Involvement of protein kinase C and not of NFκB in the modulation of macrophage nitric oxide synthase by tumor-derived phosphatidyl serine

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Abstract. Nitric oxide (NO) is one of the main cytotoxic effector molecules involved in the killing of tumor cells by macrophages. In macrophages, lipopolysaccharide (LPS) alone or in combination with IFN-γ causes the generation of NO by an inducible form of NO synthase (iNOS). We have previously reported that macrophages from mammary tumor bearers have a downregulation of their NO production leading to a diminished cytotoxic activity. Further studies lead to the isolation and characterization of phosphatidyl serine (PS) as a NO inhibitory factor produced by mammary tumor cells. Pretreatment of macrophages with PS was shown to downregulate their cytotoxic potential and NO production upon stimulation with LPS. Activation of PS-pretreated macrophages with LPS and IFN-γ resulted in higher levels of NO than those observed with LPS alone, but lower than those of untreated macrophages activated with LPS and IFN-γ. These results correlated with the levels of iNOS RNA as detected by Northern blot analyses. A study of the expression and binding activity of the transcription factor NFκB in macrophages pretreated with PS revealed no differences with untreated macrophages. Investigation of the possible signaling pathways leading to the induction of iNOS revealed that in LPS-stimulated macrophages, increases in internal calcium concentration $[Ca^{2+}]i$ were not observed, while NO was normally produced even under calcium-deprived conditions. In contrast, an effective synergism of IFN-γ with LPS in the production of NO by macrophages required an optimal increase in $[Ca^{2+}]i$ stimulated by IFN-γ. This increment in $[Ca^{2+}]i$ was significantly reduced in PS-pretreated macrophages. Further experiments demonstrated that pretreatment of macrophages with PS did not change the normal pattern of tyrosine phosphorylation stimulated by LPS but strikingly inhibited PKC activity. Combinations of LPS and IFN-γ did not alter the latter result, suggesting that IFN-γ enhances LPS-induction of iNOS through a pathway other than activation of PKC. Importantly, expression of PKC isozymes in both untreated and PS-pretreated macrophages stimulated with LPS remained constant. Out data suggest that, in tumor bearers, PKC and not NFκB is the main target for PS to exert its NO inhibitory action on LPS-activated macrophages. An excess of PS in PS-PKC interaction may be responsible, at least in part, for this type of PKC inhibition. Furthermore, PS also appears to downregulate the rise in $[Ca^{2+}]i$ promoted by IFN-γ in macrophages, reducing the synergism of this cytokine with LPS and leading to a less effective production of NO.

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Introduction

One of the multiple functions of activated macrophages is their ability to recognize and to lyse tumor cells. During this process, a number of lytic factors such as TNF-α, superoxide, hydrogen peroxide, and NO are released. NO has been recognized as one of the most efficient and non-specific lethal molecules in the repertoire of macrophage cytotoxic machinery (1). However, it is important to point out that failure of macrophages to maintain a balance in NO production could bring detrimental consequences to the host, including increase in tumor burden, stroke, and septic shock (2).

In previous studies we have shown that peritoneal macrophages (PEM) from mammary tumor-bearing BALB/c mice (T-PEM) display a diminished ability to lyse tumor cells upon stimulation with LPS, a phenomenon that is associated to a lower production of nitric oxide, and that is reversed upon costimulation with IFN-γ (3). We have also shown that PS, a factor released by mammary tumor cells, concomitantly inhibits the production of NO and the cytotoxic activity in LPS-activated macrophages (4). Furthermore, a blocking of putative LPS receptor(s) by PS is unlikely to have occurred since normal amounts of IL-1α and IL-6 are secreted by LPS-activated macrophages pretreated with PS. Similarly, superoxide production by macrophages under the latter experimental conditions did not undergo significant variations.

Several laboratories have demonstrated that IFN-γ synergizes with LPS in the induction of macrophages iNOS (5,6). In this regard, we have shown that although macrophages from tumor-bearing mice activated with LPS have significantly diminished levels of NO, dual stimulation of these cells with LPS and IFN-γ, results in their normal production of NO (3,7). Further analysis of macrophages from tumor-bearing mice have shown that these cells display a diminished PKC activity and a decreased binding activity of transcription factors such as NFκB that appear to lead to their impaired production of NO as compared to the levels seen in macrophages from normal mice (8). In this study, an investigation of the mechanisms by which PS-pretreatment affects the synthesis of iNOS by macrophages was undertaken. The data reported herein provide additional information about the signal transduction pathways involved in the synthesis of NO and suggest that inhibition of PKC activity by PS, and not an alteration of NFκB expression or binding activity by this lipid, may be responsible for the downregulation of iNOS transcription in LPS-activated macrophages. Thus, a more dynamic function of PS, i.e. as a PKC regulator during signal transduction events, is envisioned.

Materials and methods

Mice. BALB/c mice were maintained by brother-sister mating in our facilities. Our institutional animal care and use committee approved the animal experiments.

Macrophage cultures. Normal mice were injected i.p. with 1.5 ml of 3% thioglycollate (Difco Laboratories, Detroit, MI). On day 4, the peritoneal-exudate macrophages (PEM) were obtained by peritoneal lavage with 10 ml ice-cold RPMI-1640/mouse. Peritoneal cells were washed twice and resuspended in RPMI-1640 medium. The adherent population was obtained following the plastic-adherent technique as previously described (4). This procedure provides a population consisting of >95% macrophages as determined by staining with Diff-Quick differential stain (Baxter, McGaw Park, IL) and nonspecific esterase staining (Sigma). Viability of cells was routinely >95% by trypan blue exclusion. PEM were seeded into 96-wells, flat bottom microtiter plates (Costar, Cambridge, MA) at a cell density of $1.5x10^5$/well and cultured in RPMI-1640-supplemented media with 10% endotoxin-free FCS, 100 U penicillin, 100 μg/ml of streptomycin, $5x10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine, 1% nonessential amino acids, 1% essential amino acids, and 1% sodium pyruvate (all from Gibco laboratories, Grand Island, NY).

