Overexpression of Protein Kinase C Isoforms Protects RAW 264.7 Macrophages from Nitric Oxide-Induced Apoptosis: Involvement of c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase, p38 Kinase, and CPP-32 Protease Pathways

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Overexpression of Protein Kinase C Isoforms Protects RAW 264.7 Macrophages from Nitric Oxide-Induced Apoptosis: Involvement of c-Jun N-Terminal Kinase/Stress-Activated Protein Kinase, p38 Kinase, and CPP-32 Protease Pathways

Chang-Duk Jun,* Chun-Do Oh,‡ Hyun-Jeong Kwak,* Hyun-Ock Pae,* Ji-Chang Yoo,* Byung-Min Choi,* Jung-Soo Chun,‡ Rae-Kil Park,* and Hun-Taeg Chung‡

Nitric oxide (NO), a radical produced in mammalian cells from arginine in a reaction catalyzed by NO synthase (NOS), has pleiotropic biologic activities (1–3). NO is produced during inflammatory reactions and has been implicated as a signaling molecule (4–6) as well as a toxic effector (7–9). NO mediates activation or inhibition of various enzyme systems (6, 10), DNA damage (11), and oxidative reactions (12–14), with a variety of biologic effects, including killing of microorganisms (15), antiviral activity (16), and cytostasis and cell death (2, 3, 7–9).

Recently, the action of NO has been related to induction of programmed cell death, or apoptosis, in various cells including murine RAW 264.7 macrophages (17–19). Apoptosis is an active, energy-dependent mode of cell death of typical morphologic changes, such as nucleoplasmic and cytoplasmic condensation, and the formation of extensive membrane blebs and novel membranous structures known as apoptotic bodies (20). Although activation of soluble guanylyl cyclase, followed by 3′,5′-cGMP generation, has been known as a prime physiological NO action (21), toxic or apoptotic NO-signaling is still an enigma. Possible mechanisms include interactions between NO and iron-sulfur enzymes or protein thiol groups (3), the NAD(H)-dependent modification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (22), or direct DNA damage (11).

The mitogen-activated protein (MAP) kinase is an essential part of the signal transduction machinery and occupies a central position in cell growth, differentiation, and programmed cell death (23–25). To date, several mammalian MAP kinases have been identified, including the p42 and p44 (extracellular signal-regulated kinase (ERK)) (26, 27), c-Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) (28–30), and p38 kinase (31–33). All three MAP kinases phosphorylate substrates on serine and threonine residues located adjacent to proline residues, and members of all MAP kinases are activated as a result of simultaneous phosphorylation on threonine and tyrosine residues by upstream dual-specificity kinases. However, the three MAP kinases are activated in response to different extracellular stimuli,
have different downstream targets, and, therefore, perform different functions. ERKs are characterized by growth factors, usually by means of a Ras-Raf-1-dependent cascade (24, 34), whereas JNK/SAPK (30) and p38 kinase (31–33) are strongly activated by UV irradiation, osmotic stress, and the inflammatory cytokines TNF and IL-1.

While several studies have demonstrated the significant role of MAP kinases in apoptotic signaling, there is limited information concerning the roles of MAP kinases in NO actions. A previous report indicated that NO and related chemical species (NOX) activate the ERK, p38, and JNK/SAPK subgroups of MAP kinases in human Jurkat T cells (35). In contrast, another report demonstrated that NO promotes PC12 cell survival and blocks JNK activation caused by trophic factor withdrawal (36).

Recently, several reports demonstrated that NO-induced apoptosis is inhibited by exposure to phorbol ester and protein kinase C (PKC) activation in murine RAW 264.7 cells (19, 37). Despite the evidence that PKC mediates down-regulation of p35 and Bax expression (37), questions regarding its inhibitory action on apoptotic cell death in RAW 264.7 remain unanswered. Therefore, the first objective of this study was to determine which MAP kinases were tyrosine-phosphorylated and activated in response to NO in murine RAW 264.7 macrophages. The second objective was to determine whether activation of PKC by phorbol ester or up-regulation of PKC isoforms (i.e., II, III, δ, and η) could block apoptosis along with concomitant inhibition of MAP kinase activation after NO addition.

