Nitric Oxide and Nitric Oxide-generating Agents Induce a Reversible Inactivation of Protein Kinase C Activity and Phorbol Ester Binding*

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Since S-nitrosylation of protein thiols is one of the cellular regulatory mechanisms induced by nitric oxide (NO), and since protein kinase C (PKC) has critical thiol residues which influence its kinase activity, we have determined whether NO could regulate this enzyme. Initial studies were carried out with purified PKC and the NO-generating agent S-nitrosocysteine. This agent decreased phosphotransferase activity of PKC in a Ca²⁺- and oxygen-dependent manner with an IC₅₀ of 75 μM. Phorbol ester binding was affected partially only at higher concentrations (>100 μM) of S-nitrosocysteine. This inactivation of PKC was blocked by the NO scavenger oxyhemoglobin or reversed by dithiothreitol. It is likely that NO initially induced an S-nitrosylation of vicinal thiols, which were then oxidized to form an intramolecular disulfide. Other NO-generating agents such as S-nitroso-N-acetylpenicillamine and sodium nitroprusside, as well as authentic NO gas, induced similar types of PKC modifications. In intact B16 melanoma cells treated with S-nitrosocysteine a rapid decrease in PKC activity in both cytosol and membrane was observed. Unlike in experiments with purified PKC, in intact cells treated with S-nitrosocysteine the phorbol ester binding also decreased to a rate equal to that of PKC activity. These modifications were readily reversed by treating the homogenates with dithiothreitol in test tubes or by removing the NO-generating source from intact cells. To determine whether the limited amounts of NO generated within the intact cells could induce this type of PKC modification, the macrophage cell line IC-21 was treated with lipopolysaccharide and Ca²⁺ ionophore A23187 to induce the NO production. With an increase in generation of NO (3-12 h period) in these cells, a parallel and irreversible decrease in PKC activity and phorbol ester binding was observed. A specific inhibitor for NO synthase, N⁵⁰-monomethyl-L-arginine, inhibited both the production of NO and PKC inactivation. In experiments using purified enzyme or intact cells there was no decrease in cAMP-dependent protein kinase activity. Conceivably, NO production for limited time induces a reversible inactivation of PKC due to the formation of a disulfide bridge(s), whereas the chronic production of NO could induce irreversible inactivation of PKC. The reversible or irreversible inactivations of PKC may in part influence NO-mediated cytoprotective or cytotoxic actions, respectively.

Nitric oxide (NO) is emerging as an important cellular regulator (1-3). It has been shown to play roles in diverse physiological processes such as vasodilation, inhibition of platelet aggregation, and neurotransmission (1-3). Moreover, high amounts of NO generated by activated macrophages and neutrophils in response to cytokines may induce tumoricidal and bactericidal actions (4-6). The important actions of NO include the activation of soluble guanylate cyclase by its interaction with heme (7). Other hemoproteins such as cytochrome P-450 or nonheme iron-centered proteins like actinase are also influenced by NO (8, 9). Furthermore, S-nitrosylation of protein thiols by NO may represent an important cellular regulatory mechanism (10-12). If two vicinal thiols in the protein are modified to S-nitrosothiols, these may oxidize to form more stable disulfides as shown for the N-methyl-L-aspartate receptor (12). S-Nitrosylated proteins may exhibit new functions as shown for the tissue-type plasminogen activator (14). Alternatively, protein S-nitrosylation could lead to increased ADP-ribosylation as demonstrated for glyceraldehyde-3-phosphate dehydrogenase and actin (15-17).

Protein kinase C (PKC), in addition to its activation by the second messengers Ca²⁺, diacylglycerol, and arachidonic acid (18-20), may also be activated by oxidative modification induced by oxidants (21-25). A selective oxidative modification of the regulatory domain results in a Ca²⁺-lipid-independent activation of the kinase with a loss of phorbol ester binding (21, 22). Alternatively, a selective oxidative modification of the kinase domain results in the generation of a modified form of PKC that exhibits only phorbol ester binding (22). Some oxidants such as H₂O₂ induce an irreversible oxidative modification (21), whereas others such as m-periodate induce a reversible modification involving the oxidation of vicinal thiols to form disulfides (22). The CI constant region present within the PKC regulatory domain contains a tandem repeat of a cysteine-rich sequence, which may be susceptible to thiol modifying agents (26). Therefore, it is possible that PKC may be regulated by S-nitrosylation at this cysteine-rich region.

