Induction of Serine and Threonine Protein Phosphorylation by Endotoxin-Associated Protein in Murine Resident Peritoneal Macrophages

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Endotoxin-associated protein (EP) from *Salmonella typhi* activated murine resident peritoneal macrophages to produce prostaglandin E2 (PGE2). Cells from both endotoxin nonresponder (C3H/HeJ) and the endotoxin responder (C3H/OuJ) mouse strains were activated by EP. This EP-induced prostaglandin E2 production was blocked by the protein kinase C (PKC) inhibitor H-7 as well as the tyrosine kinase inhibitor genistein, suggesting the involvement of both serine and threonine phosphorylation and tyrosine phosphorylation pathways in the activation of resident peritoneal macrophages by EP. Immunoblot analysis using antiphosphoserine and antiphosphothreonine antibodies showed that EP induced the serine and threonine phosphorylation of a 14-kDa protein (p14). This phosphorylation was not induced by phorbol myristic acid or by lipopolysaccharide endotoxin. Inhibitors of PKC, PKA, and PKG did not block the phosphorylation of p14. However, the tyrosine kinase inhibitor piceatannol blocked p14 serine and threonine phosphorylation, suggesting that this phosphorylation is dependent upon and preceded by a tyrosine phosphorylation step.

Protein kinases have been shown to play a major role in signal transduction during macrophage activation. Tyrosine kinase(s) (1, 2, 5, 6, 18) as well as serine and/or threonine kinase(s) (4, 9, 11, 19) were shown to be involved in this signaling process. A significant increase in protein kinase C (PKC) activity in macrophages was induced by gamma interferon as well as by a lipid A precursor from *Escherichia coli* (9, 19). The PKC inhibitor (H-7) was shown to inhibit lipopolysaccharide (LPS)-induced prostaglandin E2 (PGE2) production by Kupffer cells and the expression of interleukin-1 mRNA in murine peritoneal macrophages and human monocytes (4, 11, 12, 20). The PKC inhibitors also inhibited endotoxin-induced thromboxane B2 synthesis in rat peritoneal macrophages (7). A serine-phosphorylated protein of 65 kDa (p65) has been purified from murine macrophages stimulated with LPS, indicating the involvement of a serine kinase in macrophage activation (13). The calmodulin (CaM)-dependent kinase (hereafter termed CaM kinase) was also shown to be involved in macrophage activation by the fact that W-7 (CaM kinase inhibitor) inhibited the LPS-induced expression of interleukin-1 mRNA in murine macrophages (12) and in human monocytes (11).

In this study, we examined the role of serine and threonine phosphorylation in the release of arachidonic acid (AA) metabolites by resident peritoneal macrophages (RPM) after stimulation with endotoxin-associated protein (EP). EP has been described as a stimulant of B-cell proliferation and as a polyclonal activator (16). Unlike endotoxin, EP was shown to stimulate cells from an endotoxin nonresponder mouse strain (C3H/HeJ) as well as an endotoxin responder strain (C3H/OuJ) (8, 16). EP was also shown to stimulate tumor cytotoxicity and interferon production in thioglycolate-elicited macrophages (15). However, the signal transduction pathway induced by EP in macrophages has not been described. In order

to examine the role of protein kinases in the activation of macrophages by EP, protein kinase inhibitors were used to block EP-induced production of PGE2 by RPM from C3H/HeJ and C3H/OuJ mouse strains. Moreover, immunoblot analysis was used to detect serine and threonine phosphorylation induced by EP.

MATERIALS AND METHODS

Animals. C3H/HeJ and C3H/OuJ mouse strains were obtained from Jackson Laboratory (Bar Harbor, Maine). Five- to seven-week-old mice of either sex were purchased and maintained on water and Purina Chow ad libitum at the State University of New York—Health Science Center at Brooklyn animal facility. When the mice were used, they were 2 to 4 months old.

