

Hypercholesterolemia Abrogates Late Preconditioning via a Tetrahydrobiopterin-Dependent Mechanism in Conscious Rabbits

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Background—Although the late phase of ischemic preconditioning (PC) is known to confer cardioprotection in healthy animal models, it is unknown whether this phenomenon exists in the presence of hypercholesterolemia. The goal of this study was to determine whether the infarct-sparing effect of late PC is affected by hypercholesterolemia and, if so, whether a tetrahydrobiopterin (BH₄)-dependent mechanism is responsible for the loss of late PC.

Methods and Results—Conscious rabbits fed a normal diet or a 1% cholesterol diet for 6 weeks were subjected to ischemic PC (six 4-minute coronary occlusion/4-minute reperfusion cycles) and, 24 hours later, underwent a 30-minute occlusion followed by 3 days of reperfusion. A total of 125 rabbits were used. In normocholesterolemic rabbits, ischemic PC reduced infarct size, an effect that was abrogated by administration of the BH₄ synthesis inhibitor *N*-acetylserotonin (15 mg/kg IV) before the 30-minute occlusion. In hypercholesterolemic rabbits, however, ischemic PC failed to reduce infarct size. Myocardial BH₄ levels in the ischemic zone increased 24 hours after ischemic PC in normocholesterolemic rabbits but not in hypercholesterolemic rabbits. In addition, in normocholesterolemic rabbits, pretreatment with *N*-acetylserotonin completely abolished the ischemic PC-induced increase in myocardial BH₄ levels.

Conclusions—This study demonstrates that (1) hypercholesterolemia abrogates both the infarct-sparing effect of late PC and the concomitant upregulation of myocardial BH₄, and (2) inhibition of myocardial BH₄ synthesis in the absence of hypercholesterolemia is sufficient to abolish the infarct-sparing effect of late PC. The results support the concept that hypercholesterolemia abrogates late PC by preventing the upregulation of BH₄, an essential cofactor for inducible nitric oxide synthase. (*Circulation*. 2005;112:2149-2156.)

Key Words: infarction ■ ischemia ■ hypercholesterolemia ■ reperfusion ■ nitric oxide synthase

The late phase of ischemic preconditioning (PC) is the phenomenon whereby brief episodes of ischemia paradoxically increase the tolerance of the heart to subsequent ischemia/reperfusion injury 24 to 72 hours later.¹ The presence of this phenomenon has been well documented in a variety of healthy animal models.²⁻⁵ However, because hypercholesterolemia is one of the most prevalent risk factors for coronary artery disease and may interfere with the biochemical pathways that underlie the PC response,⁶⁻⁸ it is translationally important to determine whether late PC exists in animals with this disorder. To date, the effect of hypercholesterolemia on late PC remains largely unknown. We have recently shown that hypercholesterolemia blunts the antiinfarct effects of nitric oxide (NO) donor-induced late PC in conscious rabbits⁹; however, no study has examined whether hypercholesterolemia interferes with ischemia-induced late PC as well. Because the mechanism of late PC differs depending on the stimulus,¹ data obtained in the setting of

NO donor-induced late PC may not necessarily apply to ischemic PC.

Because late PC is mediated by the inducible isoform of NO synthase (iNOS)³ and because iNOS-dependent NO generation is critically dependent on the availability of tetrahydrobiopterin (BH₄), an essential cofactor for iNOS¹⁰ that has been found to be deficient in the presence of hypercholesterolemia,¹¹ we hypothesized that hypercholesterolemia may abrogate the late phase of ischemic PC by interfering with BH₄ synthesis. Accordingly, the aim of the present study was to determine whether the infarct-sparing effect of late PC is absent in hypercholesterolemic rabbits and, if so, whether the loss of the late PC protection is related to the effect of hypercholesterolemia on myocardial BH₄ upregulation.

Methods

The conscious rabbit model of myocardial ischemia has been described in detail previously.² Briefly, New Zealand White male

