



Ischemic Preconditioning Increases iNOS Transcript Levels in Conscious Rabbits via a Nitric Oxide-dependent Mechanism

W. Keith Jones, Michael P. Flaherty, Xian-Liang Tang, Hitoshi Takano, Yumin Qiu, Supratim Banerjee, Traci Smith and Roberto Bolli

Experimental Research Laboratory, Division of Cardiology and Jewish Hospital Heart and Lung Institute, University of Louisville, Louisville, KY 40292, USA

(Received 22 December 1998, accepted in revised form 4 May 1999)

W. K. JONES, M. P. FLAHERTY, X.-L. TANG, H. TAKANO, Y. QIU, S. BANERJEE, T. SMITH AND R. BOLLI. Ischemic Preconditioning Increases iNOS Transcript Levels in Conscious Rabbits via a Nitric Oxide-dependent Mechanism. *Journal of Molecular and Cellular Cardiology* (1999) 31, 1469–1481. Recent studies implicate iNOS as the mediator of the late phase of ischemic preconditioning (PC). However, it is unknown whether induction of iNOS activity is mediated by transcriptional, post-transcriptional, translational, or post-translational mechanisms. To address this issue, we isolated and sequenced a partial iNOS cDNA expressed in preconditioned rabbit myocardium. Using a rabbit-specific probe generated from this sequence, we measured the steady state levels of the iNOS transcript after ischemic PC [six cycles of 4-min occlusion/4-min reperfusion (O/R)]. Three hours after ischemic PC, the iNOS mRNA levels in the ischemic/reperfused region were increased approximately three-fold relative to samples from the non-ischemic region and from control rabbits. This increase in mRNA levels was completely abolished by pretreatment with the NOS inhibitor *N*^ω-nitro-L-arginine. Conversely, administration of the NO donor nitroglycerin induced an increase in iNOS mRNA levels similar to that induced by ischemic PC. We conclude that in the conscious rabbit, ischemic PC induces an increase in iNOS mRNA levels, and that this induction is triggered by increased generation of NO during the PC stimulus. These results provide direct evidence that upregulation of iNOS is a natural response of the heart to a brief ischemic stress and that NO itself, in the absence of ischemia, upregulates myocardial iNOS transcript levels, a finding that may have implications for nitrate therapy. This previously unrecognized NO-dependent upregulation of iNOS mRNA is likely to play an important role in the development of late PC as well as in many other pathophysiological conditions in which NO is implicated.

© 1999 Academic Press

KEY WORDS: iNOS; Rabbit; Ischemia.

Introduction

Exposure of the heart to a brief ischemic stress induces a delayed, relatively sustained protective response against subsequent ischemic injury, which is fully manifest 24–72 h later. This phenomenon, termed the “second window” or “late phase” of ischemic preconditioning (PC) (Kuzuya *et al.*, 1993; Sun *et al.*, 1995; Bolli, 1996; Marber and Yellon, 1996; Yang *et al.*, 1996), has recently become the focus of considerable interest because of its potential

clinical significance (Yellon and Baxter, 1995; Bolli, 1996; Marber and Yellon, 1996). Previous studies in conscious rabbits have implicated nitric oxide (NO) both as the “trigger” and as the mediator of the cardioprotective effects of late PC (reviewed in Bolli *et al.*, 1998). Specifically, administration of the non-selective nitric oxide synthase (NOS) inhibitor *N*^ω-nitro-L-arginine (L-NA) during the initial PC ischemia was found to abrogate the development of delayed protection against myocardial stunning (Bolli *et al.*, 1997a, b) and infarction (Qiu *et al.*,

Please address all correspondence to: W. Keith Jones, Division of Cardiology, 511 S. Floyd St. Rm. 128, University of Louisville, Louisville, Kentucky 40202.

1997) and conversely, administration of NO donors in the absence of ischemia was found to induce a delayed cardioprotective effect indistinguishable from that of the late phase of ischemic PC (Banerjee *et al.*, 1998; Takano *et al.*, 1998b; Guo *et al.*, 1999). Furthermore, administration of L-NA on day 2 (24 h after ischemic PC) completely abrogates the cardioprotective effects against myocardial stunning (Bolli *et al.*, 1997b) and infarction (Takano *et al.*, 1998a), indicating that NOS also plays an essential role in mediating cardioprotection on day 2. A similar abrogation of late PC is observed when rabbits are treated on day 2 with the relatively-selective iNOS inhibitors aminoguanidine (AG) and S-methylisothiurea sulfate (SMT), suggesting that the NOS isoform involved in mediating protection on day 2 is iNOS (Bolli *et al.*, 1997b; Takano *et al.*, 1998a). Collectively, these results support the hypothesis that enhanced NO generation acts as a trigger for the development of late PC on day 1, while NO generated by the inducible NOS (iNOS) acts as the mediator of the cardioprotective effects of late PC on day 2 ("NO hypothesis of late PC") (Bolli *et al.*, 1998).

Although the pharmacological studies reviewed above implicate iNOS as a critical mediator of cardioprotection in late PC, the evidence is indirect. An increase in iNOS mRNA during the development of ischemic PC has never been documented. Furthermore, the mechanism whereby brief ischemic stress upregulates iNOS activity 24 h later remains unknown. iNOS can be induced in almost every cell type and organ, including the heart (Balligand *et al.*, 1994; Wang and Marsden, 1995), and while iNOS is regulated predominantly at the level of transcription, there are specific examples of regulation at the post-transcriptional and post-translational levels (Wang, 1995; Forstermann and Kleinert, 1995). Thus, ischemic PC may induce iNOS activity by increasing iNOS transcript levels, by post-translational modification of pre-existing iNOS protein, or both. In the present study we tested the hypothesis that the increase in iNOS activity during the late phase of ischemic PC is due, at least in part, to an increase in iNOS transcript levels. To this end, we measured myocardial steady state levels of iNOS mRNA in the same conscious rabbit model of late PC in which the NO hypothesis was previously developed (Bolli *et al.*, 1997a, b). Since no clones or nucleic acid sequence data exist for rabbit iNOS, we cloned and characterized a partial rabbit iNOS cDNA generated by RT-PCR. Because NO triggers late PC (Qiu *et al.*, 1997; Bolli *et al.*, 1997a; Takano *et al.*, 1998b), we also investigated the role of NO in inducing the

accumulation of iNOS mRNA. The results demonstrate, for the first time, that brief ischemia causes a rapid accumulation of iNOS mRNA in the heart, and that this phenomenon is triggered by the increased generation of NO associated with the ischemic stress.

Materials and Methods

Conscious rabbit preparation

The conscious rabbit model of ischemic PC has been described in detail previously (Maldonado *et al.*, 1997; Qiu *et al.*, 1997; Bolli *et al.*, 1997a, b; Takano *et al.*, 1998a, b). Briefly, male New Zealand White rabbits (2.0–2.5 kg) were instrumented under sterile conditions with placement of a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal in the region to be rendered ischemic, and bipolar ECG leads on the chest wall. The chest wound was closed in layers, and a small tube was left in the thorax for three days to aspirate air and fluids postoperatively. Gentamicin was administered prior to surgery and on the first and second postoperative days (0.7 mg/kg i.m. each day). The rabbits were allowed to recover for a minimum of 10 days after surgery.

Experimental protocol for ischemia/reperfusion

Throughout the experiments, rabbits were kept in cages in a quiet dimly lit room. Left ventricular (LV) systolic wall thickening, the range gate depth, and the ECG were recorded on a thermal array chart recorder (Gould TA 6000, Valley View, OH, USA). Coronary artery occlusion was produced by inflating the balloon occluder. The performance of successful coronary occlusions was verified by observing the development of ST-segment elevation and changes in the QRS complex on the ECG and the appearance of paradoxical systolic wall thickening on the ultrasonic crystal recordings. Successful reperfusion was documented by the normalization of the ECG and by the resumption of active systolic wall thickening. No antiarrhythmic drugs were given at any time.