Since NO can produce protective or regulatory functions in the cell at a low concentration while toxic effects at higher concentrations, its synthesis may be tightly regulated in the cell. PKC has been shown to be involved in the induction of NO synthase, an enzyme involved in the synthesis of NO from...
L-arginine (27, 28). Moreover, PKC may also down-regulate the activity of NO synthase by direct phosphorylation of the enzyme (29). NO has been shown to inhibit NO synthase activity directly and phorbol 12,13-dibutyrate (PDBu) binding of PKC is irreversibly inactivated by NO generated in situ in macrophage cell lines stimulated with lipopolysaccharide and Ca2+-ionophore A23187. These results suggest that PKC may be one of the susceptible targets for NO action.

**EXPERIMENTAL PROCEDURES**

**Materials**—The SV40-immortalized mouse macrophage cell line IC-21 was obtained from American Type Culture Collection. S-Nitroso-cysteine and S-nitroso-N-acetylpenicillamine were prepared as described before (12). A saturated solution of NO (1 m) was prepared by passing NO gas (Aldrich) under a nitrogen atmosphere just before use (30). PKC from rabbit brain was purified to apparent homogeneity as described previously (21). In some experiments partially purified PKC (approximately 600 units/mg protein) was used. M-kinase was derived from native PKC by limited proteolysis using calpain II as described previously (21).

**PKC Treatment with NO-generating Agents**—Since the NO-mediated modification of PKC requires incubation in the presence of Ca2+, it is important to have PKC preparations free from contamination by calpain or other proteases, which can proteolyze PKC when it is exposed to Ca2+. Although PKC at various stages of purification exhibited a sensitivity to NO, PKC in freshly purified preparations was completely inhibited by NO. Purified PKC (1.5–5 units) was incubated with indicated concentrations of Ca2+ and NO-generating agents in a total volume of 0.4 ml at room temperature for 5 min. Then 0.1 ml of 50 mM EDTA solution containing 1 mg/ml BSA was added, and the treated samples were subjected to a “centrifuge column technique” to rapidly remove the low molecular weight compounds (31).

**Isolation of PKC from Cells Treated with NO-generating Agents**—The cytosolic and detergent-solubilized membrane fractions were prepared from cells (32). These fractions (2.5 ml) were applied to 0.5 ml DEAE-cellulose (DE-52) columns previously equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM DTT). After washing the column with 5 ml of buffer A, the bound PKC was eluted with 1.25 ml of 0.1 M NaCl in buffer A (32).

**PKC Assay**—The assays of PKC as well as cAMP-dependent protein kinase were carried out in 96-well plates with fitted filtration discs made of Durapore membranes (32). The unique aspect of this method was that both incubations and filtrations were carried out in the same well. Briefly, PKC reaction samples containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.33 mM CaCl2, 0.1 mM ATP, histone H1 (0.1 mg/ml), 0.04 mM leupeptin, and 25 pM of PKC sample in a total volume of 125 μl were incubated in the wells of a multiwell filtration plate at 30 °C for 5 min. Histone H1 was precipitated and filtered with 25% trichloroacetic acid. The filters were punched into scintillation vials, and the radioactivity associated with filters was counted. PKC activity was expressed as units, where 1 unit of enzyme transfers 1 nmol of phosphate to histone H1/min at 30 °C.

