Our previous studies have shown that human native low density lipoprotein (LDL) can be oxidized by activated human monocytes. In this process, both activation of protein kinase C (PKC) and induction of superoxide anion ($O_2^-$) production are required. PKC is a family of isoenzymes, and the functional roles of individual PKC isoenzymes are believed to differ based on subcellular location and distinct responses to regulatory signals. We have shown that the PKC isoenzyme that is required for both monocyte $O_2^-$ production and oxidation of LDL is a member of the conventional PKC group of PKC isoenzymes (Li, Q., and Cathcart, M. K. (1994) J. Biol. Chem. 269, 17508–17515). The conventional PKC group includes PKCα, PKCβII, PKCβIII, and PKCγ. With the exception of PKCγ, each of these isoenzymes was detected in human monocytes. In these studies, we investigated the requirement for select PKC isoenzymes in the process of monocyte-mediated LDL lipid oxidation. Our data indicate that PKC activity was rapidly induced upon monocyte activation with the majority of the activity residing in the membrane/particulate fraction. This enhanced PKC activity was sustained for up to 24 h after activation. PKCα, PKCβII, and PKCβIII protein levels were induced upon monocyte activation, and PKCα and PKCβII substantially shifted their location from the cytosol to the particulate/membrane fraction. To distinguish between these isoenzymes for regulating monocyte $O_2^-$ production and LDL oxidation, PKCα or PKCβ isoenzyme-specific antisense oligonucleotides were used to selectively suppress isoenzyme expression. We found that suppression of PKCα expression inhibited both monocyte-mediated $O_2^-$ production and LDL lipid oxidation by activated human monocytes. In contrast, inhibition of PKCβ expression (including both PKCβI and PKCβII) did not affect $O_2^-$ production or LDL lipid oxidation. Further studies demonstrated that the respiratory burst oxidase responsible for $O_2^-$ production remained functionally intact in monocytes with depressed levels of PKCα because $O_2^-$ production could be restored by treating the monocytes with arachidonic acid. Taken together, our data reveal that PKCα, and not PKCβII or PKCβIII, is the predominant isoenzyme required for $O_2^-$ production and maximal oxidation of LDL by activated human monocytes.

It has been suggested that monocyte-mediated low density lipoprotein (LDL) oxidation may play an important role in atherogenesis and inflammatory tissue injury (reviewed in Ref. 1). Previous studies in our laboratory have shown that human monocytes develop the ability to oxidize native LDL lipids only upon activation, thereby transforming LDL into an in vitro cytotoxin (2, 3). The mechanisms involved in monocyte-mediated LDL lipid oxidation are not fully understood. We previously reported that superoxide anion ($O_2^-$) released upon activation of human monocytes was required for monocyte-mediated LDL oxidation, because removal of $O_2^-$ by superoxide dismutase prevented LDL oxidation (2, 3). General antioxidants such as butylated hydroxytoluene, vitamin E, and ascorbate also inhibited monocyte-mediated oxidation of LDL (2, 4, 5). We also found that increases in intracellular Ca$^{2+}$ levels, cytosolic phospholipase A$_2$ activity, and protein kinase C (PKC) activity were required for monocyte-mediated LDL lipid oxidation (6–8). We routinely measure monocyte-mediated LDL oxidation at 24 h after monocyte activation, a time when considerable oxidation products have accumulated and the oxidation begins to plateau. In our earlier studies, PKC activity was shown to contribute to LDL lipid oxidation both early and late during the 24-h incubation period (7).

To date at least 12 isoenzymes of PKC having differential activator responsiveness, cellular distribution, and substrate specificity have been reported in mammalian tissue. They are divided into three major groups: conventional PKCs (α, βI, βII, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ, λ, μ, and ν) (reviewed in Ref. 9). Although only cPKCs are Ca$^{2+}$-dependent, all three groups of PKCs are believed to participate in signal transduction (9, 10), and accumulating evidence suggests that PKC isoenzymes likely play unique roles and induce different functional changes within cells.