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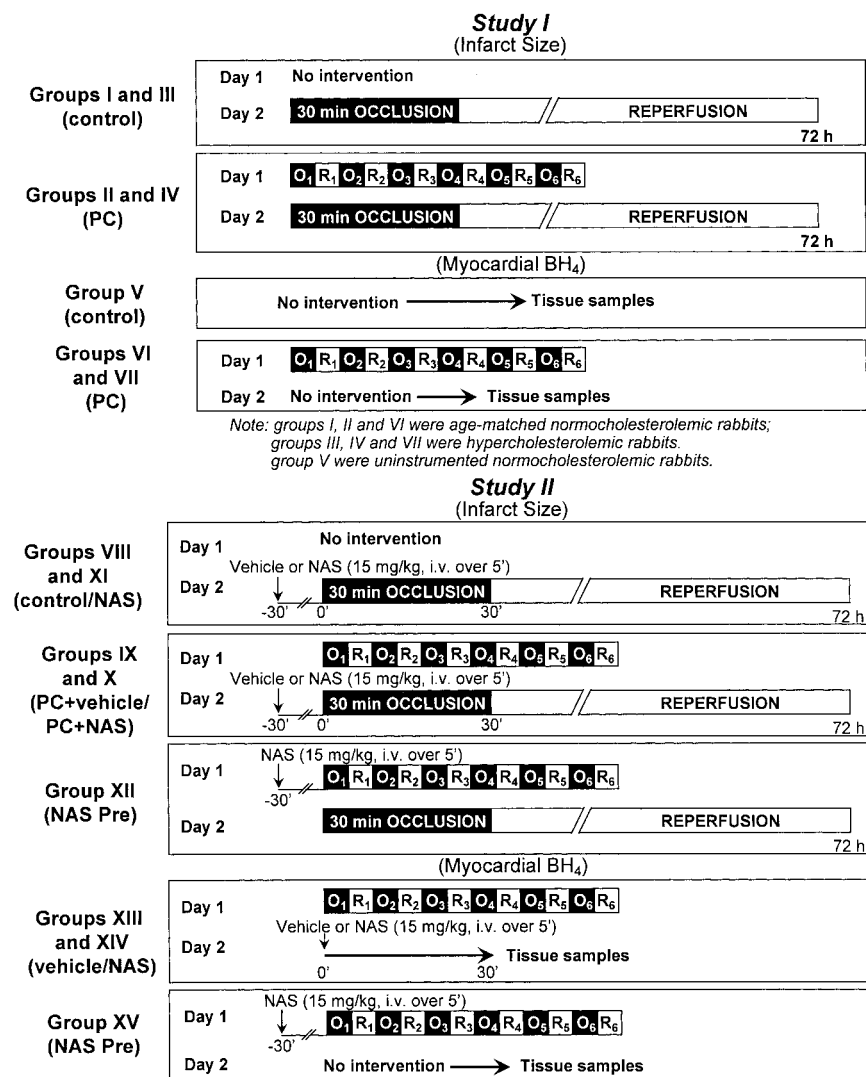


Figure 1. Experimental protocol.

rabbits (weight approximately 2.0 to 2.5 kg; age 3 to 4 months) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery and with bipolar ECG leads. The experiments consisted of 2 consecutive studies (studies I and II; Figure 1).

Study I: Effect of Hypercholesterolemia on Late PC and Myocardial BH₄

Starting on day 3 after surgery, chronically instrumented rabbits were assigned to 2 groups: an age-matched normocholesterolemic group (30 rabbits), which was fed a standard rabbit chow, and a hypercholesterolemic group (30 rabbits), which was fed a diet enriched with 1% cholesterol (Purina Test Diets, Richmond, Ind) for 6 weeks. All rabbits were restricted to 100 g of chow per day and had free access to drinking water. After the animals were euthanized, a segment of descending thoracic aorta was stained with oil red O stain¹² to verify the absence of atherosclerotic lesions.

At the end of the 6-week feeding protocol, rabbits were assigned to 6 groups (Figure 1). Groups I through IV (n=12/group) were subjected to a 30-minute coronary artery occlusion followed by 3 days of reperfusion. Groups I (normocholesterolemic control group) and III (hypercholesterolemic control group) underwent the 30-minute occlusion with no PC, whereas groups II (normocholesterolemic PC group) and IV (hypercholesterolemic PC group) were preconditioned with a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles 24 hours before the 30-minute coronary

occlusion (Figure 1). Groups VI and VII (normocholesterolemic and hypercholesterolemic, respectively; n=6/group) were studied to determine myocardial BH₄ levels 24 hours after PC (Figure 1). An additional group of 3 uninstrumented rabbits (group V) was studied to determine BH₄ levels in naïve myocardium. Tissue samples were harvested from the ischemic/reperfused (anterior wall) and nonischemic zone (posterior wall) of the left ventricle, frozen in liquid nitrogen, and stored at -80°C until use. All coronary occlusion/reperfusion studies were performed with rabbits in the conscious state.

Study II: Role of BH₄ in Late PC

Rabbits were allowed to recover for a minimum of 10 days after surgery and were assigned to 8 groups (Figure 1). To determine whether BH₄ is necessary for late PC, 5 groups of rabbits (VIII through XII; n=10/group) underwent a 30-minute coronary artery occlusion followed by 3 days of reperfusion without (groups VIII and XI) or with (groups IX, X, and XII) an ischemic PC protocol 24 hours earlier (Figure 1). Rabbits received an intravenous bolus of vehicle (groups VIII [control group] and IX [PC+vehicle group]) or the BH₄ synthesis inhibitor *N*-acetylserotonin (NAS; 15 mg/kg over 5 minutes) 30 minutes before the 30-minute coronary occlusion on day 2 (groups X [PC+NAS group] and XI [NAS group]) or 30 minutes before the ischemic PC protocol on day 1 (group XII [NAS Pre group]). To determine whether NAS inhibits PC-induced myocardial BH₄ synthesis, rabbits were preconditioned, and 24 hours

later, tissue samples were harvested 30 minutes after administration of vehicle (group XIII [control group]) or NAS (same dose; group XIV [NAS group]; Figure 1). To determine whether NAS pretreatment affects PC-induced myocardial BH₄ synthesis 24 hours later, an additional group of rabbits (group XV) received NAS (same dose) 30 minutes before the ischemic PC protocol on day 1, and tissue samples were harvested 24 hours after PC (Figure 1). NAS (Sigma Chemical Co) was dissolved in normal saline (total volume infused=15 mL), and the pH was brought up to ≈ 7.50 with 0.1 N NaOH. All solutions were filtered through a 0.2- μ m Millipore filter to ensure sterility.