**Phorbol Ester Binding**—[3H]PDBu was used as a ligand for the determination of phorbol ester binding using the multiwell filtration approach (32). For optimal PDBu binding to purified PKC, the conditions discussed in method 1 as described previously were used (32). Briefly, 25 μl of purified PKC was incubated with 20 mM Tris-HCl, pH 7.5, 0.6 mM CaCl2, 0.15 μM leupeptin, 0.66 μM pepstatin A, 20 nM [3H]PDBu (20 Ci/mmol; 200,000 dpm), 0.1 mg/ml BSA, 0.1 mg/ml bovine γ-globulin in the wells of the 96-well filter plate with fitted Durapore membrane discs at 30 °C for 10 min. The ligand bound PKC was adsorbed to DEAE-Sephadex beads, and the beads then were filtered and washed in the same multwells with 20 mM Tris-HCl, pH 7.5. The radioactivity associated with the DEAE-Sephadex beads retained on the filter was counted. To determine the optimal PDBu binding to PKC fraction isolated by DEAE-cellulose chromatography from the crude cell extracts, the conditions standardized with the cytosolic receptor (method 2) were used (32). The major differences in method 2 compared to method 1 were that the concentration of phosphatidylserine was increased by

**RESULTS**

**Inactivation of Purified PKC by S-Nitroso-cysteine**—To determine whether NO can directly modify PKC, the purified enzyme was initially treated with S-nitroso-cysteine, a NO-generating agent; after removing this compound by a centrifuge column technique, the modification of PKC was evaluated by measuring both histone phosphotransferase activity and PKC binding. When PKC was treated with S-nitroso-cysteine (200 μM) in the presence of 1 mM EDTA, there was a significant decrease in either kinase activity or PKC binding (Fig. 1). On the contrary, when the same treatment was carried out in the presence of Ca2+ (>100 μM), there was a substantial decrease in PKC activity. Nevertheless, PKC binding was affected by only 30% at higher concentration of Ca2+ (1 mM). The optimal concentration of Ca2+ required was in the range of 1 mM in absence of lipids (Fig. 1). However, in the presence of phosphatidylserine and diolene, the Ca2+ required for the S-nitroso-cysteine-induced inactivation decreased to as low as 1 μM. However, the lipids blocked inactivation of PKC binding occurring at higher concentrations of S-nitroso-cysteine. Using the optimal concentration of Ca2+ required (in the absence of lipids), the IC50 value for S-nitroso-cysteine inactivation of PKC activity was found to be 75 μM (Fig. 2).

PKC activity did not recover by subjecting the NO-treated PKC to extensive dialysis, to hydrophobic chromatography, or to DEAE-cellulose chromatography in the absence of thiol agents. Conceivably, NO induced a stable modification in PKC.

**5 When freshly (less than 1 week) purified PKC was used for treatment with S-nitroso-cysteine (10 to 100 μM) in the presence of 1 mM Ca2+, it resulted in a 20–40% increase in the PDBu binding. In contrast, using a stored (more than 10 days) preparation of PKC resulted in only a 0–10% increase in PDBu binding under these conditions.
Then the PKC activity and PDBu binding were determined in both S-nitrosocysteine solution stored at room temperature for 48 h aliquots. The absence of lipids using the required high amount of Ca^{2+} incubation was initiated by adding various concentrations of S-nitroso-cysteine, and then after incubation the excess S-nitrosocysteine was removed by using a centrifuge column technique. The desalted PKC sample was divided into two aliquots. To one aliquot, DTT was added to a 2 mM (to deplete NO) did not affect PKC activity. The scavenger of NO, oxyhemoglobin (300 pM) indeed induced PKC modification.

To gain insight into the mechanism of S-nitrosocysteine-mediated PKC inactivation, PKC inactivated by treatment with S-nitrosocysteine, after removing the excess S-nitrosocysteine, was treated with 2 mM DTT for 15 min at 4 °C. As shown in Fig. 2, PKC modified with lower concentrations of S-nitrosocysteine significantly recovered by incubation with DTT. The ready reversibility of the S-nitrosocysteine-induced modification of PKC suggests that this modification may involve disulfide bridge formation. Although other oxidatively modified amino acid residues such as methionine sulfoxides can be reduced by DTT, this requires treatment with substantially higher (20 mM) concentrations of DTT at higher temperatures (100 °C) for prolonged periods of time. At higher concentrations (200 μM) of S-nitrosocysteine used, inactivation of either kinase activity or PDBu binding was not completely reversed with DTT even after prolonged (24 h) incubation with DTT.

