

Isoform-Selective Activation of Protein Kinase C by Nitric Oxide in the Heart of Conscious Rabbits

A Signaling Mechanism for Both Nitric Oxide-Induced and Ischemia-Induced Preconditioning

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Abstract—Although isoform-selective translocation of protein kinase C (PKC) ϵ appears to play an important role in the late phase of ischemic preconditioning (PC), the mechanism(s) responsible for such translocation remains unclear. Furthermore, the signaling pathway that leads to the development of late PC after exogenous administration of NO in the absence of ischemia (NO donor-induced late PC) is unknown. In the present study we tested the hypothesis that NO activates PKC and that this is the mechanism for the development of both ischemia-induced and NO donor-induced late PC. A total of 95 chronically instrumented, conscious rabbits were used. In rabbits subjected to ischemic PC (six 4-minute occlusion/4-minute reperfusion cycles), administration of the NO synthase inhibitor *N*^w-nitro-L-arginine (group III), at doses previously shown to block the development of late PC, completely blocked the ischemic PC-induced translocation of PKC ϵ but not of PKC η , indicating that increased formation of NO is an essential mechanism whereby brief ischemia activates the ϵ isoform of PKC. Conversely, a translocation of PKC ϵ and $-\eta$ quantitatively similar to that induced by ischemic PC could be reproduced pharmacologically with the administration of 2 structurally unrelated NO donors, diethylenetriamine/NO (DETA/NO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP), at doses previously shown to elicit a late PC effect. The particulate fraction of PKC ϵ increased from 35 \pm 2% of total in the control group (group I) to 60 \pm 1% after ischemic PC (group II) (P <0.05), to 54 \pm 2% after SNAP (group IV) (P <0.05) and to 52 \pm 2% after DETA/NO (group V) (P <0.05). The particulate fraction of PKC η rose from 66 \pm 5% in the control group to 86 \pm 3% after ischemic PC (P <0.05), to 88 \pm 2% after SNAP (P <0.05) and to 85 \pm 1% after DETA/NO (P <0.05). Neither ischemic PC nor NO donors had any appreciable effect on the subcellular distribution of PKC α , $-\beta$ 1, $-\beta$ 2, $-\gamma$, $-\delta$, $-\mu$, or $-\nu$; on total PKC activity; or on the subcellular distribution of total PKC activity. Thus, the effects of SNAP and DETA/NO on PKC closely resembled those of ischemic PC. The DETA/NO-induced translocation of PKC ϵ (but not that of PKC η) was completely prevented by the administration of the PKC inhibitor chelerythrine at a dose of 5 mg/kg (group VI) (particulate fraction of PKC ϵ , 38 \pm 4% of total, P <0.05 versus group V; particulate fraction of PKC η , 79 \pm 2% of total). The same dose of chelerythrine completely prevented the DETA/NO-induced late PC effect against both myocardial stunning (groups VII through X) and myocardial infarction (groups XI through XV), indicating that NO donors induce late PC by activating PKC and that among the 10 isozymes of PKC expressed in the rabbit heart, the ϵ isotype is specifically involved in the development of this form of pharmacological PC. In all groups examined (groups I through VI), the changes in the subcellular distribution of PKC ϵ protein were associated with parallel changes in PKC ϵ isoform-selective activity, whereas total PKC activity was not significantly altered. Taken together, the results provide direct evidence that isoform-selective activation of PKC ϵ is a critical step in the signaling pathway whereby NO initiates the development of a late PC effect both after an ischemic stimulus (endogenous NO) and after treatment with NO-releasing agents (exogenous NO). To our knowledge, this is also the first report that NO can activate PKC in the heart. The finding that NO can promote isoform-specific activation of PKC identifies a new biological function of this radical and a new mechanism in the signaling cascade of ischemic PC and may also have important implications for other pathophysiological conditions in which NO is involved and for nitrate therapy. (*Circ Res.* 1999;84:587-604.)

Key Words: diethylenetriamine ■ nitric oxide ■ *S*-nitroso-*N*-acetylpenicillamine ■ *N*^w-nitro-L-arginine ■ protein kinase C ϵ isoform ■ translocation

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The late phase of ischemic preconditioning (PC) is the phenomenon whereby a brief ischemic stress increases the tolerance of the heart to myocardial stunning and infarction 24 to 72 hours later.^{1–13} Because of its sustained nature, this endogenous cardioprotective mechanism may have considerable clinical relevance and is the focus of intense investigation.^{12,13} Recent studies^{8,10,14} have identified a critical role of NO as a trigger for the development of the late phase of ischemic PC. Specifically, it has been demonstrated that administration of NO synthase (NOS) inhibitors during the initial ischemic stimulus abolishes late PC against both myocardial stunning⁸ and myocardial infarction¹⁰ and, conversely, that administration of NO donors in the absence of ischemia can fully reproduce the cardioprotective effects of the late phase of ischemic PC.¹⁴ These results support the concept that enhanced biosynthesis of NO during the first ischemic stress mobilizes an adaptive mechanism that culminates in increased resistance to ischemic injury 24 to 72 hours later. However, the intracellular signaling events that are activated by NO and the molecular mechanisms whereby this radical triggers late PC remain unknown. Elucidation of the signaling pathway(s) that transduces augmented NO formation into cardioprotection is essential not only to unravel the cellular mechanism of ischemic PC but also to develop clinical therapies that can reproduce this phenomenon with PC-mimetic drugs.

One of the better-characterized intracellular signaling events during ischemic PC is the activation of protein kinase C (PKC).^{15,16} Considerable evidence supports the involvement of this family of enzymes in both the early and the late phases of ischemic PC.^{6,17–33} Using the same rabbit model in which NO has been shown to trigger late PC,^{8,10,14} we have found that the initial ischemic stimulus induces selective translocation of 2 novel PKC isoforms (ϵ and η) from the cytosolic to the particulate fraction³² and that inhibition of ϵ isoform translocation with the PKC inhibitor chelerythrine results in abrogation of late PC,³³ indicating that the development of this cardioprotective phenomenon involves a PKC ϵ -mediated signaling pathway. Since both NO and PKC are necessary for the occurrence of the late phase of ischemic PC, it is plausible to postulate that NO may evoke the late PC response via activation of PKC. At present, however, virtually nothing is known regarding the role of PKC in NO signaling in the heart, not only in the setting of ischemia, but also in general. Specifically, no information is available regarding (1) whether NO can activate PKC in the myocardium, either in the absence or in the presence of ischemia; (2) if so, whether such PKC activation is generalized or restricted to selected isoforms within the PKC family; and (3) whether activation of PKC is a necessary signaling step in the development of NO-induced late PC. Because of the ubiquitous functions of NO, demonstration of NO-induced activation of PKC in the heart would have mechanistic implications not only for ischemic PC but also for many other pathophysiological processes that are modulated by NO.

In the present study, we tested the central hypothesis that NO activates PKC and that this is the mechanism for the development of both ischemia-induced and NO donor-induced late PC. The 4 specific goals were to determine (1) whether inhibitors of

NOS (at doses known to block ischemic PC) block ischemic PC-induced translocation of PKC; (2) whether administration of NO donors (at doses known to induce late PC) induces isoform-selective translocation of PKC in a manner congruous with that observed during ischemic PC; (3) whether NO donor-induced translocation of PKC isozymes can be blocked by in vivo administration of PKC inhibitors; and (4) if so, whether the same doses of PKC inhibitors also block NO donor-induced late PC. To achieve these goals, a comprehensive study consisting of 4 consecutive phases was designed to interrogate the mechanism of NO-dependent signaling in a conscious rabbit model of late PC by combining direct measurements of PKC activity and subcellular distribution in cardiac tissue with physiological measurements of protection against both myocardial stunning and myocardial infarction in the intact animal.

In phase I, we first examined the effect of the NOS inhibitor *N*^o-nitro-L-arginine (L-NA) on ischemic PC-induced translocation of PKC ϵ and η . Having established that L-NA blocks this translocation, in phase II we explored whether administration of NO donors, in the absence of ischemia, mimics the translocation of PKC ϵ and η induced by ischemia. To achieve this goal, the effect of NO donors on total myocardial PKC activity, as well as on the subcellular distribution of all 10 PKC isoforms expressed in the adult rabbit myocardium,³² was systematically examined. In an effort to exclude nonspecific effects of NO donors, 2 structurally unrelated agents, diethylenetriamine/NO (DETA/NO) and *S*-nitroso-*N*-acetylpenicillamine (SNAP), were investigated. The effect of the specific PKC inhibitor chelerythrine on DETA/NO-induced translocation of PKC ϵ and η was also determined. To verify that the translocated ϵ proteins were in their active state, measurements of ϵ protein subcellular distribution were combined with measurements of ϵ isoform-selective phosphorylation activity. Having obtained direct evidence that NO donors activate PKC and that this is blocked by chelerythrine in vivo, in the subsequent 2 phases (III and IV) we investigated whether activation of PKC ϵ plays a causative role in the development of the cardioprotection or is simply an epiphenomenon. To achieve this goal, we determined whether the same dose of chelerythrine that blocks NO-dependent activation of PKC ϵ also blocks NO-dependent cardioprotection against stunning (phase III) and infarction (phase IV). The experiments were conducted in the same rabbit model in which previous studies have demonstrated both the role of NO and that of PKC ϵ and η in the late phase of ischemic PC.^{8,10,14,32,33} This enabled us to correlate the present results with those prior studies.^{8,10,14,32,33} A conscious-animal preparation was used in all studies to avoid the confounding influence of factors associated with open-chest preparations (anesthesia, surgical trauma, fluctuations in temperature, excessive catecholamine levels, abnormal hemodynamics, exaggerated generation of reactive oxygen species, release of cytokines, etc^{34–40}), which might interfere with NO production or PKC activity.

The results demonstrate, for the first time, that the activation of PKC ϵ associated with ischemic PC is prevented by inhibition of endogenous NO production, that a similar activation of PKC ϵ is elicited by exogenous NO in the absence of ischemia, and that inhibition of PKC ϵ activation completely abrogates the devel-

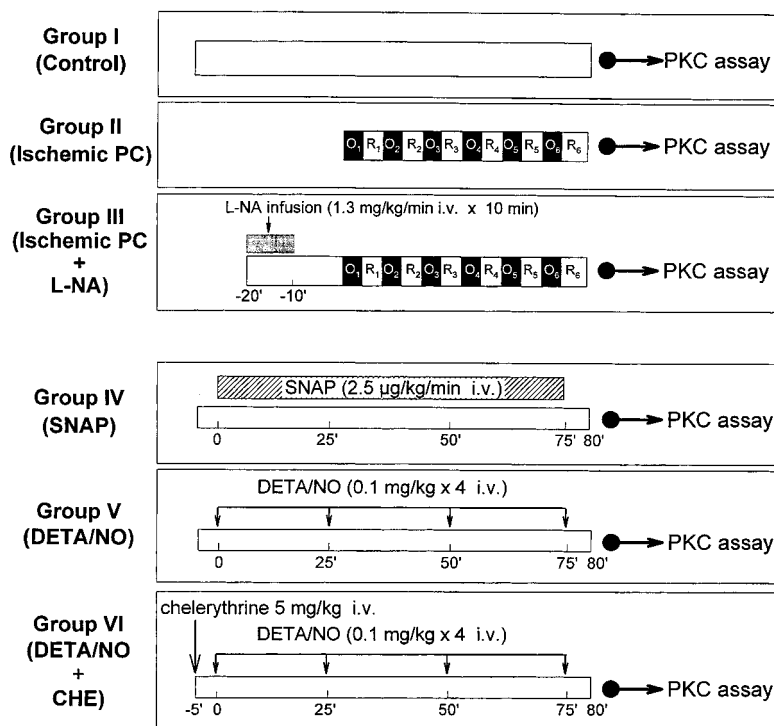


Figure 1. Diagram of the experimental protocol for the studies of PKC (phases I and II). PKC assays were performed immediately after collecting the tissue samples. O indicates 4-minute coronary occlusion; R, 4-minute reperfusion. CHE, chelerythrine.

opment of NO donor-induced late PC against both stunning and infarction. These data provide direct evidence supporting the concept that NO preconditions the heart by activating the ϵ isoform of PKC and that NO-dependent activation of PKC ϵ is an essential step in the cellular signaling cascade underlying the late phase of ischemic PC.

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). The conscious rabbit model of ischemic PC has been described in detail previously^{7-11,14,33,41} and will be briefly summarized here.