Materials and Methods

Materials

Glutathione S-transferase (GST)-c-Jun N-terminal protein, JNK/SAPK, and anti-JNK1 Abs were purchased from Stratagene (La Jolla, CA). Anti-ERK-1 or -2 mAbs were purchased from Transduction Laboratories (Lexington, KY). Abs specific to phosphorylated ERK-1, -2, and p38 kinase were obtained from New England Biolab (Beverly, MA). SB203580, PD098059, or -2 mAbs were purchased from Transduction Laboratories (Lexington, KY).

Cell lysates (50 μg) from RAW 264.7 cells and PKC overexpressed cells were separated by SDS-PAGE and transferred to a nitrocellulose membrane. PKC isoforms were detected with isoform-specific anti-PKC mAb for δ (Transduction Laboratories, Lexington, KY) or with polyclonal Abs for βII (Santa Cruz Biotechnology, Santa Cruz, CA) and η (Biomol, Plymouth Meeting, PA). PKC isoforms were visualized using a peroxidase-conjugated secondary Ab and the enhanced chemiluminescence system (39, 40).

Protein kinase assay for JNK/SAPK

JNK/SAPK activity was assayed as described previously (41). Cells were stimulated according to experimental protocols and lysed using buffer A containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS. Cell lysates were subjected to centrifugation at 12,000 × g for 10 min at 4°C. The soluble fraction was incubated 1 h at 4°C with Abs against JNK/SAPK. After the addition of protein G-agarose, the reaction mixtures were incubated for 1 h at 4°C and then subjected to microcentrifugation. The immunopellets were rinsed three times with buffer A, then twice with 20 mM HEPES, pH 7.4. Immunocomplex kinase assays were performed by incubating the immunopellets for 30 min at 30°C with a GST-c-Jun protein (2 μg) in 20 μl of the reaction buffer containing 0.2 mM sodium orthovanadate, 2 mM DTT, 10 mM MgCl2, 2 μl (γ-32P)ATP, and 20 mM HEPES, pH 7.4. The reaction was terminated by adding 5 μl of 5× sample buffer and heating the solution at 80°C for 3 min. The reaction mixture was subjected to electrophoresis on 12% polyacrylamide gel. The phosphorylated substrates were visualized by autoradiography.

Assay of ERK-1, -2, or p38 kinase

Cells were stimulated according to experimental protocols. Proteins were extracted with a buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl; 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM 4-nitrophenyl phosphate, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin A, 1 μM of 4-(2-aminoethyl) benzenesulfonyl fluoride). The activation of MAP kinase was examined by determining its phosphorylation state using Ab specific to phosphorylated ERK-1, -2, and p38 kinase.

CPP32-like protease activity assay

CPP32-like protease activity was measured as described in detail previously (42). RAW 264.7 cells (5 × 106 cells) were harvested from the cultured 6-well plates, washed with ice-cold PBS, and resuspended in 200 μl of buffer A’ (100 mM HEPES, pH 7.4, 140 mM NaCl, and the protease inhibitors, including 0.5 mM PMSF, 5 μg/ml pepstatin, and 10 μg/ml leupeptin). The cell suspension was lysed by three cycles of freezing and thawing. The crude cytosol was obtained as the supernatant from centrifugation at 12,000 × g for 20 min at 4°C. Assays were set up in flat-bottom 96-well plates containing 400 μg Ac-DEVD-pNA in buffer B’ (100 mM HEPES, pH 7.4, 20% glycerol, and protease inhibitors) and 200 μg of cytosol in a total volume of 200 μl. The CPP32-like protease activity was determined by measuring absorbance at 405 nm for 3 h. The reaction mixture without substrate was used as a control.

