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The Role of Tyrosine Phosphorylation in Lipopolysaccharide- and Zymosan-Induced Procoagulant Activity and Tissue Factor Expression in Macrophages

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The expression of surface procoagulants by exudative macrophages represents an important mechanism underlying local fibrin deposition at sites of extravascular inflammation. The present studies investigated the contribution of tyrosine phosphorylation to the generation of macrophage procoagulant activity (PCA) and tissue factor expression in response to proinflammatory stimuli. Both lipopolysaccharide (LPS) and zymosan rapidly stimulated tyrosine phosphorylation in elicited murine peritoneal macrophages. This effect was prevented by the tyrosine kinase inhibitors genistein and herbimycin and augmented by the addition of the phosphotyrosine phosphatase inhibitor vanadate. The vanadate-mediated rise in phosphotyrosine accumulation was abrogated by the use of diphenylene iodonium, an inhibitor of the respiratory burst oxidase, suggesting a role for peroxides of vanadate as contributors to the tyrosine phosphorylation. This notion was supported by the finding that vanadyl hydroperoxide markedly increased the accumulation of phosphotyrosine residues. To define the role of tyrosine phosphorylation in the induction of macrophage PCA by LPS, the effects of tyrosine kinase inhibition by genistein and herbimycin were investigated. Both agents inhibited the expression of macrophage PCA. Further, Northern blot analysis with the cDNA probe for murine tissue factor indicated that the inhibition occurred at the mRNA level or earlier. Since vanadate augmented phosphotyrosine accumulation, it was hypothesized that it might enhance generation of macrophage products. However, vanadate reduced induction of PCA in response to LPS. By contrast, vanadate augmented basal prostaglandin E_2 (PGE_2) release and stimulated PGE_2 release by macrophages. Indomethacin prevented the increase in PGE_2 but only partially restored normal levels of PCA. The effect of vanadate on tissue factor expression appeared to be posttranscriptional. These studies thus demonstrate, by functional Western blotting and Northern blotting techniques, that tyrosine phosphorylation plays a role in the regulation of macrophage PCA and tissue factor expression in response to proinflammatory stimuli.

Fibrin deposition is a ubiquitous response to inflammation. Studies have implicated a role for macrophage-mediated coagulation (i.e., procoagulant activity [PCA]) in the pathogenesis of a variety of pathological processes. For example, the accumulation of fibrin in delayed-type hypersensitivity (DTH) responses is responsible for the induration observed at these sites (14, 15). Fibrin deposition in these lesions is mediated by the influx of macrophages and subsequent expression of the surface procoagulant, tissue factor (TF). Antibodies inhibiting the interaction between TF and factor VII/VIIa prevent the development of the DTH response by precluding the initiation of the coagulation cascade (35). Extravascular coagulation also appears to contribute to the development of intra-abdominal abscesses containing a mixed bacterial flora. Reduction in local fibrin deposition effected by the use of anticoagulants or fibrinolytics or, alternatively, by systemic defibrinogenation abrogates abscess formation in part by augmenting bacterial clearance by host phagocytic cells (12, 25, 54, 65). While gram-negative enteric bacteria are known to induce macrophage PCA, both in vivo and in vitro, (1, 2, 61) the cellular mechanisms leading to the expression of PCA remain incompletely defined.

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Lipopolysaccharide (LPS) is an integral component of the cell wall of gram-negative enteric bacilli. It is a potent stimulus for the expression of macrophage PCA as well as for the release of other macrophage-derived mediators such as tumor necrosis factor (TNF), both in vitro and in vivo (4, 55). LPS binds to the CD14 receptor on the surface of macrophages after associating with the LPS-binding protein (LBP) (69, 82) or the septin complex (81). The contribution of the various components of the signalling pathway to the ultimate effector functions of macrophages has been studied extensively. Studies with pertussis toxin have implicated a role for guanine nucleotide-binding proteins in mediating LPS-stimulated cell activation (19, 36, 83). Inhibitors of Ca^{2+} and calmodulin cause variable effects on the transcription of mRNAs for a number of cytokines (39, 59), while agents which impair phospholipase A_2 activity have been shown to block the induction of TNF transcripts (56). Initial studies examining the ability of LPS to induce protein phosphorylation suggested that this event was mediated via protein kinase C (PKC) (60, 80). However, subsequent studies have indicated no significant increase in PKC activity following cell stimulation with LPS (22). Further, inhibitors of PKC have varied effects on the ability of LPS to stimulate the production of mediator molecules by macrophages or to effect macrophage priming (10, 11, 39, 40, 62), suggesting the existence of PKC-independent signalling pathways in these cells.

Recent studies have implicated a role for tyrosine phosphor-

ylation in the generation of mediator molecules by macrophages in response to a variety of stimuli (3, 5, 21, 22, 27, 66, 67, 76, 77). For example, tyrosine phosphorylation is required for induction of several cytokines including TNF, interleukin-1, and interleukin-6 following stimulation of monocytes by LPS, as evidenced by the ability of tyrosine kinase inhibitors to abrogate release of these products (3, 27). The early signalling mechanisms contributing to LPS-induced tyrosine phosphorylation have, in part, been characterized. Although CD14, the glycosyl-phosphatidylinositol-linked protein receptor for the LPS–LPS-binding protein complex, has no intrinsic tyrosine kinase activity (33), there is physical coupling and activation of one member of the *src* family of tyrosine kinases, *lyn*, and activation of two others, *hck* and c-*fgr*, following LPS treatment (70). Studies have also shown that tyrosine kinase inhibitors prevent activation of the transcription factor NF-κB by LPS (31).