Reagents. Lipopolysaccharide (LPS) [*Escherichia coli* 055:B5] from Difco laboratories was used as indicated. The calcium ionophore A23187, pepstatin A, chymostatin, aprotinin, PMSF, and DTT (dithiothreitol) were obtained from Boehringer Mannheim, Indianapolis, IN. Synthetic 1-stearoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine] was obtained from Avanti Polar Lipids, Inc., Alabaster, AL. Murine r-IFN-γ was purchased from Genzyme, Cambridge, MA. Indo-1-AM was purchased from Molecular Probes, Inc., Eugene, OR. Polyclonal anti-mouse horseradish peroxidase was purchased from Transduction Laboratories, Lexington, KY.

Nitrite (NO_2^-) determination. PEM from normal mice were preincubated for 24 h in the presence or absence of PS. In some experiments, the cells were treated with calcium chelators as indicated in Results. Following this, the macrophages were washed twice and further incubated for 24 h with or without LPS to elicit the production of NO. In some experiments IFN-γ or the calcium ionophore A23187 were used alone or in combination with LPS as activators. Nitrite concentration in the cell-free macrophage supernatants served as a reflection of NO production and was measured by the colorimetric Griess reaction as described by Stuehr and Nathan (1). Briefly, 100 μl of sample aliquots were mixed with 100 μl of Griess reagent (1% sulfanilamide; 0.1% N-[naphthyl] ethyl-enediamine dihydrochloride and 2% H_3PO_4) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured via Rainbow reader, Tecan (SLT Lab Instruments), Research Triangle Park, NC. NO_2^- levels were determined using $NaNO_2$ as standard, and double-distilled water was blank.

Northern blotting. Murine peritoneal exudate macrophages were plated and incubated as described above. Total RNA was isolated utilizing Trizol Reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instruction. RNA samples (15 μg) were electrophoresed in 0.5% formaldehyde-agarose gel, transferred onto 0.45 μm nitrocellulose membranes (Costar), and baked at 80˚C for 2 h. The membranes were pre-hybridized for 4 h at 42˚C (5X Denhardt's Reagent, 100 μg/ml salmon sperm DNA, 50% formamide, 5X SSC, and 01% SDS) (7). iNOS cDNA probes, a generous gift from S.H. Snyder and C. Lowenstein, were radiolabeled with [^{32}P]-dCTP μCi/mmol; New England Nuclear, Boston, MA) by random primer labeling (Amersham Corporation, Arlington Heights, IL). Blots were hybridized at 42˚C overnight in the pre-hybridization solution and the labeled blots were autoradiographed with Amersham Hyperfilm at -70˚C for 8 h. ß-actin levels were similarly examined and served as controls to normalize RNA quantity.

Electrophoretic mobility shift assay. Isolation of nuclear extracts, sequences and preparation of oligonucleotide probes, EMSA and supershift assays were conducted as previously described (8); all antibodies used were from Santa Cruz Biotechnologies (Santa Cruz, CA). Briefly, ten million N-PEM pretreated or not with PS were activated with LPS (10 μg/ml) for 4 h prior to the extraction of nuclear proteins. Nuclear proteins (6 μg) were then incubated at room temperature with the labeled double-stranded oligonucleotides (10^5 cpm) matching the $NF\kappa B_D$ transcription factor site (8) of the iNOS promoter for 30 min prior to separation on 5% PAGE. Gels were dried, autoradiographed and exposed overnight at -80˚C. For supershifts, nuclear proteins were incubated with 2 μg polyclonal antibodies specific for each of the NFκB family proteins for 30 min at 4˚C prior to the addition of the labeled double-stranded oligonucleotides matching the NFκB site of the iNOS promoter for further 30 min at room temperature.

Western blotting. Whole cell extracts from normal peritoneal exudate macrophages untreated or treated with PS were used.

To isolate whole cell extracts, PEM were lysed using cold radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, following manufacturer's instructions) and sodium vanadate (Roche, 1 mM final concentration). Whole-cell lysates were then cleared by centrifugation (30 min at 13,000 rpm) and the supernatants containing total cellular proteins were stored at -80˚C. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL), and Western blotting was performed as described (9,10), using 20 μg protein per lane. Bands were visualized using the Supersignal West Pico chemiluminescent substrate (Pierce), and blots were exposed to BioMax autoradiographic film (Kodak, Rochester, NY). The primary antibody used (against NFκB p65) was a rabbit anti-mouse polyclonal from Santa Cruz Biotechnologies, and the presence of actin was detected by the use of a rabbit anti-mouse polyclonal antibody (Sigma-Aldrich, St. Louis, MO). For the mouse monoclonal antibody utilized, secondary antibody was a goat anti-mouse isotype matched to the corresponding primary antibody.

Densitometric quantitation of blots. Images were scanned on a Howtek Scanmaster 3$^+$ scanner (Howtek, Inc. Hudson, NH). Band intensity was assessed on a Sun Sparc station 5 computer (Sun Microsystems Computer Corp., Mountain View, CA) utilizing BioImage whole band analyzer software (BioImage Systems Corp., Ann Arbor, MI). In both Western and Northen blot analyses, band intensity was normalized to control ß-actin signals to account for any differences in total protein or RNA content for each sample. Band intensity was reported in relative densitometric units. The units were calculated as a fraction relative to the maximum sample density for each blot which was provided an arbitrary value of one.

