



Review Article

Cardioprotective Function of Inducible Nitric Oxide Synthase and Role of Nitric Oxide in Myocardial Ischemia and Preconditioning: an Overview of a Decade of Research

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R. BOLLI. Cardioprotective Function of Inducible Nitric Oxide Synthase and Role of Nitric Oxide in Myocardial Ischemia and Preconditioning: an Overview of a Decade of Research. *Journal of Molecular and Cellular Cardiology* (2001) 33, 1897–1918. Over the past decade, an enormous number of studies (>100) have focused on the role of nitric oxide (NO) in myocardial ischemia. It is important to distinguish the function of NO in unstressed (non-preconditioned) myocardium from its function in preconditioned myocardium (i.e. myocardium that has shifted to a defensive phenotype in response to stress). Of the 92 studies that have examined the role of NO in modulating the severity of ischemia/reperfusion injury in non-preconditioned myocardium, the vast majority [67 (73%)] have concluded that NO (either endogenous or exogenous) has a protective effect and only 11 (12%) found a detrimental effect. The proportion of studies supporting a cytoprotective role of NO is similar *in vivo* [35 (71%) out of 49] and *in vitro* [32 (74%) out of 43]. With regard to the delayed acquisition of tolerance to ischemia [late preconditioning (PC)], overwhelming evidence indicates a critical role of NO in this phenomenon. Specifically, enhanced biosynthesis of NO by eNOS is essential to trigger the late phase of ischemia-induced and exercise-induced PC, and enhanced NO production by iNOS is obligatorily required to mediate the anti-stunning and anti-infarct actions of late PC elicited by five different stimuli (ischemia, adenosine A₁ agonists, opioid δ_1 agonists, endotoxin derivatives and exercise). Thus, NO plays a dual role in the pathophysiology of the late phase of PC, acting initially as the trigger and subsequently as the mediator of this adaptive response (“NO hypothesis of late PC”). The diversity of the PC stimuli that converge on iNOS implies that the upregulation of this enzyme is a central mechanism whereby the myocardium protects itself from ischemia. The NO hypothesis of late PC has thus revealed a cytoprotective function of iNOS in the heart, a novel paradigm which has recently been extended to other tissues, including kidney and intestine. Other corollaries of this hypothesis are that the heart responds to stress in a biphasic manner, utilizing eNOS as an immediate but short-term response and iNOS as a delayed but long-term defense, and that the fundamental difference between non-preconditioned and late preconditioned myocardium is the tissue level of iNOS-derived NO, which is tonically higher in the latter compared with the former. Hence, late PC can be viewed as a state of enhanced NO synthesis.

The NO hypothesis of late PC has important therapeutic implications. In experimental animals, administration of NO donors in lieu of ischemia can faithfully reproduce the molecular and functional aspects of ischemia-induced late PC, indicating that NO is not only necessary but also sufficient to induce late PC. The recent demonstration that nitroglycerin also induces late PC in patients provides proof-of-principle for the concept that nitrates could be used as a PC-mimetic therapy for the prophylaxis of ischemic injury in the clinical arena. This novel application of nitrates could be as important as, or perhaps even more important than, their current use as antianginal and preload-reducing agents. In addition, gene transfer of either eNOS or iNOS has been shown to replicate the infarct-sparing actions of ischemic PC, suggesting that NOS gene therapy could be an effective strategy for alleviating ischemia/reperfusion injury. Ten years of research have demonstrated that NO plays a fundamental biological role in protecting the heart against ischemia/reperfusion injury. The time has come to

translate this enormous body of experimental evidence into clinically useful therapies by harnessing the cytoprotective properties of NO.

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KEY WORDS: Nitric oxide synthase; Nitrates; Preconditioning; Myocardial ischemia; Gene therapy; Nitroglycerin.

Introduction

The discovery that nitric oxide (NO), an air pollutant, serves as a mediator of biological processes has been one of the most remarkable advances in biomedical research in the 20th century. Over the past 15 years, a deluge of articles have implicated this ubiquitous molecule in virtually every physiological and pathophysiological process. It is thus not surprising that the role of NO in myocardial ischemia and in myocardial preconditioning (PC) has become the focus of considerable interest. Indeed, this has been one of the fastest growing areas in basic cardiovascular research in recent times, and one that has yielded important new insights into the molecular basis of myocardial ischemia/reperfusion injury. More than 100 articles have focused on this issue over the past decade. The purpose of this essay is to review this enormous body of information and distill it to few, essential concepts that are widely supported by the literature. Particular emphasis is placed upon the cardioprotective properties of inducible NO synthase (iNOS), an enzyme whose function in the cardiovascular system was enigmatic until recently and that has now been identified as a major defensive mechanism against ischemia.

Conceptually, it is important to distinguish the role of NO in modulating ischemia/reperfusion injury in unstressed (non-preconditioned or naïve) myocardium from the role of NO in preconditioned myocardium (i.e. myocardium that has shifted to a defensive phenotype in response to stress). Because the phenomenon of PC encompasses two temporally and pathophysiologically distinct phases with widely different clinical implications,^{1,2} the role of NO in the early and late phases of PC needs to be examined separately.

Role of Nitric Oxide in Modulating Ischemia-Reperfusion Injury in Non-preconditioned Myocardium

Over the past 10 years, an enormous number of studies (at least 92) have examined the role of NO in modulating the severity of ischemia/reperfusion

injury in non-preconditioned myocardium (Tables 1 and 2).³⁻⁹⁴ Most of these investigations have utilized a pharmacological approach to either inhibit endogenous NOS activity with L-arginine analogs during myocardial ischemia/reperfusion or enhance NO availability by administering NO donors (e.g. SNAP, DETA/NO, nitroglycerin) or precursors (L-arginine) prior to, during, or after ischemia; a minority of studies have utilized genetically engineered mice deficient in eNOS or iNOS.

Even a cursory analysis of these studies dispels two widely held misconceptions. First, it has been asserted that the role of NO in myocardial ischemia is deleterious (due to its putative pro-oxidant actions) and that the literature on this topic is controversial, due to the inconsistency of the results obtained by different investigators. The review of the literature summarized in Tables 2 and 3, however, does not support this interpretation. Of the 92 studies that have examined the role of NO in myocardial ischemia,³⁻⁹⁴ 67 (73%) have concluded that NO (either endogenous or exogenous) has a protective effect (Tables 1 and 2; Fig. 1). This conclusion was based upon the observation that inhibition or genetic ablation of endogenous NOS activity exacerbated myocardial ischemia/reperfusion injury and that exogenous supplementation of NO (via NO donors or NO precursors) ameliorated the severity of such injury. The protective effect of NO was apparent in the setting of both regional and global ischemia, in different species, and using either infarction or stunning as the endpoint. Inhibition or genetic ablation of NOS was found to have no discernible effect on ischemia/reperfusion injury in 18 studies (20%) and to be detrimental in 11 studies (12%). (In one investigation,⁹⁵ L-NA reduced heart rate significantly during ischemia; therefore, conclusions regarding the effect of L-NA on infarct size in this study cannot be made. This study is not included in Table 1 or in Figure 1.)

