Rapid Communication

Nuclear Factor-κB Plays an Essential Role in the Late Phase of Ischemic Preconditioning in Conscious Rabbits

Yu-Ting Xuan, Xian-Liang Tang, Supratim Banerjee, Hitoshi Takano, Richard C.X. Li, Hui Han, Yumin Qiu, Jian-Jun Li, Roberto Bolli

Abstract—Although it is recognized that late preconditioning (PC) results from upregulation of cardioprotective genes, the specific transcription factor(s) that govern this genetic adaptation remains unknown. The aim of this study was to test the hypothesis that the development of late PC is mediated by nuclear factor-κB (NF-κB) and to elucidate the mechanisms that control the activation of NF-κB after an ischemic stimulus in vivo. A total of 152 chronically instrumented, conscious rabbits were used. A sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles, which elicits late PC, induced rapid activation of NF-κB, as evidenced by a marked increase in p65 content (+164%; Western immunoblotting) and NF-κB DNA binding activity (+306%; electrophoretic mobility shift assay) in nuclear extracts isolated 30 minutes after the last reperfusion. These changes were attenuated 2 hours after ischemic PC and resolved by 4 hours. Competition and supershift assays confirmed the specificity of the NF-κB DNA complex signals. The mobility of the NF-κB DNA complex was shifted by anti-p65 and anti-p50 antibodies but not by anti–c-Rel antibodies, indicating that the subunits of NF-κB involved in gene activation after ischemic PC consist of p65-p50 heterodimers. Pretreatment with the NF-κB inhibitor diethyldithiocarbamate (DDTC; 150 mg/kg IP 15 minutes before ischemic PC) completely blocked the nuclear translocation and increased DNA binding activity of NF-κB. The same dose of DDTC completely blocked the cardioprotective effects of late PC against both myocardial stunning and myocardial infarction, indicating that NF-κB activation is essential for the development of this phenomenon in vivo. The ischemic PC-induced activation of NF-κB was also blocked by pretreatment with *N*$^{\omega}$-nitro-L-arginine (L-NA), a nitric oxide synthase (NOS) inhibitor, *N*-2-mercaptopropionyl glycine (MPG), a reactive oxygen species (ROS) scavenger, chelerythrine, a protein kinase C (PKC) inhibitor, and lavendustin A, a tyrosine kinase inhibitor (all given at doses previously shown to block late PC), indicating that ischemic PC activates NF-κB via formation of NO and ROS and activation of PKC- and tyrosine kinase–dependent signaling pathways. A subcellular redistribution and increased DNA binding activity of NF-κB quantitatively similar to those induced by ischemic PC could be reproduced pharmacologically by giving the NO donor diethylenetriamine/NO (DETA/NO) (at a dose previously shown to elicit late PC), demonstrating that NO in itself can activate NF-κB in the heart. Taken together, these results provide direct evidence that activation of NF-κB is a critical step in the signal transduction pathway that underlies the development of the late phase of ischemic PC in conscious rabbits. The finding that four different pharmacological manipulations (L-NA, MPG, chelerythrine, and lavendustin A) produced similar inhibition of NF-κB suggests that this transcription factor is a common downstream pathway through which multiple signals elicited by ischemic stress (NO, ROS, PKC, tyrosine kinases) act to induce gene expression. To our knowledge, this is the first demonstration that NO can promote NF-κB activation in the heart, a finding that identifies a new biological function of NO and may have important implications for various pathophysiological conditions in which NO is involved and for nitrate therapy. **(*Circ Res*. 1999;84:1095-1109.)**

Key Words: inducible nitric oxide synthase ■ myocardial ischemia/reperfusion ■ postischemic myocardial dysfunction ■ peroxynitrite ■ myocardial adaptation

The late phase of ischemic preconditioning (PC) is a delayed adaptive response that renders the heart relatively resistant to ischemia/reperfusion injury 12 to 24 hours after a brief ischemic stress.[1–13] Recent studies suggest that the initial signals responsible for triggering the development of late PC after the ischemic stimulus (on day 1) involve the generation of nitric oxide (NO)[5,6] and reactive oxygen species (ROS),[14,15] and the subsequent activation of protein kinase C (PKC)[13,16–18] and protein tyrosine kinases.[19–21] In addition, mounting evidence suggests that the cardioprotective effects

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of late PC observed 24 hours later (on day 2) are due to upregulation of NO synthase (NOS) and, specifically, of the inducible isoform (iNOS).[8,11] The facts that late PC requires more than 6 hours to become manifest[9,10] and that it is blocked by cycloheximide[22] indicate that it is dependent on the synthesis of new proteins. Nevertheless, there is still a major gap in our understanding of the mechanism of late PC: namely, the downstream signaling pathways that transduce the activation of cellular kinases soon after ischemia into increased expression of iNOS (and possibly after genes) 24 hours later remain enigmatic. If de novo protein synthesis is involved, it seems likely that the signaling cascade of late PC would include the activation of one or more transcription factors that direct the expression of the protein(s) responsible for cardioprotection (such as iNOS). The identity (or even the existence) of such factor(s), however, is unknown.

One of the transcription factors that could activate gene expression in response to ischemic PC is nuclear factor-κB (NF-κB). This oxidant-sensitive transcription factor plays a critical role in the immediate-early activation of a multitude of genes encoding signaling and defense proteins expressed in response to various stressful situations and therefore appears to be a general mediator of cellular responses to stress (reviewed in References 23 and 24). The fact that iNOS mediates late PC[8,11] supports the hypothesis that NF-κB may participate in this phenomenon, since it is well established that the 5′ flanking region of the iNOS gene contains a consensus sequence that binds to NF-κB[25] and that activation of NF-κB is a central mechanism controlling the induction of iNOS in several cell types, including cardiac myocytes.[25–28] However, the role of NF-κB in the delayed myocardial adaptations to ischemic stress remains poorly understood.

The overall goal of the present study was to test the hypothesis that the development of late PC is mediated by NF-κB and to elucidate the cellular mechanisms that control the activation of this transcription factor after an ischemic stimulus in vivo. Four fundamental specific questions were addressed: (1) Does ischemic PC activate NF-κB? (2) If so, does NF-κB activation play a causative role in the development of late PC, or is it an epiphenomenon? (3) What is the chemical signal that triggers the activation of NF-κB during ischemic PC? (4) Which cellular kinase(s) is/are responsible for the ischemic PC-induced activation of NF-κB? To address these issues, a series of molecular analyses were combined with functional studies in a well-characterized rabbit model of late PC.[5,6,13,15,17,18,20–22,29] All experiments were performed in conscious animals to obviate the confounding effects of factors associated with open-chest preparations, such as anesthesia, surgical trauma, fluctuations in temperature, elevated catecholamine levels, excessive ROS formation, release of cytokines, which could interfere with myocardial stunning and infarction,[30–36] with PC,[37–40] and/or with NF-κB.[23,24] The results demonstrate, for the first time, that NF-κB plays a necessary role in the development of the late phase of ischemic PC and that its activation is modulated by NO, ROS, PKC, and tyrosine kinases.

Materials and Methods

This study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine (Louisville, Ky) and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, National Institutes of Health, Publication No. 86-23).

Experimental Preparation

The experimental preparation has been described in detail previously.[5–8,10,11,13,18,29] Briefly, New Zealand White male rabbits (2.2±0.1 kg; Myrtles Rabbitry, Thompson Station, Tenn) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal[41] in the center of the region to be rendered ischemic, and bipolar ECG leads on the chest wall. All rabbits were allowed to recover for a minimum of 10 days after surgery. Throughout the experiments, rabbits were kept in a cage in a quiet, dimly lit room. No antiarrhythmic agents were given at any time.

Experimental Protocol

The study consisted of three successive phases (A, B, and C) (Figures 1 through 3).

Phase A: Effect of Ischemic PC on NF-κB

Rabbits were assigned to 7 groups (Figure 1). Group I (control) did not receive any treatment and did not undergo coronary artery occlusion. The rabbits were given heparin (1000 U IV), after which they were anesthetized with sodium pentobarbital (50 mg/kg IV) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples (≈0.5 g) were rapidly removed from the anterior and posterior left ventricular (LV) wall and stored in liquid nitrogen until used. Groups II, III, IV, and V underwent a sequence of six 4-minute coronary occlusions interspersed with 4 minutes of reperfusion and were euthanized at 30, 60, 120, or 240 minutes after the last reperfusion, respectively. Myocardial samples were rapidly removed from the ischemic-reperfused region (whose boundaries had been marked with sutures at the time of instrumentation) and from the nonischemic region (posterior LV wall), frozen in liquid nitrogen, and stored at −140°C until used. In group VI (diethyldithiocarbamate [DDTC] treatment), rabbits underwent 6 coronary occlusion/reperfusion cycles and received DDTC (150 mg/kg IP) 15 minutes before the first coronary occlusion. The rabbits were euthanized 30 minutes after the sixth reperfusion. DDTC (diethyldithiocarbamic acid, sodium salt; Sigma Chemical Co) was dissolved in normal saline (total volume injected, 10 mL). In group VII (DDTC pretreatment), rabbits received the same dose of DDTC without undergoing occlusion/reperfusion cycles and were euthanized at a corresponding time (89 minutes after DDTC). In both groups VI and VII, myocardial samples were obtained as described above.

Phase B: Effect of DDTC on Ischemic PC

Studies of Myocardial Stunning

The experimental protocol consisted of 2 or 3 consecutive days of coronary occlusions (days 1, 2, and 3, respectively). On each day, the rabbits were subjected to a sequence of six 4-minute occlusion/4-minute reperfusion cycles (Figure 2). Rabbits were assigned to 3 groups: group VIII (control), group IX (DDTC treatment), and group X (DDTC pretreatment). On day 1, group IX underwent 6 occlusion/reperfusion cycles and received DDTC at the same dose that was used in group VI in phase A (150 mg/kg IP 15 minutes before the first occlusion), whereas group VIII received no treatment. On day 1, group X received the same dose of DDTC as group IX without undergoing coronary occlusion/reperfusion; these rabbits then underwent a sequence of 6 occlusion/reperfusion cycles on days 2 and 3.