Rabbits were assigned to six groups (Fig. 1). Group I (ischemic PC) underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles. This protocol induces late PC against both myocardial stunning (Maldonado *et al.*, 1997; Bolli *et al.*, 1997a, b; Takano *et al.*, 1998b) and myocardial

infarction (Qiu *et al.*, 1997; Takano *et al.*, 1998a, b). Group IV (L-NA + ischemic PC) underwent the same sequence of occlusion/reperfusion cycles and received an i.v. infusion of L-NA at a rate of 1.3 mg/kg/min for 10 min, starting 20 min before and ending 10 min before the first coronary occlusion (total dose 13 mg/kg). This dose of L-NA has previously been shown to block the development of late PC against stunning (Bolli *et al.*, 1997a, b) and infarction (Qiu *et al.*, 1997) in conscious rabbits. L-NA (Sigma Chemical Co., St Louis, MO, USA) was dissolved in normal saline (total volume infused, 20 ml). In both groups I and IV, the rabbits were given heparin (1000 U i.v.) 3 h after the last reperfusion, after which they were anesthetized with sodium pentobarbital (50 mg/kg i.v.) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples (~20 mg) were rapidly removed from the center of the ischemic-reperfused region (whose boundaries had been marked with sutures at the time of instrumentation) and from the non-ischemic region (posterior LV wall) while the heart was maintained on ice-cold RNase-free PBS. Tissue samples were stored in liquid nitrogen until used. Group II (non-ischemic control) underwent the same instrumentation as the other groups but did not undergo coronary occlusion and did not receive any treatment. At 10–14 d after surgery (time corresponding to the interval elapsed between instrumentation and euthanasia in the other groups), the rabbits were euthanized and samples were obtained from the anterior and posterior LV walls as described above. Group III (non-instrumented control) did not undergo any surgical procedure and did not receive any treatment. Rabbits were euthanized and tissue samples harvested from the anterior and posterior LV walls as described for group II. Group VI (NTG) received a continuous i.v. infusion of nitroglycerin [NTG (2 µg/kg/min)] for 60 min. This dose of NTG has previously been shown to induce late PC in conscious rabbits (Banerjee *et al.*, 1998). The rabbits were euthanized 3 h after the end of the infusion and tissue samples were harvested from the anterior and posterior LV walls as described above. Neither L-NA nor NTG, at the doses used in this study, produces haemodynamic abnormalities in rabbits (Qiu *et al.*, 1997; Takano *et al.*, 1998b). Group V (permanent occlusion) underwent coronary occlusion without reperfusion. These rabbits were euthanized 60 h after the occlusion and tissue samples obtained from the center of the infarct. All samples were treated as described below for the isolation and analysis of iNOS mRNA levels.

Reverse transcription polymerase chain reaction (RT-PCR)

Human, mouse and rat iNOS DNA sequences were analysed for regions of high interspecific sequence conservation. Two oligodeoxynucleotides from highly conserved regions of the iNOS cDNA, located approximately 918 bp apart, were chosen as PCR primers. The oligodeoxynucleotide primers were synthesized to the consensus iNOS sequence (mouse-human-rat) in this region and were designated as primer 1 and primer 2. Primer 1 is homologous to the region 500–523, and primer 2 to the region 1392–1416 of the published mouse iNOS cDNA sequence (Kone *et al.*, 1995). Primer 1 is 100% homologous to the mouse, rat and human iNOS cDNAs, while primer 2 is 100% homologous to the rat and mouse, and 92% homologous to the human cDNA. The region of the iNOS cDNA between 500–1416 is rather well conserved (relative to the mouse sequence), with 94.3% homology at the nucleic acid level between mouse and rat, 84.4% between rat and human, and 83.6% between mouse and human. Overall, the entire iNOS cDNA is 86.9% conserved between mouse and rat, 64.1% between rat and human, and 71.6% between mouse and human. The sequences of primers 1 and 2 are 5'-TAGAAACAACAGGAACCTACCAAC-3', and 5'-CTGGTGGAAACACAGGGGTGATGCTC-3', respectively.

Conscious rabbits underwent the standard PC protocol (six cycles of 4-min occlusion/4-min reperfusion) and were euthanized 3 h later. Samples of ischemic-reperfused and non-ischemic tissue were rapidly obtained while the heart was maintained on ice-cold RNase-free PBS. Total RNA was isolated (see below) and 5 µg of total RNA was used in the RT reactions using primer 2 as the reverse primer. The RT was performed using MMLV reverse transcriptase and the reverse primer at 42°C for 60 min. A fraction (one-fifth) of the RT reaction was then used with primer 1 as the forward primer and primer 2 as the reverse primer in the PCR reaction. The PCR program used consisted of a 4-min 94°C cycle followed by 35 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C. The program ended with a 10-min incubation at 72°C. The reaction mixtures were chilled to 4°C, run on a 2.5% agarose gel, and visualized by UV transillumination.

Cloning and sequence analysis of a partial rabbit iNOS cDNA

An RT-PCR product approximately 918 bp in length was isolated by gel purification from Seaplaque

