sup>9</sup> Whether endogenous cardioprotective signal transduction known to be disrupted by diabetes and hyperglycemia may be restored by pharmacologic therapy is unknown. Therefore, we tested the hypothesis that the 3-hydroxyl-3-methylglutaryl coenzyme A inhibitor simvastatin restores the beneficial effects of IPC during hyperglycemia through a nitric oxide-mediated mechanism.

## Materials and Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin. Furthermore, all conformed to the Guiding Principles in the

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

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Received from the Departments of Anesthesiology, Pharmacology and Toxicology, and Medicine (Division of Cardiovascular Diseases), the Medical College of Wisconsin, and the Clement T. Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin; and the Department of Biomedical Engineering, Marquette University, Milwaukee, Wisconsin. Submitted for publication February 8, 2007. Accepted for publication November 20, 2007. Supported in part by National Institutes of Health grant Nos. HL 063705 (to Dr. Kersten), GM 066730 (to Dr. Warltier), and HL 054820 (to Dr. Warltier) from the US Public Health Service, Bethesda, Maryland.

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Care and Use of Animals<sup>10</sup> of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.<sup>11</sup>

#### General Preparation

Implantation of instruments has been previously described in detail.<sup>12</sup> Briefly, mongrel dogs of either sex were anesthetized with sodium barbital (200 mg/kg) and sodium pentobarbital (15 mg/kg) and ventilated using positive pressure with an air-and-oxygen mixture after tracheal intubation. Arterial blood gases were maintained within a physiologic range by adjustment of tidal volume and respiratory rate. Temperature was maintained with a heating blanket. A 7-French, dual micromanometer-tipped catheter was inserted into the aorta and left ventricle for measurement of aortic and left ventricular (LV) pressures and the maximum rate of increase of LV pressure ( $+dP/dt_{max}$ ). Heparin-filled catheters were inserted into the left atrial appendage and the right femoral artery for administration of radioactive microspheres and withdrawal of reference blood flow samples, respectively. A catheter was also inserted in the right femoral vein for saline administration. A 1-cm segment of the left anterior descending coronary artery (LAD) was isolated immediately distal to the first diagonal branch, and a silk ligature was placed around the vessel for production of coronary artery occlusion and reperfusion. Hemodynamics were continuously monitored on a polygraph during experimentation and digitized using a computer interfaced with an analog-to-digital converter.

#### Experimental Protocol

Baseline systemic hemodynamics were recorded 90 min after instrumentation was completed and calibrated. All dogs were subjected to a 60-min LAD occlusion followed by 3 h of reperfusion (fig. 1). In four experimental groups, dogs were randomly assigned to receive 0.9% saline or 15% dextrose in water to increase blood glucose concentrations to 300 mg/dl with or without IPC (four 5-min LAD occlusions interspersed with 5-min reperfusion conducted immediately before prolonged LAD occlusion). Additional groups of dogs pretreated with oral administration of simvastatin for 3 days (20 mg daily; 24 and 48 h before and on the day of experimentation) were studied in the presence or absence of IPC and hyperglycemia. This dose and duration of simvastatin exposure was based on evidence in the literature that short-term statin use increases endothelial nitric oxide synthase expression.<sup>13</sup> Three final groups of dogs received the nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) with or without IPC or the combination of IPC, hyperglycemia, and simvastatin. L-NAME (30 mg)<sup>14</sup> was infused intracoronary (0.1 ml/min *via* a small catheter [PE-20]) over 10 min immediately before brief IPC stimuli. Regional myocardial blood flow was measured under baseline conditions, during LAD

occlusion, and after 1 h of reperfusion. Dogs were excluded from the analysis if (1) intractable ventricular fibrillation occurred or (2) subendocardial coronary collateral blood flow exceeded  $0.15 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ .<sup>15</sup>

#### Measurement of Myocardial Infarct Size

At the end of each experiment, myocardial infarct size was measured as previously described in detail.<sup>5,16</sup> Briefly, the LV area at risk for infarction (AAR) was separated from the normal area, and the two regions were incubated at 37°C for 20–30 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH 7.4. After overnight storage in 10% formaldehyde, infarcted and noninfarcted myocardium within the AAR were carefully separated and weighed. Infarct size was expressed as a percentage of the AAR.

#### Determination of Regional Myocardial Blood Flow

Carbonized plastic microspheres ( $15 \pm 2 \mu\text{m}$  [SD] in diameter) labeled with <sup>141</sup>Ce, <sup>103</sup>Ru, or <sup>95</sup>Nb were used to measure regional myocardial perfusion as previously described.<sup>17</sup> Transmural tissue samples were selected from the ischemic region (distal to the LAD occlusion) and were subdivided into subepicardial, midmyocardial, and subendocardial layers of approximately equal thickness. Samples were weighed and placed in scintillation vials, and the activity of each isotope was determined. Similarly, the activity of each isotope in the reference blood flow sample was assessed. Tissue blood flow ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ ) was calculated as  $Q_r \times C_m/C_r$ , where  $Q_r$  = rate of withdrawal of the reference blood flow sample (ml/min),  $C_m$  = activity (cpm/g) of the myocardial tissue sample, and  $C_r$  = activity (cpm) of the reference blood flow sample. Transmural blood flow was considered as the average of subepicardial, midmyocardial, and subendocardial blood flows.