Studies of Myocardial Infarction

On day 2, all rabbits were subjected to a 30-minute coronary artery occlusion followed by 3 days of reperfusion (Figure 2). Diazepam was administered 20 minutes before the onset of ischemia (4 mg/kg IP) to relieve the stress caused by the coronary occlusion. Rabbits were assigned to 4 groups (Figure 2). Group XI (control group)

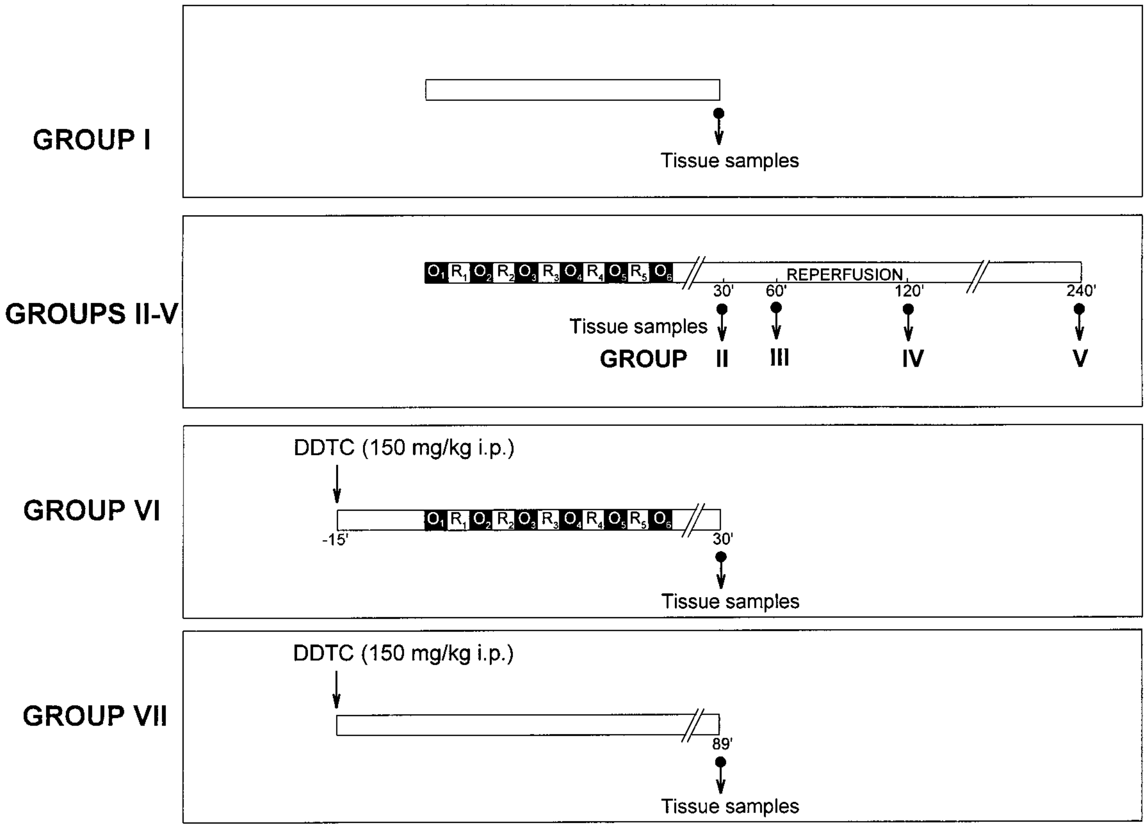

Figure 1. Experimental protocol for phase A.

underwent the 30-minute occlusion with no PC and no drug pretreatment on day 1. Group XII (PC group) was preconditioned with a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles 24 hours before the 30-minute coronary occlusion (day 1). Group XIII (DDTC treatment) underwent the same protocol as group XII except that the rabbits received DDTC (150 mg/kg IP) 15 minutes before the first coronary occlusion on day 1 (this is the same dose that was used in group VI in phase A). Group XIV (DDTC pretreatment) underwent the same protocol as group XIII except that the rabbits were not preconditioned.

Phase C: Effects of L-NA, MPG, Chelerythrine, Lavendustin A, and DETA-NO on NF-κB

Rabbits were assigned to 6 groups (groups XV through XX) (Figure 3). Five of these groups (groups XV through XIX) underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles. Group XV (N^{ω}-nitro-L-arginine [L-NA] group) received an intravenous infusion of L-NA at a rate of 1.3 mg · kg^{-1} · min^{-1} for 10 minutes, starting 20 minutes before and ending 10 minutes before the first coronary occlusion (total dose, 13 mg/kg; total volume infused, 20 mL). Group XVI (*N*-2-mercaptopropionyl glycine [MPG] group) received a continuous intravenous infusion of MPG (0.42 mg · kg^{-1} · min^{-1}), beginning 60 minutes before the first coronary occlusion and ending 30 minutes after the sixth reperfusion (total dose, 56 mg/kg; total volume infused, 5.4 mL/kg). Group XVII (chelerythrine group) received an intravenous bolus of chelerythrine (5 mg/kg) 5 minutes before the first coronary occlusion. Group XVIII (lavendustin A group) received an intravenous bolus of lavendustin A (1 mg/kg) 10 minutes before the first coronary occlusion. L-NA (Sigma) was dissolved in normal saline. MPG (Sigma) was dissolved in sterile water, and the pH was adjusted to 7.5 using 0.1 mmol/L NaOH. Chelerythrine chloride (Research Biomedicals International) was dissolved in 0.5 mL/kg of DMSO+0.5 mL/kg of normal saline. Lavendustin A (CalBiochem Co) was dissolved in a 10% (vol/vol) solution of DMSO in normal saline.

Because both chelerythrine and lavendustin A were dissolved in DMSO, an additional group of rabbits (group XIX [DMSO control group]) was studied to examine any possible influence of DMSO itself on NF-κB. Five minutes before the first coronary occlusion, these rabbits received an intravenous bolus of the vehicle used in group XVII for chelerythrine (0.5 mL/kg of DMSO+0.5 mL/kg of saline) (Figure 3). (The dose of DMSO used in group XVII to dissolve chelerythrine [0.5 mL/kg] was higher than that used in group XVIII to dissolve lavendustin A [0.1 mL/kg]; consequently, group XIX received the dose used in group XVII). Group XX (diethylenetriamine/NO [DETA/NO] group) received 4 consecutive intravenous boluses of DETA/NO (0.1 mg/kg each) every 25 minutes (total dose, 0.4 mg/kg) (Figure 3). DETA/NO (Alexis Corp) was dissolved in PBS immediately before the infusion; to remove oxygen, the PBS solution was bubbled with nitrogen for at least 30 minutes before dissolving DETA/NO.[29] All rabbits were euthanized 30 minutes after the sixth reperfusion (groups XV through XIX) or 30 minutes after the last bolus of DETA/NO (group XX).

Preparation of Cytosolic Proteins

Cytosolic proteins were prepared using a modification of the method described by Balligand et al[26] and Garcia-Cardena et al.[42] Samples were powdered and homogenized in homogenization buffer containing 25 mmol/L Tris-HCl [pH 7.4], 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 1 mmol/L PMSF, 1 mmol/L DTT, 25 μg/mL leupeptin, 25 mmol/L NaF, and 1 mmol/L Na_3VO_4. The homogenates were centrifuged at 14 000*g* for 15 minutes, and the resulting supernatants were collected as cytosolic proteins for Western blot analysis. Protein contents were determined by a Bio-Rad protein assay kit.

Preparation of Nuclear Extracts

Nuclear extracts were prepared using a modification of the method described by Dignam et al.[43] The samples were homogenized in

