Selective Inhibition of Cytosolic Phospholipase A$_2$ in Activated Human Monocytes

REGULATION OF SUPEROXIDE ANION PRODUCTION AND LOW DENSITY LIPOPROTEIN OXIDATION*

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Our previous studies have shown that monocyte activation and release of O$_2^-$ are required for monocyte-mediated low density lipoprotein (LDL) lipid oxidation. We have also found that intracellular Ca$^{2+}$ levels and protein kinase C activity are requisite participants in this potentially pathogenic process. In these studies, we further investigated the mechanisms involved in the oxidation of LDL lipids by activated human monocytes, particularly the potential contributions of the cytosolic phospholipase A$_2$ (cPLA$_2$) signaling pathway. The most well-studied cPLA$_2$, has a molecular mass of 85 kDa and has been reported to be regulated by both Ca$^{2+}$ and phosphorylation. We found that cPLA$_2$ protein levels and cPLA$_2$ enzymatic activity were induced upon activation of human monocytes by opsonized zymosan. Pharmacologic inhibition of cPLA$_2$ activity by AA-COCF$_3$, which has been reported to be a specific inhibitor of cPLA$_2$ as compared with sPLA$_2$, caused a dose-dependent inhibition of cPLA$_2$ enzymatic activity and LDL lipid oxidation induced by activated human monocytes, whereas sPLA$_2$ activity was not affected. To corroborate these findings, we used specific antisense oligonucleotides to inhibit cPLA$_2$. We observed that treatment with antisense oligonucleotides caused suppression of both cPLA$_2$ protein expression and enzymatic activity as well as monocyte-mediated LDL lipid oxidation. Furthermore, antisense oligonucleotide treatment caused a substantial inhibition of O$_2^-$ production by activated human monocytes. In parallel experimental groups, cPLA$_2$ sense oligonucleotides did not affect cPLA$_2$ protein expression, cPLA$_2$ enzymatic activity, O$_2^-$ production, or monocyte-mediated LDL lipid oxidation. These studies support the proposal that cPLA$_2$ activity is required for activated monocytes to oxidize LDL lipids.

Human native low density lipoprotein (LDL)$^1$ can be oxidized by activated human monocytes, neutrophils, and cells of the monocyteid cell line U937 (1, 2) as well as endothelial cells and smooth muscle cells (3). Once oxidized, LDL is chemotactic for monocytes (4), serves as a cytotoxin for target cells (1, 5–7), and hinders the movement of macrophages (8). It is recognized by scavenger and oxidized LDL receptors on macrophages and is taken up by these receptors in an unregulated fashion (9–12). Oxidized LDL has been detected in atherosclerotic lesions (13, 14). Macrophages trapped in the artery wall may take up oxidized LDL, thus contributing to the formation of foam cells and fatty streak lesions. Cell-mediated oxidation of LDL has therefore been suggested to be a key event in atherogenesis as well as in inflammatory tissue injury (15).

In our culture system, human monocyte oxidation of LDL is dependent on monocyte activation. Since activation of monocytes is a complex process, one that involves a series of secondary messengers that mediate signal transduction and alter cell function, we have begun to identify several key signaling pathways that are required for converting a blood monocyte to an activated monocyte that can mediate LDL lipid oxidation. We have found that superoxide anion (O$_2^-$) production is required for this process and that intracellular Ca$^{2+}$ levels are integrally involved in oxidation of LDL lipids by activated human monocytes. Both the influx of extracellular Ca$^{2+}$ and the release of intracellular Ca$^{2+}$ are involved (16). Recently, we demonstrated that a Ca$^{2+}$-regulated, intracellular signaling pathway, protein kinase C (PKC), was required. Our experimental results showed that depletion of PKC activity by phorbol 12-myristate 13-acetate, inhibition of PKC activity by pharmacologic inhibitors, or suppression of PKC levels by antisense oligonucleotides caused an inhibition of LDL lipid oxidation by activated human monocytes (17). The isoenzyme of PKC, required for oxidation of LDL by activated monocytes, was shown to be a member of the cPKC group of isoenzymes.

The rise of intracellular Ca$^{2+}$ levels and activation of PKC elicit a variety of cellular responses including phosphorylation of target proteins which are located throughout the cell, on the plasma membrane, in the cytosol, and in the nucleus. This can initiate a cascade of other second messengers to transmit intracellular signals that ultimately alter cell function (18). Ca$^{2+}$-, and PKC-dependent signaling, therefore, provide exceptionally versatile signaling mechanisms. Downstream effects of Ca$^{2+}$ and PKC have been reported to be related to the induction of several other intracellular signal transduction pathways, one of these pathways involves phospholipase A$_2$ (PLA$_2$) which hydrolyzes the sn-2 fatty acid on phospholipids producing free fatty acid and lysophospholipid (18, 19). Both free fatty acid and lysophospholipid serve as lipid mediators to regulate cell functions. PLA$_2$ also plays a critical role in providing substrate for the biosynthesis of prostaglandins and leukotrienes by releasing arachidonic acid (AA) from membrane phospholipids.