#### Statistical Analysis

Statistical analysis of data within and between groups was performed with analysis of variance for repeated measures followed by the Student-Newman-Keuls test. All data are expressed as mean  $\pm$  SEM. Each hemodynamic measurement in table 1 (heart rate, mean aortic blood pressure, LV systolic pressure, LV end-diastolic pressure, maximal rate of increase of LV pressure), blood glucose, and transmural myocardial perfusion in the ischemic region were analyzed separately by repeated-measures analysis of variance. First, an overall factorial model was fitted, and the presence of any group, time, or group  $\times$  time interaction effect was evaluated; the result of this analysis is presented with each table. The Huynh-Feldt correction for sphericity was used when testing the within animal effects. Whenever statistically significant effects ( $P < 0.05$ ) were found, we proceeded with more detailed *post hoc* comparisons. Whenever significant interactions were found, each experimental group and each time point were analyzed separately. The

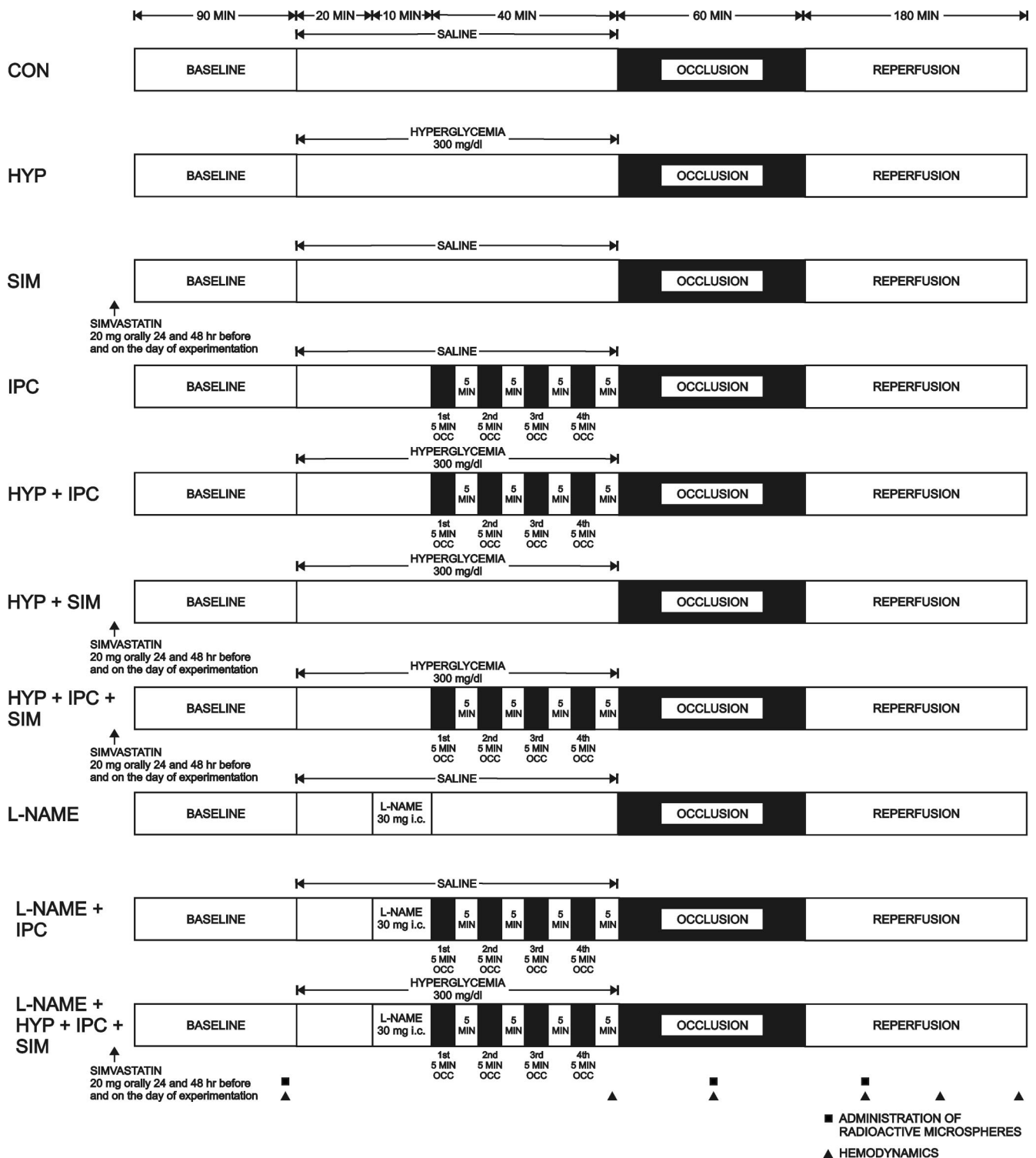


Fig. 1. Schematic diagram illustrating the experimental protocol. CON = control; HYP = hyperglycemia; IPC = ischemic preconditioning; L-NAME = *N*-nitro-L-arginine methyl ester; OCC = occlusion; SIM = simvastatin.

