rapid communication

In vitro knockout of human p47phox blocks superoxide anion production and LDL oxidation by activated human monocytes

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Abstract **We previously reported that superoxide dismutase (SOD) blocked human monocyte oxidation of LDL and therefore concluded that superoxide anion (O_2^-) was required for oxidation. Others, however, have suggested that SOD may inhibit by mechanisms alternative to the dismutation of O_2^-. This study definitively addresses the involvement of O_2^- in monocyte oxidation of LDL. Using an antisense ODN designed to target p47phox mRNA, we found that treatment of monocytes with antisense ODN caused a substantial and selective decrease in expression of p47phox protein, whereas sense ODN was without effect. Corresponding functional assays demonstrated that antisense ODN inhibited production of O_2^-. As sense ODN caused no inhibition of O_2^- production, these results suggested that inhibition of p47phox expression caused reduction in O_2^- production. Evaluation of the contribution of O_2^- production to monocyte-mediated oxidation of LDL lipids confirmed that O_2^- production is required for LDL lipid oxidation as antisense ODN treatment significantly inhibited LDL oxidation whereas sense ODN treatment caused no inhibition. This is the first report of the reduction of NADPH oxidase activity in intact human monocytes by directly targeting the mRNA of a significant member of this enzyme complex. Our results provide convincing data that O_2^- is indeed required for monocyte-mediated LDL oxidation.**—Bey, E. A., and M. K. Cathcart. **In vitro knockout of human p47phox blocks superoxide anion production and LDL oxidation by activated human monocytes.** ***J. Lipid Res.*** **2000.** 41: **489–495.**

Supplementary key words lipid oxidation • LDL oxidation • atherosclerosis • monocyte-mediated LDL oxidation • superoxide anion • NADPH oxidase • inflammation • antisense oligonucleotides • monocyte activation • oxygen-free radicals • respiratory burst oxidase

Oxidation of LDL by activated monocytes is believed to contribute to the pathogenesis of atherosclerosis (1). Our laboratory has focused on defining the mechanisms of monocyte-mediated LDL oxidation (2–14). We have reported that monocyte activation and production of O_2^- are required for LDL oxidation. Studies implicating a requisite role for O_2^- relied in part on results obtained using superoxide dismutase (SOD) to remove O_2^-. Other laboratories have questioned whether SOD might inhibit this process by means other than the dismutation of O_2^- (15, 16). Furthermore, results from other laboratories are conflicting regarding the ability of cells that are defective in O_2^- production to oxidize LDL (17, 18). We therefore conducted the following studies to more rigorously assess the contribution of O_2^- in the process of monocyte-mediated LDL lipid oxidation.

Human monocytes, upon activation, generate a burst of O_2^- production. The activation-induced release of this reactive oxygen radical is believed to be primarily due to the activation of the NADPH oxidase enzyme also referred to as the respiratory burst oxidase. The components of the NADPH oxidase complex have been defined in recent years through studies with intact or lysate fractions of neutrophils and much of what we know about how the function of this enzyme complex has been derived from studies of neutrophils from patients with defective NADPH oxidase activity, a clinical disorder called chronic granulomatous disease (CGD) (19–21). The phagocyte NADPH oxidase consists of a membrane-associated b-type cytochrome b_{559} (that copurifies with the GTP binding protein RAP1A), and three cytosolic components p47phox, p67phox, and the small G protein rac 1/2 (22–24). The membrane-bound cytochrome consists of two subunits: a glycoprotein termed gp91phox and a 22-kDa protein, p22phox. The cytochrome is responsible for accepting electrons

Abbreviations: BCS, bovine calf serum; HPLC, high performance liquid chromatography; HPODE, hydroperoxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; LDL, low density lipoprotein; ODN, oligodeoxyribonucleotide; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbol myristate acetate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; ZOP, opsonized zymosan.

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from NADPH. (25, 26) Another cytosolic component, a 40-kDa protein (p40phox), associates with p47phox and p67phox in a cytosolic high molecular mass complex (27). The monocyte NADPH oxidase seems to have the same components as the well-studied neutrophil enzyme complex although regulation of the complex appears to differ.