Measurement of intracellular calcium mobilization. The mobilization of intracellular calcium was done following a previously described method (11) with modifications. Briefly, experimental macrophages were washed twice with HBSS without calcium, resuspended in loading buffer (HBSS, 1 mM $MgSO_4$, 10 μM indo-1/AM, 0.08 pluronic F-127, and 20 mM HEPES, pH 7.4) at 3x10^5 cells/ml and incubated at 37˚C for 45 min. The cells were then pelleted and resuspended in loading buffer without indo-1/AM and pluronic F-127 to be further incubated for 20 min. Then, the cells were washed twice and suspended in working medium (HBSS, 1 mM $MgSO_4$, and 20 mM Hepes, pH 7.4) at 3x10^6 cells/ml. Oscillation of $[Ca^{2+}]i$ was assessed as changed in the 405 nM (emission wavelength of calcium-bound indo-1)/485 (maximum emission of calcium-free indo-1) fluorescence ratio (indo-1 ratio). Cells (200 μl) were challenged with 10 μg/ml of LPS, 3.0 μM A23187 or ionomycin, 50 U/ml of IFN-γ, and analyzed on a FACStar plus (Beckton Dickinson, San Jose, CA). Indo-1 ratio values were collected and plotted as linear histograms.

Detection of tyrosine phosphorylation by Western blotting. PEM lysates for detection of tyrosine phosphorylation were prepared as descried for iNOS protein determination, and a protocol previously described was followed with minor modifications (12). Briefly, protein (40 μg) was separated on 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. Filters were blocked with 5% BSA in washing buffer (T-TBS:20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and then probed for 1 h at room temperature with recombinant IgG anti-phosphotyrosine antibody conjugated to horseradish peroxidase (1:1000 in blocking buffer). Tyrosine phosphorylated proteins were detected with an ECL kit (Amersham Corporation).

Protein kinase C assay. The activity of membrane protein kinase C was assayed by minor modifications of published methods (13). Adherent PEM (30x10^6) in 20 ml culture media were incubated in the presence or absence of PS (30, 130 or 160 μg/ml) for 18 h. The cells were washed twice with cold HBSS, and incubated for 7 min in 10 ml HBSS in the presence or absence of stimulators as stated in Results. The reaction was quenched by adding a 4-fold excess of ice cold buffer, and the cell monolayer was washed with cold HBSS. The macrophages were scraped into 240 μl of extraction buffer (50 mM Tris, pH 7.5, 0.15 mM EGTA, 2.0 mM EDTA, 2.0 mM PMSF, and 20 mM DTT). The cell suspension was centrifuged at 100,000 x g for 60 min (4˚C) and the supernatant was discarded. The remaining pellet was then resuspended in 240 μl of extraction buffer plus 0.1% Triton X-100 and incubated at 4˚C for 1 h with gentle agitation. When the incubation period was completed, the suspension was centrifuged at 100,000 x g for 1 h (4˚C), and the supernatant was collected and labeled as membrane or particulate PKC. This fraction was diluted prior to the assay with diluting buffer (25 mM Tris, pH 7.2, 0.15 M NaCl and 50% glycerol) to reach 0.02% final concentration of Triton X-100. Concentrations of Triton X-100 >0.02% in the sample interfere with the assay method used in this experiment.

Enzyme activity was immediately determined using a colorimetric protein kinase C assay kit (Pierce). Briefly, 10 μl of samples were incubated at 37˚C for 1 h with 15 μl pre-mixed reaction mixture (25 mM Tris, pH 7.2, 0.15 M NaCl, 50% glycerol, 2 mM ATP, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$ 0.002% Triton X-100, 0.2 mg/ml PS, 0.02 mg/ml DG and 0.36 mM of myelin basic protein labeled with a fluorescent probe) and after this time, 20 μl of the reaction mixture were applied onto the affinity membrane of an individual separation unit. The membrane specifically bound the phosphorylated substrate while the non-phosphorylated substrate pass through the support when washed with binding buffer (0.1 M sodium acetate, 0.5 M NaCl, and 0.02% sodium azide, pH 5.0). The phosphorylated myelin basic protein was then eluted from the membrane with elution buffer (0.1 M ammonium bicarbonate, pH 8.0, containing 0.02% sodium azide) and quantitated by measuring its absorbance at 570 nm. Known protein kinase C concentrations were used to generate a standard curve, which was used to determine the protein kinase C activity of unknown samples. In order to eliminate the phosphorylating contribution of other kinases that are not members of the protein kinase C isoforms, we assayed PKC activity in the presence and absence of PS in the reaction mixture. Protein kinase C activity was then defined as follows: PKC activity (PS present in reaction mixture) - PKC activity (PS absent in reaction mixture).

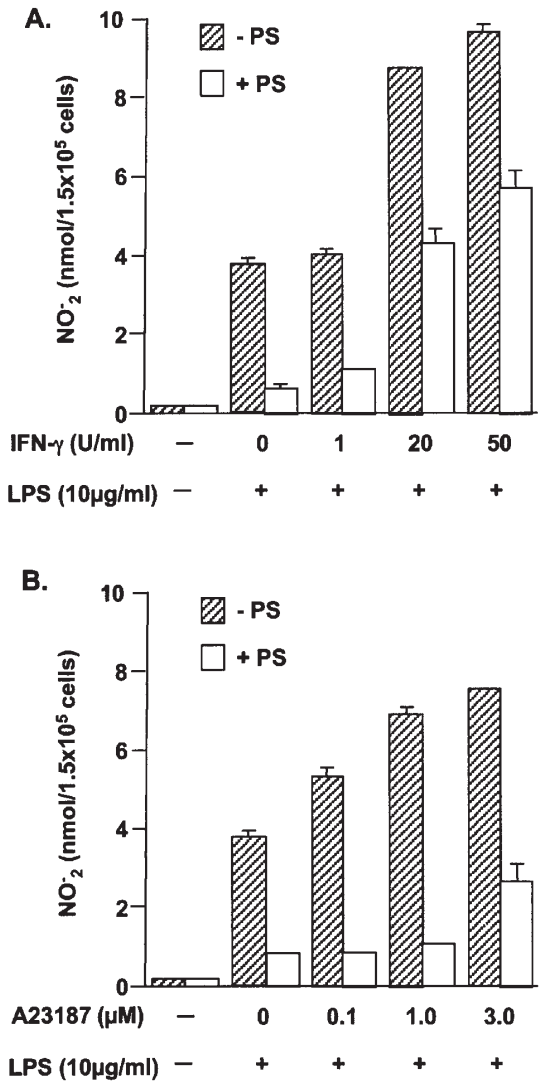

Figure 1. Inhibitory effect of PS on the production of NO_{2^-} by macrophages. Macrophages from normal mice (1.5x10^5/well) were incubated for 24 h in plain medium or medium containing 160 μg/ml PS. The macrophage cultures were further incubated for 24 h with fresh medium supplemented with LPS (10 μg/ml) alone or in combination with either IFN-γ (A) or A23187 (B). The supernatants were collected and NO_{2^-} was measured as described in Materials and methods. Values represent mean ± SE of triplicate wells from 3 separate experiments.