Experimental Preparation

New Zealand White male rabbits (2.0 to 2.5 kg, age 3 to 4 months) 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 in the center of 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 3 days to aspirate air and fluids postoperatively. Gentamicin was administered before surgery and on the 1st and 2nd postoperative days (0.7 mg/kg IM each day). The animals were allowed to recover for a minimum of 10 days after surgery.

Experimental Protocol

Throughout the experiments, rabbits were kept in a cage in a quiet, dimly lit room. Left ventricular (LV) systolic wall thickening (WTh), range gate depth, and ECG were recorded on a thermal array chart recorder (Gould TA6000). 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 thinning on the ultrasonic

crystal recordings. Successful reperfusion was documented by the normalization of the ECG and by the resumption of active systolic WTh. The experimental protocol is illustrated in Figures 1 through 3. The study consisted of 4 successive phases (phases I through IV).

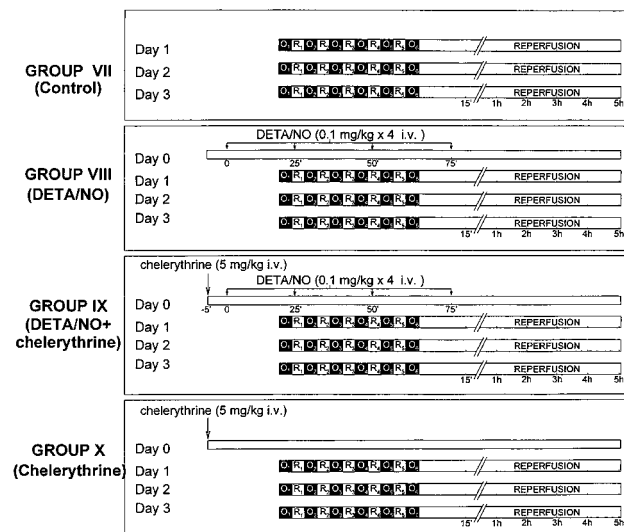


Figure 2. Experimental protocol for the studies of myocardial stunning (phase III). Four groups of rabbits underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles followed by a 5-hour observation period for 3 consecutive days (days 1, 2, and 3). Twenty-four hours before the first coronary occlusion (day 0), rabbits in group VII (n=5, control) received no pretreatment, rabbits in group VIII (n=5, DETA/NO) received 4 intravenous boluses of DETA/NO (0.1 mg/kg each) every 25 minutes (total dose, 0.4 mg/kg), rabbits in group IX (n=5, DETA/NO+chelerythrine) received the same dose of DETA/NO given to group VIII and an intravenous bolus of chelerythrine (5 mg/kg) 5 minutes before the first DETA/NO bolus, and rabbits in group X (n=5, chelerythrine) received the same dose of chelerythrine given to group IX (5 mg/kg). O indicates 4-minute coronary occlusion; R, 4-minute reperfusion.

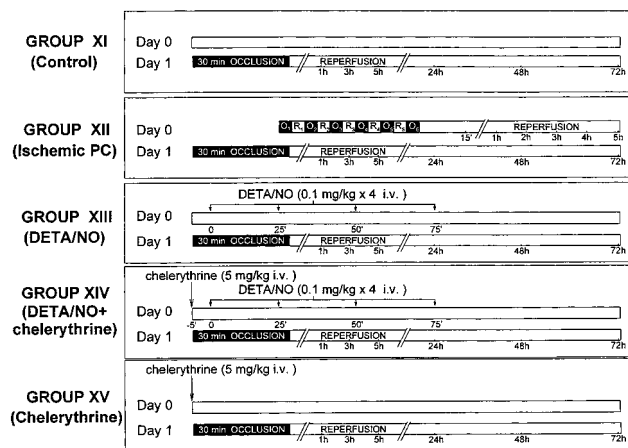


Figure 3. Experimental protocol for the studies of myocardial infarction (phase IV). On day 1, 5 groups of rabbits were subjected to a 30-minute coronary occlusion followed by 3 days of reperfusion. Twenty-four hours earlier (day 0), rabbits in group XI ($n=8$, control) received neither ischemic PC nor drug pretreatment, rabbits in group XII ($n=8$, ischemic PC) underwent six 4-minute coronary occlusion/4-minute reperfusion cycles, rabbits in group XIII ($n=8$, DETA/NO) received 4 intravenous boluses of DETA/NO (0.1 mg/kg each) every 25 minutes (total dose, 0.4 mg/kg), rabbits in group XIV ($n=7$, DETA/NO+chelerythrine) received the same dose of DETA/NO given to group XIII and an intravenous bolus of chelerythrine (5 mg/kg) 5 minutes before the first DETA/NO bolus, and rabbits in group XV ($n=7$, chelerythrine) received the same dose of chelerythrine given to group XIV (5 mg/kg).

Phase I: Effect of L-NA on Ischemic PC-Induced PKC Translocation

The goal of phase I was to determine whether the translocation of PKC ϵ and η induced by ischemic PC is blocked by the NOS inhibitor L-NA. Rabbits were assigned to 3 groups (Figure 1). Group I (nonischemic control) did not receive any treatment and did not undergo coronary occlusion. At 10 to 14 days after surgery (time corresponding to the interval elapsed between instrumentation and euthanasia in the other groups), the rabbits were given heparin (1000 units intravenously), after which they were anesthetized with sodium pentobarbital (50 mg/kg intravenously) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples (≈ 0.5 g) were rapidly removed from the anterior LV wall and stored in liquid nitrogen until used. Group II (ischemic PC) underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles without any treatment. This protocol induces late PC against both myocardial stunning^{8,9,14,33} and myocardial infarction.^{10,11,14} Group III (ischemic PC+L-NA) underwent the same sequence of occlusion/reperfusion cycles and received an intravenous infusion of L-NA at a rate of 1.3 mg/kg per minute for 10 minutes, starting 20 minutes before and ending 10 minutes 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 stunning⁸ and infarction¹⁰ in conscious rabbits. L-NA (Sigma) was dissolved in normal saline (total volume infused, 20 mL). No sedative or antiarrhythmic agents were given at any time. In both groups II and III, the rabbits were euthanized 5 minutes after the last reperfusion (a time point at which marked translocation of PKC has previously been found in this model³²). The heart was immediately excised, and myocardial samples (≈ 0.5 g) were rapidly removed from the ischemic-reperfused region (the boundaries of which had been marked with sutures at the time of instrumentation) and stored in liquid nitrogen until used.

Phase II: Effect of NO Donors on PKC

The goal of phase II was to determine whether administration of NO donors, in the absence of ischemia, induces translocation of PKC

similar to that induced by ischemic PC and whether such translocation can be prevented by PKC inhibitors. Rabbits were assigned to 3 groups (Figure 1). Group IV (SNAP) received a continuous intravenous infusion of SNAP (2.5 μ g/kg/min) for 75 minutes. The rabbits were euthanized 5 minutes after the end of the infusion, and tissue samples were obtained as described above. Group V (DETA/NO) received 4 consecutive intravenous boluses of DETA/NO (0.1 mg/kg each) every 25 minutes (total dose, 0.4 mg/kg). The rabbits were euthanized 5 minutes after the last bolus, and tissue samples were obtained as described above. These doses of SNAP and DETA/NO have previously been shown to induce late PC against myocardial stunning and infarction in conscious rabbits.¹⁴ SNAP (Sigma) was dissolved in normal saline (total volume infused, ≈ 11 mL); DETA/NO (Alexis Corp) was dissolved in PBS (total volume infused, 4 mL). SNAP and DETA/NO were dissolved immediately before the infusion; to remove oxygen from the solution, both the PBS and the normal saline solutions were bubbled with nitrogen for at least 30 minutes before dissolving SNAP or DETA/NO. Rabbits in group VI (DETA/NO+chelerythrine) were given the same dose of DETA/NO as in group V; in addition, they were given an intravenous bolus of chelerythrine (5 mg/kg) 5 minutes before the first DETA/NO injection. This dose of chelerythrine was chosen because it has previously been shown to be effective in abrogating the late phase of ischemic PC against stunning and the concomitant translocation of PKC ϵ in conscious rabbits.³³ Chelerythrine chloride (Research Biochemicals International) was dissolved in 2 mL of DMSO+2 mL of normal saline (total volume infused, 2 mL). The rabbits were euthanized 5 minutes after the last bolus of DETA/NO, and tissue samples were obtained as described above.

Phase III: Studies of Myocardial Stunning

The goal of phase III was to determine whether NO donor-induced late PC against myocardial stunning is abrogated by the administration of PKC inhibitors. The experimental protocol consisted of 3 consecutive days of coronary artery occlusions (days 1, 2, and 3, respectively); on each day, the rabbits were subjected to a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles (Figure 2). No sedative or antiarrhythmic agents were given at any time. Rabbits were assigned to 4 groups (Figure 2). Group VII (control) underwent the coronary occlusion/reperfusion protocol on days 1, 2, and 3 without any treatment. In group VIII (DETA/NO), rabbits received 4 consecutive intravenous boluses of DETA/NO (0.1 mg/kg each) every 25 minutes (total dose, 0.4 mg/kg) 24 hours before the first sequence of coronary occlusion/reperfusion cycles (this is the same dose that was used in group V of phase II and in group XIII of phase IV). In group IX (DETA/NO+chelerythrine), rabbits received the same dose of DETA/NO as in group VIII; in addition, they were given an intravenous bolus of chelerythrine (5 mg/kg) 5 minutes before the first DETA/NO injection (this is the same dose that was used in group VI of phase II and in group XIV of phase IV). In group X (chelerythrine), rabbits received chelerythrine alone (5 mg/kg intravenously) 24 hours before the first sequence of occlusion/reperfusion cycles.

Phase IV: Studies of Myocardial Infarction

The goal of phase IV was to determine whether NO donor-induced late PC against myocardial infarction is abrogated by the administration of PKC inhibitors. The experimental protocol consisted of a 30-minute coronary artery occlusion followed by 3 days of reperfusion. Diazepam was administered 20 minutes before the onset of ischemia (4 mg/kg IP) to relieve the stress caused by the coronary occlusion. No antiarrhythmic agents were given. Rabbits were assigned to 5 groups (Figure 3). Group XI (control) underwent the 30-minute occlusion with no PC protocol or drug pretreatment. Group XII (ischemic PC) was preconditioned with a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles 24 hours before the 30-minute coronary occlusion. Group XIII (DETA/NO) was given 4 consecutive intravenous boluses of DETA/NO (0.1 mg/kg) every 25 minutes (total dose, 0.4 mg/kg) 24 hours before the 30-minute coronary occlusion. In group XIV (DETA/NO+chelerythrine), rabbits were given the same dose of DETA/NO

as in group XIII; in addition, they received an intravenous bolus of chelerythrine (5 mg/kg) 5 minutes before the first DETA/NO injection. Group XV (chelerythrine) was given chelerythrine alone (5 mg/kg intravenously) 24 hours before the 30-minute coronary occlusion. In phases III and IV, all solutions used for intravenous injection (DETA/NO and chelerythrine) were filtered through a 0.2 μm Millipore filter to ensure sterility.

Tissue Sample Preparation

In phases I and II, frozen myocardial tissue samples were powdered in a prechilled stainless steel mortar and pestle. Total cellular proteins were obtained by glass-glass homogenization of the powdered tissue in sample buffer containing (in mmol/L) Tris-HCl (pH 7.5) 50, EDTA 5, EGTA 10, and benzamide 10; (in $\mu\text{g}/\text{mL}$) phenylmethylsulfonyl fluoride 50, aprotinin 10, leupeptin 10, and pepstatin A 10; and 0.3% β -mercaptoethanol.³² The cytosolic and particulate portions of total cellular proteins were separated by a 30-minute centrifugation at 45 000g. Protein concentration was determined by the method of Bradford (Bio-Rad). Using similar methods, the cytosolic and particulate fractions have been found to yield equivalent amounts of proteins.³² To ensure the most accurate assessment of PKC protein expression and to avoid any decay in PKC phosphorylation activity, protein samples were processed by either Western immunoblotting or phosphorylation assays immediately after tissue sample preparation. Each Western immunoblotting and activity assay was performed in all 5 rabbits in each group.