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1 The abbreviations used are: LDL, low density lipoprotein(s); MDA, malondialdehyde; ZOP, oposnized zymosan; TBA, thiobarbituric acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; cPLA$_2$, cytosolic phospholipase A$_2$; sPLA$_2$, secretory PLA$_2$; iPLA$_2$, Ca$^{2+}$-independent PLA$_2$; lyso-PC, lysophosphatidylcholine; PKC, protein kinase C; AA, arachidonic acid; DEDA, 7,7-dimethylheptacosadienoic acid; 4-Bbp, 4-bromophenacyl bromide; LPO, lipid peroxide; HPLC, high performance liquid chromatography; AACOCF$_3$, arachidonyl trifluoromethyl ketone.
Consequently, PLA$_2$s have been implicated in many cellular processes and disease states, such as maintenance of cellular phospholipid pools, participation in inflammatory reactions and host defense, and involvement in myocardial ischemia. Furthermore, AA has been reported to induce O$_2^·$ production in human neutrophils (20) and monocytes (21) by activation of NAPDH oxidase or by its metabolism via lipoxigenase pathways (22). Our laboratory has previously shown that both O$_2^·$ production and lipoxigenase are involved in monocyte-mediated LDL lipid oxidation (5, 16, 17), suggesting that PLA$_2$s might participate in this process.

Phospholipases A$_2$ are a diverse family of enzymes with a growing number of members. Among the mammalian enzymes, the most well-characterized are the 14-kDa secretory PLA$_2$ (sPLA$_2$) and the 85-kDa cytosolic PLA$_2$ (cPLA$_2$) (19). The sPLA$_2$ is Ca$^{2+}$-dependent and requires mM levels of Ca$^{2+}$ for activity. It also has seven disulide bonds that are required for activity and therefore is sensitive to treatment with reducing agents such as dithiothreitol (DTT). In contrast, the 85-kDa cPLA$_2$ requires only μM levels of Ca$^{2+}$, levels that can be reached intracellularly, and it does not have disulide bonds so its activity is not susceptible to reducing agents. Although cPLA$_2$ is 85 kDa, it migrates as an 110-kDa protein in SDS gels. Unlike the sPLA$_2$, cPLA$_2$ shows a preference for arachidonic acid in the sn-2 position of substrate phospholipid. The activity of this latter enzyme is induced by protein phosphorylation and Ca$^{2+}$-dependent translocation to membranes from the cytosol. In addition to these two enzymes several Ca$^{2+}$-independent PLA$_2$s (iPLA$_2$s) have been described, including the canine myocardial 40-kDa iPLA$_2$ (23), the murine macrophage-like cell line P388D$_1$, 80-kDa iPLA$_2$ (24), bovine brain 100-kDa iPLA$_2$ (25), and an 80-kDa iPLA$_2$ from CHO cells (46). These iPLA$_2$s are Ca$^{2+}$-independent and activated by ATP or detergent. To date, only the latter iPLA$_2$ has been cloned (46).

The 85-kDa cPLA$_2$ is believed to be an important regulator of arachidonic acid availability and thereby controls the production of potent lipid mediators. We were particularly interested in this enzyme because its activity has been shown to be regulated by PKC phosphorylation and by Ca$^{2+}$ levels, and both PKC and Ca$^{2+}$ have been shown to be key participants in monocyte oxidation of LDL. We therefore designed a series of experiments to test the hypothesis that cPLA$_2$ participates in regulating the activation-dependent oxidation of LDL lipids by monocyte monocytes.

MATERIALS AND METHODS

Chemicals—DEDAA (7,7-dimethylxocadoseic acid), ONO-RS-082 (2-p-amylcinnamolylamino-4-chlorobenzoic acid), 4-BpB (4-bromo-phenacyle bromide), aristolochic acid, and AACOCF$_3$ (arachidonyl trifluoromethyl ketone) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). DEDAA was dissolved in ethanol. ONO- RS-082, 4-BpB, and AACOCF$_3$ were dissolved in dimethyl sulfoxide. Aristolochic acid was dissolved in water. Arachidonic acid (AA) and 1,α-lysophosphatidylcholine (lyso-PC, Sigma) were dissolved in ethanol. All these reagents were made as 100-fold stock solutions and stored at −20°C prior to use.

Zymosan, obtained from ICN Biochemicals (Cleveland, OH), was opsonized (26) and used at a concentration of 2 mg of protein to activate human monocytes and U937 cells. Opsonized zymosan (ZOP) was suspended in phosphate-buffered saline as a 20-fold stock solution and stored at −70°C prior to use.