type I error rate was controlled at 0.05 within each group and each time point by the use of the Student-Newman-Keuls test. Although this application of the Student-Newman-Keuls test does not guarantee overall control of the type I error rate by itself, these tests were protected by the significant result of the global test.

## Results

Seventy-six dogs were instrumented to obtain 67 successful experiments. Four dogs were excluded because of intractable ventricular fibrillation (1 in the simvastatin alone group, 1 in the L-NAME alone group, and 2 in

Table 1. Systemic Hemodynamics

	Baseline	Preocclusion	30 min CAO	Reperfusion		
				1 h	2 h	3 h
HR, beats/min						
Control	135 ± 6	131 ± 7	133 ± 6	122 ± 5	125 ± 6	124 ± 4
HYP	137 ± 5	147 ± 4	131 ± 4	128 ± 7	129 ± 7	133 ± 6
SIM	143 ± 4	134 ± 4*	135 ± 4*	122 ± 3*	122 ± 2*	121 ± 2*
IPC	133 ± 4	128 ± 5	128 ± 6	122 ± 4	119 ± 6*	118 ± 7*
HYP + IPC	135 ± 6	131 ± 6	138 ± 5	135 ± 4	134 ± 6	135 ± 6
HYP + SIM	137 ± 6	136 ± 6	120 ± 10	133 ± 6	133 ± 7	134 ± 8
HYP + IPC + SIM	138 ± 7	138 ± 3	138 ± 3	125 ± 4*	128 ± 5	128 ± 6
L-NAME	135 ± 9	117 ± 10*	114 ± 8*	112 ± 6*	115 ± 9*	115 ± 10*
L-NAME + IPC	124 ± 4	110 ± 7	115 ± 8	110 ± 6	110 ± 3	105 ± 6*
L-NAME + HYP + IPC + SIM	128 ± 5	116 ± 4*	118 ± 5*	112 ± 4*	111 ± 4*	112 ± 4*
MAP, mmHg						
Control	99 ± 4	100 ± 3	91 ± 5	97 ± 5	104 ± 6	102 ± 5
HYP	100 ± 4	122 ± 12*	93 ± 4	100 ± 5	103 ± 4	98 ± 5
SIM	113 ± 3	110 ± 1	100 ± 5*	98 ± 5*	107 ± 5	105 ± 5
IPC	91 ± 3	91 ± 4	87 ± 4	97 ± 5	101 ± 5	96 ± 3
HYP + IPC	106 ± 7	111 ± 4	100 ± 5	117 ± 2	115 ± 4	115 ± 1
HYP + SIM	108 ± 4	108 ± 4	77 ± 7*	82 ± 3*	88 ± 5*	88 ± 6*
HYP + IPC + SIM	109 ± 6	102 ± 5	95 ± 5	101 ± 4	104 ± 4	99 ± 6
L-NAME	103 ± 6	122 ± 5*	104 ± 8	105 ± 9	108 ± 9	112 ± 9
L-NAME + IPC	111 ± 8	112 ± 7	102 ± 6	89 ± 4*	103 ± 5	101 ± 9
L-NAME + HYP + IPC + SIM	98 ± 1	105 ± 4	94 ± 3	100 ± 6	104 ± 6	100 ± 4
LVSP, mmHg						
Control	110 ± 5	111 ± 4	97 ± 5	101 ± 6	112 ± 5	109 ± 5
HYP	115 ± 5	137 ± 13*†	100 ± 5	108 ± 4	111 ± 5	104 ± 4
SIM	126 ± 4	126 ± 2	109 ± 5*	105 ± 6*	114 ± 5	112 ± 6*
IPC	100 ± 5	100 ± 5	91 ± 5	102 ± 6	104 ± 5	100 ± 2
HYP + IPC	121 ± 9	125 ± 4	108 ± 6	124 ± 3	122 ± 4	124 ± 3
HYP + SIM	119 ± 4	119 ± 5	88 ± 6*	92 ± 3*	99 ± 5*	98 ± 5*
HYP + IPC + SIM	120 ± 7	112 ± 6	100 ± 7*	105 ± 5	110 ± 3	102 ± 5*
L-NAME	115 ± 6	134 ± 5*	110 ± 8	111 ± 9	114 ± 9	117 ± 9
L-NAME + IPC	120 ± 10	121 ± 8	108 ± 8	92 ± 5*	108 ± 5	106 ± 9
L-NAME + HYP + IPC + SIM	109 ± 2	115 ± 5	100 ± 3	104 ± 6	107 ± 6	103 ± 5
LVEDP, mmHg						
Control	5 ± 1	5 ± 1	15 ± 2*	18 ± 3*	16 ± 3*	16 ± 2*
HYP	5 ± 2	8 ± 2	11 ± 2	11 ± 1	13 ± 2*	10 ± 2
SIM	6 ± 1	6 ± 1	14 ± 1*	19 ± 3*	16 ± 2*	16 ± 2*
IPC	6 ± 1	6 ± 1	9 ± 1	10 ± 2*	11 ± 2*	12 ± 2*
HYP + IPC	7 ± 1	7 ± 1	9 ± 2	10 ± 2	12 ± 3	13 ± 2*
HYP + SIM	10 ± 1	9 ± 1	16 ± 3*	14 ± 2*	13 ± 2	13 ± 2
HYP + IPC + SIM	7 ± 1	9 ± 1	12 ± 2	20 ± 2*	14 ± 3	13 ± 2
L-NAME	9 ± 1	11 ± 2	22 ± 2*†	18 ± 1*	21 ± 4*	20 ± 4*
L-NAME + IPC	6 ± 2	10 ± 2	13 ± 3	16 ± 3*	15 ± 4*	16 ± 3*
L-NAME + HYP + IPC + SIM	8 ± 1	7 ± 1	12 ± 1	14 ± 2*	16 ± 2*	15 ± 2*
LV + dP/dt <sub>max</sub> , mmHg/s						
Control	1,860 ± 200	1,970 ± 230	1,540 ± 140*	1,360 ± 130*	1,350 ± 110*	1,220 ± 50*
HYP	1,870 ± 130	2,340 ± 320*	1,520 ± 90	1,580 ± 120	1,540 ± 70	1,420 ± 70
SIM	2,170 ± 110	2,030 ± 110	1,720 ± 130*	1,450 ± 80*	1,500 ± 80*	1,440 ± 80*
IPC	1,680 ± 130	1,640 ± 150	1,600 ± 140	1,420 ± 150*	1,280 ± 90*	1,190 ± 90*
HYP + IPC	2,090 ± 190	2,210 ± 110	1,950 ± 100	1,680 ± 90*	1,540 ± 100*	1,540 ± 70*
HYP + SIM	1,750 ± 60	1,920 ± 70	1,390 ± 150*	1,430 ± 80*	1,470 ± 90*	1,430 ± 100*
HYP + IPC + SIM	1,970 ± 160	1,930 ± 120	1,770 ± 100*	1,530 ± 90*	1,520 ± 90*	1,460 ± 120*
L-NAME	1,780 ± 130	1,460 ± 110*	1,230 ± 100*	1,190 ± 110*	1,240 ± 100*	1,250 ± 110*
L-NAME + IPC	1,840 ± 300	1,430 ± 110	1,460 ± 110	1,100 ± 30*	1,170 ± 40*	1,100 ± 150*
L-NAME + HYP + IPC + SIM	1,740 ± 110	1,600 ± 110	1,360 ± 100*	1,220 ± 50*	1,160 ± 50*	1,110 ± 40*