Naturally occurring mutations in one of several components of the NADPH oxidase can lead to CGD. Among these are mutations in the gene coding for p47phox resulting in defective production of $O_2^{\cdot-}$ (19). We therefore chose to target p47phox with antisense ODN to inhibit expression of this protein. We demonstrate that antisense ODN treatment selectively inhibited p47phox protein levels. We then monitored the effects of decreased p47phox expression on $O_2^{\cdot-}$ production and LDL oxidation. We found that $O_2^{\cdot-}$ production by intact human mononuclear phagocytes was substantially inhibited by this treatment and, furthermore, we showed that NADPH oxidase-derived $O_2^{\cdot-}$ production was required for monocyte-mediated LDL oxidation. These results settle the controversy over the requirement for $O_2^{\cdot-}$ in this process.

MATERIALS AND METHODS

Isolation of human monocytes

Whole blood (240 cc) was collected from donors into heparinized syringes. The mononuclear cell layer was isolated by centrifugation of diluted blood over a Ficoll-Paque density solution. The isolation of monocytes was performed using a modification (6, 13) of the method of Kumagai et al. (28). After washing the mononuclear layer three times with PBS, contaminating platelets were removed by 2 centrifugations through 4 ml of bovine calf serum (BCS, Hyclone, Logan, UT) after overlaying the serum with the mononuclear cells (13). Monocytes were isolated from the platelet-free mononuclear cells by adherence to 75 cm^2 flasks (Costar, Cambridge, MA) pre-coated with 100% BCS and containing DMEM and 10% BCS (BCS/DMEM). The flasks were incubated for 2 h at 37°C/10% CO_2. The nonadherent cells were removed by washing the flasks 3 times with BCS/DMEM. The nonadherent cells were centrifuged at 200 *g*, resuspended in BCS/DMEM, and plated in a separate flask. The flasks were allowed to incubate at 37°C/10% CO_2 for 24 h. The adherent cells were collected from both flasks by removing the media and placing the cells in the presence of 5 mm EDTA. The cells were allowed to incubate for 15 min at 37°C/10% CO_2. The flasks were then shaken and vigorous pipetting was used to release the cells. EDTA was removed by washing the the monocytes 3 times with BCS/DMEM. The cells were resuspended in BCS/DMEM and counted. Although these "monocytes" are cultured for 3–4 days during the course of the experiments, we refer to them as monocytes to distinguish them from monocyte-derived macrophages which are typically cultured for 7–10 days prior to use and are more fully differentiated.

Treatment with ODN

The sense and antisense ODN sequences of human monocyte p47phox were chosen from a unique area of mRNA that is near the 5′ end of the message (Accession # M25665), but does not recognize the translation start site. We preferentially chose ODN in areas that were predicted by the software program Mulfold© to lack 2° structure (29). The choice of sequences was screened for uniqueness using Blast©. The ODN sequences were also tested for lack of secondary structure and oligo pairing, again using Mulfold©.

The antisense oligomer was complementary to nucleotides 394–413 of human monocyte p47phox mRNA. The antisense sequence was 5′-TTTGTCTGGTTGTCTGTGGG-3′. The sense sequence was 5′-CCCACAGACAACCAGACAAA. The ODN were synthesized with phosphorothioate-modified bases to limit degradation and HPLC purified to limit contamination by incomplete synthesis products (Genosys Biotechnologic Inc., Woodlands, TX).

For $O_2^{\cdot-}$ and TBARS experiments involving treatment of monocytes with antisense ODN, monocytes (1.0×10^5/0.1 ml/well) were pre-treated (as indicated in individual experiments) with antisense or sense ODN. For $O_2^{\cdot-}$ experiments, after the pretreatment period, the medium was changed to RPMI 1640 without phenol red and the $O_2^{\cdot-}$ assay was performed as described. For LDL oxidation experiments, after the treatment period, the medium was changed to RPMI 1640, LDL and ZOP were added, and the lipid oxidation assays were performed as described below and in several of our prior publications (2, 10–12, 14).