Lipoprotein Preparation—Low density lipoprotein (LDL) was prepared according to previously described methods which minimize oxidation and exposure to endotoxin (2). All reagents used for LDL isolation were prepared with Chelex-treated MilliQ water. Each batch of LDL was assayed for endotoxin contamination by the limulus amoebocyte lysate assay (kit QCL-1000, Whittaker Bioproducts Inc., Walkersville, MD). Final endotoxin contamination was always <0.03 unit/mg LDL cholesterol. LDL was stored at 0.5 mg/ml EDTA. Immediately before use, LDL was dialyzed at 4°C against phosphate-buffered saline with-
tion, cells were harvested and resuspended in 200 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, and 0.5% Nonidet P-40). The cells were vortexed for 15 s, and cellular debris and nuclei were removed by centrifugation in a microcentrifuge at 1000 × g for 10 min. The supernatants were collected, and 100 μg of cell lysate protein was prepared for 7% SDS-PAGE (31). The SDS-PAGE gel was transferred to a polyvinylidene difluoride membrane by the semi-dry method (32). After blocking the nonspecific binding sites with 10% milk in Tris buffer (20 mM Tris-base, pH 7.4, 1.5 mM NaCl, 1% Nonidet P-40) for 1 h at room temperature, cellular cPLA₂ protein was detected with a 1:1000 dilution of rabbit anti-human recombinant cPLA₂ monoclonal antibody (generously provided by Dr. J. Clark, Genetic Institute, Inc., Andover, MA), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution). The polyvinylidene difluoride membrane was developed using enhanced chemiluminescence (Amersham Corp.).

For immunoprecipitation of cPLA₂ protein, the total cell lysate from 10 × 10⁶ cells was incubated with 30 μl of polyclonal antibody prebound to protein A beads for 1 h at 4°C. After incubation, cell lysates were centrifuged at 1000 × g for 10 min. Pellets were collected and prepared for 7% SDS-PAGE. After transfer to a polyvinylidene difluoride membrane, cPLA₂ protein was detected by Western blotting using anti-human recombinant cPLA₂ monoclonal antibody (generously provided by Dr. J. Clark, Genetic Institute, Inc., Andover, MA).

**Treatment of Cells with Oligonucleotides**—The sense and antisense sequences of cPLA₂ were selected from a unique area of the mRNA that is near the 5' end of the message. Prior to selection, the sequences were selected by screening for uniqueness using Blast© and were also tested for lack of secondary structure and oligo pairing using Mfold© (33).

The antisense oligomer was complementary to nucleotides 219–238 of cPLA₂, which code for amino acids 27–34 of the protein. The sequence was 5'GCC CTT TGT CCA CTT TGG TGC 3'. The sequence of the cPLA₂ sense oligomer was 5'CAC CAA AGT GAC AAA GGG GGC 3'. Phosphothioate-modified oligonucleotides were used for these studies to limit degradation. The oligonucleotides were synthesized and purified by HPLC prior to use (Genosys Biotechnologic, Inc., Woodlands, TX).

For these experiments, human monocytes (1 × 10⁶ cells/ml) were incubated with 0.5 mg of cholesterol/ml, and ZOP (2 mg/ml) were cultured in RPMI 1640 without serum in the presence or absence of ZOP (2 mg/ml) for 24 h. After incubation, cells were lysed. Cellular debris was removed by centrifugation, and cellular proteins (100 μg of protein/lane) were assessed by Western blotting analysis with cPLA₂-specific antibody as described under “Materials and Methods.” Lane 1, unactivated human monocytes; lane 2, ZOP-activated human monocytes; lane 3, unactivated U937 cells. Results from one of two similar experiments are shown. B, direct detection of cPLA₂ protein in cell lysate. Lane 1, cPLA₂ in unactivated human monocytes; lane 2, cPLA₂ in ZOP-activated human monocytes. Results from one of three similar experiments are shown.

cPLA₂ involvement in human monocyte oxidation of LDL

cPLA₂ protein is induced in ZOP-activated human monocytes. Human monocytes (2.5 × 10⁶ cells/ml) were incubated in the presence or absence of ZOP (2 mg/ml) for 24 h. After incubation, cells were lysed. Cellular debris was removed by centrifugation, and cellular proteins (100 μg of protein/lane) were assessed by Western blotting analysis with cPLA₂-specific antibody as described under “Materials and Methods.” Left side arrows indicate the position of cPLA₂ protein. A, immunoprecipitation of cPLA₂ protein. Lane 1, unactivated human monocytes; lane 2, ZOP-activated human monocytes; lane 3, unactivated U937 cells. Results from one of two similar experiments are shown. B, direct detection of cPLA₂ protein in cell lysate. Lane 1, cPLA₂ in unactivated human monocytes; lane 2, cPLA₂ in ZOP-activated human monocytes. Results from one of three similar experiments are shown.