To further determine whether the formation of a disulfide bridge(s) is required to induce the inactivation of PKC, the enzyme modification was carried out in an anaerobic (low oxygen) atmosphere. Under a nitrogen atmosphere, treatment with S-nitrosocysteine (75 μM) showed no appreciable decrease in PKC activity (Fig. 3). This suggested that S-nitrosylation of thiols, which can also occur in an oxygen-free atmosphere, was not sufficient to induce inactivation of PKC. Conceivably, initial S-nitrosylation of vicinal thiol residues in the Ca^{2+}-induced site in PKC may lead to oxidation to form an intramolecular disulfide bridge within this region, resulting in inactivation of the enzyme. This type of S-nitrosylation-induced disulfide bridge formation and inactivation has been recently reported for N-methyl-D-aspartate receptors in neurons (12). At intermediate concentrations (200 μM) of S-nitrosocysteine tested, PKC was inactivated in both oxygen and nitrogen atmospheres. However, PKC modified in only aerobic conditions (an oxygen atmosphere) was regenerated with DTT (Fig. 3A), whereas PKC inactivated in the nitrogen atmosphere failed to regenerate (Fig. 3B). It is possible that in an oxygen atmosphere initial formation of a disulfide bridge(s) made PKC refractory to further modification by NO, thus preventing subsequent irreversible modification of the enzyme. In a nitrogen atmosphere, alternatively, inhibiting the formation of a disulfide bridge allowed the NO-mediated irreversible modification of PKC at this region. At higher concentrations (1 mM), however, NO induced inactivation of PKC in both oxygen and nitrogen atmospheres, which was not appreciably reversed with DTT.

Calpain-digested PKC (M-kinase) was also inactivated by S-nitrosocysteine with an IC_{50} value of 50 μM. Ligands such as ATP, Mg^{2+}, and histone H1 either alone or in combination did not protect either PKC or M-kinase from S-nitrosocysteine-induced inactivation. Furthermore, the direct addition of S-nitrosocysteine to the PKC assay mixture also inhibited PKC activity suggesting that all the components present in the PKC reaction mixture did not decrease the NO-mediated inhibition of kinase activity. Histone H1 phosphotransferase activity of cAMP-dependent protein kinase was unaffected by S-nitrosocysteine even at the highest concentration (1 mM) tested.

Inactivation of PKC by Other NO-generating Agents and NO Gas—Other NO-generating agents such as S-nitroso-N-acetyl-

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The removal of excess S-nitrosocysteine was important before adding DTT to reverse PKC modification, which otherwise resulted in a reaction of S-nitrosocysteine with DTT. The resulting S-nitrosylated thiol agent induced further modification of PKC.

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**Table 1**

| Agent | PKC activity (units) |
|-------|----------------------|
|       | Without DTT | With DTT |
| Control, exposed for 5 min | 1.74 | 1.83 |
| Control, exposed for 20 min | 1.61 | 1.69 |
| S-nitroso-N-acetylpenicillamine (100 μM) for 5 min | 0.69 | 1.76 |
| SNP (200 μM) alone for 20 min | 1.31 | 1.48 |
| SNP (200 μM) ± 1 mM DTT for 20 min | 0.86 | 0.98 |
| NO (100 μM) for 5 min | 0.41 | 1.23 |
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penicillamine also induced both reversible and irreversible modifications of PKC in a rapid manner similar to S-nitrosocysteine (Table I). Sodium nitroprusside (SNP)-mediated inactivation of PKC required longer (30 min) periods of treatment and was also less pronounced. Furthermore, the presence of 1 mM DTT during the SNP treatment enhanced the PKC inactivation. The reasons for the requirement of DTT in mediating the SNP-mediated modification of PKC was not clear. Spontaneous decomposition of SNP produces not only NO, but also Fe2+ and cyanide, each of which may react with thiol groups. A similar type of DTT requirement for SNP-mediated S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase has been recently reported (15). Furthermore, this SNP-modification of PKC was not reversed when the desulted modified enzyme was incubated with DTT. Authentic NO gas (100 μM) also induced the inactivation of PKC which was reversed by approximately 60% with 2 mM DTT. This further supports the theory that NO gas by itself may modify PKC.