Induction of PCA in LPS-treated murine peritoneal macrophages is due to increased expression of TF, a 47-kDa surface expressed glycoprotein (50). This process is regulated both transcriptionally and posttranscriptionally (7, 28). The promoter region of the murine TF gene has been shown to contain consensus binding sequences for Sp1, egr-1, AP-1, and NF-κB. Functional analysis of the promoter region indicates that ligation of both the AP-1 and the NF-κB binding sequences is required for optimal gene transcription. Since tyrosine kinase inhibitors have been shown to impair NF-κB activation, it was hypothesized that these agents might impair induction of macrophage PCA and TF expression by reducing LPS-induced transcription of the TF gene.

MATERIALS AND METHODS

Animals. Six- to 8-week-old female Swiss Webster mice were obtained from Charles River Laboratories. Following delivery to our animal facility, the animals were allowed to acclimatize for 2 to 4 days prior to use in these studies. Animals were then maintained in colonies of no more than five animals per cage and fed standard mouse chow and water ad libitum.

Materials. Fetal bovine serum, Hanks' balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free), glutamine, penicillin or streptomycin, genistein, and herbimycin A were from Gibco. RPMI 1640 medium, zymosan, molecular mass standards, Coomassie blue, Nonidet P-40, HEPES, rabbit brain thromboplastin, and dimethyl sulfoxide (DMSO) were from Sigma. Bisindolylmaleimide (BIM) was purchased from Calbiochem. Staurosporine was from Biomol. Sodium orthovanadate was from Aldrich Chemicals, and hydrogen peroxide was from Fisher Scientific. The monoclonal antiphosphotyrosine antibody PY20 immunoglobulin G2B (IgG2B) was obtained from ICN. The polyclonal antiphosphotyrosine antibody was from Transduction Laboratories. The goat anti-rabbit IgG and sheep anti-mouse IgG conjugated to horseradish peroxidase were from Amersham. *Escherichia coli* LPS and powdered Brewer's thioglycolate were obtained from Difco.

Solutions. Supplemented RPMI contained RPMI 1640 with 10% heat-inactivated fetal bovine serum, L-glutamine (4 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and 20 mM HEPES (pH 7.4). Zymosan and LPS were suspended in HBSS.

Cell preparation. Murine macrophages were harvested by peritoneal lavage, with 10 ml of sterile HBSS with 1 U of heparin per ml, 4 days following intraperitoneal injection of 2 ml of thioglycolate medium. Lavage fluids were pooled and centrifuged at 750 × *g* for 10 min. The cell-containing pellet was then washed in sterile HBSS and centrifuged again at 750 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 5 ml of sterile water for 15 s to lyse any erythrocytes and then diluted to 45 ml with sterile HBSS. The suspension was sedimented again, and the cells were resuspended in RPMI and then counted with a hemocytometer. The cell population was then diluted to a concentration of 1×10^6 to 2×10^6 cells per ml in supplemented RPMI and aliquoted into polypropylene tissue culture tubes. This technique generated a cell suspension with a viability in excess of 95%, as assessed by trypan blue exclusion, and a cell population of 80 to 90% macrophages, as assessed by nonspecific esterase staining, Wright's staining, and transmission electron microscopy.

Cells were stimulated for 0.5 to 4.0 h at 37°C in 5% CO_2 with either LPS or zymosan. In some experiments, cells were pretreated with inhibitors of signalling pathways as indicated in the text. Control cells were exposed to 0.1% DMSO during the pretreatment period. At the end of the incubation period, cells were sedimented by centrifugation at 750 × *g* for 10 min. Supernatants were aspirated and frozen at −70°C for later measurement of prostaglandin E_2 (PGE_2). Cell pellets were resuspended in RPMI 1640 and frozen at −70°C for measurement of PCA. For phosphotyrosine detection, cells were rapidly sedimented, and pellets were immediately solubilized in boiling Laemmli sample buffer.

Detection of tyrosine phosphorylation. Tyrosine phosphorylation was determined by immunoblotting with antiphosphotyrosine antibodies (30). Following electrophoresis on 10% polyacrylamide gels, the samples were blotted onto nitrocellulose by using the Bio-Rad Mini Trans-Blot system for 1 h at 100 V. The blot was then incubated with 10 ml of blocking solution containing the monoclonal antibody for 2 h with shaking at room temperature. The blot was then washed three times with blocking solution and incubated in 2 μCi of ^{125}I-protein A in blocking solution for 1 h at room temperature. The blots were washed and dried, and autoradiography was performed with intensifying screens at −70°C. In some experiments, electrophoresed samples were blotted onto Immobilon by using the Bio-Rad Mini Trans-Blot system for 1 h at 100 V. The blot was incubated with 10 ml of blocking solution and then exposed to a 1/1,000 dilution of the primary antibody for 2 h with shaking at room temperature. The blot was then washed three times with antibody buffer solution and incubated with a 1/5,000 dilution of anti-IgG antibody conjugated to horseradish peroxidase. The blots were washed, dried, and quantitated by using an enhanced chemiluminescence detection system (79).

Measurement of macrophage PCA. PCA in freeze-thawed macrophages was determined by measuring their capacity to shorten the spontaneous clotting time of citrated plasma from healthy humans in a one-stage clotting assay (47). An 80-μl sample of macrophage cell lysates obtained by freeze-thawing was added to 80 μl of citrated normal human platelet-poor plasma, and then 80 μl of 25 mM $CaCl_2$ was added to initiate the reaction. The time required for the appearance of a fibrin gel at 37°C with gentle agitation was recorded. Clotting times were converted to milliunits of PCA by comparison with the clotting times of a rabbit brain thromboplastin standard in which 36 mg (dry weight) per ml was assigned a value of 100,000 mU of PCA. Results of previous studies have demonstrated that LPS-induced mouse macrophage PCA is only slightly less effective in shortening the clotting time of human plasma than in shortening the clotting time of mouse plasma (43). The induction of PCA from a baseline of 300 mU/10^6 macrophages to 2,800 mU/10^6 macrophages in cells stimulated by LPS alone represented a shortening of the clotting time from 70 to 52 s. The assay was used over the range of 10 to 10,000 mU of PCA, this range being linear with a normal plasma substrate. Previous studies have shown that PCA induced with *E. coli* LPS has TF-like activities, making comparison with a thromboplastin standard valid (64).

