Oxidized Low-Density Lipoprotein Stimulates Protein Kinase C (PKC) and Induces Expression of PKC-Isotypes via Prostaglandin-H-Synthase in P388D$_1$ Macrophage-Like Cells†

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ABSTRACT: Treatment of cells with LPS-free oxLDL significantly enhanced protein kinase C (PKC) activity in cell extracts from P388D$_1$ macrophage-like cells as determined by phosphorylation of histone H1 or Ac-MBP[4-14] substrate peptide. This effect was abolished by the PKC inhibitors H-7 and bisindolylmaleimide I while pertussis toxin failed to block stimulation. The phosphotransferase activity was also increased by acetylated LDL (acLDL) and maleylated albumin (malBSA), the oxLDL effect was inhibited by chloroquine which also blocked oxLDL-induced stimulation of tyrosine kinase activity. Marginal stimulation of PKC activity was observed when lipid extracts from oxLDL were used, indicating that uptake via scavenger receptors (SR) is mandatory. Polyinosinic acid (poly I) exhibited a concentration-dependent inhibition of the oxLDL-induced effect suggesting that SR II/I but not CD36 interactions are critical to PKC activation. Modified (lipo)proteins increased the concentration of diacylglycerol and differentially affected the levels of individual PKC isoenzymes predominantly in the cytosolic fraction. Changes of activity induced by oxLDL could be primarily assigned to alterations of the activities and levels of the isoenzymes β and δ. Treatment with oxLDL, acLDL, and malBSA was also accompanied by increased production of prostaglandins as well as by an enhanced level of cyclooxygenase 2 (COX 2) as determined by Western blot analysis. Effecs of oxLDL on PKC activity/expression was supressed by the cyclooxygenase inhibitors indomethacin, by pre-exposure to the inhibitor of both lipoxygenase and cyclooxygenase, 2,2-dimethyl-6-(4-chlorophenyl)-7-phenyl-2,2-dihydro-1*H*-pyrrolizine-5-ylacetic acid (ML 3000), and by treatment with the specific COX 2-inhibitor *N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398). These results indicate that oxLDL, acLDL, and malBSA exhibit a COX 2-dependent and isotype specific effect on PKC in P388D$_1$ cells following uptake via SR II/I and subsequent lysosomal degradation.

Since Goldstein and Brown (Goldstein et al., 1979) reported the uncontrolled uptake of acetylated LDL[1] (acLDL) by macrophages, there is a large body of evidence which now supports the involvement of oxidatively modified LDL (oxLDL) in the development of atherosclerotic lesions (Rosenfeld, 1991; Witztum & Steinberg, 1991). OxLDL is detectible in human arteries (Ylä-Herttuala et al., 1989) and on the basis of present knowledge represents a critical feature in atherogenesis (Steinberg et al. 1989). The oxidation of the lipoprotein is initiated by transition metal ions *in vitro* (Lynch & Frei, 1993) or by interactions with cells, such as endothelial cells (Steinbrecher et al. 1984) and monocytes/macrophages (Parthasarathy et al., 1986). Modification of LDL by oxidative processes results in uncontrolled, receptor-mediated uptake by macrophages (Steinbrecher et al. 1989) which in turn causes a great variety of biological effects including the accumulation of cholesterol ester transformation to foam cells, enhanced synthesis of prostanoids, changes in gene expression, and cell toxicity to mention a few (Ross, 1993; Esterbauer, 1993; Lusis & Navab, 1993).

Native LDL has been reported to induce translocation of protein kinase C activity in smooth muscle cells, and it has been suggested that activation of PKC, together with the intrinsic kinase characteristic of growth factor receptors, may be crucial to the signal transduction pathway for LDL (Scott-Burden et al., 1989). However, despite phosphorylation being a central event in signal transduction and cell regulation, the knowledge about the effects of lipoproteins on protein phosphorylation is limited. In particular, little is known about the role of PKC in relation to oxLDL-induced effects. PKC is a family of isoenzymes varying in peptide sequence and in enzymological properties [for review see Nakamura and Nishizuka (1994)]. Recent studies emphasize the isoenzyme-specific control of cellular events, likely to involve specific mechanisms for activation of distinct isoforms (Hug & Sarre, 1993). PKC-dependent regulatory events with potential implications in atherogenesis comprise

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[1] Abbreviations: COX, cyclooxygenase (= prostaglandin H synthase); DAG, diacylglycerol; LDL, low-density lipoprotein; oxLDL low-density lipoprotein; acLDL, acetylated low-density lipoprotein; LOX, lipoxygenase; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; malBSA, maleylated bovine serum albumin; MBP, myelin basic protein; NDGA, nordihydroguajaretic acid; PAF, platelet-activating factor (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine); PGE_2, prostaglandin E_2; PIP_2 phosphatidylinositol 4,5-bisphosphate; IP_3, inositol triphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; SD, standard deviation; SR, scavenger receptor; poly I, polyinosinic acid; TNBS, trinitrobenzenesulfonic acid.

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the regulation of LDL-receptor activity (Auwerx et al., 1989a) and synthesis of prostaglandins (Pfannkuche et al., 1989) in macrophages as well as the expression of scavenger receptors (Auwerx et al., 1989b). The aim of this study was to examine the effects of LDL, oxidized LDL, and modified lipoproteins on the activity of protein kinase C with special emphasis on isotype-specific effects. The PKC family comprises at least 11 subspecies of serine/threonine protein kinase (Hug & Sarre, 1993). These isoenzymes have been grouped into classes depending on their requirements for calcium. They can be further subdivided on the basis of structural features and their requirements for lipid activators. The term conventional PKC designates the four Ca^{2+}-, phosphatidylserine-, and diacylglycerol (DAG)-dependent protein kinases (PKC α, β1, β2, and γ); the PKCβ_1 and β_2 proteins are derived from one gene via alternative splicing and differ only in the sequence of several C-terminal amino acids. The novel PKCs (PKC δ, ϵ, θ, η, the last being also known as L) comprise phospholipid-, diacylglycerol-, and phosphatidylserine-dependent but Ca^{2+}-independent subtypes; atypical PKCs consist of ζ and the recently described ι subspecies.

In this study we evaluate the ability of several scavenger receptor ligands to affect the levels and the distribution of four selected PKC isoenzymes in the membrane and cytosolic fraction of the murine cell line $P388D_1$. Furthermore, during our attempts to elucidate the mechanism leading to changes in enzyme activity or distribution, we examined the consequences of receptor−ligand interactions and receptor-mediated uptake with regard to phosphotransferase activity. We found that PKC activity in $P388D_1$ macrophage-like cells is stimulated by oxLDL and other scavenger receptor ligands causing cyclooxygenase-dependent changes in the levels of α, β, δ, and ζ PKC isoenzymes.

EXPERIMENTAL PROCEDURES

Reagents. (2,2-Dimethyl-6-(4-chlorophenyl)-7-phenyl-2,2-dihydro-1*H*-pyrrolizine-5-yl)acetic acid (ML 3000) was obtained from Merckle (Blaubeuren, Germany). Protein kinase C (PKC) from rat brain, monoclonal antibodies to PKC isoenzymes α, β, δ, and ζ, anti-rabbit IgG conjugated with alkaline phosphatase, 4-nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate, and ATP-Na_2 were obtained from Boehringer Mannheim (Mannheim, Germany). Antibodies against inducible cyclooxygenase were obtained from Cascade (Reading, U.K.), and tyrosine kinase substrate peptide (RRLTEDNEYTARG) was from Bachem (Bubendorf, Switzerland). Ac-MBP[4-14] peptide (APRTPGGRR) was a product of Upstate Biotechnology (Lake Placid, NY), anti-pan protein kinase C (rabbit polyclonal IgG) was from Biomol (Hamburg, Germany). *sn*-1,2-Diacylglycerol kinase (*Escherichia coli*, 2 units/mg), 1-[6-((17β,3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U-73122), and CV 3988 were obtained from Calbiochem-Novabiochem (Bad Soden, Germany). Radioiodination of LDL was performed by Immun Diagnostic GmbH (Bensheim, Germany) according to a published protocol using *N*-bromosuccinimide as oxidizing reagent (Sinn et al., 1988). [^{125}]LDL (7.70 TBq/mM) was diluted with unlabeled LDL to attain a specific activity of 100 kBq/mg of protein; endotoxin tests were performed with E-Toxate from Sigma.

Cell Culture. The $P388D_1$ macrophage-like cell line (clone 3124, American Type Culture Collection, Rockville, MD) was cultured at 37 °C in RPMI 1640 supplemented with glutamine (2 mM), 10% heat-inactivated FCS, penicillin (50 units/mL), and streptomycin (50 μg/mL) in a humidified atmosphere (5% CO_2 in air). Cells were plated at the indicated density, washed with serum-free medium or buffer as indicated to remove non-adherent cells and then incubated with the respective stimuli for various time periods in serum-free medium.

