Sites of Action of Protein Kinase C and Phosphatidylinositol 3-Kinase Are Distinct in Oxidized Low Density Lipoprotein-induced Macrophage Proliferation*

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Oxidized low density lipoprotein (Ox-LDL) can induce macrophage proliferation in vitro. To explore the mechanisms involved in this process, we reported that activation of protein kinase C (PKC) is involved in its signaling pathway (Matsumura, T., Sakai, M., Kobori, S., Biwa, T., Takemura, T., Matsuda, H., Hakamata, H., Horiuchi, S., and Shichiri, M. (1997) Arterioscler. Thromb. Vasc. Biol. 17, 3013–3020) and that expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) and its subsequent release in the culture medium are important (Biwa, T., Hakamata, H., Sakai, M., Miyazaki, A., Suzuki, H., Kodama, T., Shichiri, M., and Horiuchi, S. (1998) J. Biol. Chem. 273, 28305–28313). However, a recent study also demonstrated the involvement of phosphatidylinositol 3-kinase (PI3K) in this process. In the present study, we investigated the role of PKC and PI3K in Ox-LDL-induced macrophage proliferation. Ox-LDL-induced macrophage proliferation was inhibited by 90% by a PKC inhibitor, calphostin C, and 50% by a PI3K inhibitor, wortmannin. Ox-LDL-induced expression of GM-CSF and its subsequent release were inhibited by calphostin C but not by wortmannin, whereas recombinant GM-CSF-induced macrophage proliferation was inhibited by wortmannin by 50% but not by calphostin C. Ox-LDL activated PI3K at two time points (10 min and 4 h), and the activation at the second but not first point was significantly inhibited by calphostin C and anti-GM-CSF antibody. Our results suggest that PKC plays a role upstream in the signaling pathway to GM-CSF induction, whereas PI3K is involved, at least in part, downstream in the signaling pathway after GM-CSF induction.

Macrophage-derived foam cells are the key cellular elements in the early stages of atherosclerosis (1). Macrophages take up oxidized low density lipoprotein (Ox-LDL)1 through the scavenger receptor pathways and transform into foam cells in vitro (2). Foam cells producing various bioactive molecules, such as cytokines and growth factors, are believed to play an important role in the development and progression of atherosclerosis (1).

One of the characteristic events in the atherosclerotic lesion is the proliferation of cellular components of arterial walls. In addition to the growth of vascular smooth muscle cells (1), several reports emphasize the presence of macrophages and macrophage-derived proliferating foam cells in the early stages of human and rabbit atherosclerotic lesions (3–5). A pioneering study using stanch-elicted mouse peritoneal macrophages by Yui et al. (6) first demonstrated the Ox-LDL-induced macrophage proliferation in vitro. Subsequent studies showed the growth-stimulating capacity of Ox-LDL for other macrophages, such as mouse resident peritoneal macrophages (7, 8), rat resident peritoneal macrophages (9), murine bone marrow-derived macrophages (10), human monocyte-derived macrophages (11), and THP-1-derived macrophages (12). Since macrophage-derived foam cells play an important role in the development of atherosclerotic lesions (1), it is possible that macrophage proliferation may modulate the progression of atherosclerosis. Thus, clarification of the mechanism of macrophage activation, proliferation, and survival process is expected to enhance our understanding of the pathogenesis of atherosclerosis. In this regard, our recent study revealed that Ox-LDL can induce a rise in intracellular calcium concentration and activate protein kinase C (PKC) in mouse peritoneal macrophages (13). Subsequently, it was shown that activation of PKC leads to release into the culture medium of granulocyte/macrophage colony-stimulating factor (GM-CSF), which plays a priming role in the Ox-LDL-induced macrophage proliferation (14). In a recent study using human macrophage-derived cells (THP-1 cells) and mouse peritoneal macrophages, however, Martens et al. (12) provided evidence that phosphatidylinositol 3-kinase (PI3K) is also involved in the Ox-LDL-induced macrophage proliferation. The present study was undertaken to determine the relationship between PI3K and PKC on one hand and induction of GM-CSF on the other, in the signaling pathway for Ox-LDL-induced macrophage proliferation.