Materials. RPMI 1640 cell culture medium was obtained from American Bioorganics Inc. (Niagara Falls, N.Y.), and fetal calf serum (endotoxin level, 0.06 ng/ml) was obtained from GIBCO BRL (Grand Island, N.Y.). EP was prepared from *Salmonella typhi* (strain 0901) in our laboratory by Raymond Castagna, as previously described (16). Leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and piceatannol (3,4,3',5'-tetrahydroxy-*trans*-stilbene) were obtained from Boehringer Mannheim Co. (Indianapolis, Ind.). PGE2 and leukotriene C₄ (LTC₄) enzyme immunoassay (EIA) systems were purchased from Cayman Chemicals (Ann Arbor, Mich.). The interleukin-1 α assay system was obtained from Genzyme (Cambridge, Mass.). H-7 [1-(5-isoquinoline sulfonyl)-2-methylpiperazine dihydrochloride] and genistein [5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one; 4',5,7-trihydroxyisoflavone] were purchased from Calbiochem (San Diego, Calif.). Tyrphostin-23 [(3,4-dihydroxybenzylidene)malononitrile], herbimycin A (benzoquinoid ansamycin antibiotic), chelerythrine chloride [1,2-dimethoxy-dimethyl-12-methyl(1,3)benzodioxolo(5,6-C)phenanthridiniumchloride], H-89 [*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline-sulfonamide · 2 HCl], and HA1004 [*N*-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride] were all obtained from Biomol Research Inc. (Plymouth Meeting, Pa.). The ascites-derived antiphosphoserine monoclonal antibody, antiphosphothreonine monoclonal antibody, and the alkaline phosphatase conjugates of goat anti-mouse immunoglobulin G and goat anti-rabbit immunoglobulin G were all purchased from Sigma Chemical Co. (St. Louis, Mo.).

Macrophage isolation and purification. Resident peritoneal cells were obtained from C3H/HeJ and C3H/OuJ mice by peritoneal lavage. The mice were killed with CO₂. Approximately 6 to 8 ml of RPMI 1640 containing 10% fetal calf serum was injected intraperitoneally into each mouse and was then withdrawn. The collected cells were then centrifuged, and the cell pellet was washed two times with RPMI 1640. After the final wash, the pellet was reconstituted with 5 to 10 ml of medium. The cells were counted, appropriately diluted, and then seeded into wells of either a 24-well plate or a 96-well microtiter plate according to the experiment being performed. The plates were then incubated at 37°C for 2 to 3 h. Nonadherent cells were removed by repeated rinsing of the wells with

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RPMI 1640. Adherent cells were reconstituted in medium and then reincubated for 24 h. As determined by esterase staining, 90 to 95% of these cells were macrophages.

Assay for AA metabolite release. The effect of EP on the release of two AA metabolites was examined. PGE2 and LTC4 were chosen as examples of the cyclooxygenase pathway and the lipooxygenase pathway of the AA metabolism, respectively. The assay for both products is an EIA, which is based on the competition between PGE2 or LTC4 in the sample and an acetylcholinesterase-labeled PGE2 or LTC4 for binding to a limited amount of the corresponding monoclonal antibody (Cayman Chemicals). The resulting color intensity measured at 420 nm is inversely proportional to the amount of PGE2 or LTC4. A standard curve was plotted with known amounts of PGE2 or LTC4 and was used to determine the amount of the metabolite in the culture supernatants.

Macrophages from either C3H/HeJ or C3H/OuJ mice were prepared as described above, with 1.25×10^5 peritoneal cells per well in a 96-well microtiter plate. After the nonadherent cells were washed, EP was added to the wells simultaneously with or without the addition of a protein kinase inhibitor at 37°C in a 10% CO₂ atmosphere for 24 h. After incubation, the culture supernatants were removed and centrifuged to remove cell debris and then the amounts of AA metabolites were estimated by EIA as described above. LPS endotoxin (0.1 µg) was used as a positive control for stimulating PGE2 production by C3H/OuJ macrophages.

Experiments were carried out in replicates. The mean and the standard error of the mean of the results from three experiments were calculated (see the tables). When inhibitors were used, and the inhibition was questionable (20% or less), the statistical significance of inhibition was determined by using the Student *t* test to compare the results from experiments with inhibitor to the control results (without inhibitor).

