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NITRIC OXIDE INHIBITS THE EXPRESSION OF PROTEIN KINASE C δ GENE IN THE MURINE PERITONEAL MACROPHAGES

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Summary : Since there is increasing evidence that protein kinase C (PKC) has a crucial role in the production of nitric oxide (NO) from activated macrophages, this study was undertaken to adress whether NO could regulate the expression of the gene of this enzyme. Stimulation of the cells with lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA) after treatment with recombinant interferon-γ (rIFN-γ) resulted in the increased production of NO in the medium. rIFN-γ in combination with either LPS or PMA showed marked inhibition of the expression of PKCδ gene, whereas rIFN-γ alone showed modest inhibition. The inhibition of gene expression was correlated with the amount of NO produced by activated macrophages. The inhibitory effect of NO on the expression of PKCδ gene is mimicked by the treatment of NO generating agent, sodium nitroprusside (SNP). On the other hand, a specific inhibitor for NO synthase, N^G-monomethyl-L-arginine (N^GMMA), blocked the inhibition of the expression of PKCδ gene by blocking the NO production in the rIFN-γ and LPS-stimulated cells. However, production of NO did not affect the expression of both TNF-α and TGF-β gene which were induced by the stimulation of macrophages, as well as β-actin gene, which was constitutively expressed in the macrophages. In conclusion, these findings show that NO has a regulatory role for the expression of the gene of PKC δ which is crucially involved in the process of NO synthesis.

Nitric oxide (NO) has emerged as an important intra- and inter- cellular regulatory molecule with functions as diverse as vasodilatation, neural communication or host defense (1-3). The important action of NO include the activation of soluble guanylate cyclase by its interaction with heme (4). Other hemoproteins such as cytochrome P-450 or non-heme iron-centered proteins like aconitase are also influenced by NO (5,6). The production of NO is catalysed by the enzyme NO synthase (NOS), of which there are at least two different types(1). One is constitutive and is Ca^{2+}/calmodulin-dependent. The other is inducible by cytokines and bacterial lipopolysaccharide (LPS) and is Ca^{2+}-independent. The activity of these enzymes can be specially and stoichiometrically inhibited by the structural analogues of L-arginine such as N^G-monomethyl L-arginine (N^GMMA).

Since NO can do protective or regulatory function in the cells at a low concentration while toxic effect at higher concentration, its synthesis may be tightly regulated in the cells. Indeed, a number of cytokines such as TGF-β, IL-4, and TNF-α are effective in the regulation of inducible NOS (iNOS) expression (7-9). Furthermore, recent studies show that there may be

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also self-regulatory mechanism modulating the process of induction and activity of iNOS (10). NO has been shown to inhibit NOS activity and thus may act as negative feedback modulator of its own synthesis. It is also known that NO and NO generating agent induce a reversible inactivation of PKC activity by S-nitrosylation (11). Nevertheless, it is not known whether NO can directly modulate the expression of the gene of PKC which is deeply involved in the process of iNOS induction.

PKC is involved in the regulation of a variety of cellular processes including proliferation, differentiation and metabolism (12,13). PKC also plays an important role in the cellular response to growth factors, neurotransmitters, and hormones (13). Normal activation of the kinase activity of PKC protein appears to be mediated by diacylglycerol, which is produced in the course of inositol phospholipid breakdown. The PKC kinase activity can also directly activated by exogenous phorbol esters such as phorbol 12-myristate 13-acetate (PMA). In macrophages, PKC seems to be important second messenger for transduction of signals affecting macrophage activation and NO production (14). Increased PKC activity has been implicated in the induction of NO synthesis in rat hepatocytes, peritoneal macrophages, and murine microglial cells (14-16). Moreover PKC may down-regulate the activity of NOS by direct phosphorylation of the enzyme (17). Recent study reports that the most abundant PKC isoform mRNA in peritoneal macrophages is PKCδ and other PKC isoforms such as PKCα and PKCβ are pooly expressed in these cells (18).