Cell viability and apoptosis assay

Cell viability was determined by PI exclusion test. Cells were treated with different inducers and/or inhibitors, washed with PBS twice, resuspended in PBS containing 20 μg/ml PI, and then immediately analyzed on a FACStar (Becton Dickinson, Rutherford, NJ). Cells that permitted PI uptake, interpreted as nonviable, were expressed as a percentage of the total cell number. For apoptosis assay, cells after agent treatment were fixed in 4% neutral-buffered paraformaldehyde, permeabilized with PBS/0.5% Triton X-100, and nuclei were stained for 20 min with the chromatin-staining Hoechst dye 33342. The coverslips were then washed, mounted onto slides, and viewed with a fluorescence microscope.

Quantitation assay of apoptosis

To quantify the number of cells undergoing apoptosis, cells were fixed with 4% neutral-buffered formalin. Apoptotic cells were stained using the terminal deoxynucleotidyl transferase (TdT) method (Apoptag). Endogenous peroxidase was first quenched with 2% hydrogen peroxide, and the cells were permeabilized using company-supplied equilibration buffer. The 3’ OH ends of degraded DNA were reacted with Tdt and digoxigenin-labeled ATP for 30 min. After washing with PBS, slides were reacted with an anti-digoxigenin mAb conjugated to peroxidase, washed, and developed with 3,3’-diaminobenzidine tetrahydrochloride. Stained cells were then counted using a light microscope.

DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described (43). Briefly, genomic DNA was purified with Wizard Genomic DNA purification kit. After ethanol precipitation, samples of 10 μg in each lane were subjected to electrophoresis on a 1.4% agarose at 50 V for 3 h. DNA was stained with ethidium bromide.
affected by SNP (Fig. 1). However, the total amounts of JNK/SAPK protein were not elevated JNK/SAPK activity within 30 min. This increase reached

**Results**

**NO activates JNK/SAPK and p38 kinase but does not affect ERK-1 and -2.** Initially, we wished to determine which MAP kinases were tyrosine-phosphorylated and activated in response to NO in murine RAW 264.7 macrophages. As shown in Fig. 1A, SNP significantly elevated JNK/SAPK activity within 30 min. This increase reached a maximum level of approximately 10-fold by 1 h after SNP addition. However, the total amounts of JNK/SAPK protein were not affected by SNP (Fig. 1A, bottom). We then attempted to determine whether NO also activate other members of MAP kinase family, the p38 kinase and ERK-1 and -2. Cultures of RAW 264.7 cells were incubated for various times (0–4 h) with SNP (1 mM), and then the cell lysates were prepared and phosphoryrosine-containing bands were visualized by immunoblot analysis (Fig. 1, B and C). Phosphorylation of p38 kinase was detected after 4 h of incubation with SNP, but not detected at the early time periods (0–2 h) (Fig. 1B). However, SNP induced neither phosphorylation of ERK-1 and -2 nor alteration of total amounts of ERK protein (Fig. 1C). In addition, although RAW 264.7 cells were treated with SNP for longer time periods, there were no detectable changes of phosphorylation of ERK-1 and -2 (data not shown). To determine whether the selective inhibitors of p38 kinase and MAP/ERK1 kinase, SB203580 (44) and PD098059 (45), respectively, could affect NO-induced cytotoxicity, RAW 264.7 cells were treated for 8 h with either SB203580 (10 μM) or PD098059 (10 μM) in the presence of SNP (0.5–1 mM). We found that SB203580, which by itself had no effect on cell morphology or viability, slightly reduced the cytotoxic action of SNP while PD098059 had no effect (Fig. 2), suggesting that p38 kinase is involved in the induction of programmed cell death by NO in RAW 264.7 macrophages. Although we have not currently determined the functional involvement of JNK/SAPK, activation of JNK/SAPK might be important for NO-induced signaling in RAW 264.7 macrophages.