Low-Density Lipoprotein (LDL) Preparation and Oxidation. LDL was isolated from pooled human plasma containing EDTA (1 mM) by sequential flotation in an ultracentrifuge at preselected densities (between 1.019 and 1.063 g/mL) as described (Havel et al., 1955) and dialyzed extensively against Tris-buffer (20 mM, pH 7.8, 150 mM NaCl, 0.2 mM EDTA). LDL samples were kept sterile under nitrogen in Tris-buffer at 4 °C and used within 2 weeks. Prior to oxidation, LDL was stored over polymyxin B agarose for at least 24 h, the polymyxin suspension was removed by centrifugation, the supernatant was passed through a polymyxin B agarose column (2 mL) using sterile, non-pyrogenic single-use material for intravenous application, and the eluate was dialyzed against Tris-buffer (except EDTA). All preparations were then tested, also subsequent to oxidation for absence of endotoxin. Oxidation of LDL and ^{125}I-labeled LDL (100 kBq/mg of protein, 1 mg of protein/mL) was initiated by addition of $CuSO_4$ (20 μM), followed by incubation continued for up to 24 h at 37 °C under sterile conditions. Reactions were terminated by addition of EDTA (0.2 mM).

Preparation of Acetyl-LDL and Maleylated BSA. Acetyl-LDL (acLDL) was prepared according to the method of Basu et al. (1979). Briefly, 2.5 mg of LDL (3 mg/mL) in phosphate buffer (50 mM, pH 8.0) was reacted with acetic anhydride (45 μg/mg of protein) on ice. The reagent was added in 3 portions within 40 min, keeping the pH adjusted to 8.0 by addition of 1 M NaOH. Albumin was modified with maleic anhydride as reported by Butler and Hartley (1972). Maleic anhydride in dioxane (80 μL, 1 M) was added to 20 mg of BSA (10 mg/mL) in 0.1 M potassium pyrophosphate buffer (pH 8.5). The mixture was stirred on ice, and the pH was adjusted to 9.0 with NaOH (1 M). After 5 min, 2 mL of 0.5 M sodium phosphate buffer (pH 7.4) was added. Acetylated LDL and maleylated BSA (malBSA) were dialyzed against PBS or passed through Bio-Rad P-6DGel columns and checked for absence of endotoxin as described above. The term (lipo)proteins in the following text includes acLDL, oxLDL, and malBSA. The modification of all proteins was controlled by TNBS titration of reactive lysine groups and by agarose gel electrophoresis. Electrophoretic mobility (30 mA, 50 V, 1.5 h) of the lipoproteins was determined on an agarose gel (1.0%) in Tris/CH_3COOH (pH 8.3, 8 mM) supplemented with EDTA (0.2 mM). Protein staining was carried out with Coomassie Blue R250 or with the Silverstain kit from Bio-Rad.

^{125}I-Labeled LDL Uptake. Uptake of radioiodinated LDL was determined according to a published protocol (Tokuda et al., 1993) using cells cultured in 50 mm dishes (3×10^6/dish). Where indicated, the macrophages were pretreated with lipopolysaccharides (LPS, *E. coli*, serotype 026:B6, 100 ng/mL) for 1 h at 37 °C. Two hours after seeding, they were washed three times with 2 mL of HBSS and incubated with another 2 mL of HBSS for 20 min at 37 °C. The supernatant was removed, 1.5 mL of HBSS containing 0.1%

of BSA and ^{125}I-labeled (lipo)protein (80 μg of protein/dish) was added, and incubation continued for 30 min at 37 °C. The cells were then washed three times with 1.5 mL of ice-cold HBSS and solubilized with 1.0 mL of sodium hydroxide (0.3 M) and Triton X-100 (0.1%) for 1 h at 37 °C. Aliquots were taken for determination of radioactivity (500 μL) and protein content (10 μL).

Determination of Reactive Lysines. The number of lysine residues was measured by titration with TNBS as described (Habeeb, 1966) using valine as standard. LDL (5 μg) was mixed with 100 μL of $NaHCO_3$ (4%, w/v) and 5 μL of TNBS (0.1%, w/v). After 1 h of incubation (37 °C) and the addition of 10 μL of HCl (1 M) and 10 μL of SDS (10%) the number of modified lysines was determined by measuring the absorbance at 335 nm.

PKC Assay. The macrophage (3×10^6/dish) were treated with the lipoproteins and other agents for various times in serum-free RPMI 1640 medium as described above. Following incubation, the cells were washed and scraped in 1 mL of medium, isolated by centrifugation at 1500*g* for 0.5 min at 4 °C, and resuspended in 50 μL of homogenization buffer (buffer H: 20 mM HEPES, pH 7.4, containing 2 mM EDTA, 2 mM EGTA, 2 mM DTT, 300 mM sucrose, 10 μg of aprotinin/mL, 10 μg of leupeptin/mL, 10 μg of PMSF/mL, and 10 μg of benzamidine/mL). The homogenates were fast-frozen in liquid nitrogen and stored at −80 °C for up to 2 weeks. In several experiments, the homogenates were separated into cytosolic and membrane fractions by centrifugation (100 000*g*, 60 min at 4 °C). The supernatants (cytosolic fractions) were removed, and the pellets were resuspended by brief sonication in homogenization buffer containing 0.3% Triton X-100 and incubated on ice for 45 min. They were centrifuged again (100 000*g*, 60 min at 4 °C) to yield the soluble membrane fraction. Cytosolic and soluble membrane fractions were fast-frozen in liquid nitrogen and stored at −80 °C until assayed. After being thawed, samples were sonicated at 0 °C for 30 s and PKC activity determined by measuring the incorporation of [γ-^{32}P]-ATP into histone H1 or Ac-MBP[4-14] peptide in the presence of phosphatidylserine (160 μg/mL), dioleoylglycerol (20 μg/mL), and $CaCl_2$ (1 mM) in a final volume of 50 μL. The reaction was started by addition of a mixture containing 20 mM HEPES, pH 7.4, 1 mg of histone H1 or Ac-MBP-[4-14] peptide/mL, 12.5 mM $MgCl_2$, 0.4 mM EDTA, 20 μM ATP (37 000 Bq of [γ-^{32}P]ATP/sample) to 20 μL of cell lysate (80 μg of protein). After incubation for 5 min at 37 °C, phosphorylation was terminated by transferring an aliquot (40 μL) to a Whatman phosphocellulose filter (P81, 4 cm^2). The filters were air-dried, washed four times in 75 mM *o*-phosphoric acid (5 mL/sample), dried again, and Cerenkov radiation counted.

Tyrosine Kinase Assay. The cells were homogenized, and activity was determined as described for the PKC assay using the substrate peptide (RRLTEDNEYTARG) except for the addition of phosphatidylserine, dioleylglycerol, and calcium.

Partial Purification of PKC. PKC was prepared according to a published protocol (Hauschildt et al., 1988) with some modifications: $P388D_1$ macrophages (5×10^7/mL of serum-free RPMI 1640 medium) were washed twice with medium and suspended in ice-cold extraction buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, 330 mM sucrose, 0.01% leupeptin). They were then disrupted by sonication for 1 min. The homogenate was ultracentrifuged (100 000*g*, 1 h, 4 °C), the supernatant cytosolic fraction was harvested, and the pellet was resuspended in extraction buffer containing 1% Triton X-100. The latter fraction was incubated for 60 min at 4 °C and centrifuged again as described to give a detergent-soluble fraction. Aliquots (500 μL) of the cytosolic and soluble membrane fractions were loaded on a 6 mL anion exchange column (Resource Q, Pharmacia-Biotech, Sollentuna, Sweden), equilibrated with extraction buffer (without sucrose). After being washed with 72 mL of this buffer, the enzyme was eluted with a linear gradient (0−0.4 M NaCl, 48 mL) of 2 mL/min. Fractions of 2 mL were collected, and 20 μL aliquots were assayed as described. Peak PKC activity eluted at a NaCl concentration of 130−170 mM.

Phosphorylation of Endogenous Substrates. Cellular extracts (30 μg of protein in 20 mM Tris-HCl, pH 7.4) were incubated with 10 mM magnesium acetate, 200 μM EDTA, 50 μM EGTA, 1.5 mM $CaCl_2$, and [γ-^{32}P]ATP (150 000 Bq/tube) in a total volume of 50 μL in the presence of phosphatidylserine/dioleoylglycerol. After incubation for 4 min at 27 °C, the reaction was terminated by adding 25 μL of Laemmli buffer (Laemmli, 1970) containing 10% SDS (w/v) followed by heating to 100 °C for 3 min. One-dimensional polyacrylamide electrophoresis was performed as described by Laemmli (1970) in a 4%−15% gradient gel. The gels were stained, destained, dried, and exposed to Kodak XAR film for 24 h at −80 °C. Concurrent electrophoresis of molecular weight standards allowed assignment of approximate molecular weights to the phosphorylated substrates.

PGE_2 and LTB_4 Quantitation. Eicosanoids were determined in the medium upon stimulation with the (lipo)proteins and was carried out with enzyme immunoassay kits from Amersham (Braunschweig, Germany) and Cascade (Reading, U.K.).