TF mRNA induction. TF mRNA induction was assessed by Northern blot analysis with the cDNA probe for murine TF (63) (kindly provided by Michael Getz, Mayo Clinic, Rochester, Minn.). Briefly, 10×10^6 cells were pelleted, and total RNA was extracted by the method described by Chomczynski and Sacchi (13). After electrophoresis, RNA was transferred to GeneScreen and hybridized with a ^{32}P random-labelled cDNA probe for murine TF. The loading of comparable amounts of RNA between lanes was assured by probing with a cDNA probe for rat B tubulin (44) or ribosomal 18S.

Macrophage chemiluminescence. LPS- and zymosan-stimulated chemiluminescence was monitored during a 240-min period with a luminometer (Automat model LB 953; EG & G Berthold, Bad Wildbad, Germany). Chemiluminescence was integrated during this interval with software provided by the manufacturer whereby the area under the curve correlates with the release of reactive oxygen species.

Measurement of PGE_2. PGE_2 in cell supernatants was measured by using a PGE_2 ^{125}I-labelled radioimmunoassay system from Amersham International, Markham, Ontario, Canada.

Endotoxin contamination. RPMI 1640, HBSS, fetal calf serum, sterile water, sterile saline, and all other reagents were tested for endotoxin contamination by the standard *Limulus* amoebocyte lysate assay (Association of Cape Cod, Woods Hole, Mass.) and were found to contain <0.1 ng of endotoxin per ml, which constituted the lower limit of the test.

Statistics. Statistics were calculated by one-way analysis of variance and Neumann-Keuls for comparison between groups. Data are expressed as the means and standard errors for *n* observations.

RESULTS

Tyrosine phosphorylation in stimulated macrophages. Stimulation of cells with LPS (10 μg/ml) caused a time-dependent increase in phosphotyrosine accumulation in peritoneal macrophages (Fig. 1). The specificity of the PY20 IgG2B monoclonal antibody for tyrosine-phosphorylated polypeptides had been previously established by competitive displacement from the blots by phosphotyrosine but not by phosphoserine or phosphothreonine (29). Tyrosine phosphorylation occurred rapidly (as early as 2 min) and persisted for up to 4 h. The most prominent bands had molecular masses of approximately 38, 41, 47, 55, 61, 73, 80, and 102 kDa. The rapid induction of

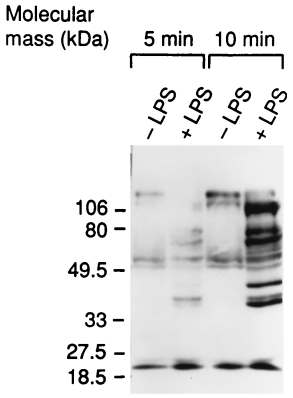

FIG. 1. Effect of LPS on phosphotyrosine accumulation in macrophages. Macrophages (2×10^6/ml) were incubated with LPS (10 μg/ml). At the indicated time points, cells were rapidly sedimented, and pellets were immediately solubilized in boiling Laemmli sample buffer. Tyrosine phosphorylation was determined as described in Materials and Methods. A representative blot is shown.

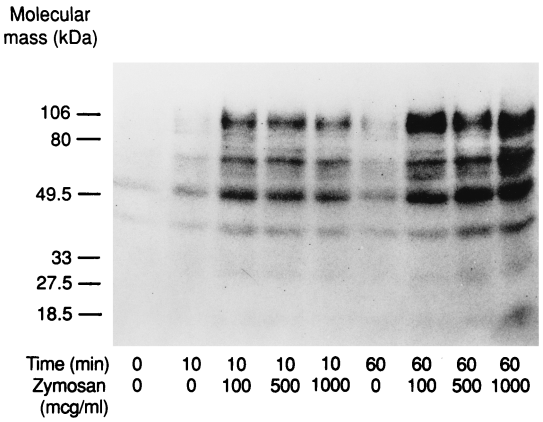

FIG. 3. Effect of zymosan on phosphotyrosine accumulation in macrophages. Macrophages (2×10^6/ml) were incubated with zymosan (0 to 1,000 μg/ml) for up to 60 min. At the indicated time points, cells were rapidly sedimented, and pellets were immediately solubilized in boiling Laemmli sample buffer. Tyrosine phosphorylation was determined as described in Materials and Methods. A representative blot is shown.

tyrosine phosphorylated proteins, particularly in the molecular mass range of 38 to 47 kDa, has previously been reported (31, 77, 78). Pretreatment of cells for 1 h with the tyrosine kinase inhibitor genistein (10 μg/ml) reduced the accumulation of phosphotyrosine (Fig. 2). Genistein also reduces baseline phosphotyrosine activation induced by thioglycolate-stimulated migration of cells into the peritoneal cavity.

Incubation of peritoneal macrophages with the particulate stimulus zymosan similarly resulted in a time- and dose-dependent increase in tyrosine phosphorylation of several proteins (Fig. 3). Tyrosine phosphorylation increased as early as 10 min and peaked at 60 to 120 min, with an optimal concentration of zymosan (100 to 500 μg/ml). The major bands had molecular masses of approximately 39, 51, 71, and 94 kDa. Several other minor bands were observed, particularly at the higher molecular masses. Preincubation of cells with genistein impaired phosphotyrosine accumulation (data not shown).