Repeated-Measures Analysis of Variance Results for Systemic Hemodynamics

Variable	Group	Time	Group × Time
HR	$F_{9,57} = 2.55, P = 0.0154$	$F_{5,285} = 19.53, P < 0.0001$	$F_{45,285} = 1.64, P = 0.0298$
MAP	$F_{9,57} = 2.38, P = 0.0234$	$F_{5,285} = 13.68, P < 0.0001$	$F_{45,285} = 2.06, P = 0.0007$
LVSP	$F_{9,53} = 2.52, P = 0.0175$	$F_{5,265} = 23.42, P < 0.0001$	$F_{45,265} = 1.69, P = 0.0140$
LVEDP	$F_{9,53} = 2.10, P = 0.0462$	$F_{5,265} = 48.70, P < 0.0001$	$F_{45,265} = 1.69, P = 0.0521$
LV + dP/dt <sub>max</sub>	$F_{9,53} = 3.44, P = 0.0021$	$F_{5,265} = 84.33, P < 0.0001$	$F_{45,265} = 1.63, P = 0.0210$

Data are mean ± SEM.

\* Significantly ( $P < 0.05$ ) different from baseline. † Significantly ( $P < 0.05$ ) different from the respective value in the control group.CAO = coronary artery occlusion; HR = heart rate; HYP = hyperglycemia; IPC = ischemic preconditioning; L-NAME = *N*-nitro-*L*-arginine methyl ester; LV + dP/dt<sub>max</sub> = maximal rate of increase of left ventricular pressure; LVEDP = left ventricular end-diastolic pressure; LVSP = left ventricular systolic pressure; MAP = mean aortic blood pressure; SIM = simvastatin.