EXPERIMENTAL PROCEDURES

Materials—[3H]Thymidine (80 Ci/mmol) and enzyme-linked immunosorbent assay (ELISA) kit for determination of mouse GM-CSF levels were purchased from Amersham Life Science (Buckinghamshire, United Kingdom). Calphostin C and wortmannin were purchased from Sigma and stored at −20 °C as stock solutions in dimethyl sulfoxide (Me2SO). The final concentrations of Me2SO were <0.1% in the culture medium. At this concentration, Me2SO did not affect cell viability and macrophage proliferation. Calphostin C at <500 nM and wortmannin at <100 nM did not show any cytotoxic effect, as determined by 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Other chemicals were of the highest grade available from commercial sources. Lipoproteins and Their Modifications—Human LDL (d = 1.019 to 1.063 g/ml) was isolated by sequential ultracentrifugation from the plasma of consenting normolipidemic subjects after overnight fasting (15). Monocytes were separated from diluted human Ethical Committee (15). LDL was dialyzed against 0.15 mM NaCl and 1 mM EDTA (pH 7.4). Ox-LDL was prepared as incubation of 0.1 mg/ml LDL in phosphate-buffered saline (PBS) with 5 μM CuSO₄ for 20 h, followed by the addition of 1 mM EDTA and cooling on ice (16). Protein concentrations were determined by BCA protein assay reagent (Pierce) using BSA as a standard, and were expressed in milligrams of protein/ml (17). The levels of exogenous associated with these lipoproteins were < 1 μg/μl of protein, which were measured by a commercially available kit (Toxicolor system, Seikagaku Corp.). Moreover, macrophage proliferation was not affected by exotoxin at a concentration <1 ng/ml in our experimental system.

Cell Culture—Peritoneal macrophages were collected from anesthetized male C57/He mice (25–30 g) by peritoneal lavage with 8 ml of ice-cold PBS, centrifuged at 200 × g for 5 min, and suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc.), streptomycin (0.1 mg/ml), and penicillin (100 units/ml) (medium A) (18). The experimental protocol was approved by the Ethics Review Committee for Animal Experiments of our institution. Cells were dispersed in each well of appropriate tissue culture plates and incubated for 90 min in medium A. Nonadherent cells were removed by washing three times with prewarmed medium A. After washing, cell number decreased to ~80%. More than 98% of adherent cells were considered to be macrophages based on four criteria, including (i) adhesion to culture plates, (ii) morphological features resembling mononuclear cells after Giemsa staining, (iii) the capacity to take up carbon particles, and (iv) positive immunohistochemistry with antibody for CD 68 (6, 19). The cells were >95% viable as determined by trypan blue staining and lactic dehydrogenase release. Unless otherwise specified, all cellular experiments were performed at 37 °C in a humidified atmosphere.

Tritiated Thymidine Incorporation and Cell-counting Assays—Peritoneal cells were adjusted to 5 × 10⁵ cells/ml, and 1 ml of cell suspensions in medium A were dispensed in each well of 24-well tissue culture plates (15.5 mm in diameter, Corning Glass Works, Corning, NY) and incubated for 90 min. Nonadherent cells were removed by washing three times with 1 ml of medium A. Macrophage monolayers thus formed were cultured with 1 ml of medium A in the presence of the lipoproteins to be tested. For thymidine incorporation assay, 18 h before the termination of the experiments, 20 μl of 50 μCi/ml [³²P]thymidine was added to each well and incubated for 18 h. After discarding the medium, each well was washed three times with 1 ml of PBS and the cells were lysed with 0.5 ml of 0.5% NaOH by incubation on ice for 10 min. Cell lysates were neutralized with 0.25 ml of 1 M HCl, further precipitated with 0.25 ml of 40% trichloroacetic acid by incubation on ice for 20 min, and then washed three times with 1 ml of ice-cold PBS. The resulting trichloroacetic acid-insoluble material was collected on filters (Millipore PVDF filter; 0.45-μm pore size) and washed three times with 1 ml of 99.5% ethanol. Filters were dried under air, and their radioactivity was counted in a liquid scintillation counter (14).

Macrophage monolayers were incubated in 1 ml of medium A with the lipoproteins to be tested. After incubation for 7 days, 18 h were lysed in 1% (v/v) Triton X-100, and naphthol blue-black-stained nuclei were counted in a hemocytometer as described previously (14).

ELISA for GM-CSF—Macrophage monolayers (5 × 10⁵ cells/plate, 1 cm in diameter, Falcon) were cultured in 15 ml of medium A with or without the lipoproteins to be tested for indicated times, and then 300 μl of the medium were collected. The concentration of GM-CSF protein was determined according to the instructions provided by the manufacturer of mouse GM-CSF-specific ELISA system (sensitivity, 5 pg/ml, Amersham Pharmacia Biotech) using recombinant murine GM-CSF as a standard (14).