The level of phosphotyrosine accumulation represents a balance between tyrosine kinase activity and the opposing tyrosine phosphatase activity. Vanadate, an inhibitor of tyrosine phosphatase activity, was tested for its effect on tyrosine phosphorylation induced by zymosan and LPS (Fig. 4A and B, respectively). While vanadate treatment alone caused little effect on phosphotyrosine accumulation (data not shown), its addition to zymosan resulted in a marked augmentation (Fig. 4A). Similarly, vanadate stimulated phosphotyrosine accumulation in LPS-treated cells, albeit to a lesser extent (Fig. 4B). Peroxides of vanadate were recently shown to be potent inhibitors of tyrosine phosphatases (74). Since the reaction of vanadate with superoxide and hydrogen peroxide leads to the generation of peroxovanadyl and vanadyl hydroperoxide, respectively (48, 49), and macrophages are known to release these reactive oxygen species in response to various stimuli (75), studies were performed to discern whether peroxides of vanadate may be contributing to the accumulation of tyrosine-phosphorylated proteins in macrophages exposed to vanadate. The effect of diphenylene iodonium (DPI), an inhibitor of the respiratory burst oxidase (24), on phosphotyrosine accumulation in stimulated cells exposed to vanadate was studied. Figure 4C illustrates the chemiluminescent response of macrophages as a measure of the respiratory burst. Stimulation was observed with both agents but was more profound with zymosan than with LPS. DPI reduced stimulated chemiluminescence levels to below control levels. DPI also caused a slight reduction in the zymosan- and LPS-induced phosphotyrosine accumulations in the absence of vanadate. The addition of DPI abrogated the vanadate-induced increase in tyrosine phosphorylation in cells exposed to both zymosan and LPS (Fig. 4A and B, respectively). Control levels of phosphotyrosine accumulation were again below those observed in zymosan- or LPS-treated cells. In addition, vanadyl hydroperoxide, prepared by mixing equimolar concentrations of sodium orthovanadate and hydrogen peroxide, markedly enhanced phosphotyrosine accumulation (Fig. 4A). DPI had no effect on phosphotyrosine accumulation induced by vanadyl hydroperoxide, indicating that its inhibition of tyrosine phosphorylation by zymosan in the presence of vanadate was unlikely to be artifactual.

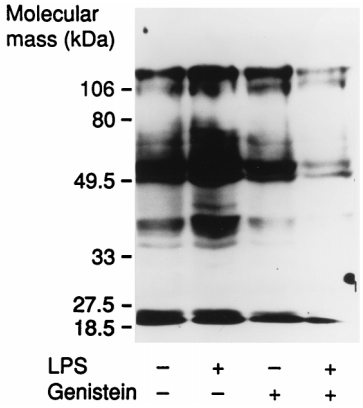

FIG. 2. Effect of genistein on LPS-induced tyrosine phosphorylation. Cells (2×10^6/ml) were preincubated with genistein (10 μg/ml) or a vehicle (0.1% DMSO) for 1 h and then stimulated with LPS (10 μg/ml) for 240 min. Cells were then sedimented, and pellets were immediately solubilized in boiling Laemmli sample buffer. Tyrosine phosphorylation was then detected by immunoblotting as described in Materials and Methods. A representative blot is shown.

Role of tyrosine phosphorylation in induction of PCA. Inhibitors of tyrosine kinase activity were used to determine whether LPS-induced tyrosine phosphorylation participated in the signalling pathways for the generation of macrophage PCA. At concentrations shown to impair LPS-induced phosphotyrosine accumulation, both genistein (10 μg/ml with a 1-h