Detection of PKC isozymes by Western blotting. Membrane PKC was extracted as described for the protein kinase C assay. Protein (58 μg) from each lysate were added per well of a 10% SDS-PAGE gel. The proteins were transferred to nitrocellulose, blocked for 1 h with 5% BSA in PBS-T, washed 3 times with PBS-T, and then probed for 1 h at room temperature with dilutions of IgG monoclonal anti-PKC isozymes (Transduction Laboratories) in PBS-T, 5% BSA as follows: α (1:5000), ß (1:2500) γ (1:250), ε (1:250), ζ (1:500), λ (1:250), and δ (1:1000). Positive controls provided by Transduction Laboratories, and molecular markers were used to identify the proper PKC isozyme. After washing, the membranes were incubated for 1 h at room temperature with polylconal anti-mouse horseradish peroxidase conjugate (1:000). Blots were detected with an ECL system (Amersham Corporation).

Results

Partial reversal of the depressed levels of NO in PS-pretreated macrophages activated with LPS by adding IFN-γ or the calcium ionophore A23187. We have previously shown that the decreased NO production observed in LPS-stimulated macrophages from tumor-bearing mice could be completely overcome by the stimulation of LPS in conjunction with high levels of IFN-γ (3,7). PS-pretreated macrophages have lower levels of NO than untreated macrophages upon activation with LPS. Activation of untreated macrophages with 10 μg/ml LPS and increasing amounts of IFN-γ, promoted higher levels of NO production in a concentration-dependent manner. In PS-pretreated macrophages, the levels of NO release are downregulated even in the presence of high levels of IFN-γ (Fig. 1A). To obtain further information about the differences in the synergistic action of IFN-γ in untreated and PS-pretreated macrophages, we replaced IFN-γ with the calcium ionophore A23187, an agent known to cooperate with LPS in the production of NO by macrophages (14,15). In Fig. 1B it can be seen that addition of 0.1, 1.0 or 3.0 μM A23187 in combination with LPS to untreated macrophages resulted in higher levels of NO in parallel to increasing levels of calcium ionophore. However, pretreatment of macrophages with 160 μg/ml PS before activation, resulted in downregulated levels of NO production, even in the presence of increasing amounts of A23187. In the absence of LPS, A23187 was unable to induce NO production in either untreated or PS-pretreated macrophages (data not shown). Comparison of the data presented in Fig. 1A and B indicate that IFN-γ synergizes with LPS more efficiently than A23187 for the synthesis of NO.

PS reduces iNOS-mRNA levels. To evaluate whether the decreased NO production observed in pretreated macrophages was a consequence of alterations of iNOS mRNA expression, we performed Northern blot analyses. The results shown in Fig. 2 revealed that in untreated macrophages, both IFN-γ and A23187 synergized with LPS in the induction of iNOS-mRNA. The amplification of the signal induced with LPS plus IFN-γ was stronger than that observed with LPS plus A23187. Importantly, in all cases in which macrophages were activated to produce iNOS-RNA, their RNA levels were downregulated when macrophages were pretreated with PS before activation.

The binding activity and the expression of NFκB in macrophages are not altered by pretreatment with PS. Binding of NFκB to the $NF\kappa B_D$ site in region I to the iNOS promoter has been shown to be crucial for the LPS-induced iNOS expression (7). Nuclear extracts from untreated or PS-treated macrophages stimulated by LPS were tested for their potential to bind the transcription factor NFκB using electrophoretic mobility shift assays (Fig. 3A). Competition experiments with cold NFκB and irrelevant Oct-1 binding sites showed the specificity of the binding to the NFκB site. The results of our experiments showed that NFκB binding of LPS stimulated macrophages from normal mice is not modified by pretreatment with PS. In further analyses we evaluated the expression of NFκB p65, one of the main NFκB family members with

