Role of Cyclic Guanosine Monophosphate in Late Preconditioning in Conscious Rabbits
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Background—Although NO has been shown to serve both as the trigger and the mediator of the late phase of ischemic preconditioning (PC), it is unknown whether NO acts via activation of soluble guanylate cyclase (sGC). The objective of this study was to investigate the role of sGC in late PC in conscious rabbits using the selective sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ).

Methods and Results—A total of 172 conscious rabbits were used. When nonpreconditioned rabbits were subjected to a sequence of 4-minute coronary occlusion/4-minute reperfusion cycles, myocardial cyclic guanosine monophosphate (cGMP) levels increased significantly at the end of the third and sixth occlusions. In rabbits preconditioned 24 hours earlier (on day 1) with six occlusion/reperfusion cycles, myocardial cGMP levels on day 2 were significantly higher than in nonpreconditioned rabbits even before ischemia but did not increase further during a second sequence of 4-minute occlusion/reperfusion cycles. Administration of ODQ before the six occlusion/reperfusion cycles on day 1 did not prevent the development of late PC against either stunning or infarction on day 2. In contrast, administration of ODQ on day 2 completely ablated the late PC effect against both stunning and infarction.

Conclusions—These results indicate that enhanced synthesis of cGMP by sGC is not necessary for ischemia to trigger a late PC effect but is required for the protection to become manifest 24 hours later. This implies that NO participates in late PC via two distinct mechanisms; ie, it triggers late PC on day 1 via a cGMP-independent mechanism and it mediates late PC on day 2 via a cGMP-dependent mechanism. (Circulation. 2002;105:3046-3052.)

Key Words: ischemia ■ reperfusion ■ guanylate cyclase ■ cyclic guanosine monophosphate ■ oxadiazoles

The late phase of ischemic preconditioning (PC) is a protective adaptation whereby brief episodes of ischemia enhanced the tolerance of the heart to subsequent ischemia 24 to 72 hours later.1-5 In recent years, numerous studies have demonstrated that NO plays a dual role in late PC, acting both as the trigger of this phenotypic shift during the initial ischemic stress (on day 1)1-5 and as the mediator of cardioprotection 24 hours later (on day 2).6-7 (NO hypothesis of late PC). Two different isoforms of NO synthase (NOS) are sequentially involved in the pathophysiological cascade of late PC, with calcium-dependent NOS (cNOS, most likely endothelial NOS) generating the NO that initiates the development of the PC response on day 1 and inducible NOS (iNOS) then generating the NO that protects against recurrent ischemia on day 2.6 However, despite the impressive body of evidence implicating NO in late PC, the mechanism(s) whereby enhanced NO biosynthesis triggers and mediates this phenomenon remain(s) essentially unknown.

In particular, a critical issue to be elucidated is whether NO acts via its second messenger, cyclic guanosine monophosphate (cGMP). Many of the biological actions of NO occur via the activation of soluble guanylate cyclase (sGC) and the resulting increase in cGMP tissue levels. cGMP has been shown to exert a number of actions that would be expected to be beneficial during myocardial ischemia9-12 (reviewed in Reference 2). On the other hand, NO can directly modulate the function of numerous proteins via cGMP-independent pathways, eg, via reaction with Fe2+hemoproteins such as cyclooxygenase-2, nitrosation of thiols, ADP-ribosylation, tyrosine nitration, etc. An additional cGMP-independent mechanism is the rapid reaction of NO with superoxide anion (·O2-) leading to the formation of peroxynitrite (ONOO-), which in turn decomposes to form the hydroxyl radical (·OH) or another oxidant species with similar reactivity.14 Both ONOO- and ·OH-derived reactive oxygen species have been shown to alter the function of numerous proteins.13 At present, virtually nothing is known regarding whether the involvement of NO in late PC is mediated via cGMP-dependent or independent mechanisms.