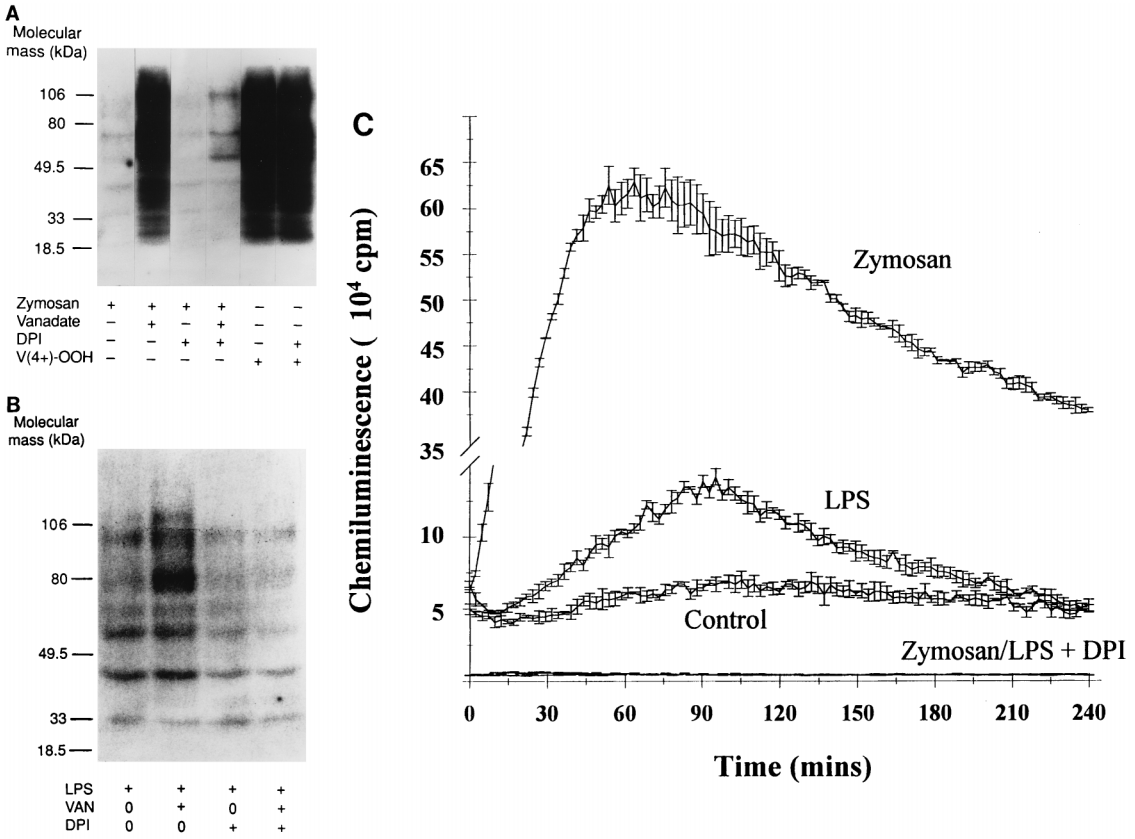

FIG. 4. (A and B) Effects of vanadate and DPI on zymosan-induced phosphotyrosine accumulation in macrophages. Macrophages (2×10^6/ml) were treated with either zymosan (100 μg/ml) for 30 min (A) or LPS (10 μg/ml) for 1 h (B) in the presence or absence of vanadate (100 μM) and DPI (10 μg/ml). Cells were then sedimented, and the pellets were immediately solubilized in boiling Laemmli sample buffer. Tyrosine phosphorylation was determined as described in Materials and Methods. Representative blots are shown. (C) Effect of DPI on zymosan- or LPS-induced chemiluminescence. Macrophages (2×10^6/ml) were stimulated with zymosan (500 μg/ml) or LPS (10 μg/ml) in the presence or absence of DPI (10 μg/ml). Cells were preincubated with DPI for 45 min. The data shown, representative of six experiments, are the means and standard errors for triplicate samples at each time point. The area under the curve correlates with the release of reactive oxygen species. The mean areas ± standard errors of the means for six experiments were 1.19 ± 0.16 (control), 1.96 ± 0.13 (LPS), 0.19 ± 0.02 (LPS plus DPI), 11.93 ± 0.06 (zymosan), and 0.11 ± 0.01 (zymosan plus DPI).

pretreatment [Fig. 5A]) and herbimycin A (1 μg/ml with a 2-hour pretreatment [Fig. 5B]) reduced PCA expression by macrophages following stimulation with LPS. Similar inhibition was observed when PCA was stimulated by the addition of zymosan (data not shown). Inhibition was not due to the use of DMSO (0.1%) as a vehicle or to cell toxicity since viability remained greater than 84% as determined by trypan blue exclusion.

PCA induced by LPS treatment of monocytes/macrophages has been shown to be due to increased surface expression of the surface glycoprotein TF. TF gene transcription has been shown to regulated by the presence of AP-1 and NF-κB binding sites in the promoter region. Since tyrosine kinase inhibitors have been shown to prevent NF-κB translocation in LPS-stimulated cells (31), it was hypothesized that their effect on PCA expression might be due to reduced TF gene transcription. To examine this possibility, Northern blot analysis of TF mRNA in LPS-treated cells was performed. Both genistein and herbimycin reduced the level of TF mRNA transcripts observed in LPS-treated cells to control levels (Fig. 6A and B, respectively).

Since vanadate augmented phosphotyrosine accumulation in stimulated cells, it was postulated that it might increase production of macrophage PCA in response to LPS. As shown in Fig. 7, vanadate (100 μM) unexpectedly reduced PCA expression by macrophages stimulated with LPS. This result was not secondary to a nonspecific effect of vanadate itself in the clotting assay as the direct addition of vanadate to LPS-stimulated cells during the clotting assay had no effect (data not shown). The mechanism underlying the vanadate-induced inhibition of LPS-stimulated PCA production was examined in further detail. Recent studies have reported a correlation between arachidonic acid release and tyrosine phosphorylation in LPS-stimulated macrophages (76). Since several products of arachidonic acid metabolism, including those of the PGE series, have been shown to impair PCA in response to LPS (17), it was hypothesized that vanadate effected inhibition by augmenting PGE_2 production. Vanadate increased basal PGE_2 release by cells (54 ± 6 pg/ml for control versus 136 ± 34 pg/ml for vanadate; $n = 6$; $P < 0.05$ [Fig. 8A]). Vanadate also increased LPS-induced PGE_2 release by ~89% (range, 8 to 273%; $n = 6$, $P < 0.05$). To determine whether this rise in PGE_2 could account for the reduction in PCA, cells were simultaneously treated with indomethacin (3 μM). This agent completely prevented the release of PGE_2 by LPS-stimulated cells. Consistent with our previous reports (40), indomethacin had no effect on LPS-stimulated PCA (Fig. 8B). In the presence of vanadate, indomethacin caused a partial restoration (~38%) of LPS-stimulated PCA (Fig. 8B). This effect was observed in all six experiments performed. To further examine