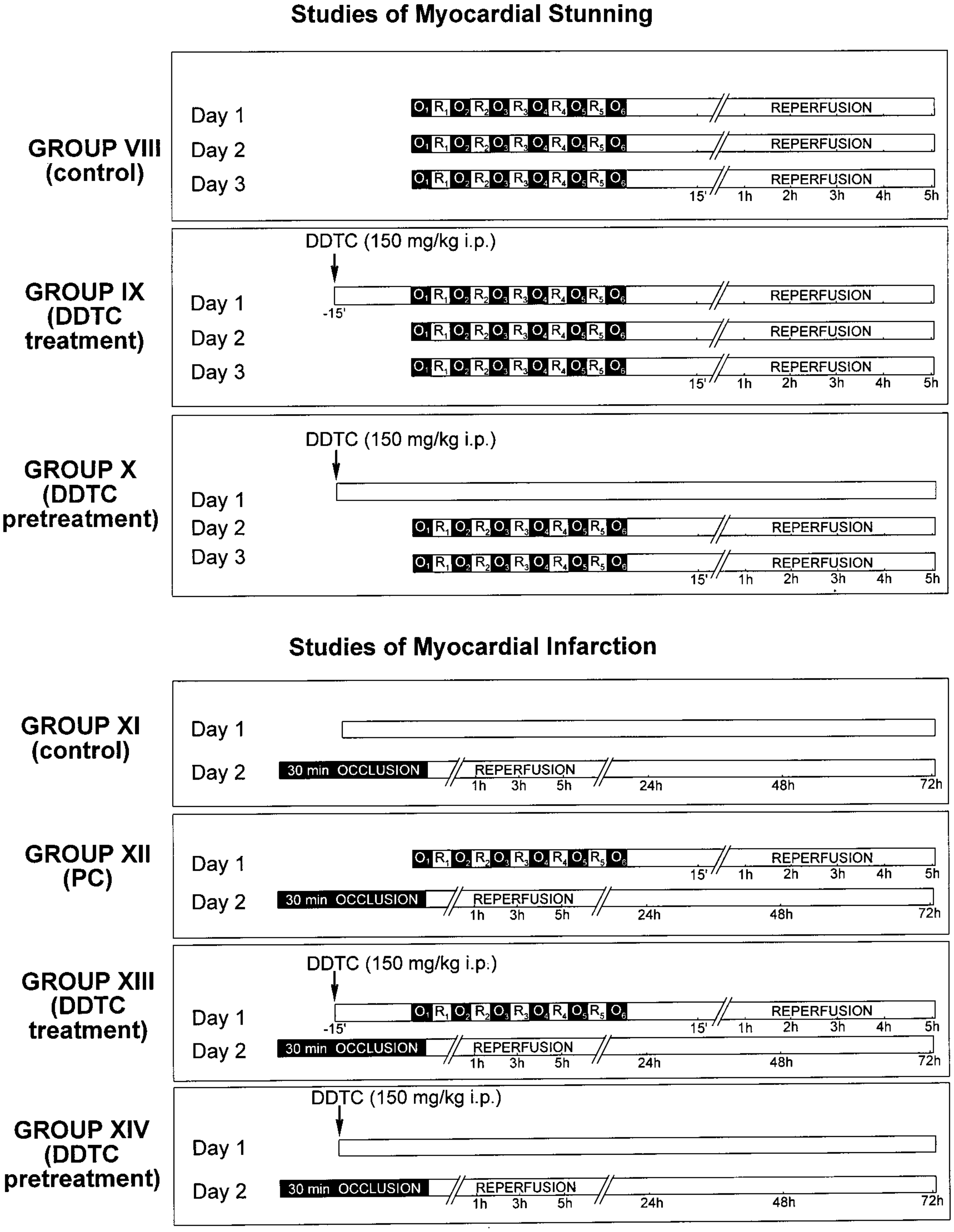

Figure 2. Experimental protocol for phase B.

buffer A (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 1 mmol/L DTT, 25 μg/mL leupeptin, and 1 mmol/L PMSF). After a 10-minute incubation on ice, the samples were centrifuged at 1850*g* for 10 minutes at 4°C. The pellets were dissolved in buffer B (buffer A+0.1% Triton X-100), incubated on ice for 10 minutes, and then centrifuged as above. The crude nuclear pellets were washed once with buffer A and resuspended in buffer C (20 mmol/L HEPES [pH 7.9], 25% glycerol (vol/vol), 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and 1 mmol/L PMSF) for 30 minutes at 4°C. Nuclear proteins were recovered after centrifugation at 25 000*g* for 30 minutes. The resulting clear supernatants were dialyzed against 100 volumes of buffer D (20 mmol/L HEPES [pH 7.9], 4% glycerol, 50 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L $MgCl_2$, 0.5 mmol/L PMSF, and 0.5 mmol/L DTT) for 6 hours at 4°C. The dialysates were centrifuged again at 25 000*g* for 30 minutes. The resulting supernatants were designated as the nuclear protein extracts. The purity of the nuclear extracts was examined using lactate dehydrogenase (LDH) as a cytosolic marker, and it was found that 1.8% of total myocardial tissue LDH was present in the nuclear fraction.

Western Immunoblotting Analysis

The subcellular distribution of the p65 subunit of NF-κB was assessed using standard SDS-PAGE Western immmunoblotting

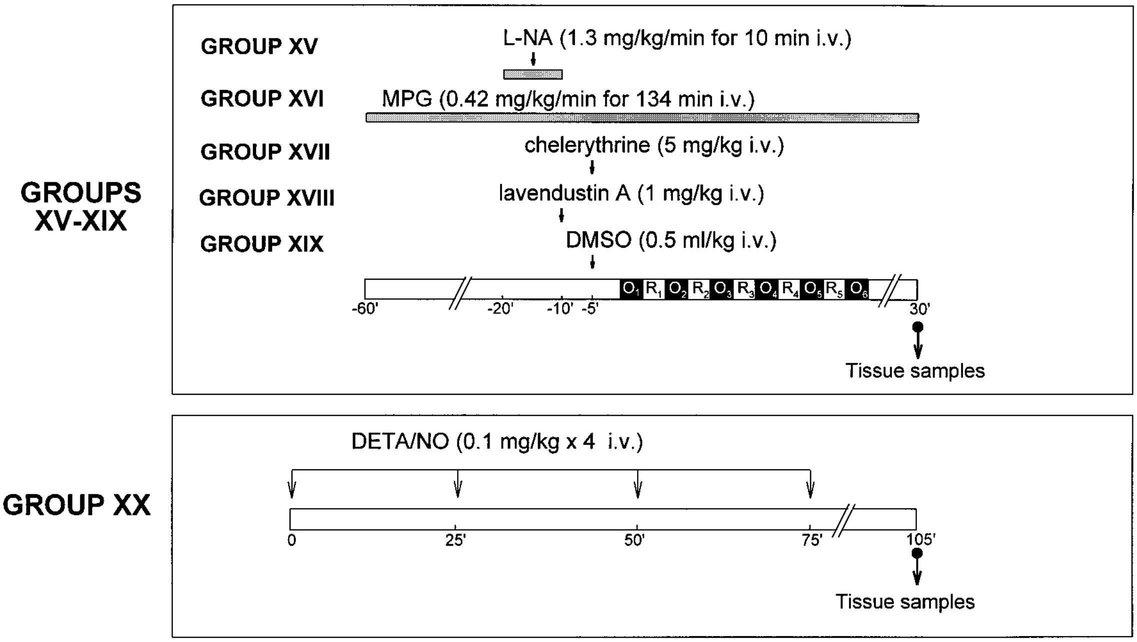

Figure 3. Experimental protocol for phase C.

techniques. Briefly, 70 μg of cytosolic proteins and 60 μg of nuclear proteins were electrophoresed on a 12% denaturing SDS gel for 2 to 3 hours. After electrophoresis, the proteins on the gel were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) overnight at 4°C. Gel transfer efficiency was carefully recorded by making photocopies of membranes dyed with reversible Ponceau staining[17]; gel retention was determined by Coomassie blue staining as previously described.[17] The membranes were incubated in 5% nonfat dry milk in TBST buffer (10 mmol/L Tris-HCl [pH 7.2], 0.15 mol/L NaCl, and 0.05% Tween-20) followed by incubation with specific polyclonal anti–NF-κB p65 antibodies (Santa Cruz Biotechnology, Inc). After extensive rinsing with TBST buffer, the blots were incubated with HRP-conjugated anti-rabbit secondary antibodies and developed with the use of an enhanced chemiluminescence

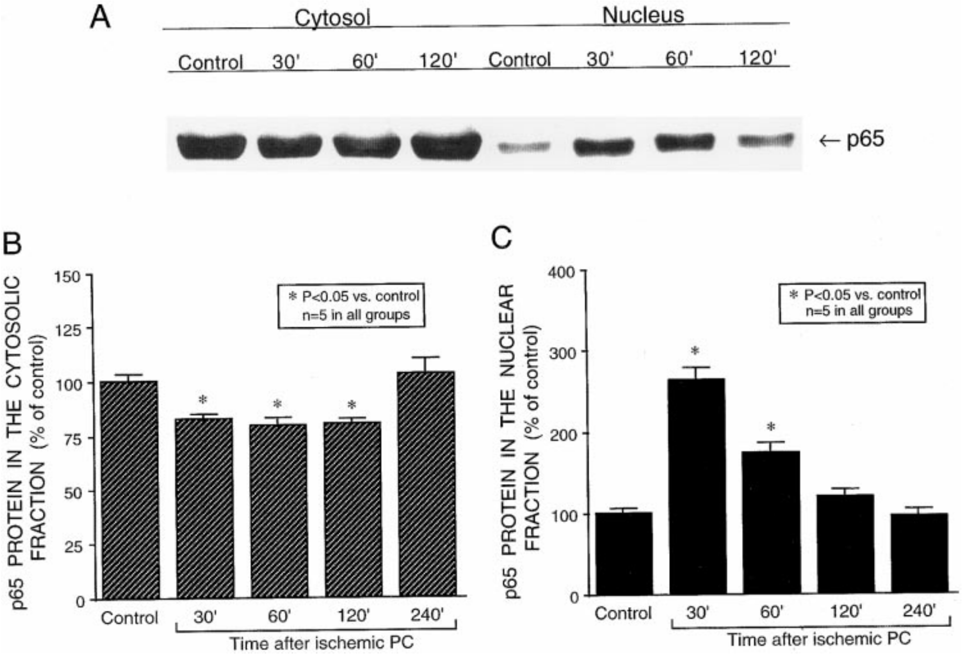

Figure 4. Effect of ischemic PC on the subcellular distribution of the p65 subunit of NF-κB. Conscious rabbits were subjected to a sequence of 6 cycles of 4-minute coronary occlusion/4-minute reperfusion; samples of ischemic-reperfused myocardium were obtained at 30, 60, 120, or 240 minutes after the sixth reperfusion (groups II, III, IV, and V, respectively). Control rabbits (group I) did not undergo coronary occlusion/reperfusion. A, Western blot analysis of p65. Cytosolic and nuclear proteins were separated by SDS-PAGE, transferred, and immunoblotted with a specific anti-p65 antibody followed by ECL. The position of p65 is indicated by the arrow. Data shown are representative of 5 separate experiments with similar results. The level of p65 in the nuclear fraction was very low in control tissue but increased markedly at 30 and 60 minutes after ischemic PC. B, Time-dependent changes in cytosolic p65 protein content after ischemic PC. C, Time-dependent changes in nuclear p65 protein content after ischemic PC. Data are mean±SEM (n=5).