RT-PCR Analysis—After incubation of murine peritoneal macrophage monolayers (2 × 10⁶ cells/well in six-well plate, 3.5 cm in diameter, Nunc) in medium A with 40 μg/ml Ox-LDL for 1 h, total RNA was extracted (TRIzol; Life Technologies Inc.). The first strand DNA synthesis containing 1 μg of total RNA was primed with oligo(dT). Primers used for PCR amplification of GM-CSF and β-actin were designed on the basis of murine GM-CSF cDNA (20) and murine β-actin cDNA (21). PCR was performed as described previously (14). The sizes of RT-PCR products of GM-CSF and β-actin were expected to be 368 and 540 base pairs, respectively. To verify that the amplification products were consistent with the reported sequences of murine GM-CSF and β-actin, they were ligated into pGEM-T (Promega, Madison, WI), trans­ferred to Echerichia coli XLI-Blue and sequenced by using 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Lipoproteins and Their Modifications—Human LDL (d = 1.019 to 1.063 g/ml) was isolated by sequential ultracentrifugation from the plasma of consenting normolipidemic subjects after overnight fasting (15). Monocytes were separated from diluted human Ethical Committee (15). LDL was dialyzed against 0.15 mM NaCl and 1 mM EDTA (pH 7.4). Ox-LDL was prepared as incubation of 0.1 mg/ml LDL in phosphate-buffered saline (PBS) with 5 μM CuSO₄ for 20 h, followed by the addition of 1 mM EDTA and cooling on ice (16). Protein concentrations were determined by BCA protein assay reagent (Pierce) using BSA as a standard, and were expressed in milligrams of protein/ml (17). The levels of exogenous associated with these lipoproteins were < 1 μg/μl of protein, which were measured by a commercially available kit (Toxicolor system, Seikagaku Corp.). Moreover, macrophage proliferation was not affected by exotoxin at a concentration <1 ng/ml in our experimental system.

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Involvement of PKC and PI3K in Macrophage Proliferation

Peritoneal macrophages from C3H/He mice (5 \times 10^5 cells/well in 24-well tissue culture plates) were incubated for 6 days with medium A alone (\(\bullet\)) or with 40 \(\mu\)g/ml Ox-LDL in 1 ml of medium A in the presence of indicated concentrations of calphostin C (\(\circ\)). B, peritoneal macrophages (5 \times 10^6 cells/well) were incubated for 6 days with medium A alone (\(\bullet\)) or with 40 \(\mu\)g/ml Ox-LDL in 1 ml of medium A in the presence of indicated concentrations of wortmannin (\(\circ\)). During the last 18 h of incubation, cells in each well were chased with \([^{3}H]\)thymidine, harvested, and radioactivity was determined as described under “Experimental Procedures.” Each experiment was performed in triplicate. Data are expressed as mean \pm S.D. of three separate experiments.

Peritoneal macrophages from C3H/He mice (5 \times 10^5 cells/well in 24-well tissue culture plates) were incubated for 7 days in medium A with 40 \(\mu\)g/ml Ox-LDL in the presence of 50 nM wortmannin or 250 nM calphostin C. On day 7, the number of cells was counted as described under “Experimental Procedures.” Each experiment was performed in triplicate. Data are expressed as mean \pm S.D. of three separate experiments.

**Table 1**

| Sample                          | Cell no.         |
|--------------------------------|------------------|
| Medium alone                    | \(\times 10^4\)/well |
| Wortmannin (50 nM)              | 3.52 \pm 0.52 (100%) |
| Calphostin C (250 nM)           | 3.57 \pm 0.33 (101%) |
| Ox-LDL (40 \(\mu\)g/ml)         | 3.61 \pm 0.46 (103%) |
| Ox-LDL + wortmannin             | 7.24 \pm 0.92 (206%)# |
| Ox-LDL + calphostin C           | 5.23 \pm 0.50 (149%)# |

GM-CSF was determined by mouse GM-CSF-specific ELISA as described under “Experimental Procedures.” Each experiment was performed in triplicate. Data are expressed as mean \pm S.D. of three separate experiments. Statistical analyses were performed using Student’s \(t\) test. *, \(p < 0.05\), compared with cells incubated with Ox-LDL alone.

