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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,* Jang-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 cytoxicity 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 (JNK), and p38 kinase (28–30), and members of all MAP kinases are activated as a result of the simultaneous phosphorylation on threonine and tyrosine residues by upstream dual-specificity kinases. However, the three MAP kinases (JNK, p38, and c-Jun) 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 (33–35) 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 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 isomers (i.e., 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). BD203580, PD98059, N-acetyl-Asp-Glu-Val-p-nitroanilide (Ac-DEVD-pNA), N-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), and propidium iodide (PI) were purchased from Calbiochem-Behring (La Jolla, CA). Sodium nitroprusside (SNP), potassium ferricyanide (PFC), Hoechst 33342, PMA, and staurosporine (STSN) were purchased from Biochem-Behring (La Jolla, CA). Sodium nitroprusside (SNP), potassium ferricyanide (PFC), Hoechst 33342, PMA, and staurosporine (STSN) were purchased from Ac-DEVD-pNA, Ac-DEVD-CHO, and propidium iodide (PI) were purchased from Calbiochem-Behring (La Jolla, CA). Sodium nitroprusside (SNP), potassium ferricyanide (PFC), Hoechst 33342, PMA, and staurosporine (STSN) were purchased from Sigma (St. Louis, MO). N-sitotroglutathione (GSNO) was purchased from Alexis (San Diego, CA). Molecular size marker of DNA was purchased from Bethesda Research Laboratories (Bethesda, MD). Genomic DNA purification kit was obtained from Promega (Madison, WI). All reagents used for in situ nick translation were obtained from Oncor (Gaithersburg, MD).

Generation of PKC overexpressing cell lines

The expression vector (i.e., MTH vector containing cDNA for PKC-δ, III, and η) that was used has been described previously (38). Clones of RAW 264.7 cells that overexpress one of the PKC isomers or a control plasmid lacking the PKC genes (empty vector (EV)-4) were generated by the transfection of expression vector using lipofectamine (Life Technologies, Gaithersburg, MD) with the procedure recommended by the manufacturer. The transfected cells were subsequently grown in selection medium (G418 at 800 μg/ml of complete medium). Following 10–20 days in selection medium, single colonies were picked and subsequently examined for the presence of PKC proteins by Western blotting. The cells were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics.

Western blot analysis of PKC isoforms

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 δ (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 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 μCi [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 × 105 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 μM 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 quantitate 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 (Apotag). Endogenous peroxidase was first quenched with 2% hydrogen peroxide, and the terminal deoxynucleotidyl transferase (TdT) method (Apotag) was used to detect DNA strand breaks. Stained cells were then washed, mounted onto slides, and viewed with a fluorescence microscope.

DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described previously (43). Briefly, genomic DNA was purified by 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.
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). SNP significantly increased p38 kinase activity within 30 min. This increase reached a maximum level of approximately 10-fold by 30 min after SNP addition. This increase reached a maximum level of approximately 10-fold by 1 h after SNP addition. However, the total amounts of p38 kinase protein were not affected by SNP (Fig. 1A).

**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). Cell lysates were blotted with Abs specific for the tyrosine-phosphorylated form of ERK or Abs for ERK-1 and -2 protein and visualized as in B.

**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 DNA laddering (19, 37). To determine the specific down-modulatory 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. A4) 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, -δ, and η, which contained higher levels of PKC isoforms, remained.

**FIGURE 1.** NO activates JNK/SAPK and p38 kinase but does not affect ERK-1 and -2. A, RAW 264.7 cells were incubated for various times (0–2 h) with SNP (1 mM). Then, the cells were harvested, lysed, and immunoprecipitated with a specific Ab against JNK-1 and analyzed for their catalytic activities to phosphorylate GST-c-Jun-NT, as described in Materials and Methods. The reaction mixtures were separated by SDS/PAGE, and phosphorylated GST-c-Jun protein was visualized by autoradiography. B, RAW 264.7 cells were incubated for various times (0–4 h) with SNP (1 mM). Cell lysates were blotted with Abs specific for the tyrosine-phosphorylated form of p38 kinase and were visualized using a peroxidase-conjugated secondary Ab and the enhanced chemiluminescence system. C, RAW 264.7 cells were incubated for various times (0–4 h) with SNP (1 mM). Cell lysates were blotted with Abs specific for the tyrosine-phosphorylated form of ERK or Abs for ERK-1 and -2 protein and visualized as in B.

**FIGURE 2.** Induction of apoptosis by SNP and its inhibition by SB203580. RAW 264.7 cells were incubated for 8 h with SNP (0.5–1 mM) in the presence or absence of SB203580 or PD098059. For the combination treatment, cells were incubated with SB203580 or PD098059 for 2 h before SNP addition. Quantitative analysis of apoptosis was determined by in situ TdT-apoptosis assay method. Results are expressed means ± SE of three independent experiments. *, p ≤ 0.001 vs the value obtained in the control.
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. 4 A).

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 and 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 Fig. 1B). However, phosphorylation of ERK-1 and -2 was not changed (Fig. 6C, bottom and Fig. 1C). Similar results were obtained from other PKC-overexpressed cells including PKC-βII-4 and PKC-η-6 clones (data not shown).
Previously, we reported that endogenously generated or exogenously applied NO markedly inhibits expression of PKC isoforms, such as PKC-δ, in murine macrophages (46). To determine whether SNP has any effect on PKC isoform expression in transfected 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 involvement 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 activation of the three known MAP kinase subfamilies; it strongly activates 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-βII, -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 concomitant 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 NOS inhibitor, N^3^-monomethyl-L-arginine, thereby relating endogenous NO generation to macrophage 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 altered NO-target interaction, scavenging of NO, or efficient repair
Three independent experiments. For example, PKC activation blocks apoptotic cell death in countries. Such as ERK, p38, and JNK/SAPK in human Jurkat T cells. For example, JNK/SAPK expression of atypical PKC-ε protects human leukemia cells against drug-induced apoptosis. On the other hand, we previously reported that phorbol ester, a PKC activator, synergistically augments NO-induced apoptosis in human leukemic HL-60 cells. 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-βII, -δ, and -η) neither blocked IFN-γ/LPS signaling pathways resulting in inducible NOS expression nor endogenous NO formation (data not shown). Our results corroborate previous reports that the transfection of RAW 264.7 cells with plasmids harboring PKC-ε isotype but not with PKC-α, -βII, or -δ isotypes resulted in the expression of NOS (56). Obviously, therefore, our results further suggest that PKC (at least in such isotypes as PKC-βII, -δ, and -η) 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-δ-5) suppressed NO-induced activation of CPP32-like protease. Although we do not know whether the suppression of CPP32-like protease activity in PKC transfecants is due to the direct interaction of PKC with CPP32-like protease, PKC function might be required at a step before CPP32-like protease activation 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 of any mechanisms account for the inhibition of NO-mediated signaling pathways in PKC-overexpressing RAW 264.7 macrophages.

**References**