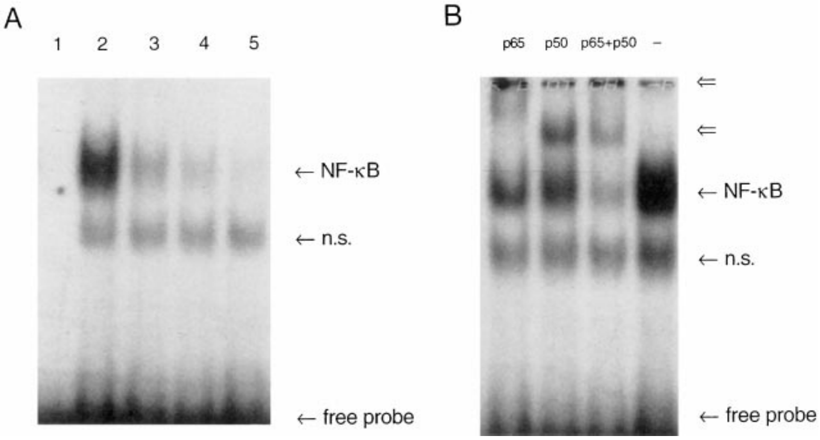

Figure 5. Competition assay and supershift assay of NF-κB DNA binding activity in nuclear extracts of preconditioned myocardium. Nuclear extracts were prepared from ischemic-reperfused tissue obtained 30 minutes after 6 cycles of coronary occlusion/reperfusion. An EMSA was performed using a ^{32}P-labeled NF-κB consensus oligonucleotide probe, as described in Materials and Methods. A, Competition assay of NF-κB DNA binding activity. The binding activity assay was performed in the absence (lane 2) or presence of increasing amounts of unlabeled NF-κB consensus oligonucleotide (lane 3, 2.5-fold; lane 4, 5-fold; and lane 5, 10-fold). Lane 1 shows the reaction mixture alone without nuclear extracts. The specific bands of NF-κB DNA complexes, the nonspecific (n.s.) activity, and the free probe are indicated by the arrows. When increasing folds of unlabeled NF-κB oligonucleotide were preincubated with the nuclear extracts before the addition of the labeled probe, the NF-κB DNA complex signal disappeared progressively (lanes 3 through 5). B, Supershift assay of NF-κB DNA binding activity. The binding activity assay was performed in the presence of anti-p65 antibodies (lane 1), anti-p50 antibodies (lane 2), a combination of both (lane 3), or no antibodies (lane 4). The shifted bands of specific NF-κB DNA complexes are indicated by the arrow. The open arrows indicate the NF-κB DNA complexes that were supershifted by anti-p65 antibodies, by anti-p50 antibodies, and by their combination.

system (ECL kit, Amersham). After washing with TBST buffer, the blots were air-dried and exposed to Kodak films in x-ray cassettes with intensifying screens. The p65 signals detected by immunoblotting and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated using an image scanning densitometer (Personal PI, Molecular Dynamics). As elaborated previously,[17] despite a careful attempt to achieve equal protein loading in all lanes of the gel, the total amounts of proteins transferred from each lane to the nitrocellulose membranes during blotting are rarely identical. Therefore, given the critical importance of quantitating p65 as accurately as possible, each p65 signal was normalized to the corresponding Ponceau stain signal determined by densitometric analysis of the Ponceau stain record, as previously described.[17] To ensure consistency in the data analysis, the cytosolic and nuclear fractions of the same sample were run on the same gel (Figure 4).

Electrophoretic Mobility Shift Assay (EMSA)

A double-stranded 22-mer oligonucleotide with the sequence 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega Corp) was end-labeled using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase according to the manufacturer's protocol. This oligonucleotide has the consensus sequence for NF-κB binding, as indicated by underlines. After the labeling reaction, the ^{32}P-labeled oligonucleotide was purified with a G-25 Sephadex column (Pharmacia). The binding reactions were performed in a final volume of 10 μL that contained nuclear proteins (8 μg), 10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 4% glycerol (vol/vol), and 1 μg Poly(dI-dC) · Poly(dI-dC) (Sigma).[44] After a 10-minute preincubation at 4°C, the labeled probe (50 000 to 60 000 cpm) was added to the mixture and incubated for an additional 20 minutes at 22°C. After the binding reactions, the DNA protein complexes were separated on 4%

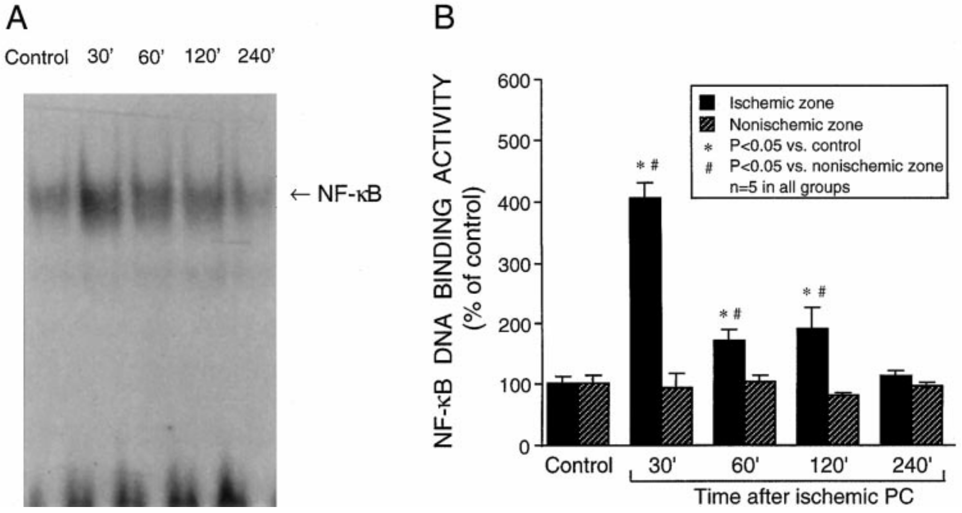

Figure 6. Time course of increased NF-κB DNA binding activity after ischemic PC. A, Nuclear extracts were prepared from control samples (group I) and ischemic-reperfused samples obtained at 30, 60, 120, and 240 minutes after 6 coronary occlusion/reperfusion cycles (groups II, III, IV, and V, respectively). The extracts were incubated with the ^{32}P-labeled NF-κB oligonucleotide probe to determine the binding activity in an EMSA. The shifted bands of specific NF-κB DNA complexes are indicated by the arrow. Data shown are representative of 5 separate experiments in each group. B, The specific signals of the NF-κB DNA complexes in the nuclear extracts isolated from the ischemic-reperfused region and the nonischemic region (posterior LV wall) of control and ischemic-reperfused hearts were scanned and expressed as a percentage of control. Data are mean±SEM.

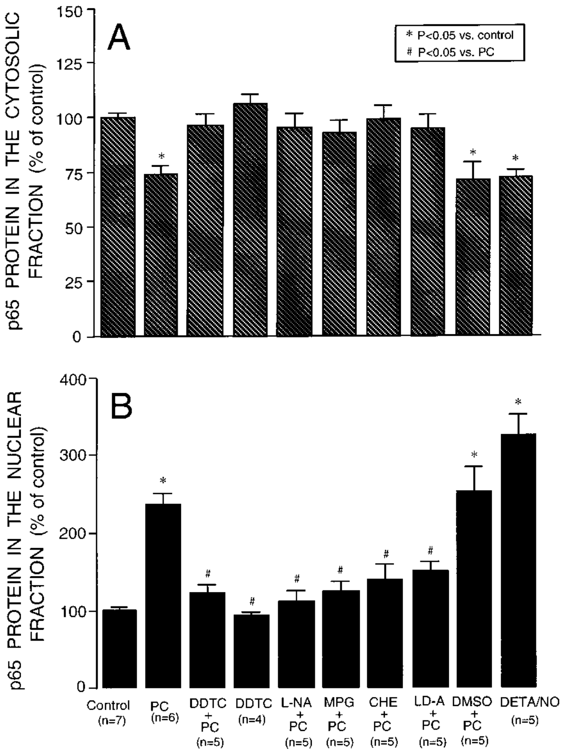

Figure 7. NF-κB p65 subunit content in the cytosolic (A) and nuclear (B) fractions of myocardial samples obtained from the ischemic/reperfused region of the heart of control rabbits (this group included the 5 rabbits in group I plus 2 other control rabbits), of rabbits subjected to six 4-minute coronary occlusion/4-minute reperfusion cycles without any treatment (PC [group II]) (this group included the 5 rabbits in group II plus 1 additional rabbit), of rabbits subjected to the six 4-minute occlusion/4-minute reperfusion cycles in conjunction with the administration of DDTC (DDTC+PC [group VI]), L-NA (L-NA+PC [group XV]), MPG (MPG+PC [group XVI]), chelerythrine (CHE+PC [group XVII]), lavendustin A (LD-A+PC [group XVIII]), or DMSO (DMSO+PC [group XIX]), and of rabbits given DETA/NO or DDTC without ischemic PC (DETA/NO [group XX] and DDTC [group VII]). Cytosolic or nuclear proteins were separated by SDS-PAGE, transferred, and immunoblotted with a polyclonal anti-p65 antibody followed by ECL. The specific p65 signals from 5 separate experiments were scanned and expressed as a percentage of control. Data are mean±SEM.

nondenaturing polyacrylamide gels in 0.5× Tris borate-EDTA (TBE) buffer. Gels were vacuum-dried and exposed to x-ray film at −70°C using an intensifying screen. Specific band intensities were quantified by an image scanning densitometer, as described above.