**Table 2. Blood Glucose Concentrations (mg/dl)**

	Baseline	Preocclusion	30 min CAO	Reperfusion	
				1 h	3 h
Control	83 ± 6	—	83 ± 6	76 ± 6	72 ± 7
HYP	83 ± 4	307 ± 8*	108 ± 12*	73 ± 7	77 ± 8
SIM	81 ± 6	—	68 ± 4*	60 ± 3*	56 ± 3*
IPC	71 ± 7	—	59 ± 6	67 ± 5	63 ± 8
HYP + IPC	77 ± 5	293 ± 13*	106 ± 25	82 ± 11	76 ± 6
HYP + SIM	93 ± 6	309 ± 47*	160 ± 49	92 ± 11	91 ± 3
HYP + IPC + SIM	75 ± 3	309 ± 13*	77 ± 13	78 ± 10	81 ± 7
L-NAME	88 ± 6	—	72 ± 7	86 ± 15	76 ± 7
L-NAME + IPC	74 ± 7	—	65 ± 7	68 ± 6	63 ± 4
L-NAME + HYP + IPC + SIM	78 ± 3	313 ± 16*	85 ± 10	63 ± 8	74 ± 3

Repeated-Measures Analysis of Variance Results for Blood Glucose

Variable	Group	Time	Group × Time
Blood glucose	$F_{7,38} = 15.86, P < 0.0001$	$F_{4,152} = 161.0, P < 0.0001$	$F_{28,152} = 14.89, P < 0.0001$
Blood glucose without preocclusion	$F_{9,46} = 2.66, P = 0.0143$	$F_{3,138} = 3.04, P = 0.0645$	$F_{27,138} = 0.94, P = 0.5202$

Data are mean ± SEM.

\* Significantly ( $P < 0.05$ ) different from baseline.

CAO = coronary artery occlusion; HYP = hyperglycemia; IPC = ischemic preconditioning; L-NAME = *N*-nitro-L-arginine methyl ester; SIM = simvastatin.

the L-NAME + IPC group). Nine dogs were excluded because subendocardial collateral blood flow exceeded  $0.15 \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  (1 in the control group, 1 in the hyperglycemia alone group, 1 in the hyperglycemia + IPC group, 3 in the simvastatin alone group, and 3 in the L-NAME + IPC group).

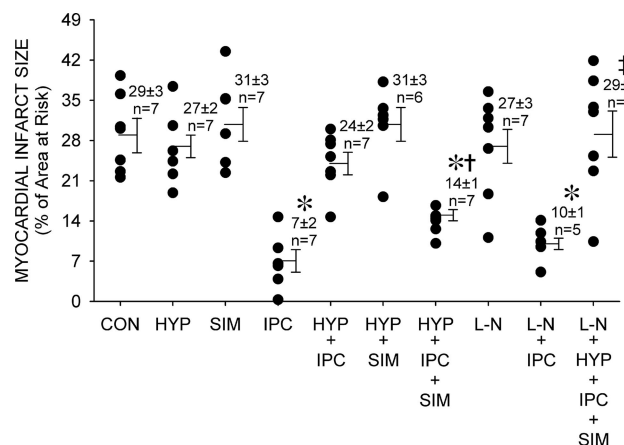
#### Hemodynamics and Blood Glucose Concentrations

There were no differences in baseline hemodynamics among experimental groups (table 1). Hyperglycemia increased mean arterial and LV systolic pressures and LV  $\text{dP/dt}_{\text{max}}$  before coronary occlusion. L-NAME caused transient increases in mean arterial and LV systolic pressures and decreases in heart rate and LV  $\text{dP/dt}_{\text{max}}$ . LV end-diastolic pressure increased to a greater extent during coronary occlusion in dogs pretreated with L-NAME as compared with 0.9% saline. There were no other significant differences in hemodynamics among groups during coronary artery occlusion and reperfusion. There were no differences in baseline blood glucose concentrations among groups. Blood glucose concentrations (table 2) were unchanged over time in dogs receiving 0.9% saline. Infusion of dextrose increased blood glucose concentration before coronary occlusion. Blood glucose concentrations returned to baseline values in hyperglycemic dogs during coronary occlusion and reperfusion. Blood glucose concentrations were modestly decreased during LAD occlusion and reperfusion in simvastatin-pretreated dogs in the absence of hyperglycemia.