**Overexpression of PKC protects against NO-induced apoptosis and reduces both JNK/SAPK and p38 kinase activity**

NO releasing compounds permit the investigation of NO signaling irrespective of NOS involvement. Within 8 h after exposure to NO donors, RAW 264.7 macrophages responded with apoptotic cell death, characterized by chromatin condensation and by DNA laddering (19, 37). To determine the specific down-regulatory role of PKC on NO-induced apoptotic cell death in murine RAW 264.7 macrophages, we produced clones of RAW 264.7 cells that over-express individual PKC isoforms such as βII, δ, and η. Parental RAW 264.7 cells expressed α, δ, ε, λ/θ, μ, and η isoforms of PKC, while PKC-βII was basically not detectable by Western blotting (Fig. 3, A and B). As expected, RAW 264.7 cells that had been stably transfected with expression vectors containing cDNA for PKC isoforms (βII, δ, and η) expressed substantial amounts of the appropriate isoforms (Fig. 3B).

With the use of SNP and GSNO, we elicited DNA cleavage in RAW 264.7 macrophages (Fig. 4, A–C). Internucleosomal DNA degradation determined qualitatively by agarose gel electrophoresis (Fig. 4A) or quantitatively by in situ TdT-apoptosis assay method (Fig. 4C) was selected as a reliable apoptotic parameter. SNP or GSNO, exposed for 12 h, elicited 45–50% DNA degradation in RAW 264.7 parent cells (Fig. 4C). Similar results were obtained with EV-transformed cells (EV-4). In contrast, exposure of PKC transfectants (PKC-βII, δ, and η) to SNP or GSNO resulted in substantially less DNA cleavage. Clones PKC-βII-4, δ-5, and η-6, which contained higher levels of PKC isoforms, remained viable. However, SB203580 and PD098059 protected these cells against NO-mediated DNA cleavage (Fig. 4C). Hence, both JNK/SAPK and p38 kinase are involved in NO-induced apoptosis in RAW 264.7 macrophages.
viable with little evidence of apoptosis within the 12-h incubation period. Clone PKC-Δ-3, which expressed the lowest amount of PKC-Δ among the stable transfectants, showed no protection (Fig. 4, A and C). To verify the involvement of NO on SNP-induced DNA fragmentation, we evaluated the effect of PFC, which is structurally similar to SNP except for the absence of a nitroso group. As expected, PFC (0.5 mM) alone did not induce DNA fragmentation in RAW 264.7 parent or EV-transformed cells as determined by gel electrophoresis (Fig. 4A).

To test the regulatory role of PKC on NO-induced apoptosis, we observed the effect of STSN, a potent PKC inhibitor, in RAW 264.7 parent or PKC transfectants. STSN (20 nM) significantly increased NO-induced apoptosis in both RAW 264.7 parent and PKC transfectants (Fig. 5).

To elucidate the mechanism that caused the resistance of PKC transfectants against death induced by the exposure to NO donors, the activities of three MAP kinase subfamilies were investigated in RAW 264.7 parent and PKC transfectants. Treatment of PKC-Δ-5 cells with SNP (1 mM) significantly suppressed JNK/SAPK activity as compared with parental RAW 264.7 cells (Fig. 6A). In addition, activation of JNK/SAPK by SNP was slightly reduced after treatment of parental RAW 264.7 cells with PMA (200 nM), which was already known to inhibit NO-induced apoptosis (19), but increased after treatment with STSN (100 nM), implying the regulatory roles of PKC on NO-induced JNK/SAPK pathway (Fig. 6B). Phosphorylation of p38 kinase was also decreased in PKC-Δ-5 cells as compared with RAW 264.7 parent cells (Fig. 6C, top and...
Previously, we reported that endogenously generated or exog-

enously applied NO markedly inhibits expression of PKC iso-

forms, such as PKC-δ, in murine macrophages (46). To determine
whether SNP has any effect on PKC isoform expression in trans-
fected RAW 264.7 cells, the cells were incubated for various times
(0–24 h) with SNP (0.5 mM). As expected, treatment of PKC
transfectants with SNP significantly decreased the expression of
PKC isoforms in a time-dependent manner (Fig. 7).