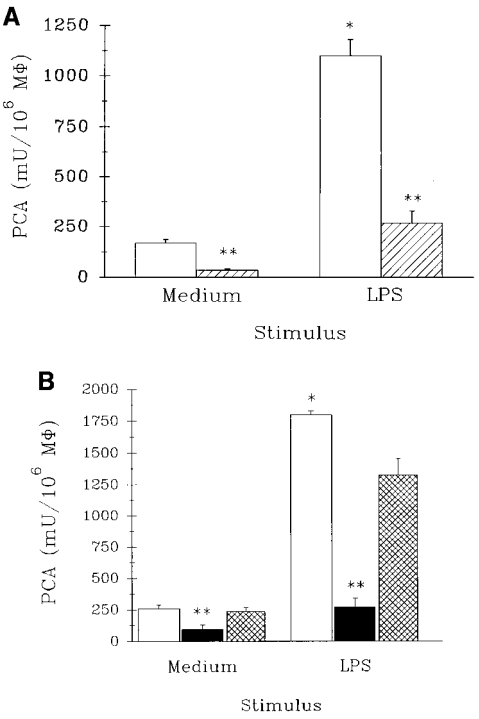

FIG. 5. (A) Effect of genistein on induction of PCA activity by LPS. Macrophages (Mϕ) (10^6/ml) were stimulated with LPS (10 μg/ml) in the presence (▨) or absence (□) of genistein (10 μg/ml) for 4 h. Cells were preincubated in genistein for 1 h prior to stimulation. At 4 h, cells were pelleted, resuspended in RPMI, and frozen at −70°C for later assay of PCA activity. The data represent the means and standard errors of the means for three separate experiments, each performed in duplicate. *, P of <0.05 versus control; **, P of <0.05 versus untreated for each group. (B) Effect of herbimycin on induction of PCA activity by LPS. Macrophages (Mϕ) (10^6/ml) were stimulated with LPS (10 μg/ml) in the presence of herbimycin (1 μg/ml) or DMSO (0.1%) for 4 h. Cells were preincubated in herbimycin for 2 h prior to stimulation. At 4 h, cells were pelleted, resuspended in RPMI, and frozen at −70°C for later assay of PCA activity. The data are the means and standard errors of the means for four separate experiments, each performed in duplicate. □, untreated macrophages; ■, herbimycin-treated macrophages; ▨, macrophages treated with 0.1% DMSO; *, P of <0.05 versus control; **, P of <0.05 versus untreated for each group.

this phenomenon, the effect of vanadate on LPS-stimulated macrophage TF mRNA was examined. The addition of vanadate to LPS-stimulated cells caused a slight increase in the level of TF mRNA compared to that of LPS alone, suggesting that the observed inhibitory effect on LPS-induced macrophage PCA is primarily posttranscriptional (Fig. 8C).

The role of PKC activation in LPS-induced PCA. Previous studies have reported an important role for PKC stimulation in the induction of monocyte/macrophage PCA by LPS (10, 73). The PKC signalling pathway may overlap with that defined for tyrosine phosphorylation in several ways. First, tyrosine phosphorylation of the γ-1 isoform of phospholipase C may lead to phosphatidylinositol hydrolysis and subsequent activation of PKC (32). Second, treatment of cells with phorbol esters may induce phosphotyrosine accumulation (30, 68). After the determination that tyrosine phosphorylation participated in the induction of PCA and TF by LPS, the relative contribution of PKC to the signalling pathway was evaluated with inhibitors of PKC activation. The specific PKC inhibitor BIM (2.5 μM) was tested for its ability to inhibit LPS-induced PCA. BIM did not inhibit PCA induction by LPS (Fig. 9A), despite its ability to totally abrogate phorbol ester-stimulated chemiluminescence (data not shown), thereby confirming its ability to inhibit PKC

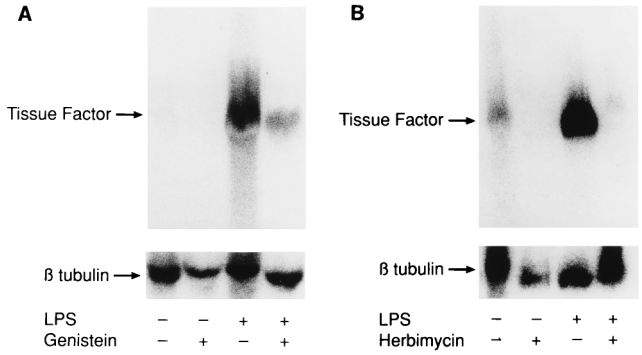

FIG. 6. Effect of tyrosine kinase inhibitors on levels of TF mRNA transcripts. Macrophages (10×10^6/ml) were pretreated with genistein (10 μg/ml for 1 h) (A) or herbimycin (1 μg/ml for 2 h) (B) and then stimulated with LPS. At 4 h, posttreatment, mRNA was extracted and subjected to Northern blot analysis with ^{32}P-labelled cDNA probes for murine TF and β-tubulin. The blots shown are representative of three similar experiments.

activation. Similarly, another PKC inhibitor, staurosporine, caused only partial inhibition of PCA induction (Fig. 9A). Thus, inhibition of LPS-induced PCA correlated more closely with a reduction in tyrosine phosphorylation than it did with the inhibition of PKC activation.

DISCUSSION

The present studies investigated the role of tyrosine phosphorylation in the induction of macrophage PCA following exposure to proinflammatory stimuli. Both LPS and zymosan induced phosphotyrosine accumulation and a rise in PCA. The functional relevance of the tyrosine phosphorylation was demonstrated by the ability of two distinct tyrosine kinase inhibitors, genistein and herbimycin, to inhibit PCA induction in murine peritoneal macrophages. Northern blot analysis of TF mRNA expression revealed that the effect of tyrosine phosphorylation occurred at the mRNA level or earlier. Considered together, these functional, Western immunoblotting and Northern blotting data support the conclusion that tyrosine phosphorylation is an important component of the signalling pathway leading to the expression of PCA and TF in stimulated murine peritoneal macrophages.

Several studies have suggested a role for PKC-dependent

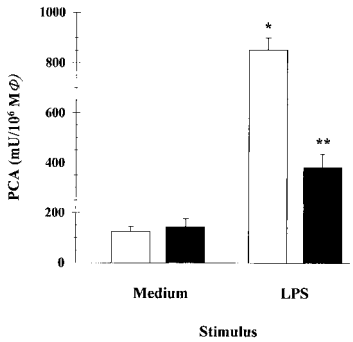

FIG. 7. Effect of vanadate on LPS-stimulated PCA activity in macrophages (Mϕ). Cells (10^6/ml) were stimulated with LPS (10 μg/ml) with 100 μM vanadate (■) or without vanadate (□) for 4 h. At 4 h posttreatment, cells were pelleted, resuspended in RPMI, and frozen at −70°C for later assay of PCA activity. The data are the means and standard errors of the means for six separate experiments, each performed in duplicate. *, P of <0.01 versus control; **, P of <0.05 versus LPS alone.