Reversible Modification of PKC in Intact Cells Treated with NO-generating Agents—Whether NO can modify PKC in intact cells was evaluated in the B16 melanoma cell line (F10 subline). As shown in Fig. 4, when the S-nitrosocysteine-treated (5 min) cell extracts were processed for isolation and determination of PKC activity in the absence of DTT, there was a decrease in PKC activity both in the cytosol (65%) and in the membrane fraction (90%). Unlike in isolated intact PKC, S-nitrosocysteine treatment of intact cells decreased PDBu binding from the control value (0.92 pmol/mg protein) in proportion to a decrease in PKC activity (data not shown). Treatment of cell extracts with DTT, however, reversed this NO-induced inactivation of PKC activity (Fig. 4). There was no appreciable change in PKC activity in the cells when reduced hemoglobin (500 μM) was included in the medium during treatment with S-nitrosocysteine. Alternatively, cell treatment with an equal concentration of cysteine, cystine, or NO-depleted preparation of S-nitrosocysteine did not induce any changes in PKC activity. This suggested that NO generated from S-nitrosocysteine in fact induced the PKC modification. The activity of cAMP-dependent protein kinase did not decrease in these treated cells.

To understand whether the NO-induced modification of PKC occurring in intact cells could be reversed by any endogenous mechanism, further studies were carried out. Initially, the B16 melanoma cells were treated with S-nitrosocysteine for 5 min, and then the treated cells were washed and incubated in a fresh medium without any S-nitrosocysteine. To prevent new protein synthesis, cycloheximide (10 μg/ml) was included in the medium. In the washed cells, PKC activity in both the cytosol and membrane started to recover within 15 min after removal of the NO source, and it returned to the control untreated level within 45 min (Fig. 4). A similar type of reversible inactivation of PKC in intact cells was also noted with S-nitroso-N-acetylpenicillamine.

**Nitric Oxide-induced Inactivation of PKC in Macrophage Cell Line Stimulated with Lipopolysacharide—**To test whether the smaller concentrations of NO generated in situ could induce the inactivation of PKC, the SV40-immortalized mouse macrophage cell line IC-21 was stimulated with lipopolysacharide (LPS) (1 μg/ml) in the presence of Ca2+ ionophore A23187 (1 μM). This treatment within a few h increased the release of NO as measured as nitrite using Greiss reagent (Fig. 5A). A concomitant decrease in PKC activity was observed (Fig. 5B). The DTT treatment failed to regenerate the inactivated PKC activity. PDBu binding also proportionately decreased from the control value (0.78 pmol/mg protein). Since NO can inhibit protein synthesis (33), whether the decrease in PKC activity after the induction of NO synthesis was caused by the inhibition of PKC synthesis was evaluated. Protein synthesis inhibitor cyclohex-

**Fig. 4. Reversible inactivation of PKC in B16 melanoma cells treated with S-nitrosocysteine.** Confluent B16 melanoma cells (F10) were washed with Hank’s balanced salt solution (HBSS) and then treated for 5 min with 250 μM S-nitrosocysteine solutions diluted in HBSS just before use. During this treatment period, the medium was changed every minute with freshly diluted S-nitrosocysteine in HBSS. Then the treated cells were washed (as indicated with arrow) twice with HBSS and kept in minimum essential medium containing 10% fetal calf serum and cycloheximide (10 μg/ml). At various intervals of time, each set of cells was homogenized in a buffer without DTT and divided into two aliquots. To one aliquot DTT was added to a 2 mM final concentration. These samples containing DTT and no DTT were separately processed while maintaining this difference in DTT in all the buffers used for PKC isolation by DEAE-cellulose chromatography.