Overexpression of PKC isoforms blocks activation of
CPP32-like protease

Recently, CPP32, a member of IL-1-converting enzyme (ICE)
family cysteine proteases, has emerged as one of the key proteases
in spontaneous (47), anti-Fas- (48), and STSN-mediated apoptosis
in various cell types (49). To gain further insights for the protective
role of PKC on NO-induced apoptosis, we investigated the in-
volved role of CPP32-like protease in RAW 264.7 parent,
EV-transformed (EV-4), and PKC-δ-5 cells. Interestingly, SNP
significantly activated CPP32-like protease in the parent and EV-
transformed cells but not in PKC-δ-5 cells (Fig. 8A). In addition,
NO-induced apoptosis was significantly reduced in the presence of
CPP32-like protease inhibitor, Ac-DEVD-CHO, in RAW 264.7
parent cells (Fig. 8B).

Discussion

Our present results show that NO affects differentially the activa-
tion of the three known MAP kinase subfamilies; it strongly activ-
vates both JNK/SAPK and p38 kinase, but does not activate ERK-1 and -2 in RAW 264.7 macrophages. Activation of both
JNK/SAPK and p38 kinase can be effectively antagonized by the
transfer of various PKC isoforms (PKC-βⅠ, -δ, and -η). Previous
investigations established that RAW 264.7 macrophages are highly
susceptible to endogenously generated or exogenously supplied
NO (19, 37). The cellular response to NOS induction, with con-
comitant massive and sustained NO formation, is compatible with
apoptosis, as characterized by chromatin condensation and DNA
laddering. In those experiments all apoptotic alterations were
blocked by the addition of the NO inhibitor, N^3-monomethyl-L-
arginine, thereby relating endogenous NO generation to macro-
phage apoptosis (19). Cytotoxic and/or cytostatic actions of NO
are not only directed against invading pathogens but also can affect
susceptible host cells. Therefore, the existence of cellular defense
mechanisms that oppose the damaging potential of these radicals
and that account for differential cellular susceptibilities to NO
seem likely. Protective mechanisms may be attributable to an al-
tered NO-target interaction, scavenging of NO, or efficient repair

![FIGURE 5](image-url) Effect of STSN on NO-induced apoptosis in RAW 264.7
parent and PKC transfectants. RAW 264.7 parent (WT), EV-transformed
(EV-4), or PKC-overexpressed cells were incubated for 12 h with SNP (0.5
mM) in the presence or absence of STSN (20 nM). Quantitative analysis of
apoptosis was determined by in situ TdT-apoptosis assay method. Results
are expressed means ± SE of three independent experiments.

![FIGURE 6](image-url) Overexpression of PKC suppresses NO-induced JNK/
SAPK and p38 kinase activation but does not affect ERK-1 and -2. A. RAW
264.7 parent (WT) or PKC-δ-5 cells were cultured as in Fig. 1A. Then,
JNK/SAPK activity was examined, as described in Materials and Methods.
B. Up- or down-regulation of NO-induced JNK/SAPK activation by PKC
modulator. RAW 264.7 cells were incubated for 2 h with SNP (1 mM),
PMA (200 nM), STSN (100 nM), SNP plus PMA, or SNP plus STSN.
Then, JNK/SAPK activity was examined as in A. C. PKC-overexpressed
cells (PKC-δ-5) were incubated for various times (0–4 h) with SNP
(1 mM). Cell lysates were blotted with Abs specific for the tyrosine-phos-
phorylated form of p38 kinase (top) or Abs for ERK (bottom) and were
visualized using a peroxidase-conjugated secondary Ab and the enhanced
chemiluminesence system. Note: both Fig. 6C and Fig. 1, B and C are
representative of the same blot.