PKC Western Immunoblotting Analysis

Assessment of PKC isoforms was conducted using standard SDS-PAGE Western immunoblotting techniques, as previously described.³² Briefly, 100 μg of proteins derived from the homogenate or from the cytosolic fraction or the particulate fraction of the homogenate was electrophoresed on a 10% denaturing gel for 4 to 6 hours at 30 mA per gel. Proteins were electroblotted onto nitrocellulose membranes (Amersham). Gel transfer efficiency was carefully recorded by making photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining.³² Adequate background blocking was accomplished by incubating the nitrocellulose membranes with 5% nonfat dry milk in Tris-buffered saline. Antibodies against PKC isoforms α , β , γ , ϵ , ζ , θ , ι/λ , and μ (Transduction Laboratories); PKC isoforms β_1 and β_2 (Sigma); and PKC isoforms η and δ (Santa Cruz Biotechnology) were used to assess the expression of each individual PKC isoform. The PKC immunoblots were developed with the use of a chemiluminescent system (enhanced chemiluminescence kit, Amersham). The specificity of the PKC antibody binding was confirmed by the use of recombinant PKC isoform peptides. Although the β_1 , β_2 , and γ isoforms have identical molecular weight (80 kDa), the antibodies to these isotypes have no detectable cross-reactivity with one another.³² In view of the extremely high homology between the sequences of PKC isoforms ι and λ ^{42,43,44} and between the sequences of their antigenic peptides (A. Recupero, Transduction Laboratories, personal communication, November 1997), the determinations of these 2 isoforms were combined; the data presented are the average of the measurements obtained with the ι and λ antibodies.

The PKC signals detected by immunoblotting and the corresponding records of Ponceau stains of nitrocellulose membranes were quantified by using an image-scanning densitometer (Personal PI, Molecular Dynamics). To ensure consistency in the data analysis, the cytosolic and particular fractions of all 5 tissue samples in each group were run on the same gel. Each immunoblotting experiment was performed in duplicate, and the results were averaged. In this study, valid comparisons among samples required that PKC isoform expression be normalized to total protein content. However, as elaborated previously,³² 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 quantifying PKC isoforms as accurately as possible, each PKC isoform signal was normalized to the corresponding Ponceau stain signal determined by densitometric analysis of the Ponceau stain record.³²

Measurement of Total PKC Activity

Total PKC activity (the sum of the activities of all 10 isoforms expressed in the rabbit heart³²) was quantitated using a PKC enzyme assay system (Amersham), as described previously.³² In prior studies,³² we performed pilot experiments using protein samples ranging from 5 to 150 μg and identified a window of linear relationship on the dose-response curve where sample proteins ranged from 10 to 50 μg ; the optimal sample dose was found to be 25 μg of proteins. Therefore, in the current experiments, 25 μg of proteins from either the cytosolic or the particulate fraction were incubated with 0.2 μCi of [γ -³²P]ATP and (in mmol/L) ATP 0.1, HEPES 2.3, MgCl₂ 5.5, and DTT 2.9; 2.3 $\mu\text{g}/\text{mL}$ phorbol 12-myristate 13-acetate (PMA); 28.8 $\mu\text{g}/\text{mL}$ L- α -phosphatidyl-L-serine; 86.5 $\mu\text{mol}/\text{L}$ substrate peptide (VRKRTLRL); and 600 $\mu\text{g}/\text{mL}$ lysine-rich histone type III_S in 50 mmol/L Tris-HCl buffer (pH 7.5) for 15 minutes at 37°C. The reaction was carried out both in the presence and in the absence of 1.15 mmol/L calcium acetate. The reaction was terminated by the addition of stop solution (containing 300 mmol/L orthophosphoric acid). The phosphorylated substrates were transferred to binding paper, washed in 5% acetic acid, and counted with a β scintillation counter. Washing conditions were optimized to achieve low nonspecific counts (<5% of total counts). Both calcium-dependent and calcium-independent PKC activities were calculated from the specific counts (total minus nonspecific). Each sample was assayed in triplicate. Data are expressed as pmol of phosphate transferred per minute per mg of sample proteins.

Measurement of PKC ϵ Isoform-Selective Activity

To specifically determine the phosphorylation activity of the ϵ isoform of PKC, 50 μg of proteins from either the cytosolic or the particulate fraction were immunoprecipitated overnight with PKC ϵ isoform monoclonal antibodies (Upstate Biotechnology). The immunoprecipitates were then subjected to a phosphorylation assay using a PKC ϵ -selective substrate (ERM_RPRKRQGSVRRRV). The cytosolic and particulate fractions of the tissue samples were prepared as previously described.³² The optimal substrate concentration, 1 nmol/L, was determined from dose-response (substrate versus phosphorylation activity) curves generated in pilot experiments.

Measurement of Regional Myocardial Function

Regional myocardial function was assessed as systolic thickening fraction using the pulsed Doppler probe, as previously described.⁸ In the studies of myocardial stunning (phase III), the total deficit of systolic WTh (an integrative assessment of the overall severity of myocardial stunning) was 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.^{8,9,14,33,41} In the studies of myocardial infarction (phase IV), the total deficit of systolic WTh was calculated by the same method during the 72-hour recovery phase after the 30-minute occlusion.^{10,11,14} In all animals, measurements from at least 10 beats were averaged at baseline, and from at least 5 beats at all subsequent time points.

Measurement of Region at Risk and Infarct Size

At the conclusion of the protocol in phases III and IV, the rabbits were given heparin (1000 units intravenously), after which they were anesthetized with sodium pentobarbital (50 mg/kg intravenously) and euthanized with KCl. The heart was excised and the size of the ischemic-reperfused region (region at risk) was determined by tying the coronary artery at the site of the previous occlusion and by perfusing the aortic root for 2 minutes with a 5% solution of phthalo blue dye in normal saline at a pressure of 70 mm Hg using a Langendorff apparatus. The heart was then cut into 6 or 7 transverse slices, which were incubated for 10 minutes at 37°C in a 1% solution of triphenyltetrazolium chloride in phosphate buffer (pH=7.4). All atrial and right ventricular tissues were excised. In the studies of myocardial stunning (phase III), the region at risk (which was identified by the absence of blue dye) was separated from the rest of the left ventricle, and both components were weighed. In the studies

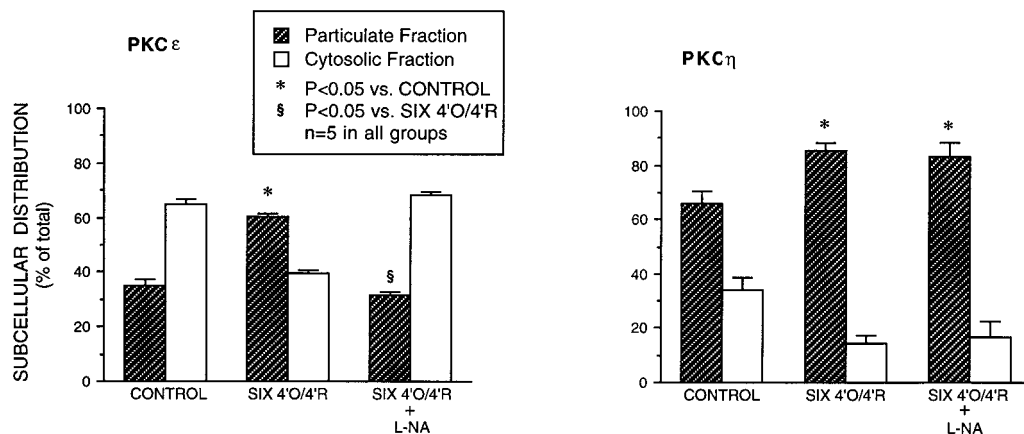


Figure 4. Subcellular distribution of PKC ϵ (left panel) and η (right panel) isoforms in nonischemic control rabbits (group I), in rabbits preconditioned with the protocol of 6 cycles of 4-minute coronary occlusion/4-minute reperfusion (six 4'O/4'R, group II), and in rabbits preconditioned with the same protocol after the administration of L-NA (group III). In rabbits subjected to ischemic PC in the absence of L-NA (group II), there was a significant translocation of both PKC ϵ and PKC η to the particulate fraction ($P < 0.05$ vs control). The subcellular distribution of PKC ϵ in group III (ischemic PC+L-NA) was virtually indistinguishable from that in group I, indicating that L-NA completely abolished ischemic PC-induced translocation of PKC ϵ . In contrast, the particulate fraction of PKC η in group III (ischemic PC+L-NA) was similar to that in group II (ischemic PC) and significantly greater than that in group I ($P < 0.05$), indicating that L-NA did not attenuate ischemic PC-induced translocation of PKC η . Data are mean \pm SEM ($n = 5$ in all groups).

of myocardial infarction (phase IV), the slices were weighed, fixed in a 10% neutral buffered formaldehyde solution, and photographed (Nikon AF N6006). Transparencies were projected onto a paper screen at a 10-fold magnification, and the borders of the infarcted, ischemic-reperfused, and nonischemic regions were traced. The corresponding areas were measured by computerized planimetry (Adobe Photoshop, version 4.0), and from these measurements infarct size was calculated as a percentage of the region at risk.^{10,11,14}

Statistical Analysis

Data are reported as mean \pm SEM. In phases I and II, differences among groups with respect to total PKC activity, PKC ϵ isoform-selective activity, and subcellular distribution of individual PKC isoforms were analyzed using a 1-way ANOVA. If the ANOVA showed an overall difference, post hoc contrasts were performed with Student t tests for unpaired data using the Bonferroni correction.⁴⁵ In phases III and IV, for intragroup comparisons, hemodynamic variables and WTh were analyzed by a 2-way repeated-measures ANOVA (time and day) followed by Student t tests for paired data with the Bonferroni correction.⁴⁵ For intergroup comparisons, data were analyzed by either a 1-way or a 2-way repeated-measures (time and group) ANOVA, as appropriate, followed by unpaired Student t tests with the Bonferroni correction.⁴⁵ The relationship between infarct size and risk-region size was compared among groups with an ANCOVA using the size of the risk region as the covariate.^{11,14} The correlation between infarct size and risk-region size was assessed by linear regression analysis using the least-squares method. All statistical analyses were performed using the SAS software system.

Results

A total of 95 conscious rabbits were used in this study (15 for phase I, 15 for phase II, 20 for phase III, and 45 for phase IV).

Phase I: Effect of L-NA on Ischemic PC-Induced PKC Translocation

Previous investigations have documented that the ischemic PC protocol used in the present study (6 cycles of 4-minute coronary occlusion/4-minute reperfusion) induces selective translocation of PKC isoforms ϵ and η ³² and that the NOS inhibitor L-NA completely abolishes ischemic PC.^{8,10} To determine whether generation of NO is responsible for the

ischemic PC-induced translocation of PKC, in phase I of the present study we measured the subcellular distribution of the ϵ and η isoforms in control rabbits not subjected to ischemic PC (group I) and in rabbits undergoing the ischemic PC protocol (six 4-minute occlusion/4-minute reperfusion cycles) in the absence (group II) or presence (group III) of L-NA. A total of 15 conscious rabbits were instrumented for phase I. Of these, 5 were assigned to group I (nonischemic control), 5 to group II (ischemic PC), and 5 to group III (ischemic PC+L-NA group). All rabbits completed the protocol successfully.

As expected,³² a significant translocation of PKC ϵ and η to the particulate fraction was observed in group II (ischemic PC) (Figure 4). In group III (ischemic PC+L-NA), however, the particulate fraction of PKC ϵ was significantly ($P < 0.05$) less than in group II (ischemic PC) ($32 \pm 1\%$ versus $60 \pm 1\%$ of total, respectively) and was similar to that measured in group I (nonischemic control) ($35 \pm 2\%$) (Figure 4). Thus, administration of L-NA completely blocked the translocation of the ϵ isoform induced by ischemic PC. In contrast, L-NA did not attenuate the translocation of the η isoform associated with ischemic PC (Figure 4). The particulate fraction of η in group III (ischemic PC+L-NA) ($83 \pm 5\%$ of total) did not differ significantly from that in group II (ischemic PC) ($86 \pm 3\%$ of total) and was significantly ($P < 0.05$) greater than that in group I (nonischemic control) ($66 \pm 5\%$ of total). Thus, despite pretreatment with L-NA, the ischemic PC protocol still induced significant translocation of the η isoform to the particulate fraction.

As expected,³² ischemic PC (group II) had no appreciable effect on total myocardial PKC activity (Table 1). Administration of L-NA during ischemic PC also had no significant effect on total myocardial PKC activity (Table 1). Both the calcium-stimulated and the calcium-independent PKC activity remained unaltered in group III (ischemic PC+L-NA) compared with groups I (nonischemic control) and II (ischemic PC) (Table 1).