Another commonly held misconception is that *in vitro* studies tend to favor a detrimental role of NO in myocardial ischemia/reperfusion injury whereas *in vivo* studies suggest a protective role. Again, the analysis of the literature does not support this concept. As shown in Tables 1 and 2 and in

Table 1 Studies examining the role of NO in modulating ischemia/reperfusion injury in non-preconditioned myocardium

<i>In vivo</i> studies					
Authors	Source of NO tested		Conclusion regarding the role of NO	Notes	Reference
	Endogenous	Exogenous			
Johnson <i>et al.</i> , <i>J Pharmacol Exp Ther</i> 1990; 252 : 35–41		Exogenous	Beneficial		3
Johnson <i>et al.</i> , <i>Am Heart J</i> 1990; 119 : 530–537		Exogenous	Beneficial		4
Johnson <i>et al.</i> , <i>Crit Care Med</i> 1991; 19 : 244–252		Exogenous	Beneficial		5
Weyrich <i>et al.</i> , <i>Circulation</i> 1992; 86 : 279–288		Exogenous	Beneficial		6
Siegfried <i>et al.</i> , <i>J Pharmacol Exp Ther</i> 1992; 260 : 668–675		Exogenous	Beneficial		7
Nakanishi <i>et al.</i> , <i>Am J Physiol</i> 1992; 263 : H1650–H1658		Exogenous	Beneficial		8
Matheis <i>et al.</i> , <i>Am J Physiol</i> 1992; 262 : H616–H620	Endogenous		Detrimental	Global ischemia	9
Yao and Gross, <i>Circ Res</i> 1993; 73 : 1193–1201	Endogenous		No effect		10
Lefer <i>et al.</i> , <i>J Cardiovasc Pharmacol</i> 1993; 22 : S34–S43		Exogenous	Beneficial		11
Patel <i>et al.</i> , <i>Biochem Biophys Res Commun</i> 1993; 194 : 234–238	Endogenous		Detrimental		12
Lefer <i>et al.</i> , <i>Circulation</i> 1993; 88 : 2337–2350		Exogenous	Beneficial		13
Hasebe <i>et al.</i> , <i>Circulation</i> 1993; 88 : 2862–2871	Endogenous		Beneficial		14
Martorana <i>et al.</i> , <i>Eur J Pharmacol</i> 1994; 257 : 267–273		Exogenous	Beneficial		15
Hartman <i>et al.</i> , <i>J Pharmacol Exp Ther</i> 1994; 270 : 1071–1076	Endogenous		No effect		16
Pernow <i>et al.</i> , <i>Eur Heart J</i> 1994; 15 : 1712–1719		Exogenous	Beneficial		17
Ehring <i>et al.</i> , <i>Circulation</i> 1994; 90 : 1368–1385	Endogenous		No effect		18
Richard <i>et al.</i> , <i>Br J Pharmacol</i> 1995; 115 : 1532–1538	Endogenous		No effect		19
Hoshida <i>et al.</i> , <i>J Pharmacol Exp Ther</i> 1995; 274 : 413–418	Endogenous		Beneficial		20
Williams <i>et al.</i> , <i>Cardiovasc Res</i> 1995; 30 : 79–86	Endogenous		Beneficial		21
Hartman, <i>Ann Thorac Surg</i> 1995; 60 : 789–792		Exogenous	No effect		22
Pabla <i>et al.</i> , <i>Am J Physiol</i> 1995; 269 : H1113–H1121		Exogenous	Beneficial		23
Engelman <i>et al.</i> , <i>Ann Thorac Surg</i> 1995; 60 : 1275–1281		Exogenous	Beneficial	Global ischemia	24
Sato <i>et al.</i> , <i>J Thorac Cardiovasc Surg</i> 1995; 110 : 302–314		Exogenous	Beneficial	Global ischemia	25
Mizumura <i>et al.</i> , <i>Cardiovasc Res</i> 1995; 29 : 482–489		Exogenous	Beneficial		26
Zhu <i>et al.</i> , <i>Am Heart J</i> 1996; 132 : 91–100		Exogenous	No effect		27
Liu <i>et al.</i> , <i>Hypertension</i> 1996; 27 : 7–13	Endogenous		No effect		28
Hoshida <i>et al.</i> , <i>J Am Coll Cardiol</i> 1996; 27 : 902–909		Exogenous	No effect		29
Hoshida <i>et al.</i> , <i>J Pharmacol Exp Ther</i> 1996; 278 : 741–746		Exogenous	Beneficial		30
Mizumura <i>et al.</i> , <i>Cardiovasc Res</i> 1996; 32 : 274–285		Exogenous	Beneficial		31

continued

Table 1 Studies examining the role of NO in modulating ischemia/reperfusion injury in non-preconditioned myocardium—*continued*

Authors	<i>In vivo</i> studies		Conclusion regarding the role of NO	Notes	Reference
	Source of NO tested				
	Endogenous	Exogenous			
Carrier <i>et al.</i> , <i>Ann Thorac Surg</i> 1996; 61 : 1651–1657		Exogenous	Beneficial	Global ischemia	32
Zhao <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1997; 29 : 1567–1576	Endogenous		Beneficial		33
Bolli <i>et al.</i> , <i>Circ Res</i> 1997; 81 : 1094–1107	Endogenous		Beneficial§		34
Node <i>et al.</i> , <i>Circulation</i> 1997; 96 : 1953–1963	Endogenous		Beneficial		35
Mizuno <i>et al.</i> , <i>J Thorac Cardiovasc Surg.</i> 1997; 113 : 379–389	Endogenous	Exogenous	Beneficial	Global ischemia	36
Mori <i>et al.</i> , <i>Cardiovasc Res</i> 1998; 40 : 113–123	Endogenous		Detrimental		37
Lee <i>et al.</i> , <i>Ann Thorac Surg</i> 1998; 65 : 1353–1359		Exogenous	Detrimental		38
Yang <i>et al.</i> , <i>Hypertension</i> 1999; 34 : 24–30	Endogenous	Exogenous	Beneficial		
Hoshida <i>et al.</i> , <i>Circulation</i> 1999; 99 : 434–440	Endogenous		No effect	eNOS KO	39
Jones <i>et al.</i> , <i>Am J Physiol</i> 1999; 276 : H1567–H1573	Endogenous		Beneficial	eNOS KO	40
Xi <i>et al.</i> , <i>Circulation</i> 1999; 99 : 2157–2163	Endogenous		Beneficial		41
Kis <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1999; 31 : 1229–1241	Endogenous		Beneficial		42
Shinmura <i>et al.</i> , <i>Am J Physiol</i> 1999; 277 : H2495–H2503		Exogenous	Beneficial		43
Xi <i>et al.</i> , <i>Am J Physiol</i> 1999; 277 : H2418–H2424	Endogenous		Beneficial		44
Wang <i>et al.</i> , <i>Am J Hypertens</i> 1999; 12 : 174–182	Endogenous		Detrimental		45
Heusch <i>et al.</i> , <i>Circ Res</i> 2000; 87 : 146–152	Endogenous		Beneficial		46
Zhao <i>et al.</i> , <i>Circulation</i> 2000; 102 : 902–907	Endogenous		Beneficial		94
Gonon <i>et al.</i> , <i>J Cardiovasc Pharmacol</i> 2000; 36 : 405–412	Endogenous		Beneficial		47
Post <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 725–733	Endogenous		No effect		48
Gourine <i>et al.</i> , <i>Am J Physiol</i> 2001; 280 : H1105–H1112	Endogenous		Beneficial		49

§ Although L-NA did not exacerbate stunning in unpaced hearts, it did so when heart rate was kept constant by pacing. In one investigation,⁹⁵ L-NA reduced heart rate significantly during ischemia; therefore, conclusions regarding the effect of L-NA on infarct size in this study cannot be made. This study is not included in Table 1 or in Figure 1.

Figure 1, 71% of the *in vivo* studies (35 out of 49) have concluded that NO is protective (i.e. that inhibition of NO biosynthesis is detrimental and/or that enhanced NO availability is beneficial); only four *in vivo* studies have concluded that NO is detrimental. This breakdown is not different among the *in vitro* studies, where 74% (32 out of 43) have concluded that NO is protective during myocardial ischemia/reperfusion.

In summary, the overwhelming majority of the 92 studies published in the past decade supports a

cytoprotective role of NO (either endogenous or exogenous) in myocardial ischemia/reperfusion injury, both *in vitro* and *in vivo*. The fact that only a small fraction (12%) of the published articles reported a deleterious effect of NO suggests that this finding is not widely reproducible and may be model-dependent. Considering the numerous differences among investigations with respect to experimental settings, species, experimental protocols, dosages of drugs etc., a certain degree of variance in outcomes should be expected.

