Nitric Oxide (NO) Induces Nitration of Protein Kinase Cε (PKCε), Facilitating PKCε Translocation via Enhanced PKCε-RACK2 Interactions

A NOVEL MECHANISM OF NO-TRIGGERED ACTIVATION OF PKCε

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Activation of protein kinase C (PKC) by nitric oxide (NO) has been implicated in the development of cardioprotection. However, the cellular mechanisms underlying the activation of PKC by NO remain largely unknown. Nitrination of protein tyrosine residues has been shown to alter functions of a variety of proteins, and NO-derived peroxynitrite is known as a strong nitrating agent. In this investigation, we demonstrate that NO donors promote translocation and activation of PKCε in an NO- and peroxynitrite-dependent fashion. NO induces peroxynitrite-mediated tyrosine nitrination of PKCε in rabbit cardiomyocytes in vitro, and nitrotyrosine residues were also detected on PKCε in vivo in the rabbit myocardium preconditioned with NO donors. Furthermore, coimmunoprecipitation of PKCε and its receptor for activated C kinase, RACK2, illustrated a peroxynitrite-dependent increase in PKCε-RACK2 interactions in NO donor-treated cardiomyocytes. Moreover, using an enzyme-linked immunosorbent assay-based protein-protein interaction assay, PKCε proteins treated with the peroxynitrite donor SIN-1 exhibited enhanced binding to RACK2 in an acellular environment. Our data demonstrate that post-translational modification of PKCε by NO donors, namely nitrination of PKCε, facilitates its interaction with RACK2 and promotes translocation and activation of PKCε. These findings offer a plausible novel mechanism by which NO activates the PKC signaling pathway.

Protein kinase C (PKC) is a family of serine-threonine kinases that participate in numerous biological processes (1, 2). In the heart, activation of PKC reduces the myocardial ischemic injury, whereas inhibition of PKC abolishes ischemic preconditioning (3–5). Recently, it has been shown that this cardioprotective effect can be fully mimicked by modulating the activity of a single isozyme of this family, the ε isozyme of PKC (6–9). Multiple molecular events have been shown to have an activating effect on this enzyme, among which, of particular interest, is nitric oxide (NO). Although the effects of NO on PKC depend on its biological functions and on the cell types (10–14), NO-induced activation of PKC is well documented in the heart (15, 16). Several investigations have demonstrated that at doses that produce a cardioprotective effect, exogenous NO (released by NO donors) activates PKC in an isoform-specific manner (17–20). Furthermore, activation of this isozyme has been demonstrated to play an essential role in orchestrating the signal transduction events during NO-induced cardioprotection against ischemic injury (15, 21). However, the exact molecular mechanism(s) whereby NO activates PKCε in the heart remain largely unknown.

As a relatively stable hydrophobic free radical gas, NO can readily diffuse through cell membranes (22). Within cells, NO itself and NO-derived reactive nitrogen species are capable of reacting with various molecular targets that include complex biological molecules, such as proteins, lipids, and DNA, as well as low molecular weight compounds (23). One of the important molecular targets of NO are protein tyrosine residues, which can be modified to fairly stable 3-nitrotyrosines upon reacting with nitrating species. Protein nitrination is believed to be a selective process with respect to both the proteins and the specific protein tyrosine residues that can undergo this post-translational modification (24). Nitrination of protein tyrosine residues has been shown to alter the functions of a variety of proteins under physiological and pathophysiological conditions both in vitro and in vivo (23, 25). Posttranslational modification of tyrosine residues has been shown to play an important role in modulating the activity of several PKC isozymes (26–28), and analysis of the molecular sequence of PKCε reveals that it harbors multiple tyrosine residues. Therefore, we hypothesized that nitrination of PKCε on tyrosine residues may contribute to NO-induced activation of PKCε.

Peroxynitrite (ONOO-) is a well characterized nitrating species that is produced under physiological conditions when NO reacts with superoxide anion (O2-) (29). Because the reaction of NO with O2- to form ONOO- occurs at a near diffusion-limited rate, generation of ONOO- will predominate in any setting where NO and O2- are released concomitantly. Considering the fact that that cardiac myocytes have several potential sources of O2-, which among others include mitochondria and NADPH
oxidase (30), it stands to reason that ONOO− can play a role as a mediator of NO donor-induced nitration of PKCe.