**Table 2**

Effect of calphostin C and wortmannin on expression of GM-CSF mRNA induced by Ox-LDL

| Sample                          | GM-CSF (pM)     |
|--------------------------------|----------------|
| Ox-LDL (40 \(\mu\)g/ml)         | 3.58 \pm 0.23 (100%) |
| Wortmannin (50 nM)              | 3.57 \pm 0.22 (101%) |
| Calphostin C (250 nM)           | 3.61 \pm 0.24 (103%) |
| Ox-LDL + wortmannin             | 7.24 \pm 0.92 (206%)# |
| Ox-LDL + calphostin C           | 5.23 \pm 0.50 (149%)# |

GM-CSF expression. Incubation of cells with the medium alone was not associated with GM-CSF mRNA expression under the experimental conditions, whereas incubation with 40 \(\mu\)g/ml Ox-LDL resulted in the appearance of a significant band of GM-CSF mRNA (Fig. 2). This band was significantly reduced by coincubation with calphostin C, while coincubation with wortmannin had no effect (Fig. 2). We next compared the effects of calphostin C and wortmannin on Ox-LDL-induced GM-CSF release into the culture medium by using ELISA specific for mouse GM-CSF. As shown in Fig. 3A, macrophages spontaneously released GM-CSF into the medium at a concentration of 0.15 pM. Incubation of macrophages with 40 \(\mu\)g/ml LDL resulted in a slight increase in GM-CSF release into the culture medium, but its concentration returned to basal level at 24 h (Fig. 3A).

In contrast, when cells were incubated with the same concentration of Ox-LDL, the concentration of GM-CSF in the culture medium markedly increased and reached a peak level at 4 h, followed by a gradual decrease to the basal level at 24 h (Fig. 3A). When cells were incubated for 4 h with Ox-LDL together with 250 nM calphostin C or 50 nM wortmannin, Ox-LDL-induced GM-CSF secretion was inhibited by 70% by calphostin C, whereas wortmannin had no effect (Fig. 3B), as was the case with GM-CSF expression (Fig. 2). The above results indicated that both calphostin C and wortmannin significantly inhibited macrophage proliferation (Fig. 1 and Table 1), whereas only calphostin C effectively inhibited GM-CSF expression (Fig. 3) as well as GM-CSF release into the medium (Fig. 3). Thus, it seems relevant to assume that the principal site of action of PKC is upstream in the signaling pathway for GM-CSF expression, but that of PI3K is downstream in the signaling pathway after GM-CSF expression, or a signaling pathway unrelated to GM-CSF.
Involvement of PKC and PI3K in Macrophage Proliferation

Effects of Calphostin C and Wortmannin on GM-CSF-induced Macrophage Proliferation—In order to test our notion that PI3K might be involved in the signaling pathway after GM-CSF release, we examined the effect of wortmannin on macrophage proliferation induced by recombinant GM-CSF. As shown in Fig. 4A, the inhibitory effect of calphostin C on recombinant GM-CSF-induced thymidine incorporation into macrophages was negligible (up to 250 nM) or very small (15% inhibition at 500 nM) (Fig. 4A). In sharp contrast, GM-CSF-induced thymidine incorporation into macrophages was significantly inhibited by wortmannin in a dose-dependent fashion, although the extent of inhibition was incomplete, amounting to 45% at 50 nM wortmannin. The cell-counting assay also showed that macrophage proliferation induced by recombinant GM-CSF was significantly inhibited by 52% by wortmannin, but not by calphostin C (Table II). These results suggested that PI3K acts, at least in part, after GM-CSF release in the signaling pathway of macrophage proliferation.

PI3K Activity in Ox-LDL-treated Macrophages—We finally compared the serial changes in PKC and PI3K activities of peritoneal macrophages during incubation with Ox-LDL or LDL. Incubation of macrophages with LDL did not change the membrane PKC activity. However, incubation of cells with Ox-LDL resulted in a rapid activation of PKC, reaching a peak activity at 10 min after the addition of Ox-LDL to the culture medium. In contrast to PKC activity, however, PI3K activity gradually increased again to 2.3-fold above the basal level, with a second peak at 4 h after the addition of Ox-LDL (Fig. 5B). Incubation with LDL did not change PI3K activity in these macrophages (data not shown). These results indicate that Ox-LDL-induced activation of PI3K occurs at two time points, 10 min and 4 h after the addition of Ox-LDL to the culture medium. In order to specify which time point is most important for Ox-LDL-induced macrophage proliferation, we determined the effects of calphostin C and anti-GM-CSF antibody on Ox-LDL-induced PI3K activation. As shown in Fig. 6, Ox-LDL-induced PI3K activation at 10 min was not affected by anti-mouse GM-CSF antibody and calphostin C, whereas PI3K activation at 4 h after the addition of Ox-LDL was significantly inhibited (by 70%) not