Table 2 Studies examining the role of NO in modulating ischemia/reperfusion injury in non-preconditioned myocardium

<i>In vitro</i> studies					
Authors	Source of NO tested		Conclusion regarding the role of NO	Notes	Reference
	Endogenous	Exogenous			
Woditsch and Schror, <i>Am J Physiol</i> 1992; 263 :H1390–H1396	Endogenous		No effect		51
Depre <i>et al.</i> , <i>Circulation</i> 1995; 92 : 1911–1918	Endogenous		Detrimental	Blood-perfused hearts	52
Hiramatsu <i>et al.</i> , <i>J Thorac Cardiovasc Surg</i> 1995; 109 : 81–86	Endogenous	Exogenous	Beneficial		53
Weselcouch <i>et al.</i> , <i>Am J Physiol</i> 1995; 268 : H242–H249	Endogenous		No effect		54
Woditsch and Schror, <i>Agents Actions Suppl</i> 1995; 45 : 189–194		Exogenous	Beneficial		55
Naseem <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1995; 27 : 419–426	Endogenous		Detrimental		56
Massoudy <i>et al.</i> , <i>J Cardiovasc Pharmacol</i> 1995; 25 : 440–447		Exogenous	Beneficial		57
Takeuchi <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1995; 27 : 1405–1414		Exogenous	Beneficial		58
Hiramatsu <i>et al.</i> , <i>Ann Thorac Surg</i> 1995; 60 : 1187–1192		Exogenous	Beneficial	Blood-perfused hearts	59
Hiramatsu <i>et al.</i> , <i>J Thorac Cardiovasc Surg</i> 1995; 110 : 172–179		Exogenous	Beneficial		Blood-perfused hearts
Konorev <i>et al.</i> , <i>J Pharmacol Exp Ther</i> 1995; 274 : 200–206		Exogenous	Beneficial		61
Amrani <i>et al.</i> , <i>Cardiovasc Res.</i> 1995; 30 : 200–204	Endogenous		Beneficial		62
Schultz and Wambolt, <i>Cardiovasc Res</i> 1995; 30 : 432–439	Endogenous	Exogenous	Beneficial		63
Woolfson <i>et al.</i> , <i>Circulation</i> 1995; 91 : 1545–1551	Endogenous	Exogenous	Detrimental		64
Wanna <i>et al.</i> , <i>J Thorac Cardiovasc Surg</i> 1995; 110 : 1054–1062	Endogenous		Beneficial		65
Beresewicz <i>et al.</i> , <i>Cardiovasc Res</i> 1995; 30 : 1001–1008	Endogenous		Beneficial		66
Engelman <i>et al.</i> , <i>J Thorac Cardiovasc Surg</i> 1995; 110 : 1047–1053	Endogenous		Beneficial		67
Pabla <i>et al.</i> , <i>Circ Res</i> 1996; 78 : 65–72	Endogenous	Exogenous	Beneficial		68
Bugge and Ytrehus, <i>J Mol Cell Cardiol</i> 1996; 28 : 2333–2341	Endogenous		No effect		69
Engelman <i>et al.</i> , <i>Circulation</i> 1996; 94 : II407–II411		Exogenous	Beneficial/ Detrimental#		70
Ferdinandy <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1997; 29 : 3321–3333	Endogenous		No effect		71
Massoudy <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1997; 29 : 535–544	Endogenous		No effect/ Beneficial*		72
Luo <i>et al.</i> , <i>Ann Thorac Surg</i> 1997; 64 : 993–998	Endogenous		No effect		73
Wang <i>et al.</i> , <i>J Cardiovasc Pharmacol</i> 1997; 29 : 291–296		Exogenous	Beneficial†		74
Yasmin <i>et al.</i> , <i>Cardiovasc Res</i> 1997; 33 : 422–432	Endogenous		Detrimental		75
Brunner, <i>J Mol Cell Cardiol</i> 1997; 29 : 2363–2374	Endogenous	Exogenous	Beneficial		76
duToit <i>et al.</i> , <i>Br J Pharmacol</i> 1998; 123 : 1159–1167		Exogenous	Beneficial		77

continued

Table 2 Studies examining the role of NO in modulating ischemia/reperfusion injury in non-preconditioned myocardium—*continued*

<i>In vitro</i> studies					
Authors	Source of NO tested		Conclusion regarding the role of NO	Notes	Reference
	Endogenous	Exogenous			
Izhar <i>et al.</i> , <i>J Cardiovasc Surg</i> 1998; 39 : 321–329		Exogenous	Beneficial		78
Das <i>et al.</i> , <i>Ann NY Acad Sci</i> 1998; 865 : 297–308		Exogenous	Beneficial		79
Weinbrenner <i>et al.</i> , <i>Cardiovasc Res</i> 1998; 38 : 678–684	Endogenous		No effect		80
Agullo <i>et al.</i> , <i>Am J Physiol</i> 1999; 276 : H1574–H1580		Exogenous	Beneficial		81
Baker <i>et al.</i> , <i>Ann NY Acad Sci</i> 1999; 874 : 236–253		Exogenous	Beneficial		82
Csonka <i>et al.</i> , <i>Circulation</i> 1999; 100 : 2260–2266	Endogenous		No effect/ Beneficial§		83
Flogel <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1999; 31 : 827–836	Endogenous		Detrimental	eNOS KO	84
Csont <i>et al.</i> , <i>Br J Pharmacol</i> 1999; 128 : 1427–1434		Exogenous	Beneficial		85
Nakano <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 1159–1167	Endogenous		No effect		86
Sumeray MS <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 35–42	Endogenous		Beneficial		87
Kanno <i>et al.</i> , <i>Circulation</i> 2000; 101 : 2742–2748	Endogenous		Beneficial	eNOS KO	88
Horimoto <i>et al.</i> , <i>J Surg Res</i> 2000; 92 : 56–63		Exogenous	Beneficial		89
Hampton <i>et al.</i> , <i>Am J Physiol</i> 2000; 279 : H260–H268		Exogenous	Beneficial	iNOS KO	90
Hannan <i>et al.</i> , <i>J Surg Res</i> 2000; 93 : 127–132	Endogenous		Beneficial	eNOS KO	91
Young <i>et al.</i> , <i>Am J Physiol</i> 2000; 279 : H1453–H1459	Endogenous		Beneficial		92
Lochner <i>et al.</i> , <i>Am J Physiol</i> 2000; 279 : H2752–H2765	Endogenous		No effect		93

† Spontaneous NO release had no effect; VEGF-induced NO release protective.

L-arginine beneficial before or during ischemia, detrimental during reperfusion.

§ L-NA had no effect at 4.6 $\mu\text{mol/l}$ but was protective at 46 $\mu\text{mol/l}$.

* NOS inhibitors had no effect but abolished endothelin-1 protection.

Role of Nitric Oxide in the Early Phase of Preconditioning

The early phase of PC is a protective mechanism that develops immediately (within minutes) after the stimulus, which can be either brief ischemia, rapid pacing, pharmacologic manipulations (i.e. administration of G-coupled receptor agonists or NO donors), or other interventions.^{1,96} The 15 studies that have examined the role of NO as a trigger of the development of the early phase of PC^{10,19,44,49,54,64,69,71,83,86,89,93,97–99} are summarized in Table 3. A total of eight studies have addressed ischemia-induced early PC.^{49,54,64,83,86,93,97,98} Of these, five have

concluded that inhibition of endogenous NO synthesis with NOS antagonists does not abrogate the protective effects of ischemic PC against cell death,^{49,54,64,86} post-ischemic dysfunction,⁵⁴ and arrhythmias.⁹⁸ The lack of a necessary role of NO in triggering the early phase of ischemia-induced PC was observed both *in vitro*^{54,64,86} and *in vivo*^{49,98} In contrast, three studies concluded that NOS inhibitors abrogate early PC. Vegh *et al.*⁹⁷ found that L-NAME attenuated the antiarrhythmic effects of the early phase of ischemic PC in open-chest dogs. Arrhythmias, however, are not a conventional endpoint of PC studies and their accurate assessment requires careful analysis of collateral perfusion and

Table 3 Studies examining the role of NO as a trigger of the early phase of preconditioning