Translocation and subcellular redistribution of PKCe have been recognized as important molecular events for the activation of this isozyme (1, 2, 31). The particulate translocation of PKCe is known to be facilitated by its interaction with a specific anchoring protein termed receptor for activated C kinase 2, or RACK2 (32). Importantly, the interaction of PKCe with RACK2 is crucial for mediating PKCe activation and function (6, 8, 33). Accordingly, we postulated that tyrosine nitration may serve to promote the interaction between PKCe and its RACK2, thereby leading to enhanced expression and activity of PKCe in the particulate fraction.

In the present study, we investigated whether NO induces PKCe activation and translocation in adult cardiac myocytes and elucidated the cellular mechanisms of this event. Cardiomyocytes were treated with the NO donor S-nitrosoglutathione (SNAP), which was previously shown to induce PKCe activation and cardiac protection in vivo (15). The translocation and activation of PKCe, as well as the posttranslational modification of this isozyme such as tyrosine nitration, were characterized both in cultured cardiac cells in vitro and in a rabbit model of NO preconditioning in vivo. We found that NO induces activation, translocation, and nitration of PKCe. Furthermore, nitration of PKCe enhances PKCe-RACK2 interactions and activates PKCe translocation.

EXPERIMENTAL PROCEDURES

The present study was performed in accordance with guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, publication NIH 86–23).

Materials and Reagents—M199 medium, fetal calf serum, penicillin, and streptomycin were obtained from Invitrogen. Type II collagenase was from Worthington. SNAP, SIN-1, and Ebselen were from Calbiochem. Mouse monoclonal antibody against PKCe and horseradish peroxidase-conjugated goat anti-mouse secondary antibody were obtained from Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-nitrotyrosine antibody and nitrotyrosine immunoblotting control were from Upstate Biotechnology (Lake Placid, NY). Rat monoclonal anti-TCP-1α (anti-RACK2) and horseradish peroxidase-conjugated rabbit anti-rat IgG were obtained from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Protein A/G PLUS-agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL)-detecting reagents were obtained from Amersham Biosciences. Reagents for SDS-PAGE were from Bio-Rad Laboratories. All other reagents were from Sigma.

Isolation of Adult Rabbit Cardiac Myocytes—Adult rabbit cardiac myocytes were isolated using a modification of the method of Haddad et al. (34) using collagenase type II digestion. This method yielded 80–85% rod-shaped cardiac myocytes, generating an average total of 4–6 × 10⁶ cells per rabbit heart. This method has been employed previously to study PKCe-induced activation of nitogen-activated protein kinases in rabbit cardiomyocytes (35). In brief, isolated myocytes were plated onto laminin-coated 100-mm dishes at subconfluence (2 × 10⁶ cells/dish) and cultured overnight at 37 °C in M199 medium with 2% fetal bovine serum, penicillin, and streptomycin. The medium was replaced with serum-free M199 medium supplemented with taurine (5 mM), creatine (5 mM), and carnitine (5 mM), and the cells were cultured under serum-starved conditions for 3 h prior to the performance of the treatments with a nitric oxide donor.

Experimental Protocol—Cardiomyocytes obtained from the same rabbit were divided into the following groups: a control group, in which only the vehicle (Me₂SO) was added to the medium; three groups in which the cells were incubated for 40 min with different concentrations of the NO donor SNAP obtained by diluting the stock solution of SNAP in the culture medium (2, 20, or 100 μM final concentrations of SNAP); a group in which the cells were pretreated for 10 min with the NO scavenger oxyhemoglobin at a 50 μM concentration before the treatment with 20 μM SNAP; and a group in which the peroxynitrite scavenger Ebselen was added at a concentration of 2 mM 10 min prior to the treatment with 20 μM SNAP. The SNAP stock solution was prepared freshly by dissolving it in Me₂SO prior to each experiment. After the treatment, the medium was removed, the cells were rinsed twice with ice-cold PBS, scraped off the bottoms of the dishes, and frozen at −80 °C.