The quinoxalin derivative 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) is a potent and selective inhibitor of NO-stimulated sGC activity, without actions on particulate
GC or adenylyl cyclase. The objective of this study was to use ODQ to investigate the role of NO-dependent activation of sGC in the phenomenon of late PC. Three specific questions were addressed, as follows. (1) Does ischemic PC increase myocardial cGMP levels in vivo and, if so, is this the result of increased sGC activity? (2) Do cGMP levels differ in late preconditioned versus nonpreconditioned myocardium before or during an ischemic challenge? (3) Does inhibition of NO-dependent sGC activity interfere either with the development of the late PC response after the first ischemic stress (day 1) or with the manifestation of cardioprotection during the second ischemic challenge (day 2)? To address these issues, a well-established rabbit model was used in which the role of NO has previously been well documented. To comprehensively examine the role of sGC, both late PC against myocardial stunning and late PC against infarction were examined. All studies were performed in conscious animals in an effort to test the role of sGC under conditions that are as physiological as possible.

Methods
This study was conducted in New Zealand White male rabbits (Myrtles Rabbitry, Thompson Station, Tenn) instrumented under sterile conditions and allowed to recover for a minimum of 10 days after surgery. The experimental preparation and the protocols for the studies of myocardial stunning and infarction have been described in detail previously. The experimental protocols for phase I (measurements of cGMP), phase II (studies of myocardial stunning), and phase III (studies of myocardial infarction) are illustrated in Figures 1 and 2 and detailed in the Data Supplement.

Results
A total of 172 conscious rabbits were used (20 for the pilot studies, 65 for phase I [measurements of cGMP], 25 for phase II [studies of myocardial stunning], and 62 for phase III [studies of myocardial infarction]). The exclusions are summarized in Table I.

Phase I: Measurements of cGMP

Nonpreconditioned Groups (Groups I to VI)
When rabbits were subjected to a sequence of 4-minute coronary occlusion/4-minute reperfusion cycles, myocardial cGMP levels increased significantly (~22% [P<0.05]) in the ischemic zone at the end of the third occlusion (3 O/R group), but did not rise further at the end of the sixth occlusion (6 O/R group [Figures 3A and 4]). Administration of ODQ under baseline conditions (ODQ group) had no effect on cGMP (Figure 3A), indicating that basal cGMP production occurs...
mainly via mechanisms independent of NO-driven sGC. However, administration of ODQ before the sequence of 4-minute occlusion/reperfusion cycles (ODQ/3 O/R group) completely abrogated the increase in cGMP at the end of the third occlusion (Figure 3A), indicating that this dose of ODQ effectively inhibits ischemia-induced activation of sGC in vivo. Administration of S-nitroso-N-acetylpenicillamine (SNAP) in the absence of ischemia (SNAP group) resulted in a robust (>91%) increase in myocardial cGMP levels compared with group I (Figure 3A), providing a positive control for the cGMP measurements.

**Preconditioned Groups (Groups VII to XIII)**

In preconditioned rabbits, baseline myocardial cGMP levels 24 hours after PC (on day 2) were 27% higher than the corresponding baseline levels in nonpreconditioned hearts (P<0.05 [Figures 3 and 4]), indicating that ischemic PC resulted in increased myocardial cGMP content 24 hours later, even before the second ischemic insult. This increase in baseline cGMP levels in preconditioned myocardium was completely abrogated by administration of ODQ on day 2 (ODQ group [Figure 3B]). In contrast to nonpreconditioned rabbits (Figure 3A), when preconditioned rabbits were subjected to a sequence of occlusion/reperfusion cycles on day 2, the cGMP levels did not increase appreciably above baseline levels either at the end of the third occlusion (3 O/R group) or at the end of the sixth occlusion (6 O/R group [Figures 3B and 4]). When preconditioned rabbits were given ODQ before the occlusion/reperfusion cycles on day 2 (ODQ/3 O/R group), the myocardial cGMP levels measured at the end of the third occlusion were significantly (P<0.05) lower than in the corresponding group not given ODQ (3 O/R group [Figure 3B]). DMSO had no effect on cGMP levels (DMSO/3 O/R group [Figure 3B]). When rabbits were given ODQ before the occlusion/reperfusion cycles on day 1 (ODQ on day 1 group), the myocardial cGMP levels measured 24 hours later (on day 2) were similar to the baseline levels in preconditioned hearts and were 25% higher than the baseline levels in nonpreconditioned hearts (P<0.05 [Figure 3]).