Western Blot Analysis and Immunoprecipitation. Western blotting of PKC isoforms was performed according to Burnette (1981). All compounds in contact with the gel were prewetted in 20 mM Tris, 150 mM glycine, and 20% methanol (degassed in vacuo before use). Equal concentrations of sample (20 μg/lane) were subjected to SDS−PAGE using a 4%−15% gradient gel. The proteins were transferred to nitrocellulose membranes (0.45 μm pore size) at 30 V for 18 h using a Bio-Rad minitransblot apparatus. The nitrocellulose then was blocked with BSA, 1% (w/v) in Tris, (pH 7.5, 50 mM, NaCl 150 mM, 0.05% Tween 20) at room temperature by gently rocking on a plate shaker. After 4 h, the blocking solution was replaced by anti-PKC, α-, β-, δ-, or ζ-solution (2 μg of antibody/mL of blocking solution), and incubation was continued for 4 h. The nitrocellulose then was washed three times with 20 mL of Tris-buffer (50 mM/L), 0.15 M NaCl, and Tween 20 (0.05%, v/v), pH 7.5, followed by treatment with anti-rabbit IgG conjugated with alkaline phosphatase (Ig-AP, 400 milliunits/mL) in washing buffer for 1 h. After three times washing and subsequent incubation with 4-nitro blue tetrazolium chloride (0.3 mM) and 5-bromo-4-chloro-3-indolyl phosphate (0.7 mM), in Tris (pH 9.5, 0.1 M; NaCl, 0.1 M; $MgCl_2$, 50 mM) for 15 min at room temperature, the staining reaction was stopped by rinsing with PBS (pH 7.4) containing 20 mM EDTA. In some experiments, determination of PKC isoenzyme level was performed by densitometry using a Bio-Rad densitometer. The anti-PKC β and δ antibodies also served for

immunoprecipitation in cell extracts; furthermore, rabbit polyclonal antibodies (anti pan PKC) were used. Precipitation was carried out with cell lysate (5 μg/mL) in homogenization buffer which was treated with the antibodies (5−15 μg/sample) for 2 h at 4 °C. The immunocomplex was captured with 100 μL of protein A agarose (2 h incubation) and isolated by brief centrifugation at 14 000*g*. PKC activity was determined in the supernatant as outlined above. In some experiments, the pellet was washed three times with ice-cold PBS (7.4), resolubilized in 40 μL of the same buffer (0.2% Triton X-100), and centrifuged, and phosphotransferase activity of the supernatant was measured.

Western blot analysis of prostaglandin H synthase 2 (cyclooxygenase 2, COX 2) was carried out essentially as described for the PKC isoforms using rabbit polyclonal antiserum against human and murine COX 2; the antiserum did not cross-react with murine COX 1. Each immunoblot analysis of PKC isotypes or COX 2 was carried out at least in duplicate, and data presented in figures and tables are representative of typical results reproduced at least twice.

Quantification of Diacylglycerol. Diacylglycerol content was measured as described (Preiss et al., 1987) with some modifications: 3×10^6 macrophages were stimulated with the (lipo)proteins; after 30 min, the supernatant was removed and all reactions were stopped by addition of 2.5 mL of methanol. Water (1.0 mL) was then added, and the cells were scraped and transferred to a 10 × 75-mm glass culture tube. Addition of chloroform (1.0 mL) gave a monophase, and the addition of water (1.5 mL), chloroform (1.5 mL), and subsequent centrifugation according to a protocol by Bligh and Dyer (1959) then gave a two-phase system. The lower chloroform phase was removed, and 500 μL was used for phosphate determination according to the method of Aimes and Dubin (1960). The remaining chloroform phase was evaporated under a stream of nitrogen, and the amount of diacylglycerol was measured as described (Whatley et al., 1993): the dry residue was solubilized in 25 μL of 7.5% octyl β-D-glucopyranoside and cardiolipin (5 mM in 1 mM DETAPAC, pH 6.6). The mixture was sonicated for 30 s and then allowed to stand for 30 min at room temperature. To the mixture was then added 50 μL of DTT (0.7 mg/mL) in reaction buffer (100 mM imidazole HCl, pH 6.6, 100 mM NaCl, 25 mM MgCl, 2 mM EGTA) and 10 units of diacylglycerol kinase. The reaction was started by the addition of 10 μL of 10 mM [γ-^{32}P]ATP (300 000 Bq/sample) and was vortexed and incubated 30 min at room temperature. Phosphorylation was terminated by the addition of 1.9 mL of a mixture of chloroform/methanol/water (1.5:2.5:1, v/v). The addition of 0.5 mL of water and 0.5 mL of chloroform then gave a two-phase system. The aqueous phase was removed, and the organic phase was washed three times with 1 mL of $HClO_4$ (0.4 M). A 50 μL aliquot was counted to estimate the total radioactivity. Another 50 μL aliquot was mixed with authentic unlabeled phosphatidic acid and then spotted on a TLC plate (eluent: chloroform/methanol/glacial acetic acid, 325:75:25, v/v). The product was identified by staining with Phospray (Supelco, Bad Homburg, Germany), and lanes containing [^{32}P]phosphatidic acid were then scraped off and Cerencov counted. The recovery and extent of conversion were verified using a known amount of dioleoylglycerol and were routinely found to be greater than 95%.

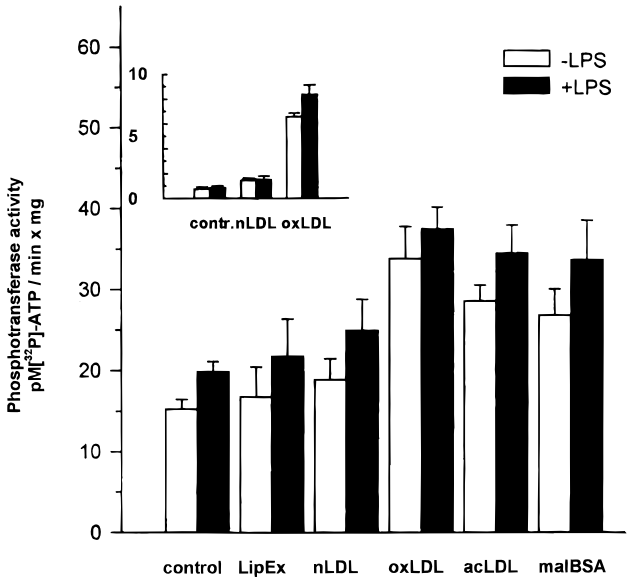

FIGURE 1: PKC activity changes induced by lipoproteins, lipid extract, and malBSA. P388D$_1$ cells were incubated with the respective (lipo)proteins (30 min, native LDL = nLDL, acetylated LDL = acLDL, oxidized LDL = oxLDL, all 80 μg/3mL of medium ≅ 50 nM; maleylated BSA = malBSA ≅ 400 nM) or with an extract of oxidized LDL (LipEx, equivalent to 80 μg of oxLDL as determined by quantification of total lipid phosphate) as indicated. Enzyme activity was determined using histone H1 or Ac-MBP[4-14] (inset) as a substrate as described under Experimental Procedures. The solid bars designate results obtained after pretreatment with LPS (100 ng/mL, 1 h, washing twice with 2 mL of medium) prior to addition of the (lipo)proteins or the extract. Results are presented as the mean ± standard deviation (SD) for triplicate determinations of a representative experiment.

RESULTS

Stimulation of PKC Activity by Oxidized LDL, Acetylated LDL, and Maleylated Albumin. The macrophage-like cell line P388D$_1$ was used to study effects of modified lipoproteins on protein kinase C. This murine cell line shows characteristics of peritoneal macrophages/human monocytes and expresses a great number of scavenger receptors (SR) (Via et al., 1985a,b), thus representing a useful tool to study receptor-dependent events likely to proceed in the early states of atherogenesis. We found that oxidized LDL (oxLDL), prepared by incubation with Cu^{2+}, significantly enhanced phosphorylation of the exogenous peptide histone H1 by P388D$_1$ cell extracts, whereas native LDL (nLDL) increased PKC activity by only 24%. Typically, the phosphotransferase activity was approximately doubled by 30 min of pretreatment with oxLDL (Figures 1 and 2a). The PKC specificity of the effect is supported by phosphorylation of the specific substrate peptide Ac-MBP[4-14] (Yasuda et al., 1990), by the inhibitory effect of H-7 (Hidaka & Hagiwara, 1987), and in particular by inhibition with the specific PKC inhibitor bisindolylmaleimide I (Davis et al., 1992) (Table 4). We found that the effect on the phosphorylation of the Ac-MBP[4-14] peptide was more pronounced than the phosphorylation of histone: oxidized LDL produced a stimulation up to 10-fold the level of the control samples (Figure 1, inset). Besides variation of relative kinase activities with different protein substrates and PKC isoenzymes, the differences in the degree of phosphorylation obtained could be due to the lower k_m and higher V_{max} of the acetylated peptide, which cannot be dephosphorylated by the most phosphatases (Farrar er al., 1991). Moreover,