TABLE 1. Total Myocardial PKC Activity

	Total PKC Activity, pmol/min/mg		Particulate Fraction, % of Total		Cytosolic Fraction, % of Total	
	PMA+Ca ²⁺	PMA	PMA+Ca ²⁺	PMA	PMA+Ca ²⁺	PMA
Group I (control)	185±8	133±3	15±3	12±4	85±3	88±4
Group II (ischemic PC)	182±15	141±8	15±6	11±3	85±6	89±3
Group III (ischemic PC+L-NA)	170±15	130±7	9±4	11±5	91±4	89±5
Group IV (SNAP)	184±9	128±11	13±1	16±1	87±1	84±1
Group V (DETA/NO)	198±9	146±7	14±2	17±2	86±2	83±2
Group VI (DETA/NO+chelerythrine)	194±10	143±4	11±5	12±3	89±5	88±3

Total myocardial calcium-stimulated (PMA+Ca²⁺) and calcium-independent (PMA) PKC activity in nonischemic control rabbits (group I), in rabbits preconditioned with 6 cycles of 4-minute coronary occlusion/4-minute reperfusion (group II), in rabbits preconditioned with the same ischemic protocol after the administration of L-NA (group III), in SNAP-treated rabbits (group IV), in DETA/NO-treated rabbits (group V), and in rabbits treated with the same dose of DETA/NO as in group V after the administration of chelerythrine (group VI). PMA-stimulated PKC activity was measured in the presence and absence of calcium (see Materials and Methods). Ischemic PC, ischemic PC+L-NA, SNAP, DETA/NO, and DETA/NO+chelerythrine had no significant effect on either calcium-stimulated or calcium-independent total PKC activity. Data are mean±SEM (n=5 in all groups).

Phase II: Effect of NO Donors on PKC

The results of phase I demonstrate that the NOS inhibitor L-NA blocks the translocation of PKC ϵ induced by ischemic PC, implying that this signaling event is caused by generation of NO. However, the possibility of nonspecific actions of L-NA cannot be ruled out. Furthermore, since NOS can produce both NO and $\cdot\text{O}_2^-$,⁴⁶⁻⁴⁸ inhibition of NOS may prevent PKC activation by preventing generation of $\cdot\text{O}_2^-$ rather than generation of NO. Accordingly, in phase II we sought to obtain direct evidence that NO in itself, without any increase in $\cdot\text{O}_2^-$, can activate PKC ϵ . To this end, we tested whether exogenous NO, in the absence of ischemia, can mimic the effects of ischemic PC on PKC. Since the ischemic PC-induced translocation of the ϵ isoform of PKC is inhibited by chelerythrine,³³ we further tested whether the NO donor-induced translocation of PKC ϵ can also be inhibited by chelerythrine.

A total of 15 conscious rabbits were instrumented for phase II. Of these, 5 were assigned to group IV (SNAP), 5 to group V

(DETA/NO), and 5 to group VI (DETA/NO+chelerythrine). All rabbits completed the protocol successfully.

Effect of NO Donors on the Subcellular Distribution of PKC Isoforms and on Total PKC Activity

We have previously reported that the adult rabbit myocardium expresses 10 isoforms of PKC (α , β_1 , β_2 , γ , δ , ϵ , η , μ , ζ , ι , and λ).³² In the present study, the subcellular distribution of all of these 10 isoforms was determined in groups I (nonischemic control), IV (SNAP), and V (DETA/NO). Neither DETA/NO nor SNAP had any discernible effect on the subcellular distribution of conventional PKCs (α , β_1 , β_2 , and γ) (Figures 5 and 6). The proportion of isoform ζ in the particulate fraction increased significantly ($P<0.05$) after SNAP treatment (Figure 5) but not after DETA/NO treatment (Figure 6). The other atypical PKCs (ι/λ) were unaffected by either SNAP (Figure 5) or DETA/NO (Figure 6).

In contrast to the conventional and atypical PKC isoforms, the subcellular distribution of PKC ϵ and η , 2 novel PKC isoforms, was significantly altered by the NO donors.

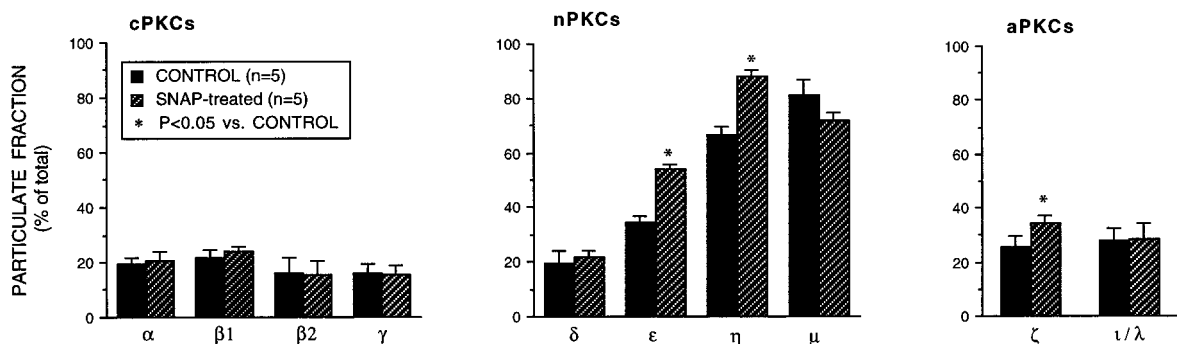


Figure 5. Effect of SNAP on the subcellular distribution of all 10 PKC isoforms expressed in the rabbit myocardium. Illustrated in this figure is the particulate fraction (expressed as a percentage of total PKC isoform) for the 3 subgroups of PKC isozymes, which are conventional PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs). SNAP induced a significant increase in the particulate fractions of the ϵ and η isoforms in the novel PKC subgroup ($P<0.05$ vs control) and also a significant translocation of the ζ isoform in the atypical PKC subgroup ($P<0.05$ vs control). SNAP had no demonstrable effect on the subcellular distribution of the other PKC isoforms. Data are mean±SEM (n=5 in all groups).

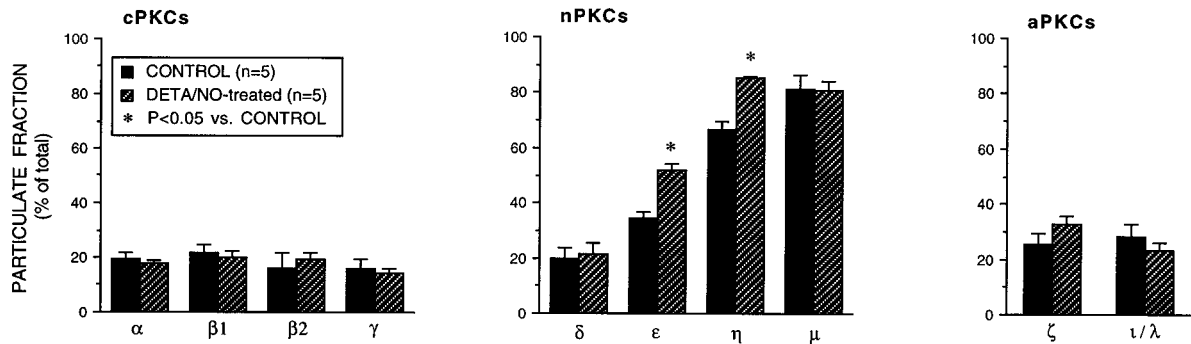


Figure 6. Effect of DETA/NO on the subcellular distribution of all 10 PKC isoforms expressed in the rabbit myocardium. Illustrated in this figure is the particulate fraction (expressed as a percentage of total PKC isoform) for the 3 subgroups of PKC isozymes, which are conventional PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs). Similar to the results obtained with SNAP, DETA/NO induced a significant increase in the particulate fractions of ϵ and η ($P < 0.05$ vs control) but, unlike SNAP, had no significant effect on the ζ isoform. DETA/NO had no demonstrable effect on the subcellular distribution of the other PKC isoforms. Data are mean \pm SEM ($n=5$ in all groups).

The expression of PKC ϵ in the particulate fraction increased from $35 \pm 2\%$ of total in untreated control rabbits (group I) to $54 \pm 2\%$ in SNAP-treated rabbits (group IV) (Figure 5) and to $52 \pm 2\%$ in DETA/NO-treated rabbits (group V) (Figure 6). An example of an immunoblot illustrating DETA/NO-induced translocation of PKC ϵ is presented in Figure 7. The expression of PKC η in the particulate fraction increased from $66 \pm 5\%$ in control rabbits to $88 \pm 2\%$ in SNAP-treated rabbits (group IV) (Figure 5) and to $85 \pm 1\%$ in DETA/NO-treated rabbits (group V) (Figure 6). The subcellular distribution of the remaining 2 novel PKC isoforms (δ and μ) was not affected by either SNAP (Figure 5) or DETA/NO (Figure

6). Interestingly, the degree of translocation of the ϵ and η isoforms elicited by SNAP and DETA/NO was comparable with that elicited by ischemic PC in group II (Figure 8).

SNAP and DETA/NO did not produce any significant change in total myocardial PKC activity, either in the presence of calcium and PMA or in the presence of PMA alone (Table 1). The subcellular distribution of PKC activity also remained unaltered after NO donor treatment (Table 1).

Effect of Chelerythrine on DETA/NO-Induced Translocation of PKC

Having established that DETA/NO induces translocation of PKC ϵ and η isoforms, we examined whether this effect could be prevented by PKC inhibitors. To this end, the subcellular distribution of the ϵ and η isoforms was compared in rabbits receiving DETA/NO in the absence (group V) or in the presence (group VI) of chelerythrine. In group VI (DETA/NO+chelerythrine), the particulate fraction of PKC ϵ was significantly ($P < 0.05$) less than in group V (DETA/NO) ($38 \pm 4\%$ versus $52 \pm 2\%$ of total, respectively) and was similar to that measured in group I (nonischemic control) ($35 \pm 2\%$) (Figure 8). Thus, administration of chelerythrine completely inhibited the translocation of the ϵ isoform induced by DETA/NO. In contrast, chelerythrine was less effective in attenuating the translocation of the η isoform associated with DETA/NO administration (Figure 8). The particulate fraction of η in group VI (DETA/NO+chelerythrine) ($79 \pm 2\%$ of total) did not differ significantly from that in group V (DETA/NO) ($85 \pm 1\%$ of total), and was significantly ($P < 0.05$) greater than that in group I (nonischemic control) ($66 \pm 5\%$ of total). Thus, despite pretreatment with chelerythrine, administration of DETA/NO still induced significant translocation of the η isoform to the particulate fraction.

DETA/NO (group V) had no appreciable effect on total myocardial PKC activity (Table 1). Administration of chelerythrine in conjunction with DETA/NO also had no significant effect on total myocardial PKC activity (Table 1). Both the calcium-stimulated and the calcium-independent PKC activity remained unaltered in group VI (DETA/

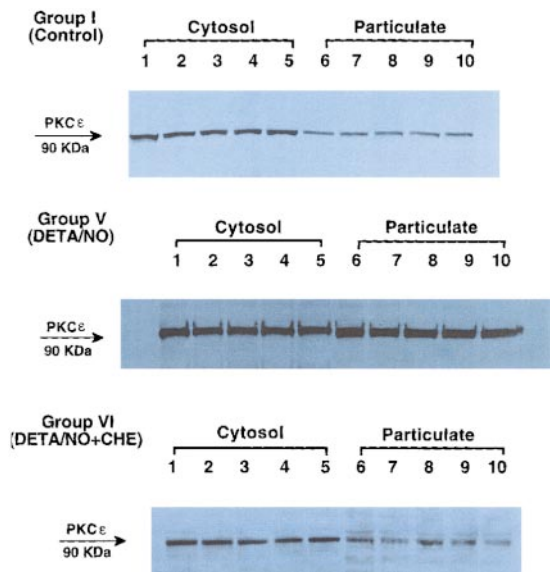


Figure 7. Western immunoblots showing the subcellular distribution of the PKC ϵ isoform in 5 nonischemic control rabbits (group I), in 5 rabbits treated with DETA/NO (group V), and in 5 rabbits treated with DETA/NO in conjunction with chelerythrine (group VI). Note that in group I, most of the ϵ isoform resides in the cytosolic fraction, whereas in group V, most of the ϵ isoform resides in the particulate fraction. In group VI, most of the ϵ isoform is in the cytosolic fraction, indicating that chelerythrine completely blocked DETA/NO-induced translocation of this isoform.