In previous studies, we found that a Ca$^{2+}$-dependent cPKC was involved in the process of $O_2^-$ production and required for LDL lipid oxidation by activated monocytes (7), we therefore initiated studies to identify which cPKC isoenzyme(s) was involved in these processes. In the present studies, PKC isoenzyme-specific antisense oligonucleotides (ODN) were carefully designed and then used to suppress the expression of individual isoenzymes. After establishing efficacy and selectivity of antisense inhibition by Western analysis, the ODN were then tested for their effects on monocyte-mediated $O_2^-$ production and LDL lipid oxidation. Our data demonstrate that PKCα, but not PKCβII or PKCβIII, regulates monocyte $O_2^-$ production as well as monocyte-mediated LDL lipid oxidation.
EXPERIMENTAL PROCEDURES

Materials—Cytochrome c and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma. SOD was dissolved in phosphate-buffered saline as a 100-fold stock solution and stored at −20 °C prior to use. Anti-human PKC isoenzyme antibodies directed to synthetic isoenzyme peptides and recombinant PKC isoenzymes were purchased from Oxford Biomedical Research, Inc. (Oxford, MI) and BioGenex (San Ramon, CA). Secondary antibodies were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Zymosan obtained from ICN Biochemicals (Cleveland, OH) was opsonized (11) and used at a concentration of 2 mg/ml to activate human monocytes. Opsonized zymosan (ZOP) was suspended in phosphate-buffered saline as a 20-fold stock solution and stored at −70 °C prior to use. PVDF membranes were from Micron Separations, Inc. (Westborough, MA).

Isolation of Human Monocytes—Human monocytes were isolated from heparinized whole blood by sequential centrifugation over a Ficoll-Paque density solution and adherence to serum-coated cell culture flasks as described previously (2, 3). After washing away nonadherent cells, the adherent cells were released with 5 mM EDTA and plated into multiwell dishes at 1.0–2.5 × 10⁶ cells/ml and plated into multiwell dishes at 1.0–2.5 × 10⁶ cells/ml. This cell population consisted of greater than 95% monocytes (3). The isolated human monocytes were then cultured overnight in Dulbecco’s modified Eagle’s medium with 10% bovine serum before use in experiments. The monocytes were then washed twice with RPMI 1640 (Whittaker, Walkersville, MD) and incubated in RPMI 1640 with or without LDL (0.5 mg cholesterol/ml) in the presence or absence of ZOP (2 mg/ml).

Isolation of Human LDL—LDL was prepared from human plasma by sequential density ultracentrifugation as described previously (12). During the ultracentrifugation procedure, LDL was protected from exposure to light and oxidation (6, 7). LDL was filter sterilized and stored in 0.5 mM EDTA. The concentration of LDL was adjusted to 10 mg cholesterol/ml. Each batch of LDL was also assayed for endotoxin contamination by the limulus amebocyte lysate assay (kit QCL-1000, Whittaker Bioproducts Inc., Walkersville, MD). Final endotoxin contamination was always <0.03 unit/ml LDL cholesterol. Immediately before use, LDL was dialyzed against phosphate-buffered saline without magnesium (Life Technologies, Inc.) in the dark. 1 g/liter of Chelex was added to the dialysis buffer. In all experiments, LDL was used at a final concentration of 0.5 mg cholesterol/ml.

Measurement of LDL Lipid Oxidation—Human monocytes (1 × 10⁶ cells/ml) pretreated with ODN diluted or with ODN (see "Treatment of cells with ODN") were incubated with LDL (0.5 mg cholesterol/ml) in the presence or absence of activator (ZOP, 2 mg/ml) in RPMI 1640 in 96-well flat bottomed culture plates (Costar, Cambridge, MA) for 24 h. After incubation, cell-mediated LDL lipid oxidation was determined by a modified thiobarbituric acid (TBA) assay described by Schuh et al. (13). This assay detects malondialdehyde (MDA) and MDA-like compounds reacting with TBA in oxidized LDL. Briefly, 5 μl of 1 mM butyraldehyde, 5 μl of 100 mM EDTA, and 50 μl of 25% trichloroacetic acid were added to the samples, followed by 10 μl of 10% TBA. The samples were then incubated at 60 °C for 40 min. After incubation, fluorescence at 515 nm excitation and 553 nm emission was determined. Malondialdehyde bis (dimethyl acetal), i.e. 1,1,3,3-tetramethoxypropane (Aldrich), was used as the standard at concentrations of 0–10 nmol MDA/ml. Samples were assayed in triplicate and expressed in terms of MDA equivalents (nmol of MDA/ml).

