Published in final edited form as:

Am J Physiol Heart Circ Physiol. 2007 October ; 293(4): H2557–H2564. doi:10.1152/ajpheart.00858.2007.

The cardioprotection of the late phase of ischemic preconditioning is enhanced by postconditioning via a COX-2-mediated mechanism in conscious rats

Hiroshi Sato, Roberto Bolli, Gregg D. Rokosh, Qiuli Bi, Shujing Dai, Gregg Shirk, and Xian-Liang Tang

Institute of Molecular Cardiology, Department of Medicine, University of Louisville, Louisville, Kentucky

Abstract

The present study sought to determine whether the combination of late preconditioning (PC) with postconditioning enhances the reduction in infarct size. Chronically instrumented rats were assigned to a 45-min (*subset 1*) or 60-min (*subset 2*) coronary occlusion followed by 24 h of reperfusion. In each subset, rats received no further intervention (control) or were preconditioned 24 h before occlusion (PC), post-conditioned at the onset of reperfusion following occlusion, or pre-conditioned and postconditioned without (PC + postconditioning) or with the COX-2 inhibitor celecoxib (3 mg/kg ip; PC + postconditioning + celecoxib) 10 min before postconditioning. Myocardial cyclooxygenase-2 (COX-2) protein expression and COX-2 activity (assessed as myocardial levels of PGE₂) were measured 6 min after reperfusion in an additional five groups (control, PC, postconditioning, PC + postconditioning, and PC + postconditioning + celecoxib) subjected to a 45-min occlusion. PC alone reduced infarct size after a 45-min occlusion but not after a 60-min occlusion. Postconditioning alone did not reduce infarct size in either setting. However, the combination of late PC and postconditioning resulted in a robust infarct-sparing effect in both settings, suggesting additive cardioprotection. Celecoxib completely abrogated the infarct-sparing effect of the combined interventions in both settings. Late PC increased COX-2 protein expression and PGE₂ content. PGE₂ content (but not COX-2 protein) was further increased by the combination of both interventions, suggesting that postconditioning increases the activity of COX-2 induced by late PC. In conclusion, the combination of late PC and postconditioning produces additive protection, likely due to a postconditioning-induced enhancement of COX-2 activity.

Keywords

myocardium; ischemia; infarct size; cyclooxygenase-2

THE PHENOMENON OF ISCHEMIC PRECONDITIONING (PC) has been well documented to be a powerful endogenous mechanism of cardioprotection. PC is a biphasic phenomenon, with an early (or classic) phase that develops within minutes from the initial ischemic insult and lasts 2–3 h (11, 28) and a late (or delayed) phase that becomes apparent 12–24 h later and lasts 3–4 days (5). Despite extensive studies, the actual mechanism of protection remains unclear. The current consensus is that early PC is mediated by the activation of preexisting signaling kinases (such as PKC, phosphatidylinositol 3-kinase/Akt,

etc.), whereas the cardioprotection of late PC is conferred by the induction of new proteins [such as inducible nitric oxide (NO) synthase (7, 33), cyclooxygenase-2 (COX-2) (31), Mn superoxide dismutase (17), and aldose reductase (29)].

More recently, Vinten-Johansen and colleagues (44) have described a cardioprotective phenomenon, termed ischemic postconditioning, in which brief intermittent episodes of myocardial ischemia applied at the onset of reperfusion result in a significant reduction in infarct size. Postconditioning has been subsequently confirmed to be cardioprotective by several groups in various experimental models (2, 8, 9, 13, 16, 18, 21, 37, 41–43). Despite these reports, our recent study (35) in conscious rats found that the protection afforded by postconditioning is modest relative to that afforded by early and late PC, being observed only when the index ischemic insult is <45 min. Because postconditioning is thought to be more clinically relevant than PC (38), exploring interventions that enhance the cardioprotective effect of postconditioning may be useful.

As postconditioning has been suggested to share many of the signaling mechanisms of early PC (14, 15), it is possible that there may be redundancy between these two forms of protection, which would limit potential synergy if both were performed to protect the heart from ischemia-reperfusion injury. Indeed, previous studies (13, 37) have found that the infarct-sparing effect of postconditioning is not enhanced by early PC.

Given that late PC provides cardioprotection via the induction of new proteins (5), we hypothesized that postconditioning may lead to additive cardioprotection. COX-2 has been implicated as a mediator of the cardioprotection of late PC induced by ischemia (31), heat stress (3), and pharmacological stimuli such as opioids (19, 25), nicorandil (36), atorvastatin (4), and anesthetics (34). Therefore, the present study sought to determine whether combining late PC and postconditioning enhances the infarct-sparing effect and, if so, whether this additive effect is mediated by a COX-2-related mechanism.