In this study, we examined the effect of NO on the expression of PKCδ gene. NO and NO generating agent inhibited the expression of PKCδ gene and the blocking agent of NO production blocked the inhibition of the expression of this gene. This is the first evidence that NO affect the transcriptional level of gene expression of the enzyme which is involved in NO synthesis.

MATERIALS AND METHODS

Mice : The original stock of C57BL/6 mice was purchased from the Jackson Laboratory (Bar Harbor, ME) and the mice were maintained in the Department of Microbiology and Immunology, Wonkwang University School of Medicine (Iri, Korea). To obtain peritoneal macrophages, mice were used at 8 to 12 wks of age.

Reagents : Murine rIFN-γ (1 x 10^6 units/mg) was purchased from Genzyme (Munchen, F.R.G.). LPS from *Escherichia coli*, PMA, LiCl, urea, N-(1-naphthyl)-ethylenediamine dihydrochloride, sodium nitrite and sulfanilamide were purchased from Sigma Chemical Co. (St, Louis, MO). Agarose, phenol, MMLV reverse transcriptase, RNasin, DNA polymerase I, Taq polymerase, and deoxynucleotide triphosphate were purchased from GIBCO-Bethesda Research Laboratories (Gaithersburg, MD). DuPont New England Nuclear (Boston, MA) was the source of [α-^{32}P]dCTP. pBluescript II KS(-) plasmid was purchased from Stratagene Inc. (San Diego, CA). All reagents and media for tissue culture experiments were tested for their LPS content with a colorimetric Limulus amoebocyte lysate assay (detection limit 10 pg/ml ; Whittaker Bioproducts, Walkersville, MD). 100 mm diameter petri dish was purchased from Nunc Inc. (North Aurora Road, Illinois). DMEM containing L-arginine (84 mg/L), HBSS, and FCS and other tissue culture reagents were purchased from GIBCO (Gaithersburg, MD).

Cell cultures : Thioglycollate(TG)-elicited macrophages were harvested 3 days after i.p. injection of 2.5 ml TG to mice 8 to 12 wks of age and isolated as reported previously (19). Peritoneal lavage was performed using 8 ml of HBSS containing 10 U/ml heparin. Cells were

then distributed in DMEM supplemented with 10 % (v/v) FCS in100 mm-diameter plastic petri dishes (1 x 10^7 cells/dish), incubated for 3 hours at 37°C in an atmosphere of 5 % CO_2 and washed three times with HBSS to remove nonadherent cells, and then equilibrated with DMEM containing 10 % FCS before treatment as indicated in the text.

Measurement of nitrite concentration : NO synthesis in cell cultures was measured by a microplate assay method as described (20). Briefly, 100 µl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent (1 % sulfanilamide / 0.1 % N-(1-naphthyl)-ethylenediamine dihydrochloride / 2.5 % H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek multiskan (Flow Laboratories, Australia). NO_2^- was determined by using sodium nitrite as standard. Cell-free medium alone contained 5-8 µM of NO_2^- ; this value was determined in each experiment and subtracted from the value obtained with cells.

Preparation of probe : Probes specific for the transcripts encoding murine PKCδ, TNF-α, TGF-β, and β-actin used in this study have been described in detail previously (21-24). For detection of iNOS mRNA transcripts, sense and antisense oligonucleotide primers specific for the coding regions of those genes were synthesized by conventional technology. The following oligonucleotide primers were used ; forward primer, 5' - GGCCTTGGCTCCAGCATGT AC - 3', 1856 through 1876, reverse primer, 5' - GCTGCCGCTCTCATCCAGAAC - 3', 2395 through 2415. The numbers represent the nucleotide numbers on the complementary strands of each cDNA sequence (25). Total cellular RNA (5 µg) from rIFN-γ- and LPS-stimulated macrophages of C57BL/6 mice was used as a template and the single-strand cDNA was synthesized with down-stream antisense primers by reverse transcriptase. cDNA was amplified in a 12 µl reaction mixture by a Dae Han Medical Co. DNA thermal cycler (Seoul, Korea) using Taq polymerase in 45 cycles of 5 second of denaturation at 94°C, 5 seconds of annealing at 55°C, and 20 seconds of synthesis at 72°C. Polymerase chain reaction (PCR) products were examined on 1% agarose gel. For the analysis of DNA sequence, PCR products were gel purified, treated with T4 polynucleotide kinase, and then with the Klenow fragment of DNA polymerase I. The products were subcloned into the *EcoR* V site of the pBluescript II KS(-) plasmid. One µg of plasmid DNA was radiolabeled by random priming with [α-^{32}P] dCTP. The resultant specific activity was approximately 1 x 10^8 cpm/µg and was used at 1 x 10^7 cpm/blot.