#### Myocardial Infarct Size and Coronary Collateral Blood Flow

No differences in LV AAR were observed between groups (control:  $n = 7, 41 \pm 1\%$ ; hyperglycemia alone:  $n =$

7,  $36 \pm 2\%$ ; simvastatin alone:  $n = 7, 36 \pm 2\%$ ; IPC alone:  $n = 7, 43 \pm 3\%$ ; hyperglycemia + IPC:  $n = 7, 33 \pm 3\%$ ; hyperglycemia + simvastatin:  $n = 6, 36 \pm 2\%$ ; hyperglycemia + IPC + simvastatin:  $n = 7, 39 \pm 2\%$ ; L-NAME alone:  $n = 7, 37 \pm 3\%$ ; L-NAME + IPC:  $n = 5, 35 \pm 4\%$ ; hyperglycemia + IPC + simvastatin + L-NAME:  $n = 7, 37 \pm 2\%$ ). IPC significantly ( $P < 0.05$ ) reduced infarct size ( $7 \pm 2\%$  of the LV AAR) as compared with control ( $29 \pm 3\%$ ; fig. 2). Hyperglycemia, simvastatin, L-NAME alone, and hyperglycemia with simvastatin did not alter infarct size ( $27 \pm 2, 31 \pm 3, 27 \pm 3, \text{ and } 31 \pm 3\%$ , respectively). Hyperglycemia ( $24 \pm 2\%$ ), but not L-NAME ( $10 \pm 1\%$ ), blocked the protective effects of IPC. Simvastatin re-



**Fig. 2.** Myocardial infarct size expressed as a percentage of the left ventricular area at risk during control (CON) conditions, hyperglycemia (HYP), and simvastatin pretreatment (SIM) in the presence or absence of ischemic preconditioning (IPC) with or without *N*-nitro-L-arginine methyl ester (L-N). Data are mean ± SEM. \* Significantly ( $P < 0.05$ ) different from CON. † Significantly ( $P < 0.05$ ) different from HYP + IPC. ‡ Significantly ( $P < 0.05$ ) different from HYP + IPC + SIM.

**Table 3. Transmural Myocardial Perfusion in the Ischemic (LAD) Region ( $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ )**

	Baseline	30 min CAO	1 h Reperfusion
Control	1.23 ± 0.17	0.08 ± 0.02*	1.79 ± 0.20*
HYP	0.75 ± 0.12	0.09 ± 0.02*	1.92 ± 0.24*
SIM	1.36 ± 0.10	0.04 ± 0.01*	1.68 ± 0.10*
IPC	0.80 ± 0.04	0.09 ± 0.01*	1.62 ± 0.29*
HYP + IPC	1.16 ± 0.14	0.08 ± 0.02*	1.84 ± 0.17*
HYP + SIM	0.96 ± 0.11	0.10 ± 0.03*	1.54 ± 0.27*
HYP + IPC + SIM	1.46 ± 0.26	0.05 ± 0.02*	1.64 ± 0.12
L-NAME	0.73 ± 0.10	0.05 ± 0.01*	1.45 ± 0.43*
L-NAME + IPC	0.76 ± 0.09	0.06 ± 0.02*	1.14 ± 0.24
L-NAME + HYP + IPC + SIM	0.92 ± 0.07	0.05 ± 0.01*	1.25 ± 0.27

Repeated-Measures Analysis of Variance Results for Transmural Myocardial Perfusion

Variable	Group	Time	Group × Time
Myocardial perfusion	$F_{9,57} = 1.80, P = 0.0878$	$F_{2,114} = 245.36, P < 0.0001$	$F_{18,114} = 1.57, P = 0.0936$

Data are mean ± SEM.

\* Significantly ( $P < 0.05$ ) different from baseline.

CAO = coronary artery occlusion; HYP = hyperglycemia; IPC = ischemic preconditioning; LAD = left anterior descending coronary artery; L-NAME = *N*-nitro-L-arginine methyl ester; SIM = simvastatin.

stored the protective effect of IPC in the presence of hyperglycemia ( $14 \pm 1\%$ ), and this beneficial effect was blocked by L-NAME ( $29 \pm 4\%$ ). Transmural myocardial blood flow was similar in each experimental group during baseline conditions (table 3). LAD occlusion produced similar reductions in transmural myocardial perfusion. There were no differences in coronary collateral blood flow among groups.

## Discussion

Many of the adverse consequences of diabetes and hyperglycemia are thought to result from the combination of reduced nitric oxide activity and increased generation of reactive oxygen species.<sup>18</sup> However, the potentially adverse interactions between hyperglycemia, nitric oxide, and myocardial signal transduction responsible for protection against ischemic injury have not been fully defined. Ischemic preconditioning is a powerful endogenous mechanism that protects myocardium against infarction.<sup>19</sup> Many diverse preconditioning stimuli are known to elicit protection of ischemic myocardium by activating common signal transduction pathways including membrane bound receptors, reactive oxygen species, intracellular kinases, regulatory G proteins, and mitochondrial  $K_{ATP}$  channels.<sup>20</sup> Nitric oxide derived from endothelial nitric oxide synthase has also been identified as a critical trigger of cardioprotection. Prolonged ischemia and reperfusion was associated with the loss of cardiac endothelial nitric oxide synthase protein, but IPC completely prevented this endothelial nitric oxide synthase deficit concomitant with enhanced nitric oxide synthase activity and intracellular cyclic guanosine 3',5'-monophosphate concentration.<sup>21</sup> Re-