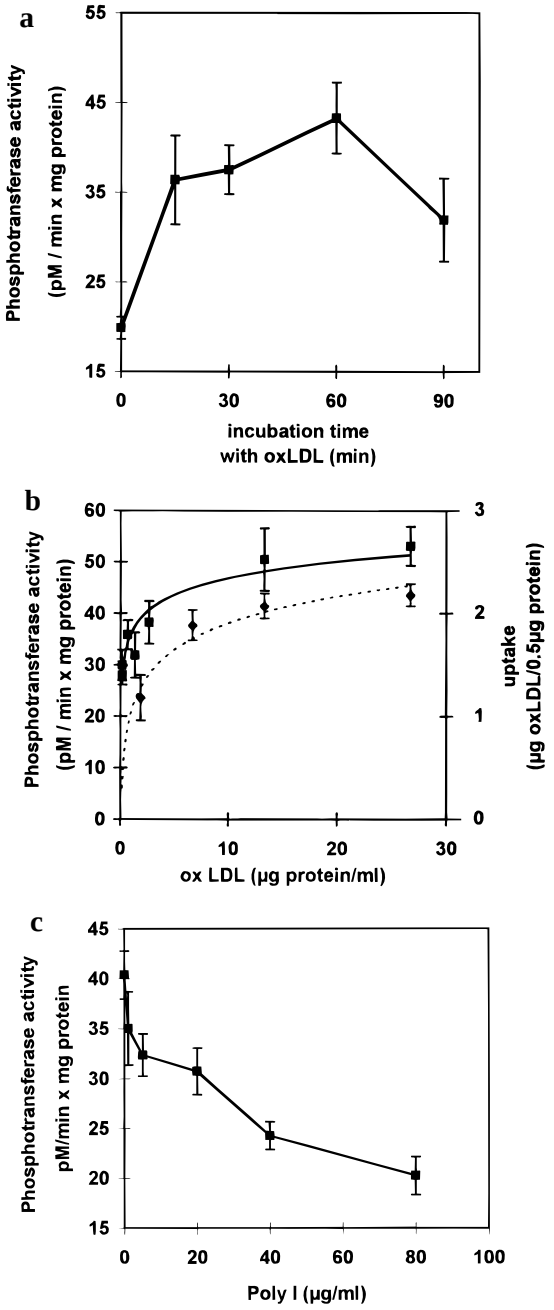

FIGURE 2: Concentration and time-dependent uptake and activation of PKC by oxidized LDL, competition by polyinosinic acid. (a) Increase of phosphotransferase activity with the duration of oxLDL treatment; P388D$_1$ cells were incubated with oxLDL (27 μg/mL), and histone phosphorylation was assayed at the indicated times. (b) Cells were treated with increasing amounts of LDL (oxidized for 24 h), and PKC activity from cell extracts was determined as phosphorylation of histone (−, pM phosphate transferred to histone/min × mg of cell protein), and the concentration-dependent uptake of oxidized [^{125}I]LDL was determined (- - -). (c) Effect of increasing amounts of polyinosinic acid on oxLDL (80 μg/3 mL)-induced PKC activity is given; control samples (not treated with lipoprotein or poly I) exhibited a phosphorylating activity of 17.5 ± 5 pM phosphate transferred to histone/min × mg of cell protein; results of a and b are presented as the mean ± SD for triplicate determinations of a representative experiment.

histone is known to enhance phosphorylation of endogenous proteins catalyzed by casein kinases (Yamamoto et al., 1979).

The activity of protein kinase C was further increased when the cells were pretreated with LPS. As shown in Figure 1, LPS pretreatment (100 ng/mL) further stimulated PKC activity up to 35% (see also Table 1), and a stimulatory effect similar to oxLDL (27 μg/mL) was obtained when cells were treated with 1 μg of LPS/mL for 30 min (not shown). Lipoproteins, however, are known as endotoxin carriers (van Lenten et al., 1986). To preclude a LPS-mediated effect, the endotoxin level of all preparations was assessed prior to addition to the cells, and we found that lipoprotein-bound endotoxin is not mandatory for oxLDL-induced PKC activation.

We next tried to analyze the molecular basis of the activatory effect of the oxLDL particle. It has been reported that PAF mediates PKC activation (Huang et al., 1988) as well as the fact that PAF-like compounds or PAF are generated during LDL oxidation (Liapikos et al., 1994). Moreover, arachidonic acid and oxidation products of unsaturated fatty acids (which are components of oxidized LDL) are capable of activating PKC (O'Brian et al., 1988). To assess the importance of the SR pathway for the incorporation of lipids, we examined the effect of chloroform−methanol extracts from oxLDL, thus probing the whole assembly of non-proteic substances. With extracts containing lipid material equivalent to the amount of intact oxLDL, only minor increases of activity were observed (10%, using histone as a substrate) (Figure 1). Higher lipid concentrations (greater than 2-fold the lipid phosphate content of 80 μg of oxLDL) were found to be cytotoxic (data not shown). Stimulation of the kinase activity produced by oxLDL could not be reduced by pretreatment with the PAF-receptor antagonist CV-3988, 40 μM (data not shown). To evaluate the contribution of protein−receptor interactions to PKC-activation, the effects of malBSA, a lipid-free scavenger receptor ligand, and acLDL, a ligand containing unoxidized (phospho)lipids, were determined. AcLDL was found to be similarly effective as oxLDL in terms of histone phosphorylation. The same effect was achieved with malBSA at elevated concentrations (400 nM, calculated on the basis of 67 kDa M_r) (Figure 1).

As depicted in Figure 2a, phosphotransferase activity markedly increased already after 15 min of exposure to oxidized LDL. Prolongation up to 1 h produced only minor further increases, therefore an exposure time of 30 min was chosen in all experiments. We next used oxLDL to examine the saturability of PKC activation. When increasing amounts of oxLDL were applied, a concomitant increase of PKC activity was observed and apparent saturation was found at concentrations around 40 μg/3 mL (25 nM) (Figure 2b). A similar concentration dependence was found when the uptake of radioiodinated oxLDL was monitored (Figure 2b). All (lipo)proteins as well as oxLDL failed to induce significant changes of enzyme activity when tested with broken cells or with the isolated enzyme (data not shown). As shown in Table 1, prolongation of the oxidation time led to enhanced degrees of LDL modification, characterized by diminished TNBS-reactive lysine groups, as well as to enhanced incorporation of radioactivity into the cells. The increases of phosphotransferase activity paralleled the amount of labeled protein found in the cells. In unprimed cells, doubling of the oxidation time from 12 to 24 h produced a 36% increase in the uptake of the labeled protein and a 60% enhancement of phosphotransferase activity. In LPS-primed cells the effect of increasing oxidation times of LDL on histone phosphorylation was similar: prolongation from 12 to 24 h caused an additional uptake of 37% more protein and enhanced PKC activity by 35%. While in LPS-primed

Table 1: Effect of Increasing Oxidative LDL Modification on Cellular Uptake of Labeled Lipoprotein and PKC Activity[a]

lipoprotein	uptake of ^{125}I-labeled LDL/ 500 μg of cell protein [%]		PKC activation/mg of cell protein [pM [^{32}P]ATP/min]		reactive NH_2 [mol of NH_2/mol of apoB][b]
	−LPS	+LPS	−LPS	+LPS	
control			15.21	19.84 ± 1.26	
nLDL	1.34 ± 0.05	3.73 ± 1.47	18.90 ± 2.57	23.61 ± 3.29	325 ± 18
oxLDL (12 h)	2.14 ± 0.21	4.91 ± 0.68	21.36 ± 2.26	27.78 ± 4.86	264 ± 36
oxLDL (18 h)	2.49 ± 0.32	5.72 ± 0.38	28.73 ± 3.51	36.11 ± 3.22	212 ± 41
oxLDL (24 h)	2.90 ± 0.15	6.73 ± 1.06	33.83 ± 3.02	37.50 ± 2.71	181 ± 17

[a] The effect of the prolongation of Cu^{2+}-initiated LDL oxidation on cellular uptake of ^{125}I-labeled protein by $P388D_1$ cells and on PKC activity (determined as phosphorylation of histone H1) was measured as described under Experimental Procedures. Priming was performed as described in the Figure 1 legend. Cells or LPS-primed (100 ng/mL) cells were treated with 80 μg of LDL in 3 mL of medium [≅50 nM apoB (M_r 500 kDa), 30 min] and oxidized for the time indicated, and incorporation of radioactivity was determined by γ-counting. [b] Estimated as TNBS reactivity. Each value is the mean ± standard deviation (SD) of triplicate determinations of a representative experiment.

Table 2: Effect of LDL on Distribution of PKC Activity in Cytosolic and Membrane Fractions [pM [^{32}P]ATP/min × mg of protein][a]

	homogenate		cytosolic fraction		membrane fraction	
	−LPS	+LPS	−LPS	+LPS	−LPS	+LPS
control	10.54 ± 7.56	9.92 ± 2.60	6.51 ± 3.66	6.20 ± 1.28	2.17 ± 0.43	3.60 ± 1.18
nLDL	13.07 ± 3.41	12.79 ± 2.14	8.72 ± 2.98	7.39 ± 1.96	1.98 ± 0.45	2.91 ± 1.49
oxLDL	23.96 ± 4.21	26.47 ± 3.69	16.99 ± 2.87	18.10 ± 3.41	3.79 ± 1.10	5.12 ± 1.51

[a] Cells were primed with LPS as indicated and stimulated with native (nLDL) and oxidized LDL (oxLDL) for 30 min, broken, and fractionated as described under Experimental Procedures. LPS treatment was carried out as described under the legend to Figure 1. Phosphotransferase activity was measured using histone H1 as substrate. The data are expressed as the means ± SD of two experiments with triplicate determinations.

cells the uptake of protein was more than double at all oxidation times monitored (compared to unprimed cells), the relative difference in PKC activation was much smaller.