Cell Sample Preparation—The cell samples were processed for the assessment of protein expression and phosphorylation activity of PKCe. The myocytes were resuspended and homogenized by glass-glass homogenization in sample buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 50 μg/ml phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A). The total cellular proteins in the homogenates were subjected to centrifugation at 45,000 × g for 40 min; the supernatant (cytosolic fractions) were removed and proteins were suspended in the above sample buffer (particulate fractions). The protein concentrations in the cytosolic and particulate fractions of the samples were determined using the method of Bradford.

Western Immunoblotting Analysis—The subcellular distribution and translocation of PKCe were assessed by standard Western immunoblotting techniques as previously described (15). Briefly, 100 μg of proteins RACK2 antibody and the particulate fraction of each cell sample were subjected to SDS-PAGE on a 10% denaturing gel and were electroblotted onto nitrocellulose membrane. Gel transfer efficiency and equal loading of protein were monitored by Ponceau staining of the nitrocellulose membranes. Background blocking was performed by incubating the membranes with 5% nonfat milk in Tris-buffered saline. Monoclonal antibodies against PKCe isoform 6 were used to assess its subcellular distribution and translocation. Monoclonal anti-RACK2 antibodies were used to detect protein tyrosine nitration. Monoclonal anti-TCP-1α antibodies were used to determine RACK2 protein expression. The protein signal was visualized using standard ECL methods.

Immunoprecipitations—To carry out immunoprecipitations, 5 μg of anti-PKCε antibodies were incubated with 50 μl of protein A/G-agarose beads for 40 min at 4 °C as described previously (36). The anti-PKCε antibodies were substituted with IgG in controls. The protein A/G-agarose-purified anti-PKCε complex was washed three times with phosphate-buffered saline containing 0.1% Triton X-100 and incubated with 1000 μg of proteins from the particulate fractions of the cell samples overnight at 4 °C, after which the beads were washed three times with PBS containing 0.1% Triton X-100. The immunoprecipitates were subsequently subjected to Western immunoblotting using either anti-nitrotyrosine antibodies (to detect tyrosine nitration of PKCe protein), anti-nitrotyrosine antibodies (to assess its co-immunoprecipitation with PKCe), or anti-PKCε antibodies.

Measurement of PKCe Isoform-selective Phosphorylation Activity—The phosphorylation activity of PKCe was determined using a previously employed method (15, 21). Briefly, 50 μg of proteins from the particulate fraction were immunoprecipitated overnight with PKCe monoclonal antibodies. Subsequently, the immunoprecipitates were subjected to SDS-PAGE followed by a phosphorylation assay using a PKCe-selective peptide substrate (ERMPPRKQGVSRRV). ELISA for the Assessment of PKCe-RACK Interactions—Recombinant PKCe and RACK2 proteins were generated as described previously (36, 37), and the interactions of PKCe and RACK2 were determined. For the PKCe-RACK2 in vitro binding assay, 96-well flat-bottomed ELISA plates were coated with either 10, 100, 500, 5, 10, or 50 ng of recombinant purified RACK2 dissolved in PBS (50 μl/well) and incubated overnight at 4 °C. The wells were washed three times with PBS and blocked with 200 μl of 4% bovine serum albumin (w/v) blocking buffer for 2 h at 37 °C to minimize any nonspecific binding. After washing the ELISA plates three times with PBS, 40 ng of recombinant purified PKCe was added to each well and incubated for 2 h at 37 °C (final volume, 50 μl per well). PKCe protein had either been left unpretreated (control), pretreated with 1 μM concentration of the NO donor SIN-1 in 15 mM HEPES buffer (pH 7.4) for 30 min (SIN-1-treated), or pretreated with 60 μM l-glutathidylserine and 100 mM phosphoramidate (PS plus PMA-treated) for 30 min at room temperature. After allowing binding of PKCe to RACK2, the wells were washed three times with PBS, and 50 μl of anti-PKCe primary antibody (1:400 in bovine serum albumin blocking buffer) were added to each treated (control) and PS plus PMA-treated (PBST) wells. The PBST was used to wash the plates three times to remove any weakly, nonspecifically bound proteins, after which 50 ng of horseradish peroxidase-conjugated anti-mouse antibodies (1:3500) were added to each well. The plates were again incubated for 1 h at 37 °C and then washed 3 times with PBST. The bound antibodies were detected by incubating the reaction wells with TMB detection reagent for 30 min at room temperature and, for a greater sensitivity, subsequent acidifying using
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50 μl of 2 M H₂SO₄. The optical density was measured on an ELISA plate reader at a single wavelength of 450 nm.