**TABLE II**

| Sample                          | Cell no.      |
|--------------------------------|---------------|
| Medium alone                   | 4.28 ± 0.19 (100%) |
| Wortmannin (50 nM)             | 4.31 ± 0.21 (101%) |
| Calphostin C (250 nM)          | 4.29 ± 0.23 (100%) |
| GM-CSF (5 nM)                  | 13.62 ± 0.83 (318%) |
| GM-CSF + wortmannin            | 8.76 ± 0.82 (205%)§ |
| GM-CSF + calphostin C          | 12.16 ± 0.62 (280%) |

**FIG. 4** Effect of calphostin C (A) and wortmannin (B) on recombinant GM-CSF-induced thymidine incorporation into macrophages. A, Peritoneal macrophages (5 × 10^4 cells/well) in 24-well tissue culture plates) were incubated for 4 days with medium A alone (●) or with 5 nM recombinant GM-CSF in the presence of indicated concentrations of calphostin C (○). B, peritoneal macrophages (6 × 10^4 cells/well) were incubated for 4 days with medium A alone (●) or with 5 nM recombinant GM-CSF in the presence of indicated concentrations of wortmannin (○). During the last 18 h of incubation, cells in each well were chased with [3H]thymidine and harvested, and radioactivity was determined as described under “Experimental Procedures.” Each experiment was performed in triplicate. Data are expressed as mean ± S.D. of three separate experiments. Statistical analyses were performed using Student’s t test. §, p < 0.05, compared to medium alone; #, p < 0.05, compared to GM-CSF (Student’s t test).

**FIG. 5** Effect of Ox-LDL on membrane PKC activity (A) and PI3K activity (B). A, peritoneal macrophages (1 × 10^6 cells/well) in 10 ml of serum-free RPMI 1640 medium were incubated for indicated times with 40 μg/ml Ox-LDL (closed column) or LDL (hatched column). The membrane PKC activity was determined as described under “Experimental Procedures.” Data represent the mean ± S.D. of three separate experiments. Statistical analyses were performed using Student’s t test. §, p < 0.05, compared with control (time 0). B, peritoneal macrophages (1 × 10^6 cells/well) in 10 ml of serum-free RPMI 1640 medium were incubated for the indicated time intervals with 40 μg/ml Ox-LDL. PI3K was immunoprecipitated and incubated with phosphatidylinositol and [32P]ATP. Then, labeled PI 3-phosphate was detected by thin layer chromatography and autoradiography (inset). To quantify PI3K activity, the amounts of labeled PI 3-phosphate were analyzed using a BioImage Analyzer. Data represent the mean ± S.D. of three separate experiments. Statistical analyses were performed using Student’s t test. §, p < 0.05, compared with the control (time 0).
Involvement of PKC and PI3K in Macrophage Proliferation

![Graph](Image)

**FIG. 6.** Effect of calphostin C and anti-GM-CSF antibody on Ox-LDL-activated PI3K activity. Peritoneal macrophages (1 × 10⁷ cells/well) in 10 ml of serum-free RPMI 1640 medium were incubated with 40 μg/ml Ox-LDL for 10 min or 4 h with 5 μg/ml non-immune IgG or anti-GM-CSF antibody, or 250 nm calphostin C. PI3K was immunoprecipitated and incubated with phosphatidylinositol and [32P]ATP. Then, labeled PI 3-phosphate was detected by thin layer chromatography and autoradiography (inset). To quantify PI3K activity, the amounts of labeled PI 3-phosphate were analyzed using a BioImage Analyzer. Data represent the mean ± S.D. of three separate experiments. Statistical analyses were performed using the Student’s t test. #, p < 0.05, compared with cells incubated with Ox-LDL alone for 4 h.

only by calphostin C but also by an anti-mouse GM-CSF antibody (Fig. 6). These results strengthened our contention that PI3K is functional, at least in part, after PKC-mediated GM-CSF expression.