**Fig. 5. Irreversible inactivation of PKC in macrophage cell line (IC-21) stimulated with LPS and Ca2+ ionophore.** Confluent cells were treated with LPS (1 μg/ml) and Ca2+ ionophore A23187 (1 μM) in DMEM in the absence of NMA for various intervals of time. In another set of Petri dishes, the LPS/A23187 treatment was carried out in the presence of NO synthase-specific inhibitor NMA (1 mM) in either regular DMEM or DMEM containing excess L-arginine (4 mM). NO was determined as nitrite released into the medium. To 100 μl of medium 100 μl of Greiss reagent (47) was added, and after 10 min the absorbance was read at 550 nm using a 96-well microplate reader (Thermo Max, Molecular Devices). Total PKC (cytosol and membrane) was extracted from the cells with a buffer containing detergent and 1 mM DTT.
imide (10 μg/ml) caused only a 50% decrease in PKC activity in 24 h. Therefore, the rapid rate of decrease in PKC activity in LPS-treated cells during a 3–12-h period was not primarily due to a decrease in protein synthesis. Furthermore, the immunoreactive PKC was not decreased as measured by immunoblotting using antibodies that recognize all three forms of Ca²⁺-dependent isoenzymes (α, β, and γ) supporting the inactivation of PKC (data not shown). However, NO synthase-specific inhibitor N⁵-monomethyl-L-arginine (NMA), when added at 0.4 mM concentration in regular Dulbecco’s modified Eagle’s medium (DMEM), substantially decreased the rate of inactivation of PKC observed in LPS-stimulated cells. Nonetheless, NMA was less effective in preventing the rate of inactivation of PKC in cells treated in DMEM with excess L-arginine (4 mM). Under these conditions, the activity of cAMP-dependent protein kinase did not diminish.

**DISCUSSION**

While having less affect on the PDBu binding site, NO selectively inactivated the phosphotransferase activity of purified PKC. In this aspect the NO-induced modification in PKC differs from that of our previously reported observations with oxidants such as H2O2 and m-periodate, as well as agents like calphostin C, which affected both kinase activity and PDBu binding more or less equally (21, 22, 34). Furthermore, NO-induced modification of PKC is characterized by a lack of Ca²⁺/lipid-independent activation of the enzyme, whereas oxidants such as H2O2 and m-periodate induced oxidative activation of PKC under conditions that selectively modify the regulatory domain (21, 22). Nevertheless, PKC inactivation by NO resembled that of other oxidants in Ca²⁺ dependence (21, 22). The C1 constant region present within the regulatory domain contains a tandem repeat of a cysteine-rich sequence that is analogous to the “zinc-finger motif” found in some metalloproteins (35-38). This zinc-finger region is resembled that of other oxidants in Ca²⁺ dependence (21, 22). The formation of a disulfide bridge ($) after undergoing S-nitrosylation and oxidation susceptible site in the catalytic domain is exposed only after Ca²⁺ binding to PKC and is readily accessible in M-kinase without any requirement for cofactors (21). The NO-modified site in PKC or M-kinase was not protected by ATP and Mg²⁺. Thus, this site may be unique compared to the site modified by H2O2 and m-periodate, which was protected by ATP and Mg²⁺.

**Physiological Significance of PKC Inactivation by NO**—NO has been shown to induce in the inhibition of platelet aggregation, smooth muscle relaxation, and desensitization of the neurons to excess stimulation by glutamate (1–3). PKC may play an important role in platelet aggregation, in smooth muscle contraction, and in mediating the cellular actions in response to activation of glutamate receptors in neurons (43–45). Previous studies revealed that some of the protective effects of NO seem to be mediated by the elevation of cGMP, which can inhibit the phosphatidylinositol breakdown (43) and thereby indirectly inhibit the activation or translocation of PKC (46). Our current studies suggest that NO can also induce reversible inactivation of PKC activity by direct modification of the enzyme involving the formation of a disulfide bridge(s). This change in PKC may be readily repaired in <1 h by an intracellular mechanism. Mercapto compounds such as DTT and 2-mercaptoethanol are often added to buffers used in cell homogenization. The oxidative changes that occur in PKC by treatment with NO-generating agents may not be noticed if these reducing agents are present in the buffers. NO may produce cytotoxic actions especially when overproduced for a prolonged period of time by the induction of NO synthase in response to inflammation (4–6). Under these conditions PKC is permanently inactivated. Therefore, NO may induce, depending on the extent and duration of its production, either a reversible and temporary inactivation of PKC to desensitize the cell to second messengers, thereby eliciting protective effects or permanent inactivation of PKC, which may have cytotoxic effects.

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