MATERIALS AND METHODS

The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 86-23).

The conscious rat model of myocardial ischemia and reperfusion has been described in detail previously (35). Briefly, Fischer-344 male rats (Harlan Sprague Dawley, 9–12 wk of age) were anesthetized and instrumented under sterile conditions with a balloon occluder around the left anterior descending coronary artery and with bipolar leads anchored to the chest. Rats were allowed to recover for a minimum of 7 days after surgery.

Myocardial infarct size

Chronically instrumented rats were assigned to two subsets (Fig. 1); myocardial infarction was induced in conscious rats by performing a 45-min (*subset 1*) or 60-min (*subset 2*) coronary occlusion (as the index ischemia) followed by 24 h of reperfusion. In each subset, rats were further divided into 5 groups as follows: no further intervention [controls (*groups I* and *VI*)], PC with 12 cycles of 2-min occlusion/2-min reperfusion 24 h before the index ischemia [PC (*groups II* and *VII*)], postconditioning with 20 cycles of 10-s occlusion/10-s reperfusion at the onset of reperfusion following the index ischemia [postconditioning (*groups III* and *VIII*)], or both PC and postconditioning plus vehicle [PC + postconditioning (*groups IV* and *IX*)] or the COX-2 inhibitor celecoxib [PC + postconditioning + celecoxib (*groups V* and *X*)]. Celecoxib (Searle) was dissolved in 20% DMSO in normal saline and

administered intraperitoneally 10 min prior to the end of the index ischemia at a dose of 3 mg/kg (Fig. 1). All rats received diazepam (4 mg/kg ip) 10 min before the index ischemia to relieve the stress caused by the sustained coronary occlusion. No antiarrhythmic agents were given at any time. At the conclusion of the study, rats were killed. The occluded/reperfused vascular bed and the infarct were identified by postmortem perfusion of the heart with triphenyltetrazolium and Phthalo blue dye, as previously described (35). Infarct size was calculated by computerized videoplanimetry (35).

Myocardial levels of COX-2

An additional five groups of rats in *subset 3* underwent a 45-min coronary occlusion and were killed 6 min after reperfusion (Fig. 2). *Group XI* (control) received no further intervention, whereas *groups XII, XIII, XIV, and XV* received PC, postconditioning, PC + postconditioning, and PC + postconditioning + celecoxib, respectively, as in *subset 1*. At the end of 6 min of reperfusion, rats were euthanized, and myocardial samples were rapidly removed from the ischemic-reperfused region and from the nonischemic region [posterior left ventricular (LV) wall], frozen in liquid N₂, and stored at -140°C until used. Myocardial COX-2 protein expression was analyzed by Western immunoblot analysis using rabbit polyclonal anti-COX-2 antibody (Cayman Chemical) and normalized to the corresponding Ponceau S signal determined by densitometric analysis of the Ponceau S stain record, as previously described (31). To assess the enzymatic activity of COX-2, myocardial PGE₂ content was measured. PGE₂ was extracted from tissue samples, purified by using the PGE₂ affinity sorbent, and measured using an enzyme immunoassay kit (Cayman Chemical) as previously described (31).

Statistical analysis

Data were analyzed by a one-way ANOVA followed by Student's *t*-tests with the Bonferroni correction using SigmaStat 2.0 for Windows. The relationship between infarct size and risk region size was compared among groups with analysis of covariance (ANCOVA) using the size of the risk region as the covariate (27, 33, 35) and was assessed by linear regression analysis using the least-squares method. ANCOVA was performed using SPSS 8.0 for Windows. *P* < 0.05 was considered significant. Results are reported as means ± SE.

RESULTS

A total of 102 conscious rats were used (40, 39, and 23 rats, respectively, for the experiments of *subsets 1, 2, and 3*). Table 1 summarizes the initial assignments and exclusions. Rats that developed ventricular fibrillation but cardioverted spontaneously were included in the final analysis. There were no significant differences in heart rate throughout the experimental protocol among groups (Table 2). In addition, body weight, total LV weight, and the size of the region at risk did not differ among the groups (Table 3).

Myocardial infarct size

In *subset 1* (rats subjected to a 45-min coronary occlusion), the myocardial infarct size was $64.5 \pm 2.9\%$ of the risk region in the control group (*group I*) and was reduced significantly in the PC group (*group II*: $43.0 \pm 5.0\%$, *P* < 0.05 vs. *group I*) but not in the postconditioning group (*group III*: $57.0 \pm 2.4\%$, *P* = not significant) (Figs. 3 and 4), confirming our previous observation that the cardioprotection of postconditioning is weaker than that of PC (35). The infarct size was $32.8 \pm 6.4\%$ in the PC + postconditioning group (*group IV*; *P* < 0.05 vs. *groups I and III*) (Figs. 3 and 4). The infarct size in *group IV* was ~25% smaller compared with *group II*, although the difference was not statistically significant, suggesting that the combined interventions produced a more robust infarct-sparing effect.