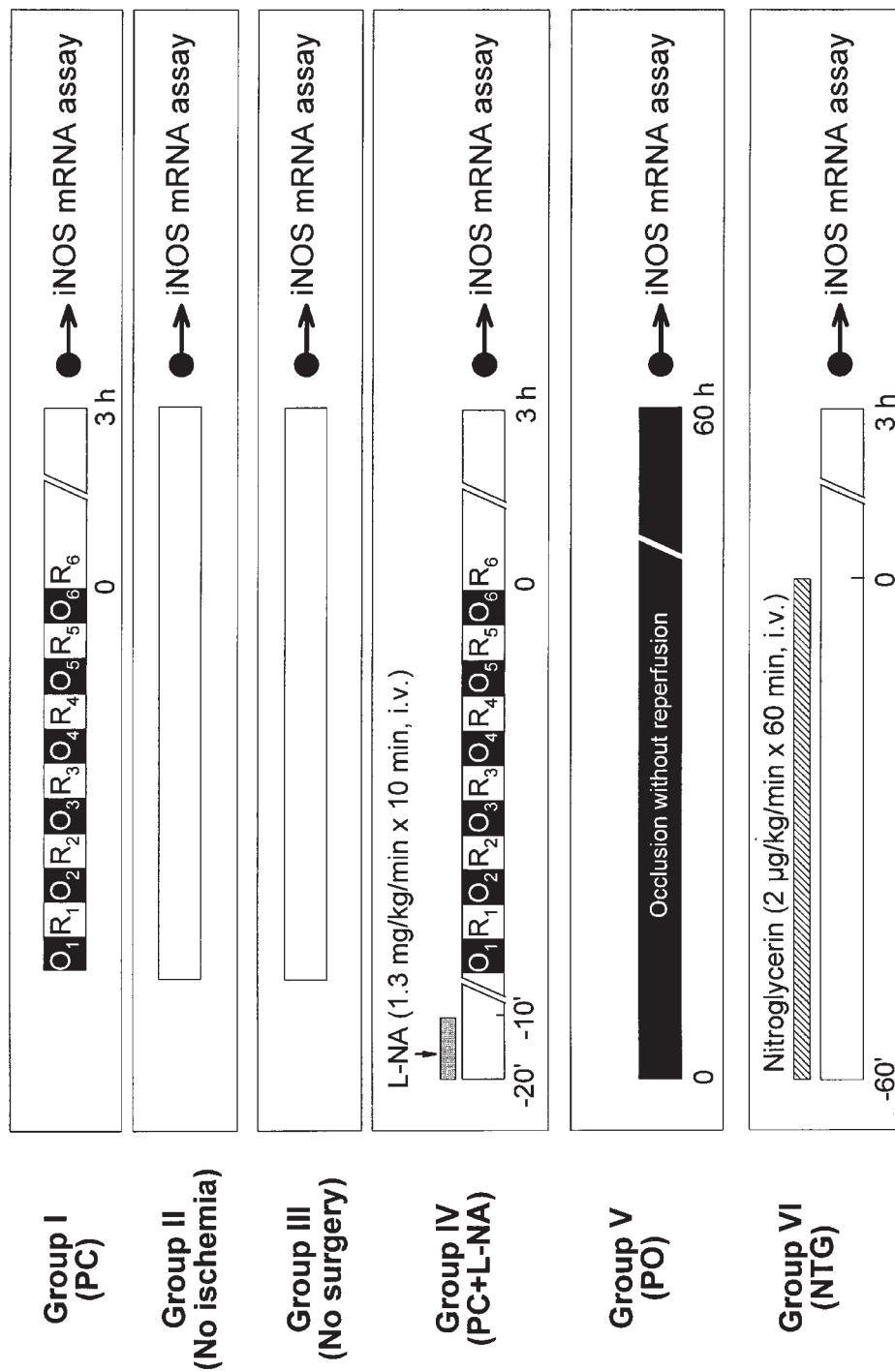


Figure 1 Experimental protocol. Six groups of rabbits were used. All groups were instrumented and allowed to recover for a minimum of 10 days prior to initiating the protocols (Materials and Methods). On day 1, rabbits in group I [*n* = 6 (ischemic PC)] underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles. Group IV [*n* = 6 (L-NA + ischemic PC)] underwent the same sequence of occlusion/reperfusion cycles and received an i.v. infusion of L-NA at a rate of 1.3 mg/kg/min for 10 min, starting 20 min before and ending 10 min before the first coronary occlusion (total dose 13 mg/kg). In both groups I and IV, the rabbits were euthanized 3 h after the last reperfusion. Group II [*n* = 6 (non-ischemic control)] did not receive any treatment and did not undergo coronary occlusion. Group III [*n* = 3 (non-instrumented control)] was not instrumented and did not undergo ischemia or pharmacological treatment. Group V [*n* = 3 (permanent occlusion)] underwent coronary occlusion without reperfusion. These rabbits were euthanized 60 h after the occlusion and tissue samples obtained from the center of the infarct. Rabbits in group VI [*n* = 6 (NTG)] received a continuous i.v. infusion of nitroglycerin [NTG (2 µg/kg/min)] for 60 min and were euthanized 3 h later. Tissue samples were treated as described in Materials and Methods for the isolation and analysis of iNOS mRNA levels.

low-melting point agarose according to the manufacturer's specifications (Seaplaque, FMC Bio-products, Rockland, ME, USA). The cDNA fragment was cloned into the pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA, USA) and sequenced by dideoxy chain termination using primer 1 and the T7 primer (from the plasmid), yielding 210 bp of nucleic acid sequence from the 5' end of the RT-PCR product. Three independent clones were sequenced twice using each primer. A 52 bp sequence was selected with appropriate characteristics (predicted T_m , consideration of potential secondary structures) for quantitative RNA dot blot analysis. This sequence was used to synthesize a 52 bp oligodeoxynucleotide which constituted the rabbit iNOS probe used in the RNA dot blot analysis. To determine whether the rabbit iNOS oligodeoxynucleotide was mono-specific, it was end-labeled with γ [^{32}P]-ATP and T4 polynucleotide kinase, hybridized to a Southern blot containing rabbit genomic DNA restricted with three different restriction enzymes, and washed under stringent conditions. A single discreet band was observed in each lane (data not shown) using conditions that had previously been employed to establish specificity for several other oligodeoxynucleotide probes (Robbins *et al.*, 1990; Jones *et al.*, 1996).

Quantitative RNA dot blot hybridization

Total RNA was isolated from tissue samples using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and an Ultraturax T25 tissue homogenizer (Janke & Kunkel, GMBH & Co., IKA Labortechnik, Staufenm, Germany) according to the manufacturer's protocol. Among the techniques available for mRNA quantitation, dot blot hybridization was selected because of its greater sensitivity and accuracy in measuring relatively small changes in mRNA (Subramaniam *et al.*, 1991). The procedures for quantitative dot blot hybridization were as described previously (Robbins *et al.*, 1990; Jones *et al.*, 1996) with modifications. Briefly, RNA was resuspended in DEPC-treated water, quantitated by spectrophotometry at an optical density of 260 nm, diluted and denatured by boiling. Three micrograms of total RNA per dot was blotted to nitrocellulose filters using a dot-blot filtration manifold (Bio-Rad, Melville, NY, USA). Filters were baked at 80°C *in vacuo* at a pressure of 760 mmHg for 90 min. The iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) oligonucleotide probes underwent 5' end-labeling with $[\gamma$ - ^{32}P]ATP using the Promega T4 polynucleotide kinase DNA 5'-end

labeling system (Promega, Milwaukee, WI, USA). Filters were prehybridized for 4–6 h and hybridized for 10–15 h at 50°C in a hybridization oven (Robbins Scientific, Model 1000). Filters were washed at 55°C once with $2.0 \times \text{SSC}/1.0\%$ SDS for 15 min, then twice at 55°C with $0.5 \times \text{SSC}/1.0\%$ SDS for 15 min. Quantitation of hybridization signals was with a Storm 480 phosphorimaging system and Imagequant software (Molecular Dynamics, Sunnyvale, CA, USA). The signal intensity of each dot was normalized to the signal produced by the GAPDH oligonucleotide probe (Jones *et al.*, 1996).

Statistical analysis

Data are reported as means \pm s.e.m. Differences in iNOS mRNA levels between the ischemic-reperfused and non-ischemic regions in the same group were analysed by paired Student's *t*-tests. Intergroup differences in iNOS mRNA levels in corresponding regions (ischemic-reperfused/anterior wall or non-ischemic/posterior wall) were analysed using a one-way ANOVA. If the ANOVA showed an overall difference, *post hoc* contrasts were performed with unpaired Student's *t*-tests using the Bonferroni correction (Wallenstein *et al.*, 1980).

Results

Cloning and sequencing of a partial rabbit iNOS cDNA via RT-PCR in preconditioned rabbit myocardium

Because the nucleotide sequence of the rabbit iNOS is unknown, it was necessary to obtain a rabbit iNOS cDNA clone from which probes could be developed for quantitative RNA dot blot analysis. The results of RT-PCR using RNA extracted from the ischemic-reperfused and non-ischemic zone of preconditioned hearts and oligonucleotide primers homologous to highly conserved regions of iNOS (see Materials and Methods) showed that there was expression of an iNOS related transcript in the ischemic-reperfused zone (Fig. 2). No attempt was made to draw quantitative conclusions from the results of the RT-PCR analyses; rather, they served as a source of material for the isolation of a partial rabbit iNOS cDNA as described (see Materials and Methods).

Sequence analysis showed the cloned partial rabbit iNOS cDNA to be 79.6, 80.3 and 83.8% homologous to the mouse, rat and human iNOS transcripts, respectively (Fig. 2). This is comparable