covery of contractile function was enhanced and cyclic guanosine 3',5'-monophosphate concentration was increased by IPC in isolated rat hearts, and these actions were attenuated by nitric oxide synthase inhibitors or the guanylate cyclase inhibitor 1H-<sup>1,2,4</sup>oxadiazolol-[4,3-a]quinoxaline-1-one.<sup>22</sup> Preconditioning with simulated ischemia also attenuated death of isolated cardiomyocytes, and this effect was blocked by the nitric oxide synthase inhibitor *N*<sup>G</sup>-monomethyl-L-arginine monoacetate.<sup>23</sup> The nitric oxide donor *S*-nitroso-*N*-acetyl-L,L-penicillamine mimicked preconditioning. This protection was also antagonized by 1H-<sup>1,2,4</sup>oxadiazolol-[4,3-a]quinoxaline-1-one.<sup>23</sup>

Genetic models have demonstrated a role for endothelial nitric oxide synthase and nitric oxide during early IPC. Myocardial ischemia and reperfusion injury was attenuated in mice with myocyte-specific overexpression of endothelial nitric oxide synthase.<sup>24</sup> In addition, infarct size was reduced in transgenic mice overexpressing either bovine or human endothelial nitric oxide synthase.<sup>25</sup> These data are supported by findings indicating marked increases in infarct size in endothelial nitric oxide synthase knockout mice.<sup>26</sup> Additional intriguing results implicating an important role for endothelial nitric oxide synthase in cardioprotection were reported by Bell and Yellon,<sup>27</sup> who demonstrated that endothelial nitric oxide synthase-derived nitric oxide reduced the threshold of preconditioning in endothelial nitric oxide synthase knockout mice. Four cycles of brief ischemia and reperfusion were required to produce IPC and decrease infarct size, but a less intense preconditioning stimulus did not protect the heart against infarction in this genetic deletion model.<sup>27</sup> Therefore, the contribution of nitric oxide to cardioprotection may be especially

critical when the intensity of the IPC stimulus borders on the threshold.

Investigations from our laboratory have demonstrated that experimentally induced diabetes and exogenous hyperglycemia block reductions in infarct size after prolonged coronary occlusion and reperfusion in response to ischemia,<sup>5,6</sup> volatile anesthetics,<sup>7,8</sup> and the selective mitochondrial  $K_{ATP}$  channel agonist diazoxide.<sup>9</sup> The current results confirm and extend these previous findings and demonstrate that a moderate degree of hyperglycemia abolishes myocardial protection produced by IPC. The results further indicate that the ability of IPC to reduce infarct size is restored by short-term administration of simvastatin during hyperglycemia. In addition, the current data indicate that these beneficial effects of simvastatin are dependent on nitric oxide synthase activity.

Statins exert beneficial effects independent of blood cholesterol-lowering action,<sup>4,28</sup> in part by increasing nitric oxide bioavailability. Simvastatin up-regulated endothelial nitric oxide synthase expression by posttranscriptional stabilization of endothelial nitric oxide synthase messenger RNA (mRNA) in human endothelial cells in the presence of oxidized low-density lipoprotein.<sup>29</sup> A 29% increase in endothelial nitric oxide synthase protein was observed in coronary microvessels harvested from dogs chemically treated (2 weeks) with oral simvastatin concomitant with enhanced basal and stimulated nitrate and nitrite production.<sup>30</sup> Simvastatin also increased endothelial nitric oxide synthase activity by stimulating endothelial nitric oxide synthase phosphorylation through activation of the prosurvival enzyme Akt (protein kinase).<sup>31</sup> In contrast to endothelial nitric oxide synthase activation by simvastatin, hyperglycemia seems to inhibit endothelial nitric oxide synthase activity by enhancing *N*-acetylglucosamine modification and decreasing phosphorylation of the Akt phosphorylation site (ser<sup>1177</sup>).<sup>32</sup>

Statins have previously been shown to produce cardioprotective effects *in vivo*<sup>13,33</sup> and *in vitro*.<sup>34-36</sup> Simvastatin improved recovery of contractile function and decreased polymorphonucleocyte accumulation in isolated rat hearts.<sup>35</sup> Similar results were observed in intact mice<sup>33</sup> when statins were administered 18 h before ischemia and reperfusion. Perfusion of rat hearts with simvastatin before global ischemia and reperfusion also enhanced LV function; increased endothelial nitric oxide synthase protein, endothelial nitric oxide synthase mRNA, and coronary effluent nitrite concentration; and reduced inducible nitric oxide synthase expression and ultrastructural mitochondrial injury.<sup>36</sup> Moreover, chronic administration (5 days) of simvastatin markedly decreased infarct size and reduced neutrophil accumulation in a murine model of type II diabetes. These salutary actions occurred in the absence of change in serum cholesterol levels.<sup>13</sup>