Gel Supershift and Competition Assays

Gel supershift assays and competition assays were performed to ensure that the signal was specific for NF-κB. For the supershift assays, 0.3 μg of either anti-p65 or anti-p50 antibodies (Santa Cruz Biotechnology, Inc) was added, separately or together, to the reaction mixtures immediately after addition of radiolabeled probe. For the competition assays, increasing fold molar excess of unlabeled NF-κB oligonucleotide was added into separate reaction mixtures.

Measurements of Regional Myocardial Function, Region at Risk, and Infarct Size

Regional myocardial function was assessed as systolic thickening fraction using the pulsed Doppler probe, as previously described.[41] The total deficit of systolic wall thickening (WTh) (an integrative assessment of the overall severity of myocardial stunning) was

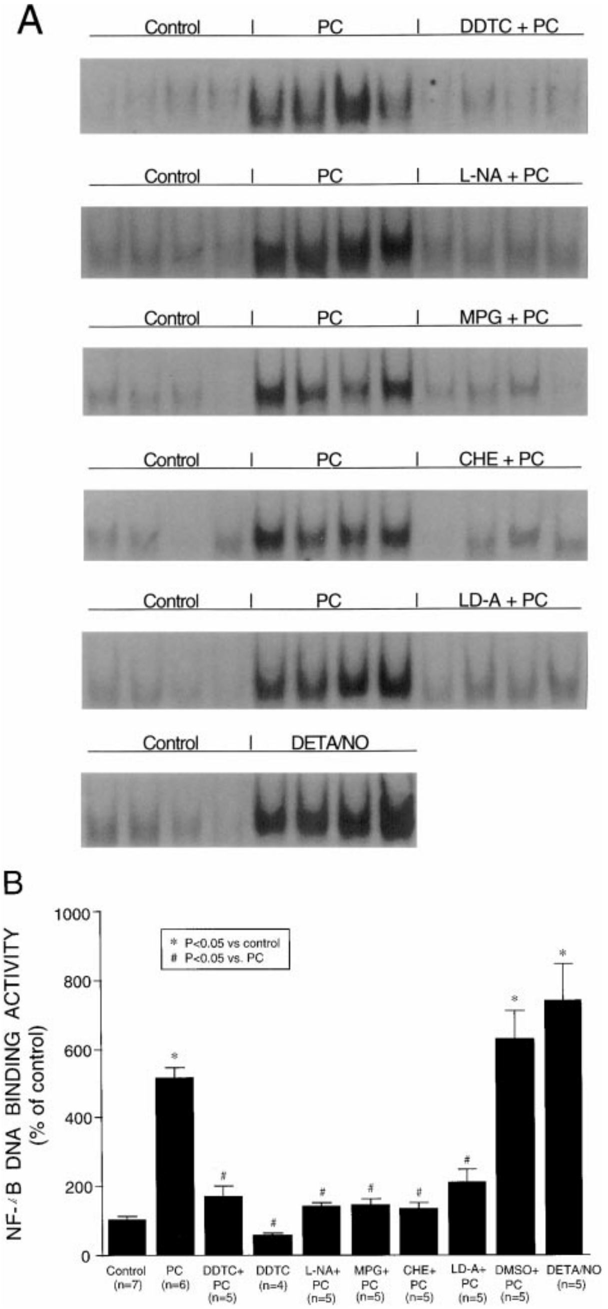

Figure 8. NF-κB DNA binding activity in the nuclear fraction of myocardial samples obtained from the ischemic/reperfused region of the heart of control rabbits (this group included the 5 rabbits in group I plus 2 other control rabbits), of rabbits subjected to six 4-minute coronary occlusion/4-minute reperfusion cycles without any treatment (PC [group II]) (this group included the 5 rabbits in group II plus 1 additional rabbit), of rabbits subjected to the six 4-minute occlusion/4-minute reperfusion cycles in conjunction with the administration of DDTC (DDTC+PC [group VI]), L-NA (L-NA+PC [group XV]), MPG (MPG+PC [group XVI]), chelerythrine (CHE+PC [group XVII]), lavendustin A (LD-A+PC [group XVIII]), or DMSO (DMSO+PC [group XIX]), and of rabbits given DETA/NO or DDTC without ischemic PC (DETA/NO [group XX] and DDTC [group VII]). The nuclear extracts were incubated with the ^{32}P-labeled NF-κB oligonucleotide probe to determine the DNA binding activity as described in Materials and Methods. A, EMSAs performed in the various groups (samples from 4 rabbits in each group are shown). B, Specific NF-κB signals were scanned and expressed as a percentage of control. Data are mean±SEM.

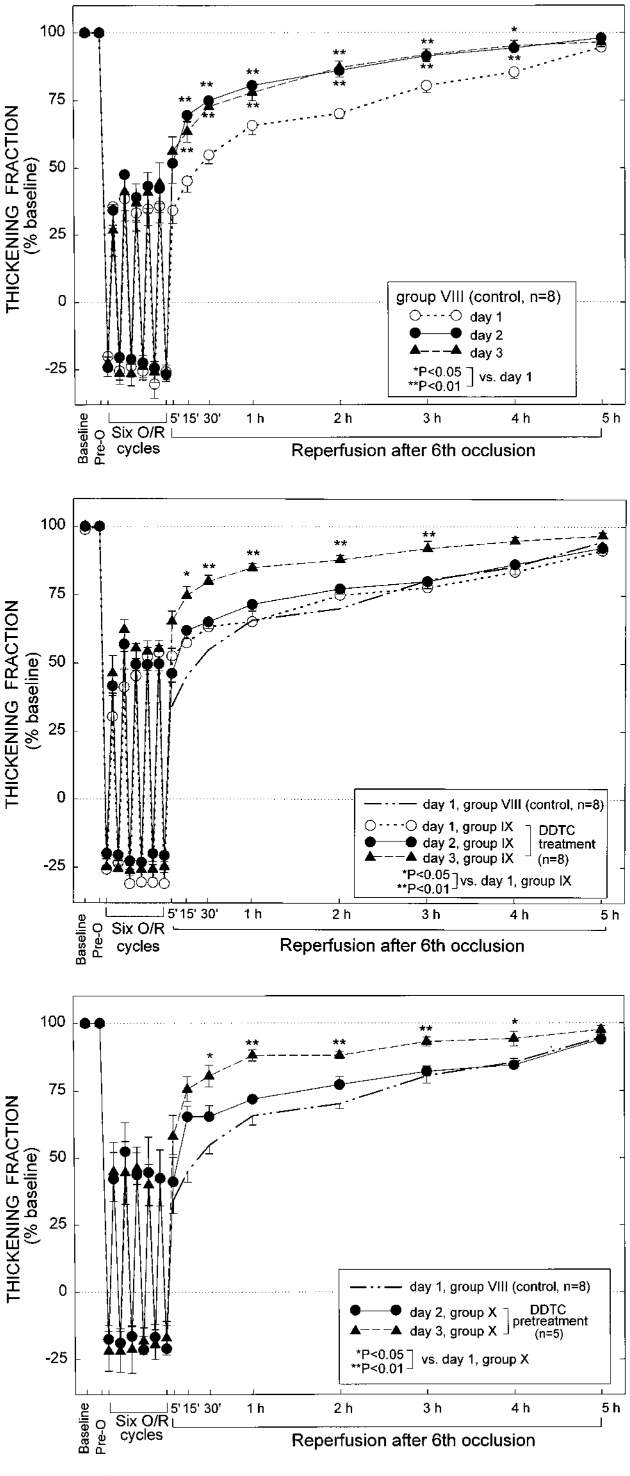

Figure 9. Systolic thickening fraction in the ischemic-reperfused region 5 minutes before the first occlusion or before DDTC (baseline), immediately before the first occlusion (Pre-O), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. Top, Measurements obtained in group VIII (control, n=8). Middle, Measurements obtained in group IX (DDTC treatment, n=8). Bottom, Measurements obtained in group X (DDTC pretreatment, n=5). Baseline measurements were taken 5 minutes before the first occlusion in groups VIII and X and 5 minutes before administration of DDTC in group IX. In all 3 groups, measurements taken on day 1 are represented by the dashed line with open circles; measurements taken on day 2 are represented by the continuous line with solid circles; and measurements taken on day 3 are represented by the interrupted line with solid triangles. To facilitate comparisons, the data pertaining to day 1 of group VIII (control) are shown again in the middle and bottom panels (dashed and dotted line without symbols, n=8). Thickening fraction is expressed as a percentage of baseline values. Baseline systolic thickening fraction did not differ among the 3 groups on the same day or among different days within the same group (36.3±2.8%, 37.0±3.0%, and 36.3±3.1% on days 1, 2, and 3, respectively, in group VIII; 40.0±0.9%, 39.6±1.4%, and 38.7±1.2% in group IX; and 43.7±3.3%, 42.7±2.6%, and 41.5±3.2% in group X). Data are mean±SEM.

calculated by measuring the area comprised between the systolic WTh-versus-time line and the baseline (100% line) during the 5-hour recovery phase after the sixth reperfusion.[5,7,8,10,13,18,29] The size of the ischemic-reperfused region (region at risk) and the size of the infarct were determined by postmortem perfusion followed by staining with triphenyltetrazolium chloride, as previously detailed.[6,11,18,29]

Statistical Analysis

Data are reported as mean±SEM. In phases A and C, differences among groups with respect to p65 cytosolic content, p65 nuclear content, and NF-κB DNA binding activity were analyzed using one-way ANOVA. If the ANOVA showed an overall difference, post hoc contrasts were performed with Student *t* tests for either paired or unpaired data, and the resulting probability values were adjusted according to the Bonferroni correction.[45] In phase B, data were analyzed by either one-way or two-way repeated-measures (time and group) ANOVA followed by paired or unpaired Student *t* tests, as appropriate, with the Bonferroni correction.[45]

Results

A total of 152 conscious rabbits were used in the present study.