RNA extraction and Northern blotting : Total RNA was prepared by the LiCl-urea method (26), electrophoresed in 1.2 % agarose-formaldehyde gels and transferred to nylon membranes by capillary action in 20 x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.2). After prehybridization, the filters were hybridized with random [α-^{32}P] dCTP labeled probes with specific activities of 1 to 5 x 10^8 cpm/µg in 10 % dextran sulfate, 50 % formamide, 4 x SSC, 1 x Denhardt's solution, and 10 µg/µl salmon sperm DNA for 24 hours at 42°C. The filters were then washed, dried, and examined by autoradiography.

RESULT

Initially we wished to determine whether murine macrophages could be stimulated by PMA, a PKC activator, either alone or in combination with rIFN-γ to induce the expression of iNOS gene. LPS in combination with rIFN-γ were used as a control for maximal induction of iNOS gene. TG-elicited macrophages were cultured for 6 hours either in medium alone or in medium containing rIFN-γ (5 units/ ml). The cells were then stimulated with LPS (10 ng/ml) or PMA (200 nM) for 18 hours and NO release was measured by the method of Griess (nitrite). The content of iNOS mRNA was also analysed by Northern hybridization with radiolabeled cDNA encoding iNOS. As Fig. 1 A and B shows, LPS or PMA synergized with rIFN-γ for the expression of iNOS gene as well as NO production and the content of iNOS mRNA in the cells was correlated with the amount of nitrite in the medium.

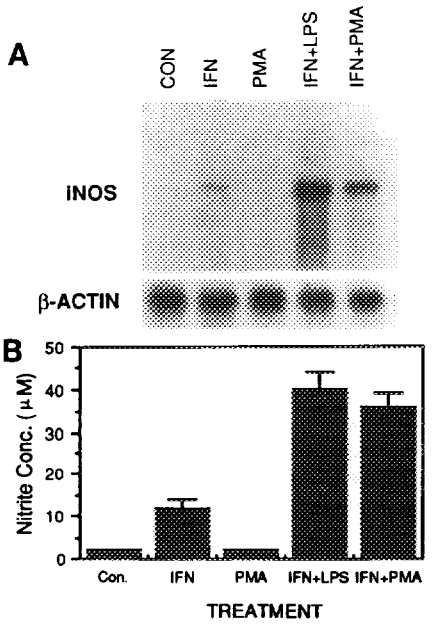

Figure 1. PMA or LPS induced NO production in rIFN-γ-stimulated murine peritoneal macrophages. Thioglycollate-elicited macrophages (1 x 10^6 cells/well) were cultured for 6 hours either in medium alone or in medium containing rIFN-γ (5 U/ml). The cells were then treated with either LPS (10 ng/ml) or PMA (200 nM) as indicated, and cultured for 18 hours. (A) iNOS mRNA was analyzed by Northern hybridization. Blots were hybridized with the indicated [^{32}P] dCTP-radiolabeled cDNA and exposed to x-ray film for 24 hours. (B) NO release was measured by the method of Griess (nitrite) . Results are presented as the means ±SD of three experiments.