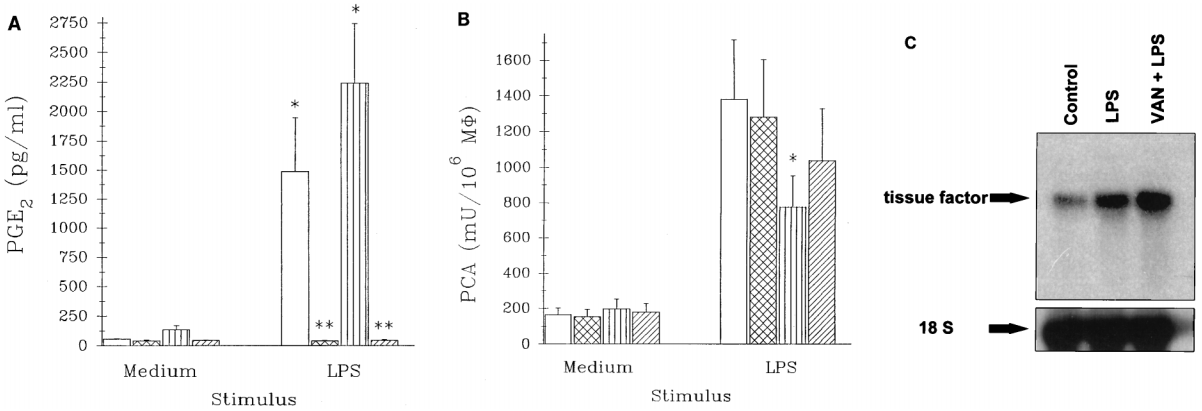

FIG. 8. (A) Effect of vanadate on PGE_2 release from macrophages. Control or LPS-stimulated macrophages were incubated with vanadate (100 μM) in the presence (▨) or absence (▥) of indomethacin (3 μM). At 4 h posttreatment, cells were sedimented, and the supernatant was aspirated for determination of PGE_2 by radioimmunoassay as described in Materials and Methods. The data are the means and standard errors of the means for five or six experiments each done in duplicate. *, P of <0.05 versus control; **, P of <0.05 versus LPS and vanadate groups. □, no treatment; ▨, indomethacin treatment alone. (B) Effect of indomethacin on LPS-stimulated PCA activity. Cells were treated as described for panel A and the cell pellets were pelleted, resuspended in RPMI, and frozen at −70°C for later assay of PCA activity. The data are the means and standard errors of the means for six experiments, each performed in duplicate. *, P of <0.05 versus LPS alone. Symbols are as described for panel A. (C) Effect of vanadate (VAN) (100 μM) on levels of TF mRNA transcripts. Cells (10×10^6/ml) were stimulated with LPS (10 μg/ml) plus vanadate (100 μM) or LPS alone for 4 h. At 4 h posttreatment, mRNA was extracted and subjected to Northern blot analysis with ^{32}P-labelled cDNA probes for murine TF and 18S. The blot shown is representative of two similar experiments.

signalling in the stimulation of monocyte/macrophage PCA by LPS. These conclusions were based on the ability of PKC agonists to stimulate PCA (10, 37, 38, 51, 52, 73) as well as the observation that various PKC inhibitors were able to abrogate LPS-stimulated PCA (10, 73). However, these findings have not been uniformly reported. Brozna and Carson (8) demonstrated that phorbol myristate acetate, at concentrations sufficient to translocate PKC activity from the cytosolic to the particulate fraction in adherent human monocytes, blocked adherence-induced PCA expression and suppressed PCA expression in cells activated by adherence or LPS treatment. Our laboratory has previously shown that PMA alone had little direct effect on PCA in thioglycolate-elicited murine peritoneal macrophages, although it primed cells for increased PCA expression in response to LPS and live bacteria (40, 41). The variability observed with respect to the role of PKC in LPS-induced PCA in monocytes/macrophages may be related to the source of the cell studied as well as the degree of cellular activation. In the present studies, thioglycolate-elicited murine peritoneal macrophages were used. These cells are considered to be in a state of heightened activation, by virtue of the fact that they are induced to migrate into the peritoneal cavity by the initiation of sterile inflammation. Presumably, these cells are representative of those which might be recruited into the peritoneal cavity during intraabdominal infection. In these cells, two tyrosine kinase inhibitors, genistein and herbimycin, completely prevented the stimulation of PCA by LPS, while BIM, a PKC inhibitor which completely abrogated phorbol ester-stimulated chemiluminescence, had no effect on LPS-induced PCA. These findings are consistent with those recently reported by Han and colleagues (31). These investigators reported that herbimycin A, but not the PKC inhibitor GF 109203X, prevented the induction of tyrosine phosphorylation and activation of NF-κB by LPS. Given that LPS-stimulated TF gene expression requires NF-κB activation (9, 53), these data support the notion that signalling may occur through a pathway requiring tyrosine phosphorylation but one independent of PKC activation. Another PKC inhibitor, staurosporine, caused partial inhibition of PCA. This agent is known to have tyrosine kinase inhibitor activity, and thus, its partial inhibitory effect may be related to this action (34). The inhibition of LPS-induced PCA reported for other PKC inhibitors, such as H7, might be explained on a similar basis (10, 73). Alternatively, under the experimental conditions used in those studies, PKC-dependent signalling pathways, possibly via induction of tyrosine phosphorylation, may have participated in PCA induction (66).