Authors	Setting	PC stimulus	Endpoint	Results	Reference
Ischemia-induced early PC					
Vegh <i>et al.</i> , <i>Br J Pharmacol</i> 1992; 107 : 648–652	<i>In vivo</i>	Ischemia	Arrhythmias	PC abolished	97
Weselcouch <i>et al.</i> , <i>Am J Physiol</i> 1995; 268 :H242–H249	<i>In vitro</i>	Ischemia	Post-ischemic dysfunction, LDH	PC not abolished	54
Lu <i>et al.</i> , <i>J Cardiovasc Pharmacol</i> 1995; 25 : 524–530	<i>In vivo</i>	Ischemia	Arrhythmias	PC not abolished	98
Woolfson <i>et al.</i> , <i>Circulation</i> 1995; 91 : 1545–1551	<i>In vitro</i>	Ischemia	Infarct size	PC not abolished	64
Csonka <i>et al.</i> , <i>Circulation</i> 1999; 100 : 2260–2266	<i>In vitro</i>	Ischemia and pacing	Function, LDH, arrhythmias	PC abolished	83
Post <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 725–733	<i>In vivo</i>	Ischemia	Infarct size	PC not abolished	49
Nakano <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 1159–1167	<i>In vitro</i>	Ischemia	Infarct size	PC not abolished	86
Lochner <i>et al.</i> , <i>Am J Physiol</i> 2000; 279 : H2752–H2765	<i>In vitro</i>	Ischemia	Function	PC abolished	93
Pacing-induced early PC					
Ferdinandy <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1997; 29 : 3321–3333	<i>In vitro</i>	Pacing	Function, LDH	PC abolished	71
Pharmacologically-induced early PC					
Yao and Gross, <i>Circ Res</i> 1993; 73 : 1193–1201	<i>In vivo</i>	Acetylcholine	Infarct size	PC not abolished	10
Richard <i>et al.</i> , <i>Br J Pharmacol</i> 1995; 115 : 1532–1538	<i>In vivo</i>	Acetylcholine	Infarct size	PC abolished	19
Bugge and Ytrehus, <i>J Mol Cell Cardiol</i> 1996; 28 : 2333–2341	<i>In vitro</i>	Bradykinin	Infarct size	PC not abolished	69
Shinmura <i>et al.</i> , <i>Am J Physiol</i> 1999; 277 :H2495–H2503	<i>In vivo</i>	Exogenous NO (SNAP)	Stunning	PC not induced	44
Lochner <i>et al.</i> , <i>Am J Physiol</i> 2000; 279 : H2752–H2765	<i>In vitro</i>	Exogenous NO (SNP, SNAP, L-arginine)	Function	PC induced	93
Hill <i>et al.</i> , <i>Circulation</i> 2001; 104 : 694–699	<i>In vivo</i>	Exogenous NO (NTG)	Infarct size	PC induced	99
Horimoto <i>et al.</i> , <i>J Surg Res</i> 2000; 92 : 56–63	<i>In vitro</i>	NO(L-arginine)	Infarct size	PC induced	89
Nakano <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 1159–1167	<i>In vitro</i>	Exogenous NO (SNAP)	Infarct size	PC induced	86

ischemic zone size. Csonka *et al.*⁸³ reported in isolated rat hearts that L-NA blocked the protective effects of ischemia- and pacing-induced PC on LDH release, arrhythmias, and post-ischemic ventricular dysfunction, concomitant with enhanced NO accumulation during the test ischemia/reperfusion. Lochner *et al.*⁹³ found that L-NAME abrogated the beneficial effects of ischemic PC in isolated rat hearts and that these effects could be mimicked by pretreatment with nitroprusside or SNAP (but not L-arginine). With respect to pacing-induced early PC, Ferdinandy *et al.*⁷¹ reported in isolated rat hearts that the protective effects of this form of early PC

on post-ischemic dysfunction and LDH release were blocked in the presence of NOS inhibitors. Only few studies are available in the area of pharmacologically-induced early PC. Yao and Gross¹⁰ and Bugge and Ytrehus⁶⁹ found that acetylcholine-induced and bradykinin-induced PC, respectively, were not blocked by NOS inhibitors in open-chest dogs¹⁰ and isolated rat hearts,⁶⁹ in contrast, Richard *et al.*¹⁹ reported that acetylcholine-induced early PC was blocked by L-NA in open-chest rats.

One important form of pharmacologically-induced early PC is that elicited by NO donors. Although the studies reviewed above indicate that

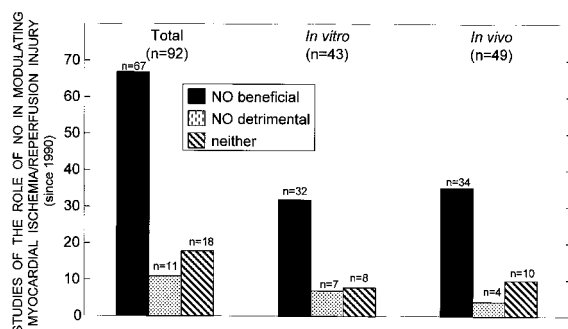


Figure 1 Summary of the original papers that have examined the role of nitric oxide (NO) in modulating myocardial ischemia/reperfusion injury in non-preconditioned myocardium. A total of 92 papers have been published since 1990. Of these, 67 (73%) have concluded that NO (either endogenous or exogenous) protects against ischemia/reperfusion injury, as evidenced by the fact that inhibition or genetic ablation of endogenous NOS activity exacerbated ischemia/reperfusion injury and/or that exogenous supplementation of NO (via NO donors or NO precursors) ameliorated the severity of such injury. (In one investigation,⁹⁵ L-NA reduced heart rate significantly during ischemia; therefore, conclusions regarding the effect of L-NA on infarct size in this study cannot be made. This study is not included in Table 1 or in Figure 1.). The percentage of studies supporting a protective role of NO is similar in the *in vitro* setting [32 out of 43 (74%)] and in the *in vivo* setting [35 out of 49 (71%)]. Among the *in vitro* studies, the numbers do not add up because two investigations^{63,75} concluded that endogenous NO is detrimental whereas exogenous NO is beneficial, one study⁷⁰ concluded that exogenous NO can be either beneficial or detrimental depending on the timing of treatment, and one investigation⁷³ concluded that spontaneous NO release has no effect but VEGF-induced NO release is protective (see Table 2). Therefore, each of these four studies^{63,70,73,75} is counted twice under the *in vitro* category.

endogenous NO is not necessary for ischemia-induced early PC, considerable evidence suggests that administration of *exogenous* NO can elicit an early PC-like protection. Nakano *et al.*⁸⁶ demonstrated in isolated rabbit hearts subjected to a 30-min coronary occlusion followed by 2 h of reperfusion that pretreatment with SNAP in lieu of ischemia elicited a powerful infarct-sparing effect comparable to that elicited by ischemic PC. The protective effects of SNAP were unlikely to be due to continued presence of the drug during ischemia, as SNAP was removed 10 min before the coronary occlusion. SNAP-induced early PC was abrogated by MPG and chelerythrine, indicating that its cardioprotective effects are mediated by generation of ROS and activation of PKC—a signaling pathway analogous to that involved in NO-induced late PC.^{100–102} Similarly, Hill *et al.*⁹⁹ found that i.v. infusion of nitroglycerin ending 1 h prior to a 30-min coronary

occlusion elicited an infarct-sparing effect in conscious rabbits, although the magnitude of this early PC effect was less than that of the late PC effect observed 24–72 h after nitroglycerin. Interestingly, Banerjee *et al.*¹⁰¹ found that the same dose of i.v. nitroglycerin failed to induce an early PC effect against myocardial stunning, which is consistent with the notion that ischemia itself does not induce early PC against stunning.¹⁰³

In summary, the preponderance of the evidence indicates that endogenous biosynthesis of NO is not required for the development of the early phase of PC, at least when ischemia is the PC stimulus. However, supplementation of exogenous NO can elicit an early PC-like protective effect against infarction (but not against stunning). The conclusion that NO is sufficient but not necessary for early PC is not surprising, since it is now appreciated that early PC is a multifactorial adaptation that can be triggered by a number of different chemical signals which are released during the initial ischemic stress, including adenosine, bradykinin, opioid agonists, ROS, catecholamines, etc.¹ The redundancy of the signals that can trigger early PC helps to rationalize why elimination of one of these stimuli (NO) is not sufficient to block the development of protection in the presence of the other stimuli.