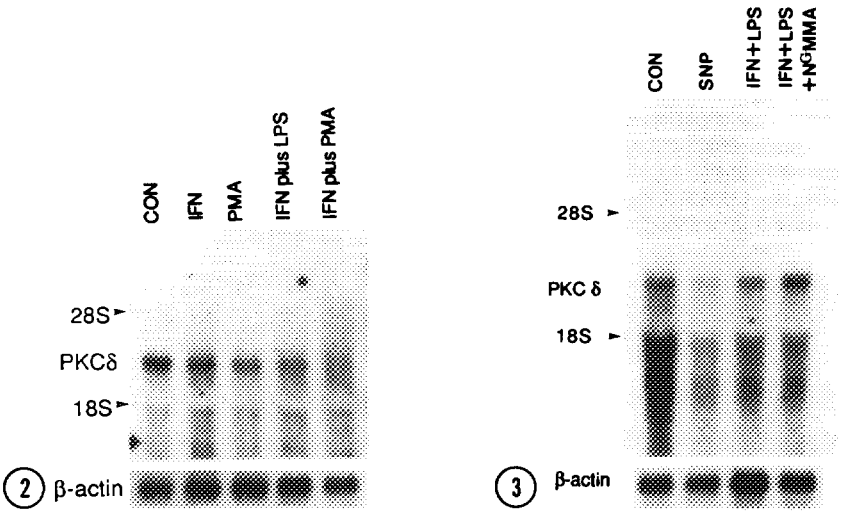

Figure 2. NO induced inhibition of PKCδ gene expression. Thioglycollate-elicited macrophages (1 x 10^6 cells/well) were cultured for 6 hours either in medium alone or in medium containing rIFN-γ (5 U/ml). The cells were then treated with either LPS (10 ng/ml) or PMA (200 nM) as indicated and cultured for 18 hours and PKCδ mRNA was analyzed by Northern hybridization. Blots were hybridized with the indicated [^{32}P] dCTP-radiolabeled cDNA and exposed to x-ray film for 24 hours. Results are presented one of the three similar experiments.

Figure 3. Effect of SNP or N^GMMA on the expression of PKCδ gene. Thioglycollate-elicited macrophages (1 x 10^6 cells/well) were cultured for 24 hours in medium alone or in medium containing as indicated reagent and PKCδ mRNA was analyzed by Northern hybridization. Blots were hybridized with the indicated [^{32}P] dCTP-radiolabeled cDNA and exposed to x-ray film for 24 hours. Results are presented one of the three similar experiments.

Next, we measured the amount of PKCδ mRNA in the cells which had been stimulated with rIFN-γ. As Fig. 2 shows, rIFN-γ showed small inhibitory effect, whereas rIFN-γ in combination with either LPS or PMA showed marked inhibitory effect on the expression of PKCδ gene. The degree of inhibition was correlated with the amount of NO produced by the cells. Although PMA alone could not produce NO, PMA alone inhibited the expression of this gene. To further confirm the inhibitory effect of NO on the expression of PKCδ gene, SNP, *in vitro* NO generating agent, was added in the wells containing the cells. As Fig. 3 shows, the expression of PKCδ gene was markedly inhibited by the treatment of 200 μM SNP without the loss of viability (above 99% of the cells were viable by trypan blue dye exclusion test). When the production of NO from rIFN-γ plus LPS-stimulated macrophages was blocked by the treatment with N^GMMA (1 mM), the expression of PKCδ gene recovered to the level of unstimulated cells.

As Fig. 4 shows, the expression of both TNF-α and TGF-β genes increased by stimulation of the cells and the expression of these genes was not affected by NO produced by activated cells. In addition, NO production had no effect on the expression of β-actin gene which is constitutively expressed in macrophages. Although these results do not cover the effects of NO on the expression of all genes, this is the first evidence that NO specifically inhibit the expression of the PKCδ gene which is crucially involved in the process of macrophage activation.