PKC Activity Assay—Human monocytes (2.5 × 10⁶ cells/ml, a total of 10 × 10⁶ cells/group) and ZOP (2 mg/ml) were incubated for 24 h. After incubation, cell lysates were prepared using a buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, 25 μg/ml leupeptin, and 25 μg/ml aprotinin. Following centrifugation at 1000 × g for 15 min, postnuclear supernatants were collected, and protein concentrations of supernatants were determined by the Lowry assay (7). PKC activity was determined in 20 μg of cell lysate protein using a PKC assay kit (Life Technologies, Inc.). This assay is based on measurement of the phosphorylation of acetylated myelin basic peptide (amino acids 4–14) in the presence and absence of the PKC pseudosubstrate inhibitor peptide, PKC (amino acids 19–36). The PKC activity measurements were predetermined to be in the linear range of the assay.

In studies of intracellular location of PKC activity, human monocytes (2.5 × 10⁶ cells/ml, a total of 10 × 10⁶ cells/group) and ZOP (2 mg/ml) were incubated for 24 h. After incubation, cell lysates were prepared by sonication in hypotonic buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgSO₄, 0.5 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml leupeptin) as described previously (14). Postnuclear lysates were centrifuged at 100,000 × g for 1 h at 4 °C, and supernatants were collected and are referred to as the cytosol fractions. The pellets were resuspended in the same buffer and incubated at 4 °C for 1 h. After centrifugation at 1000 × g for 10 min, supernatants were collected and are referred to as the particulate/membrane fractions. The protein concentrations in both fractions were determined by the Lowry assay (15). After being pelleted all fractions were frozen at −70 °C. Protein concentration in both cytosol and particulate/membrane fractions was as described above, and data are expressed as pmol phosphate incorporated per 10⁶ monocytes.

Western Blotting Analysis—Human monocytes (2.5 × 10⁶ cells/ml, a total of 10 × 10⁶ cells/group) were incubated in the presence or absence of ZOP at the highest reagents and as indicated in the figure legend. After incubation, cells were harvested, and for total cell analysis the cells were resuspended in 100 μl of hypotonic lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgSO₄, 0.5 mM EGTA, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 100 μg/ml DNase, 100 μg/ml RNase, and 0.5% Nonidet P-40), and the cells were vortexed for 15 s and then subjected to centrifugation at 1000 × g for 10 min. For analysis of proteins in the cytosolic and membrane/particulate fractions, cell lysates were prepared by sonication in the above buffer but in the absence of detergent. Fractions were obtained as described under “PKC Activity Assay.” The samples were then prepared for 8% SDS-PAGE (16). The proteins from the SDS-PAGE gels were transferred to PVDF membranes (17). After blocking the nonspecific binding sites with 5% milk in Tris buffer (20 mM Tris-base, 150 mM NaCl, 0.1% Nonidet P-40, pH 7.4) for 1 h at room temperature, PKC isoenzymes were detected using rabbit anti-human PKC isoenzyme-specific polyclonal antibodies (1:1000 dilution), followed by incubation with goat anti-rabbit IgG (1:1000 dilution) conjugated with horseradish peroxidase. The PVDF membrane was then developed by enhanced chemiluminescence (Amersham Pharmacia Biotech). The identity of the different PKC isoforms was confirmed by testing the isoenzyme specificity of the antibodies using recombinant isoforms of PKC and by confirming appropriate migration of cell-derived, immunoreactive bands on SDS-PAGE. Appropriate migration was determined by running molecular weight markers in adjacent lanes of the gel and calculating the predicted molecular weight of immunoreactive bands.