Role of Nitric Oxide in the Late Phase of Preconditioning

In addition to an early phase of protection that lasts 1–2 h, ischemic PC elicits a late phase of cardioprotection that appears 12–24 h after the stimulus and persists for 72 h (reviewed in reference 2).^{104–106} Unlike the early phase, which protects against infarction but not against stunning, the late phase of ischemic PC protects against both infarction and stunning.^{2,103} In view of this, and in view of the sustained duration of the protective effect, the late phase of PC may have greater clinical relevance than the early phase. The late phase of PC can be elicited not only by ischemia but also by various pharmacologic and non-pharmacologic manipulations, including activation of adenosine A₁ and A₃ receptors, opioid δ_1 receptors, bradykinin B₂ receptors,¹⁰⁷ administration of NO donors, endotoxin, endotoxin derivatives and cytokines, and exposure to exercise.² Although early and late PC can be triggered by the same stimuli, the mechanisms involved are fundamentally different: early PC results from a rapid (and short-lived) post-translational modification of pre-existing proteins,

whereas late PC is due to *de novo* synthesis of cardioprotective proteins secondary to genetic reprogramming of the heart.²

In recent years, considerable evidence has accumulated which indicates a pivotal role of NO in the late phase of PC induced by ischemia, pharmacologic manipulations, and exercise (Fig. 2).^{2,108} Since these studies have been reviewed in detail elsewhere,^{2,108} only a synopsis of the salient concepts will be presented here. In discussing this topic, it is important to bear in mind the distinction between the “triggers” of late PC (i.e. the molecular species that initiate this adaptation when a PC stimulus is applied) and the “mediators” of late PC (i.e. the molecular species that confer cardioprotection 24–72 h after the PC stimulus²). The essential aspects of the role of NO in late PC can be summarized as follows (Fig. 2):

- (i) Enhanced biosynthesis of NO is necessary to trigger both ischemia-induced^{109,110} and exercise-induced¹¹¹ late PC (Table 4), indicating that NO is an important chemical signal whereby the heart shifts to a defensive phenotype upon exposure to ischemic or exercise stress.
- (ii) The source of the NO that triggers late PC during ischemia or exercise is most likely eNOS, because (1) the development of late PC is blocked by pretreatment with the non-selective NOS inhibitor L-NA, but not with the relatively selective iNOS inhibitors aminoguanidine and S-methylisothiourea,³⁴ and (2) direct measurements of NOS activity have shown a burst of calcium-dependent NOS (eNOS and/or nNOS) activity immediately after the PC ischemia, which is abolished by the same dose of L-NA that abrogates the development of late PC.¹¹²
- (iii) Administration of NO donors (DETA/NO, SNAP, nitroglycerin) in lieu of ischemia elicits a delayed cardioprotection against both myocardial stunning and infarction which is indistinguishable from that elicited by ischemia (Table 4).^{99–102,113} Thus, NO is not only necessary, but also sufficient to induce late PC.
- (iv) The signaling pathway whereby NO (either endogenous or exogenous) induces late PC involves the generation of reactive oxygen species,¹⁰⁰ the activation of the ϵ isotype of protein kinase C,^{102,114} the subsequent recruitment of Src and Lck tyrosine kinases,^{114–116} and the activation of the transcription factor NF- κ B,¹¹⁷ culminating in transcriptional activation of the *iNOS* gene.¹¹⁸ This is the same signaling pathway that is responsible for the development of ischemia-induced late PC.² Upregulation of iNOS after ischemic PC also requires activation of the JAK-STAT signaling pathway (specifically, of JAK1, JAK2, STAT1 and STAT3).¹¹⁹ Thus, at least two signaling pathways (PKC ϵ -Src/Lck-NF- κ B and JAK1/2-STAT1/3) are involved in the transcriptional activation of the *iNOS* gene following ischemic PC. Whether these two pathways act in parallel or in series remains to be determined. The fact that both NF- κ B and STAT1/3 are required to activate the *iNOS* gene is consistent with the notion that multiple transcription factors, acting in an additive or synergistic manner, are necessary to induce iNOS expression in other systems.²
- (v) The precise mechanism whereby NO activates PKC ϵ remains unclear. NO could activate the enzyme directly via nitrosylation. In addition, NO is known to react with $\cdot\text{O}_2^-$ (which is generated in response to myocardial ischemia/reperfusion) to form peroxynitrite (ONOO $^-$) which, in turn, could activate PKC ϵ either directly or via its reactive byproducts, such as $\cdot\text{OH}$.¹⁰⁸ This latter scenario is supported by the finding that NO donor-induced activation of PKC ϵ can be blocked by the ONOO $^-$ and $\cdot\text{OH}$ scavenger MPG.¹⁰⁰ Irrespective of the exact mechanism, the finding that the initiation of ischemia-induced late PC is blocked by both L-NA^{109,110} and MPG¹²⁰ suggests that both NO and $\cdot\text{O}_2^-$ are necessary to trigger this phenomenon, either by reacting with one another or via independent (parallel) mechanisms.
- (vi) Clinically relevant NO donors, such as nitroglycerin, can elicit late PC when given either by the i.v. route^{99,101} or by the transdermal route.⁹⁹ The infarct-sparing effects of nitroglycerin are robust and persist for 72 h after a single i.v. infusion or after a single transdermal patch administration.⁹⁹ Furthermore, the ability of nitroglycerin to elicit late PC is not hampered by the presence of tolerance to the hemodynamic actions of nitrates.⁹⁹ These findings support novel applications of nitrates for the prophylaxis of ischemic injury in patients.²
- (vii) In addition to its role as a trigger of ischemia-induced late PC, NO also plays a critical role as a mediator of cardioprotection 24 h later (Table 5).^{34,121–123} The NOS isoform responsible for generating the NO that confers car-