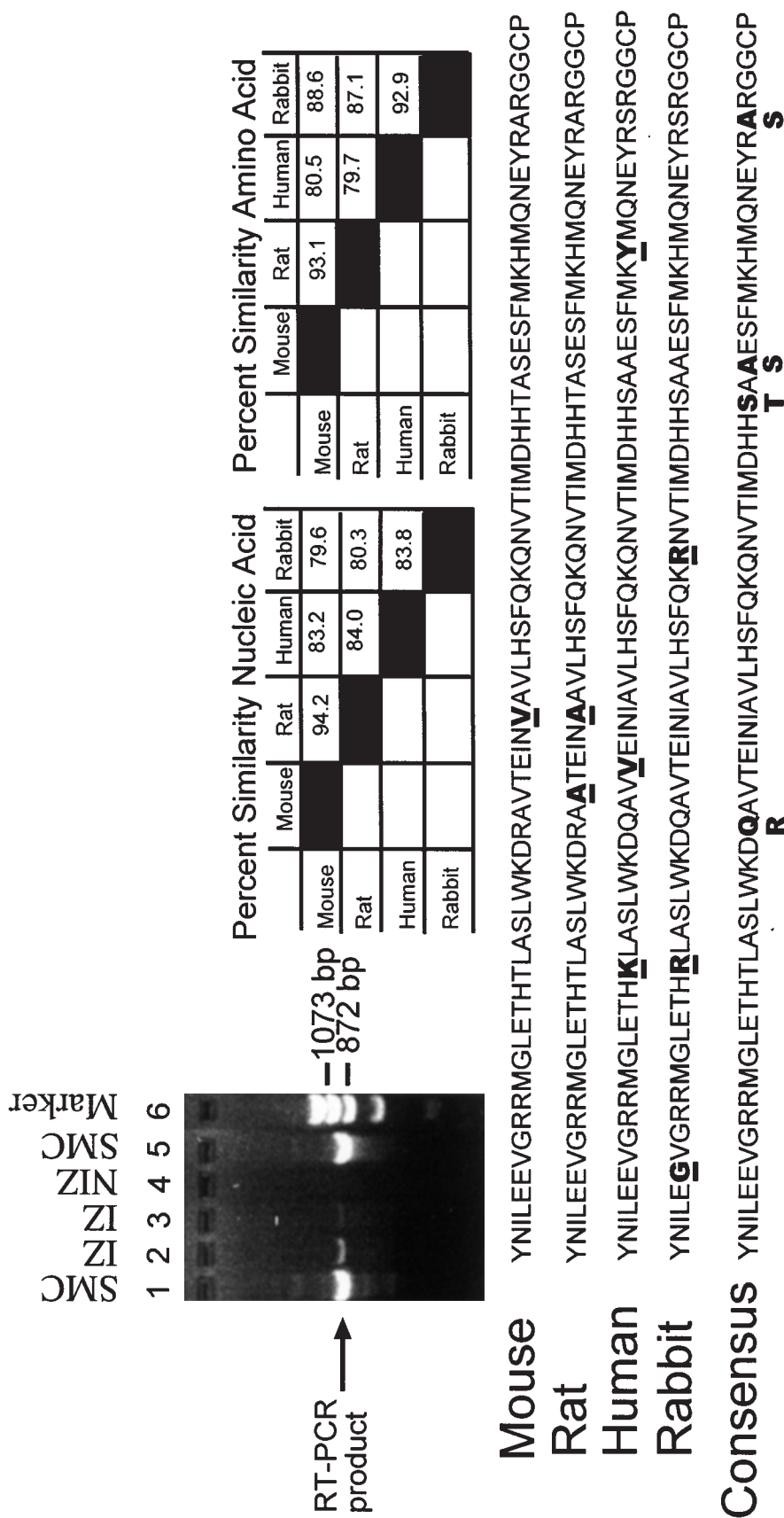


Figure 2 Detection of iNOS transcript by RT-PCR. The gel shows the results of RT-PCR using total RNA isolated 3 h after ischemic PC (six cycles of 4-min coronary occlusion/4-min reperfusion) from the ischemic-reperfusion region (lanes 2 and 3) and the non-ischemic region (lane 4) in conscious rabbits. The RNA samples used in lanes 1 and 5 were from cultured rat smooth muscle cells treated with lipopolysaccharide and interferon gamma. Lane 6 contains a DNA molecular weight marker (relevant sizes indicated). The tables show sequence comparisons at both the DNA (left) and predicted amino acid levels (right) among partial mouse, rat, human and rabbit iNOS sequences. An alignment between the predicted amino acid sequences is shown below. The consensus sequence was derived from the four sequences shown, and deviation from the consensus is shown in bold (underlined) for each of the proteins. The rabbit amino acid sequence obtained in this study is more similar to iNOS than any protein in the Genbank database, and is more closely related to the human protein sequence in this region. Note that the sequence similarity (at either the nucleic acid or amino acid levels) between the rabbit and each of the other species is comparable to that among the other species; for example, the amino acid sequence similarity between the rabbit and the mouse is 88.6%, while the amino acid sequence similarity between the rat and the mouse is 93.1%.

to the degree of nucleic acid sequence conservation between mouse and human iNOS (83.2%). Conversely, sequence conservation between the rabbit iNOS clone and the mouse, bovine and human eNOS and nNOS sequences was only 27–33%. The predicted amino acid sequence for the rabbit clone was 88.6, 87.1 and 92.9% identical to the predicted sequences for mouse, rat and human iNOS, respectively (Fig. 1). Based upon these considerations, we conclude that we have isolated a partial cDNA corresponding to a portion of the rabbit iNOS transcript.

Effect of ischemic PC on iNOS steady state mRNA levels

To determine whether ischemic PC modulates iNOS mRNA levels, six conscious rabbits (group I) underwent six cycles of 4-min coronary occlusions/4-min reperfusion and were euthanized 3 h later. RNA isolated from the ischemic–reperfused and non-ischemic regions were used for quantitative RNA dot blot hybridization (Fig. 3). Two control groups were studied: rabbits in group II (non-ischemic controls) were instrumented in the same fashion as group I but did not undergo ischemia/reperfusion, whereas rabbits in group III (non-instrumented controls) did not undergo any surgical manipulation prior to euthanasia. Another group of rabbits (group V) underwent permanent coronary occlusion to produce infarction. These animals served as a positive control, since iNOS is known to be transcriptionally upregulated in cardiac tissue after sustained ischemia resulting in infarction (Bing and Suzuki, 1996). In all samples, the steady state levels of iNOS mRNA were normalized to the levels of GAPDH mRNA. In pilot studies we found that the steady state levels of GAPDH mRNA (normalized both to 18S rRNA levels and to total mRNA levels) are unaffected by ischemia/reperfusion, as illustrated in Figure 3.

In control rabbits that were not subjected to ischemia/reperfusion (group II), the levels of iNOS mRNA were similar in the anterior and posterior LV walls; these levels were not significantly different from those measured in control rabbits that were not subjected to surgical instrumentation (group III) (Fig. 3). In rabbits subjected to ischemic PC (group I), there was a significant increase in iNOS mRNA levels in the ischemic–reperfused region [2.8 times the levels in the non-ischemic region ($P < 0.05$)]. The iNOS mRNA levels were 4.2-fold higher in the ischemic–reperfused region of preconditioned rabbits as compared with the anterior LV wall of non-ischemic control rabbits (group II)

($P < 0.05$) (Fig. 3). As expected from previous studies (Bing and Suzuki, 1996), samples derived from rabbits that had undergone permanent coronary occlusion (group V) demonstrated a marked (approximately 9-fold) increase in the iNOS mRNA levels relative to the samples from the anterior and posterior LV walls of non-ischemic control rabbits (group II) (Fig. 3).

Effect of L-NA on iNOS mRNA levels following ischemic PC

To determine whether NO is the signal causing increased iNOS steady state transcript levels after ischemic PC, six conscious rabbits underwent ischemic PC after pretreatment with L-NA (13 mg/kg) prior to the six cycles of occlusion/reperfusion (group IV). This dose of L-NA has previously been shown to abrogate late PC against both myocardial stunning and infarction in conscious rabbits (Bollini *et al.*, 1997a; Qiu *et al.*, 1997). Rabbits were euthanized 3 h after the last reperfusion and mRNA levels were determined as described above. In contrast to group I, there was no significant increase in the levels of iNOS mRNA in the ischemic–reperfused zone of group IV relative to either the non-ischemic zone of the same hearts or the anterior LV wall of control rabbits (groups II and III) (Fig. 3). The iNOS mRNA levels in the ischemic–reperfused zone of L-NA treated rabbits were significantly less than those in group I (ischemic PC; $P < 0.05$) (Fig. 3).

Effect of NTG on iNOS transcript levels

To determine whether administration of exogenous NO can reproduce the changes in iNOS mRNA effected by ischemic PC, six conscious rabbits received NTG (2 $\mu\text{g}/\text{kg}/\text{min}$ i.v. for 1 h) and were euthanized 3 h later. RNA was prepared from samples of anterior and posterior LV wall and assayed by RNA dot blot analysis as described above, along with samples from six non-ischemic control rabbits (group II) and six rabbits subjected to ischemic PC (group I). Administration of NTG (group VI) resulted in a significant increase in iNOS mRNA levels in both the anterior and the posterior LV walls relative to non-ischemic control rabbits (group II) ($P < 0.05$) (Fig. 4). This increase was comparable to the increase observed in the ischemic–reperfused region of rabbits subjected to six cycles of occlusion/reperfusion (group I) (Fig. 4).