In Vivo Rabbit Model of NO-induced Cardioprotection—To explore the functional significance of PKCe nitration, we used a well established conscious rabbit model of NO-induced cardioprotection as described previously (15, 38, 39). Briefly, male New Zealand White rabbits (Myle’s Rabbitry Inc., Thompson Station, TN; 2.0–2.5 kg, age 3–4 months) were given the NO donor diethylenetriamine/NO (DETA/NO, 0.1 mg/kg intravenously every 25 min for 75 min; total dose of 0.4 mg/kg). This dose of DETA/NO has been shown to trigger rapid activation by DETA/NO was previously demonstrated (15, 21).

Statistical Analysis—All data are reported as means ± S.E. Differences between groups were analyzed using Student’s t test for unpaired data. A p value of <0.05 was considered significant.

RESULTS

Effect of the NO Donor SNAP on the Subcellular Distribution of PKCe—We have previously reported that NO released by NO donors induces preconditioning in rabbit hearts by activating PKCe in an isozyme-specific manner (15, 21). To assess the effects of NO donors on PKCe in isolated adult rabbit cardiac myocytes, we treated the cells with SNAP, an NO donor that has been shown to afford cardioprotection in rabbits (15, 38, 39). Cardiac myocytes were incubated with one of the three increasing concentrations of SNAP (2, 20, and 100 μM) for 40 min, and the changes in the PKCe expression in the particulate fraction were subsequently determined. SNAP treatment induced a significant, concentration-dependent, translocation of PKCe from the cytosolic to the particulate fraction of the cells (Fig. 1). This effect of SNAP was similar to that induced by PMA (data not shown). Pretreatment of the cells with the NO scavenger oxyhemoglobin at a concentration of 50 μM prevented the SNAP-induced translocation of PKCe, demonstrating that this effect of SNAP was due to NO release. Similarly, pretreatment of the cells with the peroxynitrite scavenger Ebselen at a concentration of 2 μM for 10 min prior to SNAP application attenuated the translocation of ε protein, implicating a role of a secondary peroxynitrite formation in mediating the effects of SNAP on PKCe.

Effects of SNAP on PKCe Isoform-selective Phosphorylation Activity—We next examined whether SNAP treatment of cardiomyocytes led to changes in the kinase activity of particulate PKCe parallel to the changes in its subcellular distribution. The ability of PKCe to phosphorylate its specific peptide substrate was measured in the particulate fractions of samples treated with different concentrations of SNAP. The results were compared with PKCe activity in Mn²⁺SO₄-treated control samples and samples that were pretreated with oxyhemoglobin or Ebselen. We found that SNAP induced a dose-dependent increase in the phosphorylation activity of PKCs in the particulate fraction (Fig. 2). Pretreating cardiac myocytes with either oxyhemoglobin or Ebselen before treating them with 20 μM SNAP attenuated the SNAP-induced activation of PKCe, suggesting that this effect was dependent on the release of NO and on the secondary formation of ONOO⁻.

SNAP-induced Tyrosine Nitration of PKCe in Cardiac Myocytes—We then proceeded to test whether NO produced during SNAP treatment of myocytes induced a posttranslational modification of PKCe on tyrosine residues. We postulated that the activation of PKCe by SNAP, as manifested by both increased particulate PKCe expression and increased PKCe phosphorylation activity, might be associated with the secondary production of ONOO⁻, which has been shown to induce nitration of tyrosine residues on proteins (23, 25). Therefore, formation of 3-nitrotyrosines on PKCe in response to treatment with SNAP was evaluated by immunoprecipitating PKCe from the partic-
ultracentrifugation and then immunoblotting the precipitates with anti-nitrotyrosine monoclonal antibodies. A 20 μM concentration of SNAP, which significantly activated PKCe (Fig. 2), induced a greater than 3.3-fold increase in the amount of tyrosine nitration on this PKC isofrom compared with controls (Fig. 3). Scavenging of NO by oxyhemoglobin or ONOO⁻ by Ebselen markedly attenuated the effect of SNAP. These data support the concept that SNAP-induced nitration of PKCe on its tyrosine residues was mediated by release of NO and secondary formation of ONOO⁻.