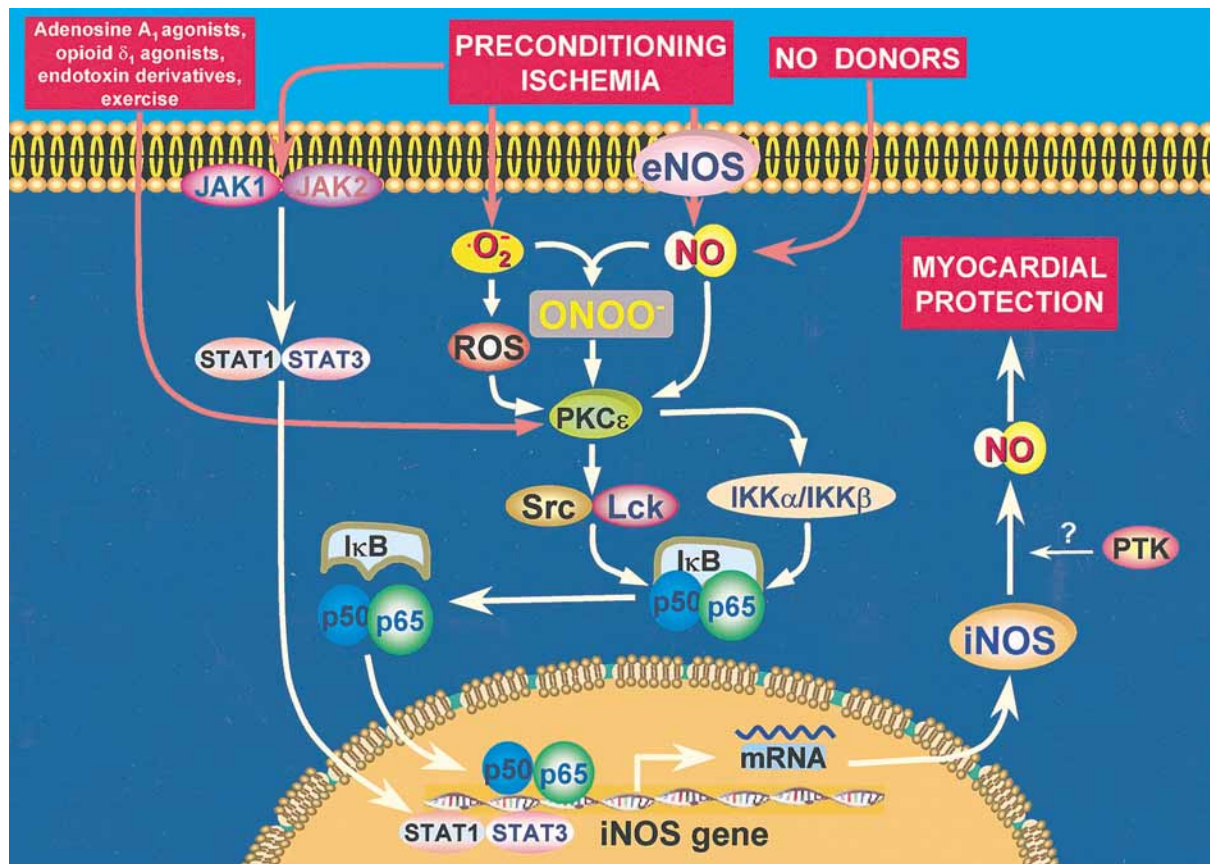


Figure 2 Schematic representation of the role of nitric oxide (NO) in the late phase of preconditioning (PC). A brief episode of myocardial ischemia/reperfusion causes increased production of NO (most likely via eNOS) and $\cdot\text{O}_2^-$ [leading to formation of secondary reactive oxygen species (ROS)], NO and $\cdot\text{O}_2^-$ activate the ϵ isoform of PKC via mechanisms that remain to be elucidated. Both NO and $\cdot\text{O}_2^-$ -derived ROS could directly activate PKC ϵ via nitrosylation and oxidative modification, respectively; alternatively, NO and $\cdot\text{O}_2^-$ are known to react to form ONOO $^-$ which, in turn, could activate PKC ϵ . PKC ϵ activation then triggers a complex signaling cascade that involves Src and/or Lck tyrosine kinases and probably other kinases, leading to phosphorylation of I κ B α and mobilization of the transcription factor NF- κ B. Both IKK-dependent serine phosphorylation (on residues 32 and 36) and IKK-independent tyrosine phosphorylation (on residue 42) of I κ B α appear to be required for ischemic PC-induced activation of NF- κ B. Other transcription factors are most likely involved as well. In particular, ischemic PC has recently been found to activate JAK1 and JAK2 with subsequent tyrosine phosphorylation and activation of STAT1 and STAT3, which is essential for iNOS upregulation. Binding of NF- κ B and STAT1/3 to the iNOS promoter results in transcriptional activation of the iNOS gene and synthesis of new iNOS proteins, which leads to a phenotype characterized by tonically enhanced NO biosynthesis (preconditioned phenotype). There is evidence that post-translational modulation of iNOS by Src and/or Lck is important for iNOS activity on day 2. iNOS-derived NO, in turn, protects the myocardium from recurrent ischemia via mechanisms that remain unclear. One plausible mechanism is the activation of COX-2-dependent synthesis of cardioprotective prostanoids (e.g. PGE $_2$ and PGI $_2$). A similar activation of PKC and downstream kinases can be elicited pharmacologically by NO donors, adenosine A $_1$ agonists, δ_1 -opioid receptor agonists, endotoxin derivatives, and physical exercise, leading to a preconditioned phenotype that is similar to that elicited by an ischemic stress. An interesting corollary of this paradigm is that the heart possesses a feed-forward mechanism whereby eNOS-derived NO can upregulate iNOS (eNOS-dependent iNOS induction).

dioprotection is iNOS, as demonstrated by both pharmacological^{34,121,122} and genetic¹²³ studies. The first documentation of the role of iNOS as an obligatory mediator of late PC was provided in 1997.³⁴ Since then, several studies have convincingly demonstrated that ischemic PC induces upregulation of iNOS protein expression and activity 24 h later¹¹².

^{115,118,119,123,124} and that inhibition of either iNOS protein expression¹²³ or iNOS activity^{34,121,122} completely abrogates the salutary effects of late PC. iNOS is specifically up-regulated in cardiac myocytes (Table 5).¹²⁴

(viii) In addition to ischemia-induced late PC, iNOS is an obligatory mediator of several forms of pharmacologic PC, including late PC induced

Table 4 Studies examining the role of NO as a trigger of the late phase of preconditioning

Authors	Setting	PC stimulus	Endpoint	Results	Reference
Ischemia-induced late PC					
Bolli <i>et al.</i> , <i>Circ Res</i> 1997; 81 : 42–52	<i>In vivo</i>	Ischemia	Stunning	PC abolished	109
Qiu <i>et al.</i> , <i>Am J Physiol</i> 1997; 273 : H2488–H2494	<i>In vivo</i>	Ischemia	Infarction	PC abolished	110
Pharmacologically-induced late PC					
Takano <i>et al.</i> , <i>Circ Res</i> 1998; 83 : 73–84	<i>In vivo</i>	DETA/NO, SNAP	Stunning and infarction	PC induced	100
Banerjee, <i>et al.</i> , <i>Am J Physiol</i> 1999; 277 : H2488–H2494	<i>In vivo</i>	Nitroglycerin	Stunning	PC induced	101
Guo <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1999; 31 :A11 (abstr.)	<i>In vivo</i>	DETA/NO	Infarction	PC induced	113
Ping <i>et al.</i> , <i>Circ Res</i> 1999; 84 : 587–604	<i>In vivo</i>	DETA/NO, SNAP	Stunning and infarction	PC induced	102
Hill <i>et al.</i> , <i>Circulation</i> 2001; 104 : 694–699	<i>In vivo</i>	Nitroglycerin	Infarction	PC induced	99
Exercise-induced late PC					
Guo <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2001; 33 : A41 (abstr.)	<i>In vivo</i>	Exercise	Infarction	PC induced	111

by stimulation of adenosine A₁ (but not A₃) receptors,^{125–127} opioid δ₁ receptors,¹²⁵ NO donors,¹²⁸ endotoxin derivatives,^{129–132} and exercise¹³³ (Table 5). Thus, iNOS-dependent biosynthesis of NO appears to be a final common pathway whereby the heart protects itself in response to diverse stresses.^{2,108}

- (ix) In contrast to the three studies mentioned above,^{125–127} one study in isolated hearts¹³⁴ has concluded that the protection afforded by adenosine A₁ receptor-induced late PC is not iNOS-dependent (Table 5).
- (x) The precise mechanism whereby iNOS-derived NO protects against ischemia 24–72 h after a PC stimulus remains unknown, although recent data indicate that this involves the activation of guanylate cyclase.¹³⁵
- (xi) The upregulation of iNOS protein expression and activity after the PC stimulus is modest,^{112,115,118,119,123,124} an order of magnitude less than that observed after a lethal dose of lipopolysaccharide.^{123,124} This is an important observation, as it may explain the apparent paradox whereby an enzyme known to produce cytotoxicity, such as iNOS, is recruited by the heart to confer protection against ischemic insults. Specifically, it has been proposed^{123,124} that a relatively modest upregulation of iNOS, such as during late PC, is cardioprotective whereas a massive upregulation of iNOS, such as during inflammation or septic shock, is detrimental.

The Nitric Oxide Hypothesis of Late Preconditioning

In summary, the studies reviewed above support a complex paradigm in which two different NOS isoforms are sequentially involved in the pathophysiologic cascade of late PC, with eNOS generating the NO that triggers late PC on day 1^{109–111} and iNOS then generating the NO that protects against ischemia 24–72 h later.^{34,121–123,125–132,133} This led to the formulation in 1998 of the “NO hypothesis of late PC”,¹⁰⁸ which states that NO plays a dual role in the pathophysiology of the late phase of PC, acting initially as the trigger and subsequently as the mediator of this adaptive response. These two roles are interconnected, for it is the burst of NO production during the PC stimulus that causes increased expression and activity of iNOS 24 h later.^{112,118} In the 3 years following its formulation,¹⁰⁸ the NO hypothesis of late PC has been convincingly validated by pharmacologic, biochemical and molecular genetic evidence accumulated in various models of cardiac adaptation,^{34,109–113,118,121–133} and thus should now be regarded as a proven hypothesis. The studies published to date on the role of NO as a trigger and mediator of late PC are categorized in Tables 4 and 5, and a schematic illustration of the NO hypothesis of late PC is presented in Figure 2. An interesting and conceptually novel corollary of this paradigm is that the heart possesses a feed-forward mechanism whereby eNOS-derived NO can upregulate iNOS

Table 5 Studies examining the role of NO as a mediator of the late phase of preconditioning