**DISCUSSION**

Macrophages and macrophage-derived foam cells are known to proliferate in atherosclerotic lesions (3–5). Recent studies showed that Ox-LDL exhibited a growth-promoting activity toward several types of macrophages in vitro (6–14, 23–25). However, to our knowledge, the signaling pathway(s) from binding of Ox-LDL to macrophage proliferation has not been fully defined. Since macrophage-derived foam cells are thought to play an important role in the development and progression of atherosclerotic lesions (1), elucidation of the mechanism of Ox-LDL-induced macrophage proliferation would be an interesting project. In this regard, we recently demonstrated that activation of PKC and subsequent release of GM-CSF play an important role in Ox-LDL-induced macrophage proliferation in vitro (13, 14). On the other hand, Martens et al. (12) recently reported the involvement of PI3K in Ox-LDL-induced macrophage proliferation. Therefore, we compared in the present study the role of PKC to that of PI3K. The major conclusions of the present study could be summarized as follows. In the signaling pathway leading to macrophage proliferation, PKC is located before GM-CSF induction, whereas PI3K is located, at least in part, after GM-CSF induction (see Fig. 7). These conclusions were supported by the following findings. (i) Ox-LDL-induced macrophage proliferation was significantly inhibited by a PKC inhibitor, calphostin C, and a PI3K inhibitor, wortmannin (Fig. 1 and Table I). (ii) Ox-LDL-induced GM-CSF expression and its subsequent release into the culture medium were inhibited by calphostin C but not by wortmannin (Figs. 2 and 3). (iii) In contrast, recombinant GM-CSF-induced macrophage proliferation was significantly inhibited by wortmannin but not by calphostin C (Fig. 4 and Table II). (iv) PI3K activation by Ox-LDL occurred at two time points (10 min and 4 h after the addition of Ox-LDL); the latter was inhibited by calphostin C and by an anti-GM-CSF antibody, whereas the former was not affected by an anti-GM-CSF antibody or by PKC inhibitor (Fig. 6).

Our previous results (13, 14) and those of the present study clearly showed that the Ox-LDL-induced GM-CSF release is mediated by activation of PKC. Extensive studies using T-lymphocytes showed that GM-CSF induction following PKC activation is mainly regulated at a transcriptional level (26) and several cis-acting elements that regulate GM-CSF gene expression were identified (27). Moreover, T-lymphocytes were shown to express and release GM-CSF in response to PKC activators, such as phorbol 12-myristate 13-acetate and A23187 (a calcium ionophore) (28–31). Furthermore, phorbol 12-myristate 13-acetate alone could significantly induce macrophage proliferation (13, 32). The PKC family is known to comprise at least 11 different isoforms of serine/threonine protein kinase, such as conventional PKC (α, β1, β2, and γ), novel PKC (δ, ε, θ, and η), and atypical PKC (ζ and λ) (33). Activation of PKC is regulated by C1 and C2 regions (34). The C1 region is composed of two tandem repeats of a cysteine-rich, zinc finger-like motif, which serves as a binding site for diacylglycerol and phorbol 12-myristate 13-acetate, and the C2 region is required for calcium sensitivity (34). Therefore, it is possible to assume that conventional PKC containing both C1 and C2 regions is a candidate signal mediator of Ox-LDL-induced GM-CSF induction, but the involvement of novel PKC having only C1 region cannot be ruled out. With regard to downstream signaling pathways from PKC activation to GM-CSF expression, our recent study using gel shift and luciferase assays showed that a putative AP-2 binding site from −169 to −160 of the murine GM-CSF promoter was a positive responsive site and GM-kB/GC box (−95 to −73) was a negative responsive site for Ox-LDL-induced GM-CSF expression in mouse peritoneal macrophages (35). Further studies are necessary to identify PKC isosform(s) specific for Ox-LDL-induced GM-CSF expression.

GM-CSF is a glycoprotein-nature cytokine that regulates the differentiation, survival, and proliferation of granulocytes/macrophages (28). The biological action of GM-CSF is mediated by its specific receptor which consists of two subunits designated α and β subunits (36, 37). The β subunit has a long intracytoplasmic tail and plays an important role in signal transmission, but has neither an intrinsic enzyme activity nor a binding site for G proteins (38). Binding of GM-CSF to its receptor in various types of cells generates several intracellular tyrosine phosphorylation pathways, such as Janus kinase/signal transducers and activators of transcription, Jun NH₂-terminal kinase/stress-activated protein kinase, Ras-Raf mitogen-activated protein kinase, PI3K-protein kinase B, and protein kinase A (39–42). In the present study, we demonstrated that Ox-LDL enhanced PI3K activity at 10 min and 4 h after the addition of Ox-LDL (Fig. 5), and that the latter was significantly inhibited by an anti-GM-CSF antibody (Fig. 6). Moreover, macrophage proliferation induced by Ox-LDL or GM-CSF was significantly inhibited by wortmannin (Tables I and II). Furthermore, an anti-GM-CSF antibody significantly inhibited Ox-LDL-induced macrophage proliferation (14). These findings strongly suggest that activation of PI3K at the late time point is involved in Ox-LDL-induced macrophage proliferation after GM-CSF expression. However, we also demonstrated that 50 nm wortmannin produced 50% inhibition of Ox-LDL-induced macrophage proliferation (Fig. 1 and Table I). Moreover, under identical conditions, 20 μM LY294002, another PI3K inhibitor, also showed 50–55% inhibition when assessed by both thymidine incorporation and cell counting assays (data not shown). The concentrations of these PI3K inhibitors used...
FIG. 7. Schematic representation of the signaling pathways of Ox-LDL-induced macrophage proliferation. The results of the present and previous studies (Refs. 13 and 14) as well as those of other investigators (Refs. 12 and 50) support the following scheme regarding the signaling pathways of Ox-LDL-induced macrophage proliferation. Ox-LDL-induced stimulation is first transmitted into cells via an unidentified pertussis toxin-sensitive G-protein-coupled receptor. This activates phospholipase C (PLC), which mediates hydrolysis of phosphatidylinositol diacylglycerol (PI3K) enzymes as well as calcium released from the endoplasmic reticulum (ER) stimulated by inositol triphosphate to activate calcium. Activated PLC then induces the expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) and its release into the medium. Interaction of GM-CSF with its receptor leads to induction of macrophage proliferation in an autocrine or paracrine fashion either via a PI3K pathway (50%) or a PI3K-independent pathway(s) (50%). Since Ox-LDL-induced macrophage proliferation is inhibited by >80% by anti-GM-CSF antibody, the major pathway is GM-CSF-dependent (>80%), whereas the remaining portion (<20%) could be mediated by a cytokine(s) distinct from GM-CSF. Ox-LDL-induced PI3K activation at 10 min plays a minor, if any, role in Ox-LDL-induced macrophage proliferation, since it does not influence GM-CSF expression.