SNAP-induced Tyrosine Nitration of PKCe in the Rabbit Model of NO-induced Cardioprotection in Vivo—To determine whether NO induces tyrosine nitration of PKCe in vivo, we examined tissue samples from hearts treated with DETA/NO and control hearts treated with the vehicle. The dose of DETA/NO was previously documented to induce activation of PKCe and protection against myocardial infarction (15, 39). 1000 μg of proteins from the particulate fractions were used to carry out immunoprecipitations utilizing anti-PKCe antibodies. We observed a significant increase in the amount of tyrosine nitration on PKCe in hearts treated with DETA/NO when compared with the controls (Fig. 4). These data demonstrate that tyrosine nitration of PKCe occurs in vivo after administration of NO donors at doses that had been shown previously to induce preconditioning by activating PKCe.

SNAP Enhances PKCe-RACK2 Interactions—The goal of the next set of experiments was to assess whether SNAP modulated the ability of PKCe to interact with its selective anchoring protein, β'-COP (RACK2). When cardiac myocytes were incubated with 20 μM SNAP, the amount of RACK2 that co-immunoprecipitated with PKCe-specific antibodies increased significantly (Fig. 5). This indicates that binding of PKCe to its selective RACK increased upon treatment with exogenous NO. To investigate whether the increase in PKCe-RACK2 interactions occurred via secondary ONOO⁻ formation, we preincubated the cells with 2 μM Ebselen prior to the application of SNAP. Scavenging this strong nitrating agent prevented the increase in the binding of PKCe to RACK2 (Fig. 5).

Lack of Effect of SNAP on RACK2—When cardiomyocytes were incubated with increasing concentrations of the NO donor SNAP, no change was observed in the subcellular distribution of RACK2 when compared with untreated controls. In control samples, 79 ± 3% of total RACK2 was present in the particulate fraction. In cardiomyocytes treated with 2, 20, and 100 μM SNAP, the amounts of RACK2 in the particulate fractions were 81 ± 1, 77 ± 4, and 80 ± 3% of total RACK2 expression, respectively. Furthermore, we did not detect nitrotyrosine signals on RACK2 in NO donor-treated cells (data not shown).

Assessment of PKCe-RACK2 Interactions by ELISA—To investigate the physical interactions between PKCe and RACK2, we generated recombinant PKCe protein using a baculovirus expression system (37) and recombinant RACK2 protein using an expression vector (a gift from Dr. Daria Mochly-Rosen). The interaction between PKCe and RACK2 was characterized using an ELISA assay. 96-well ELISA plates were precoated with increasing amounts of RACK2 protein (10, 100, 500, 5, 10, or 50 ng). PKCe recombinant protein was either untreated (control), pretreated with 2 μM ONOO⁻ donor SIN-1 (SIN-1-treated) for 30 min, or preincubated with phosphatidylycerine, at a concentration 60 μg/ml, and phorbol myristate acetate, at 100 nM concentration (PS plus PMA)-treated) for 30 min. 40 ng of either untreated, SIN-1-, or (PS plus PMA)-treated PKCe was added to each well (total volume, 50 μl). The plates were incubated for 2 h, allowing sufficient binding between PKCe and RACK2 recombinant proteins. As shown in Fig. 6, the amount of PKCe bound to RACK2 increased with RACK2 in a dose-dependent fashion. Pretreating the recombinant PKCe proteins with the ONOO⁻ donor SIN-1 significantly enhanced PKCe interactions with RACK2, shifting the dose-response curve to the left at the higher concentrations (≥10 ng). SIN-1-treated PKCe also exhibited increased maximal binding to RACK2 (Fig. 6). This latter effect of the ONOO⁻ donor was comparable with that of the known activators of PKC, PS, and PMA (Fig. 6). These data demonstrate that ONOO⁻ activates PKCe directly, that is, in the absence of other molecules that are present in the cellular environment.
DISCUSSION

Although NO-induced activation of PKCe has been well documented, the cellular and molecular mechanisms mediating this event remain virtually unknown. We hereby present evidence to demonstrate that NO may activate PKCe via posttranslational modification (nitration of tyrosine residues) of this isozyme, a phenomenon observed both in isolated cardiac cells in vitro and in a model of NO-induced cardiac protection in vivo. We also show that nitration of PKCe enhances its interaction with the selective anchoring protein RACK2, an event that is critical for PKCe translocation and activation. To the best of our knowledge, this is the first study delineating a direct molecular modification of PKC by NO in the heart. Given the ubiquitous role of NO and PKC in the regulation of cardiac function, these findings may have significant implications for a variety of NO- and PKC-mediated biological processes in the cardiovascular system.