Postmortem Analysis of Myocardial Infarct Size

At the conclusion of the study, the occluded/reperfused vascular bed and the infarct were identified by postmortem perfusion of the heart with triphenyltetrazolium and Phthalo blue dye, as described previously.⁹ Infarct size was calculated by computerized videoplanimetry.⁹

Measurement of Myocardial BH₄

Myocardial BH₄ content was determined by reverse-phase HPLC, as described previously.¹³

Statistical Analysis

Data are reported as mean \pm SEM. Measurements were analyzed with a 1-way or 2-way repeated-measures ANOVA, as appropriate, followed by paired or unpaired Student *t* tests with the Bonferroni correction. The relationship between infarct size and risk region size was compared among groups with an ANCOVA that used the size of the risk region as the covariate.⁹

Results

Exclusions

A total of 125 conscious rabbits were used. Seventeen of the 60 rabbits used for study I were excluded: 7 (2 each in groups I, II, and III and 1 in group IV) died of ventricular fibrillation during the 30-minute coronary occlusion, and 10 (3 in group III, 2 each in groups I, II, and IV and 1 in group VII) were excluded because of malfunction of the balloon occluder. Therefore, 9 rabbits in group IV, 8 each in groups I and II, 7 in group III, all 6 in group VI, and 5 in group VII completed the experimental protocol.

Eleven of the 65 rabbits used for study II were excluded: 8 (2 each in groups VIII, XI, and XII and 1 each in groups IX and X) died of ventricular fibrillation during the 30-minute coronary occlusion, and 3 (1 each in groups IX, X, and XI) were excluded because of malfunction of the balloon occluder. Therefore, 8 rabbits in groups VIII, IX, X, and XII, 7 in group XI, and all 5 in groups XIII, XIV, and XV completed the experimental protocol.

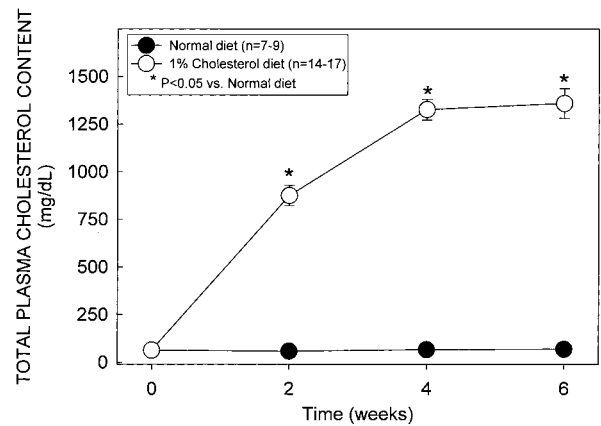


Figure 2. Total plasma cholesterol levels over the course of the study. Rabbits were fed a 0% cholesterol diet (normal diet) or a 1% cholesterol-enriched diet for 6 weeks. Blood samples were taken at 0 (baseline), 2, 4, and 6 weeks into the diet feeding.

Plasma Cholesterol Levels and Atherosclerotic Lesions

As seen in Figure 2, during the 6-week period, total plasma cholesterol levels were not changed in age-matched rabbits (63 \pm 11 mg/dL at baseline versus 69 \pm 10 mg/dL at week 6) but were markedly increased in rabbits fed a 1% cholesterol-enriched diet (64 \pm 4 mg/dL at baseline versus 1359 \pm 78 mg/dL on week 6). Despite the marked increase in plasma cholesterol, postmortem oil red O staining did not reveal any macroscopic atherosclerotic lesion in the descending thoracic aorta of any hypercholesterolemic rabbit (data not shown).

Vasodilator Response

In 8 cholesterol diet-fed rabbits, the response of arterial blood pressure to endothelium-dependent (acetylcholine and bradykinin) and endothelium-independent (nitroglycerin) vasodilators was tested at baseline (before the cholesterol diet was begun) and at the end of the experiments (just before the animal was euthanized). Arterial pressure was measured as described previously.² As shown in Figure 3, the hypotensive response to acetylcholine and bradykinin was blunted after the 6-week cholesterol diet, whereas the response to nitroglycerin was not, which suggests that NO bioactivity was impaired.

Study I: Hypercholesterolemia Abrogates Late PC

There were no appreciable differences in heart rate among groups throughout the experimental protocols (data not shown).

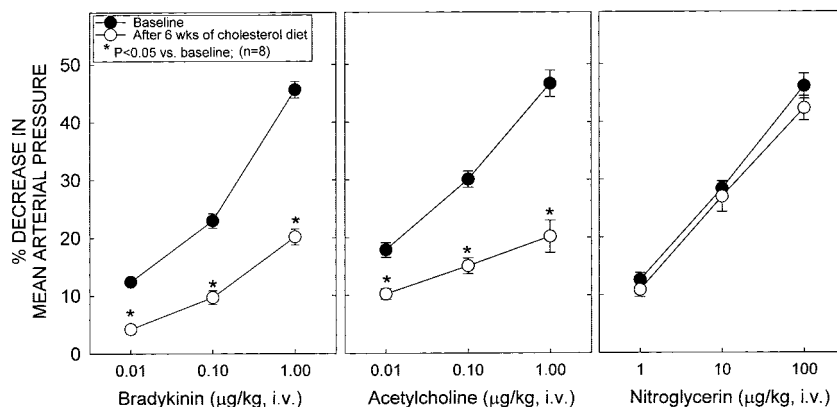


Figure 3. Change in mean arterial pressure in response to a bolus intravenous injection of 0.01, 0.10, or 1.0 μ g/kg of bradykinin (left) and acetylcholine (middle panel), and 1, 10, or 100 μ g/kg of nitroglycerin (right panel) at baseline (before cholesterol diet) and after 6 weeks of a 1% cholesterol-enriched diet (just before sacrifice; n=8).