REFERENCES

1. Ross, R. (1993) Nature 362, 801–809
2. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witzum, J. L. (1989) N. Engl. J. Med. 320, 915–924
3. Gordon, D., Reidy, M. A., Benditt, E. P., and Schwartz, S. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4609–4613
4. Rosenfeld, M. K., and Ross, R. (1990) Arteriosclerosis 10, 680–687
5. Spagnoli, L. G., Orlandi, A., and Santus, G. (1991) Atherosclerosis 88, 87–92
6. Uy, S., Sasaki, T., Miyazaki, A., Horiiuchi, S., Yamazaki, M. (1993) Arterioscler. Thromb. 13, 331–337
7. Sakai, M., Miyazaki, A., Hakamata, H., Sasaki, T., Uy, S., Yamazaki, M., Shichiri, M., and Horiiuchi, S. (1994) J. Biol. Chem. 269, 31430–31435
8. Sakai, M., Miyazaki, A., Hakamata, H., Kodama, T., Suzuki, K., Shichiri, M., and Horiiuchi, S. (1996) J. Biol. Chem. 271, 27346–27352
9. Sato, Y., Kobori, S., Sakai, M., Yano, T., Higashi, T., Matsumura, T., Morikawa, W., Terano, T., Miyazaki, A., Horiiuchi, S., Matsumura, T., Shichiri, M., and Horiuchi, S. (1996) Atherosclerosis 125, 15–26
10. Hamilton, J. A., Myers, D., Jessup, W., Cochrane, F., Byrne, R., Whitty, G., and Ross, S. (1999) Arterioscler. Thromb. 19, 98–105
11. Sakai, M., Miyazaki, A., Hakamata, H., Sato, Y., Matsumura, T., Kobori, S., Shichiri, M., and Horiuchi, S. (1996) Arterioscler. Thromb. 16, 600–605
12. Martens, J. S., Reiner, N. E., Herrera-Velit, P., and Steinbrecher, U. P. (1998) J. Biol. Chem. 273, 4915–4920
13. Matsumura, T., Sakai, M., Kobori, S., Biwa, T., Takemura, T., Matsuda, H., Hakamata, H., Horiiuchi, S., and Shichiri, M. (1997) Arterioscler. Thromb. 17, 3013–3020
14. Biwa, T., Hakamata, H., Sakai, M., Miyazaki, A., Suzuki, H., Kodama, T., Shichiri, M., and Horiiuchi, S. (1998) J. Biol. Chem. 273, 28305–28313
15. Miyazaki, A., Sakai, M., Sugino, H., Hakamata, H., Sakamoto, Y., and Horiiuchi, S. (1994) J. Biol. Chem. 269, 5264–5269
16. Sakai, M., Miyazaki, A., Sakamoto, Y., and Horiuchi, S. (1992) FEBS Lett. 314, 199–202
17. Miyazaki, A., Rahim, A. T. M. A., Araki, S., Morino, Y., and Horiuchi, S. (1991) Biochim. Biophys. Acta 1082, 143–151
18. Hakamata, H., Miyazaki, A., Sakai, M., Sugino, H., Sakamoto, Y., and Horiiuchi, S. (1994) Arterioscler. Thromb. 14, 1860–1865
19. Moore, K. J., Fabunmi, R. P., Anderson, L. P., and Freeman, M. W. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1647–1654
20. Miyatake, S., Otaka, T., Yokota, T., Lee, F., and Arai, K. (1985) EMBO J. 4, 2561–2568
21. Alonso, S., Minty, A., Bourlet, Y., and Bruckdorfer, M. (1986) J. Mol. Biol. 23, 11–22
22. Fukui, Y., and Hanafusa, H. (1989) Mol. Cell. Biol. 9, 1651–1658
23. Martens, J. S., Lougheed, M., Gomaz-Munoz, A., and Steinbrecher, U. P. (1999) J. Biol. Chem. 274, 10903–10910
24. Sakai, M., Shichiri, M., Hakamata, H., and Horiiuchi, S. (1998) Trends Cardiovasc. Med. 8, 119–124
25. Sakai, M., Biwa, T., Matsumura, T., Takemura, T., Matsuda, H., Anami, Y., Sasahara, T., Kobori, S., and Shichiri, M. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 1726–1735
26. Brunson, K. A., Beverly, B., Kang, S.-M., Lenaldo, M., and Schwartz, R. H. (1991) J. Immunol. 147, 3601–3609
27. Miyatake, S., Seiki, M., Yosida, M., Arak, K. (1988) Mol. Cell. Biol. 8, 5381–5387
28. Gasen, J. C. (1991) Blood 77, 1131–1145
29. Sugimoto, K., Kubo, A., Miyatake, S., Araki, K., and Arai, N. (1990) Int. Immunol. 2, 787–794
30. Masuda, E. S., Tomokita, H., Tsuboi, A., Shishomai, J., Hung, P., Araki, K., and Arai, N. (1993) Mol. Cell. Biol. 13, 7399–7407
31. Wang, C.-Y., Bassuk, A. G., Boise, L. H., Thompson, C. B., Bravo, R., and Richmond, J. M. (1994) Mol. Cell. Biol. 14, 1153–1159
32. Hamilton, J. A., and Dientsman, S. R. (1981) J. Cell Biol. 106, 445–450
33. Hug, H., and Sarre, T. F. (1993) Biochem. J. 291, 329–343
34. Nishizuka, Y. (1985) FASEB J. 9, 484–496
35. Matsumura, T., Sakai, M., Matsuda, K., Furukawa, N., Kaneko, K., and
Involvement of PKC and PI3K in Macrophage Proliferation