cytochrome c has an increased absorbance at 550 nm. Human monocytes (1 × 10⁶ cells/ml) and antisense oligonucleotides or sense oligonucleotide (5 μM) were preincubated for 24 h in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum. After preincubation, cells and 320 μM cytchrome c (Sigma) were incubated in the presence or absence of 150 units/ml superoxide dismutase (from bovine erythrocytes, Sigma) in 96-well cell tissue culture plates (a total volume of 100 μl/well) in RPMI 1640 without phenol red and serum (Whittaker, Walkersville, MD) at 37°C in a humidified incubator with 10% CO₂ for 1 h. ZOP (2 mg/ml) and test agents were included during the incubation. After incubation, the absorbance was measured at 550 nm. Equation 2 was used to determine the nmol of O₂⁻ produced (where SOD is superoxide dismutase).

\[ \frac{A_{550}}{m} \text{nmol/ml} = A_{550} \times (159) \text{ (in the absence of SOD)} \times (159) \quad (\text{Eq. 2}) \]

**Statistical Analyses**—The data from experiments were analyzed using the unpaired two-tailed Student’s t test. Statistical tests were performed with GraphPAD InStat software (GraphPAD Software Inc., San Diego, CA). Data points with a p < 0.05 were considered to be significantly different.

**RESULTS**

We first investigated the induction of cellular cPLA₂ by Western blotting analysis using different anti-human cPLA₂-specific antibodies. In these experiments, human monocytes were incubated with the activator (ZOP) for 24 h. After incubation, cell lysates were prepared. In one set of experiments, cPLA₂ protein was immunoprecipitated from cell lysates using anti-human cPLA₂-specific polyclonal antibodies. After immunoprecipitation, cPLA₂ protein was detected by Western blotting using an anti-human cPLA₂-specific monoclonal antibody as described under “Materials and Methods.” The result is shown in Fig. 1A. In a similar experiment, cPLA₂ cell lysates, without immunoprecipitation, were directly detected by Western blotting analysis with cPLA₂-specific antibody as described under “Materials and Methods.” Left side arrows indicate the position of cPLA₂ protein. A, immunoprecipitation of cPLA₂ protein. Lane 1, unactivated human monocytes; lane 2, ZOP-activated human monocytes; lane 3, unactivated U937 cells. Results from one of two similar experiments are shown. B, direct detection of cPLA₂ protein in cell lysate. Lane 1, cPLA₂ in unactivated human monocytes; lane 2, cPLA₂ in ZOP-activated human monocytes. Results from one of three similar experiments are shown.
cPLA₂ Involvement in Human Monocyte Oxidation of LDL

Induction of cPLA₂ protein and enzymatic activity in ZOP-activated human monocyte. Human monocytes (2.5 × 10⁶ cells/ml) were incubated in the presence or absence of ZOP (2 mg/ml) for different times as indicated. After incubation, cells were lysed. Cellular debris was removed by centrifugation. A, quantitation of cPLA₂ protein levels in unactivated human monocytes (open circles) and in ZOP-activated human monocytes (closed circles). Inset, cPLA₂ protein levels were detected by Western blotting as described under “Materials and Methods.” Left side arrow indicates the position of cPLA₂ protein. Results from one of two similar experiments are shown. B, cPLA₂ enzymatic activity was assessed in lysates of ZOP-activated human monocytes in the presence of 2 mM DTT as described under “Materials and Methods.” Data represent the mean ± S.E. obtained from three similar experiments. The significance of induction was determined by Student’s t test (* indicates p < 0.05).

After incubation, ¹⁴C-labeled free arachidonic acid and phosphatidylcholine were separated by TLC using the solvent system as described under “Materials and Methods.” The data shown in Fig. 2B indicate that there is a basal level of endogenous DTT-resistant cPLA₂ activity in unactivated human monocytes. The cPLA₂ enzymatic activity began to increase after 4 h of activation. The maximal induction of cPLA₂ enzymatic activity was 12 h after initiation of activation. Although the cPLA₂ enzymatic activity gradually decreased after 12 h of activation, significant induction of cPLA₂ enzymatic activity was still observed at 24 h of activation. Significant induction of cPLA₂ enzymatic activity is indicated by asterisks (※ indicates p < 0.05). These results indicate that human monocyte cPLA₂ enzymatic activity is induced upon activation, and the induction of cPLA₂ enzymatic activity is correlated with the rise in cPLA₂ protein levels. It should be noted that this assay may detect both the activities of the cPLA₂ and the Ca²⁺-dependent cytosolic PLA₂ (iPLA₂). The involvement of phospholipases A₂ in the process of human monocyte and U937 cell oxidation of LDL was first evaluated using several, structurally unrelated, pharmacologic inhibitors of PLA₂, including DEDA, ONO-RS-082, aristolochic acid, and 4-BpB. Freshly isolated human monocytes or U937 cells, LDL, and ZOP were incubated together in the presence or absence of the PLA₂ inhibitors for 24 h. After incubation, the lipid oxidation of LDL was assessed by the TBA assay and the LPO assay. The TBA assay is a widely used method to detect malondialdehyde and MDA-like compounds derived from lipid oxidation products (27). The LPO assay detects lipid hydroperoxide which are produced upon lipid oxidation (28). Our experimental results demonstrated that each of these PLA₂ inhibitors showed dose-dependent inhibition of LDL lipid oxidation by activated human monocytes and U937 cells (data not shown). These data, regardless of the fact that most of these pharmacologic PLA₂ inhibitors are nonselective for sPLA₂ versus cPLA₂, provided the first suggestion that PLA₂ activity was involved in LDL lipid oxidation by activated human monocytes and U937 cells.