Treatment of Cells with ODN—The sequence for PKCa isoenzyme-specific antisense ODN was 5'-CCG CGT GGA GTG GTG GCC CGG-3', and its control sense ODN sequence was 5'-CCG GCA GAG ACG ACT CCA CGG CGG-3'. The sequence of PKCβ isoenzyme-specific antisense ODN was 5'-AGC CGG CCG TGC TCT CCT CGG-3', and its control sense ODN sequence was 5'-CGA GGA GAG CAC CGG CTG-3'. The PKCβ isoenzyme-specific antisense ODN was selected from areas of conserved sequence between PKCβ and PKCζ. All of the oligomers were phosphorothioate modified and HPLC-purified. They were selected by our previously described criteria (7, 18). Briefly, the sequences were selected from areas of the mRNA that are relatively free of secondary structure as predicted by Mulford 211. In each case the translation start site was avoided as a target for antisense recognition to avoid the recognition of consensuses sites that often characterize this region of the mRNA. The selected sequences were also tested for lack of internal secondary structure and oligo pairing using Mulford 211 (18). Sequences were then screened for uniqueness using Blast 211. For experiments using ODN treatment, human monocytes (1.0–2.5 × 10⁶ cells/ml) were cultured in Dulbecco’s modified Eagle’s medium with 10% bovine serum albumin in the presence or absence of sense or antisense oligomers for 20 h. Levels of PKCa or PKCβ isoenzyme expression were determined at either 1 or 24 h post-activation by ZOP to match the times of the O2⁻ and LDL oxidation assays, respectively. For activation the medium was changed to serum-free RPMI 1640, and for the 24 h activation experiments fresh ODN were added along with ZOP. Monocytes were then assayed for PKCa or PKCβ isoenzyme expression, cell-mediated LDL lipid oxidation, and O2⁻ production as described.

Measurement of Superoxide Anion Production—Superoxide anion (O2⁻) production was measured by the cytochrome c reduction assay as described previously (19). Briefly, human monocytes (1 × 10⁶ cells/ml) and cytochrome c (320 μM; Sigma) were incubated in the presence or absence of 150 unit/ml SOD (from bovine erythrocytes; Sigma) in 96-well flat-bottomed culture plates in RPMI 1640 without phenol red (Whittaker, Walkersville, MD). ZOP (2 mg/ml) was then added for 1 h at 37 °C in a humidified incubator with 10% CO₂. After incubation, the absorbance was measured with a Thermo Max Microplate Reader at 550 nm using Softmax software (PerSeptive Biosystems, Framingham, MA). The following equation was used to determine the nmol of O2⁻ produced: O2⁻ nmol/ml = [A550 (in the absence of SOD) × 158.73] − [A550 (in the presence of SOD) × 158.73].
PKC activity is induced upon human monocyte activation. Human monocytes (2.5 × 10^6 cells/ml, a total of 10 × 10^6 cells/group) and ZOP (2 mg/ml) were incubated together for different periods of time as indicated. After incubation, cell lysates were prepared and fractionated, and PKC activity assays were performed as described under “Experimental Procedures.” Closed circles represent PKC activity in cytosol fractions. Open circles represent PKC activity in particulate/membrane fractions. Data represent the averages ± data ranges of duplicate samples obtained in one of three experiments giving very similar results.

RESULTS

PKC activity in cytosol and particulate/membrane fractions was investigated at various times after human monocyte activation. ZOP was used as the monocyte activator. The data from a representative experiment are summarized in Fig. 1. PKC activity was induced very quickly in ZOP-activated human monocytes. Activity was first detected in the cytosolic fraction and then in the particulate/membrane fraction. At 1 h post activation, the predominant activity was found in the membrane-containing particulate fraction. Although cytosolic PKC activity reached a maximum within 1 h of activation, activity dropped somewhat to a plateau level that remained stable and substantially higher than basal levels throughout the 24 h incubation. On the other hand, PKC activity in the particulate/membrane fraction accounted for the majority of the activity by 1 h after activation and maintained this higher level of activity throughout the 24-h incubation. These data indicate that upon activation monocyte PKC activity is substantially induced in both fractions but to a greater degree in the particulate fraction.