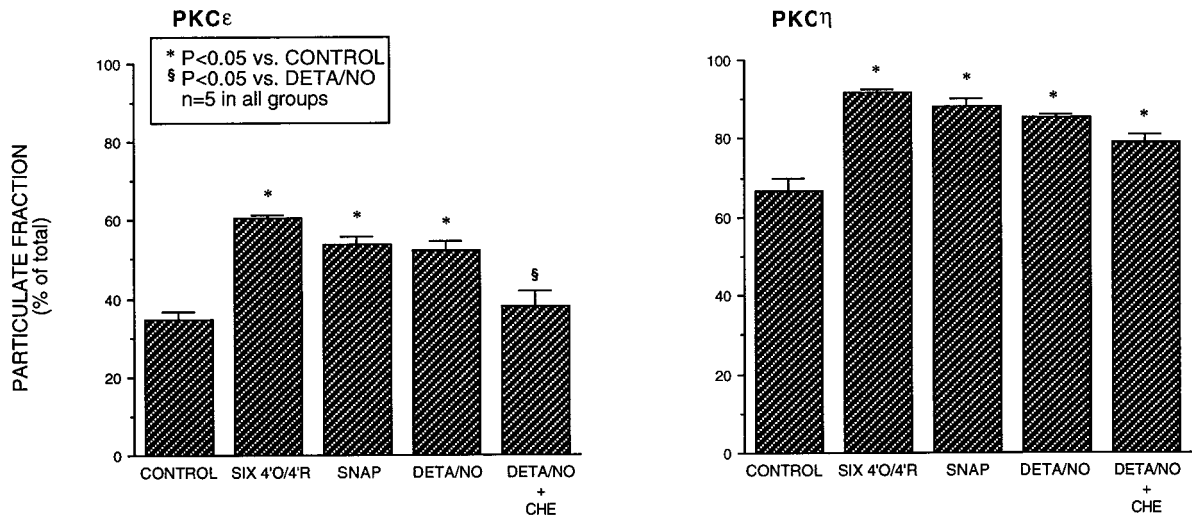


Figure 8. Expression of the PKCε and -η isoforms in the particulate fraction in nonischemic control (group I), ischemic PC (group II), SNAP-treated (group IV), DETA/NO-treated (group V), and DETA/NO+chelerythrine-treated (group VI) rabbits. Compared with control rabbits, the particulate fraction of the ε isoform (right panel) and the η isoform (left panel) was significantly increased in rabbits treated with SNAP or DETA/NO ($P < 0.05$ vs control) as well as in rabbits subjected to the ischemic PC protocol (6 cycles of 4-minute occlusion/4-minute reperfusion [4'O/4'R]) ($P < 0.05$ vs control). The extent of translocation of the ε and η isoforms induced by either SNAP or DETA/NO was similar to that induced by ischemic PC. Note that the DETA/NO-induced translocation of the ε isoform, but not that of the η isoform, was completely blocked by chelerythrine (group VI) ($P < 0.05$ vs DETA/NO-treated rabbits). Data are mean ± SEM (n=5 in all groups).

NO+chelerythrine) compared with groups I (nonischemic control) and V (DETA/NO) (Table 1).

Effect of Ischemic PC and NO Donors on the Isoform-Selective Activity of PKCε

The finding that neither L-NA (Figure 4) nor chelerythrine (Figure 5) affected the translocation of PKCη is consistent with the notion that PKCε is the critical isoform responsible for the development of late PC after ischemia.³³ Because measurements of total PKC activity are not sensitive enough to detect activation of individual isozymes,³² we performed additional studies to measure the isoform-selective phosphorylation activity of PKCε. We found that concomitant with the

translocation of the ε protein (Figure 4), ischemic PC induced a significant increase in PKCε isoform-selective activity in the particulate fraction (65.2 ± 2.7% above control, Figure 9), indicating that the translocated ε proteins were in their active state. A similar increase in PKCε isoform-selective activity in the particulate fraction was observed after the administration of either DETA/NO or SNAP (DETA/NO, 54.4 ± 5.3% above control; SNAP, 48.1 ± 4.9% above control; Figure 9). Furthermore, the ischemic PC-induced activation of PKCε was blocked by L-NA, and the DETA/NO-induced activation of PKCε was blocked by chelerythrine (Figure 9). Thus, in all groups examined (groups I through VI), the changes in the subcellular distribution of the ε protein (Figures 4 through 6)

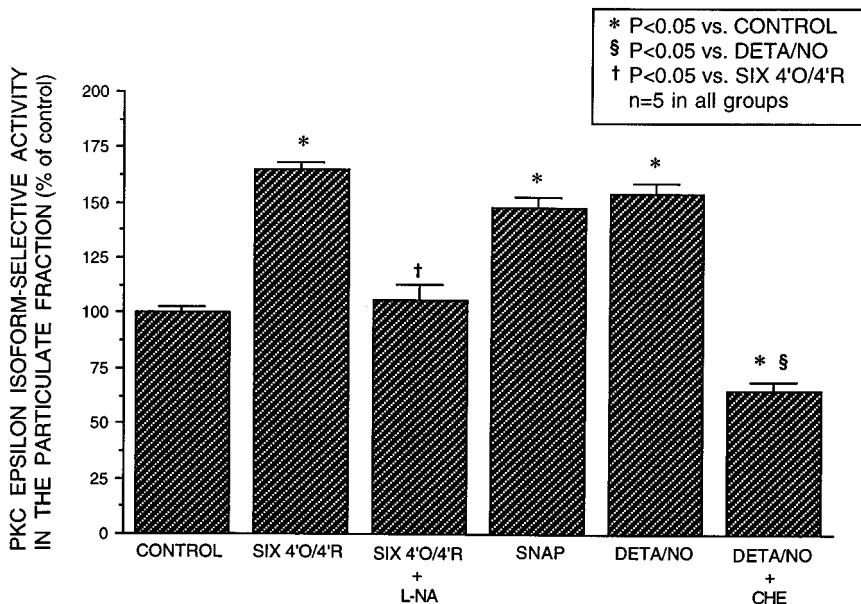


Figure 9. PKCε isoform-selective phosphorylation activity, measured after immunoprecipitation, in the 6 experimental groups (n=5 in each group). Compared with the control group (group I), the activity of PKCε in the particulate fraction was significantly increased in rabbits subjected to ischemic PC (6 cycles of 4-minute occlusion/4-minute reperfusion [six 4'O/4'R; group II]). In rabbits subjected to ischemic PC after administration of L-NA (six 4'O/4'R+L-NA; group III), the increase in PKCε activity induced by the 6 occlusion/reperfusion cycles was completely abolished. Administration of either SNAP (group IV) or DETA/NO (group V), in the absence of ischemia, induced a significant increase in PKCε activity comparable with that induced by ischemic PC. In rabbits given DETA/NO in conjunction with chelerythrine (DETA/NO+CHE; group VI), the increase in PKCε activity induced by this NO donor was abolished. Data are mean ± SEM.

were associated with parallel changes in the isoform-selective phosphorylation activity of the ϵ isozyme (Figure 9), whereas total PKC activity was not significantly altered (Table 1).

Phase III: Studies of Myocardial Stunning

Exclusions and Postmortem Analysis

Of the 20 rabbits instrumented for the studies of myocardial stunning, 5 were assigned to group VII (control), 5 to group VIII (DETA/NO), 5 to group IX (DETA/NO+chelerythrine), and 5 to group X (chelerythrine). All of the animals completed the protocol satisfactorily and were included in the data analysis. Postmortem analysis showed that the size of the occluded-reperfused vascular bed was similar in the 4 groups: 0.8 ± 0.1 g ($16.6 \pm 2.1\%$) in group VII, 0.8 ± 0.1 g ($17.8 \pm 1.8\%$) in group VIII, 0.9 ± 0.2 g ($18.5 \pm 2.5\%$) in group IX, and 0.9 ± 0.1 g ($18.8 \pm 1.2\%$) in group X. Tissue staining with triphenyltetrazolium chloride confirmed the absence of infarction in all animals. In all rabbits, the ultrasonic crystal was found to be at least 3 mm from the boundaries of the ischemic-reperfused region.

Hemodynamic Variables

Twenty-four hours before day 1 (day 0), there were no significant changes in arterial blood pressure or systolic WTh at any time during or after the administration of DETA/NO, DETA/NO+chelerythrine, or chelerythrine in groups VIII, IX, and X, respectively (representative measurements are given in Table 2). In groups VIII and X, heart rate decreased slightly ($\approx 16\%$) after the injection of chelerythrine and returned to baseline values by 3 hours (Table 2). On days 1, 2, and 3, there were no appreciable differences in heart rate among the 4 groups, either during the sequence of coronary occlusion/reperfusion cycles or during the 5-hour reperfusion period (Tables 3 and 4).

Regional Myocardial Function

Baseline systolic thickening fraction on days 1, 2, and 3 averaged $34.1 \pm 5.5\%$, $32.7 \pm 4.9\%$, and $32.4 \pm 5.3\%$, respectively, in group VII; $39.9 \pm 3.1\%$, $40.9 \pm 3.4\%$, and $39.6 \pm 3.0\%$ in group VIII; $32.5 \pm 3.3\%$, $32.1 \pm 2.9\%$, and $33.0 \pm 3.3\%$ in group IX; and $32.6 \pm 2.6\%$, $32.3 \pm 3.1\%$, and $31.9 \pm 2.6\%$ in group X. There were no significant differences among groups VII, VIII, IX, and X on the same day, or among different days within the same group. Furthermore, within the same group there were no significant differences among days 1, 2, and 3 with respect to the extent of paradoxical systolic thinning during the 6 occlusions (Figures 10 through 13).

Group VII (Control)

On day 1, thickening fraction remained significantly ($P < 0.05$) depressed for 4 hours after the sixth reperfusion and recovered by 5 hours (Figure 10), indicating that 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 after the 6 occlusion/reperfusion cycles was markedly improved compared with day 1 (Figure 11). The total deficit of WTh after the sixth reperfusion was 54% less on both days 2 and 3 compared with day 1 ($P < 0.01$) (Figure 14). Thus, as expected,^{8,9,14,33} myocardial stunning was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1.

TABLE 2. Hemodynamic Variables During and After Administration of DETA/NO, DETA/NO+Chelerythrine, or Chelerythrine

	Baseline	End of Drug Administration*	2 Hours After End of Drug Administration
Heart rate, bpm			
Group VIII	258 \pm 11	245 \pm 11	252 \pm 16
Group IX	238 \pm 4	213 \pm 17	226 \pm 4
Group X	241 \pm 8	232 \pm 16	206 \pm 11†
Group XIII	249 \pm 7	252 \pm 6	249 \pm 8
Group XIV	253 \pm 6	210 \pm 8†	220 \pm 14
Group XV	251 \pm 7	238 \pm 11	212 \pm 7†
Mean arterial pressure, mm Hg			
Group VIII	80 \pm 2	77 \pm 2	78 \pm 2
Group IX	72 \pm 3	75 \pm 9	78 \pm 8
Group X	71 \pm 3	74 \pm 6	75 \pm 7
Group XIII	73 \pm 7	71 \pm 6	70 \pm 4
Group XIV	83 \pm 7	82 \pm 4	78 \pm 5
Group XV	82 \pm 4	84 \pm 4	86 \pm 5
Systolic WTh fraction, %			
Group VIII	40.5 \pm 2.5	41.2 \pm 2.1	39.9 \pm 2.9
Group IX	32.2 \pm 3.1	34.5 \pm 3.7	32.5 \pm 3.5
Group X	35.7 \pm 4.3	35.4 \pm 3.3	36.0 \pm 4.3
Group XIII	35.3 \pm 4.4	34.7 \pm 4.5	35.2 \pm 4.9
Group XIV	34.1 \pm 2.7	34.1 \pm 2.8	33.5 \pm 2.6
Group XV	33.2 \pm 3.0	33.1 \pm 2.7	32.0 \pm 2.5

Groups VIII and XIII received 4 boluses of 0.1 mg/kg DETA/NO at 25-minute intervals. Groups IX and XIV received DETA/NO according to the same protocol used in group VIII and XIII; in addition, they were given a bolus of 5 mg/kg chelerythrine 5 minutes before the first DETA/NO treatment. Groups X and XV received a bolus of 5 mg/kg of chelerythrine alone. Data are mean \pm SEM.

In groups VIII, IX, XIII, and XIV, these measurements were taken 75 minutes after the first bolus of DETA/NO (ie, immediately after the fourth bolus). In groups X and XV, these measurements were taken immediately after the bolus of chelerythrine.

† $P < 0.05$ vs. baseline.