Western blots

For these experiments, human monocytes were plated in 6-well tissue culture plates (Costar, Cambridge, MA) at a density of 2.5×10^6/ml in 10% BCS DMEM. Cells were treated with 1–10 μm of antisense or sense ODN for up to 3 days (as indicated). After the incubation period, cells were lysed in the presence of 200 μl hypotonic lysis buffer (50 mm Tris-HCL, pH 7.5, 5 mm $MgSO_4$, 0.5 mm EGTA, 0.1% 2-mercaptoethanol, 1 mm PMSF, 20 μg/ml leupeptin, and 0.5% Nonidet P-40). The cells were vortexed for 10–15 sec and cellular debri and nuclei were removed by centrifugation at 200 *g* for 10 min. The supernatants were collected and sonicated for 3–5 sec. The supernatants were again centrifuged at 10,000 *g* at 4°C in a microcentrifuge. The supernatants were separated from pellets and 25–100 μg of lysate protein was prepared for 10% SDS-PAGE. The proteins from the SDS-PAGE gels were transferred to a PVDF membrane (0.2 μm, Bio-Rad Laboratories, Richmond, CA) by the semi-dry method. Nonspecific binding sites were blocked with 5% milk in Tris-buffered saline (20 mm Tris-base, pH 7.4, 1.5 m NaCl, 1% NP-40) at 4°C for 24 h. Human p47phox was detected with goat anti-human p47phox polyclonal antibody (1:1000, generously provided by Drs. Harry Malech and Thomas Leto) followed by incubation with a horseradish peroxidase-conjugated rabbit anti-goat IgG (1:1000, Pierce Biochemicals, Rockford, IL). The PVDF membrane was developed using enhanced chemiluminescence (Pierce, Arlington Heights, IL). Erk1/2 kinase was detected with rabbit anti-human Erk 1/2 kinase (1:1000, Upstate Biotechnology, Inc., Waltham, MA) followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000, Pierce Biochemicals, Rockford, IL). The PVDF membrane was developed as above.

Superoxide anion assay

The method used to determine $O_2^{\cdot-}$ produced by human monocytes was a modification of an assay previously reported by Pick and Mizel (30). The assay measures SOD-inhibitable cytochrome c reduction. Human monocytes were plated in flat-bottomed 96-well tissue culture plates (100 μL, 1×10^6/mL) BCS/DMEM and allowed to adhere for at least 2 h. After 3 days of antisense ODN treatment, the medium in the wells was changed to RPMI without phenol red (Biowhitaker, Walkersville, MD). Cytochrome c (160 u/mL, Sigma, St. Louis, MO), with or without superoxide dismutase (300 u/mL, Sigma, St. Louis, MO), was added to the wells. The cells were incubated for 1 h at 37°C and the plates were read in a Molecular Devices Thermomax microplate reader at 550 nm. The amount of $O_2^{\cdot-}$ produced by monocytes was calculated

using the extinction coefficient of 158.73 and expressed as nanomoles per milliliter (30).

Lipoprotein preparation

Low density lipoprotein was prepared according to previously published methods (11, 31), taking precautions to minimize exposure to light, air, metal ions, and LPS. All solutions used in the preparation of LDL were Chelex-treated. The final LDL preparation was stored in 0.5 mm EDTA. Prior to use LDL was dialyzed at 4°C against PBS without calcium or magnesium in the dark. Chelex (1 g/L) was added to the dialysis buffer. Different LDL batches gave essentially identical results in all of the experiments performed.

Lipid oxidation assays

Thiobarbituric acid assay. The TBARS assay was used to measure LDL lipid oxidation mediated by activated human monocytes. The assay was performed in 96-well tissue culture plates. The assay was kindly provided by Elliot Sigal and Craig Laughton (Syntex, Palo Alto, CA) with minor modifications. The cells (1.0×10^5/0.1 ml/well) were cultured in RPMI-1640 with LDL (0.5 mg LDL cholesterol/ml), ± SOD 150 U/ml, and ZOP (2 mg/ml) for 18–20 h prior to performing the TBARS assay. Malonaldehyde bis(dimethyl acetal) (Sigma, St. Louis, MO) was used as the standard and serial dilutions were added to empty wells on each plate. The volume of standard added was equivalent to the starting volume of all the samples. The following additions were made to each well: 10 μL of 1 mm BHT, 10 μL of 10 mg/ml EDTA, 50 μL of 50% trichloroacetic acid, and 75 μL of 1% thiobarbituric acid in 0.3% NaOH. After wrapping the plates in plastic they were placed in a 65°C water bath for 60 min. The plastic was removed from the plates, the plates were centrifuged and read from the top of the plate in a Cytofluor II fluorescence reader with the excitation at 530 nm and emission at 590. The calculations were made from the standard curve using Cytocalc II software to determine nanomole equivalents.