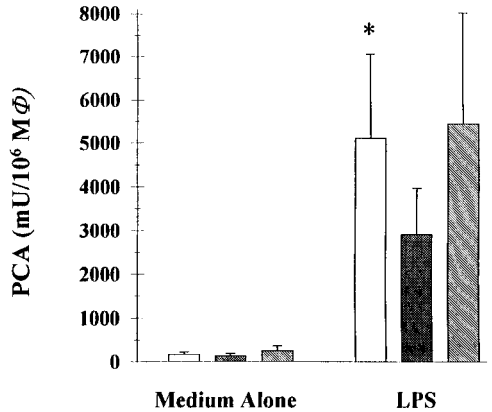

FIG. 9. (A) Effect of PKC inhibitors on induction of PCA activity by LPS. Macrophages (Mϕ) (10^6/ml) were stimulated with LPS alone (□) or LPS (10 μg/ml) in the presence or BIM (2.5 μM) (▧) or staurosporine (100 nM) (▨) for 4 h. At 4 h posttreatment, cells were washed, resuspended in RPMI, and frozen at −70°C for later assay of PCA activity. The data are the means and standard errors of the means for four separate experiments, each performed in duplicate. *, P of <0.05 versus control.

Vanadate, a tyrosine phosphatase inhibitor, augmented phosphotyrosine accumulation in stimulated cells. Recent studies performed with permeabilized HL60 cells that differentiated along granulocytic lines demonstrated that vanadate-induced tyrosine phosphorylation was mediated in part by the reaction products of reduced oxygen metabolites and vanadate (i.e., vanadyl hydroperoxide or peroxovanadyl) (74). The present studies demonstrate that a similar mechanism is active in intact macrophages. Three lines of evidence support this conclusion. First, DPI reduced the vanadate-induced increase

in phosphotyrosine accumulation in response to both zymosan and LPS, at a concentration which abrogated the respiratory burst. Secondly, vanadyl hydroperoxide, generated by the coincubation of hydrogen peroxide and sodium orthovanadate, mimicked the effect of vanadate seen in stimulated cells. The addition of DPI had no effect on this response, indicating that its ability to reduce vanadate-induced phosphotyrosine accumulation was not artifactual. Finally, the increase in phosphotyrosine accumulation seen with the addition of vanadate correlated well with the magnitude of the respiratory burst induced in the cells. Specifically, it was relatively small in LPS-treated cells and comparatively large following exposure to zymosan. The precise mechanism whereby peroxides of vanadate mediate their effect is not clear, although recent studies performed with neutrophils suggest that the redox state of the cell may directly modulate accumulation of tyrosine phosphoproteins (23, 26).

Despite increasing phosphotyrosine accumulation, vanadate unexpectedly caused an inhibition of LPS-stimulated PCA production. Cell viability, as determined by trypan blue exclusion, remained high in LPS-treated cells incubated with vanadate (>84%). More importantly, another cell product, PGE_2, was released in greater amounts in vanadate-treated cells, thus making it unlikely that cell toxicity played a significant role in the observed reduction in PCA production. Stimulation of PGE_2 release by vanadate represents one possible mechanism contributing to this effect, since this and other prostaglandins have been shown to inhibit the generation of macrophage products (18, 42). The present studies showed a rise in PGE_2 release by vanadate in both stimulated and unstimulated cells. To determine the possible contribution of prostaglandins, including PGE_2, to the inhibition of LPS-induced PCA by vanadate, the effects of indomethacin on the vanadate-induced increase in PGE_2 and reduction in PCA were examined. Although indomethacin abolished PGE_2 release, PCA was only partially restored to levels observed in the absence of vanadate. These data suggest that while PGE_2 may contribute to the depression in PCA generation, other inhibitory influences appear to be induced by the vanadate-mediated phosphotyrosine accumulation. The Northern blot analysis evaluating the effect of vanadate on levels of TF mRNA induced by LPS supports this conclusion. While previous studies have shown that prostanoids generally inhibit TF expression at the level of transcription (18), the present studies demonstrate that vanadate acts posttranscriptionally.

Vanadate has been shown to increase arachidonic acid metabolism in response to various stimuli in rat hepatocytes, dog kidney cells, and bovine aorta smooth muscle cells (46) and, in the present studies, augmented PGE_2 release in elicited peritoneal macrophages both in the presence and in the absence of a second stimulus LPS. The precise mechanism underlying this effect has not been defined. A recent report has documented the ability of p42 mitogen-activated protein (MAP) kinase to phosphorylate phospholipase A_2 and induce its activation (57). In this regard, stimulation of macrophages with LPS has recently been shown to induce tyrosine phosphorylation and activation of several different MAP kinases (21, 31, 78). LPS-induced tyrosine phosphorylation of proteins with molecular masses similar to those of proteins previously identified as MAP kinases (38 and 41 kDa) was also observed in the present studies. Thus, the ability of vanadate to augment phosphorylation of these kinases may represent one possible mechanism leading to increased arachidonic acid release and synthesis of PGE_2.

In summary, the present studies demonstrate a primary role for tyrosine phosphorylation in the induction of macrophage PCA and TF by proinflammatory stimuli. A recent report has documented that tyrosine kinase inhibition resulted in decreased human monocyte TF mRNA expression in response to LPS and phorbol myristate acetate (72). The present report identifies induction of tyrosine phosphoproteins in this process and the relatively greater role of tyrosine kinase signal transduction in contrast to signalling processes mediated via PKC. However, investigators have begun to identify further potential for cross talk between these two pathways previously believed to be exclusive. This has been described as, in effect, a "team-blue-sees-red" (6) phenomenon whereby a protein tyrosine kinase acts to relay messages from PKC and cytoplasmic calcium to Shc, (a Grb-2 adaptor protein), thus activating Ras-mediated regulation (45). The relative importance of these relay kinases in other systems remains to be examined. A recent report has documented the ability of tyrosine kinase inhibition to abrogate lethality in a murine endotoxemia model (58). Since previous studies have demonstrated that an anti-TF antibody as well as TF pathway inhibitor reduced mortality in an experimental *E. coli* septic shock model (16, 71), data presented herein suggest the possibility that the beneficial effect of tyrosine kinase inhibition may be attributed in part to inhibition of endotoxin-induced TF expression.

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