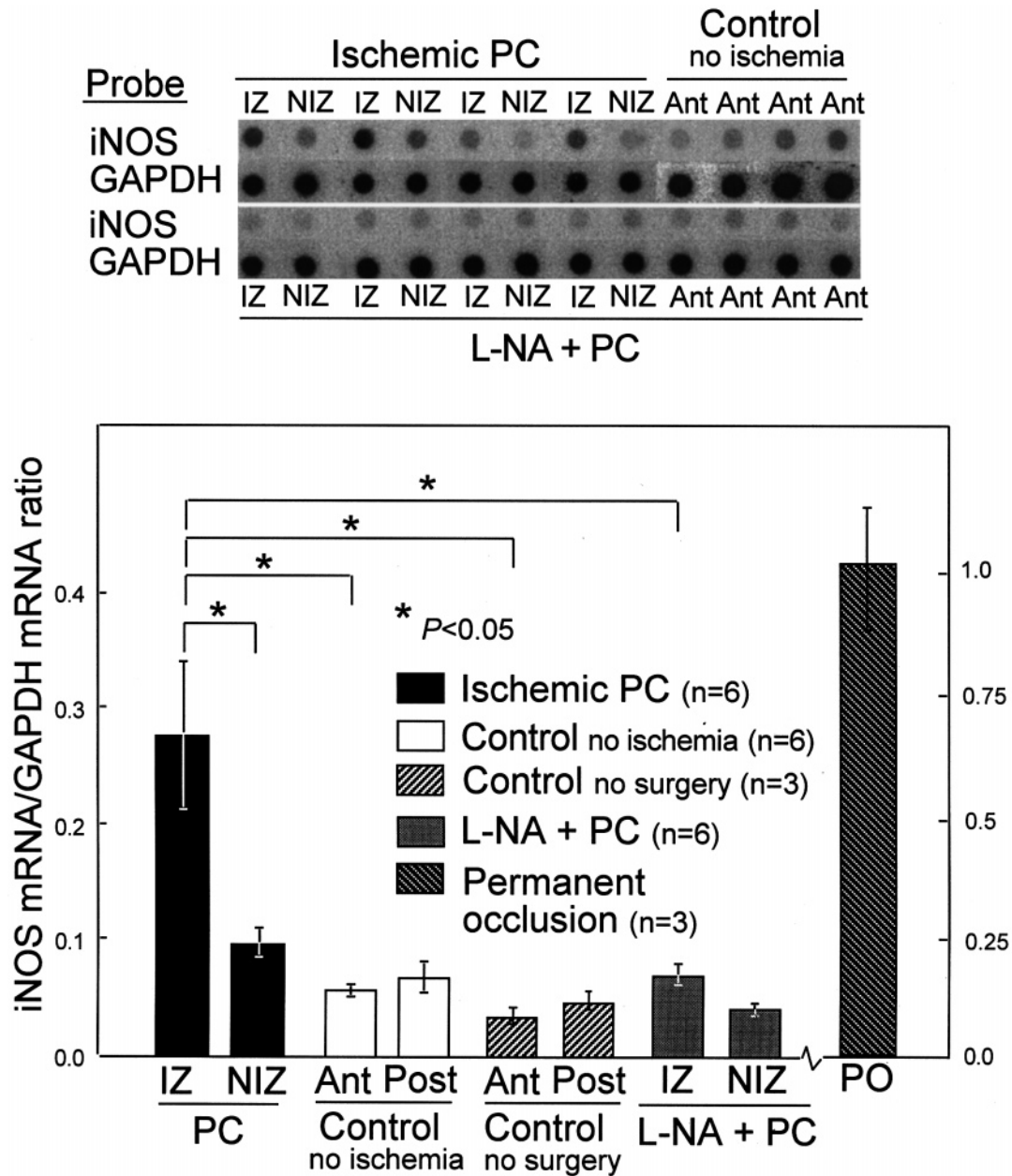


Figure 3 Quantitative dot blot hybridization analysis of iNOS steady state transcript levels. Rabbits in group I ($n=6$) were subjected to an ischemic PC protocol (six cycles of 4-min coronary occlusion/4-min reperfusion). Rabbits in group IV ($n=6$) underwent the same protocol except that they were given L-NA (13 mg/kg) prior to the six occlusion/reperfusion cycles. In both groups, tissue samples were taken after 3 h of reperfusion from the ischemic-reperfused zone (IZ) and the non-ischemic zone (NIZ). Non-ischemic control rabbits (group II, $n=6$) did not undergo coronary occlusion/reperfusion; non-instrumented control rabbits (group III, $n=3$) did not undergo surgery or coronary occlusion. Tissue samples were obtained from the anterior (Ant) and the posterior (Post) LV wall. Rabbits in group V ($n=3$) underwent a permanent coronary occlusion (PO) and tissue samples from the infarcted region were obtained 60 h later. RNA was isolated and quantitative dot blot hybridization used to determine the steady state level of the iNOS transcript. Transcript levels are expressed as arbitrary units of hybridization signal intensity normalized relative to GAPDH signal as previously described (Robbins *et al.*, 1990; Jones *et al.*, 1996). Samples from preconditioned rabbits (group I) showed a significant increase in the level of the iNOS transcript in the ischemic-reperfused zone. Pretreatment with L-NA (group IV) completely abrogated this increase. As expected, the level of iNOS mRNA was significantly increased in the infarcted zone in rabbits that had undergone permanent occlusion (group V). Note that the scale on the right applies only to group V. There were no significant differences in iNOS mRNA levels between NIZ/PC samples (group I, non-ischemic tissue samples) and Ant/Control or Post/Control samples (group II, anterior and posterior LV wall) and between samples from group III (non-instrumented controls) and the Ant/Control or Post/Control samples (group II, anterior and posterior LV wall). Data are means \pm s.e.m.

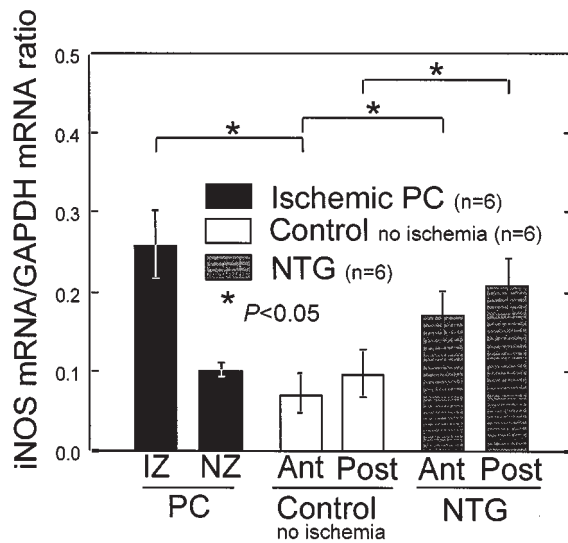


Figure 4 Quantitative dot blot hybridization analysis of iNOS steady state transcript levels. Rabbits in group I ($n=6$) were subjected to ischemic PC (six cycles of 4-min coronary occlusion/4-min reperfusion). Rabbits in group II (non-ischemic controls, $n=6$) did not undergo coronary occlusion/reperfusion. Rabbits in group VI ($n=6$) received NTG ($2 \mu\text{g}/\text{kg}/\text{min}$ for 1 h i.v.). Tissue samples were harvested after 3 h of reperfusion in group I, 3 h after the infusion of NTG in group VI, and at corresponding times in group II (non-ischemic control). RNA was isolated and quantitative dot blot hybridization used to determine the steady state level of the iNOS transcript. Transcript levels are expressed as arbitrary units of hybridization signal intensity normalized relative to GAPDH signal as previously described (Robbins *et al.*, 1990; Jones *et al.*, 1996). Samples from NTG treated rabbits (group VI) exhibited a significant increase in the steady state level of the iNOS mRNA relative to controls. This increase was similar to that observed in the ischemic zone of preconditioned rabbits (group I). Note that while ischemic PC induces an increase in the levels of iNOS mRNA only in the ischemic zone, NTG administration results in increased iNOS mRNA levels in both the anterior and posterior LV walls, as expected. Data are means \pm SEM.