Phase A: Effect of Ischemic PC on NF-κB

The goals of phase A were to determine (1) whether ischemic PC is associated with activation of NF-κB and (2) whether such activation can be blocked by DDTC in vivo. Five rabbits were assigned to each of the groups studied (groups I through VII). All of the rabbits completed the protocol successfully.

Effect of Ischemic PC on the Subcellular Distribution of p65

Figure 4A shows a representative Western blot analysis of the p65 content in the cytosolic and nuclear fractions prepared from a control tissue sample (group I) and from samples taken at serial times after the ischemic PC protocol (groups II through V). In the control sample, almost all of the p65 protein was found in the cytosolic fraction. After 6 occlusion/reperfusion cycles, however, the content of p65 protein increased in the nuclear fraction and decreased in the cytosolic fraction (Figure 4A). Quantitative analysis showed that the cytosolic content of p65 protein was 83% of control at 30 minutes, 79% at 60 minutes, and 80% at 120 minutes ($P<0.05$ versus control at all time points) and returned to control levels at 240 minutes (Figure 4B). The nuclear p65 content, on the other hand, increased 2.6-fold versus control at 30 minutes ($P<0.05$) and 1.7-fold ($P<0.05$) at 60 minutes after ischemic PC and returned to control levels at 120 minutes (Figure 4C). Thus, the kinetics of the increase in p65 in the nucleus mirrored the kinetics of its decrease in the cytosol, indicating that the ischemic PC protocol induced a

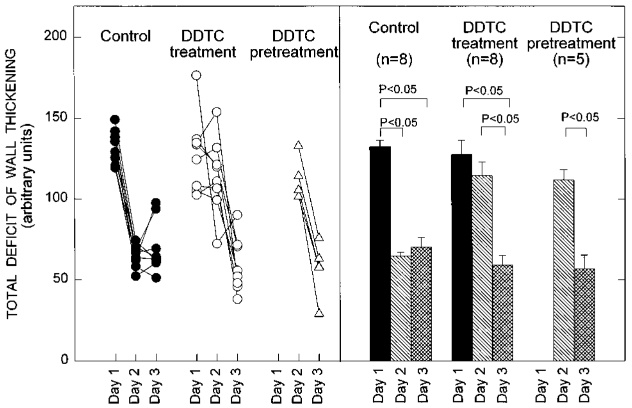

Figure 10. Total deficit of WTh after the sixth reperfusion on days 1, 2, and 3 in the control (n=8), DDTC-treated (n=8), and DDTC-pretreated (n=5) groups (groups VIII, IX, and X, respectively). Left, Values of total deficit of WTh in individual rabbits. Right, Mean±SEM. The total deficit of WTh was measured in arbitrary units, as described in the text.

rapid translocation of this subunit of NF-κB from the cytosolic to the nuclear compartment.

Specificity of NF-κB DNA Binding

To determine whether the translocated NF-κB was active, we next tested whether the increased NF-κB in the isolated nuclear fraction could specifically bind to κB DNA motifs. Nuclear extracts were prepared from tissue samples obtained 30 minutes after ischemic PC (when the translocation was maximal [Figure 4C]) and analyzed by an EMSA using a ^{32}P-labeled oligonucleotide probe with a high-affinity κB binding site. As illustrated in Figure 5A, a shifted band of NF-κB DNA complex was observed in nuclear extracts from postischemic tissues (lane 2) but not in the reaction medium alone (lane 1). Competition assays were performed to confirm that the signal was specific for the putative NF-κB DNA complex. When increasing folds of unlabeled NF-κB oligonucleotide (2.5-, 5-, and 10-fold molar excess) were preincubated with the nuclear extracts before the addition of the labeled probe, the NF-κB DNA complex signal disappeared progressively (Figure 5A, lanes 3 through 5). In contrast, when a 10-fold molar excess of unlabeled oligonucleotides containing the consensus sequence for either AP-1 or CREB was incubated with the reaction mixture, the NF-κB DNA complex signal was unaffected (data not shown). Furthermore, supershift assays demonstrated that when specific anti-p65 or anti-p50 antibodies were added to the EMSA reaction mixture, the mobility of the NF-κB DNA complex was shifted (Figure 5B). A combination of anti-p65 and anti-p50 antibodies caused further gel retardation and almost abolished the NF-κB DNA band (Figure 5B).

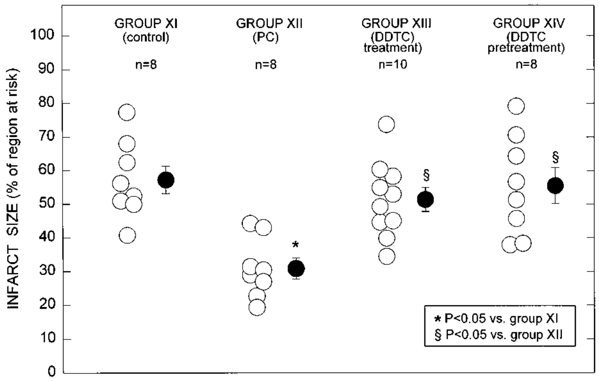

Figure 11. Myocardial infarct size in groups XI (control, n=8), XII (ischemic PC, n=8), XIII (DDTC treatment, n=10), and XIV (DDTC pretreatment, n=8). Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas solid circles represent mean±SEM. Of the 55 rabbits instrumented for the studies of myocardial infarction, 12 were assigned to the control group (group XI), 11 to the preconditioned group (group XII), 16 to the DDTC-treated group (group XIII), and 16 to the DDTC-pretreated group (group XIV). Eight rabbits died of ventricular fibrillation during the 30-minute occlusion (2 in group XI, 1 in group XII, 4 in group XIII, and 1 in group XIV). Four rabbits were excluded because of a small region at risk (<10% of the LV) (1 in group XI, 1 in group XII, 1 in group XIII, and 1 in group XIV). Five rabbits could not be used because of malfunction of the balloon occluder (1 in group XII and 4 in group XIV) and 3 rabbits because of technical problems during the postmortem perfusion (1 in group XI and 2 in group XIV). One rabbit in group XIII died overnight on day 1. Therefore, a total of 8 rabbits completed the protocol in group XI, 8 in group XII, 10 in group XIII, and 8 in group XIV.

The results of these competition studies and supershift analyses clearly demonstrate the presence of NF-κB–specific DNA binding in nuclear extracts of preconditioned myocardium. Additionally, the results of the supershift analysis with specific anti-p65 and anti-p50 antibodies indicate that both the p65 and the p50 subunits of NF-κB are involved in the increased NF-κB DNA binding activity after ischemic PC. This finding, coupled with the results of the Western blot analysis showing a time-dependent translocation of p65 from the cytosolic to the nuclear compartment (Figure 4), supports the conclusion that the subunits of NF-κB proteins involved in the activation of κB motifs consist of p65-p50 heterodimers. In contrast to anti-p65 and anti-p50 antibodies, anti–c-Rel antibodies failed to shift the mobility of the NF-κB DNA complex (data not shown), suggesting that the c-Rel subunit does not participate in the activation of κB motifs during ischemic PC in this model.

Effect of Ischemic PC on the NF-κB DNA Binding Activity

As illustrated in Figure 6A and 6B, the NF-κB DNA binding activity detected in nuclear extracts increased 4.1-fold at 30 minutes after ischemic PC (group II [$P<0.05$ versus control]), remained elevated at 60 minutes (group III; 1.7-fold [$P<0.05$]) and at 120 minutes (group IV; 1.9-fold [$P<0.05$]), and returned to values not significantly different from control by 240 minutes (group V). Thus, ischemic PC was associated with a marked increase in NF-κB DNA binding activity in the nuclear fraction, which resolved within 4 hours from the PC stimulus. In nuclear extracts prepared from the nonischemic region (posterior LV wall), the NF-κB DNA binding activity did not change appreciably after ischemic PC (Figure 6B).

Effect of DDTC on NF-κB

Administration of DDTC 15 minutes before the 6 occlusion/reperfusion cycles (group VI) prevented the ischemic PC-induced decrease in p65 in the cytosolic fraction (Figure 7A) and its increase in the nuclear fraction (Figure 7B), as well as the increase in NF-κB DNA binding activity in the nuclear fraction (Figure 8A and 8B). Thus, this dose of DDTC effectively abrogated the activation of NF-κB elicited by ischemic PC in conscious rabbits.

Phase B: Effect of DDTC on Ischemic PC

Having found that ischemic PC activates NF-κB and that this activation can be blocked by DDTC, in phase B, we determined whether DDTC blocks late PC against stunning and infarction. The aim of these studies was to establish the functional significance of NF-κB activation during ischemic PC.

Studies of Myocardial Stunning

Exclusions and Postmortem Analysis

Of the 26 rabbits instrumented for the studies of myocardial stunning, 10 were assigned to the control group (group VIII), 10 to the DDTC-treated group (group IX), and 6 to the DDTC-pretreated group (group X). One rabbit in group VIII died of ventricular fibrillation (during the second occlusion on day 1). One rabbit in group IX died 4 hours after the sixth reperfusion on day 2. Three rabbits were excluded because of balloon occluder malfunction (1 in each group). Therefore, 8 rabbits completed the protocol in group VIII, 8 in group IX, and 5 in group X. The size of the occluded-reperfused vascular bed was similar in the 3 groups: 0.75±0.06 g (17.4±1.6% of LV weight) in group VIII, 0.93±0.13 g (18.2±2.1%) in group IX, and 0.80±0.10 g (15.9±1.5%) in group X. Tissue staining with triphenyltetrazolium chloride confirmed the absence of infarction in all animals.

Regional Myocardial Function

There were no significant differences among the 3 groups with respect to heart rate (data not shown) or baseline systolic thickening fraction (see Figure 9 legend).