Detection of serine- and threonine-phosphorylated proteins: activation and preparation of cell lysates. Macrophages were prepared from 2.5×10^6 peritoneal cells per well which were seeded onto a 24-well plate, as described above. After the macrophages were purified, they were incubated in 1 ml of medium containing the stimulant at 37°C in 10% CO₂. Preliminary experiments showed that stimulation for 1 h was optimal compared with shorter and longer periods; therefore, the stimulation was carried out for 1 h throughout the study. When protein kinase inhibitors were used, they were added to the cells 2 h prior to the addition of the stimulant. After incubation, the cells were washed with phosphate-buffered saline (PBS; 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 154 mM NaCl [pH 7.4]) containing 1 mM sodium orthovanadate (Na₃VO₄), and then 125 µl of lysis buffer (20 mM Tris-HCl [pH 8], 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 20 µM leupeptin, 0.15 U of aprotinin per ml, 2 mM EDTA) was added to each well. Lysis was carried out for 30 min at 4°C followed by centrifugation at 14,000 rpm in an Eppendorf centrifuge 5415C to remove insoluble material. Approximately 100 µl of the lysate supernatant was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

SDS-PAGE and immunoblotting. Approximately 100 µl of each lysate was mixed with 20 µl of 6× sample buffer and boiled for 5 min. Then, 50-µl aliquots of this lysate were added to each lane of an SDS-10% polyacrylamide nonreducing minigel. Electrophoresis was then carried out at 35 mA and was continued until the dye ran out of the gel. Proteins were then transferred onto a nitrocellulose membrane by use of the TE 22 tank system (Hoefer Scientific Instruments, San Francisco, Calif.). The transfer was continued for 1.5 to 2 h at 500 mA. As a protein loading control, the membrane was stained with Ponceau S to determine the efficiency of the protein loading and transfer. Immunoblotting was not performed if different protein levels were seen in the different lanes as indicated by the stain. The membrane was washed with PBS and placed in blocking buffer (3% nonfat skim dry milk in PBS) for 1 h at room temperature. Subsequently, the blocking buffer was decanted and antiphosphoserine or antiphosphothreonine monoclonal antibody was added at a concentration of 1 µg/ml (in blocking buffer) and was incubated for 2 to 3 h at room temperature. The membrane was then washed five times with distilled water and was incubated for 1 h at room temperature with goat anti-mouse immunoglobulin G labeled with alkaline phosphatase. The membrane was washed with PBS containing 0.05% Tween 20 for 3 to 5 min and then was incubated at room temperature with alkaline phosphatase substrate solution until the bands were developed. Using an LKB Ultrascan XL densitometer, we obtained densitometer tracings of protein bands which were used to estimate the intensity of phosphorylation.

RESULTS

Effects of EP on the production of AA metabolites. The effect of EP on PGE2 production by resident macrophages was examined as an indicator of macrophage activation. RPM from C3H/HeJ and C3H/OuJ mouse strains were incubated with EP or LPS. After 24 h of stimulation, the culture supernatants were removed and the amount of PGE2 in picograms per milliliter was estimated by an EIA system as described in Materials and Methods. From preliminary experiments, a dose-

TABLE 1. Effects of EP on production of PGE2 in RPM from C3H/HeJ and C3H/OuJ mice

Amt of activator (µg/well)		Amt of PGE2 (mean [pg/ml] ± SEM) for strain:	
EP	LPS	C3H/HeJ	C3H/OuJ
		<312	<2,000
	0.1	<312	>100,000
	10.0	<312	ND ^a
0.001		30,000 ± 1,270	>100,000
0.01		27,600 ± 1,130	ND
1.00		28,200 ± 2,970	ND

^a ND, not determined.

response pattern was observed at picogram levels of EP and saturation levels were observed at nanogram dose levels. As shown in Table 1, a significant amount of PGE2 (30,000 pg/ml) was produced when C3H/HeJ macrophages were stimulated with 1 ng of EP. When EP was added to C3H/OuJ macrophages, more than 100,000 pg/ml was detected. As expected, when LPS was used, C3H/OuJ macrophages but not C3H/HeJ macrophages were stimulated to produce PGE2. The culture supernatants were also examined for the presence of LTC4 by an EIA system as described in Materials and Methods. As shown in Table 2, the amount of LTC4 produced by unstimulated and stimulated C3H/HeJ macrophages was below the detectable level (<1,500 pg/ml) as determined by the assay. When C3H/OuJ macrophages were used, no significant stimulation was obtained by EP or LPS with a stimulation index (SI) of less than 2 over the background level.