Discussion

Prior pharmacological studies have led to the formulation of the NO hypothesis of late PC, which postulates that NO triggers late PC on day 1 and that iNOS mediates late PC on day 2 (Bolli *et al.*, 1998). However, direct evidence supporting this paradigm is lacking. The present study provides new molecular evidence that directly supports key elements of the NO hypothesis of late PC.

The salient findings of this study can be summarized as follows. First, in conscious rabbits myocardial steady state levels of the iNOS transcript are significantly increased 3 h after ischemic PC. This is the first demonstration of an increase in iNOS mRNA after ischemic PC. Notably, the same PC

protocol employed in this study results in augmented iNOS activity 24 h later (Xuan *et al.*, 1999). Second, at doses that block the development of late PC (Qiu *et al.*, 1997; Bolli *et al.*, 1997a, b) and prevent the increase in iNOS activity 24 h after ischemic PC (Xuan *et al.*, 1999), L-NA blocks the increase in iNOS mRNA levels observed 3 h after ischemic PC. This indicates that NOS activity is necessary for the increase in cardiac iNOS mRNA to occur. Third, administration of an NO donor (NTG) [at doses previously shown to mimic late PC (Banerjee *et al.*, 1998)] produces an increase in iNOS transcript levels comparable to that observed following ischemic PC, suggesting that NO in itself (in the absence of the cellular perturbations associated with ischemia) can upregulate iNOS mRNA levels in the heart. Thus, NO appears to be both necessary and sufficient to increase cardiac iNOS mRNA levels. Taken together, these results demonstrate that brief episodes of ischemia augment myocardial iNOS mRNA levels via an NO-dependent mechanism. On the basis of the present results and of previous studies in this same model (Qiu *et al.*, 1997; Bolli *et al.*, 1997a, b; Takano *et al.*, 1998a, b; Xuan *et al.*, 1999), we propose that generation of NO on day 1 causes an increase in the steady state iNOS mRNA pool, which then results in increased iNOS activity, NO generation, and cardioprotection on day 2.

Whether the increase in iNOS steady state mRNA levels is due to transcriptional upregulation of iNOS or post-transcriptional stabilization of the transcript remains to be determined. The iNOS gene is known to be inducible in a wide variety of cell types, and regulation of iNOS activity is predominantly at the transcriptional level (Wang and Marsden, 1995; Forstermann and Kleinert, 1995). The transcription of iNOS is known to be upregulated in the heart (Bachmaier *et al.*, 1997; Chandrasekar *et al.*, 1998) and in isolated cardiac myocytes in response to infection, inflammation, and lipopolysaccharide or cytokine administration (Balligand *et al.*, 1994; LaPointe and Sitkins, 1997; Kinugama *et al.*, 1997; Buchwalow *et al.*, 1997; Bachmaier *et al.*, 1997). Furthermore, the activity and/or protein levels of iNOS have been found to increase after inflammation or after sustained myocardial ischemia resulting in infarction (Dudek *et al.*, 1994b; Wildhurl *et al.*, 1995a, b; Bing and Suzuki, 1996). Thus, transcriptional regulation of iNOS is likely to be a functionally important component of the mechanism underlying the regulation of iNOS activity 24 h after ischemic PC. This concept is also supported by the finding that brief episodes of myocardial ischemia/reperfusion activate a number of

signal transduction pathways and transcription factors that are known to orchestrate iNOS gene expression, including protein kinase C [PKC (Ping *et al.*, 1997)], mitogen activated protein kinases [MAPKs (Ping *et al.*, 1998b)], protein tyrosine kinases [PTKs (Dawn *et al.*, 1998a, b; Ping *et al.*, 1998a)], nuclear factor kappa B [NF- κ B (Xuan *et al.*, 1999)], activating protein 1 [AP-1 (Chandrasekar and Freeman, 1997)], and tumor necrosis factor alpha [TNF- α (Gurevitch *et al.*, 1996)]. The fact that monophosphoryl lipid A, which acts similarly to lipopolysaccharide to induce myocardial iNOS in rats (Tosaki *et al.*, 1998) and rabbits (Zhao *et al.*, 1997), is able to pharmacologically mimic the late phase of PC (Zhao *et al.*, 1997; Elliott, 1998; Tosaki *et al.*, 1998), also supports our hypothesis that activation of iNOS transcription underlies the increase in iNOS mRNA levels and plays an important role in late PC.

The exact mechanism whereby an increase in NO upregulates iNOS mRNA is unknown. Since the same stimuli (ischemic PC and NTG) found to increase iNOS mRNA in this study have previously been shown to activate PKC (Ping *et al.*, 1997; Banerjee *et al.*, 1998), MAPKs (Ping *et al.*, 1998b), PTKs (Dawn *et al.*, 1998a, b; Ping *et al.*, 1998a), and NF- κ B (Xuan *et al.*, 1999), it seems plausible to postulate that NO-induced activation of all or some of these signaling elements is responsible for increased transcription of the iNOS gene resulting in increased iNOS mRNA levels. Regulation of iNOS transcription by NO has previously been shown in other systems. For example, in hemorrhagic shock NO and the induction of iNOS are necessary for NF- κ B activation, and NF- κ B in turn directly regulates iNOS gene transcription in the liver and lung (Hierholzer *et al.*, 1998). Although L-arginine analogues in high concentrations (50–270-fold higher than used in this study) have been reported to generate NO non-enzymatically in highly artificial systems (Moroz *et al.*, 1998), this phenomenon would not account for the contrasting effects of NTG and L-NA upon iNOS transcript levels observed in the present study; if anything, NO generation from L-NA should have enhanced rather than suppressed the upregulation of iNOS after ischemic PC.

There is currently no direct evidence concerning the identity of the NOS isoform that produces the NO that triggers late PC on day 1. Previous studies in conscious rabbits have shown that the development of late PC against myocardial stunning is blocked by the non-selective NOS inhibitor L-NA but not by the relatively-selective iNOS inhibitors AG and SMT (Bolli *et al.*, 1997b). Though not conclusive, these results support the conclusion

that the NOS isoform responsible for generating the NO that triggers late PC is a constitutive NOS (nNOS and/or eNOS). That NO itself is capable of eliciting a cardioprotective state similar to that of ischemic PC is supported by the fact that the NO donors, given 24 h before the ischemic insult, cause a reduction in the severity of myocardial stunning and in the size of myocardial infarction similar to that caused by the late phase of ischemic PC (Takano *et al.*, 1998b). In this regard, it is important to note that infusion of NTG (at the same dose used in the present study) induces a late PC effect that is indistinguishable from that of the late phase of ischemic PC (Banerjee *et al.*, 1998). Thus, there is a direct correlation between the late PC-mimetic effect of NTG and the effect of NTG on iNOS mRNA levels.

In conclusion, this study reveals a new change in gene expression associated with brief myocardial ischemia/reperfusion. It seems reasonable to postulate that the three-fold increase in steady state iNOS mRNA levels after ischemic PC is related to the increased iNOS activity observed 24 h later (Xuan *et al.*, 1998) and to the cardioprotection afforded by this phenomenon. Although conclusive proof will require further molecular studies, the pharmacological evidence presented herein supports the concept that NO serves as a cellular signal that modulates iNOS mRNA levels, suggesting a new biological function of this radical and a new mechanism in the pathophysiology of ischemic PC. This concept may also have important implications for clinical therapies with NO-releasing agents (e.g. nitrates). The notion that NO itself can upregulate iNOS mRNA levels raises the intriguing possibility that nitrates and other NO donors may have a similar effect in the heart of patients with coronary artery disease. Because of the ubiquitous role NO plays in several cardiovascular disorders, the concept that a relatively brief increase in NO generation can lead to increased iNOS mRNA levels in the heart may have implications for several pathophysiological conditions (besides ischemic PC) in which NO is involved.