Group VIII (Control Group). On day 1, thickening fraction remained significantly ($P<0.05$) depressed for 4 hours after the sixth reperfusion and returned to values not significantly different from preocclusion values by 5 hours (Figure 9). Thus, the sequence of six 4-minute occlusion/4-minute reperfusion cycles resulted in severe myocardial stunning that lasted, on average, 4 hours. On days 2 and 3, however, the recovery of WTh was improved compared with day 1 (Figure 9). The total deficit of WTh after the sixth reperfusion was 48% and 47% less on days 2 and 3, respectively, compared with day 1 ($P<0.05$ for both) (Figure 10). Thus, as expected,[5,7,8,10,13,18] myocardial stunning was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1 (late PC against stunning).

Group IX (DDTC-Treated Group). Unlike the results obtained in control rabbits, in DDTC-treated rabbits, the recovery of WTh during the 5-hour reperfusion period was not improved on day 2 compared with day 1 (Figure 9), so that the total deficit of WTh did not differ significantly between the 2 days (Figure 10). On day 3, the recovery of WTh was markedly improved and the deficit of WTh markedly reduced compared with day 2 (Figures 9 and 10). Thus, administration of DDTC on day 1 completely blocked the development of late PC on day 2; when DDTC was not administered (day 2), a full PC effect became apparent on day 3.

Group X (DDTC-Pretreated Group). On day 2, both the recovery of WTh (Figure 9) and the total deficit of WTh (Figure 10) were similar to the corresponding values measured on day 1 in the control group. Thus, administration of DDTC did not exacerbate the severity of myocardial stunning 24 hours later, indicating that the absence of late PC against stunning observed on day 2 in group IX cannot be ascribed to a delayed deleterious action of DDTC on myocardial contractility.

Studies of Myocardial Infarction

Exclusions

Of the 55 rabbits instrumented for the studies of myocardial infarction, 21 were excluded for the reasons specified in the legend to Figure 11.

Region at Risk and Infarct Size

There were no appreciable differences among groups XI, XII, XIII, and XIV with respect to heart rate or baseline systolic thickening fraction (data not shown). The weight of the region at risk did not differ among the 4 groups (0.73±0.13 g [16.3±2.2% of LV weight]) in group XI; 0.72±0.08 g [16.0±2.0%] in group XII; 0.77±0.08 g [17.5±1.4%] in group XIII; and 0.92±0.16 g [21.5±2.8%] in group XIV). The average infarct size was 45% smaller in group XII (ischemic PC group) compared with group XI (control group) ($P<0.05$ [Figure 11]), indicating a late PC effect against myocardial infarction. In contrast, in rabbits treated with DDTC (group XIII), infarct size was similar to that measured in controls (Figure 11), indicating that DDTC abrogated the late PC effect against infarction. In group XIV, infarct size did not differ significantly from that in controls (Figure 11), indicating that pretreatment with DDTC did not affect the extent of cell death in nonpreconditioned myocardium. Analysis of covariance demonstrated that for any given size of the region at risk, the resulting infarction was greater in preconditioned rabbits treated with DDTC than in untreated preconditioned rabbits (data not shown). Thus, administration of DDTC on day 1 completely blocked the development of late PC against infarction on day 2, and this effect cannot be ascribed to delayed deleterious actions of DDTC on infarct size. In keeping with the infarct size data, the recovery of systolic WTh after the 30-minute occlusion was significantly improved in group XII versus group XI (3.2±5.7% of baseline versus −15.8±5.1%, respectively, at 72 hours [$P<0.05$]), and this improvement was abolished by DDTC (−17.6±3.9% of baseline at 72 hours).

Phase C: Effects of L-NA, MPG, Chelerythrine, Lavendustin A, and DETA/NO on NF-κB

Having found that inhibition of NF-κB blocks late PC, in phase C, we sought to elucidate the cellular mechanisms whereby ischemia induces activation of NF-κB in vivo.

Exclusions

Of the 36 rabbits instrumented for phase C, 6 were assigned to group XV (L-NA treatment), 6 to group XVI (MPG treatment), 5 to group XVII (chelerythrine treatment), 5 to group XVIII (lavendustin A treatment), 6 to group XIX (DMSO control group), and 5 to group XX (DETA/NO treatment). Two rabbits could not be used because of malfunction of the balloon occluder (one in group XV and one in group XIX). One rabbit in group XVI was excluded because of ventricular fibrillation. Therefore, a total of 5 rabbits completed the protocol in each group. Two additional rabbits were used as controls (in addition to the 5 control rabbits in group I), and 1 rabbit was added to the PC group (in addition to the 5 rabbits in group II).

Subcellular Distribution of p65 and NF-κB DNA Binding Activity

Administration of L-NA, MPG, chelerythrine, or lavendustin A before the 6 occlusion/reperfusion cycles prevented the ischemic PC-induced decrease in p65 in the cytosolic fraction (Figure 7A) and its increase in the nuclear fraction (Figure 7B), as well as the ischemic PC-induced increase in NF-κB DNA binding activity in the nuclear fraction (Figure 8A and 8B). Thus, in the conscious rabbit, the same doses of L-NA,[5,6] MPG,[15] chelerythrine,[13] and lavendustin A[20] that abrogate the cardioprotective effects of late PC also abrogate the activation of NF-κB elicited by ischemic PC. DMSO (the vehicle used for chelerythrine and lavendustin A), in itself, had no significant effect (group XIX) (Figures 7 and 8).

Administration of DETA/NO without ischemia/reperfusion (group XX) caused a decrease in p65 in the cytosolic fraction (Figure 7A), an increase in p65 in the nuclear fraction (Figure 7B), and an increase in NF-κB DNA binding activity in the nuclear fraction (Figure 8A and 8B), all of which were comparable to those elicited by ischemic PC in group II. Thus, the same dose of DETA/NO that induces cardioprotective effects similar to those of the late phase of ischemic PC[29] also induces a subcellular redistribution and activation of NF-κB similar to that induced by ischemic PC.

Discussion

It is generally agreed that the development of the late phase of ischemic PC involves a cascade of signaling events that culminates in the upregulation of cardioprotective genes.[12,46] Although the early events of this cascade have been partially deciphered (eg, formation of NO and ROS,[5,6,14,15,29] stimulation of adenosine A_1 receptors,[3] activation of PKC and tyrosine kinases[13,16–21]), little or nothing is known regarding the distal events beyond kinase activation. In particular, the identity of the transcription factor(s) downstream of PKC and tyrosine kinases remains unknown and represents an important unresolved problem.

Salient Findings

The present study provides significant new insights into this issue. The results reported herein demonstrate that, in the conscious rabbit, a sequence of six 4-minute occlusion/4-minute reperfusion cycles, which induces late PC against both myocardial stunning and myocardial infarction, induces rapid activation of NF-κB, as evidenced by its nuclear translocation and increased DNA binding activity. Pretreatment with the NF-κB inhibitor DDTC blocks the nuclear translocation and increased DNA binding activity of NF-κB and, at the same time, blocks the cardioprotective effects of late PC against both myocardial stunning and myocardial infarction, indicating that activation of NF-κB is an essential mechanism whereby brief ischemia results in delayed cardioprotection. The ischemic PC-induced activation of NF-κB is also blocked by pretreatment with the NOS inhibitor L-NA, the ROS scavenger MPG, the PKC inhibitor chelerythrine, and the tyrosine kinase inhibitor lavendustin A (all given at doses previously shown to block late PC[5,6,13,15,20]), demonstrating that the cellular mechanism whereby ischemic PC activates NF-κB involves the formation of NO and ROS and the activation of PKC- and tyrosine kinase–dependent pathways. The multiplicity of the signals that modulate NF-κB suggests that this is a common downstream effector in the genesis of late PC. Finally, a subcellular redistribution and increased DNA binding activity of NF-κB quantitatively similar to those induced by ischemic PC can be reproduced pharmacologically with the administration of the NO donor DETA/NO (at doses previously shown to elicit a late PC effect[29]), demonstrating that NO in itself, in the absence of the other cellular perturbations associated with ischemia, can induce NF-κB activation in the heart. This last finding reveals an NO-dependent signaling pathway that was heretofore unrecognized. Taken together, these results provide direct evidence, for the first time, that activation of NF-κB is a critical step in the signal transduction pathway that underlies the development of the late phase of ischemic PC. To our knowledge, this is also the first study to suggest that NO, ROS, and PKC play an important role in modulating the response of NF-κB to a brief ischemic stress.

Functional Significance of NF-κB Activation After Ischemic PC

In phase B, the functional significance of NF-κB activation in late PC was interrogated using DDTC. DDTC was selected for several reasons. Dithiocarbamates, such as DDTC, have been shown to be potent inhibitors of NF-κB activation (and iNOS induction) in various cell types,[47–49] including cardiac myocytes.[50,51] DDTC is effective at micromolar concentrations and is considerably more potent than other compounds.[47] Furthermore, dithiocarbamates prevent activation of NF-κB by many different stimuli and in different cell lines but do not interfere with other transcription factors, such as AP-1, TRE, oct-1, CRE, and Sp1.[47] We found that abrogation of NF-κB activation with DDTC resulted in abrogation of protection 24 hours later, demonstrating that the nuclear translocation and increased DNA binding activity of NF-κB observed after ischemic PC are not merely an epiphenomenon but rather are an obligatory component of the signaling cascade that underlies late PC. The fact that inhibition of NF-κB activation resulted in loss of late PC in rabbits subjected to six 4-minute occlusion/4-minute reperfusion cycles (group IX) as well as in rabbits subjected to a 30-minute occlusion (group XIII) indicates that this transcription factor plays a critical role not only in the delayed protection against mild reversible injury associated with brief ischemia (myocardial stunning) but also in the delayed protection against severe irreversible injury associated with sustained ischemia (myocardial infarction). Therefore, mobilization of NF-κB appears to be involved in the acquisition of tolerance to ischemia/reperfusion injury in general.