Effects of protein kinase inhibitors on EP-mediated PGE2 production by C3H/HeJ and C3H/OuJ macrophages. In view of the high level of PGE2 production by EP in both C3H/HeJ and C3H/OuJ mouse strains, a group of protein kinase inhibitors was used to determine the roles of PKC, PKA, PKG, and tyrosine kinase(s) in EP-mediated induced PGE2 production by resident macrophages from these mice.

RPM from C3H/HeJ and C3H/OuJ mice were incubated with EP alone or EP along with several doses of one of the following inhibitors: the PKC inhibitor H-7, the PKA-PKG inhibitor HA1004, and the tyrosine kinase inhibitors tyrphostin and genistein. After incubation, the supernatants were examined for their levels of PGE2 by EIA as described above.

As shown in Table 3, when C3H/HeJ macrophages were used, high levels of dose-related inhibition were obtained with H-7, genistein, or tyrphostin, with more than 80% inhibition when a 20 µM dose of these inhibitors was used. However, no inhibition by HA1004 was observed. When C3H/OuJ macrophages were used, a partial inhibition level of 26% was observed with 20 µM H-7 and an insignificant (*P* > 0.05) inhibition level of 11% was obtained with 20 µM HA1004. However,

TABLE 2. Effects of EP on production of LTC4 in RPM from C3H/HeJ and C3H/OuJ mice

Amt of activator (µg/well)		Amt of LTC4 (mean [pg/ml] ± SEM) for strain:	
EP	LPS	C3H/HeJ	C3H/OuJ
		<1,500	2,500 ± 200
	10.0	<1,500	2,200 ± 200
	100.0	<1,500	2,700 ± 100
10.0		<1,500	3,400 ± 300
100.0		<1,500	4,600 ± 400

TABLE 3. Effects of protein kinase inhibitors on EP-induced PGE₂ production from C3H/HeJ and C3H/OuJ RPM^a

Inhibitor and concn (μM)	% Inhibition ^b (mean ± SEM) for strain:	
	C3H/HeJ	C3H/OuJ
	0	0
H-7		
5	6 ± 1	23 ± 9
10	46 ± 3	19 ± 6
20	80 ± 1	26 ± 3
HA1004		
5	0	12 ± 7
10	0	12 ± 7
20	0	11 ± 7
Genistein		
5	77 ± 1	68 ± 10
10	>80	65 ± 10
20	>80	68 ± 9
Tyrphostin		
5	66 ± 1	0
10	>80	2 ± 2
20	>80	10 ± 5

^a The dose of EP used for induction of PGE₂ was 1 ng for each well.^b Percent inhibition (%I) is calculated as follows: %I = (PGE₂ without inhibitor - PGE₂ with inhibitor)/PGE₂ without inhibitor.

a high level of inhibition (68%) was obtained with genistein. When tyrphostin was used, little if any inhibition was observed.

Effects of PMA and ionomycin on PGE₂ production by C3H/OuJ and C3H/HeJ macrophages. In addition to determination of signal transduction pathways by using inhibitors, determination of whether a particular pathway is involved in macrophage activation can also be made by using known activators of that pathway. These activators include phorbol myristic acid (PMA), which stimulates PKC, and ionomycin, which increases intracellular Ca²⁺ levels (9, 19).

RPM from C3H/OuJ and C3H/HeJ mouse strains were incubated for 24 h with EP, PMA, or ionomycin. Optimal doses were selected on the basis of preliminary experiments using several doses of each activator. These doses ranged from 0.01 ng to 1 μg of EP, 10 to 100 nM of PMA, and 0.1 to 0.5 μM of ionomycin. The optimal doses were found to be 1 ng per well for EP and concentrations of 100 nM for PMA and 0.4 μM for ionomycin. After incubation, the culture supernatants were collected and were examined for their levels of PGE₂. As shown in Table 4, high levels of PGE₂ were produced by RPM from C3H/OuJ mice stimulated with EP (SI, 22.5) or PMA (SI, 100). RPM from C3H/HeJ mice were also responsive but to a lesser extent, with an SI of 7.9 with EP stimulation and an SI of 17.3 with PMA stimulation. However, only C3H/OuJ macrophages released PGE₂ in response to ionomycin (SI, 71).