The results obtained with the SR ligands led us to hypothesize that occupation of SR could be sufficient to initiate transduction of signals which lead to changes in PKC activity. However, we found that a ligand of type A scavenger receptors polyinosinic acid failed to affect PKC activity at concentrations up to 80 μg/mL (not shown). On the other hand, oxLDL-induced enzyme activation could be reduced by competition with increasing amounts of poly I, and addition of poly I at a concentration of 80 μg/mL blocked PKC activation almost completely (Figure 2c).

Effect of oxLDL on Cytosol Membrane Distribution of PKC Activity. To follow changes in the distribution of the total histone-phosphorylating capacity induced by oxidatively modified LDL and LPS, the phosphotransferase activity of the homogenate as well as of the membrane and the cytosolic fraction was analyzed. The results summarized in Table 2 show that in the control sample most of the PKC activity (approx. 75%) was located in the cytosol. When lipoprotein-induced changes were examined, we found that increases in enzyme activity were predominantly cytosolic. Treatment with nLDL raised levels of cytosolic activity by approximately 34% (20% after LPS treatment) while the activity of the membrane fraction remained unchanged. Oxidized LDL further enhanced PKC activity in the cytosol (161% in addition relative to the control, 71% of total activity) while a minor increase of phosphotransferase activity was found in the membrane fraction (75% in addition). Priming with LPS did not significantly change the distribution of activity.

Effect of oxLDL on Tyrosine Kinase Activity. When the cells were treated with the tyrosine kinase inhibitors genistein (15 μM) (Akiyama & Ogawara, 1991) or herbimycin A (100 nM) (Uehara & Fukazawa, 1991) prior to the addition of LPS-free oxLDL, phosphotransferase activation was reduced to only 46% in addition (increase caused by oxLDL ≡ 100%,

Table 3: Tyrosine Kinase Activity and Inhibition by Genistein, Herbimycin A, and Chloroquine[a]

treatment	tyrosine (PKC activity) [pM ^{32}P]ATP/min × mg of protein]
control	34.11 ± 3.0 (18.90 ± 2.57)
oxLDL (27 μg/mL)	47.91 ± 4.9 (33.83 ± 3.95)
chloroquine, 40 μM + oxLDL (27 μg/mL)	31.94 ± 5.4
LPS (100 ng/mL)	41.73 ± 6.2
genistein, 15 μM	17.62 ± 7.81 (18.40 ± 2.94)
genistein, 15 μM + oxLDL	21.37 ± 6.31 (27.55 ± 4.32)
herbimycin A, 100 nM	(17.21 ± 3.24)
herbimycin A, 100 nM + oxLDL	(16.90 ± 2.15)

[a] Cells were treated (30 min) with the respective stimuli/inhibitors (30 min pretreatment with genistein or chloroquine, 18 h with herbimycin A prior to oxLDL), washed (2 × 2 mL of medium), and broken, and the tyrosine kinase activity was determined with the RRLTEDNEYTARG substrate peptide as described under Experimental Procedures [values in parentheses refer to the phosphotransferase (PKC) activity assayed with histone H1]. The data are expressed as the means ± SD of two experiments with triplicate determinations.

Table 3). Furthermore, the inhibitor herbimycin A completely suppressed activation, suggesting a LPS-independent role of tyrosine kinases in oxLDL-induced PKC activation. To further assess the involvement of tyrosine kinases, we examined the phosphorylation of the synthetic tyrosine substrate peptide RRLTEDNEYTARG (Pike et al., 1982). Consistent with a tyrosine kinase-dependent activation of PKC by oxLDL, we found that PKC activation was accompanied by increased incorporation of phosphate in the peptide (Table 3). As observed for inhibition of PKC stimulation by chloroquine, enhancement of tyrosine kinase activity was abolished by this compound.

Inhibition of Lipoprotein-Induced PKC Activation. Administration of modified LDL is known to stimulate arachidonic acid metabolism in macrophages (Yokode et al., 1988), PGE_2 being the predominant metabolite released after challenge with acLDL (Hartung et al., 1986). Along with

Table 4: Inhibition of oxLDL-Induced PKC Activation by COX/LOX, PKC, PLC Inhibitors, and Inhibitors of RNA and Protein Synthesis[a]

treatment	PKC activity/mg of cell protein [pM ^{32}P]ATP/min]
control	17.49 ± 3.76 (0.89 ± 0.17)[b]
oxLDL	35.03 ± 4.83 (8.21 ± 0.73)[b]
H-7, 100 μM + oxLDL	26.42 ± 2.91
bisindolylmaleimide I, 100 nM + oxLDL	12.47 ± 1.98
ML 3000, 5 μM + oxLDL	17.10 ± 2.84
indomethacin, 1 μM + oxLDL	19.13 ± 4.42
NDGA, 4 μM + oxLDL	29.38 ± 3.21
NS-398, 2 μM + oxLDL	17.56 ± 0.98
pertussis toxin 1 μg/mL + oxLDL[c]	27.85 ± 8.49
chloroquine, 20 μM + oxLDL	18.36 ± 1.42
U-73122, 5 μM + oxLDL	14.49 ± 6.33
cycloheximide, 10 μM	23.77 ± 5.57
actinomycin D, 3 μM	21.68 ± 6.19

[a] Cells primed with LPS were treated with the inhibitors (all 30 min except for U-73122, which was treated for 10 min) and incubated with oxLDL (30 min) as described in the legend of Figure 1. Phosphotransferase activity was measured using histone H1 as substrate as described under Experimental Procedures. [b] Phosphotransferase activity was determined in the supernatant using Ac-MBP[4-14] peptide substrate. [c] Pertussis toxin (1 μg/mL) was added for 4 h, and then cells were incubated with oxLDL and PKC activity measured. The data are expressed as the means ± SD of two experiments with triplicate determinations.

Table 5: PKC Activity after Immunoprecipitation[a]

PKC (iso)enzyme	PKC activity [pM ^{32}P]ATP/min × mg of cell protein (% of control, respectively)[b]
oxLDL, anti pan PKC immunoprecipitation	158 ± 11[b]
oxLDL, anti PKC β immunoprecipitation	24.71 ± 5.87[c] (2.78 ± 0.65)[d] 144 ± 13[b]
oxLDL, anti PKC δ immunoprecipitation	27.49 ± 6.44[c] (5.88 ± 0.82)[d] 129 ± 9[b]

[a] Cells (3×10^6/dish) were stimulated with oxLDL (30 min, 27 μg/mL). [b] Phosphotransferase activity of the pellet was determined subsequent to immunoprecipitation with anti PKC antibodies [normalized to equal protein content, activity of the pellet of the control (w/o oxLDL) = 100%] as described under Experimental Procedures. [c] Residual PKC activity was determined in the supernatants of cell extracts with histone or [d] Ac-MBP[4-14] peptide substrate after immunoprecipitation with limiting amounts of antibodies; activities of samples (control, oxLDL) not treated with antibodies, see Table 4 for comparison. Data represent average results of at least two experiments performed with triplicate determinations.

the stimulation of PKC activity, a 5−7.8-fold increased level of PGE_2 and a 8.6−12.3-fold increased level of LTB_4 was produced by oxLDL, acLDL, and malBSA (PGE_2 only) in $P388D_1$ cells at typical concentrations (50 and 400 nM, respectively); treatment with native LDL (80 μg/3 mL, 50 nM) raised PGE_2 and LTB_4 to 2.6- and 4.8-fold the control values (data not shown). To assess the involvement of lipoxygenase and cyclooxygenase products in PKC activation we tested the inhibitory potency of nordihydroguajaretic acid (NDGA), indomethacin and ML 3000: NDGA is a redox-active lipoxygenase inhibitor (Salari et al., 1984), indomethacin is a selective cyclooxygenase inhibitor (Shen & Winter, 1977), and ML 3000 has been shown to inhibit both lipoxygenase (LOX) and cyclooxygenase (COX) by a redox-independent mechanism (Laufer et al., 1994). As presented in Table 4, ML 3000 and indomethacin completely abolished PKC activation induced by oxLDL, while NDGA (4 μM) at a concentration above IC-50 (1.8 μM) as determined by us for the LTB_4 formation in this system produced a reduction of the lipoprotein-mediated effect by only 15%. Measurement of PGE_2 and LTB_4 levels of samples pretreated with ML 3000 (5 μM), NDGA (4 μM), or indomethacin (1 μM) prior to exposure to oxLDL confirmed the inhibitory effects on eicosanoid formation and exhibited values close to those of control samples (ML 3000 PGE_2 and LTB_4 formation, NDGA LTB_4 formation) or below (indomethacin, PGE_2 formation) (data not shown). These results demonstrated that stimulation of arachidonic acid metabolism is essential to the (lipo)protein-induced increase of cytosolic PKC activity, suggesting a predominant involvement of cyclooxygenase products. To further substantiate this finding and to differentiate between cyclooxygenase 1 and the inducible cyclooxygenase 2 we tested the selective COX 2 inhibitor NS-398. This compound prevented the oxLDL-induced increase of PKC activity (Table 4) at a concentration (2 μM) reported to selectively affect COX 2 but not COX 1 activity (Futaki et al., 1994). Control experiments confirmed that neither compound (indomethacin, ML 3000 or NS-398, at concentrations listed in Table 4) reduced the basal phosphotransferase activity by ≥10% nor did any agent exhibit a direct inhibitory effect on the phosphorylating activity of ("isolated") partially purified PKC from $P388D_1$ cells (not shown).