In rats subjected to a 60-min coronary occlusion (*subset 2*), the myocardial infarct size was $73.6 \pm 3.0\%$ of the risk region in the control group (*group VI*) (Figs. 5 and 6). Neither late PC alone (*group VII*: $66.4 \pm 2.7\%$) nor postconditioning alone (*group VIII*: $71.7 \pm 4.5\%$) reduced the infarct size significantly compared with *group VI*. However, the infarct size was significantly reduced by combining late PC and postconditioning (*group IX*: $54.5 \pm 1.7\%$, $P < 0.05$ vs. *groups VI, VII, and VIII*, respectively) (Figs. 5 and 6), indicating that the two interventions exerted additive cardioprotection. The COX-2 inhibitor celecoxib effectively abrogated the additive infarct-sparing effect of late PC plus postconditioning in both subsets [*group V* (Figs. 3 and 4) and *group X* (Figs. 5 and 6)].

Expression of COX-2 protein

As shown in Fig. 7, a weak COX-2 signal was detected in control rats (*group I*). COX-2 expression in the ischemic-reperfused region was upregulated by PC (*group XII*) but, as expected, not by postconditioning (*group XIII*). COX-2 protein levels in the nonischemic region were not elevated and were similar among groups (data not shown).

Myocardial PGE₂ content

To determine whether the increase in COX-2 protein expression was associated with increased COX-2 enzymatic activity, myocardial PGE₂ content was measured using enzyme immunoassay. As shown in Fig. 8, PGE₂ content in the nonischemic region was similar among groups. PC (*group XII*) resulted in a significant increase in PGE₂ content in the ischemic-reperfused region ($+143 \pm 38\%$ vs. *group XI*, $P < 0.05$). Postconditioning (*group XIII*), on the other hand, slightly increased PGE₂ content ($+35 \pm 48\%$ vs. *group XI*), and the change was not significant ($P = 0.49$). Interestingly, the combination of PC and postconditioning (*group XIV*) resulted in a further increase in PGE₂ content vis-à-vis PC alone ($+48 \pm 10\%$ vs. *group XII*, $P < 0.05$). The increased PGE₂ content was completely abrogated by celecoxib (*group XV*) (Fig. 8). The fact that in *group XIV* there was a further increase in PGE₂ content (Fig. 8) without additional COX-2 protein expression (Fig. 7) suggests that postconditioning enhances the enzymatic activity of PC-induced COX-2.

DISCUSSION

The salient findings of this study can be summarized as follows: 1) late PC alone reduced the infarct size induced by a 45-min coronary occlusion but not by a 60-min coronary occlusion, whereas postconditioning alone had no significant infarct-sparing effect after either a 45- or 60-min occlusion; 2) the combination of late PC and postconditioning resulted in a robust infarct-sparing effect in rats subjected to either a 45- or 60-min occlusion, demonstrating an additive protective effect of the two interventions; 3) celecoxib completely abrogated the infarct-sparing actions of the combined interventions, suggesting that COX-2 plays a critical role in this additive effect; 4) late PC resulted in increased myocardial COX-2 protein expression with a concomitant increase in the myocardial content of COX-2-derived PGE₂; and 5) the combination of late PC and postconditioning further increased the myocardial PGE₂ content without a concomitant increase in COX-2 protein expression, implying that postconditioning enhances the enzymatic activity of the COX-2 induced by late PC. Taken together, these results demonstrate that the combination of late PC and postconditioning produces an additive cardioprotective effect via a COX-2-mediated mechanism.

Ischemic postconditioning is a newly described cardioprotective strategy (44) thought to be clinically more feasible than other therapies because it can be implemented during reperfusion (38). A number of investigations have found that postconditioning shares many signaling mechanisms with early PC (15) and exhibits similar physiological and cellular

aspects of protection (39). Although one study (42) reported that combining early PC with postconditioning induced additive protection in rabbits, two other studies found no such effect in rats (37) or dogs (13), implying potentially overlapping mechanisms. Unlike early PC, late PC provides cardioprotection via the synthesis of new proteins (5, 6). Therefore, if postconditioning is preceded by the intervention of late PC, in principle there should be an additive protective effect.