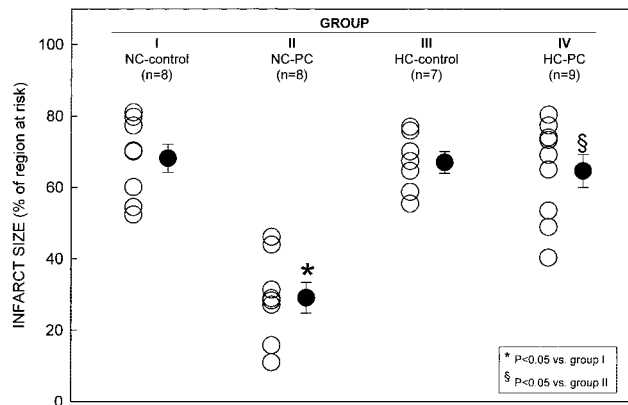


Figure 4. Myocardial infarct size after 30-minute coronary occlusion followed by 3 days of reperfusion in groups I (normocholesterolemic control group), II (normocholesterolemic PC group), III (hypercholesterolemic control group), and IV (hypercholesterolemic PC group). Groups I and III received no PC, whereas groups II and IV were preconditioned with a sequence of six 4-minute occlusion/reperfusion cycles 24 hours before the 30-minute occlusion. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles indicate individual rabbits; solid circles, mean \pm SEM; NC, normocholesterolemia; and HC, hypercholesterolemia.

In addition, there were no significant differences with respect to the weight of the region at risk (1.16 ± 0.17 g [$23.1 \pm 2.5\%$ of left ventricle weight], 1.10 ± 0.11 g [$24.0 \pm 2.3\%$], 1.19 ± 0.11 g [$25.1 \pm 2.6\%$], and 1.15 ± 0.07 g [$25.1 \pm 1.2\%$], respectively, in groups I, II, III, and IV). As expected, infarct size was smaller in group II (normocholesterolemic PC group) than in group I (normocholesterolemic control group; $29.0 \pm 6.1\%$ versus $68.2 \pm 5.7\%$ of the region at risk, respectively; $P < 0.05$; Figure 4), which indicates the occurrence of late PC in healthy rabbits. In group III (hypercholesterolemic control group; $67.0 \pm 3.1\%$ of the region at risk), infarct size was comparable to that in group I (Figure 4), which indicates that hypercholesterolemia does not affect ischemia/reperfusion injury in the unstressed state. However, despite ischemic PC, infarct size in group IV (hypercholesterolemic PC group; $64.6 \pm 4.7\%$ of the region at risk) was not smaller than in group III (Figure 4), which indicates that the PC protocol failed to induce a protective effect against myocardial infarction in hypercholesterolemic rabbits.

In all groups, the size of the infarction was positively and linearly related to the size of the region at risk ($r = 0.887$, 0.640 , 0.880 , and 0.810 in groups I through IV, respectively; Data Supplement Figure I; see <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.566190/DC1>). Analysis of the regression lines confirmed the conclusions achieved above on the basis of the average infarct size.

Hypercholesterolemia Prevents Synthesis of BH₄

In normocholesterolemic rabbits (group VI), the 6 occlusion/reperfusion cycles resulted, 24 hours later, in a robust increase in BH₄ content in the ischemic/reperfused myocardium (0.49 ± 0.06 ng/mg) compared with nonischemic myocardium (0.25 ± 0.01 ng/mg; $P < 0.05$) and naïve myocardium (group V; Figure 5). In contrast, in cholesterol-fed rabbits (group VII), the same ischemic PC protocol failed to increase myocardial BH₄ levels (0.26 ± 0.02 ng/mg in the ischemic/

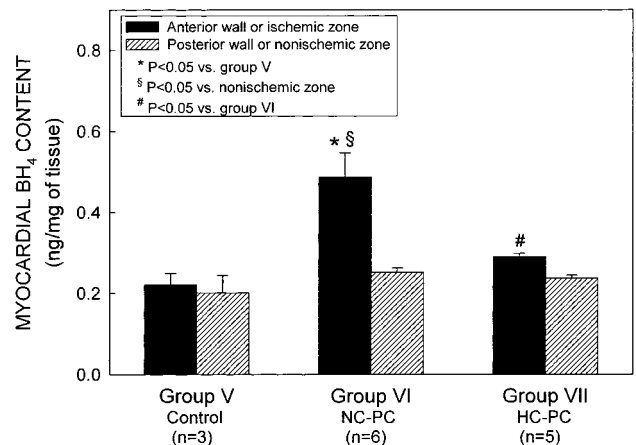


Figure 5. BH₄ levels in the anterior wall or ischemic/reperfused zone (solid bar) and in the posterior wall or nonischemic zone (hatched bar) of control (group V), age-matched normocholesterolemic (group VI [NC-PC]), or hypercholesterolemic (group VII [HC-PC]) rabbits. Both normocholesterolemic and hypercholesterolemic rabbits were preconditioned with a sequence of six 4-minute coronary occlusion/reperfusion cycles 24 hours earlier. NC indicates normocholesterolemia; HC, hypercholesterolemia.

reperfused myocardium versus 0.24 ± 0.02 ng/mg in the nonischemic myocardium; Figure 5). The BH₄ content in the posterior (nonischemic) left ventricular wall of hypercholesterolemic rabbits (group VII) was similar to that observed in the posterior wall of normocholesterolemic preconditioned rabbits (group VI) and of control rabbits (group V; Figure 5). Thus, hypercholesterolemia blunted the increase in myocardial BH₄ induced by ischemic PC but had no effect on basal BH₄ levels.