The presence of various PKC isoenzyme proteins was then evaluated in unactivated and activated human monocytes by Western blotting using PKC isoenzyme-specific antibodies. As summarized in Fig. 2, PKCα and PKCβI were detected in unactivated monocytes. Although not evident in this blot low levels of PKCβII were sometimes observed in immunoblots of unactivated monocytes. PKCβII was barely detectable in unactivated monocytes as well. Interestingly, each of the PKC isoenzymes were induced upon activation, and as expected, PKCγ was not detected in human monocytes regardless of the activation state. Because our previous studies indicated that cPKC activity was required for monocyte-mediated LDL lipid oxidation (7), in further studies we focused on the members of the cPKC group of isoenzymes that are detectable in these cells, namely PKCα, βI, and βII.

The cellular partitioning of PKCα and PKCβ isoenzymes was then examined in unactivated and activated (24 h) human monocytes. In Fig. 3, a Western blot is shown where equal amounts of protein were loaded in each lane. To adequately assess the induction and intracellular location of the isoenzymes, the relative density of each band was corrected for the total protein content in the fraction, and these data are given in Table I. Of the three isoforms of PKC that we examined, only PKCβI was predominantly located in the particulate/membrane fraction in unactivated monocytes. PKCα, PKCβI, and PKCβII all increased in the particulate/membrane fraction upon monocyte activation. When corrected for the total protein content of the fractions, it is evident that although PKCα was induced to a lesser extent than PKCβI or PKCβII, it showed the most dramatic relocation to the particulate/membrane fraction upon activation. It should be noted that the apparent induction level of the PKCβ II isoform may be inflated due to the barely detectable basal level and calculation as fold induction.

To further discriminate between PKCα and PKCβ isoenzymes as regulators of O2− production and monocyte-mediated LDL oxidation.

**Fig. 1.** PKC activity is induced upon human monocyte activation. Human monocytes (2.5 × 10^6 cells/ml, a total of 10 × 10^6 cells/group) and ZOP (2 mg/ml) were incubated together for different periods of time as indicated. After incubation, cell lysates were prepared and fractionated, and PKC activity assays were performed as described under “Experimental Procedures.” Closed circles represent PKC activity in cytosol fractions. Open circles represent PKC activity in particulate/membrane fractions. Data represent the averages ± data ranges of duplicate samples obtained in one of three experiments giving very similar results.

**Fig. 2.** Induction of PKC isoenzymes in ZOP-activated human monocytes. Human monocytes (2.5 × 10^6 cells/ml, a total of 10 × 10^6 cells/group) were incubated in the presence or absence of ZOP (2 mg/ml) for 24 h. After incubation, cell lysates were prepared and separated on an 8% SDS-PAGE. The proteins from the PAGE gel were transferred to PVDF by the semi-dry method. Individual PKC isoenzymes were detected with rabbit anti-human PKC isoenzyme-specific antibodies, followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase. The protein bands were developed by ECL. A, induction of PKCα, PKCβI, and PKCβII. 100 μg of protein was loaded on each lane. B, induction of PKCε, PKCδ, and PKCζ. Protein from 1 × 10^6 cell was loaded in each lane. Results obtained from one of three similar experiments are shown.
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**Fig. 3.** Particulate/membrane versus cytosolic location of PKCα, PKCβI, and PKCβII isoenzymes in unactivated and ZOP-activated human monocytes. Human monocytes (2.5 × 10⁶ cells/ml, a total of 10 × 10⁶ cells/group) were incubated in the presence or absence of ZOP (2 mg/ml) for 24 h. After incubation, cell lysates were made. The cytosol and the particulate/membrane were separated from postnuclear supernatants by ultracentrifugation as described under “Experimental Procedures.” A total of 100 μg of protein was loaded in each lane on 8% SDS-PAGE. Proteins, separated by SDS gels, were transferred to PVDF by the semi-dry method. Individual PKC isoenzymes were detected by Western blotting. The lanes marked with the letter c represent the cytosolic fractions, whereas those with the letter p represent the particulate/membrane fractions. The arrows indicate the predicted location of the isoenzyme as calculated from the migration of molecular weight markers within the same gel. Results from one of three similar experiments are shown.