In this study, we sought to rigorously determine whether the combination of late PC and postconditioning exerts an additive cardioprotective effect. To this end, we used the same conscious rat model used in our previous study (35). Although conscious animal models are more expensive, time consuming, and technically demanding than open-chest models, we reasoned that they yield results that are less prone to the influence of spurious factors associated with anesthesia and surgery and, therefore, are more clinically relevant (35). We confirmed our previous finding (35) that postconditioning alone fails to reduce infarct size after a 45-min ischemic period.

Using this model, we first examined the additive effects of late PC and postconditioning in rats subjected to a 45-min index ischemia (*subset 1*). We found that the combined interventions resulted in a robust limitation of infarct size, which did not differ significantly from that induced by late PC alone (Fig. 3). It is possible, however, that an additive effect may have been masked by the robust protection afforded by late PC after a 45-min occlusion, which could have made it difficult for another protective mechanism to be operative. Therefore, to further investigate whether an additive effect exists, we increased the index ischemia to 60 min (*subset 2*). The rationale was to increase infarct size so that it would exceed the limit of cardioprotection afforded by late PC. Under these conditions, the size of the infarct in control rats was large (74% of the risk region), and neither late PC alone nor postconditioning alone decreased it compared with controls (Fig. 5). When the two interventions were combined, infarct size declined to 55% of the risk region, significantly less than either late PC alone (66%) or postconditioning alone (72%) (Fig. 5). These data clearly demonstrate that, after a relatively severe ischemic insult, the combination of late PC and postconditioning produces additive cardioprotection in conscious rats.

Evidence that COX-2 plays an essential role in conferring the cardioprotection afforded by late PC was provided by studies in rabbits (31) and mice (10, 12, 23, 40) in the setting of ischemia-induced PC. Subsequent studies have shown that COX-2 mediates the delayed infarct-sparing effects of a panoply of pathophysiological stimuli and pharmacological agents, such as opioid receptor agonists (20, 22, 25), nicorandil (36), heat stress (3), anesthetics (1, 34), diazoxide (1), and atorvastatin (4). In the present study, we found that ischemic PC dramatically increased COX-2 protein expression (over 6-fold above control) 24 h later (Fig. 7), corroborating our previous findings in conscious rabbits (31). To assess COX enzymatic activity, we measured the myocardial content of PGE₂, which is the main product of COX-2 activity in PC myocardium, as shown by our previous study (31). We found that late PC was associated with a 2.4-fold increase in myocardial PGE₂ content compared with controls (Fig. 8), indicating a significant increase in COX activity, consistent with our prior results (31). A novel finding of this study is that postconditioning further increased COX enzymatic activity, as evidenced from a 3.6-fold increase in PGE₂ content under conditions in which there was no further increase in COX-2 protein expression (Fig. 7). Because this increase in PGE₂ content was associated with enhanced cardioprotection and because COX-2 inhibition completely abrogated this additive effect (Figs. 3 and 5), the present study suggests that the enzymatic activity of the COX-2 protein induced by late PC is further increased by postconditioning to provide additional cardioprotection. To our knowledge, this is the first evidence that postconditioning enhances COX-2 activity. The mechanism remains unclear but may involve increased NO availability. Previous studies

have shown that postconditioning is associated with the activation of NO synthase (37, 42) and that NO activates COX-2 protein induced by late PC (32).

Because postconditioning appears to enhance the protective effects of late PC by enhancing COX-2 activity, our findings raise the possibility that the benefits of this additive cardioprotection may be lost in patients who take the COX inhibitor acetylsalicylic acid (aspirin) (26). However, since aspirin is a relatively weak inhibitor of COX-2 activity (24), it appears that the doses used for prophylaxis of acute myocardial infarction and stroke would be unlikely to affect the additive cardioprotection of postconditioning. In this regard, we (30) have previously shown that the administration of aspirin either at an antithrombotic dose (5 mg/kg) or at an analgesic/antipyretic dose (10 mg/kg) does not interfere with the cardioprotective effects of late PC against myocardial stunning. Further studies will be necessary to definitely address this issue.

In conclusion, in conscious rats subjected to a relatively severe ischemic insult (45-min coronary occlusion), the ability of postconditioning to confer protection appears to be COX-2 dependent. That is, postconditioning does not afford protection if COX-2 is expressed at low levels (i.e., at constitutively expressed COX-2 levels), but it does confer additional protection (above and beyond that of late PC alone) when COX-2 protein is upregulated (e.g., during the late phase of PC). Thus, the combination of late PC and postconditioning produces additive protection, likely due to a postconditioning-induced enhancement of COX-2 activity. These findings have conceptual and mechanistic implications for the pathophysiology of postconditioning. Furthermore, they may facilitate the development of strategies that enhance the cardioprotection of postconditioning. For example, if patients at risk are pharmacologically preconditioned to induce expression of COX-2, they could be further protected by postconditioning.