To further investigate the requirement for cPLA₂, we used another inhibitor, AAOCOF₃, which has been reported to be a selective inhibitor of cPLA₂ (37). In these experiments, human monocytes and ZOP were incubated in the presence or absence of different concentrations of AAOCOF₃ for 24 h. After incubation, cell lysates were prepared. Then, PLA₂ activities were assessed in the presence or absence of 2 mM DTT as described under “Materials and Methods.” The experimental results are summarized in Fig. 3. AAOCOF₃ inhibited DTT-resistant PLA₂ activity in a concentration-dependent fashion, indicating that cPLA₂ activity was inhibited by AAOCOF₃ as shown in Fig. 3A. In contrast, AAOCOF₃ did not inhibit DTT-sensitive PLA₂ activity, indicating that sPLA₂ activity was not inhibited by AAOCOF₃ as shown in Fig. 3B.

In a parallel experiment, we also monitored monocyte-mediated LDL lipid oxidation in the presence or absence of AAOCOF₃. In these experiments, human monocytes were incubated with LDL and ZOP in the presence or absence of AAOCOF₃ for 24 h. After incubation, cell-mediated LDL lipid oxidation was assessed by both the TBA assay and the LPO assay. The experimental results are summarized in Fig. 4. Upon activation, cell-mediated LDL lipid oxidation was substantially increased as detected by the TBA assay (as shown in Fig. 4A) and the LPO assay (as shown in Fig. 4B). AAOCOF₃ caused a concentration-dependent inhibition of cell-mediated LDL lipid oxidation. Taken together, these data suggest that an AAOCOF₃-sensitive PLA₂ activity is required for human monocyte-mediated LDL lipid oxidation.

Fig. 2. Induction of cPLA₂ protein and enzymatic activity in ZOP-activated human monocyte. Human monocytes (2.5 × 10⁶ cells/ml) were incubated in the presence or absence of ZOP (2 mg/ml) for different times as indicated. After incubation, cells were lysed. Cellular debris was removed by centrifugation. A, quantitation of cPLA₂ protein levels in unactivated human monocytes (open circles) and in ZOP-activated human monocytes (closed circles). Inset, cPLA₂ protein levels were detected by Western blotting as described under “Materials and Methods.” Left side arrow indicates the position of cPLA₂ protein. Results from one of two similar experiments are shown. B, cPLA₂ enzymatic activity was assessed in lysates of ZOP-activated human monocytes in the presence of 2 mM DTT as described under “Materials and Methods.” Data represent the mean ± S.E. obtained from three similar experiments. The significance of induction was determined by Student’s t test (* indicates p < 0.05).
monocyte-mediated LDL lipid oxidation detected by the TBA assay. The solid bars represent LDL lipid oxidation by ZOP-activated monocytes. The hatched bars represent LDL lipid oxidation in the presence of different concentrations of AACOCF$_3$. A, DTT-resistant cPLA$_2$ activity. B, DTT-sensitive cPLA$_2$ activity (the activity in the absence of DTT minus that in the presence of DTT). PLA$_2$ activity in unactivated human monocytes (open bars), ZOP-activated human monocytes (solid bars), and ZOP-activated monocytes in the presence of different concentrations of AACOCF$_3$ (hatched bars). Data are presented as the mean ± S.E. obtained in duplicate samples of three similar experiments. The significance of inhibition was determined by Student’s t test (* indicates p < 0.05).

To investigate potential nonspecific effects of AACOCF$_3$, we also examined its toxicity to human monocytes and its ability to function as a general antioxidant. For the cytotoxicity studies, we used an assay measuring $[^{14}C]$adenine metabolite release. We have shown that the results obtained with this assay correlate well with the chromium release assay of toxicity (16).