Acknowledgements

This work was supported in part by NIH R01 grants HL-43151, HL-55757 (Dr Bolli) and HL-63034 (Dr Jones), by an AHA Fellowship award to Dr Takano (9804558), and by the Jewish Hospital Research Foundation, Louisville, KY, USA.

References

- BACHMAIER KN, NEU C, PUMMERER GS, DUNCAN TW, MAK T, MATSUYAMA T, PENNINGER JM, 1997. iNOS expression and nitrotyrosine formation in the myocardium in response to inflammation is controlled by the interferon regulatory transcription factor 1. *Circulation* **96**: 585–591.
- BALLIGAND JL, UNGUREANU-LONGROIS D, SIMMONS WW, PIMENTAL D, MALINSKI TA, KAPTURCZAK M, TAHA Z, LOWENSTEIN CJ, DAVIDOFF AJ, KELLY RA, 1994. Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. *J Biol Chem* **269**: 27580–27588.
- BALLIGAND JL, CANNON PJ, 1997. Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. *Arterioscler Thromb Vasc Biol* **17**: 1846–1858.
- BANDALETOVA TI, BROUET H, BARTSCH T, SUGIMURA H, ESUMI H, OHSHIMA H, 1993. Immunohistochemical localization of an inducible form of nitric oxide synthase in various organs of rats treated with *Propionibacterium acnes* and lipopolysaccharide. *APMIS* **101**: 330–336.
- BANERJEE S, TANG X-L, QIU Y, TAKANO H, MANCHIKALAPUDI S, DAWN B, SHIRK G, BOLLI R, 1998. Nitroglycerin induces late preconditioning against myocardial stunning via a protein kinase C mediated pathway in conscious rabbits. (Abstract). *Circulation* **98**: I–417.
- BING RJ, SUZUKI H, 1996. Myocardial infarction and nitric oxide. *Mol Cell Biochem* **161**: 303–306.
- BOLLI R, 1996. The early and late phases of preconditioning against myocardial stunning and the essential role of oxyradicals in the late phase: an overview. *Basic Res Cardiol* **91**: 57–63.
- BOLLI R, BHATTI ZA, TANG XL, QIU Y, ZHANG Q, GUO Y, JADOON AK, 1997a. Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res* **81**: 42–52.
- BOLLI R, MANCHIKALAPUDI S, TANG X-L, TAKANO H, QIU Y, ZHANG Q, JADOON AK, 1997b. The protective effects of late PC against myocardial stunning in conscious rabbits are mediated by nitric oxide synthase: evidence that nitric oxide acts as both a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ Res* **81**: 1094–1107.
- BOLLI R, DAWN B, TANG X-L, QIU Y, PING P, XUAN Y-T, JONES WK, TAKANO H, GUO Y, ZHANG J, 1998. The nitric oxide hypothesis of late preconditioning. *Basic Res Cardiol* **93**: 325–338.
- BUCHWALOW IB, SCHULZE W, KOSTIC MM, WALLUKAT G, MORWINSKI R, 1997. Intracellular localization of inducible nitric oxide synthase in neonatal rat cardiomyocytes in culture. *Acta Histochem* **99**: 231–240.
- BUTTERY LD, EVANS TJ, SPRINGALL DR, CARPENTER A, COHEN J, POLAK JM, 1994. Immunohistochemical localization of inducible nitric oxide synthase in endotoxin-treated rats. *Lab Invest* **71**: 755–764.
- CHANDRASEKAR B, FREEMAN GL, 1997. Induction of nuclear factor kappa B and activation protein 1 in post-ischemic myocardium. *FEBS Lett* **401**: 30–34.
- CHANDRASEKAR B, STREITMAN JE, COLSTON JT, FREEMAN GL, 1998. Inhibition of nuclear factor kappa B attenuates proinflammatory cytokine and inducible nitric-oxide synthase expression in postischemic myocardium. *Biochim Biophys Acta* **1406**: 91–106.
- DAWN B, QIU Y, TANG X-L, TAKANO H, BANERJEE S, BOLLI R, 1998a. Involvement of tyrosine kinases in the development of late preconditioning against myocardial stunning in conscious rabbits. (Abstract). *J Mol Cell Cardiol* **30**: A264.
- DAWN B, QIU Y, TANG X-L, TAKANO H, BANERJEE S, BOLLI R, 1998b. The protective effects of late preconditioning are mediated by tyrosine kinase activity in conscious rabbits. (Abstract). *Circulation* **98**: I–586.
- DUDEK RR, WILDHIRT S, PINTO V, GIESLER G, BING RJ, 1994a. Dexamethasone inhibits the expression of an inducible nitric oxide synthase in infarcted rabbit myocardium. *Biochem Biophys Res Commun* **202**: 1120–1126.
- DUDEK RR, WILDHIRT S, CONFORTO A, PINTO V, SUZUKI H, WINDER S, BING RJ, 1994b. Inducible nitric oxide synthase activity in myocardium after myocardial infarction in rabbit. *Biochem Biophys Res Commun* **205**: 1671–1680.
- ELLIOTT GT, 1998. Monophosphoryl lipid A induces delayed preconditioning against cardiac ischemia-reperfusion injury. *J Mol Cell Cardiol* **30**: 3–17.
- FORSTERMANN U, KLEINERT H, 1995. Nitric oxide synthase: expression and expression control of the three isoforms. *Naunyn Schmiedebergs Arch Pharmacol* **352**: 351–364.
- GUO Y, BAO W, TANG X-L, WU W-J, BOLLI R, 1998. Nitric oxide donors induce late preconditioning against myocardial infarction in mice. *J Mol Cell Cardiol* accepted.
- GUREVITCH J, FROLKIS I, YUHAS Y, PAZ Y, MATSA M, MOHR R, YAKIREVICH V, 1996. Tumor necrosis factor-alpha is released from the isolated heart undergoing ischemia and reperfusion. *J Am Coll Cardiol* **28**: 247–252.
- HIERHOLZER C, HARBRECHT B, MENEZES JM, KANE J, MACMICKING J, NATHAN CF, PEITZMAN AB, BILLIAR TR, TWEARDY DJ, 1998. Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. *J Exp Med* **187**: 917–928.
- JONES WK, GRUPP I, DOETSCHMAN T, GRUPP G, OSINSKA H, HEWETT T, BOIVIN G, GULICK J, NG W, ROBBINS J, 1996. Ablation of the cardiac MyHC chain gene leads to gene dosage effects and functional deficits in the heart. *J Clin Invest* **98**: 1906–1917.
- KAMIJO R, HARADA H, MATSUYAMA T, BOSLAND M, GERECITNO J, SHAPIRO D, LE J, KOH I, KIMURA T, GREEN SJ, MAK T, TANIGUCHI T, VILCEK J, 1994. Requirement for transduction factor IRF-1 in NO synthase induction in macrophages. *Science* **263**: 1612–1615.
- KINUGAWA K, SHIMIZU T, YAO A, KOHMOTO O, SERIZAWA T, TAKAHASHI T, 1997. Transcriptional regulation of inducible nitric oxide synthase in cultured neonatal rat cardiac myocytes. *Circ Res* **81**: 911–921.
- KONE BC, SCHWOBEL J, TURNER P, MOHAUPT MG, CANGRO CB, 1995. Role of NF-kappa B in the regulation of inducible nitric oxide synthase in an MTAL cell line. *Am J Physiol* **269**: F718–F729.
- KUZUYA T, HOSHIDA S, YAMASHITA N, FUJI H, OE H, HORI M, KAMADA T, 1993. Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* **72**: 1293–1299.
- LANDER HM, SEHAJPAL PK, NOVOGRODSKY A, 1993. Nitric oxide signaling: a possible role for G proteins. *J Immunol* **151**: 7182–7187.
- LANDER HM, OGIESTE JS, PEARCE SF, LEVI R, NOVOGRODSKY