Acknowledgments

GRANTS

This work was supported in part by National Heart, Lung, and Blood Institute Grants R01-HL-74351, R01-HL-55757, R01-HL-68088, R01-HL-70897, R01-HL-76794, and R01-HL-78825 and by American Heart Association Grant 0355391B.

REFERENCES

1. Alcindor D, Krolkowski JG, Pagel PS, Warltier DC, Kersten JR. Cyclooxygenase-2 mediates ischemic, anesthetic, and pharmacologic preconditioning in vivo. *Anesthesiology*. 2004; 100:547–554. [PubMed: 15108967]
2. Argaud L, Gateau-Roesch O, Raissy O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. *Circulation*. 2005; 111:194–197. [PubMed: 15642769]
3. Arnaud C, Joyeux-Faure M, Godin-Ribuot D, Ribaut C. COX-2: an in vivo evidence of its participation in heat stress-induced myocardial preconditioning. *Cardiovasc Res*. 2003; 58:582–588. [PubMed: 12798431]
4. Atar S, Ye Y, Lin Y, Freeberg SY, Nishi SP, Rosanio S, Huang MH, Uretsky BF, Perez-Polo JR, Birnbaum Y. Atorvastatin-induced cardioprotection is mediated by increasing inducible nitric oxide synthase and consequent S-nitrosylation of cyclooxygenase-2. *Am J Physiol Heart Circ Physiol*. 2006; 290:H1960–H1968. [PubMed: 16339820]
5. Bolli R. The late phase of preconditioning. *Circ Res*. 2000; 87:972–983. [PubMed: 11090541]
6. Bolli R. Preconditioning: a paradigm shift in the biology of myocardial ischemia. *Am J Physiol Heart Circ Physiol*. 2007; 292:H19–H27. [PubMed: 16963615]
7. Bolli R, Manchikalapudi S, Tang XL, Takano H, Qiu Y, Guo Y, Zhang Q, Jadoon AK. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is

- mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ Res.* 1997; 81:1094–1107. [PubMed: 9400391]
8. Chiari PC, Bienengraeber MW, Pagel PS, Krolikowski JG, Kersten JR, Warltier DC. Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: evidence for anesthetic-induced postconditioning in rabbits. *Anesthesiology.* 2005; 102:102–109. [PubMed: 15618793]
 9. Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K. Postconditioning via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK1/2. *Am J Physiol Heart Circ Physiol.* 2005; 289:H1618–H1626. [PubMed: 15937101]
 10. Dawn B, Xuan YT, Guo Y, Rezazadeh A, Stein AB, Hunt G, Wu WJ, Tan W, Bolli R. IL-6 plays an obligatory role in late preconditioning via JAK-STAT signaling and upregulation of iNOS and COX-2. *Cardiovasc Res.* 2004; 64:61–71. [PubMed: 15364614]
 11. Downey JM. Ischemic preconditioning: nature's own cardioprotective intervention. *Trends Cardiovasc Med.* 1992; 2:170–176. [PubMed: 21239250]
 12. Guo Y, Bao W, Wu WJ, Shinmura K, Tang XL, Bolli R. Evidence for an essential role of cyclooxygenase-2 as a mediator of the late phase of ischemic preconditioning in mice. *Basic Res Cardiol.* 2000; 95:479–484. [PubMed: 11192369]
 13. Halkos ME, Kerendi F, Corvera JS, Wang NP, Kin H, Payne CS, Sun HY, Guyton RA, Vinten-Johansen J, Zhao ZQ. Myocardial protection with postconditioning is not enhanced by ischemic preconditioning. *Ann Thorac Surg.* 2004; 78:961–969. [PubMed: 15337028]
 14. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. *Am J Physiol Heart Circ Physiol.* 2005; 288:H971–H976. [PubMed: 15358610]
 15. Hausenloy DJ, Tsang A, Yellon DM. The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. *Trends Cardiovasc Med.* 2005; 15:69–75. [PubMed: 15885573]
 16. Heusch G, Buchert A, Feldhaus S, Schulz R. No loss of cardioprotection by postconditioning in connexin 43-deficient mice. *Basic Res Cardiol.* 2006; 101:354–356. [PubMed: 16568250]
 17. Hoshida S, Yamashita N, Otsu K, Hori M. The importance of manganese superoxide dismutase in delayed preconditioning: involvement of reactive oxygen species and cytokines. *Cardiovasc Res.* 2002; 55:495–505. [PubMed: 12160946]
 18. Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E, Kremastinos DT. Protection from postconditioning depends on the number of short ischemic insults in anesthetized pigs. *Basic Res Cardiol.* 2006; 101:502–507. [PubMed: 16830072]
 19. Jiang X, Shi E, Nakajima Y, Sato S. COX-2 mediates morphine-induced delayed cardioprotection via an iNOS-dependent mechanism. *Life Sci.* 2006; 78:2543–2549. [PubMed: 16325209]
 20. Jiang X, Shi E, Nakajima Y, Sato S, Ohno K, Yue H. Cyclooxygenase-1 mediates the final stage of morphine-induced delayed cardioprotection in concert with cyclooxygenase-2. *J Am Coll Cardiol.* 2005; 45:1707–1715. [PubMed: 15893191]
 21. Kin H, Zatta AJ, Lofye MT, Amerson BS, Halkos ME, Kerendi F, Zhao ZQ, Guyton RA, Headrick JP, Vinten-Johansen J. Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. *Cardiovasc Res.* 2005; 67:124–133. [PubMed: 15949476]
 22. Kodani E, Xuan YT, Shinmura K, Takano H, Tang XL, Bolli R. δ -Opioid receptor-induced late preconditioning is mediated by cyclooxygenase-2 in conscious rabbits. *Am J Physiol Heart Circ Physiol.* 2002; 283:H1943–H1957. [PubMed: 12384473]
 23. Li Q, Guo Y, Xuan YT, Lowenstein CJ, Stevenson SC, Prabhu SD, Wu WJ, Zhu Y, Bolli R. Gene therapy with inducible nitric oxide synthase protects against myocardial infarction via a cyclooxygenase-2-dependent mechanism. *Circ Res.* 2003; 92:741–748. [PubMed: 12702642]
 24. Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, Vane JR. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci USA.* 1993; 90:11693–11697. [PubMed: 8265610]
 25. Patel HH, Hsu AK, Gross GJ. COX-2 and iNOS in opioid-induced delayed cardioprotection in the intact rat. *Life Sci.* 2004; 75:129–140. [PubMed: 15120566]