The current results extend these previous findings and demonstrate for the first time that simvastatin restores IPC-induced cardioprotection during moderate hyperglycemia. This protective effect of simvastatin occurred at a dose that alone did not alter infarct size and was dependent on nitric oxide. Therefore, this beneficial action of simvastatin during hyperglycemia may be attributed to enhancement of nitric oxide signaling elicited by IPC and not to a nonspecific effect. L-NAME also blocked the protection afforded by simvastatin during hyperglycemia, but nitric oxide synthase inhibition alone does not impair IPC during normoglycemia. These latter findings concur with the observations of Bell and Yellon<sup>27</sup> suggesting that endothelial nitric oxide synthase-derived nitric oxide may not be required for IPC if the ischemic stimulus is sufficiently robust. Our findings in addition suggest that nitric oxide may be recruited to play a more important role in IPC when endogenous signaling mechanisms are impaired by hyperglycemia. Our previous data have implicated such a relation between other signaling elements responsible for IPC and the severity of hyperglycemia. For example, the dose of the mitochondrial  $K_{ATP}$  channel agonist diazoxide required to produce pharmacologic preconditioning against infarction was dependent on blood glucose concentrations in dogs.<sup>9</sup> A higher dose of diazoxide was required to elicit protection during severe (600 mg/dl) as compared with moderate (300 mg/dl) hyperglycemia. Notably, nitric oxide modulates ion channel activity and enhances the activity of  $K_{ATP}$  channels.<sup>37</sup> Taken together, these data suggest the hypothesis that nitric oxide may be recruited by statins to counteract a decrease in myocardial  $K_{ATP}$  channel activity during hyperglycemia. Additional investigation will be required to examine this hypothesis. A recent meta-analysis demonstrated that statins markedly reduce the risk of perioperative death in patients undergoing cardiac, noncardiac, and vascular surgery.<sup>38</sup> The relation between perioperative risk reduction by statins and diabetes was not evaluated, however, and represents an important goal of future research.

The current results should be interpreted within the constraints of several potential limitations. Statins reduce plasma low-density lipoprotein concentrations, decrease production of reactive oxygen species, and diminish recruitment of inflammatory cells.<sup>4,28,39,40</sup> We did not specifically measure low-density lipoprotein or total cholesterol in healthy dogs. However, a previous study indicated that blood cholesterol levels were unchanged after 5 days of simvastatin treatment in diabetic mice.<sup>13</sup> Simvastatin, at a fourfold higher dose, induced approximately a 15% reduction in cholesterol in dogs treated with this drug for 15 days.<sup>41</sup> Therefore, it seems unlikely that differences in blood lipid concentrations among groups accounts for the protective effect of simvastatin during hyperglycemia. Blood glucose concentrations were modestly reduced by simvastatin, and a direct re-



lation between infarct size and blood glucose concentration has been previously demonstrated.<sup>6,9</sup> However, simvastatin alone did not affect infarct size as compared with control experiments, and blood glucose concentrations were not reduced by simvastatin after transient hyperglycemia. Therefore, simvastatin-induced decreases in blood glucose concentration most likely do not account for cardioprotection. The dose of simvastatin used in this investigation was based on a comparable dose in humans and on previous reports in the literature.<sup>33,35</sup> We did not examine a dose-response relation to simvastatin, nor did we specifically evaluate the influence of chronic *versus* acute administration of simvastatin on myocardial injury in the presence or absence of hyperglycemia. Hyperglycemia and L-NAME alone produced brief hemodynamic effects that may have theoretically contributed to alterations in infarct size. However, no differences in systemic hemodynamics were observed between groups during coronary artery occlusion or reperfusion, and it is therefore unlikely that hemodynamics alone contributed substantially to the results. The area of the left ventricle at risk for infarction and coronary collateral blood flow are important determinants of the extent of myocardial infarction in dogs, but no differences in these variables were observed among groups that may account for the current findings.

In summary, the current results confirm that hyperglycemia abolishes reductions of myocardial infarct size in response to IPC. The findings further indicate that this deleterious response is abolished by simvastatin in a nitric oxide-dependent fashion. The current results also suggest that nitric oxide may not be required to elicit IPC in healthy dogs, but nitric oxide may instead be recruited by statins to enhance cardioprotection. The current findings lend further support to the hypothesis that statins reduce cardiovascular risk through mechanisms that are independent of reductions in blood cholesterol.

The authors thank David Schwabe, B.S.E.E. (Department of Anesthesiology, Medical College of Wisconsin, Milwaukee, Wisconsin), for technical assistance and Nancy Just, A.A. (Department of Anesthesiology, Medical College of Wisconsin), for assistance in preparation of the manuscript.

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