Group VIII (DETA/NO)

On day 1, the recovery of WTh after the sixth reperfusion was markedly faster in group VIII than in the control group, and this improvement was sustained throughout the entire reperfusion interval (Figure 11). The total deficit of WTh in group VIII was 57% less than that observed in control rabbits on day 1 ($P < 0.05$) and similar to that observed in control rabbits on days 2 and 3 (Figure 14). On days 2 and 3, there was no further improvement in either the recovery of WTh (Figure 11) or the total deficit of WTh (Figure 14) compared with day 1. Thus, as previously reported,¹⁴ administration of DETA/NO 24 hours before the sequence of six 4-minute occlusion/reperfusion cycles resulted in an attenuation of myocardial stunning on day 1 that was essentially equivalent to that effected by ischemic PC.

Group IX (DETA/NO+Chelerythrine)

On day 1, both the recovery of WTh (Figure 12) and the total deficit of WTh (Figure 14) were similar to those noted in the

TABLE 3. Heart Rate (bpm) During Coronary Occlusion and Reperfusion (Phase III)

	Baseline	Third Occlusion	Reperfusion		
			1 Hour	3 Hours	5 Hours
Group VII (control)					
Day 1	257±12	274±9	246±12	241±9	239±5
Day 2	267±11	261±14	235±3	239±11	244±12
Day 3	266±5	246±13	251±5	247±7	234±17
Group VIII (DETA/NO)					
Day 1	261±5	253±7	248±19	230±10	227±7
Day 2	258±8	246±10	229±10	231±6	230±10
Day 3	253±11	248±7	230±15	226±11	227±8
Group IX (DETA/NO+CHE)					
Day 1	249±9	242±17	250±15	240±9	241±11
Day 2	260±13	244±17	223±12	214±11	223±6
Day 3	248±14	245±13	228±7	224±8	232±12
Group X (CHE)					
Day 1	258±12	267±9	272±12	255±13	264±10
Day 2	270±6	267±9	255±12	254±7	251±6
Day 3	268±6	277±8	261±14	250±11	244±7

In the studies of myocardial stunning (phase III, groups VII through X), all rabbits underwent a sequence of 6 cycles of 4-minute coronary occlusion/4-minute reperfusion followed by a 5-hour observation period on days 1, 2, and 3. Heart rate was measured 5 minutes before occlusion (baseline), 3 minutes into the third occlusion, and at selected times after the sixth reperfusion. CHE indicates chelerythrine. Data are mean±SEM.

control group, indicating the absence of PC against stunning. Thus, the PC effect induced by DETA/NO was completely abrogated by the concomitant administration of chelerythrine. A PC effect became apparent on days 2 and 3, as documented by the enhanced recovery of WTh (Figure 12) and the reduced deficit of WTh (Figure 14) compared with day 1.

Group X (Chelerythrine)

On day 1, both the recovery of WTh (Figure 13) and the total deficit of WTh (Figure 14) were virtually indistinguishable from those noted in the control group, indicating that administration of chelerythrine did not exacerbate the severity of myocardial stunning 24 hours later. On days 2 and 3, the recovery of WTh was enhanced (Figure 14), and the deficit of WTh was reduced (Figure 14) compared with day 1, indicating the presence of a PC effect. These results indicate that the absence of a DETA/NO-induced protective effect against

stunning in group IX cannot be ascribed to a delayed deleterious action of chelerythrine.

Phase IV: Studies of Myocardial Infarction

Exclusions and Arrhythmias

Of the 45 rabbits instrumented for the studies of myocardial infarction, 10 were assigned to group XI (control), 10 to group XII (ischemic PC), 9 to group XIII (DETA/NO), 9 to group XIV (DETA/NO+chelerythrine), and 7 to group XV (chelerythrine). Six rabbits died of ventricular fibrillation during coronary occlusion (2 in group XI, 2 in group XII, 1 in group XIII, and 1 in group XIV) and 1 after reperfusion (in group XIV). Therefore, a total of 8 rabbits completed the experimental protocol in group XI, 8 in group XII, 8 in group XIII, 7 in group XIV, and 7 in group XV. No rabbit included in the final analysis was subjected to defibrillation.

TABLE 4. Heart Rate (bpm) During Coronary Occlusion and Reperfusion (Phase IV)

	Baseline	Occlusion (15 minutes)	Reperfusion					
			1 Hour	3 Hours	5 Hours	24 Hours	48 Hours	72 Hours
Group XI (control)	251±11	277±15	275±13	260±4	265±12	266±12	262±12	258±11
Group XII (ischemic PC)	244±5	276±7	274±5	274±10	262±11	265±11	263±8	264±9
Group XIII (DETA/NO)	250±7	270±11	266±9	267±4	256±5	260±6	255±5	262±4
Group XIV (DETA/NO+CHE)	248±7	257±7	241±11	247±12	256±6	255±8	250±7	251±6
Group XV (CHE)	249±7	266±12	256±13	248±13	252±6	256±8	255±6	251±3

In the studies of myocardial infarction (phase IV, groups X through XV), all groups underwent a 30-minute coronary occlusion followed by 72 hours of reperfusion. Heart rate was measured 5 minutes before occlusion (baseline), at 15 minutes into the 30-minute coronary occlusion, and at selected times after the coronary reperfusion. CHE indicates chelerythrine. Data are mean±SEM.

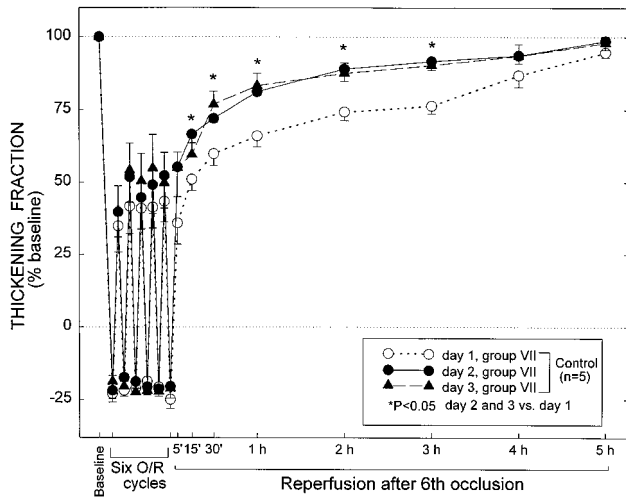


Figure 10. Systolic thickening fraction in the ischemic-reperfused region in group VII (control) 5 minutes before the first occlusion (baseline), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. Thickening fraction is expressed as a percentage of baseline values. Data are mean ± SEM.

Hemodynamic Variables

No significant changes in arterial blood pressure or systolic WTh were observed at any time during or after the administration of DETA/NO, DETA/NO+chelerythrine, or chelerythrine in groups XI, XII, and XIII, respectively, 24 hours before day 1 (day 0) (representative measurements are given in Table 2). In groups XIII and XIV, heart rate decreased slightly (~16%) after the injection of chelerythrine and returned to baseline values by 3 hours (Table 2). There were no appreciable differences in heart rate among groups XI, XII, XIII, XIV, and XV, either during the 30-minute coronary

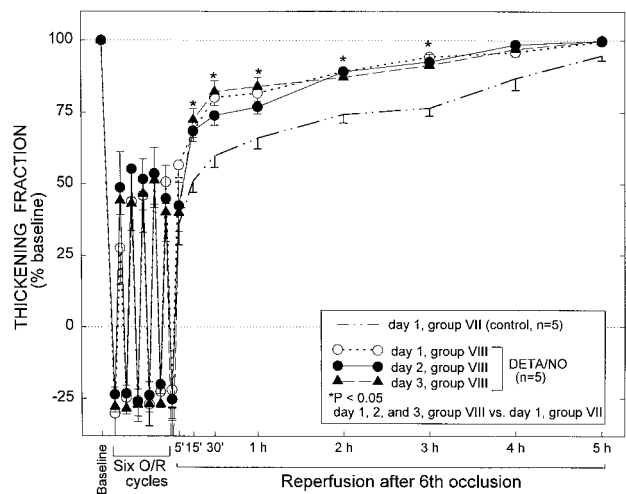


Figure 11. Systolic thickening fraction in the ischemic-reperfused region in group VIII (DETA/NO) 5 minutes before the first occlusion (baseline), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. To facilitate comparisons, the data pertaining to day 1 of group VII (control) are also shown. Thickening fraction is expressed as a percentage of baseline values. Data are mean ± SEM.

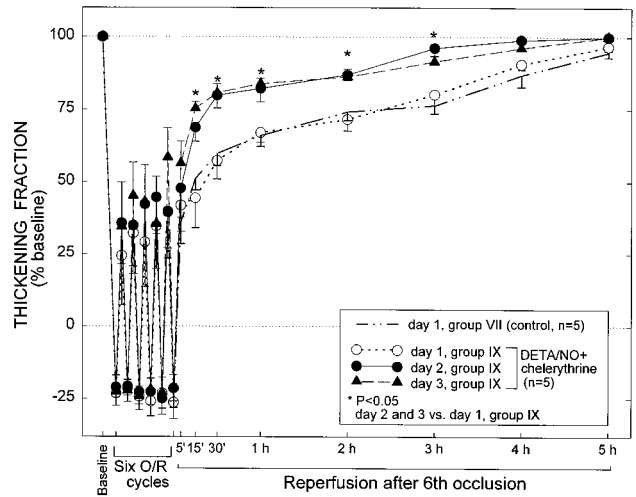


Figure 12. Systolic thickening fraction in the ischemic-reperfused region in group IX (DETA/NO+chelerythrine[CH]) 5 minutes before the first occlusion (baseline), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. To facilitate comparisons, the data pertaining to day 1 of group VII (control) are also shown. Thickening fraction is expressed as a percentage of baseline values. Data are mean ± SEM.

occlusion or during the 72-hour reperfusion period (Tables 3 and 4). Baseline systolic thickening fraction was also similar among the 6 groups (31.3 ± 3.4%, 34.2 ± 4.1%, 33.2 ± 3.6%, 34.1 ± 2.7%, and 35.6 ± 2.8% in groups XI, XII, XIII, XIV, and XV, respectively).

Region at Risk and Infarct Size

There were no significant differences among groups XI, XII, XIII, XIV, and XV with respect to the weight of the region at risk (0.7 ± 0.1 g [15.7 ± 1.2% of LV weight], 0.8 ± 0.1 g

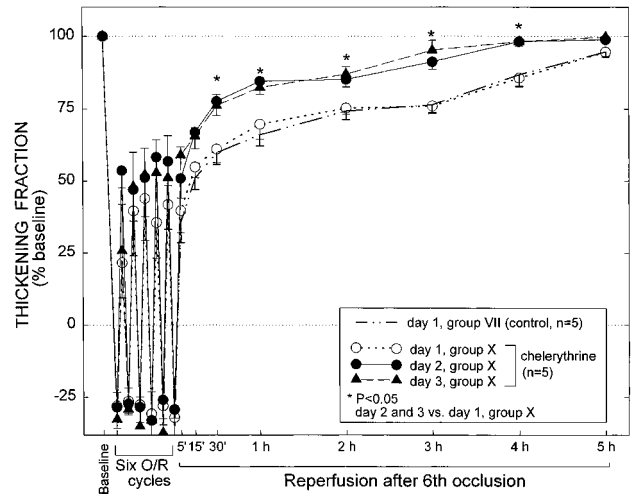


Figure 13. Systolic thickening fraction in the ischemic-reperfused region in group X (chelerythrine) 5 minutes before the first occlusion (baseline), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. To facilitate comparisons, the data pertaining to day 1 of group VII (control) are also shown. Thickening fraction is expressed as a percentage of baseline values. Data are mean ± SEM.