When the myocardial cGMP levels are expressed as a percentage of baseline nonpreconditioned levels (baseline group in Figure 3A [group I]), it is apparent that although preconditioned myocardium had higher cGMP content before ischemia, during the six occlusion/reperfusion cycles the cGMP levels were not significantly different between preconditioned and nonpreconditioned myocardium (Figure 4). ODQ had no effect under baseline conditions in nonpreconditioned myocardium; however, ODQ completely blocked the increase in cGMP noted in preconditioned myocardium both
before and during the second ischemic challenge (Figure 4), indicating that the enhanced cGMP levels associated with late PC are accounted for by enhanced sGC activity.

Phase II: Studies of Myocardial Stunning

The size of the occluded/reperfused vascular bed was similar in groups XIV to XVI (Table II). There were no appreciable differences in heart rate (Table III) or baseline systolic thickening fraction (Table IV) among the three groups on days 1, 2, and 3.

In group XIV (control), the recovery of systolic wall thickening (WTh) after the six occlusion/reperfusion cycles on days 2 and 3 was markedly improved compared with day 1 (Figure I) and the total deficit of WTh after the sixth reperfusion was correspondingly decreased (Figure 5), indicating the development of late PC against stunning.4,5,17,20 Similar results were obtained when ODQ was given before the first sequence of occlusion/reperfusion cycles on day 1 (group XV [ODQ on day 1]) (Figure 5 and Figure II), indicating that ODQ did not affect either the severity of myocardial stunning on day 1 or the development of late PC on day 2. In contrast, in group XVI (ODQ on day 2), the recovery of WTh after the six occlusion/reperfusion cycles was not improved on day 2 compared with day 1 (Figure III), and the total deficit of WTh after the sixth reperfusion on day 2 was 66% greater than the corresponding value in control rabbits (P<0.05 [Figure 5]), indicating that administration of ODQ on day 2 completely abolished the protective effect of late PC against myocardial stunning.

Figure 3. Myocardial cGMP levels. Experimental protocols are detailed in Figure 1. Illustrated are myocardial cGMP levels for the nonpreconditioned (A) and preconditioned (B) groups. In nonpreconditioned groups, tissue samples were harvested from rabbits that underwent no intervention (baseline) or from rabbits that were euthanized at the end of the third coronary occlusion (3 O/R) or at the end of the sixth occlusion (6 O/R) in a sequence of 4-minute occlusion/4-minute reperfusion cycles, with or without ODQ pretreatment. An additional group (SNAP) was euthanized at the end of a 60-minute IV infusion of SNAP at 2.5 μg·kg⁻¹·min⁻¹; this group served as a positive control. In the preconditioned groups, all rabbits underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles on day 1; 24 hours later (day 2), myocardial samples were harvested without any further intervention (baseline) or at the end of the third (3 O/R) or sixth (6 O/R) coronary occlusion in a sequence of six 4-minute occlusion/4-minute reperfusion cycles, with or without ODQ treatment. One additional group of rabbits received DMSO (DMSO·3 O/R) 30 minutes before the sequence of 4-minute occlusion/4-minute reperfusion cycles; they were euthanized at the end of the third occlusion. Another group of rabbits received ODQ (ODQ on day 1) 30 minutes before the sequence of 4-minute occlusion/4-minute reperfusion cycles on day 1; they were euthanized 24 hours later (on day 2). Data are mean±SEM.

Phase II: Studies of Myocardial Stunning

The size of the occluded/reperfused vascular bed was similar in groups XIV to XVI (Table II). There were no appreciable differences in heart rate (Table III) or baseline systolic
Phase III: Studies of Myocardial Infarction

There were no appreciable differences in heart rate (Table III) or baseline systolic thickening fraction (Table IV) among groups XVII to XXII. Similarly, there were no significant differences with respect to the size of the region at risk (Table II).