**Table I**

Induction and intracellular location of PKC isoenzymes in monocytes upon activation

| PKC isoenzyme | PKC isoenzyme protein induction upon activation (soluble + particulate) | Ratio of particulate/membrane to cytosolic PKC | Ratio of unactivated monocytes to activated monocytes |
|---------------|--------------------------------------------------------------------------|-----------------------------------------------|---------------------------------------------------|
| PKCα          |                                                                           |                                               | 2.6                                               |
| PKCβI         |                                                                           |                                               | 3.5                                               |
| PKCβII        |                                                                           |                                               | 10.8                                             |

*The apparent induction level of the PKCβII isoform may be inflated due to the barely detectable basal level and calculation as fold induction.*

LDL oxidation. PKC isoenzyme-specific antisense ODN treatment was used. The antisense ODN sequences for PKCα (recognizing only this isoenzyme) and PKCβ (recognizing both PKCβI and PKCβII) were selected as described under “Experimental Procedures.” In these experiments, human monocytes were pretreated in the presence or absence of PKC isoenzyme-specific antisense or sense ODN for 20 h. Monocytes were then exposed to fresh ODN and ZOP for 24 h in RPMI 1640. After activation, cell lysates were then prepared, and the expression of PKCα, PKCβI, and PKCβII isoenzymes was examined by Western blotting (Fig. 4A). Treatment of human monocytes with either PKCα or PKCβ antisense ODN markedly inhibited their relevant specific PKC isoenzyme expression. In each of these cases, the level of expression in antisense-treated monocytes was reduced in an isoenzyme-selective manner as compared with either the untreated or sense-treated monocytes. The lower expression of PKCβI by activated monocytes depicted in lane 2 of Fig. 4A was not characteristic of other blots that showed approximately equal levels of this isoenzyme in unactivated and activated monocytes. The PKCβ antisense ODN treatment consistently inhibited the expression of both PKCβI and PKCβII. The sense ODN control for the PKCβ antisense sometimes caused partial inhibition of PKCβ expression, but the sense control ODN did not affect either LDL lipid oxidation or O₂-production (see Fig. 6). These data, taken together, provide further support for the independence of these processes from PKCβ activity.

The PKCα antisense ODN was also examined for potential nonspecific inhibition of other isoforms of PKC as illustrated in Fig. 4B. It is evident from these results that PKCα antisense ODN treatment selectively inhibited the expression of PKCα and not PKCβI, PKCβII, PKCδ, PKCe, or PKCζ and that treatment with PKCβ antisense ODN selectively inhibited the expression of both PKCβ isoforms without inhibiting the expression of PKCα.

To investigate the potential influence of PKCα and PKCβ on LDL lipid oxidation, human monocyte-mediated LDL lipid oxidation was examined after PKC isoenzyme-specific antisense ODN treatment. Human monocytes were pretreated in the presence or absence of PKC isoenzyme-specific antisense or sense ODN. After activation with ZOP for 24 h in the presence of additional ODN, LDL lipid oxidation was determined by the TBA assay. As shown in Fig. 5, substantial LDL lipid oxidation was observed upon human monocyte activation. LDL lipid oxidation was markedly inhibited in the presence of PKCα isoenzyme-specific antisense ODN. In contrast, LDL lipid oxidation was unaffected by treatment with PKCα sense ODN or PKCβ...
isoenzyme-specific antisense or sense ODN. These data suggest that PKCα is an essential enzyme for activation-induced monocyte-mediated LDL lipid oxidation and that PKCβI and PKCβII are not involved.