26. Przyklenk K, Heusch G. Late preconditioning against myocardial stunning. Does aspirin close the “second window” of endogenous cardioprotection? *J Am Coll Cardiol.* 2003; 41:1195–1197. [PubMed: 12679221]
27. Qiu Y, Rizvi A, Tang XL, Manchikalapudi S, Takano H, Jadoon AK, Wu WJ, Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol Heart Circ Physiol.* 1997; 273:H2931–H2936.
28. Reimer KA, Jennings RB. Ischemic preconditioning: a brief review. *Basic Res Cardiol.* 1996; 91:1–4. [PubMed: 8660246]
29. Shinmura K, Bolli R, Liu SQ, Tang XL, Kodani E, Xuan YT, Srivastava S, Bhatnagar A. Aldose reductase is an obligatory mediator of the late phase of ischemic preconditioning. *Circ Res.* 2002; 91:240–246. [PubMed: 12169650]
30. Shinmura K, Kodani E, Xuan YT, Dawn B, Tang XL, Bolli R. Effect of aspirin on late preconditioning against myocardial stunning in conscious rabbits. *J Am Coll Cardiol.* 2003; 41:1183–1194. [PubMed: 12679220]
31. Shinmura K, Tang XL, Wang Y, Xuan YT, Liu SQ, Takano H, Bhatnagar A, Bolli R. Cyclooxygenase-2 mediates the cardioprotective effects of the late phase of ischemic preconditioning in conscious rabbits. *Proc Natl Acad Sci USA.* 2000; 97:10197–10202. [PubMed: 10963682]
32. Shinmura K, Xuan YT, Tang XL, Kodani E, Han H, Zhu Y, Bolli R. Inducible nitric oxide synthase modulates cyclooxygenase-2 activity in the heart of conscious rabbits during the late phase of ischemic preconditioning. *Circ Res.* 2002; 90:602–608. [PubMed: 11909825]
33. Takano H, Manchikalapudi S, Tang XL, Qiu Y, Rizvi A, Jadoon AK, Zhang Q, Bolli R. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation.* 1998; 98:441–449. [PubMed: 9714095]
34. Tanaka K, Ludwig LM, Krolkowski JG, Alcindor D, Pratt PF, Kersten JR, Pagel PS, Warltier DC. Isoflurane produces delayed preconditioning against myocardial ischemia and reperfusion injury: role of cyclooxygenase-2. *Anesthesiology.* 2004; 100:525–531. [PubMed: 15108964]
35. Tang XL, Sato H, Tiwari S, Dawn B, Bi Q, Li Q, Shirk G, Bolli R. Cardioprotection by postconditioning in conscious rats is limited to coronary occlusions <45 min. *Am J Physiol Heart Circ Physiol.* 2006; 291:H2308–H2317. [PubMed: 16815986]
36. Tang XL, Xuan YT, Zhu Y, Shirk G, Bolli R. Nicorandil induces late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol Heart Circ Physiol.* 2004; 286:H1273–H1280. [PubMed: 14684373]
37. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of “modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res.* 2004; 95:230–232. [PubMed: 15242972]
38. Vinten-Johansen J, Zhao ZQ, Jiang R, Zatta AJ. Myocardial protection in reperfusion with postconditioning. *Expert Rev Cardiovasc Ther.* 2005; 3:1035–1045. [PubMed: 16292995]
39. Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F. Postconditioning—a new link in nature’s armor against myocardial ischemia-reperfusion injury. *Basic Res Cardiol.* 2005; 100:295–310. [PubMed: 15793629]
40. Xuan YT, Guo Y, Zhu Y, Han H, Langenbach R, Dawn B, Bolli R. Mechanism of cyclooxygenase-2 upregulation in late preconditioning. *J Mol Cell Cardiol.* 2003; 35:525–537. [PubMed: 12738234]
41. Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning’s protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. *Basic Res Cardiol.* 2005; 100:57–63. [PubMed: 15614590]
42. Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. *J Am Coll Cardiol.* 2004; 44:1103–1110. [PubMed: 15337225]
43. Zatta AJ, Kin H, Lee G, Wang N, Jiang R, Lust R, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Vinten-Johansen J. Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling. *Cardiovasc Res.* 2006; 70:315–324. [PubMed: 16443207]

44. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol*. 2003; 285:H579–H588. [PubMed: 12860564]

Experimental Protocol: Myocardial Infarct Size
(Subset 1: 45-min Coronary Occlusion)

Fig. 1. Experimental protocols for the experiments of infarct size. PC, preconditioning; PostC, postconditioning; '', seconds; ', minutes; O, occlusion; R, reperfusion.

**Experimental Protocol: Myocardial COX-2 Levels
(Subset 3)**

Fig. 2.
Experimental protocols for the experiments of cyclooxygenase-2 (COX-2).

Fig. 3.

Infarct size after a 45-min coronary occlusion and 24 h of reperfusion. ○, Individual hearts; ●, group means $\pm$ SE.

Fig. 4.

Relationship between the size of the region at risk and size of myocardial infarction in rats exposed to a 45-min coronary occlusion. Both individual values and the regression lines obtained by linear regression analysis are shown for *groups I-V*. In all groups, infarct size was positively and linearly related to the size of region at risk. Analysis of covariance (ANCOVA) demonstrated that the regression lines for *groups II* and *IV* were significantly shifted downward and to the right compared with that for *group I* ($P < 0.05$ for each comparison), indicating that for any given size of the region at risk, infarct size was smaller in rats that received either late PC alone or late PC + PostC. In *group V*, the line was similar to that for *group I*, indicating that COX-2 inhibition abrogated the additive protective effect of the combined interventions.

Fig. 5.

Infarct size after a 60-min coronary occlusion and 24 h of reperfusion. ○, Individual hearts; ●, group means $\pm$ SE.

Fig. 6.

Relationship between the size of the region at risk and size of myocardial infarction in rats exposed to a 60-min coronary occlusion. Both individual values and the regression lines obtained by linear regression analysis are shown for *groups VI–X*. In all groups, infarct size was positively and linearly related to the size of region at risk. ANCOVA demonstrated that the regression line for *group IX* was significantly shifted downward and to the right compared with that for *groups VI–VIII* ($P < 0.05$ for each comparison), indicating that for any given size of region at risk, infarct size was smaller in rats that received the combined interventions of late PC and PostC. In *group X*, the line was significantly different from that in *group IX*, indicating that COX-2 inhibition abrogated the additive protective effect of the combined interventions.

Fig. 7.

Expression of COX-2 protein in rat myocardium. *A*: representative immunoblots of COX-2 expression in the ischemic-reperfused region. The Ponceau S staining reflects equal protein loading. *B*: densitometric analysis of COX-2 signals. In all samples, densitometric measurements of COX-2 immunoreactivity were expressed as percentages of the average value measured in control rats. PC, 12 cycles of 2-min ischemia/2-min reperfusion applied 24 h prior to occlusion (late PC); PostC, 20 cycles of 10-s ischemia/10-s reperfusion at the onset of reperfusion following occlusion; Celecoxib, 3 mg/kg ip celecoxib administered 10 min before reperfusion. Data were normalized to the Ponceau S signals and are expressed as means $\pm$ SE.