Average infarct size was 41% smaller in the PC group (group XVIII) than in the control group (group XVII) (Figure 6), indicating a late PC effect against myocardial infarction. A similar reduction in infarct size was observed in group XX (PC + ODQ on day 1 [Figure 6]), indicating that pretreatment with ODQ 30 minutes before the six occlusion/reperfusion cycles on day 1 did not affect the development of late PC against myocardial infarction. In group XXI (PC + ODQ on day 2), however, infarct size was similar to that measured in the control group and significantly \( P < 0.05 \) larger than that measured in the PC group (Figure 6), indicating that ODQ given 30 minutes before the 30-minute occlusion on day 2 abolished the infarct-sparing effect of late PC. When ODQ was given 30 minutes before the 30-minute occlusion in nonpreconditioned rabbits (group XXII), infarct size was not significantly different from that measured in the control group, indicating that inhibition of sGC did not have a detrimental effect on myocardial infarction in the absence of late PC.

Taken together, these results indicate that enhanced synthesis of cGMP by sGC is not necessary for ischemia to trigger a late PC effect but is required for the protection to become manifest 24 hours later. To our knowledge, this is the first study to explore the role of cGMP in the late phase of ischemic PC. It is also the first analysis of the function of sGC in an in vivo model of myocardial ischemia/reperfusion.

Methodological Considerations

ODQ was used in the present study because it is the most selective sGC inhibitor currently available. It inhibits sGC potently (IC\(_{50} = 20\) nmol/L)\(^{15,21}\). ODQ selectively antagonizes the activation of sGC by NO because it oxidizes the Fe\(^{2+}\) heme group of sGC to the Fe\(^{3+}\) form,\(^{22}\) which has poor affinity for NO. Importantly, this agent does not affect the activity of particulate GC,\(^{15,21}\) adenylyl cyclase,\(^{15}\) or NOS\(^{15,21}\); does not react directly with NO\(^{21,23}\); and does not generate superoxide anions.\(^{23}\) Thus, ODQ appears to be a useful tool to elucidate the physiological significance of the NO-cGMP pathway.

Previous studies have demonstrated that ODQ effectively inhibits NO-dependent elevations of cGMP in in vitro preparations.\(^{21,23}\) However, the ability of this agent to modulate sGC in vivo has never been reported. To our knowledge, the present study is the first to use ODQ in an in vivo animal model and to demonstrate that this agent produces both inhibition of sGC activity and a physiological effect (abrogation of PC). The dose of ODQ used herein (5 mg/kg IP) was carefully selected on the basis of pilot studies in which it was found to have no effect on arterial pressure or heart rate (Table V) while inhibiting nitroglycerin-induced hypotension for 75 minutes (Figure VI), indicating that this dose is sufficient to suppress stimulated, but not basal, sGC activity. The effects of ODQ at 5 mg/kg IP are reminiscent of those of NO-nitro-l-arginine at 13 mg/kg IV,\(^{3,5}\) which blunts the spike in cNOS activity during the PC stimulus without lowering basal cNOS activity levels.\(^{24}\) The identification of a dosage of ODQ that effectively blocks sGC activation in the intact animal without causing hemodynamic perturbations will be important to future studies aimed at elucidating the role of sGC not only in myocardial ischemia but in many other processes in which this enzyme has been implicated.
Role of cGMP as a Trigger of Late PC on Day 1

In phase I of the present study, the sequence of six 4-minute coronary occlusion/reperfusion cycles, which induces late PC3–6,16,20 and activates cNOS,3–6,16,20,24 resulted in a significant elevation in myocardial cGMP levels during the third and sixth occlusions (Figure 3), raising the possibility that cGMP may be involved in triggering late PC. The results of phases II and III, however, demonstrate that ODQ failed to block the development of delayed protection against either stunning (Figure 5 and Figures I through III) or infarction (Figure 6 and Figures IV and V) despite the fact that ODQ did block the increase in cGMP levels during the PC ischemia (Figures 3 and 4). Therefore, activation of sGC by NO is not necessary to trigger the late phase of ischemia-induced PC. In view of the fact that the development of both ischemia-induced25 and NO donor–induced26 late PC is blocked by mercaptopropionyl glycine, a scavenger of -OH and ONOO·, we propose that NO triggers delayed cardioprotection via the formation of ONOO− and/or secondary reactive oxygen species, rather than via cGMP-dependent pathways.