The results, presented in Table I, demonstrate that AACOCF$_3$, at doses used for these studies, showed less than 5% toxicity for human monocytes (shown in Table I) or U937 cells (data not shown). We also evaluated the general antioxidant activity of AACOCF$_3$, as measured by its inhibition of copper-induced LDL lipid oxidation as described previously (16, 35). The results of these studies are presented in Table II. AACOCF$_3$ showed no inhibition of copper-mediated LDL lipid oxidation at concentrations from 1 to 50 μM, indicating that AACOCF$_3$ did not exhibit antioxidant activity at concentrations used in the studies.

To corroborate our findings with AACOCF$_3$ and to determine whether, indeed, cPLA$_2$ was required, we used another approach to regulate cPLA$_2$ activity. For these studies, we used cPLA$_2$-specific antisense oligonucleotides to inhibit cPLA$_2$ expression. We also used a cPLA$_2$ sense oligonucleotide as a control in parallel cultures. In these experiments, human monocytes were incubated with sense or antisense phosphothioate-modified oligonucleotides (HPLC-purified) in the presence or absence of ZOP and LDL as described under "Materials and Methods." After incubation, both cPLA$_2$ protein expression and cPLA$_2$ enzymatic activity were determined. As shown in Fig. 5A, cPLA$_2$ protein expression in activated human monocytes was inhibited by cPLA$_2$-specific antisense oligonucleotide treatment (lane 3 of Fig. 5A) as detected by Western blotting analysis using cPLA$_2$-specific antibody. Sense oligonucleotide treatment had no effect on human monocyte cPLA$_2$ protein expression (lane 4 of Fig. 5A). We also examined the cPLA$_2$ activity in lysates of monocytes that had been treated with antisense or sense oligonucleotides. These data are shown in Fig. 5B. cPLA$_2$ activity was increased upon monocyte activation as previously observed (Fig. 2B) and antisense oligonucleotide treatment substantially inhibited cPLA$_2$ activity. In contrast, treatment with the sense oligonucleotides did not alter cPLA$_2$ activity. Furthermore, we also monitored whether oligonucleotide treatment had any nonspecific inhibitory effects on the assay. In this experiment, U937 cell lysates and oligonucleotides were included together during the cPLA$_2$ activity assay. Neither antisense oligonucleotides nor sense oligonucleotides affected the cPLA$_2$ activity assay itself (data not shown).

Next, we evaluated human monocyte-mediated LDL lipid oxidation after treatment with cPLA$_2$-specific antisense or sense oligonucleotides. Treatment with cPLA$_2$-specific anti-

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**Table I**

| Incubation additions$^a$ | $[^{14}C]$Adenine release$^b$ | Percent release$^c$ |
|-------------------------|-------------------------------|---------------------|
| Unactivated cells + LDL | 11.72 ± 0.27                 |                     |
| Activated cells + LDL + |                               |                     |
| Dimethyl sulfoxide (solvent control) | 7.10 ± 0.27 | 0                   |
| 0.2% SDS (total release) | 103.55 ± 13.2                | 100                 |
| AACOCF$_3$, 1 μM         | 8.05 ± 0.03                  | 99.8                |
| 10 μM                   | 8.22 ± 0.10                  | 99.5                |
| 25 μM                   | 10.92 ± 0.10                 | 91.4                |
| 50 μM                   | 11.72 ± 0.43                 | 91.4                |

$^a$ Human monocytes (1 × 10$^6$/ml) were labeled with $[^{14}C]$adenine and then incubated together with ZOP (2 mg/ml), LDL (0.5 mg of cholesterol/ml), and different concentrations of AACOCF$_3$, as indicated for 24 h. After incubation, the supernatants were collected.

$^b$ Release of radioactivity was determined as described under “Materials and Methods.” All experiments were performed in triplicate. The results are expressed as mean ± S.D. of data obtained in one of three similar experiments.

$^c$ Percent release of $[^{14}C]$adenine was determined by the equation described under “Materials and Methods.”
**TABLE II**

| Incubation conditions | TBA\(^a\) (nmol MDA/ml) |
|-----------------------|--------------------------|
| LDL                   | 0.40 ± 0.01              |
| LDL + 5 \(\mu M\) CuSO\(_4\) + ... |                           |
| Dimethyl sulfoxide (solvent control) | 2.76 ± 0.08              |
| AACOCF\(_3\)         |                           |
| 1 \(\mu M\)          | 2.69 ± 0.03              |
| 10 \(\mu M\)         | 2.76 ± 0.04              |
| 50 \(\mu M\)         | 2.69 ± 0.08              |

\(^a\) LDL (0.5 mg of cholesterol/ml) and 5 \(\mu M\) CuSO\(_4\) were incubated in the presence or absence of different concentrations of AACOCF\(_3\), at 37 °C for 24 h. After incubation, the supernatants were collected, and LDL lipid oxidation was determined by the TBA assay.

The TBA assay was performed as described under "Materials and Methods." LDL lipid oxidation is expressed as the mean ± S.D. in nmol of MDA/ml from one of three similar experiments.