Study II: NAS Abrogates the Infarct-Limiting Effect of Late PC

Heart rate did not differ among groups either during administration of the BH₄ synthesis inhibitor NAS or during the ischemia/reperfusion protocol (data not shown). There were no significant differences among groups VIII, IX, X, XI, and XII with respect to the weight of the region at risk (0.97 ± 0.12 g [$19.5 \pm 2.0\%$ of left ventricular weight], 0.76 ± 0.07 g [$18.0 \pm 1.1\%$], 0.90 ± 0.13 g [$18.6 \pm 2.3\%$], 0.69 ± 0.09 g [$16.1 \pm 2.0\%$], and 0.73 ± 0.07 g [$17.3 \pm 2.0\%$, respectively). The average infarct size was smaller in group IX (PC+vehicle group) than in group VIII (control group; $23.5 \pm 3.0\%$ versus $60.9 \pm 4.1\%$ of the region at risk, respectively; $P < 0.05$; Figure 6), which indicates that administration of vehicle did not interfere with late PC. In contrast, in rabbits preconditioned on day 1 and treated with NAS before the 30-minute occlusion on day 2 (group X), infarct size ($51.9 \pm 2.3\%$) was significantly greater than in group IX ($P < 0.05$) and was similar to that measured in group VIII, which indicates that NAS abrogated late PC. In group XI (NAS group), infarct size ($48.6 \pm 2.22\%$) did not differ from that in group VIII (Figure 6), which indicates that NAS did not affect the extent of cell death in nonpreconditioned myocardium. Thus, the abrogation of the infarct-sparing effect of late PC observed in group X cannot be ascribed to deleterious actions of NAS on infarct size independent of PC.

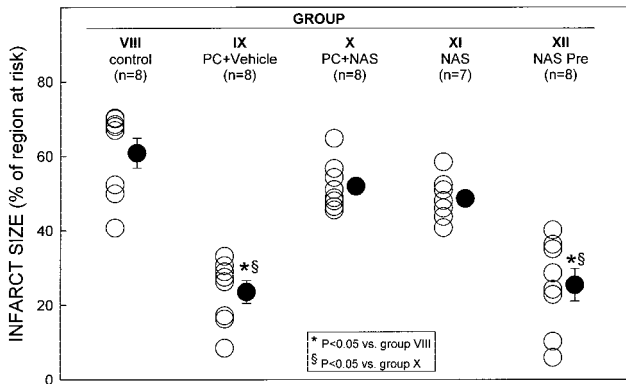


Figure 6. Myocardial infarct size after 30-minute coronary occlusion followed by 3 days of reperfusion in groups VIII through XII. Groups VIII and XI received no PC, whereas groups IX, X, and XII were subjected to ischemic PC 24 hours earlier. Rabbits received an intravenous bolus of vehicle (groups VIII [control group] and IX [PC+vehicle group]) or of the BH₄ synthesis inhibitor NAS (15 mg/kg over 5 minutes) 30 minutes before the 30-minute coronary occlusion on day 2 (groups X [PC+NAS group] and XI [NAS group]) or 30 minutes before the ischemic PC protocol on day 1 (group XII [NAS Pre group]). Infarct size is expressed as a percentage of the region at risk of infarction. Open circles indicate individual rabbits; solid circles, mean \pm SEM.

In rabbits given NAS before ischemic PC on day 1 (group XII), infarct size ($25.4 \pm 4.3\%$) did not differ significantly from that in group IX (Figure 6), which indicates that NAS pretreatment before the PC stimulus on day 1 did not affect the development of late PC. As in study I, the results obtained in study II with measurements of average infarct size were confirmed by analysis of the relation of infarct size to region at risk (Data Supplement Figure II).

NAS Abolishes the Ischemic PC-Induced Increase in BH₄ Synthesis

As shown in Figure 7, in rabbits preconditioned 24 hours earlier and given vehicle (group XIII) before euthanasia, the BH₄ content was increased in the ischemic/reperfused myocardium compared with the nonischemic myocardium (0.45 ± 0.06 versus 0.24 ± 0.03 ng/mg tissue, $P < 0.05$), which confirms the results obtained in group VI in study I. In rabbits that received NAS 24 hours after PC (group XIV), the BH₄ content was measured 30 minutes after NAS. In the nonischemic zone, BH₄ levels (0.19 ± 0.03 ng/mg tissue) were similar to those observed in group XIII, which indicates that NAS did not affect basal BH₄ content; in contrast, in the ischemic/reperfused myocardium, the ischemic PC-induced increase in BH₄ content was completely abolished (Figure 7), which indicates that the dose of NAS was sufficient to block the increased synthesis of BH₄ associated with late PC. In rabbits that received NAS pretreatment 30 minutes before the ischemic PC protocol on day 1 (group XV), the BH₄ content measured 24 hours later was similar to that observed in vehicle-treated rabbits (group XIII; Figure 7), which indicates that the inhibitory effect of NAS on ischemic PC-induced BH₄ synthesis had disappeared by 24 hours after treatment.