Chloroquine was capable to prevent PKC stimulation by oxLDL (Table 4), indicating a requirement for lysosmal degradation of the particle as observed for the stimulation of tyrosine kinase activity (Table 3). The reduction provided by U-73122, a compound which inhibits the hydrolysis of L-α-phosphatidyl-D-*myo*-inositol 4-phosphate (PI) to D-*myo*-inositol 1,4,5-triphosphate (IP_3) (Yule & Williams, 1992), indicated an involvement of phospholipases C in oxLDL-induced signal transduction leading to the enhancement of phosphotransferase activity. Pre-exposure of cells to pertussis toxin was without a pronounced effect on phosphotransferase activity whereas a significant reduction was achieved by the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide, suggesting that increased kinase synthesis is involved. The investigation with isoenzyme-specific antibodies further revealed that the oxLDL-induced increase of PKC activity (≡ 100%) was reduced by immunoprecipitation with anti-PKC β (histone by 59%, Ac-MBP[4-14] by 74%) and anti-PKC δ (histone by 43%, Ac-MBP[4-14] by 32%) antibodies (Table 5). Immunoprecipitation studies (Table 5), however, also demonstrated that the observed increase of phosphotransferase activity in the cytosolic fraction is partially due to an increase of enzymatic activity, based (at least in part) on enhanced activities of PKCs β and δ.

OxLDL-Induced Expression of Inducible Cyclooxygenase. The PKC stimulatory effect of oxidatively modified LDL could be abolished by inhibitors of the inducible cyclooxygenase NS-398 as described above. To study the effect of malBSA, LPS-free oxLDL, and NS-398 treatment on the expression of COX 2, the protein level of the enzyme was measured by Western blot analysis. As depicted in Figure 3, oxLDL (80 μg/mL, 50 nM) as malBSA (400 nM) treatment produced an increase of the COX 2 protein level. This change was detectable already at 30 min after exposure to oxLDL, and protein levels further increased along with the prolongation of the treatment as well as with the time

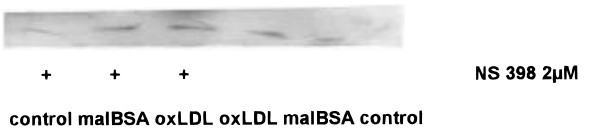

FIGURE 3: Effect of NS-398 on oxLDL and malBSA-induced COX 2 expression. After stimulation with oxLDL or malBSA (30 min, 50 or 400 nM, respectively) COX 2 protein was assayed by Western blot; +, 30 min pretreatment with NS-398 (2 μM); the immunostained band shown here is the 74 kDa band.

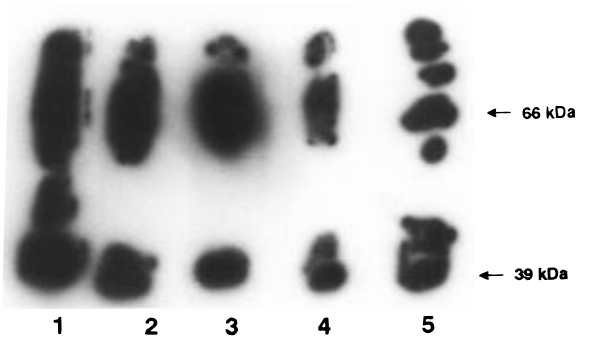

FIGURE 4: Effect of PMA, malBSA, and modified lipoproteins on endogenous substrate phosphorylation. Cells (5×10^6) were incubated with (1) 100 nM PMA, (2) malBSA, (3) oxidized LDL, (4) untreated control, and (5) native LDL. After a 30 min exposure, the cells were homogenized, and the cytosolic fraction was prepared by ultracentrifugation (100 000*g*, 4 °C, 1 h) and incubated with [γ-^{32}P]ATP in the presence of Ca^{2+}, phosphatidylserine, and dioleylglycerol. After SDS−PAGE, autoradiography was performed. Molecular mass standards are indicated to the *right* of the autoradiogram. Similar results were obtained in two other separate experiments.

after exposure (not shown). Similar results were obtained with acLDL, and a minor increase of the COX 2 protein level was found for nLDL (not shown). NS-398 pretreatment further increased the protein level in the malBSA sample, and the expression of the enzyme was also enhanced in the control sample (Figure 3). A slight increase was also observed in the oxLDL-treated sample. Under our experimental conditions no COX 2-protein was detected after preexposure to indomethacin (1 μM) prior to oxLDL.

Phosphorylation of Endogenous Substrates. To monitor changes in phosphorylation of endogenous substrates produced by oxLDL and malBSA, cellular proteins were analyzed by electrophoresis subsequent to incubation with [^{32}P]ATP. We found that treatment with oxLDL predominantly enhanced the incorporation of ^{32}P in proteins with M_r around 39 and 66 kDa (Figure 4). A similar pattern was obtained after exposure to malBSA and PMA, a modest increase of protein phosphorylation was also provoked by nLDL. Phorbol ester treatment, however, caused phosphorylation of a number of additional substrates.

Alteration of Diacylglycerol Concentration. We next examined diacylglycerol levels of lipoprotein-pretreated cells. The phorbol ester PMA caused a 2.5-fold increase at a concentration of 100 nM while nLDL exerted no significant effect (Figure 5). OxLDL approximately doubled the diacylglycerol content within 30 min after stimulation, and a similar increase was found in response to acetylated LDL (enhancement by 55%).

Effects of Lipoproteins and of malBSA on the Intracellular Level and Distribution of PKC α, β, δ, *and* ζ *Isoenzyme*. To study changes in level and distribution of PKC isoforms in response to modified (lipo)proteins, immunoblot analysis

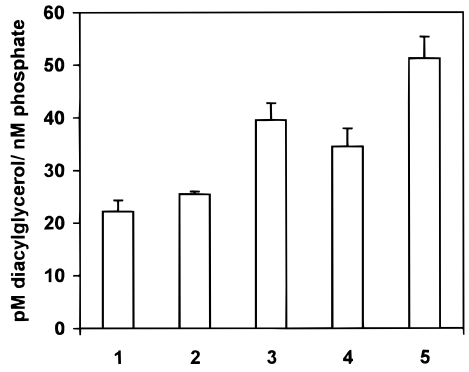

FIGURE 5: Diacylglycerol production in response to various stimuli. P388D$_1$ cells were primed with LPS (100 ng/mL, 1 h) and then incubated with the respective lipoproteins (80 μg/3 mL) as indicated: (1) untreated control, (2) native LDL, (3) oxidized LDL, (4) acetylated LDL, (5) 100 nM PMA; for conditions see Experimental Procedures. The amount of diacylglycerol was determined by scintillation spectroscopy following quantitative conversion to [^{32}P]phosphatidic acid catalyzed by 1,2-*sn*-diacylglycerol kinase in the presence of [γ-^{32}P]ATP and normalized to total lipid phosphate. Means ± SD; similar results were obtained in several independent experiments.

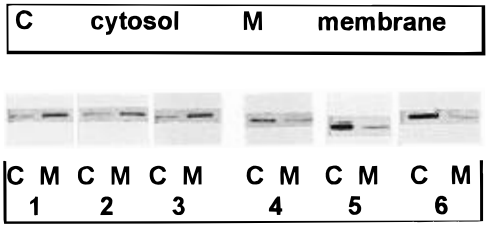

FIGURE 6: Immunoblot detection of protein kinase C β isoenzyme in P388D$_1$ cells. Cells (1×10^7/dish) were treated (1 h) with lipoproteins (27 μg/mL), and Western blotting was performed on fractionated homogenates (C, cytosolic fraction, M, membrane fraction) as described under Experimental Procedures; samples 1−3 were obtained after pretreated with LPS, and samples were obtained without pretreatment with LPS (1 h, 100 ng/mL): (1) acLDL, (2) oxLDL, (3, 4) nLDL, (5) oxLDL, (6) acLDL; for changes of the levels of other isotypes see Table 6. The immunostained band shown here is the 79 kDa band.

was carried out using isoenzyme-specific antisera. For these experiments, the cells were treated with modified lipoproteins, malBSA, and lipoxygenase/cyclooxygenase inhibitors, and the levels as well as the distribution of the α, β, δ, and ζ PKC cytosolic and membrane isotypes were determined. Prior to stimulation, proteins recognized by the antibodies were predominantly located in the cytosol, PKC β being the most abundant isoform. PKC α and δ were also present in the cytosol while PKC ζ could only be faintly detected in both membrane and cytosolic fractions. After exposure to nLDL, oxLDL, and acLDL, the cytosolic protein levels of the β and δ isotypes were significantly increased (Figure 6, Table 6) while only in the cytosol was a slight increase noted for the PKC α concentration after treatment with acLDL. When the effects of the lipoproteins were compared, oxLDL and acLDL were found to induce the most prominent changes of PKC α, β, and δ followed by modest changes caused by nLDL (PKC β and δ), indicating a prevalence of SR-dependent events (Figure 6, Table 6). The effect induced by nLDL, however, indicates that incorporation/binding of unmodified lipoprotein via LDL receptors can also cause minor changes of isotype concentrations. Priming with LPS prior to treatment with the modified lipoproteins (acLDL and oxLDL) produced enhanced recognition of the α PKC in