Previously, our laboratory has shown that \(O_2\) production is required for monocyte-mediated LDL lipid oxidation, and arachidonic acid has been shown to regulate the activity of the NADPH oxidase \(O_2\) generating complex. We therefore examined whether cPLA\(_2\) activity was essential for \(O_2\) production. In this experiment, human monocytes were preincubated with either cPLA\(_2\)-specific antisense or sense oligonucleotides for 24 h and then \(O_2\) production was quantified in response to activation. As expected, \(O_2\) production was increased upon monocyte activation as shown in Fig. 7A. Antisense oligonucleotide treatment significantly inhibited \(O_2\) production in activated human monocytes, whereas sense oligonucleotide treatment was without significant effect. Furthermore, we found that the inhibitory effect of antisense treatment could be negated by addition of arachidonic acid (AA) one product of cPLA\(_2\) activity. AA alone or with ZOP did not alter \(O_2\) production except to restore levels to normal in antisense-treated, activated monocytes.

We then conducted similar experiments to attempt to restore the ability of antisense-treated, activated monocytes to oxidize LDL lipids. In these experiments (see Fig. 7B), addition of AA restored LDL lipid oxidation by 50% in antisense-treated cells. Addition of lyso phosphatidyl choline (lyso-PC) or lyso-PC plus AA did not fully restore LDL lipid oxidation in antisense-treated cells (data not shown). Treatment with AA and/or lyso-PC did not alter levels of LDL lipid oxidation in unactivated monocytes nor in ZOP-activated monocytes that were not treated with oligonucleotides or were treated with sense oligonucleotides.

**FIG. 5.** cPLA\(_2\) antisense oligonucleotides inhibit both cPLA\(_2\) protein expression and enzymatic activity in ZOP-activated human monocytes. Human monocyte (2.5 × 10\(^6\) cells/ml) and ZOP (2 mg/ml) were incubated with either 5 \(\mu M\) cPLA\(_2\)-specific antisense or sense oligonucleotides for 24 h. After incubation, cell lysates were made. Both cPLA\(_2\) protein expression and enzymatic activity were assessed as described under "Materials and Methods." A, induction of cPLA\(_2\) proteins. Experimental results represent data obtained from one of three similar experiments. B, induction of cPLA\(_2\) enzymatic activity. Data represent the mean ± data range of the duplicate samples obtained in one of three similar experiments. The open bars represent results in unactivated human monocytes. The solid bars represent results in ZOP-activated human monocytes. The hatched bars on the left represent results in ZOP-activated monocytes treated with 5 \(\mu M\) of cPLA\(_2\)-specific antisense oligonucleotides. The hatched bars on the right represent results in ZOP-activated monocytes treated with 5 \(\mu M\) of control sense oligonucleotides. A, inset, cPLA\(_2\) Western blot of unactivated human monocytes (lane 1), activated human monocytes (lane 2), activated human monocytes treated with 5 \(\mu M\) of cPLA\(_2\)-specific antisense oligonucleotides (lane 3), and activated human monocytes treated with 5 \(\mu M\) of control sense oligonucleotides (lane 4). The significance of inhibition was determined by Student’s t test (* indicates \(p < 0.05\)).

sense oligonucleotides caused a dose-dependent decrease in monocyte-mediated LDL lipid oxidation as detected by the TBA assay (Fig. 6). In contrast, treatment with sense oligonucleotides caused no significant inhibition of LDL lipid oxidation (* indicates \(p < 0.05\)). These data suggest that cPLA\(_2\) activity is a critical regulator of the oxidation of LDL by activated human monocytes.

**FIG. 6.** Human monocyte-mediated LDL lipid oxidation is inhibited by cPLA\(_2\)-specific antisense oligonucleotides. Human monocytes (1 × 10\(^6\) cells/ml), ZOP (2 mg/ml), and LDL (0.5 mg cholesterol/ml) were incubated in the presence or absence of different concentrations of cPLA\(_2\)-specific antisense or control sense oligonucleotides for 24 h. After incubation LDL lipid oxidation was assessed by the TBA assay as described under “Materials and Methods.” The levels of monocyte-mediated LDL lipid oxidation in the presence of different concentrations of cPLA\(_2\)-specific antisense (closed circles) or control sense (open circles) oligonucleotides are shown. Data are expressed as the mean ± S.E. of triplicate samples obtained in three similar experiments. The significance of inhibition was determined by Student’s t test (* indicates \(p < 0.05\)).

**DISCUSSION**

In our previous studies, we found that human peripheral blood monocytes could oxidize LDL in an activation-dependent manner (1, 2). We also found that \(O_2\) production (2), increases in intracellular \(Ca^{2+}\) levels (16), and induction of PKC activity were required as well (17). These observations suggested that one or more \(Ca^{2+}\)- and protein phosphorylation-dependent intracellular signaling pathways regulated monocyte function and participated in the process of monocyte-mediated LDL lipid oxidation. A potential candidate for one of these pathways was the high molecular weight cPLA\(_2\). We hypothesized that cPLA\(_2\) might prove to be an important regulatory pathway in the oxidation of LDL by activated monocytes.