Discussion

Although the late phase of ischemic PC has been well documented in healthy animals,²⁻⁵ to the best of our knowl-

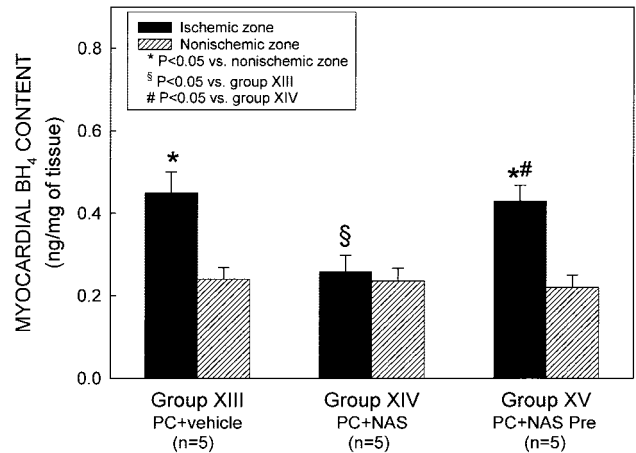


Figure 7. BH₄ levels in the ischemic/reperfused zone (solid bar) and in the nonischemic zone (hatched bar) of normocholesterolemic rabbits that received vehicle (group XIII) or NAS (group XIV) 30 minutes before being euthanized on day 2 or that received NAS pretreatment (group XV) 30 minutes before the ischemic PC protocol on day 1 and that were euthanized on day 2. All rabbits were preconditioned with a sequence of six 4-minute coronary occlusion/reperfusion cycles 24 hours earlier.

edge, no study has been performed to examine this phenomenon in animals with concurrent hypercholesterolemia. The salient findings of the present investigation, conducted in a conscious animal model, can be summarized as follows: (1) brief episodes of ischemia/reperfusion induce delayed protection against myocardial infarction in normocholesterolemic rabbits but fail to do so in rabbits fed a 1% cholesterol diet for 6 weeks, which indicates that hypercholesterolemia abrogates the infarct-sparing effect of late PC; (2) brief episodes of ischemia/reperfusion result in increased myocardial BH₄ content 24 hours later in healthy rabbits but not in hypercholesterolemic rabbits, which indicates that hypercholesterolemia prevents the upregulation of BH₄ induced by ischemic PC; and (3) in healthy rabbits, the BH₄ synthesis inhibitor NAS abolishes both the ischemic PC-induced increase in myocardial BH₄ levels and the infarct-sparing effect of late PC, which demonstrates that augmented synthesis of BH₄ is required for late PC. These data support the thesis that hypercholesterolemia interferes with late PC by preventing upregulation of myocardial BH₄ levels.

The importance of determining whether late PC is affected by hypercholesterolemia stems from the fact that this is one of the most prevalent risk factors for coronary artery disease. Given that the present study was performed to simulate the clinical setting, the use of a chronically instrumented conscious animal model was believed to be essential, because open-chest preparations are associated with a number of potentially confounding factors that may interfere with myocardial infarction¹⁴ and/or ischemic PC.¹⁵ Moreover, because BH₄ is auto-oxidized in a radical reaction¹⁶ and is oxidized by NO¹⁷ and peroxynitrite,^{18,19} it seemed important to avoid experimental conditions, such as surgical trauma, that may lead to exaggerated generation of free radicals.²⁰ Accordingly, all of the studies reported herein were performed in closed-chest, awake animals in an effort to test the effect of hypercholesterolemia on late PC under conditions that were

as physiological as possible. The rabbit was selected because it is the most commonly used species for experimental studies of hypercholesterolemia and atherosclerosis. The duration of the cholesterol-enriched diet (6 weeks) was selected to avoid the development of structural atherosclerotic lesions while achieving high and steady plasma cholesterol levels, so as to enable us to test the effect of hypercholesterolemia in the absence of obstructive vascular disease. Indeed, this protocol produced hypercholesterolemia (Figure 2) without histological evidence of atherosclerosis as demonstrated by postmortem staining of the aortas with oil red O. In addition, this hypercholesterolemic rabbit model exhibited impaired NO bioactivity as demonstrated by the blunted hypotensive response to endothelium-dependent vasodilators (Figure 3). Despite the high cholesterol levels and the impaired endothelium-dependent vasodilation, however, infarct size was virtually the same as in normocholesterolemic rabbits (Figure 4), which indicates that hypercholesterolemia does not affect the susceptibility to ischemia/reperfusion injury in naïve (unstressed) myocardium. This finding is in apparent contrast to the studies by Jung et al²¹ and Wang et al⁷ in open-chest rabbit preparations, which concluded that the hypercholesterolemic myocardium is more susceptible to ischemia/reperfusion injury. In the investigation by Jung et al,²¹ rabbits were fed an atherogenic diet (0.25% cholesterol and 3% coconut oil) for 4 weeks, after which they underwent a 30-minute coronary occlusion followed by 2 hours of reperfusion. In the study by Wang et al,⁷ rabbits were fed 0.5% cholesterol and 10% coconut oil for 8 weeks and then underwent a 30-minute coronary occlusion/4-hour reperfusion sequence. The reasons for the apparent discrepancy between these findings and the present data are unknown. The divergent results may be secondary to differences in experimental preparations (open-chest versus conscious animals), diet components (cholesterol plus coconut oil versus cholesterol only), or experimental protocols (2 to 4 hours versus 24 hours of reperfusion). As alluded to above, our decision to use a conscious rabbit model and a duration of reperfusion of 24 hours was motivated by our concern that open-chest models or shorter reperfusion intervals may lead to erroneous conclusions.