Table 6: Effects of Stimuli/Inhibitors on PKC Isoenzyme Levels and Distribution[a]

treatment	isoenzyme							
	α		β		δ		ζ	
	C	M	C	M	C	M	C	M
nLDL[b]	−	−	↑	−	↑	−	−	−
oxLDL[b]	↑	−	↑↑ (161)	−	↑↑ (147)	−	−	−
acLDL[b]	↑	−	↑↑	−	↑↑	−	−	−
nLDL	−	↓	−	↑	−	−	↑	−
oxLDL	↑	↑	−	↑ (128)	↑	−	↓	−
acLDL	↑	↑	−	↑	↑	−	↑	−
malBSA	−	−	↑↑	−	↑	−	−	−
ML 3000, 5 μM, + oxLDL	−	−	−	−	−	−	nd	nd
indomethacin, 1 μM, + oxLDL	−	−	−	−	−	−	nd	nd
NS-398, 2 μM, + oxLDL	=	−	−	−	−	−	nd	nd
PMA, 100 nM	↑↑	−	−	−	−	−	↑↑	−

[a] Effects of stimuli/inhibitors on PKC isoenzyme levels and distribution. Cells were pretreated, and the isotype levels (M_r 79 kDa) were measured by immunoblotting as described in the Experimental Procedures; treatment was carried out sequentially with the inhibitors (30 min) and then with the (lipo)proteins [1 h, nLDL, acLDL, and oxLDL, all 27 μg/mL (≅50 nM), malBSA ≅ 400 nM]. M, membrane fraction; C, cytosolic fraction, ↑ designates enhanced levels, ↓ reduced levels, and − indicates unchanged levels relative to the respective control (with LPS priming, 100 ng/mL, 1 h, followed by 2 × washing with 2 mL of medium), numbers in parentheses designate densitometric results [relative to the control (100%), average results of two separate experiments]; for LPS-induced changes see text, and for changes of PKC β also see Figure 6. [b] Cells not LPS-primed.

both fractions and of PKC δ only in the cytosol. The effect was marginal for PKC β in the cytosolic fraction; however, somewhat elevated levels were observed in the membrane fraction (not shown). Levels of PKC α in both fractions were further enhanced by subsequent addition of oxLDL or acLDL. Furthermore, LPS pretreatment significantly affected the ζ isoenzyme: with the exception of oxLDL all lipoproteins led to the detection of stronger protein bonds of this isotype (Table 6). A somewhat smaller increase of the PKC ζ level, however, was also observed in the LPS-treated cytosolic control samples (not shown). Thus, priming of the cells with lipopolysaccharides prior to lipoprotein addition induced changes primarily of PKC α and ζ, while the other isotypes were less affected. Similar to the lipoproteins, treatment with malBSA was followed by a marked increase of the levels of cytosolic β and δ isoenzyme while contrary to the treatment with modified LDLs, the level of the α isoenzyme remained unchanged (Table 6). Consistently, indomethacin, ML 3000, and NS-398 all abolished the effect of oxLDL and reduced PKC α, β, and δ to control levels (Table 6). All modified lipoproteins as well as malBSA were found to predominantly increase the cytosolic levels of the PKC isotypes. Minor changes were noted for the membrane fraction.

DISCUSSION

We have found that in $P388D_1$ macrophage-like cells exposure to oxidatively or chemically modified (lipo)proteins caused an increase of phosphotransferase activity as determined by histone H1 phosphorylation and increased incorporation of ^{32}P into endogenous substrates. The PKC specificity of this effect is supported by enhanced phosphorylation of the substrate peptide Ac-MBP[4-14] and by inhibition with the specific inhibitor bisindolylmaleimide I. Furthermore, a reduction of activity after removal of protein by immunoprecipitation as well as oxLDL-induced increase of activity in the precipitate indicates that exposure to modified (lipo)proteins leads to increased PKC activity.

Since PKC is activated by lipid peroxidation products (O'Brian et al., 1988) and oxidized LDL contains hydroperoxides and possibly compounds with PAF-like biological activity (Liapikos et al., 1994), we first hypothesized that these agents could be responsible for the observed change in phosphorylation capacity. PAF is capable of inducing PKC activation and a PKC-stimulating activity has also been attributed to lysolecithin, a compound released from oxidizing LDL (Sugiyama et al., 1994). However, stimulation of PKC activity was also observed when acLDL and malBSA were used, indicating that the internalization of preoxidized lipids is not mandatory. This can also be concluded from the results of experiments using extracts from oxLDL. However, the inhibitory effect of chloroquine, a compound that prevents the lysosomal degradation of oxLDL (Via et al., 1985b), indicates that the degradation of oxLDL or its components, respectively, is critical to PKC activation. The chloroquine effect, the stimulation by acLDL, and the minor effect induced by nLDL together suggest that scavenger receptor-dependent uptake of lipoproteins facilitates the incorporation of stimulatory agents which are released upon subsequent lysosomal degradation of the particle.

Pertussis toxin could not abolish the effect of oxLDL on PKC activity in cell extracts, a finding that rules out a direct SR signal coupling involving pertussis toxin-dependent G-proteins (Shackelford et al., 1995) as well as involvement of PAF receptors. It does, however, not exclude G-protein-sensitive changes (e.g., an increase of the Ca^{2+} level) which may lead to alterations of intracellular enzyme activity.

An SR dependency could be demonstrated by the concentration-dependent inhibition of the oxLDL effect by poly I. Polyinosinic acid alone, however, did not affect PKC activity. These findings confirm the involvement of scavenger receptors and allow the following conclusions: firstly, with the exception of (fatty-acid-free) malBSA, a ligand that essentially contains no phospholipids or fatty acids, ligand−receptor interactions are not sufficient to initiate enhanced phosphorylating activity; secondly, since poly I does not bind to CD 36 (Acton et al., 1994), a receptor that recognizes oxidized LDL (Endemann et al., 1993; Nicholson, 1995), binding/uptake of oxLDL via CD 36 is not relevant to PKC activation in our system. This interpretation is also supported by the very similar effects of oxLDL and acLDL in terms of PKC activation/expression and by the previous finding that oxLDL and acLDL did not compete for binding to CD 36 (Endemann et al., 1993). Others have reported that the murine macrophage-like cell line $P388D_1$ expresses both type I and type II scavenger receptors (Freeman et al., 1990), while most of the scavenger receptor protein expressed was the type II receptor (Ashkenas et al., 1993), which is likely to be primarily responsible for the uptake of oxLDL in our system. We found that the shape of the dose−response curves for oxLDL-induced PKC activation was similar to the curve obtained for the binding/degradation of [^{125}I]-oxLDL. Although parameters obtained from degradation assays represent metabolism of the ligand at steady state rather than binding at equilibrium (Ashkenas et al., 1993), the apparent dissociation constants at 37 °C were found to

be essentially the same for binding, uptake, and degradation (Abrams et al., 1992; Goldstein et al., 1979; Brown & Goldstein, (1979). These results are compatible with a direct dose−response relationship of SR binding/degradation and PKC activity. However, as PKC is a highly regulated enzyme and PKC activation/expression is mediated by several steps, including, e.g., activation of cyclooxygenase 2, the oxLDL-dependent modulation of PKC activity can only very roughly reflect binding/uptake of the particle.

Treatment with modified lipoproteins resulted in increased levels of diacylglycerol. A recent publication describes the hydrolysis of PIP_2 subsequent to SR ligation by oxLD (Shackelford et al., 1995). DAG, however, stabilizes an association of the enzyme with the membrane (Nakamura & Nishizuka, 1994), while the observed change of PKC activity in our experiments was predominantly cytosolic. Accumulation of DAG therefore does not appear to be a major cause for enhanced enzyme activity in our system. These data, however, have to be interpreted with caution as Triton X-100 was present in the assay of the membrane fraction and nonionic detergents have been shown to cause a loss of substrate phosphorylation of Ca^{2+}-dependent PKC isoforms (Mahoney & Huang, 1995). Others have noted an accumulation of DAG subsequent to the activation of PKC (Uhing et al., 1989), a finding which may also explain some of our results. This issue is presently being addressed by further investigations which include the monitoring of time-dependent changes.

Since arachidonic acid metabolism in macrophages is stimulated by ingestion of oxLDL and malBSA activates the cyclooxygenase pathway (Hamilton et al., 1987), inhibitors of eicosanoid metabolism were examined. Several lines of evidence support a cyclooxygenase-dependent increase of PKC activity/expression by modified LDL and malBSA: complete inhibition of the oxLDL effects could be achieved with the non-redox-active cyclooxygenase inhibitor indomethacin and with the dual inhibitor of both enzymes ML 3000, compounds which also reduced the formation of arachidonic acid metabolites. On the contrast, the lipoxygenase inhibitor NDGA which abrogated the increase of LTB_4 in response to oxLDL only faintly reduced the oxLDL-induced PKC activation. The inhibition of both effects of oxLDL, the enhanced formation of PGE_2, and the increase of activity/expression of PKC with the selective COX 2-inhibitor NS-398 suggest a COX 2 selective effect. The analysis of the COX 2 protein level revealed that malBSA, acLDL, and oxLDL enhanced the expression of COX 2, probably due to an auto-amplification by stimulated arachidonic acid metabolism. The induction of the production of new cyclooxygenase (and also of new PKC), however, is likely to contribute little to stimulation of phosphotransferase activity found after only 15 min of oxLDL treatment. Indomethacin reduced the oxLDL effect on COX 2 expression while NS-398 exhibited the opposite effect. These observations are in accordance with those reported by others (Tordjman et al., 1995) and are compatible with inhibition by NS-398 exclusively at the enzymatic level.