Fig. 8.

Content of PGE₂ in rat myocardium. PGE₂ was extracted from the ischemic-reperfused and nonischemic regions and expressed as picograms per milligram of protein. Groups were as described in Fig. 7. Data are means $\pm$ SE.

Table 1
Exclusions from the study

	Initial Assignment	Exclusions		Final Analysis
		Died of VF During Occlusion	Technical Failure	
Subset 1: 45-min occlusion				
Group I (control)	8	1		7
Group II (PC)	8		1	7
Group III (PostC)	8	1		7
Group IV (PC + PostC)	8		1	7
Group V (PC + PostC + celecoxib)	8	1	1	6
Subset 2: 60-min occlusion				
Group VI (control)	8			6
Group VII (PC)	8	1	1	6
Group VIII (PostC)	8	1	1	6
Group IX (PC + PostC)	8			6
Group X (PC + PostC + celecoxib)	7	1		6
Subset 3: myocardial levels of COX-2				
Group XI (control)	5	1		4
Group XII (PC)	4			4
Group XIII (PostC)	5	1		4
Group XIV (PC + PostC)	5	1		4
Group XV (PC + PostC + celecoxib)	4			4
Total	102	13	5	84

Values are numbers of rats excluded per group. VF, ventricular fibrillation; PC, late preconditioning (12 cycles of 2-min ischemia/2-min reperfusion applied 24 h prior to occlusion); PostC, postconditioning (20 cycles of 10-s ischemia/10-s reperfusion at the onset of reperfusion following occlusion); COX-2, cyclooxygenase-2.

Table 2

Heart rate

	n	Baseline	Occlusion			Reperfusion		
			15 min	30 min	45 min	60 min	5 min	30 min
Subset 1								
Group I	7	477±10	443±16	443±15	440±12		457±8	453±7
Group II	7	490±9	437±15	433±13	432±14		450±10	440±12
Group III	7	463±16	447±20	430±24	427±25		410±25	427±22
Group IV	7	473±13	440±7	450±7	420±5		423±10	423±10
Group V	6	457±12	433±8	430±11	417±14		420±12	410±11
Subset 2								
Group VI	6	473±14	442±5	428±9	431±7	435±8	441±8	438±10
Group VII	6	480±9	437±15	427±7	437±16	440±15	460±9	450±7
Group VIII	6	486±13	463±13	443±14	440±9	442±12	470±10	463±6
Group IX	6	470±11	453±7	440±9	443±11	451±11	453±7	457±8
Group X	6	490±10	460±9	450±13	466±7	468±8	467±7	467±7
Subset 3								
Group XI	4	482±10	462±9	448±15	450±9		443±6	
Group XII	4	473±9	454±11	462±9	456±13		460±9	
Group XIII	4	474±10	470±8	445±12	467±8		442±10	
Group XIV	4	468±13	440±12	438±13	425±15		431±9	
Group XV	4	480±15	460±7	462±9	455±10		462±8	

Values are means±SE (in beats/min); *n*, no. of animals/group. Subsets and groups were as described in Table 1 and Figs. 1 and 2.

Table 3

Body weight, total LV weight, and area at risk

	n	Body Weight, g	Total LV Weight, g	Area at Risk	
				Weight, g	%LV
Subset 1					
Group I	7	244±5	0.61±0.04	0.16±0.02	27.0±1.9
Group II	7	245±7	0.57±0.04	0.16±0.01	28.8±2.1
Group III	7	264±8	0.64±0.03	0.18±0.03	28.7±3.1
Group IV	7	275±3	0.64±0.03	0.23±0.01	33.4±1.7
Group V	6	262±10	0.68±0.05	0.22±0.01	32.4±2.4
Subset 2					
Group VI	6	260±5	0.65±0.06	0.18±0.01	28.5±2.3
Group VII	6	243±7	0.59±0.04	0.19±0.02	29.6±2.8
Group VIII	6	271±9	0.58±0.03	0.16±0.01	26.7±1.4
Group IX	6	269±7	0.69±0.03	0.21±0.01	30.2±2.1
Group X	6	272±4	0.70±0.02	0.20±0.01	28.6±1.8
Subset 3					
Group XI	4	279±6			
Group XII	4	270±8			
Group XIII	4	265±4			
Group XIV	4	246±11			
Group XV	4	268±10			

Values are means±SE; *n*, no. of animals/group. Subsets and groups were as described in Table 1 and Figs. 1 and 2. LV, left ventricle.