Role of cGMP as a Mediator of Late PC on Day 2

Although there is convincing evidence that the cardioprotective effects of late PC against both myocardial stunning4 and infarction6,7 are mediated by upregulation of iNOS on day 2, virtually nothing is known regarding the mechanism whereby iNOS-generated NO alleviates the severity of ischemia. In the present investigation the administration of ODQ on day 2 ablated the elevation in myocardial cGMP levels observed in preconditioned myocardium, both at baseline (before ischemia) (Figures 3 and 4) and at the end of the third coronary occlusion (Figures 3 and 4). The same dose of ODQ completely abrogated the antistunning (Figure 5 and Figure III) and anti-infarct (Figure 6 and Figure IV) actions of late PC, demonstrating that these actions are dependent on the activity of NO-driven sGC. Thus, iNOS-derived NO confers cardioprotection, at least in part, via activation of sGC.

Distinctive Roles of cGMP in Preconditioned Versus Nonpreconditioned Myocardium

One of the most important findings of the present study is the identification of heretofore-unrecognized differences between late preconditioned and nonpreconditioned myocardium with respect to the role of cGMP in modulating ischemia/reperfusion injury. As shown in Figure 3, the basal levels of cGMP were higher in preconditioned than in nonpreconditioned myocardium. However, when the heart was subjected to six occlusion/reperfusion cycles, these differences disappeared because cGMP levels increased in the latter but remained flat in the former (Figure 4). Thus, it appears that when the myocardium is subjected to a mild ischemic stress, cGMP levels are elevated to the same extent irrespective of whether or not the heart has been preconditioned. Given these facts, it was surprising to find that lowering cGMP levels with ODQ had a detrimental effect on stunning and infarction only in preconditioned myocardium and not in nonpreconditioned myocardium (Figures 5 and 6; Figures III and IV). The obvious question that arises is, Why does cGMP play a protective role after PC but not in the absence of PC?

We believe there are two main hypotheses to explain this apparent paradox. The first is that the tonically elevated cGMP levels that distinguish preconditioned from nonpreconditioned myocardium produce molecular adaptations before the ischemic challenge, which enables the heart to enter this challenge in a protected state. These adaptations may not take place if cGMP rises only during ischemia. The second possibility is that the cardioprotective actions of late PC are multifactorial, requiring both cGMP-dependent and cGMP-independent mechanisms. In this scenario, a relatively modest increase in myocardial cGMP levels is insufficient to alleviate ischemia in itself and becomes protective only when it is associated with other changes (eg, enhanced prostanooid synthesis17). On the other hand, a marked increase in cGMP, such as that observed after SNAP (Figure 3), could be sufficient to elicit protection in itself, as suggested by recent data.26

Conclusions

This study advances our understanding of the mechanism of late PC by revealing a differential involvement of sGC in the initiation (trigger phase) and mediation (effector phase) of this cardioprotective adaptation. The results reported herein demonstrate that, on day 1, ischemic PC triggers delayed cardioprotection via cGMP-independent pathways. In contrast, enhanced synthesis of cGMP via sGC is necessary for the attenuation of myocardial stunning and infarction by late PC to become manifest on day 2. Because NO is known to play an obligatory role both as a trigger (day 1) and as a mediator (day 2) of late PC, the present results imply that NO participates in this phenomenon via two distinct mechanisms, a cGMP-independent mechanism on day 1 and a cGMP-dependent mechanism on day 2. The present study also demonstrates that preconditioned and nonpreconditioned myocardia differ with respect to cGMP levels before ischemia, but not during ischemia, and that inhibition of sGC worsens ischemic injury only in preconditioned myocardium, revealing a fundamental difference in the role of sGC in the naive vis–à-vis the defensive (preconditioned) cardiac phenotypes.

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