Shichiri, M. (1999) J. Biol. Chem. 274, 37665–37672
36. Mitajima, A., Mui, A. L., Ogorochi, T., and Sakamaki, K. (1993) Blood 82, 1960–1974
37. Bagley, C. J., Woodcock, J. M., Hercus, T. R., Shannon, M. F., and Lopez, A. F. (1995) J. Leukocyte Biol. 57, 739–746
38. Rapoport, A. P., Abboud, C. N., and DiPersio, J. F. (1992) Blood Rev. 6, 43–75
39. Miike, S., Hiraguri, M., Kurasawa, K., Saito, Y., and Iwamoto, I. (1998) J. Leukocyte Biol. 65, 700–706
40. Hiraguri, M., Miike, S., Sano, H., Kurasawa, K., Saito, Y., and Iwamoto, I. (1997) J. Allergy Clin. Immunol. 100, S45–S51
41. Hinton, H. J., and Welham, M. J. (1998) J. Immunol. 162, 7002–7009
42. Coleman, D. L., Liu, J., and Bartiss, A. H. (1989) J. Immunol. 145, 4134–4140
43. Herrera-Veit, P., and Reiner, N. E. (1996) J. Immunol. 156, 1157–1165
44. Aagaard-Tillery, K. M. (1996) J. Immunol. 156, 4543–4554
45. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
46. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 3563–3567
47. Yao, R., and Cooper, G. M. (1995) Science 267, 2003–2006
48. McLeish, K. R., Knall, C., Ward, R. A., Gerwins, P., Coxon, P. Y., Klein, J. B., and Jonson, G. L. (1996) J. Leukocyte Biol. 64, 537–549
49. Al-Shami, A., and Naccache, P. H. (1999) J. Biol. Chem. 274, 5333–5338
50. Shackelford, R. E., Misra, U. K., Florine-Casteel, K., Sheau-Fung, T., Pizzo, S. V., and Adams, D. O. (1995) J. Biol. Chem. 270, 3475–3478
Sites of Action of Protein Kinase C and Phosphatidylinositol 3-Kinase Are Distinct in Oxidized Low Density Lipoprotein-induced Macrophage Proliferation

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