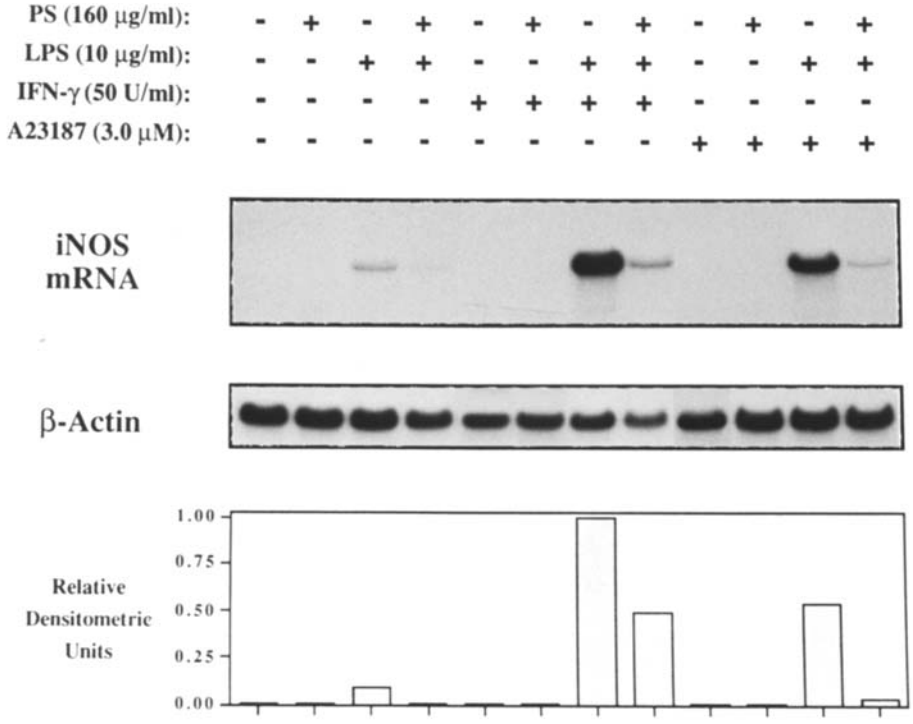

Figure 2. Effect of PS on the iNOS mRNA levels in macrophages. Macrophages from mice were pretreated for 24 h with medium containing 160 μg/ml PS. After this time, the macrophage cultures were further incubated with plain medium or medium supplemented with LPS alone or in combination with either IFN-γ or A23187. Total cellular RNA was isolated and the levels of iNOS mRNA were assessed by Northern blot analysis as described in Materials and methods. Blots were autoradiographed for 8 h. Following densitometric analysis, iNOS levels were normalized to their respective ß-actin controls and presented in the accompanying histograms as densitometric units relative to the maximum iNOS signal for this blot. Results are representative of 2 different experiments.

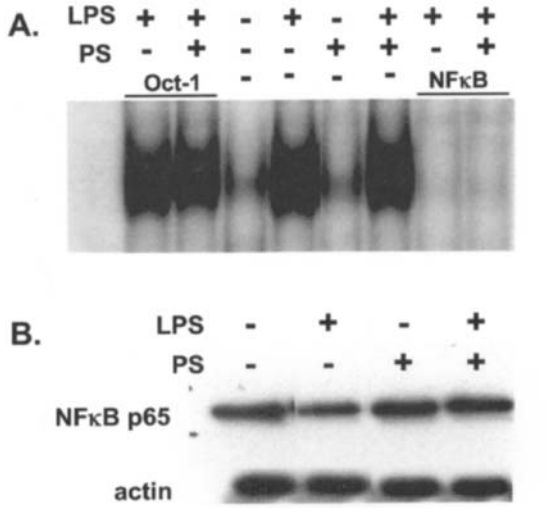

Figure 3. The lack of effect of PS-pretreatment on NFκB binding activity and expression. Macrophages from mice were pretreated for 24 h with medium alone or with 160 μg/ml of PS. (A) Cells were further treated with LPS for 4 h and nuclear extracts were tested for NFκB binding activity to the iNOS murine promoter; specificity was obtained by competition with either non-radioactive NFκB or irrelevant transcription factor Oct-1. (B) Determination of the levels of NFκB p65 protein by Western blotting in untreated or PS-pretreated macrophages. In each case, results are representative of 3 different experiments.

transactivational activity, after PS treatment using Western blots. As seen in Fig. 3B no changes were observed in the NFκB protein levels of PS-treated macrophages in comparison to those observed in PS-untreated macrophages.

Effect of activating agents on intracellular calcium mobilization in untreated and pretreated macrophages. Variations in the $[Ca^{2+}]i$ of macrophages may regulate the induction of iNOS (15). Thus, an investigation of the potential of LPS, IFN-γ, and A23187 to promote internal calcium mobilization in untreated and PS-pretreated macrophages was undertaken. When 10 μg/ml of LPS were added to either untreated or PS-pretreated macrophages, the indo-1 ratio (as indicator of fluctuations in internal calcium concentrations) remained stationary at the base line in both types of cultures (Fig. 4A). Similar results were obtained when either 1 μg/ml or 100 μg/ml LPS were used to stimulate the cells (data not shown). These findings indicate that under our experimental conditions, LPS does not promote changes in $[Ca^{2+}]i$. The indo-1 ratio in control macrophages activated with 50 U/ml IFN-γ (Fig. 4B), experimented a gradual enhancement of peak amplitude followed by a slow response decay.

In the case of PS pretreated macrophages, IFN-γ generated a similar fluorescence trace, however, the amplitude of the peak was considerably decreased. This lowering in the fluorescence intensity is indicative of a reduced accumulation of $[Ca^{2+}]i$ in the cells. Similarly, when control macrophages were challenged with A23187, an immediate and substantial rise in the indo-1 ratio was observed before declining (Fig. 4C). Pretreatment of macrophages with PS reduces the increase in $[Ca^{2+}]i$ promoted by A23187, in a more dramatic way than that observed with IFN-γ. Similar responses as those shown in Fig. 4B and C were observed when LPS plus IFN-γ and LPS plus A23187 were respectively used (data not shown). These results, together with those shown in previous figures,