No previous study has assessed the impact of hypercholesterolemia on late PC induced by ischemia. With regard to the effect of hypercholesterolemia on late PC induced by stimuli other than ischemia, only 2 reports^{22,23} are available besides our recent study.⁹ In the first report, Szekeres et al²² concluded that the protection induced by rapid ventricular pacing against the hemodynamic and electrophysiological changes caused by subsequent rapid overpacing was still present in cholesterol-fed rabbits, but a greater PC stimulus was required. These results were interpreted to suggest that hypercholesterolemia elevates the threshold for the PC response. In another study, Szilvassy et al²³ reported that the delayed protective effect induced by lipopolysaccharide against the hemodynamic and electrophysiological changes caused by ventricular overpacing was not abrogated in cholesterol-fed conscious rabbits. In both of these studies,^{22,23} however, the experiments were performed in hypercholesterolemic rabbits with atherosclerotic lesions, which may have confounded the effects of hypercholesterolemia per se (ie, the presence of

obstructive vascular disease may have caused ischemia); furthermore, the end points used were ST-segment elevation, left ventricular end-diastolic pressure, and shortening of the ventricular effective refractory period, not infarct size. These end points are not commonly used in studies of PC.^{1,24} Therefore, the findings are difficult to evaluate because the animal model was not one of pure hypercholesterolemia and because the significance of the end points selected is unclear. In addition, unlike the present study, neither of these investigations^{22,23} used ischemia as the PC stimulus. The clinical relevance of tachycardia and lipopolysaccharide as PC stimuli is uncertain. Using infarct size as the end point, we have found that administration of the NO donor DETA/NO, which induces delayed protection in healthy rabbits, fails to induce delayed protection in rabbits fed a 1% cholesterol-enriched diet for 4 weeks,⁹ which demonstrates that hypercholesterolemia blunts NO donor-induced late PC. It is unknown, however, whether these conclusions apply to late PC induced by ischemia, because the mechanism of late PC may differ depending on the stimulus used.¹ The present study is the first to demonstrate that hypercholesterolemia abrogates ischemia-induced late PC.

Late PC requires the activity of both eNOS, which triggers the transition from a naïve to a protected phenotype after the PC ischemia,^{2,25} and iNOS, which mediates the late PC effect during subsequent ischemia/reperfusion injury.^{25,26} If hypercholesterolemia impairs only the activity of eNOS, one would expect that exogenous NO (via administration of an NO donor) should restore the late PC effect. However, our previous finding⁹ that administration of the NO donor DETA/NO was unable to induce the late PC effect in hypercholesterolemic rabbits suggests that hypercholesterolemia interferes with the activity of iNOS in addition to or in lieu of the activity of eNOS. That is, even though exogenous NO (via administration of an NO donor) initiates the PC response on day 1, cardioprotection is not manifest, because hypercholesterolemia also impairs the iNOS-derived NO generation that mediates late PC on day 2. BH₄ is known to be an essential cofactor for both eNOS and iNOS.¹⁰ iNOS activity, however, may be particularly sensitive to BH₄ levels, because upregulation of this isoform is thought to result in continuous, high-level NO generation,²⁷ which in turn would require continuous resynthesis of BH₄ to support the activity of this enzyme. Because hypercholesterolemia is associated with increased production of superoxide anion,²⁸ which may either inhibit the biosynthesis or prevent the recycling of BH₄,¹⁸ we postulated that hypercholesterolemia may decrease the bioavailability of BH₄, leading to impairment of the iNOS-derived NO formation that is required to mediate late PC protection. Indeed, we found that BH₄ levels are upregulated in the preconditioned myocardium of healthy rabbits (Figure 5, group VI) but not in that of hypercholesterolemic rabbits (Figure 5, group VII). To the best of our knowledge, this is the first demonstration that ischemic PC upregulates myocardial BH₄ and that this phenomenon is blocked by hypercholesterolemia. Unlike a previous study in which hypercholesterolemia reduced BH₄ levels in rabbit aortas,¹¹ in the present study, hypercholesterolemia did not reduce BH₄ levels in nonpreconditioned myocardium (left ventricular

posterior wall in the hypercholesterolemic-PC group; Figure 5). Thus, in this rabbit model, hypercholesterolemia blunts the increase in myocardial BH₄ that is observed at 24 hours after ischemic PC but does not affect the basal myocardial content of BH₄. The difference between the present findings and those of the aforementioned study¹¹ could be due to the different tissues examined (myocardium versus aorta) and/or to the different duration of hypercholesterolemia (6 weeks versus 8 to 10 weeks).