Authors	Setting	PC stimulus	Endpoint	Results	Reference
Ischemia-induced late PC					
Bolli <i>et al.</i> , <i>Circ Res</i> 1997; 81 : 1094–1107	<i>In vivo</i>	Ischemia	Stunning	PC abolished	34
Takano <i>et al.</i> , <i>Circulation</i> 1998; 98 : 441–449	<i>In vivo</i>	Ischemia	Infarction	PC abolished	121
Imagawa <i>et al.</i> , <i>Br J Pharmacol</i> 1999; 126 : 701–708	<i>In vivo</i>	Ischemia	Infarction	PC abolished	122
Guo <i>et al.</i> , <i>Proc Natl Acad Sci USA</i> 1999; 96 : 11507–11512	<i>In vivo</i>	Ischemia	Infarction	PC abolished	123
Pharmacologically-induced late PC					
Bell <i>et al.</i> , <i>Circulation</i> 1999; 100 :I–242 (abstr.)	<i>In vitro</i>	Adenosine A ₁ agonist	Infarction	PC not abolished	134
Zhao <i>et al.</i> , <i>Circulation</i> 2000; 102 : 902–907	<i>In vitro</i>	Adenosine A ₁ agonist	Infarction	PC abolished	126
Takano <i>et al.</i> , <i>Circ Res</i> 2001; 88 : 520–528*	<i>In vivo</i>	Adenosine A ₁ agonist	Infarction	PC abolished	127
	<i>In vivo</i>	Adenosine A ₃ agonist	Infarction	PC not abolished	
Guo <i>et al.</i> , <i>Circulation</i> 2000; 102 :II–121 (abstr.)	<i>In vivo</i>	Adenosine A ₁ agonist	Infarction	PC abolished	125
Guo <i>et al.</i> , <i>Circulation</i> 2000; 102 :II–121 (abstr.)	<i>In vivo</i>	Opioid δ_1 agonist	Infarction	PC abolished	125
Guo <i>et al.</i> , <i>Circulation</i> 1999; 100 :I–562 (abstr.)	<i>In vivo</i>	Nitroglycerin; SNAP	Infarction	PC abolished	128
Xi <i>et al.</i> , <i>Am J Physiol</i> 1999; 277 : H2418–H2424	<i>In vitro</i>	RC-552	Infarction	PC abolished	129
Zhao <i>et al.</i> , <i>J Mol Cell Cardiol</i> 1997; 29 : 1567–1576	<i>In vivo</i>	Monophosphoryl lipid A	Infarction	PC abolished	130
Xi <i>et al.</i> , <i>Circulation</i> 1999; 99 : 2157–2163	<i>In vitro</i>	Monophosphoryl lipid A	Infarction	PC abolished	131
Elliott <i>et al.</i> , <i>J Mol Cell Cardiol</i> 2000; 32 : 1327–1339	<i>In vivo</i>	RC-552	Infarction	PC abolished	132
Exercise-induced late PC					
Guo <i>et al.</i> , <i>Circulation</i> (in press) (abstr.)	<i>In vivo</i>	Exercise	Infarction	PC abolished	133

* Since the non-selective NOS inhibitor L-NA was used, the role of cNOS v iNOS cannot be discerned.

(eNOS-dependent iNOS induction).² This is the opposite of what has been reported in other cell types, such as neutrophils and glial cells, where cNOS-derived NO has been found to suppress the transcription of the iNOS gene via a negative feedback mechanism.¹³⁶

Conceptual Implications of the Dual Role of Nitric Oxide in Late Preconditioning

The recognition that NO serves both as a trigger and a mediator of late PC¹⁰⁸ was somewhat surprising, as there is no *a priori* reason why the same molecule should perform both of these unrelated tasks. Nevertheless, this unexpected discovery has served to

focus attention on the pervasive role of NO in cardioprotection (Fig. 2). Furthermore, the NO hypothesis of late PC has led to a new understanding of the wisdom with which specific NOS isoforms are used for specific purposes. This hypothesis predicts that the heart assigns different tasks to eNOS and iNOS in a manner that fits the different personalities of these two enzymes.¹⁰⁸ Since eNOS is constitutively expressed and its activity can be recruited almost instantaneously by both calcium-dependent and calcium-independent mechanisms,¹³⁷ this protein is well suited for an immediate but brief reaction. iNOS, on the other hand, is dormant in unstressed myocardium (where it is expressed at low levels)^{112,115,119,123,124} but can be upregulated by stress; its activity is tonic (rather

Table 6 Mechanisms for the cardioprotective effect of NO

More likely
<ul style="list-style-type: none"> ● Inhibition of Ca²⁺ influx ● Antagonism of β-adrenergic stimulation ● Reduced contractility ● Reduced MVO₂ ● Opening of K_{ATP} channels ● Antioxidant actions (inhibition of $\cdot\text{O}_2^-$ and ONOO⁻ effects) ● Activation of COX-2 with synthesis of cytoprotective prostanoids
Less likely
<ul style="list-style-type: none"> ● Preserved endothelium-dependent vasodilation ● Reduced "no reflow" ● Reduced leukocyte infiltration ● Reduced release of cytokines ● Reduced expression of vascular cell adhesion molecule-1

than pulsatile) and does not necessitate perturbation of Ca⁺⁺ levels or cellular homeostasis.¹³⁷ Thus, iNOS is well suited for a delayed but sustained reaction. The mechanism of late PC has been found to be consistent with the most effective utilization of the specific properties of eNOS and iNOS. Indeed, the studies reviewed above indicate that when non-preconditioned myocardium is exposed to a stress (i.e. to a perceived threat) such as ischemia, it mobilizes the already available eNOS for an immediate burst of NO biosynthesis to help deal with the emergency. At the same time, the heart puts in place a strategy for dealing with the possible recurrence of this threat; specifically, it shifts to a preconditioned (defensive) phenotype by expressing iNOS, which will maintain elevated myocardial NO levels for a sustained period of time, thereby keeping the tissue protected from any possible similar threat, whose precise timing is unknown to the heart. In summary, the heart responds to stress in a biphasic manner, utilizing eNOS as an immediate but short-term response and iNOS as a delayed but long-term defense.¹⁰⁸

Role of iNOS as a Cardioprotective Protein and as a Ubiquitous Mediator of Late Preconditioning

An important consequence of the NO hypothesis of late PC has been the identification of a novel function for iNOS as a cytoprotective protein. The role of this enzyme in the cardiovascular system has

been poorly understood, but is generally thought to be detrimental because of its involvement in conditions such as septic shock, inflammation, and allograft rejection. The NO hypothesis of late PC has impelled a radical reassessment of this view.¹⁰⁸ As indicated above, there is now solid evidence that iNOS is a common mediator of the cardioprotective effects of many different types of cardiac adaptation, including late PC induced by ischemia,^{34,121-123} adenosine A₁ receptor agonists,¹²⁵⁻¹²⁷ opioid δ_1 receptor agonists,¹²⁵ NO donors,¹²⁸ endotoxin derivatives,¹²⁹⁻¹³² and exercise¹³³ (Table 5; Fig. 2). The diversity of the PC stimuli that converge on iNOS implies that: (i) the upregulation of this protein is a ubiquitous response of the heart to perceived threats; and (ii) generation of NO via iNOS is a central mechanism whereby the myocardium protects itself from impending ischemic challenges.¹⁰⁸

Importantly, it is becoming apparent that the role of iNOS as a common pathway of late PC is not limited to the heart, since recent reports have extended this paradigm to other organs. Specifically, studies of late PC in the kidney¹³⁸ and intestine¹³⁹ indicate that induction of iNOS is a critical mechanism whereby ischemia triggers delayed protection in these tissues as well, suggesting that the iNOS hypothesis, which was originally formulated in the context of cardiac PC,¹⁰⁸ may be broadly applicable outside of the cardiovascular system. A fundamental role of iNOS in protecting against ischemia is further supported by the finding that low oxygen tension induces iNOS in hepatocytes,¹⁴⁰ that iNOS expression increases in response to hypoxia in cardiac myocytes^{141,142} and in macrophages,¹⁴³ and that iNOS activity and expression are up-regulated in atrial tissue obtained from cyanotic children with congenital heart defects.¹⁴⁴ Collectively, the evidence emerging from studies of late PC and from the other studies reviewed above suggests that iNOS is a hypoxia-responsive gene and that the activity of this enzyme is a generalized mechanism of defense against ischemic injury.