Immunoblot analysis of EP-induced serine- and threonine-phosphorylated proteins in RPM. The use of protein kinase inhibitors to study signal transduction (Table 3) indicated that stimulation of resident macrophages by EP appeared to involve serine and threonine phosphorylation, since the PKC inhibitor H-7 suppressed EP-induced PGE₂. To gain more insight into the involvement of serine and threonine phosphorylation, immunoblot analysis was used to detect any EP-induced serine- and threonine-phosphorylated proteins. These proteins could then be compared with those induced by other

TABLE 4. Effects of other activators on PGE₂ production by C3H/OuJ and C3H/HeJ RPM

Activator ^a	Amt of PGE ₂ (mean [pg/ml] ± SEM) (SI ^b) for strain:	
	C3H/OuJ	C3H/HeJ
None	<400	<400
EP	9,000 ± 100 (22.5)	3,150 ± 35 (7.9)
PMA	40,000 ± 100 (100)	6,900 ± 71 (17.3)
Ionomycin	28,500 ± 354 (71.3)	<400

^a The following doses were used: 1 ng of EP, 100 nM PMA, and 0.4 μM ionomycin.^b SI, stimulation index or ratio of experimental to background levels.

activators. In order to determine which kinase(s) might be involved in the EP induced serine and threonine phosphorylation, a group of protein kinase inhibitors was used to inhibit EP-induced serine and threonine phosphorylation. RPM from C3H/HeJ and C3H/OuJ mice were stimulated with 100-μg quantities of EP as described in Materials and Methods. After stimulation, the cells were lysed and the proteins were separated by SDS-PAGE before transfer onto a nitrocellulose membrane. Serine- and threonine-phosphorylated proteins were detected by the antiphosphoserine and antiphosphothreonine immunoblotting system as described in Materials and Methods. Figure 1 shows immunoblots of serine-phosphorylated proteins (A) and threonine-phosphorylated proteins (B) in C3H/HeJ (lanes 1 and 2) and C3H/OuJ (lanes 3 and 4) macrophages. EP induced greater phosphorylation of a 14 kDa protein on both the serine and threonine residues than that produced in unstimulated cells (lanes 1 and 3). It also appears that two other protein bands, which were not well resolved, of about 36 and 38 kDa were phosphorylated but only on the serine residues.

In order to determine whether other macrophage activators can also induce serine and threonine phosphorylation of the 14-kDa protein, the serine- and threonine-phosphorylated proteins induced by LPS and PMA were examined and compared with those induced by EP in resident macrophages. As shown in Fig. 2, serine-phosphorylated proteins (A) and threonine-phosphorylated proteins (B) of both C3H/HeJ macrophages (lanes 1 to 4) and C3H/OuJ macrophages (lanes 5 to 8) were analyzed. In comparisons of unstimulated cells (lanes 1 and 5)

FIG. 1. Induction of serine and threonine phosphorylation by EP in RPM. RPM from either C3H/HeJ mice (lanes 1 and 2) or C3H/OuJ mice (lanes 3 and 4) were stimulated with 100 μg of EP per ml. After 1 h of stimulation, the cells were lysed and the proteins in each lysate were separated by SDS-PAGE before being transferred onto a nitrocellulose membrane. Serine-phosphorylated proteins (A) and threonine-phosphorylated proteins (B) were then detected by the antiphosphoserine and antiphosphothreonine immunoblotting system described in Materials and Methods. Lanes 1 and 3, unstimulated cells; lanes 2 and 4, EP-stimulated cells. The 14- and 31-kDa protein standards are indicated (arrows).

FIG. 2. Effects of other activators on serine and threonine phosphorylation in RPM. RPM from either C3H/HeJ mice (lanes 1 to 4) or C3H/OuJ mice (lanes 5 to 8) were stimulated with EP (100 μ g/ml), LPS (100 μ g/ml), or PMA (100 nM). After 1 h of stimulation, the serine-phosphorylated proteins (A) and threonine-phosphorylated proteins (B) were detected by the antiphosphoserine and antiphosphothreonine immunoblotting system. Lanes 1 and 5, unstimulated cells; lanes 2 and 6, EP-stimulated cells; lanes 3 and 7, LPS-stimulated cells; lanes 4 and 8, PMA-stimulated cells. The 14-kDa protein band is indicated (arrows).

and activated cells, only EP (lanes 2 and 6) was shown to induce phosphorylation of the 14-kDa protein. Neither LPS (lanes 3 and 7) nor PMA (lanes 4 and 8) stimulated serine or threonine phosphorylation of the 14-kDa protein band. Furthermore, PMA and LPS did not stimulate serine phosphorylation of the 36- and 38-kDa protein bands.