LPS is known to be quite a potent inflammatory stimulus, a ligand for the scavenger receptor, and an activator of antibacterial responses by macrophages (Wright, 1991; Landmann et al., 1995). It has been shown to prime $P388D_1$ cells in a concentration dependent manner, but apparently it requires further stimulation to alter the activities of enzymes involved in arachidonic acid metabolism (Glaser et al., 1990). Others have reported that priming with LPS suppresses the expression of scavenger receptors (van Lenten et al., 1985), a finding that is not necessarily contrary to our results. As reported in a previous study, removal of LPS resulted in the return and even enhancement of scavenger-receptor activity (van Lenten et al., 1992). Under our experimental conditions, LPS priming apparently results in a stimulation of receptor expression and/or activity as assessed by enhanced incorporation of radioactivity. The determination of internalized protein showed that LPS pretreatment significantly enhanced the incorporation of oxLDL; however, activation of PKC was found not to correlate with the uptake of labeled lipoprotein when cells were pretreated with endotoxin. One likely explanation of the enhanced stimulatory effect of oxLDL following LPS pretreatment is provided by the finding that endotoxin increases cyclooxygenase 2 protein and activity (Akarasereenont et al., 1995).

Bacterial lipopolysaccharide stimulates tyrosine phosphorylation (Weinstein et al., 1991) and produces myristoylation of cellular proteins which are also substrates of protein kinase C (Aderem et al., 1988). Tyrosine kinases are also involved in the induction of cyclooxygenase by endotoxin (Akarasereenont et al., 1994). Our data provide evidence that the stimulation of tyrosine kinases is an integral part of the action of oxLDL. The results are compatible with an obligatory lysosomal degradation of oxLDL leading to stimulation of tyrosine kinases followed by activation of PKC by downstream signal transduction (Figure 7). LPS treatment (100 ng/mL), although producing an effect on tyrosine kinase activity similar to oxLDL (27 μg/mL), exhibited only a marginal effect on PKC activity, and pretreatment typically enhanced (lipo)protein-mediated PKC activation by approximately 30−35 additional percent. Significant activation of PKC activity by endotoxin, however, required a high dosage (1 μg/mL), and it is of interest that other investigators have reported that a similar concentration of LPS or lipid A was necessary to produce significant PLC activation in $P388D_1$ cells. (Chang et al., 1990).

Apparently both enzyme activation and de novo protein synthesis contribute to enhanced PKC activity in response to oxLDL. Activation of PKC as de novo synthesis of protein (although not shown explicitly for PKC) has been also reported to occur in response to endotoxin (Geisel et al., 1991). Signal transduction pathways stimulated by oxLDL therefore may be partially similar to those activated in response to LPS. Differences emerging so far comprise the requirement for uptake via scavenger receptors and subsequent lysosomal degradation as prerequisites for the action of oxLDL. A preliminary model of events stimulated by oxLDL is provided in Figure 7.

Modified lipoproteins and malBSA both apparently change the expression of individual PKC isoenzymes. The possibility that protein is being redistributed from an unextractable to an extractable compartment, however, has to be considered as well. Treatment with oxLDL or malBSA enhanced the cytosolic concentration of PKC α, and in this context it is of interest that PKC α has been reported to participate in the regulation of the metabolism of arachidonic acid (Huwiler & Pfeilschifter, 1993). When the effects of acLDL with those of malBSA are compared at the isoenzyme level both ligands differ in the pattern of isotypes affected. Whereas oxLDL and acLDL in combination with LPS

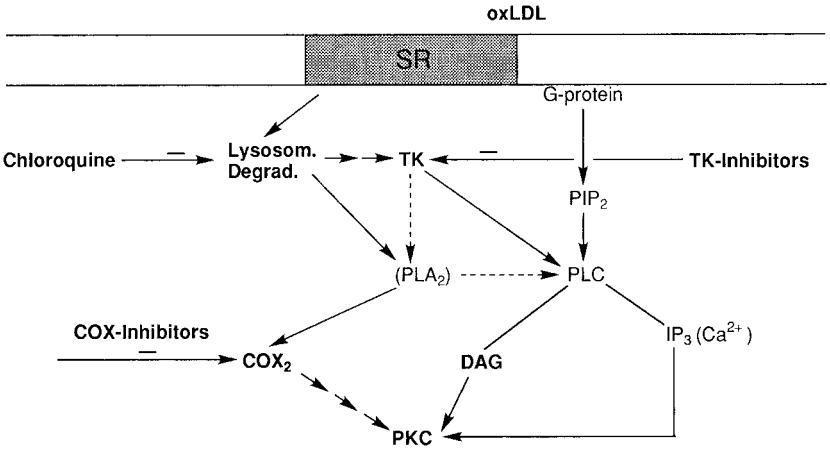

FIGURE 7: PKC-stimulating pathways. This hypothetic scheme illustrates pathways involved in oxLDL-induced stimulus−response coupling [→ designates stimulation, enhanced activity; ⇒ designates inhibition; factors that were measured (in this study) are designated by bold letters]. Binding of oxidized LDL to the scavenger receptor (SR) provokes the breakdown of phosphatidylinositol to diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) via G-protein-dependent activation of PLC (Shackelford et al., 1995). IP_3 mobilizes Ca^{2+} from intracellular stores, leading to an increase in the concentration of intracellular free Ca^{2+}. Diacylglycerol and Ca^{2+} activate protein kinase C. Lysosomal degradation subsequent to receptor-mediated uptake apparently is a prerequisite for activation of tyrosine kinase(s). Tyrosine kinase activation may activate PLA_2, $PLC_{\gamma 1}$, and $PLC_{\gamma 2}$ [for review see Nakamura and Nishizuka (1994) and Nishizuka (1995)]. Furthermore, degradation in the lysosomes may release PKC stimulatory lipids (not shown) and provide additional substrate for phospholipases. The release of arachidonic acid by phospholipases A_2 controls the production of prostaglandins (Balsinde et al., 1994). As suggested by this study, enhanced metabolism of eicosanoids is related to increased PKC activity, in particular by cyclooxygenase-dependent pathways. Accordingly, an interruption of cellular responses initiated by SR-mediated uptake of oxLDL can be achieved by chloroquine, tyrosine kinase inhibitors, or cyclooxygenase inhibitors as indicated.

priming elevate the cytosolic levels of PKC α, β, and δ, the first isotype is not affected by malBSA. This could be a reflection of deviations in signal transduction due to additional effects of the lipoprotein functioning as a carrier of bioactive lipids (or its precursors, respectively). An alternative explanation is provided by the finding that malBSA has been found to occupy an additional binding site in macrophages (Haberland et al., 1989).

LPS pretreatment increased the cytosolic level of PKC; however, the concentration of PKC β and δ remained unchanged in comparison to unprimed controls. This observation is, as for the β isotype, in agreement with a previous report (Novotney et al., 1991). Thus, while priming with LPS prior to treatment with (lipo)proteins makes only a minor contribution to the (lipo)protein-induced increase of total phosphotransferase activity, it does, however, alter the (extractable) concentrations of individual PKC isoforms.

We found that variations in phosphotransferase activity occur at least in part as a consequence of changes in the activity/expression of PKCs β and δ. However, an enhancement of protein synthesis, although detectable after 1 h of exposure to oxLDL, is likely to contribute little to short-term effects. The capability of oxLDL to cause activation of PKC (iso)enzymes provides an explanation for the observation that a short (15 min) exposure to oxLDL is sufficient for stimulation of phosphotransferase activity, and inhibition by cycloheximide/actinomycin D (30 min of oxLDL stimulation) may reflect additional effects besides the inhibition of protein synthesis. Due to the addition of activators such as Ca^{2+} and lipids to the assay, the identification of changes potentially caused by (altered concentrations of) endogenous activatory agents was excluded, although those variations may be partially responsible for an increase of enzymatic activity as determined by us. The relative contribution of (isoenzyme-specific) de novo protein synthesis and changes of (iso)enzyme activity (e.g., caused by proteolytic modification or phosphorylation of the enzyme(s) and in response to messengers with various half-lives) is likely to vary along with the duration of the exposition to oxLDL and the time passed prior to analysis.

In conclusion, we have found that modified (lipo)proteins induce a stimulation of PKC activation/expression, an event that requires binding/internalization via scavenger receptors, lysosomal degradation, and subsequent enhanced activity (expression) of inducible cyclooxygenase. These findings are of potential physiological relevance, and COX 2 inhibitors could possibly interfere with stimulated proliferation of macrophages observed in atherosclerotic lesions (Gordon et al., 1990).

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