Next, in study II, we sought to elucidate the significance of BH₄ synthesis in late PC, an issue about which nothing is currently known. We reasoned that if the ischemic PC-induced upregulation of BH₄ is essential for iNOS to mediate the late PC protection, inhibition of BH₄ synthesis in healthy animals should mimic the effects of hypercholesterolemia and block late PC. We used NAS, an inhibitor of sepiapterin reductase, which is a critical enzyme in the biosynthesis of BH₄.^{10,29} NAS has been shown to effectively inhibit BH₄ synthesis *in vivo*.³⁰ The ability of the dose of NAS used in the present study to block the increased BH₄ synthesis induced by ischemic PC was confirmed by the measurements of myocardial BH₄ levels (Figure 7). The results of study II demonstrate that in normocholesterolemic rabbits, the protection of late PC was abrogated by administration of NAS on day 2 (group X, Figure 6; Data Supplement Figure II), mimicking the results observed in hypercholesterolemic rabbits (group IV, Figure 4; Data Supplement Figure I). Our finding that preventing the increase in BH₄, in itself, is sufficient to prevent late PC supports the concept that hypercholesterolemia abrogates late PC, at least in part, by interfering with BH₄ availability. To the best of our knowledge, this is the first evidence that BH₄ synthesis is required for late PC to occur.

The ability of NAS to decrease myocardial BH₄ levels rapidly (within 30 minutes after its administration; Figure 5) is due to the fact that the active (reduced) form of BH₄ (which was measured in the present study) is constantly oxidized to the inactive form and constantly resynthesized by the BH₄ biosynthetic pathway, in which the enzyme sepiapterin reductase plays a key role.^{10,29,31} Accordingly, inhibition of sepiapterin reductase by NAS would be expected to result in a rapid decline in myocardial BH₄ levels, such as that observed in the present study at 30 minutes after NAS (Figure 7).

The finding that administration of NAS before ischemic PC failed to abrogate the infarct-sparing effects of late PC 24 hours later (Figure 6) suggests that inhibition of sepiapterin reductase by NAS does not persist for 24 hours after treatment. This was confirmed by our observation that myocardial BH₄ levels in rabbits pretreated with NAS 24 hours earlier were similar to those observed in control rabbits (Figure 7). The disappearance of sepiapterin reductase inhibition allows resynthesis of BH₄, such that the activity of iNOS can be supported and the protection afforded by late PC can be observed despite administration of NAS 24 hours earlier.

It is noteworthy that the development of late PC was not affected by inhibition of BH₄ synthesis with NAS on day 1 (group XII, Figure 6; Data Supplement Figure II), despite the fact that BH₄ is a necessary cofactor for eNOS,³² which triggers late PC on day 1.²⁵ Because NAS did not reduce the

basal levels of BH₄ (Figure 7), it is plausible that these levels may be sufficient to support the relatively brief increase in eNOS activity that generates the NO responsible for triggering the development of late PC.²⁵ In contrast to eNOS activity, which is pulsatile, iNOS activity is continuous²⁷ and therefore may not be possible without a sustained increase in BH₄ formation.

In conclusion, we have demonstrated that hypercholesterolemia completely abrogates not only the cardioprotective effects of late PC but also the concomitant upregulation of myocardial BH₄ synthesis. In addition, the present study establishes BH₄ levels as an essential determinant of the ability of the heart to shift to a preconditioned phenotype, because inhibition of BH₄ formation in healthy animals was sufficient to prevent late PC. Our results also indicate that BH₄ availability is a limiting factor for the activity of iNOS, which mediates late PC on day 2, but not for the activity of eNOS, which triggers late PC on day 1. Finally, this is the first study to demonstrate that severe hypercholesterolemia has no effect on infarct size in the unstressed (nonpreconditioned) state. Collectively, the results of the present study support the conclusion that hypercholesterolemia abrogates late PC by interfering with the upregulation of BH₄ synthesis, which leads to dysfunction of iNOS.

Conclusions

Ischemic PC is a well-documented endogenous, cardioprotective phenomenon. The underlying molecular mechanisms of this phenomenon have been investigated intensively over the last decade. The progress of research on ischemic PC has offered hope of developing new rational approaches to therapeutic protection of the ischemic myocardium; however, ischemic heart disease in humans is a complex disorder, often associated with other systemic diseases or cardiovascular risk factors, such as hypercholesterolemia/hyperlipidemia, hypertension, and diabetes, that exert multiple biochemical effects on the heart, independently of ischemia. In the present study, we have demonstrated that the infarct-sparing effect of the late phase of ischemic PC conferred in normocholesterolemic rabbits is lost in diet-induced hypercholesterolemic animals, which suggests a negative effect of hypercholesterolemia on this phenomenon. Further evidence indicates that hypercholesterolemia blunts the ischemic PC-induced increase in myocardial levels of tetrahydrobiopterin, an essential component of the biochemical pathway for generating nitric oxide, which plays a pivotal role in the late phase of ischemic PC. These results suggest that the coexistence of other diseases and risk factors impairs the biochemical mechanisms and protective effects of PC. It is important that future preclinical studies specifically examine PC in relation to complicating disease states. Broader investigation of PC in diseased models might shed more light on the underlying biochemical mechanisms of this intriguing and potentially exploitable endogenous, cardioprotective response.

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Hypercholesterolemia Abrogates Late Preconditioning via a Tetrahydrobiopterin-Dependent Mechanism in Conscious Rabbits

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