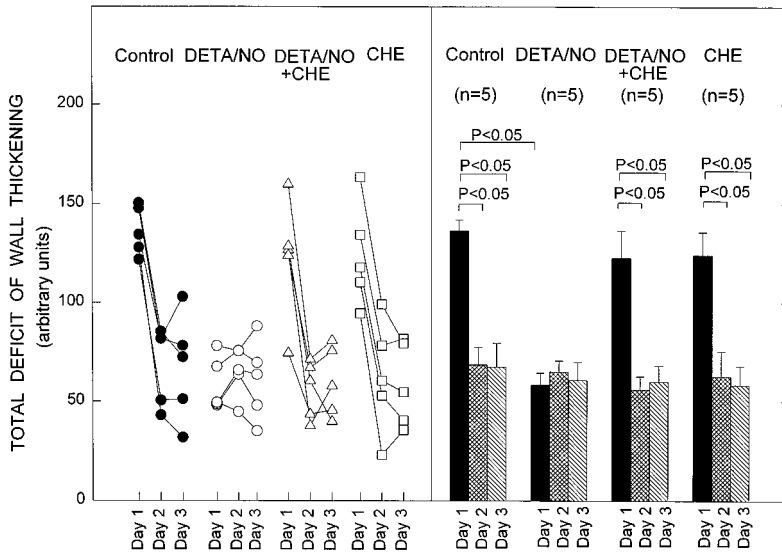


Figure 14. Total deficit of WTh after the sixth reperfusion on days 1, 2, and 3 in the control (n=5), DETA/NO (n=5), DETA/NO+chelerythrine [CHE] (n=5), and chelerythrine (n=5) groups (groups VII, VIII, IX, and X, respectively). The values of total deficit of WTh in individual rabbits are illustrated in the left panel; the means±SEM are depicted in the right panel. The total deficit of WTh was measured in arbitrary units, as described in the text.

[17.0±1.7%], 0.7±0.1 g [16.2±1.9%], 0.8±0.1 g [17.3±2.2%], and 0.8±0.1 g [19.3±1.4%], respectively). The average infarct size was 46% smaller in group XII (ischemic PC) compared with group XI (control) (58.4±3.7% versus 31.3±3.0% of the region at risk, respectively; $P<0.05$ [Figure 15]), indicating a late PC effect against myocardial infarction. A quantitatively similar PC effect was observed in group XIII (DETA/NO); the average infarct size in this group (28.7±3.8% of the region at risk) was similar to that measured in group XII and significantly ($P<0.05$) smaller than that measured in group XI (Figure 15), indicating that pretreatment with this NO donor 24 hours before the 30-minute occlusion resulted in a protective effect that was equivalent to that induced by ischemic PC. In group XIV, infarct size was indistinguishable from that measured in the control group and significantly ($P<0.05$) larger than that measured in group XIII (Figure 15), indicating that chelerythrine completely blocked the infarct-sparing effect of DETA/NO pretreatment. In group XV, infarct size was

similar to that measured in the control group, indicating that chelerythrine did not exacerbate the severity of myocardial infarction 24 hours later and that the abrogation of the infarct-sparing effect of DETA/NO could not be ascribed to a delayed deleterious action of chelerythrine.

In all groups, the size of the infarction was positively and linearly related to the size of the region at risk ($r=0.93$ in group XI, 0.79 in group XII, 0.72 in group XIII, 0.82 in group XIV, and 0.84 in group XV) (Figure 16). As expected, the regression line was shifted to the right in the ischemic PC group (group XII) as compared with the control group (group XI, $P<0.05$ by ANCOVA) (Figure 16). In the DETA/NO group (group XIII), the regression line was virtually indistinguishable from that of the ischemic PC group and was significantly shifted to the right compared with the control group ($P<0.05$ by ANCOVA) (Figure 16), indicating that for any given size of the region at risk, the resulting infarct size was reduced by pretreatment with DETA/NO and that the magnitude of this effect was similar to that induced by ischemic PC. In contrast, in the DETA/NO+chelerythrine group (group XIV) and in the chelerythrine group (group XV), the regression line did not differ from that observed in the control group (Figure 16).

Regional Myocardial Function

Because of Doppler probe malfunction, complete measurements of WTh for 3 days after reperfusion could be obtained in only 6 of 8 rabbits in group XI, 6 of 8 rabbits in group XII, 5 of 8 rabbits in group XIII, 5 of 7 rabbits in group XIV, and 5 of 7 rabbits in group XV. After release of the 30-minute occlusion, control rabbits (group XI) exhibited essentially no recovery of WTh, even at 3 days (Figure 17). In rabbits preconditioned with ischemia (group XII), the recovery of WTh was significantly ($P<0.05$) improved compared with controls at 3, 5, 24, and 72 hours after reperfusion (Figure 17). The total deficit of WTh during the 72 hours of reperfusion was 12% less in group XII than in group XI ($P<0.05$) (Figure 17). In the DETA/NO group (group XIII), the recovery of WTh was similar to that noted in the rabbits

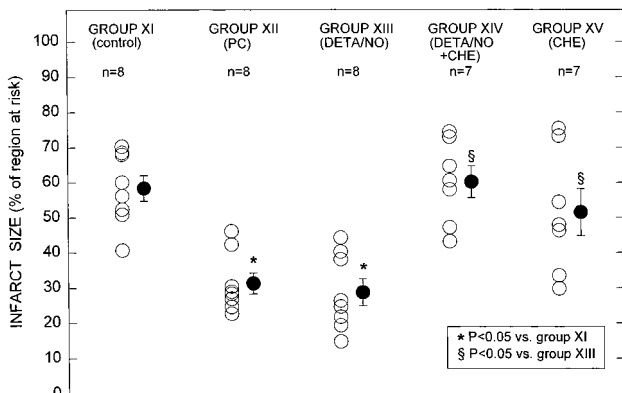


Figure 15. Myocardial infarct size in groups XI (n=8, control group), XII (n=8, ischemic PC), XIII (n=8, DETA/NO), XIV (n=7, DETA/NO+chelerythrine [CHE]), and XV (n=7, chelerythrine). Infarct size is expressed as a percentage of the region at risk of infarction. ○ represents individual rabbit; ●, mean±SEM. * $P<0.05$ vs group XI (control group); § $P<0.05$ vs group XIII (DETA/NO).

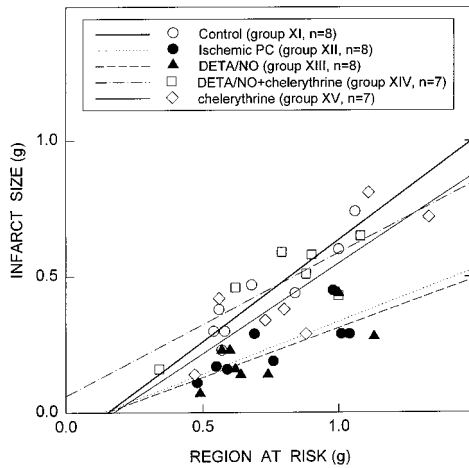


Figure 16. Relationship between size of region at risk and size of myocardial infarction. The figure illustrates both individual values and regression lines obtained by linear regression analysis for the control group (group XI, $n=8$), the ischemic PC group (group XII, $n=8$), the DETA/NO group (group XIII, $n=8$), the DETA/NO+chelerythrine group (group XIV, $n=7$), and the chelerythrine group (group XV, $n=7$). In all groups, infarct size was positively and linearly related to risk-region size. The linear regression equations were follows: control group, $y=0.75x-0.11$ ($r=0.93$); ischemic PC group, $y=0.39x-0.05$ ($r=0.79$); DETA/NO group, $y=0.37x-0.05$ ($r=0.72$); DETA/NO+chelerythrine group, $y=0.53x+0.06$ ($r=0.82$); and chelerythrine group, $y=0.66x-0.12$. ANCOVA demonstrated that the regression lines for the ischemic PC and DETA/NO groups were significantly different from that for the control group ($P<0.05$ for each comparison), indicating that for any given risk-region size, infarct size was smaller in the ischemic PC and NO donor-pretreated rabbits compared with control rabbits; in contrast, the lines for the DETA/NO+chelerythrine group and the chelerythrine group were similar to that for the control group.

preconditioned with ischemia and significantly ($P<0.05$) improved compared with controls at 1 hour and 3, 5, 24, and 72 hours after reperfusion (Figure 17). The total deficit of WTh during the 72-hour reperfusion period was 12% less in NO donor-pretreated rabbits than in control rabbits ($P<0.05$) (Figure 17). Thus, pretreatment with an NO donor resulted in enhanced recovery of myocardial contractile function, which became evident soon after reperfusion (1 hour) and was sustained throughout the 72-hour reperfusion interval; the magnitude of this effect was similar to that observed after ischemic PC in group XII. In the DETA/NO+chelerythrine group, both the recovery of WTh and the total deficit of WTh were similar to those observed in the control group (Figure 17), indicating that the salutary actions of NO donor pretreatment on the recovery of myocardial function were abrogated by the concomitant administration of chelerythrine. The results obtained in the chelerythrine group (XV) were similar to those in the control group, indicating that chelerythrine did not exacerbate myocardial dysfunction after the 30-minute occlusion 24 hours later. Because the effects of ischemic PC, DETA/NO, and chelerythrine on WTh paralleled those on infarct size (Figure 15), the WTh data provide an independent confirmation of the results obtained with tetrazolium staining.

Discussion

Although isoform-selective activation of PKC ϵ appears to play a critical role in the genesis of the late phase ("second

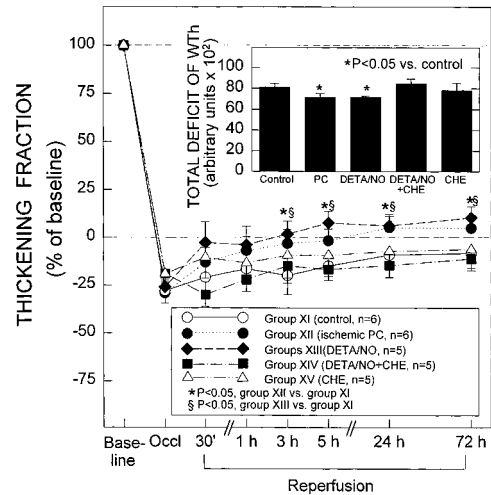


Figure 17. Systolic thickening fraction in the ischemic-reperfused region in the control group (group XI, $n=6$), the ischemic PC group (group XII, $n=6$), the DETA/NO group (group XIII, $n=5$), the DETA/NO+chelerythrine (CHE) group (group XIV, $n=5$), and the chelerythrine group (group XV, $n=5$) 5 minutes before the 30-minute occlusion (baseline), 15 minutes into the 30-minute coronary occlusion (Occl), and at selected times during the 72-hour reperfusion interval. Because of Doppler-probe malfunction, complete measurements of thickening fraction could not be obtained in all of the rabbits in groups XI, XII, XIII, XIV, and XV. Thickening fraction is expressed as a percentage of baseline values. The total deficit of WTh after infarction is depicted in the inset. The total deficit of WTh was calculated by measuring the area between systolic WTh-vs-time line and baseline (100% line) during the 3-day reperfusion period after the 30-minute occlusion (see text). Data are mean \pm SEM. * $P<0.05$ between groups XI and XII; § $P<0.05$ between groups XI and XIII. Inset, * $P<0.05$ vs the control group (group XI).

window") of ischemic PC,^{32,33} the mechanism(s) responsible for such activation remains unclear. Furthermore, the signal transduction pathway that leads to the development of late PC after administration of NO-releasing agents in the absence of ischemia (NO donor-induced late PC) is unknown. The present study provides significant new insights into these issues. First, in the conscious rabbit, administration of the NOS inhibitor L-NA (at doses previously shown to block the development of late PC^{8,10}) completely blocks the ischemic PC-induced increase in PKC ϵ protein content and the concomitant increase in PKC ϵ -selective activity in the particulate fraction, indicating that increased formation of NO is an essential mechanism whereby brief ischemia activates the ϵ isoform of PKC. Second, a translocation of PKC ϵ and η proteins and an increase in PKC ϵ activity quantitatively similar to those induced by ischemic PC can be reproduced pharmacologically with the administration of 2 structurally unrelated NO donors (at hemodynamically inactive doses previously shown to elicit a late PC effect¹⁴), demonstrating that NO in itself (in the absence of the other cellular perturbations associated with ischemia) can trigger PKC activation in the heart. Third, in vivo administration of the PKC inhibitor chelerythrine at 5 mg/kg completely prevents the DETA/NO-induced translocation and activation of the ϵ isoform but not the translocation of the η isoform, indicating that chelerythrine is a useful tool for interrogating the role of the ϵ isoform of PKC in NO donor-induced late PC. Finally,

the same dose of chelerythrine that blocks the DETA/NO-induced activation of PKC ϵ also blocks the DETA/NO-induced late PC effect against both myocardial stunning and infarction, indicating that the ϵ isozyme plays a pivotal role in the genesis of the delayed cardioprotection elicited by NO-releasing agents.

Taken together, the results of the present investigation provide direct evidence, for the first time, that isoform-selective activation of PKC ϵ is a critical signaling step in the cellular pathway whereby NO initiates the development of a late PC effect, both after an ischemic stimulus (endogenous NO) and after NO-donor treatment (exogenous NO). To our knowledge, this is also the first report that NO can activate PKC in the heart.