We found that low levels of cPLA\(_2\) protein were detectable in...
production is inhibited by suppression of cPLA₂ alone is not sufficient for LDL oxidation plus some unidentified pharmacologic inhibitors of PLA₂, results indicated LDL lipid oxidation (5).

LDL lipid oxidation (data not shown). To confirm this observation, our studies demonstrated that cPLA₂ activity was required for monocyte-mediated LDL lipid oxidation; recently, however, it has been reported that AACOCF₃ can inhibit cytosolic iPLA₂ activity in a mouse macrophage cell line, P388D1 (38). Technically, it is difficult to distinguish cPLA₂ enzymatic activity from iPLA₂ activity in this assay, because both cPLA₂ and iPLA₂ are insensitive to DTT and both activities would be detected in our assay system (19, 39).

To more specifically address the participation of cPLA₂ in this process, cPLA₂-specific antisense and sense oligonucleotides were developed. The sequence was carefully chosen from a region lacking substantial homology with other sequenced human genes. The oligonucleotides were phosphorothioate-modified to limit degradation and purified by HPLC prior to use to remove all incomplete synthesis products thereby limiting nonspecific effects. We have found this latter step to be critical in rendering specificity to antisense oligonucleotide regulation in human monocytes. The finding that antisense treatment, but not treatment with sense oligonucleotides, resulted in decreased cPLA₂ protein expression and decreased enzymatic activity leads us to believe that the decrease in monocyte oxidation of LDL was indeed due to inhibition of cPLA₂. Recent reports have also shown that cPLA₂ protein expression and enzymatic activity can be inhibited by cPLA₂ antisense oligonucleotide treatment in human monocytes (40, 41). In these studies, different cPLA₂ antisense oligonucleotide sequences were used including sequences directly recognizing the initiation site of transcription (40) and sequences recognizing cPLA₂ mRNA downstream from the initiation site (41).

An important mechanistic finding of this study is that monocyte-mediated O₂⁻ production is inhibited by suppression of cPLA₂ activity (Fig. 7A). Although numerous studies report that AA and phospholipase A₂ appear to regulate O₂⁻ production, this is the first report that specific suppression of cPLA₂ protein expression and enzymatic activity, using cPLA₂-specific antisense oligonucleotide treatment, inhibited O₂⁻ production by activated human monocytes. The finding that cPLA₂ regulates LDL lipid oxidation is also novel. Interestingly, both O₂⁻ production and LDL lipid oxidation were inhibited to the same extent, but as discussed below, neither was inhibited completely (see Fig. 7).

We have recently reported that O₂⁻ is required for monocyte oxidation of LDL, yet it is clear from data from our laboratory and others that O₂⁻ alone is not sufficient for LDL oxidation (42).² Activated monocytes must provide O₂⁻ plus some unidentified cofactor for oxidation to proceed. In this regard, it is interesting that the addition of AA completely restored O₂⁻ production by antisense-treated, activated monocytes (Fig. 7A), whereas AA only partially restored the capacity of antisense-treated, activated monocytes to oxidize LDL (Fig. 7B). We also examined whether the addition of lyso-PC or both lyso-PC + AA could restore the LDL oxidation mediated by antisense-treated, activated monocytes, but some inhibition of LDL oxide production is inhibited by suppression of cPLA₂ activity as reported to be a selective inhibitor of cPLA₂ in that it is 500-fold more potent as an inhibitor of cPLA₂ as compared with sPLA₂ (37). Since sPLA₂ has seven disulfide bridges and is inactivated by DTT, we could distinguish between sPLA₂ and cPLA₂ activities. AACOCF₃ only inhibited DTT-resistant PLA₂ activity but not DTT-sensitive PLA₂ activity, and inhibition was concentration-dependent, demonstrating that cPLA₂ activity was selectively inhibited. Importantly, AACOCF₃ also inhibited human monocyte-mediated LDL lipid oxidation in a dose-dependent fashion. In concert, these data supported our hypothesis that cPLA₂ activity was required for monocyte-mediated LDL lipid oxidation; recently, however, it has been reported that AACOCF₃ can inhibit cytosolic iPLA₂ activity in a mouse macrophage cell line, P388D1 (38). Technically, it is difficult to distinguish cPLA₂ enzymatic activity from iPLA₂ activity in this assay, because both cPLA₂ and iPLA₂ are insensitive to DTT and both activities would be detected in our assay system (19, 39).

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² A. Zendedel-Haghighi and M. K. Cathcart, unpublished observations.
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