Effects of protein kinase inhibitors on serine and threonine phosphorylation of p14. To examine which protein kinase(s) may be involved in the phosphorylation of p14, a group of protein kinase inhibitors were used to inhibit the EP-mediated phosphorylation of p14. These inhibitors included chelerythrine chloride (which inhibits PKC), H-89 (which inhibits PKA), and HA1004 (which inhibits PKG). Inhibitors were added to the culture 2 h prior to stimulation with EP. After stimulation, the cells were lysed and immunoblotted as described above. Figure 3 shows an immunoblot analysis of serine and threonine phosphorylation of C3H/HeJ macrophages (A) and C3H/OuJ

FIG. 3. Inhibition of serine and threonine phosphorylation of p14 by protein kinase inhibitors. RPM from either C3H/HeJ mice (A) or C3H/OuJ mice (B) were stimulated with EP (100 μ g/ml) with or without protein kinase inhibitors. After stimulation, the serine- and threonine-phosphorylated proteins were then detected by the antiphosphoserine and antiphosphothreonine immunoblotting system (antibodies were combined in this experiment). In both panels, lane 1 represents unstimulated cells and lane 2 represents EP-stimulated cells. The following inhibitors were added 2 h prior to the addition of EP: 5 μ M chelerythrine chloride (lane 3), 10 μ M H-89 (lane 4), 10 μ M HA1004 (lane 5), and 100 μ M piceatannol (lane 6). The 14- and 31-kDa protein standards are indicated (arrows).

macrophages (B). In C3H/HeJ macrophages, significant phosphorylation of p14 is induced by EP (Fig. 3A, lane 2) compared with that induced in unstimulated cells (lane 1). This phosphorylation was not inhibited by any of the following inhibitors: chelerythrine chloride (lane 3), H-89 (lane 4), and HA1004 (lane 5). However, piceatannol (lane 6) inhibited more than 90% of the p14 phosphorylation. In C3H/OuJ, significant phosphorylation of p14 is induced by EP (Fig. 3B, lane 2) compared with that induced in unstimulated cells (lane 1). This phosphorylation was not inhibited by any of the following inhibitors: chelerythrine chloride (lane 3), H-89 (lane 4), and HA1004 (lane 5). Instead, HA1004 enhanced serine phosphorylation of the 36- and 38-kDa protein bands (lane 5), while piceatannol (lane 6) partially inhibited p14 phosphorylation (40% inhibition).

DISCUSSION

EP has been described previously as a protective antigen, an adjuvant, a potent B-cell activator, and a macrophage activator. As a macrophage activator, EP was shown to stimulate cellular cytotoxicity in thioglycolate-induced peritoneal macrophages from CBA mice (15). EP was also shown to stimulate interferon gamma production by spleen cells from C57BL/6 and C3H/HeJ mice (15). In this study, the characterization of the stimulatory activity of EP was expanded to include its effect on the production of AA metabolites by RPM. The study showed that EP has the ability to stimulate the release of PGE₂ but not the release of LTC₄ in macrophages from C3H/OuJ and C3H/HeJ mouse strains. LPS, on the other hand, stimulated the release of PGE₂ only in macrophages from the LPS responder strain C3H/OuJ. These results indicate that there is a difference between the requirements for the activation of the cyclooxygenase pathway and those for the activation of the lipooxygenase pathway. Indeed, previous studies have shown that in macrophages the lipooxygenase pathway requires higher calcium levels than the cyclooxygenase pathway (3). Also, the stimulation of C3H/HeJ macrophages by EP but not LPS may indicate that EP and LPS activate different signaling pathways and, therefore, that EP can bypass whatever signaling defect exists in the LPS nonresponder strain.