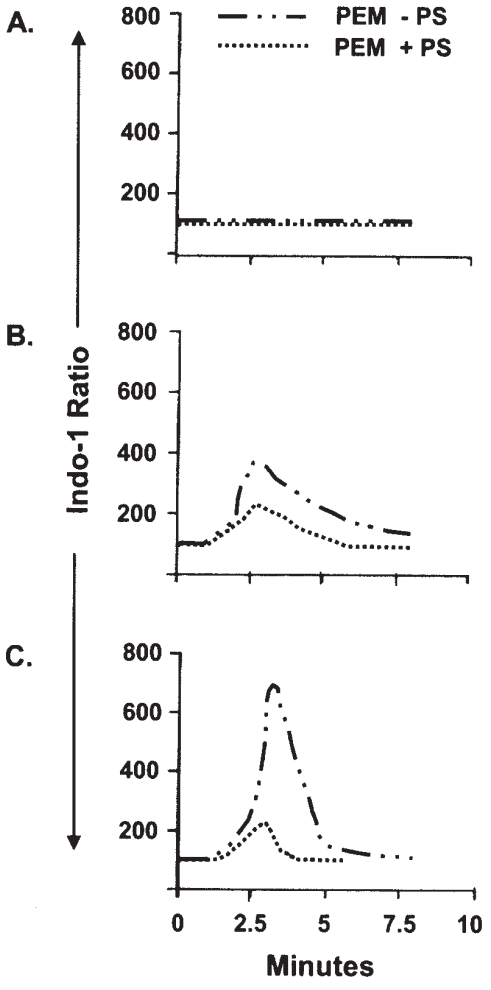

Figure 4. Effect of PS on the intracellular calcium mobilization in macrophages. Untreated or pretreated (PS, 160 μg/ml) macrophages were loaded with indo-1. At time zero, 10 μg/ml LPS (A), 50 U/ml IFN-γ, (B) or 3.0 μM A23187 (C) were added to the macrophage suspension and $[Ca^{2+}]i$ was monitored as described in Materials and methods. Indo-1 ratio represents changes in the 405 nm (emission wavelength of calcium-bound indo-1)/485 (maximum emission of calcium-free indo-1). Results are representative of 3 different experiments.

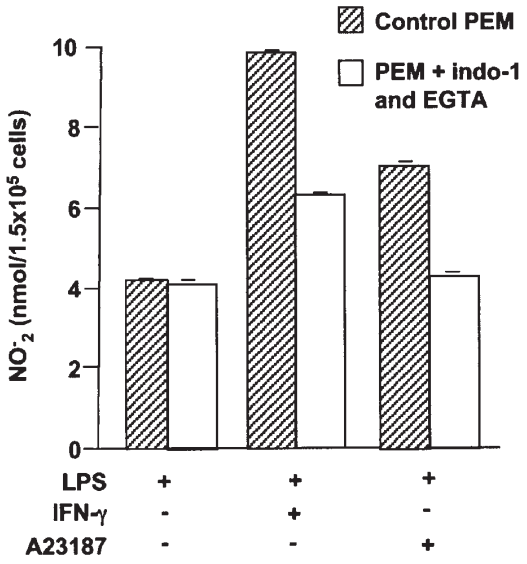

Figure 5. Calcium requirements for the induction of NO production by macrophages. Macrophages were loaded with indo-1 and cell cultures were incubated for 24 h with medium containing 3 μM EGTA and supplemented with 10 μg/ml LPS alone or in combination with 50 U/ml IFN-γ or 3.0 μM A23187. NO_2–levels in the macrophage supernatants were determined as described in Materials and methods. Results are representative of 3 different experiments.

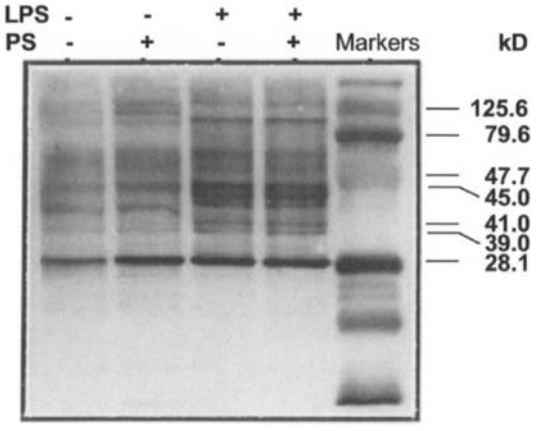

Figure 6. Protein-tyrosine phosphorylation in untreated or PS-pretreated macrophages upon activation with LPS. Macrophages from mice were pretreated for 24 h with medium alone or supplemented with 160 μg/ml PS. The macrophage cultures were further incubated for 15 min with 10 μg/ml LPS. Total cellular protein was separated on 12.5% SDS-PAGE, transferred onto nitrocellulose membranes, and probed with recombinant IgG anti-phosphotyrosine antibody conjugated to horseradish peroxidase (1:1000 in blocking buffer as described in Materials and methods). Tyrosine phosphorylated proteins were detected with an ECL kit. Results are representative of 4 different experiments.

suggest that elevation of $[Ca^{2+}]i$ is not required for the LPS-induction of iNOS. In addition, it appears that an increase in $[Ca^{2+}]i$ triggered by the co-activators IFN-γ or A23187, is involved in the amplification of the macrophage response to LPS.

Involvement of calcium in the induction of NO production by macrophages. In further experiments, we reduced internal and external calcium concentrations in macrophages before they were subjected to activation. Macrophages were loaded with 10 μM indo-1/AM to chelate-free internal calcium. To augment this effect, the cells were cultured in calcium-free media supplemented with 3 μM EGTA. Fig. 5 shows that both control and calcium-deprived macrophages produced similar levels of NO upon activation with LPS. However, the cooperative effect of IFN-γ or A23187 in the induction of NO by LPS was partially diminished in both cases. These results demonstrate that induction of NO production by LPS-stimulated macrophages does not require an elevation of $[Ca^{2+}]i$. However, the amplification of NO production in macrophages activated with LPS and co-activators could be explained at least partially by an increase of $[Ca^{2+}]i$ (Figs. 4B and C, and 5).

Pretreatment of macrophages with PS does not inhibit protein-tyrosine phosphorylation in response to LPS. It has been reported that inhibition of protein-tyrosine phosphorylation by incubation of macrophages with protein tyrosine kinase

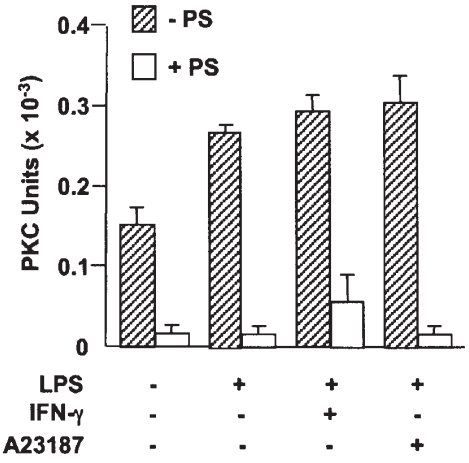

Figure 7. Effect of PS on the membrane PKC activity of macrophages. Untreated or PS (160 μg/ml) pretreated macrophages were incubated for 7 min with plain medium or medium supplemented with 10 μg/ml LPS alone or in combination with either 50 U/ml IFN-γ or 3.0 μM A23187. Membrane PKC was extracted and its activity was assessed using a PKC assay kit as described in Materials and methods. Values represent the mean ± SE of triplicate samples from 3 separate experiments.