In previous studies, we found that O$_2^-$ production was required for the process of monocyte-mediated LDL lipid oxidation, therefore, the effects of PKC isoenzyme-specific antisense ODN treatment on O$_2^-$ production were also examined. Human monocytes were preincubated with either PKCα or PKCβ isoenzyme-specific antisense or sense ODN for 20 h. After preincubation, monocytes were activated with ZOP for 1 h during which time monocyte-mediated O$_2^-$ production was quantified as described under “Experimental Procedures” or lysates were prepared for analysis of isoenzyme expression. As expected, O$_2^-$ production was induced in ZOP-activated human monocytes (Fig. 6A). PKCα antisense ODN treatment substantially inhibited O$_2^-$ production by activated monocytes, whereas other ODN were without effect. Data presented in Fig. 6B indicate that antisense ODN treatment caused isoenzyme inhibition under these conditions. The levels of PKC isoenzymes in the sense ODN pretreated and 1 h activated monocytes was similar to that in untreated, 1 h activated monocytes (data not shown). Additionally, antisense ODN to PKCα did not inhibit expression of PKCβ isoforms and vice versa (data not shown), thus confirming similar results illustrated in Fig. 4.

Even though the treatment with PKCα antisense ODN did not damage the components of the respiratory burst oxidase (NADPH oxidase) complex were derived from experiments in which superoxide anion production by antisense pretreated, activated monocytes was restored by inclusion of the anionic amphiphile, arachidonic acid. Arachidonic acid has been shown to directly activate the NADPH oxidase and circumvent requirements for PKC activity in guinea pig macrophages activated by Fcγ (20). The results of one of three experiments illustrating this restoration of O$_2^-$ production are depicted in Fig. 7. Treatment with antisense ODN significantly inhibited superoxide anion production (p < 0.05). This inhibition was ablated by the inclusion of arachidonic acid resulting in enhanced superoxide anion production to levels that were not significantly different from the amount produced by activated monocytes or activated monocytes treated with sense ODN.

**DISCUSSION**

Previously, our laboratory published studies indicating that the activity of a member of the cPKC isoenzyme family was
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required for human monocyte-mediated LDL lipid oxidation and for the production of superoxide anion (7). The cPKC group of PKC isoenzymes consists of four individual isoenzymes, including PKCa, PKCβI, PKCβII, and PKCy. Although PKCy is not present in human monocytes, the other three cPKC isoenzymes remained as viable candidates for the requisite cPKC activity required in the process of monocyte-mediated LDL oxidation and O2 production.

Activation of PKC is believed to involve the translocation of the enzyme from a cytosolic location in unstimulated cells to new subcellular sites present in the nucleus or particulate/membrane fractions of stimulated cells (reviewed in Ref. 21). Intracellular translocation is believed to be mediated by intracellular receptors for PKC termed RACKs because inhibition of the RACK binding site of a specific isoenzyme can block its translocation and relevant biologic response (22). Our studies presented in Fig. 1 reveal that PKC activity is substantially induced in both the cytosol and particulate fractions of activated human monocytes and that the predominant activity resides in the particulate fraction after 1 h of activation. These findings are similar to those reported by Kadri-Hassani et al. (23) in studies of human monocytes stimulated with PMA or diacylglycerol. We did not detect PKC activity in unactivated monocytes, a finding that appears to differ from several other studies indicating detectable levels of PKC activity in unstimulated cells (23–27). A substantial difference between those studies and the ones presented here is the assay that was used to measure PKC activity. We used an assay that measured PKC activity based on measurement of the phosphorylation of acetylated myelin basic peptide in the presence and absence of the PKC pseudosubstrate inhibitor peptide that is believed to inhibit all isoforms of PKC. Only the pseudosubstrate-inhibitable activity was considered to be mediated by PKC. Studies that have detected activity in unactivated monocytes used assays measuring the total phosphorylation of a typical PKC substrate, such as histones I and III, that might be phosphorylated by other kinases. Indeed, we observed phosphorylation of the acetylated myelin basic peptide substrate in our studies that was not inhibited by the pseudosubstrate, thus implicating phosphorylation of this substrate by kinases that are not inhibited by the pseudosubstrate peptide. Despite these differences in base-line levels of PKC activity in unactivated monocytes, all of these studies agree in finding that activation by a variety of stimuli induces PKC activity and relocation of the majority of the PKC activity to an insoluble fraction.