Cardiac Signaling Mechanisms Underlying NO-induced Activation of PKC—The effect of NO on the activity of PKC isozymes is controversial. The specific effects of NO on its biological molecular targets appear to be dependent upon the species, model, cell types, and the concentration of NO achieved (22). In the heart, NO donors given at doses that produce a cardiac protective effect have been shown to activate the ε isozyme of PKC (15, 21). However, the mechanisms of this event have not been characterized. Covalent posttranslational modification, namely phosphorylation, of PKC along with binding of PKC to the lipid second messenger diacylglycerol are recognized as the two equally important mechanisms that regulate PKC activity (1, 40). The recently discovered 3-phosphoinositide-dependent kinase (PDK)-1 has been demonstrated to induce phosphorylation of the activation loop in conventional, novel, and atypical PKCs (41–43). Moreover, posttranslational modification of tyrosine residues by phosphorylation has emerged as an important mechanism modulating PKC activity (26–28).

Our study shows that NO released by the NO donor SNAP activates PKCe in cultured myocytes in a dose-dependent fashion. PKCe activation was manifested by both translocation of this isozyme from the cytosolic to the particulate fraction and by its increased phosphorylation activity in the particulate fraction (39). Furthermore, pretreatment with the ONOO⁻ scavenger Ebselen prevented this effect of SNAP (lane 4). No significant changes in the amount of PKCe in the immunoprecipitates were detected. B, histogram depicting the amount of RACK2 bound to PKCe as percent of control.

FIG. 5. Co-immunoprecipitation of RACK2 and PKCe. A, PKCe-RACK2 interactions were assessed by immunoprecipitating PKCe and subsequent probing of the precipitates with anti-RACK2 antibody, as described under "Experimental Procedures." Cardiac myocytes treated with SNAP exhibited enhanced PKCe-RACK2 protein-protein interactions in the particulate fraction (lane 3) when compared with control (lane 2). Pretreatment with the ONOO⁻ scavenger Ebselen prevented this effect of SNAP (lane 4). No significant changes in the amount of PKCe in the immunoprecipitates were detected. B, histogram depicting the amount of RACK2 bound to PKCe as percent of control.
effect of SNAP on PKCs was due to NO production because scavenging of NO with oxyhemoglobin prevented PKC activation. In addition to the release of NO by SNAP, secondary production of ONOO⁻ was shown to be important because Ebselen, an ONOO⁻ scavenger, attenuated the activation of ε protein by SNAP.

**Nitration as a Mechanism for NO-dependent Modulation of Protein Function**—Both exogenously and endogenously produced NO have been shown to nitrate tyrosine residues on a number of proteins (25). Such covalent posttranslational modification modulates protein functions. NO’ is a relatively stable free radical molecule that is characterized by high membrane diffusibility and ability to react with biochemical molecules, which confers to NO an important role in cell signaling. Various physiological and pathophysiological actions of NO or NO-generated species depend on the NO concentration and local redox state and include reactions with heme-containing proteins, iron-sulfur clusters, lipids, DNA bases, oxygen, superoxide, water, and nitrosation of thiols and amines (44). One of the characteristic and more irreversible reactions of NO is nitration of DNA nucleotides, lipids, and aromatic amino acids. The aromatic amino acid tyrosine seems to be especially susceptible to nitration. Consequently, formation of 3-nitrotyrosines as a result of the covalent modification of tyrosines by either endogenous or exogenous NO and NO-derived species, ONOO⁻ results in the nitration of DNA nucleotides, lipids, and aromatic amino acids. The characteristic and more irreversible reactions of NO is nitration of DNA nucleotides, lipids, and aromatic amino acids. The aromatic amino acid tyrosine seems to be especially susceptible to nitration. Consequently, formation of 3-nitrotyrosines as a result of the covalent modification of tyrosines by either endogenous or exogenous NO and NO-derived species, ONOO⁻ in particular, has received much attention.