- A, 1995. Nitric oxide-stimulated guanine nucleotide exchange on p21ras. *J Biol Chem* **270**: 7017–7020.
- LAPointe MC, SITKINS JR, 1996. Mechanisms of interleukin-1 β regulation of nitric oxide synthase in cardiac myocytes. *Hypertension* **27**: 709–714.
- LIU P, HOCK CE, NAGELE R, WONG PY, 1997. Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia–reperfusion injury in rats. *Am J Physiol* **272**: H2327–H2336.
- MALDONADO C, QIU Y, TANG X-L, COHEN MV, AUCHAMPACH J, BOLLI R, 1997. Role of adenosine receptors in late preconditioning against myocardial stunning in conscious rabbits. *Am J Physiol* **273**: H1324–H1332.
- MARBER MS, YELLON DM, 1996. Myocardial adaptation, stress proteins, and the second window of protection. *Ann NY Acad Sci* **793**: 123–141.
- McKENNA TM, LI S, TAO S, 1995. PKC mediates LPS- and phorbol-induced cardiac cell nitric oxide synthase activity and hypocontractility. *Am J Physiol* **269**: H1891–H1898.
- MOROZ LL, NORBY SW, CRUZ L, SWEEDLER JV, GILLETTE R, CLARKSON RB, 1998. Non-enzymatic production of nitric oxide (NO) from NO synthase inhibitors. *Biochem Biophys Res Commun* **253**: 571–576.
- MURRY CE, JENNINGS RB, REIMER KA, 1986. Preconditioning with ischemia; a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**: 1124–1136.
- NAGASAKI A, GOTOH T, TAKEYA M, YU Y, TAKIGUCHI M, MATSUZAKI H, TAKATSUKI K, MORI M, 1996. Co-induction of nitric oxide synthase, argininosuccinate synthetase, and argininosuccinate lyase in lipopolysaccharide-treated rats. RNA blot, immunoblot, and immunohistochemical analyses. *J Biol Chem* **271**: 2658–2662.
- NODE K, KITAKAZE M, KOSAKA H, KOMAMURA K, MINAMINO T, TADA M, INOUE M, HORI M, KAMADA T, 1995. Plasma nitric oxide end products are increased in the ischemic canine heart. *Biochem Biophys Res Commun* **211**: 370–374.
- PAN J, BURGHER KL, SZCZEPANIK AM, RINGHEIM GE, 1996. Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation. *Biochem J* **314**: 889–894.
- PENG M, HUANG L, XIE ZJ, HUANG WH, ASKARI A, 1995. Oxidant-induced activations of nuclear factor-kappa B and activator protein-1 in cardiac myocytes. *Cell Mol Biol Res* **41**: 189–197.
- PING P, LI RCX, ZHANG J, TANG X-L, QIU Y, BANERJEE S, ZHENG Y-T, BOLLI R, 1998a. Ischemic preconditioning (PC) induces selective activation of Src and Lck tyrosine kinases in conscious rabbits via a PKC-dependent pathway. (Abstract). *Circulation* **98**: I-71.
- PING P, ZHANG J, CAO X, KONG D, TANG X-L, QIU Y, MANCHIKALAPUDI S, LI RC-X, BOLLI R. PKC-dependent activation of P44/P42 during myocardial ischemia/reperfusion in conscious rabbits. *Am J Physiol* (In press).
- PING P, ZHANG J, QIU Y, TANG X-L, MANCHIKALAPUDI S, CAO X, BOLLI R, 1997. Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits. *Circ Res* **81**: 404–413.
- QIU Y, R A, TANG X-L, MANCHIKALAPUDI S, TAKANO H, JADOON AK, WU W-J, 1997. Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol* **273**: H2931–H2936.
- ROBBINS J, GULICK J, SÁNCHEZ A, HOWLES P, DOETSCHMAN T, 1990. Mouse embryonic stem cells express the cardiac myosin heavy chain genes during development in vitro. *J Biol Chem* **265**: 11905–11909.
- SEKI T, HAGIWARA H, NARUSE K, KADOWAKI M, KASHIWAGI M, DEMURA H, HIROSE S, NARUSE M, 1996. In situ identification of messenger RNA of endothelial type nitric oxide synthase in rat cardiac myocytes. *Biochem Biophys Res Commun* **218**: 601–605.
- SHINDO T, IKEDA U, OHKAWA F, KAWAHARA Y, YOKOYAMA M, SHIMADA K, 1995. Nitric oxide synthesis in cardiac myocytes and fibroblasts by inflammatory cytokines. *Cardiovasc Res* **29**: 813–819.
- SCHULZ R, NAVA E, MONCADA S, 1992. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. *Br J Pharmacol* **105**: 575–580.
- SUBRAMANIAM A, JONES WK, GULICK J, WERT S, NEUMANN J, ROBBINS J, 1991. Tissue-specific regulation of the α -myosin heavy chain gene promoter in transgenic mice. *J Biol Chem* **266**: 24613–24620.
- SUN J-Z, TANG X-L, KNOWLTON AA, PARK SW, QIU Y, BOLLI R, 1995. Late preconditioning against myocardial stunning: an endogenous protective mechanism that confers resistance to postischemic dysfunction 24 h after brief ischemia in conscious pigs. *J Clin Invest* **95**: 388–403.
- SUZUKI H, WILDHIRT SM, DUDEK RR, NARAYAN KS, BAILEY AH, BING RJ, 1996. Induction of apoptosis in myocardial infarction and its possible relationship to nitric oxide synthase in macrophages. *Tissue Cell* **28**: 89–97.
- TAKANO H, MANCHIKALAPUDI S, TANG X-L, QIU Y, RIZVI A, JADOON AK, ZHANG Q, BOLLI R, 1998a. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* **98**: 441–449.
- TAKANO H, TANG X-L, QIU Y, GUO Y, FRENCH B, BOLLI R, 1998b. Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant sensitive mechanism. *Circ Res* **83**: 73–84.
- TOSAKI A, MAULIK N, ELLIOTT GT, BLASIG IE, ENGELMAN RM, DAS DK, 1998. Preconditioning of rat heart with monophosphoryl lipid A: a role for nitric oxide. *J Pharmacol Exp Ther* **285**: 1274–1279.
- WALLENSTEIN S, ZUCKER CL, FLEISS JL, 1980. Some statistical methods useful in circulation research. *Circ Res* **47**: 1–9.
- WANG Y, MARSDEN PA, 1995. Nitric oxide synthases: gene structure and regulation. *Adv Pharmacol* **34**: 71–90.
- WANG P, ZWEIR JL, 1996. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart: evidence for peroxynitrite-mediated reperfusion injury. *J Biol Chem* **271**: 29223–29230.
- WILDHIRT SM, DUDEK RR, SUZUKI H, BING RJ, 1995a. Involvement of inducible nitric oxide synthase in the inflammatory process of myocardial infarction. *Int J Cardiol* **50**: 253–261.
- WILDHIRT SM, DUDEK RR, SUZUKI H, PINTO V, NARAYAN KS, BING RJ, 1995b. Immunohistochemistry in the identification of nitric oxide synthase isoenzymes in myocardial infarction. *Cardiovasc Res* **29**: 526–531.
- XUAN Y-T, TANG X-L, QIU Y, BANERJEE S, TAKANO H, HAN H, BOLLI R, 1998. Direct evidence that NOS is the mediator of late preconditioning in conscious rabbits. (Abstract). *Circulation* **98**: I-417.
- XUAN Y-T, TANG X-L, QIU Y, BANERJEE S, TAKANO H, HAN

- H, BOLLI R, 1999. Nuclear factor kappa B plays an essential role in the late phase of ischemic preconditioning in the conscious rabbit. *Circ Res* **31**: 1095–1109.
- YANG XM, BAXTER GF, HEADS RJ, YELLON DM, DOWNEY JM, COHEN MV, 1996. Infarct limitation of the second window of protection in a conscious rabbit model. *Cardiovasc Res* **31**: 777–783.
- ZHAO L, WEBER PA, SMITH JR, COMERFORD ML, ELLIOTT GT, 1997. Role of inducible nitric oxide synthase in pharmacological “preconditioning” with monophosphoryl lipid A. *J Mol Cell Cardiol* **29**: 1567–1576.