![FIGURE 7](image-url) Down-regulation of PKC isoforms after NO addition. PKC-
overexpressed cells were incubated for various times (0–12 h) with SNP
(0.5 mM). Total cell lysates extracted from clones of RAW 264.7 cells
transfected with individual PKC isoforms (βⅠ, δ, and η) were used to
detect PKC isoforms by Western blotting.
mechanisms. Our results strongly suggest that the anti-apoptotic mechanism of PKC is closely related with the inhibition of JNK/SAPK and p38 kinase, which play critical intermediary roles in mediating signal transduction from external stimuli to the nucleus (28–33).

Previous investigations established that NO and related chemical species (NOx) activate all of the three MAP kinase subfamilies such as ERK, p38, and JNK/SAPK in human Jurkat T cells (35). It is particularly interesting that NO does not affect ERK activity in RAW 264.7 macrophages. This result indicates that activation of MAP kinases are cell-type specific. For example, JNK/SAPK was not activated in response to stimulation of macrophages by the phorbol ester, in contrast to what has been seen in a number of other cell types including T lymphocytes (28, 29). In addition, NO promoted PC12 cell survival and blocked JNK/SAPK activation caused by trophic factor withdrawal (36).

Although the factors regulating apoptosis remain obscure, the involvement of PKC has been implied by numerous lines of evidence. For example, PKC activation blocks apoptotic cell death in rat thymocytes exposed to Ca\(^{2+}\) ionophores and glucocorticoids (50), suppresses radiation-induced sphingomyelin hydrolysis and apoptosis in aortic endothelial cells (51), and prevents ceramide-induced programmed cell death in U937 monoblastic leukemic cells (52). In addition, recent report demonstrated that overexpression of PKC-\(\epsilon\) suppresses apoptosis and induces \(bcl-2\) expression in human IL-3-dependent cells (53). It also reported that overexpression of atypical PKC-\(\epsilon\) protects human leukemia cells against drug-induced apoptosis (54). On the other hand, we previously reported that phorbol ester, a PKC activator, synergistically augments NO-induced apoptosis in human leukemic HL-60 cells (55). Taken together, these findings suggest that PKC-dependent signaling processes may, in some instances, depend on the diverse stimuli and specific cell types. Our results provide evidence that PKC overexpression completely suppressed NO-mediated apoptosis and DNA laddering within the first few hours (~12 h) after NO donor application in RAW 264.7 macrophages. PKC overexpression (PKC-\(\beta\II, -\delta, \text{and } -\eta\)) neither blocked IFN-\(\gamma\)/LPS signaling pathways resulting in inducible NOS expression nor endogenous NOS induction (data not shown). Our results corroborate previous report that the transfection of RAW 264.7 cells with plasmids harboring PKC-\(\epsilon\) isotype but not with PKC-\(\alpha, -\beta I, \text{or } -\delta\) isotypes resulted in the expression of NOS (56). Obviously, therefore, our results further suggest that PKC (at least in such isotypes as PKC-\(\beta\II, -\delta, \text{and } -\eta\)) blocks NO-mediated cell death events through direct or indirect regulation of MAP kinase subfamilies in RAW 264.7 macrophages.

Recently, studies have implicated CPP32, a member of the ICE family cysteine proteases, as an obligate component of the cell death pathway in various cell types (47–49). In this report, we showed that a CPP32-like protease is also involved in NO-induced apoptosis as assessed by colorimetric assay. In addition, overexpression of PKC isotype (PKC-\(\delta\)-5) suppressed NO-induced activation of CPP32-like protease. Although we do not know whether the suppression of CPP32-like protease activity in PKC transfectants is due to the direct interaction of PKC with CPP32-like protease, PKC function might be required at a step before CPP32-like protease activity to protect NO-induced apoptosis signaling.

Collectively, although the results of this study provide strong evidence that either activation of PKC or overexpression of PKC isoforms inhibit NO-mediated signaling pathways such as JNK/SAPK, p38 kinase, and CPP32-like protease and the resulting induction of apoptosis, the point in the pathway at which PKC is involved is not clear. Additional experiments will be required to establish whether any mechanisms account for the inhibition of NO-mediated signaling pathways in PKC-overexpressing RAW 264.7 macrophages.

References