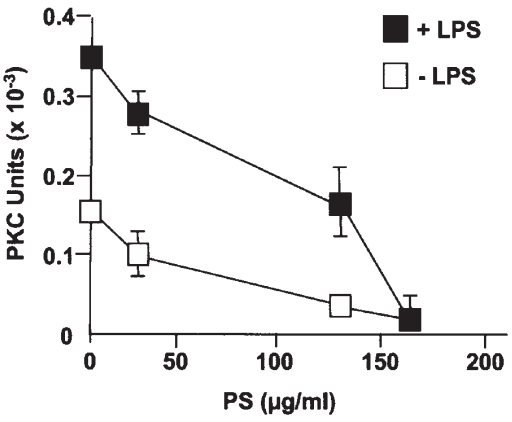

Figure 8. Concentration-dependent inhibitory effect of PS on the membrane PKC activity of macrophages. Untreated and PS-pretreated macrophages were activated for 7 min with μg/ml LPS. Membrane PKC was extracted and assessed as described in Materials and methods. Values represent the mean ± SE of triplicate samples from 3 different experiments.

inhibitors reduces their cytocidal properties (16) and release of NO (17) in response to LPS alone or in combination with IFN-γ. In particular, phosphorylation of tyrosine residues in proteins with molecular weight 39, 41, and 45 seems to play an important role in the activation of macrophages (12). To determine whether pretreatment of macrophages with PS downregulates protein-tyrosine phosphorylation induced by LPS, the lysate of these cells were subjected to Western blot analysis by using specific phosphotyrosine antibodies. Fig. 6 shows that intensity and pattern of protein-tyrosine phosphorylation in untreated and PS-pretreated macrophages were similar. Activation of untreated macrophages with LPS enhance and promote further protein tyrosine phosphorylation, including p39, p41, and p45. The same result was observed when macrophages were pretreated with PS before LPS activation.

PKC activity is inhibited in macrophages pretreated with PS. A number of studies have shown a significant correlation between PKC activation and tumoricidal properties of macrophages upon LPS stimulation (18-20). We tested whether membrane PKC activity was altered in PS-pretreated macrophages. Fig. 7 shows that membrane-bound PKC is constitutively activated in macrophages, and it is upregulated upon activation with LPS. In macrophages pretreated with PS whether activated with LPS or not, membrane-bound PKC activity was substantially inhibited. Fig. 7 also shows that combinations of LPS with the co-activators A23187 or IFN-γ did not significantly increase membrane PKC activity, as compared to the levels observed in untreated macrophages activated with LPS alone. However, PS pretreatment resulted in a profound decrease in PKC activity even when IFN-γ or the calcium ionophore were added in conjunction with LPS. The correlation between inactivation of PKC and the inhibition of NO production by macrophages pretreated with PS was further examined. Since in a previous study we showed that LPS-activated macrophages pretreated with increasing concentrations of PS had reduced levels of NO in a concentration-dependent manner (4), we evaluated whether these increases in PS concentration parallel the levels of membrane-bound PKC inactivation. Fig. 8 shows that the degree of inhibition of membrane-bound PKC activity in both unstimulated or LPS stimulated macrophages increases as a function of higher levels of PS.

The amounts of PKC isozymes are not altered in PS pretreated macrophages. A plausible explanation for the inhibition of membrane-bound PKC activity seen in macrophages pretreated with PS is that the expression of one or more PKC isozymes could have been diminished under these conditions. To investigate this possibility, we performed PKC-specific Western blot analyses of membrane lysates derived from untreated and PS-pretreated macrophages activated with LPS. These analyses included both calcium-dependent or conventional PKC (cPKC) and calcium-independent, including both novel PKC (nPKC) and atypical PKC (aPKC) isozymes (21). Fig. 9 shows that the levels of membrane cPKC isozymes (α, ß, and γ), nPKC isozymes (ε, and δ) and aPKC isozymes (ζ and λ) derived from untreated macrophages were not significantly different from those of PS-pretreated macrophages as estimated by the relative optical density of their blots. These results indicate that the downregulation of membrane PKC activity in macrophages pretreated with PS does not appear to be due to the downregulation in the expression of one or more PKC isozymes.

Discussion

Our previous studies have shown that upon stimulation with LPS, PEM from mammary tumor-bearing mice display a diminished ability to produce NO and lyse tumor targets (3). In contrast, when these cells are stimulated with LPS in combination with IFN-γ, they perform these functions at normal levels. Kinetic studies revealed that these defects became more pronounced with tumor progression. Since the