The activator used in our studies is a yeast cell wall (zymosan) that was boiled (1 h), extensively washed, and then opsonized with fresh human serum. It is known that ZOP is a broad spectrum activator. We have previously shown that ZOP induces human monocyte O2− production and LDL lipid oxidation by stimulating PKC and cPLA2 activity (7, 8). We have also found that other signal transduction pathways, e.g. phospholipase Cγ, are activated by ZOP but not required for superoxide anion production or ZOP-induced monocyte oxidation of LDL (28). Using this broad activator, we observed substantial induction of numerous PKC isoforms upon activation by ZOP (Fig. 2). In unactivated cells, PKCa was located predominantly in the cytosol and PKCβI in the particulate membrane fraction (Fig. 3), a finding similar to other analyses of PKC isoform distribution in monocytes (29, 27, 29). Both PKCa and PKCβI levels in the particulate/membrane fraction increased as a result of monocyte activation (Fig. 3). Similarly, Zheng et al. (25) have reported the translocation of PKCα and PKCβ in human monocytes with OAG and PMA, but ZOP activation was not investigated. In our studies, we detected expression of PKCβ that was not detected in studies by others (25, 27, 29). This difference may be due to the fact that we used ECL development of our Westerns, a method that likely enhances the sensitivity and detection of isoforms present at lower levels.

Although it has previously been reported that human monocyte-mediated O2− production is correlated with PKC expression, PKC activity, or PKC translocation, the identity of the relevant PKC isoenzyme was not investigated (7, 23, 30–33). Furthermore, no studies have to date identified the relevant isoenzyme of PKC that participates in monocyte-mediated LDL oxidation, although our previous studies implicated a member of the cPKC group of isoenzymes (7). To address this issue, we designed and used PKC isoenzyme-specific antisense ODN to selectively suppress the expression of different PKC isoforms. The antisense approach has proven quite successful in this and other studies by our laboratory (e.g. see Refs. 7, 8, and 34). Two factors likely contributing to the effectiveness of this approach are the use of monocytes as the target cells and the careful selection and purity of the ODN. Monocytes rapidly equilibrate with their environment through endocytic mechanisms (35), thereby maximizing uptake of ODN. Monocytes are also resting, G0 cells, and many of the target proteins that we are studying are induced upon activation, thus minimizing the need to ablate high constitutive levels of already expressed protein. We have also found that phosphorothioate ODN cause minimal nonspecific effects on monocytes at the dose ranges employed in these studies. Antisense ODN sequences were carefully selected to target sequences of mRNA devoid of predicted secondary structure while avoiding the translation start site that may contain concensus sequences. Finally, the use of HPLC-purified antisense ODN avoids variable levels of contamination of the ODN preparation with incomplete synthesis products that will diminish the predicted concentration and
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Although our data indicate that PKCα is involved in the monocyte-mediated O$_2^-$ production, whether or not PKCα regulates or directly mediates phosphorylation of p47$^{phox}$ or other components of the respiratory burst complex remains to be determined. Currently, the relationship among phosphorylation of p47$^{phox}$, O$_2^-$ production, and PKCs is under study in our laboratory. Recent studies suggest that more than one kinase may be required to accomplish the phosphorylation of p47$^{phox}$ (32, 43, 47), thus PKCα may be only one of several kinases involved. We have also found that PKCα is involved in regulating pPLA$_2$, an enzyme that generates free arachidonic acid, an anionic amphiphile that regulates superoxide anion production by NADPH oxidase.

In summary, our studies demonstrate that suppression of PKCα expression, but not PKCβI or PKCβII expression, results in inhibition of O$_2^-$ production and LDL lipid oxidation by activated human monocytes. These results suggest that PKCα is a critical regulator of both of these inflammatory processes.

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