Clinical Implications of the Nitric Oxide Hypothesis of Late Preconditioning

Besides its conceptual and pathophysiological reverberations, the NO hypothesis of late PC has important therapeutic implications. As reviewed above, administration of NO donors, in the absence of ischemia, can faithfully recapitulate both the molecular and the functional aspects of the late phase of ischemia-induced late PC in experimental

animals.^{99-102,113} In keeping with these data, recent studies performed in patients undergoing percutaneous transluminal coronary angioplasty have demonstrated that i.v. infusion of nitroglycerin protects the myocardium against ischemia 24 h after its administration,¹⁴⁵ providing proof-of-principle for the concept that nitrates exert a late PC-mimetic action in humans.¹⁴⁶ Thus far, nitrates have been used mainly for their antianginal and preload-reducing properties. The finding that nitroglycerin elicits late PC in patients¹⁴⁵ supports the novel idea that nitrates could also be used as a PC-mimetic therapy for the prophylaxis of ischemic myocellular injury in such settings as stable and unstable angina, cardiac surgery, cardiac transplantation and coronary angioplasty. This new application of nitrates could be as important as, or perhaps even more important than, their current use in patients with coronary artery disease. Most agents that elicit a late PC-like protection in experimental studies are not clinically applicable, for various reasons.¹⁴⁷⁻¹⁵² In contrast, nitrates are generally well tolerated. Although numerous clinical trials have examined the effect of nitrates in acute coronary syndromes, in all of these studies treatment was started either during or immediately after the index ischemic insult (see, for example, references 153 and 154). In view of the experimental and clinical evidence pointing to the ability of nitrates to trigger late PC, it might be fruitful to re-explore the role of nitrate therapy given *before* the onset of ischemia.

In addition, the discovery that iNOS is a common mediator of the protection afforded by so many different types of late PC supports the idea that genetic or pharmacologic strategies that upregulate this protein could be fruitfully exploited to protect the ischemic heart in patients. In this context, recent experimental data indicate that gene transfer of either eNOS¹⁵⁵ or iNOS¹⁵⁶ can replicate the powerful infarct-sparing actions of ischemic PC, suggesting that NOS gene therapy could be an effective strategy for combating ischemia/reperfusion injury by increasing local myocardial NO levels without the need for continuous administration of NO donors and without systemic NO-dependent hemodynamic changes.

Mechanism for the Cardioprotective Effect of Nitric Oxide

The precise mechanism(s) whereby NO protects the myocardium against ischemia/reperfusion injury remains unclear. As always is the case when a

mechanism is unknown, many hypotheses have been put forth (Table 6). NO or its second messenger, cGMP, has been shown to exert a number of actions that would be expected to be beneficial during myocardial ischemia, including inhibition of Ca^{2+} influx into myocytes,¹⁵⁷⁻¹⁵⁹ antagonism of the effects of β -adrenergic stimulation,^{160,161} decrease in myocardial contractility,¹⁶¹⁻¹⁶⁷ reduction in myocardial oxygen consumption,¹⁶⁸⁻¹⁷³ and opening of sarcolemmal K_{ATP} channels.¹⁷⁴⁻¹⁷⁶ The reduced Ca^{2+} current may alleviate the Ca^{2+} overload associated with acute myocardial ischemia, which is one of the major mechanisms of ischemic injury.¹⁷⁷ The recent demonstration that NO donors facilitate the activation of mitochondrial K_{ATP} channel opening by diazoxide and partially activate the channel directly¹⁷⁸ supports the hypothesis that the cardioprotective effects of NO may be mediated, at least in part, by increased activity of the mitochondrial channels. Mitochondrial K_{ATP} channel activity, however, cannot entirely account for the salutary actions of NO because K_{ATP} channel antagonists fail to block the anti-stunning effects of iNOS-dependent late PC in rabbits.¹⁷⁹ An interesting mechanism of action was recently proposed by Heusch *et al.*⁹⁴ who found that endogenous NO preserves contractile function during ischemia through preservation of calcium responsiveness at no additional energetic cost. Another possibility is that NO protects by virtue of its antioxidant properties, specifically, its ability to attenuate the deleterious free radical actions of $\cdot\text{O}_2^{-180}$ and to terminate ONOO⁻-mediated lipid radical chain propagation¹⁸¹ (Table 6).

A previously unrecognized mechanism by which NO protects the ischemic myocardium has recently emerged, namely, stimulation of cyclooxygenase-2 (COX-2) activity with consequent production of cytoprotective prostanoids such as prostaglandin (PG)_{E₂} and PGI₂.¹⁸² This mechanism was identified by Shinmura *et al.*^{182,183} in the setting of late PC, where inhibition of iNOS was found to abrogate prostanoid synthesis¹⁸² whereas inhibition of COX-2 did not affect iNOS activity¹⁸² but resulted in loss of protection,¹⁸³ indicating that COX-2 activity is driven by iNOS-derived NO and is obligatorily required for iNOS to exert its cardioprotective effects. Whether COX-1 or COX-2 activity also mediates the cardioprotective effects of NO in non-preconditioned myocardium is unknown.

NO has also been suggested to protect against lethal ischemia/reperfusion injury by preventing the impairment of endothelium-dependent coronary vasodilation¹⁸⁴ and by reducing the "no reflow" phenomenon,⁶ the infiltration of leukocytes,¹⁸⁵ the release of cytokines,¹⁸⁶ and the ex-

pression of vascular cell adhesion molecule-1 (VCAM-1)¹⁸⁷ (Table 6). These latter hypotheses, however, are less plausible because the “no reflow” phenomenon and the inflammatory response to ischemia/reperfusion are likely to be a consequence, rather than a cause, of ischemic myocellular death. Clearly, considerable additional work will be necessary to identify the mechanism of action of this ubiquitous mediator.

Conclusions

An overwhelming body of evidence accumulated over the past decade demonstrates that biosynthesis of NO by constitutive NOS (endogenous NO) plays an important role in alleviating the severity of both reversible (myocardial stunning) and irreversible (myocardial infarction) damage incurred during ischemia/reperfusion in the unstressed (non-preconditioned) heart. A similar salubrious effect can be produced by exogenous NO, given by means of NO-releasing agents or NO precursors. In addition, NO has been found to play a critical bifunctional role (as a trigger and a mediator) in late PC, a paradigm referred to as the NO hypothesis of late PC.¹⁰⁸ Specifically, enhanced generation of NO by eNOS is essential to trigger the late phase of ischemia-induced and exercise-induced PC, and enhanced NO production by iNOS is obligatorily required to mediate the anti-stunning and anti-infarct actions of late PC induced by five different stimuli (ischemia, adenosine A₁ agonists, opioid δ_1 agonists, endotoxin derivatives, and exercise). The NO hypothesis of late PC has thus revealed a previously unrecognized cytoprotective function of iNOS in the cardiovascular system.

The diversity of the PC stimuli that converge on iNOS implies that the upregulation of this enzyme is a central mechanism whereby the heart protects itself from ischemia, a concept congruous with emerging evidence indicating that iNOS is a hypoxia-inducible protein. Recent data demonstrating that iNOS mediates delayed protection in kidney and intestine have extended the NO hypothesis of late PC beyond the cardiovascular system. A corollary of this hypothesis is that the heart responds to ischemia by utilizing two different NOS isoforms in a manner that is congruous with their properties: it recruits pre-existing eNOS for an immediate burst of NO production and, at the same time, it upregulates iNOS to switch to a defensive phenotype characterized by a sustained enhancement of NO biosynthesis. In the ultimate

analysis, the fundamental difference between non-preconditioned and late preconditioned myocardium appears to be the tissue level of NO, which is tonically higher in the latter (as a result of iNOS upregulation) compared with the former. Thus, late PC can be viewed as a state of enhanced NO output. In contrast to the late phase of PC, generation of NO is not necessary for the development of the early phase of ischemia-induced PC; nevertheless, exogenous NO can elicit an early protective effect similar to the early phase of ischemic PC.

Taken together, the evidence reviewed in this article indicates that NO plays a fundamental biological role in protecting the heart against ischemia/reperfusion injury. Far from being detrimental, as sometimes has been proposed, biosynthesis of NO is instead critical to preserve tissue function and viability during an ischemic insult. The recognition that production of NO represents nature's own protective mechanism against ischemia offers fertile practical implications. Many opportunities loom on the horizon for enhancing NO availability in a manner that would be therapeutically desirable. Experimental studies have shown that NO donors in themselves, in the absence of ischemia, can faithfully reproduce the molecular and functional aspects of ischemia-induced late PC. NO-releasing agents (i.e. nitrates) are widely used in patients with coronary artery disease and are generally well tolerated, and recent studies have demonstrated the ability of nitrates to elicit a state of late PC in patients as well.¹⁴⁵ Furthermore, recent experimental data indicate that gene transfer of either eNOS¹⁵⁵ or iNOS¹⁵⁶ can replicate the powerful infarct-sparing actions of ischemic PC. The time has come to translate the enormous body of experimental work reviewed herein into clinically useful therapies by harnessing the cytoprotective properties of NO.

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