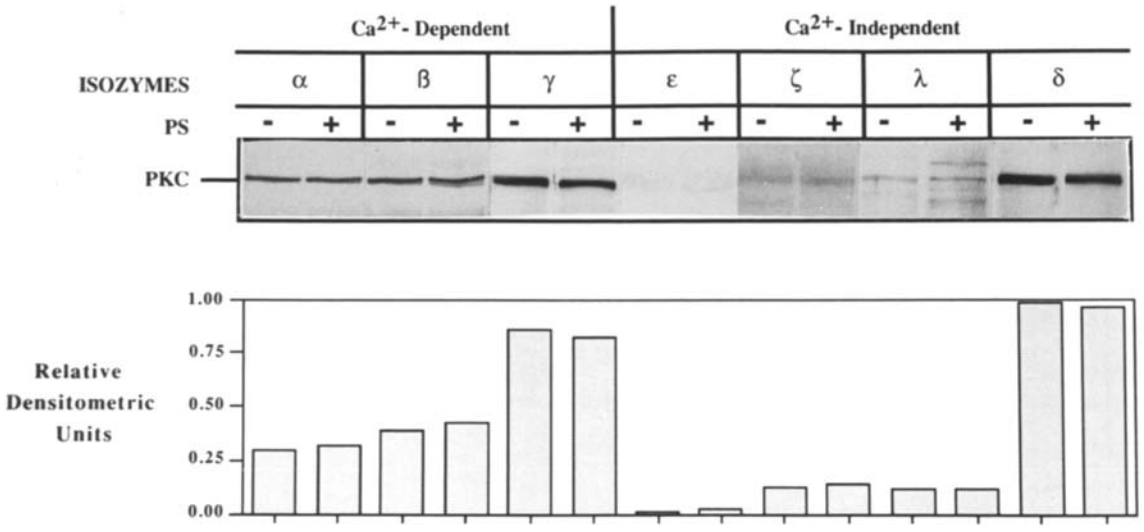

Figure 9. Levels of PKC isozymes in the membrane of macrophages either untreated or pretreated with PS. Macrophages were pretreated for 24 h with medium alone or supplemented with 160 μg/ml PS. Then, the cultures were activated for 7 min with 10 μg/ml LPS. Membrane PKC was isolated and the levels of PKC isozymes were assessed by Western blot analysis with specific anti-PKC antibodies as described in Materials and methods. Levels of membrane PKC isozymes were quantitated with a densitometer, and the results are shown in the histogram as densitometric units relative to the maximum PKC isozyme signal obtained for these blots.

mammary tumor used in these studies is known to produce PGE2 (22), granulocyte-macrophage CSF (GM-CSF) (23) and phosphatidyl serine (4), we evaluated the effects of these products on NO production and cytolytic activity. Pretreatment of normal PEM with PGE2 or recombinant GM-CSF had negligible effects on NO production and cytolytic capacity (24). In contrast, phosphatidyl serine caused a concentration-dependent inhibition of these functions in response to LPS, which could be partially overcome by the addition of IFN-γ.

The activation of macrophages is a complex cellular process in which the participation of specific kinases and cross-talking among them result in the production of a number of cytokines (25). In this regard, our unpublished observations and the work published by other investigators (17,18), provide evidence that inhibitors of either PKC or PTK are capable of inhibiting NO production by LPS-activated macrophages. In this study, we have shown that while PS inhibits membrane-PKC activity in LPS-activated macrophages, tyrosine phosphorylation activity remains unchanged. These data provide further evidence that LPS-induction of macrophage-iNOS requires the participation of both PKC and PTK. Thus, failure of either type of kinases to be activated will result in the inhibition of NO production by macrophages. A number of PKC isozymes have been implicated in this process. For instance, the ß, ε, and δ (26), and the ßII, ε, and ζ PKC (20) isozymes have been found to be constitutively present in thioglycollate-elicited C3H/HeN mouse macrophages, and J774 macrophage cell line respectively. Our results confirm a constitutive PKC activity in peritoneal elicited macrophages from BALB/c mice.

The partial recovery of NO production by PS-pretreated macrophages activated with LPS and IFN-γ implies that IFN-γ may not require the participation of PKC to enhance the message originated by LPS. Alternatively, the PKC check point may be bypassed in cells receiving the dual stimulation. Regardless of the transducing pathway followed by IFN-γ, it is known that this cytokine activates the IFN regulatory factor 1 (IRF-1) which binds directly to the IFN-regulatory factor element (IRF-E) in the iNOS promoter (27). In this manner, the LPS signal may be amplified by addition of this cytokine. Another aspect of the synergistic effect that IFN-γ exerts on macrophage-iNOS induction by LPS is related to calcium mobilization. Thus, a decrease in the intensity of $[Ca^{2+}]i$ promoted by IFN-γ was observed in PS-pretreated macrophages activated with LPS and IFN-γ as compared to that of untreated macrophages. Our data suggest that this decline in $[Ca^{2+}]i$ may be responsible for the lesser effectiveness of IFN-γ to synergize with LPS in the production of NO by PS-pretreated macrophages. These results correlate with the decline of the levels in iNOS-mRNA that we observed. In this regard, it is worth pointing out that calcium signals are known to influence gene transcription by modulating the function of various transcription factors (28). The mechanisms by which PS downregulates $[Ca^{2+}]i$ in macrophages activated with IFN-γ may be related to the inhibition of PKC activity by PS. This statement is supported by the recognized regulatory activity of PKC on calcium mobilization in a number of cells (29-31).

Taking into account our results and the evidence found in the literature, it is possible to explain the likely modulation of macrophage iNOS induction by PS. Upon activation of untreated macrophages with LPS, calcium mobilization is not promoted. Therefore, calcium independent PKC isoforms (nPKC) are the most likely candidates to be activated; yet, we were unable to show any differences in their expression levels upon PS treatment. Thus, total PKC activity is diminished by PS, but not due to a decreased expression of the different PKC isozymes. Moreover, LPS stimulates protein tyrosine phosphorylation. Activation of both PKC and PTK by LPS may in turn switch on specific kinases, leading to the activation of transcription factors responsible for the transcription of iNOS genes (17,18), one of which is

NFκB (8). Inhibition of either PKC or PTK activity may block this iNOS inductive process. However, PS does not seem to modulate PTK activity in macrophages. Thus, in PS-pretreated macrophages, PKC but not PTK activity is diminished, bringing about the inhibition of iNOS induction. PKC is known to activate IκB kinase and NFκB in T lymphocytes (32). Therefore, it is interesting that the PS-induced decrease in PKC activity observed in macrophages does not result in diminished NFκB activity in these cells. It is likely that in macrophages, PKC is not an upstream regulator of NFκB activity, as occurs in T cells. Alternatively, in PS-treated macrophages, other factors might directly or indirectly compensate, and other pathways may account for normal NFκB activity. Our results suggest a more dynamic participation of PS as a regulator of PKC activity, possibly acting as a second messenger in a variety of cell responses.

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