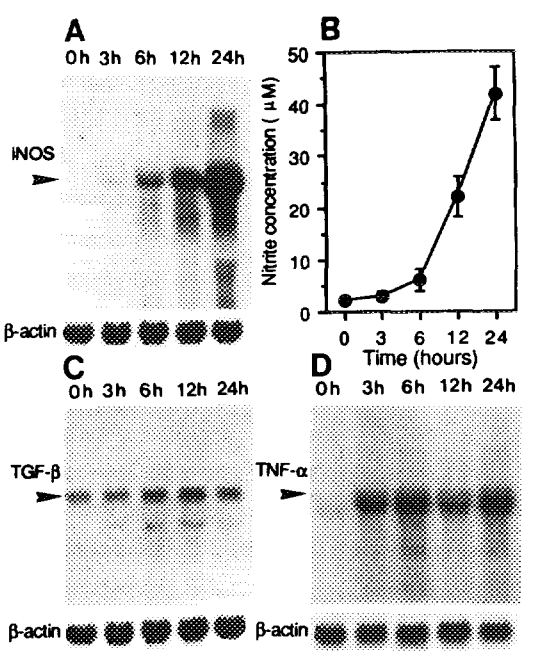

Figure 4. Effect of NO on the expression of both TGF-β and TNF-α gene. Thioglycollate-elicited macrophages (1 x 10^6 cells/well) were cultured with rIFN-γ and LPS and at various times, TGF-β and TNF-α mRNA was analyzed by Northern hybridization. Blots were hybridized with the indicated [^{32}P] dCTP-radiolabeled cDNA and exposed to x-ray film for 24-48 hours. Results are presented one of the two similar experiments.

DISCUSSION

Several reports demonstrate that production of NO by macrophages tightly regulated (7-10). This suggest that there might be a self regulatory mechanism modulating production of NO. It is also reported that NO or NO generating agent inhibit the enzyme activity of either iNOS or PKC which is directly or indirectly involved in the production of NO (10,11). However, there is no report which adresses whether NO can affect the expression of PKC gene at the transcriptional level.

The results of this study demonstrate that NO production by macrophages is regulated by the pathway of PKC signals. Stimulation of the cells with PMA after treatment with rIFN-γ resulted in increased production of NO. However, prolonged incubation of the cells with PMA, which is known to down-regulate PKC activity, before rIFN-γ treatment abolished the synergistic action of PMA on NO production (not shown). PMA-stimulated NO production was also inhibited by the treatment of the cells with PKC inhibitors such as staurosporine and polymyxin B (not shown).

Furthermore, this study shows that NO produced by the activated macrophages specifically inhibited the expression of PKCδ gene whose mRNA is known to be the most abundant isoform among PKC mRNAs in macrophages (18). This result is supported by the following observations. First, rIFN-γ alone had small inhibitory effect on the expression PKCδ gene, whereas rIFN-γ in combination with either LPS or PMA had a marked inhibitory effect. The degree of inhibition of the gene expression was correlated with the amount of NO produced by activated macrophages. Second, the addition of SNP also markedly inhibited the expression of PKCδ gene. Third, blocking of NO production by treatment of the cells with N^GMMA recovered the expression of PKCδ gene to the level of unstimulated cells. Forth, NO produced by activated cells did not affect the expression of other inducible and constitutive genes (TNF-α , TGF-β and β-action genes respectively).

PMA alone inhibited the expression of PKCδ gene. In addition, NO produced by activated cells in combination with PMA completely inhibited the expression of PKCδ gene. It may be accounted by down-regulation of PKC after prolonged incubation of the cells with PMA (11-13). So, PMA may have some inhibitory effects on the expression of PKC gene as well as the enzyme activity of PKC in macrophages.

NO has been known to induce inhibition of platelet aggregation, smooth muscle relaxation, and desensitization of the neurons to excess stimulation by glutamate (27-29). On the contrary, PKC might be involved in platelet aggregation, smooth muscle contraction, and mediating the cellular actions in response to activation of glutamate receptors in neurons (30). Previous studies revealed that NO could induce reversible inactivation of PKC by direct modification of the enzyme involving the formation of a disulfide bridge. Our current studies demonstrate that NO can also directly inhibit the expression of PKCδ gene at the transcriptional level.

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