Nitration of tyrosine residues is considered to be a stable (or even irreversible) posttranslational modification of proteins as opposed to another cellular mechanism of NO action, S-nitrosylation of protein cysteine residues. Tyrosine nitration of proteins is also a selective process with respect to both specific proteins and specific tyrosine residues that can undergo such modification (24). Importantly, tyrosine nitration has been demonstrated to be capable of altering protein functions both in vivo and in vitro, thus modulating protein functions. For example, nitration of manganese superoxide dismutase leads to its enzymatic inactivation in human renal allografts (45). ONOO⁻ dependent formation of 3-nitrotyrosines on surfactant protein A decreases the ability of this protein to aggregate lipids (46). Likewise, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase) in macrophages has been found to be nitrated on tyrosine residues by both ONOO⁻ and NO donors, and this nitration has been implicated in abrogating the interaction of different subunits of PI3-kinase (47). ONOO⁻ has been reported to induce tyrosine nitration and differential activation of the mitogen-activated protein kinases p38, JNK1/2, and ERK1/2 (48) and Src tyrosine kinases (49, 50).

In this study, we present evidence that nitration of PKCε contributes to the activating effect of NO on PKCε. Treatment of cardiac myocytes with NO donors resulted in the detection of nitrotyrosine residues on myocardial PKCε concomitant with its activation. The nitration effect appeared to be selective, as the tyrosine-bearing protein RACK2 did not exhibit any detectable nitrotyrosine signal. Both formation of nitrotyrosines on PKCε and the activation of the enzyme could be prevented by pretreating the cells with Ebselen, supporting the role of ONOO⁻ as a major nitrating agent. These data indicate that generation of ONOO⁻ secondary to SNAP treatment and subsequent nitration of tyrosine residues on PKCε may play a pivotal role in mediating the activation of this novel PKC isoform by exogenous NO.

**Activation and Translocation of PKCε via Its Interaction with RACK**—Isozyme-specific subcellular localization of each individual PKC isoform is important for the specific activity and, therefore, functions of each member of the PKC family of enzymes. A plausible theory has been put forth stating that isozyme-selective anchoring proteins, or receptor proteins, target activated PKC isofoms to their specific subcellular locations in close proximity to their corresponding substrates, thus allowing substrate phosphorylation to occur (51). Specifically, RACK2 was identified as a selective anchoring protein for activated PKCε (52). Disruption of RACK2 interactions with PKCε inhibits norepinephrine- or PMA-induced negative chronotropic effects (33) and abrogates PKC-mediated protection against ischemic injury in several species (6, 8, 9). These findings stress the importance of PKCε-RACK2 protein-protein interactions for mediating various cardiac functions that involve the ε isoform of PKC.

We found that SNAP treatment resulted in increased binding of the activated PKCε to its selective anchoring protein RACK2 in the particulate fraction of cardiomyocytes. The increase in PKCε-RACK2 interaction was mediated by generation of ONOO⁻ because it was attenuated by Ebselen. Because SNAP treatment did not result in increased expression of RACK2 in the particulate fraction, the enhancement of PKCε-RACK2 binding cannot be ascribed to increased availability of the anchoring protein for PKCε. Importantly, SIN-1 promoted the interaction of isolated recombinant PKCε and SIN-1, demonstrating that ONOO⁻ can activate PKCε directly, in the absence of other cellular components. Taken together, these results support our hypothesis that exogenous NO induces nitration of PKCε via the production of ONOO⁻ and that this event plays an important role in mediating the increase in active PKCε-RACK2 binding.

**Conclusions**—Regulation of kinase activity via posttranslational modification has been recently recognized as an important means for facilitating signal transduction in a variety of biological systems. Consistent with this concept, our results show that nitration of PKCε protein by NO donors modulates its interaction with the receptor binding protein RACK2, thereby promoting particulate translocation of PKCε and activating the PKCε signaling pathway. The findings reported herein delineate a novel signaling mechanism by which NO activates PKC ε isozymes and illustrate an example of how a cascade of molecular reactions in signal transduction can be initiated by chemical modifications of an individual element.

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