To determine if a particular protein kinase is involved in EP-mediated activation, a group of protein kinase inhibitors were used to suppress PGE₂ production stimulated by EP. The PKC inhibitor (H-7) and the tyrosine protein kinase inhibitor (genistein) inhibited EP-mediated PGE₂ in macrophages from either the C3H/HeJ or the C3H/OuJ mouse strain. These results suggest that both PKC and tyrosine protein kinase pathways are involved in the EP-mediated production of PGE₂. When the pattern of inhibition in C3H/OuJ macrophages was compared with that in C3H/HeJ macrophages, the protein kinase inhibitors only partially inhibited EP mediated-activation of the C3H/OuJ macrophages. This suggests that signaling pathways other than those involved in C3H/HeJ cells are involved in C3H/OuJ cells.

In another approach to determine the involvement of PKC in macrophage activation, we used PKC activator PMA to stimulate PGE₂ production in RPM from C3H/HeJ and C3H/OuJ mouse strains. As described above, both PMA and EP induced the production of PGE₂ in C3H/OuJ macrophages, and to a lesser extent in C3H/HeJ macrophages. This result indicates that PKC activation is sufficient to trigger PGE₂ production and that the PKC pathway is functional in C3H/HeJ cells. However, the lower-level response to PMA and EP of C3H/HeJ compared with that of C3H/OuJ macrophages indicates that C3H/HeJ hyporesponsiveness is not restricted to

LPS; rather, it reflects a signaling defect which may be common to stimulants other than LPS. Indeed, when ionomycin was used as an activator, PGE₂ production was stimulated in C3H/OuJ macrophages but not C3H/HeJ macrophages. Since ionomycin acts to increase intracellular calcium levels, these results indicate that a calcium-dependent pathway which is needed for PGE₂ production is either missing or altered in the C3H/HeJ macrophages. This finding confirmed earlier results by the Nakano group (14, 17) which demonstrated that C3H/HeJ macrophages were unresponsive to calcium ionophore A23187 (14) and that an altered CaM system may exist in the C3H/HeJ macrophages (17). Although C3H/HeJ cells were shown to contain a functional CaM system, a species of CaM-binding protein was hardly detected in cell extracts from LPS stimulated C3H/HeJ macrophages compared with the levels detected in cell extracts from LPS-responsive macrophages (17). Terada et al. concluded that the generation and/or recruitment of some of the CaM-binding proteins in C3H/HeJ macrophages were genetically altered (17).

In view of our finding that EP-mediated PGE₂ production was inhibited by the PKC inhibitor (H-7), a search for serine- and threonine-phosphorylated proteins was initiated. The results showed that EP induced the serine and threonine phosphorylation of a 14-kDa protein band (p14) and the serine phosphorylation of two protein bands of approximately 36 and 38 kDa. This phosphorylation pattern was unique to EP, since other activators such as PMA and LPS did not induce the phosphorylation of p14 or the phosphorylation of the 36- and 38-kDa protein bands. Similar results were obtained with both C3H/HeJ macrophages and C3H/OuJ macrophages. Serine and threonine phosphorylation of p14 is not directly dependent on any of the major serine-threonine kinases such as PKC, PKA, or PKG, since inhibitors of these kinases did not suppress the p14 phosphorylation. It should be noted that chelerythrine chloride inhibits PKC with a 50% inhibition constant (K_i) of 0.66 μ M, which makes it more selective than other PKC inhibitors (10). These results indicate that other serine-threonine kinases may be involved. Possibilities include calcium-CaM-regulated kinases and the microtubule-associated protein kinases. However, unexpectedly, EP-induced phosphorylation of p14 was inhibited by the tyrosine kinase inhibitor piceatannol. This result suggests that the serine and threonine phosphorylation of p14 is preceded by a tyrosine phosphorylation step which may involve p72^{syk} tyrosine kinase or a p72^{syk}-like protein which is also shown to be induced in resident macrophages by EP and inhibited by piceatannol (2). Interestingly, serine phosphorylation of the 36- and 38-kDa protein bands seemed to be enhanced when the PKG inhibitor HA1004 was used, suggesting the involvement of a G protein of the inhibitor type (Gi protein).

So far, we have detected tyrosine phosphorylation of a PTK72^{syk}-like protein (2) and serine and threonine phosphorylation of p14 in RPM activated by EP. Future studies may include an investigation of signaling pathways which are upstream and downstream of the phosphorylation p14 and the PTK72-like protein. It is also of a great interest to identify these two proteins, as well as their cellular location, function,

and role in the induction of cytokine production by macrophages.

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