Although NF-κB plays an essential role in late PC, little is known regarding the cellular mechanisms whereby a brief ischemic stress leads to the nuclear translocation and DNA binding of this protein. Accordingly, in phase C, we tested the hypothesis that NO, ROS, PKC, and tyrosine kinases are involved in ischemic PC-induced NF-κB activation.

Role of NO in Ischemic PC-Induced Activation of NF-κB

To test the hypothesis that NO formation is an important mechanism whereby ischemic PC induces NF-κB activation, we examined the effects of L-NA, a nonselective inhibitor of all three isoforms of NOS,[27] given at the same dose that has previously been shown to abrogate late PC in this model.[5,6] L-NA completely prevented NF-κB activation (Figures 7 and 8), demonstrating that NO formation is necessary for ischemic PC to mobilize this transcription factor. These results are corroborated by those obtained when the NO donor DETA/NO was administered in the absence of ischemia. DETA/NO induced nuclear translocation of p65 (Figure 7) and increased the nuclear NF-κB DNA binding activity (Figure 8) in a manner similar to that observed after ischemia, indicating that NO in itself can trigger NF-κB activation, without the need for any concomitant stimuli. Thus, increased availability of NO is not only necessary, but also sufficient to activate NF-κB in the heart of conscious animals. The dose of DETA/NO we used is the same that has previously been found to induce late PC against both myocardial stunning and infarction in conscious rabbits.[29]

Taken together, the results obtained with L-NA and DETA/NO point to a central role of NO in modulating NF-κB in the setting of myocardial ischemia/reperfusion. To our knowledge, this is the first demonstration that NO can promote NF-κB activation in the heart. This finding identifies a new biological function of NO and a new mechanism in the signaling cascade of ischemic PC. Because both NO and NF-κB participate in numerous biological processes,[23,24,27,28] this finding has potentially wide implications for many cardiovascular conditions besides myocardial ischemia. Furthermore, the notion that NO donors (eg, nitroglycerin) induce NF-κB activation could have significant implications for nitrate therapy.

The finding that NO activates NF-κB in the heart may appear surprising, since the opposite effect has been observed in isolated vascular (noncoronary) endothelial cells[52,53] and in purified preparations of recombinant NF-κB,[54] in which NO has been shown to inhibit NF-κB. Because of the many obvious differences (eg, species, cell types, stimulus for NF-κB, and in vitro versus in vivo setting), a direct comparison is not feasible. Nevertheless, the discordance between these prior results[52–54] and the present observations raises the possibility that the effect of NO on NF-κB may differ in different cell types (eg, endothelial cells versus cardiac myocytes), particularly in view of the fact that NO has been reported to activate NF-κB in T cells.[55] An alternative hypothesis is that the modulation of NF-κB by NO may be concentration dependent, as suggested by the finding that, in murine endothelial cells, NO enhances NF-κB activation at low levels but inhibits it at high levels.[56] Additional studies will be necessary to elucidate the cell-type specificity and concentration dependence of the effects of NO on NF-κB.

Role of ROS in Ischemic PC-Induced Activation of NF-κB

NO and $\cdot O_2^-$ react very rapidly to form peroxynitrite ($ONOO^-$), which then can generate hydroxyl radical (·OH) or another oxidant with similar reactivity.[57] To test the hypothesis that formation of NO- and $\cdot O_2^-$-derived oxidants (eg, $ONOO^-$ and/or ·OH) is an important mechanism whereby ischemic PC activates NF-κB, we examined whether MPG, a cell-permeant antioxidant, interferes with NF-κB activation. Because MPG reacts avidly with both $ONOO^-$ and ·OH by virtue of its thiol group,[58] and because it blocks DETA/NO-induced late PC,[29] we reasoned that it would be a useful tool to interrogate the role of NO- and $\cdot O_2^-$-derived reactive species in the activation of NF-κB. We used the same dose of MPG that has been shown to abrogate late PC after an ischemic stimulus in this model.[15] Our results demonstrate that MPG completely eliminated both the nuclear translocation (Figure 7) and the increased DNA binding activity (Figure 8) of NF-κB after ischemic PC, indicating that MPG-sensitive oxidants (such as $ONOO^-$ and/or ·OH) play an important role in ischemic PC-induced activation of NF-κB. Accordingly, on the basis of the present results and of previous studies,[5,6,14,15,29] we propose that brief myocardial ischemia mobilizes NF-κB via generation of NO and $\cdot O_2^-$ and subsequent formation of secondary reactive species, most likely $ONOO^-$ and/or ·OH.

Role of PKC and Tyrosine Kinases in Ischemic PC-Induced Activation of NF-κB

To interrogate the role of PKC in NF-κB activation, we examined the effects of chelerythrine, given at a dose that prevents translocation of PKC as well as late PC in this model.[13,18] Chelerythrine is a very potent inhibitor of PKC (IC_{50} ≈0.7 μmol/L) and reportedly has very high selectivity for PKC compared with PKA (250:1), Ca^{2+}/calmodulin-dependent protein kinase (150:1), and tyrosine kinases (150:1).[59] The finding that chelerythrine completely blocked NF-κB activation after ischemic PC (Figures 7 and 8) indicates that this phenomenon occurs via a PKC-dependent signaling pathway. To our knowledge, this is the first evidence that PKC controls the activity of NF-κB during myocardial ischemia.

To test the hypothesis that tyrosine kinases may also be involved in NF-κB activation, we examined the effects of lavendustin A, given at a dose that was previously found to abrogate late PC in this conscious rabbit model.[20] Lavendustin A is a potent and extremely selective inhibitor of tyrosine kinases (IC_{50} for epidermal growth factor receptor tyrosine kinase=0.011 μmol/L[60]; IC_{50} for $pp60^{src}$=0.5 μmol/L[61]). Even at concentrations of 100 μmol/L, lavendustin A does not inhibit PKC, PKA, or Ca^{2+}/calmodulin-dependent protein kinase.[60,61] The finding that lavendustin A abrogated the nuclear translocation (Figure 7) as well as the increased DNA binding activity (Figure 8) of NF-κB after ischemic PC indicates that tyrosine kinases are important modulators of the activation of NF-κB in this setting.

Possible Role of NF-κB as the Common Distal Pathway of Late PC

One of the most striking findings of our study is the remarkable consistency with which inhibition of NF-κB resulted in abrogation of protection. Every time NF-κB activation was blocked, late PC was also blocked. Five different agents were tested (DDTC, L-NA, MPG, chelerythrine, and lavendustin A). These agents are totally unrelated to one another and target different components of the signal transduction cascade of late PC. They were chosen to interrogate the signaling elements currently known to be involved in late PC in conscious rabbits (ie, NO, ROS, PKC, and tyrosine kinases).[5,6,13,15–21,29] All five agents inhibited NF-κB activation (Figures 7 and 8), and all five agents consistently blocked the cardioprotective effects of late PC, as documented by our present findings (Figures 10 and 11) and by previous studies.[5,6,13,15,18,20] Therefore, the present study not only demonstrates an essential role of NF-κB in late PC but also strongly suggests that this transcription regulatory protein is a common downstream pathway through which multiple signals elicited by ischemic stress (NO, ROS, PKC, and tyrosine kinases) act to induce gene transcription. Because in the setting of late PC the activation of PKC is NO dependent[18] and the activation of tyrosine kinases is PKC dependent,[21,62] these signals appear to operate in series, forming a cascade that culminates in NF-κB activation.

Previous Studies of NF-κB in Myocardial Ischemia

No previous study has examined the role of NF-κB in the late phase of either ischemic PC or NO donor–induced PC. Two prior investigations have addressed the effect of myocardial ischemia/reperfusion on NF-κB in vivo.[63,64] In the first study, Chandrasekar and Freeman[63] reported in open-chest rats that a 15-minute coronary occlusion followed by reperfusion induced an increase in NF-κB DNA binding activity and p65 protein levels in total tissue homogenates. Unlike our findings, a 5-fold increase in p65 content was reported at 15 minutes of reperfusion.[63] In a subsequent study from the same group,[64] DDTC was found to inhibit the activation of NF-κB and the concomitant induction of iNOS. An important difference between these studies and the present investigation is that we analyzed NF-κB selectively in nuclear extracts rather than in the total tissue homogenate. Because of this, and because of the numerous differences between the studies (eg, species, ischemic protocols, and time points examined), it is difficult to compare our results with those of Chandrasekar et al.[63,64] Maulik et al[65] have recently reported in isolated rat hearts that ischemic PC (4 cycles of 5-minute ischemia/10-minute reperfusion) activated NF-κB, and that this phenomenon was inhibited by the tyrosine kinase inhibitor genistein and the p38 mitogen-activated protein kinase (MAPK) inhibitor SB 203580, suggesting that NF-κB activation is regulated by both tyrosine kinase activity and p38 MAPK activity.

Conclusions

In summary, the present findings identify a new, important component of the signal transduction cascade of late PC. Our results demonstrate that activation of NF-κB is required for the development of late PC against both myocardial stunning and myocardial infarction in conscious animals, and that this signaling event is controlled by NO, ROS, PKC, and tyrosine kinases. Thus, the initial signals elicited by a brief ischemic stress are transduced into protective changes in gene expression via an NF-κB–dependent mechanism. On the basis of these results and of previous studies,[5,6,13,15–21,29] we propose a pathophysiological paradigm in which brief ischemia causes increased formation of NO and $\cdot O_2^-$, which then leads to the sequential activation of PKC, tyrosine kinases, NF-κB, and iNOS. Because of the ubiquitous roles of NO, ROS, PKC, tyrosine kinases, and NF-κB, the present results have implications not only for ischemic PC but also for a variety of other pathophysiological conditions in which NF-κB is involved. Furthermore, the rapid activation of NF-κB observed after injection of the NO donor DETA/NO may have significant implications for nitrate therapy.

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