To assess the actions of NO on PKC, we used two different approaches, ie, we examined the role of PKC in NO donor-induced late PC and, conversely, the effect of endogenous NO formed during ischemic PC on PKC. These two approaches will be discussed separately.

Isoform-Selective Activation of PKC by NO

Despite the multiplicity of NO-dependent process in the cardiovascular system, essentially nothing is known regarding the influence of NO on PKC in the heart. To determine whether NO in itself (in the absence of ischemia) affects PKC, we analyzed the influence of exogenous NO on total myocardial PKC activity, on PKC ϵ isoform-selective activity, and on the subcellular distribution of all 10 PKC isoforms expressed in the adult rabbit myocardium.³² Two different NO donors were tested. DETA/NO is a relatively long-acting NO donor that spontaneously and nonenzymatically releases NO with predictable first-order kinetics.^{49–51} SNAP is a direct NO donor that lacks tolerance-producing effects.^{52,53} The fact that the effects of DETA/NO and SNAP were similar makes it very unlikely that they may have been caused by nonspecific, NO-independent actions of these molecules. By analyzing the changes in PKC after the administration of the same doses of DETA/NO and SNAP that induce late PC against myocardial stunning and infarction,¹⁴ we were able to correlate the effects of NO donors on PKC isoforms with their effects on the development of delayed cardioprotection. It is important to stress that the doses of DETA/NO and SNAP used in this study have no hemodynamic effects,¹⁴ thus excluding indirect actions secondary to hemodynamic perturbations.

Both DETA/NO and SNAP induced significant translocation of two novel PKC isoforms, ϵ and η , but neither of them significantly altered the subcellular distribution of any of the other PKC isoforms (α , β 1, β 2, γ , δ , μ , and ν / λ) (Figures 5 and 6). Measurements of PKC ϵ isoform-selective activity confirmed that the translocated ϵ protein was activated (Figure 9). SNAP, but not DETA/NO, also induced significant translocation of PKC ζ (Figures 5 and 6). Thus, the effects of NO donors were quite selective, being restricted to only 3 of the 10 isozymes of PKC expressed in the rabbit heart. Despite their effects on PKC ϵ , $-\eta$, and $-\zeta$, the NO donors had no significant effect on total myocardial PKC activity, either calcium-stimulated or calcium-independent (Table 1). This result is consistent with previous reports from our laboratory³² and others^{54,55} indicating that ischemic PC

can occur without demonstrable changes in total PKC activity. Specifically, using the same conscious rabbit model used herein, we have observed that translocation of PKC ϵ and $-\eta$ after ischemic PC was not reflected in changes in total myocardial PKC activity.³⁴ This is not surprising, given that PKC ϵ and $-\eta$ account for a small fraction (<13%) of all PKC-isoform proteins expressed in the rabbit heart.³²

In conclusion, the results obtained with NO donors indicate that NO induces isoform-selective activation of PKC ϵ , $-\eta$, and $-\zeta$ and that measurements of total PKC activity are not sufficiently sensitive to detect the involvement of the PKC system in NO signaling. Determination of individual PKC isoform translocation and/or activity appears therefore essential to assess the role of PKC in NO-dependent processes.

Functional Significance of the Activation of PKC by NO

The translocation of PKC ϵ and $-\eta$ observed after administration of DETA/NO and SNAP could be important for the development of NO donor-induced late PC or, alternatively, could be an epiphenomenon. To resolve this issue, we examined the effect of chelerythrine on the translocation of PKC as well as on late PC after the administration of DETA/NO. Chelerythrine was selected for this study because it is reported to be more selective for PKC than other widely used inhibitors, such as staurosporine and polymyxin B,¹⁹ and because it has been shown to block both the early¹⁹ and the late^{6,33} phases of PC after an ischemic stimulus. Chelerythrine is a very potent inhibitor of PKC (IC_{50} , ≈ 0.7 μ mol/L) and reportedly has very high selectivity for PKC compared with protein kinase A (250:1), calcium/calmodulin-dependent protein kinase (150:1), or tyrosine protein kinase (150:1).⁵⁶ We found that pretreatment with chelerythrine (at a dose of 5 mg/kg) completely inhibited the DETA/NO-induced translocation of PKC ϵ (and the associated increase in PKC ϵ activity) but not the DETA/NO-induced translocation of PKC η (Figures 8 and 9), indicating that this agent could be used to investigate the role of the ϵ isoform of PKC in NO donor-dependent late PC. The same dose of chelerythrine that prevented the DETA/NO-induced activation of PKC ϵ completely prevented the development of the delayed cardioprotection against both myocardial stunning and infarction (Figures 12, 14, and 15), demonstrating that mobilization of PKC ϵ is essential for NO donor-induced late PC. These results, obtained after administration of exogenous NO (phases II through IV), are congruent with the results obtained after blockade of endogenous NO production with L-NA in the setting of ischemic PC (phase I), which demonstrate that prevention of PKC ϵ activation by L-NA results in abrogation of late PC (Figures 4 and 9).

Thus, taken together, the present observations demonstrate that the ϵ isoform of PKC plays an obligatory role both in NO donor-induced late PC (exogenous NO) and in ischemia-induced late PC (endogenous NO). Interestingly, recent studies by other investigators have identified PKC ϵ activation as an important event underlying ethanol-induced cardioprotection in isolated guinea pig hearts²⁷ and hypoxia-induced early PC in neonatal rat myocytes,²⁸ in agreement with our conclusions regarding the role of isoform-selective activation

of PKC ϵ during ischemia- or NO donor-induced late PC. The downstream targets of PKC ϵ -mediated phosphorylation remain to be elucidated but may include, among others, tyrosine kinases (specifically, Src and Lck⁵⁷) and, in the case of early PC, the mitochondrial KATP channel.⁵⁸

Whether the η isoform of PKC is also necessary for NO donor-induced late PC remains to be determined. In the setting of ischemic PC, previous studies³³ have already demonstrated that translocation of PKC η is not required for late PC to occur. Our finding that chelerythrine completely abrogated DETA/NO-induced late PC despite the fact that the translocation of PKC η was not prevented (Figure 8) indicates that mobilization of PKC η , in itself, is not sufficient to produce the late PC effect after exposure to exogenous NO. Nevertheless, the possibility that PKC η might be necessary for NO-induced PC, even though it is not sufficient, cannot be ruled out. Manipulations that selectively block η translocation without affecting ϵ translocation will be needed to discern whether PKC η plays an obligatory role in the late PC effect induced by NO donors.

The identification of PKC ϵ as a critical downstream effector of NO-dependent actions has implications that transcend ischemic PC. From a broader perspective, the finding that NO donor-induced PC is PKC dependent suggests that PKC may play an important role in NO-dependent signaling in the heart in general. Because NO is known to modulate multiple physiological and pathological processes,^{59,60} this finding may have considerable implications not only for the mechanism of myocardial ischemia/reperfusion injury but also for a host of other conditions and for nitrate therapy.

Activation of PKC During Ischemic PC Is NO Dependent

The observation that exogenous NO translocates PKC ϵ and $-\eta$ in the absence of ischemia (Figures 5 through 7) is consistent with the hypothesis that ischemic PC-induced activation of PKC is caused by the generation of NO, but does not prove it. Although NO is sufficient to induce PKC translocation, it may not be necessary, because other stimuli associated with ischemic PC may also activate PKC independently of NO.

To resolve this issue, in phase I of the present study we determined whether inhibition of NO generation with L-NA results in abrogation of PKC ϵ and $-\eta$ translocation after six 4-minute occlusion/reperfusion cycles. We were particularly interested in PKC ϵ because this appears to be the specific isoform responsible for the development of the late phase of ischemic PC in conscious rabbits.³³ We reasoned that if NO plays an obligatory role in the activation of the ϵ isoform associated with ischemic PC, L-NA should block this phenomenon. If, on the other hand, redundant signaling pathways exist whereby multiple triggers produced during the ischemic stimulus can activate PKC independently of NO, then blunting NO production with L-NA should have no effect. The finding that L-NA completely prevented the activation of PKC ϵ (Figures 4 and 9) demonstrates that NO formation is necessary for this phenomenon to occur in the conscious rabbit model of ischemic PC induced by six 4-minute occlusion/reperfusion cycles. Thus, rather surprisingly, de-

spite the fact that we used a robust ischemic stimulus (six 4-minute occlusions) that should cause the release of multiple activators of PKC, NO still appears to be essential for the activation of PKC-dependent signaling in this model.

It should be stressed, however, that the present results do not in any way exclude an important role of other mechanisms in the stimulation of PKC that occurs during ischemia. The concept that NO is necessary for PKC to be translocated in our model of ischemic PC is not in conflict with the existing body of evidence indicating that, in other experimental models, ischemic PC activates PKC via the release of different triggers (eg, adenosine, norepinephrine, bradykinin, and reactive oxygen species).^{12,15,16,61-65} It seems likely that PKC can be activated by several stimuli and that the predominant stimulus may vary depending on the animal model and the type of PC protocol used.¹⁶

NO donor-Induced PC Versus Ischemia-Induced PC

The signaling mechanisms underlying late PC after an ischemic stimulus and late PC after administration of NO-releasing agents have not been previously compared. The present study provides direct evidence to support the concept that these 2 mechanisms are similar, at least at the level of PKC. The results of phases I and II demonstrate that NO donor-induced PC and ischemia-induced PC share a common signaling event, the activation of PKC ϵ and $-\eta$. The magnitude of the changes in PKC ϵ and $-\eta$ elicited by DETA/NO and SNAP was similar to the magnitude of the changes elicited by ischemic PC (Figures 8 and 9), again supporting a common mechanism. Similarly to ischemic PC,³² DETA/NO and SNAP had no discernible effect on the α , β 1, β 2, γ , δ , μ , and ι/λ isoforms of PKC (Figures 5 and 6). Thus, in general, the effects of exogenous NO administration on the subcellular distribution of PKC isoforms mimicked those of ischemic PC. The only difference between NO donor-induced PC and ischemic PC was the translocation of the ζ isoform, which was observed in the former (only after SNAP) (Figure 5) but not in the latter.³² It seems probable, however, that the translocation of PKC ζ is not necessary for the genesis of late PC, because it occurred after the administration of SNAP but not of DETA/NO (Figures 5 and 6), whereas late PC occurs after administration of either SNAP or DETA/NO (Figures 11, 14, and 15).¹⁴

The precise mechanism by which NO activates PKC is a complex problem that will require extensive investigation. Many possibilities (and combinations thereof) must be considered. NO can directly modulate the functions of many proteins by reacting with heme groups, sulfur-iron clusters, and thiol moieties.^{59,60} Thus, NO could influence PKC activity directly or indirectly by binding to proteins involved in PKC modulation (eg, receptor for activated C kinases [RACKs] or phospholipases). Furthermore, cGMP is known to be an important second messenger for NO-dependent signaling.^{59,60} In addition, NO reacts very rapidly with superoxide anion to form peroxynitrite (ONOO⁻), which then can decompose to generate hydroxyl radical (\bullet OH) or another oxidant with similar reactivity.⁶⁶ ONOO⁻, \bullet OH, or their reactive byproducts could activate PKC either directly^{15,67} or

via activation of phospholipase D.^{68,69} Each of these mechanisms may contribute to PKC activation and will have to be interrogated in both cellular and acellular systems with combined molecular, cellular, and biochemical approaches.

Conclusions

The results of this study demonstrate that, in the conscious rabbit, the activation of PKC ϵ associated with ischemic PC requires NO generation and that a similar activation can be reproduced by increasing NO availability in the absence of ischemia. Thus, NO is both necessary and sufficient to produce the changes in PKC that are induced by a brief ischemic stress. These findings support the novel idea that NO formation represents an important cellular signal whereby ischemic PC leads to the rapid mobilization of the PKC system in the heart. Furthermore, the present results demonstrate that PKC is a critical downstream effector in the development of late PC induced pharmacologically with NO donors and that among the 10 PKC isoforms expressed in the rabbit heart, ϵ plays an obligatory role in the genesis of this phenomenon. Thus, NO (either endogenous NO produced during ischemia or exogenous NO generated during NO donor administration) induces late PC by activating PKC. In both cases, the specific isoform involved appears to be PKC ϵ . The finding that NO can promote isoform-specific activation of PKC identifies a new biological function of this radical and a new mechanism in the signaling cascade of ischemic PC. Given that NO is an important modulator of many biological processes, this finding has potentially wide implications for numerous cardiovascular conditions besides myocardial ischemia and also for nitrate therapy.

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