Detection of cholesterol-HPODE and cholesterol-HODE by reversed-phase HPLC. After the incubation of activated monocytes (ODN or mock pretreated) with LDL, BHT was added to each well to give a final concentration of 100 μm to retard autooxidation. Wells were scraped and lipids were extracted using a previously reported chloroform–methanol extraction procedure (5). After extraction, samples were dried under nitrogen and stored at −20°C until HPLC analysis.

Lipids were separated using a Rainin Microsorb C18 column (250 mm × 4.6 mm, Varian Chromatography Systems, Walnut Creek, CA), a Beckman Model 421A controller, Beckman Model 114M pumps, and a Beckman Model 163 Variable Wavelength detector. Oxidized fatty acids were eluted with a gradient solvent system as described previously (5) and were detected by absorbance at 236 nm. Standards of cholesteryl-HPODE and cholesteryl-HODE were purchased from Cayman Chemical Co.

Statistical analyses

The data from $O_2^{\cdot-}$ and LDL oxidation experiments were analyzed using the unpaired, one-tailed Student's *t* test to test the hypothesis that antisense ODN treatment might inhibit these responses. All tests were perfomed with GraphPAD software (GraphPAD Software Inc., San Diego, CA). Data points with a $P < 0.05$ were considered to be significantly different.

RESULTS

Our initial studies investigated the role of NADPH oxidase in the generation of $O_2^{\cdot-}$ upon monocyte activation with opsonized zymosan. To eliminate the known nonspecific effects of pharmacologic inhibitors, such as diphenylene iodonium, which in these cells causes dose-dependent injury concomitant with inhibition of $O_2^{\cdot-}$, we chose to utilize hu-

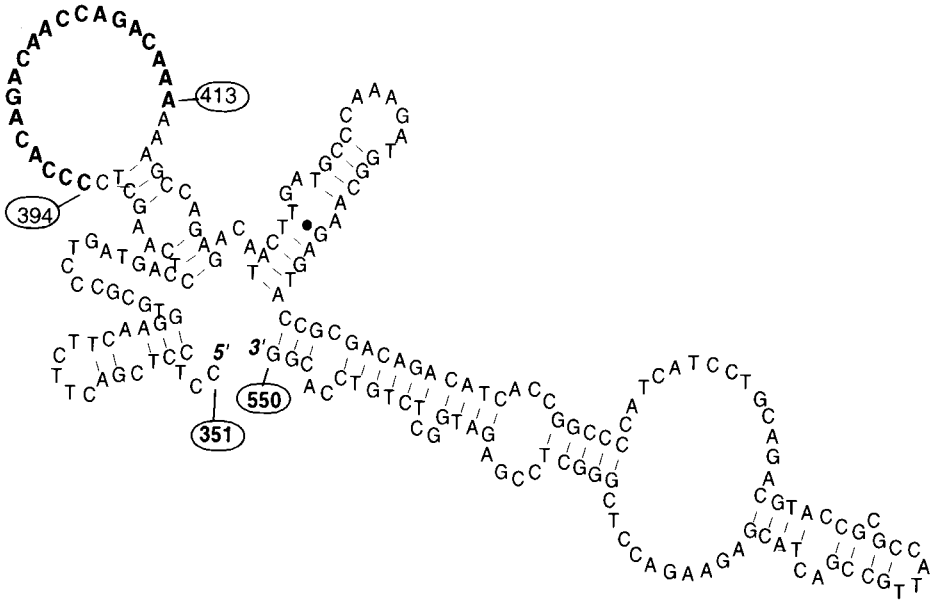

Fig. 1. Predicted secondary structure of bases 394–413 of p47phox mRNA. The p47phox antisense and sense oligonucleotide sequences were chosen after using the software program Mulfold to predict areas of the mRNA lacking substantial secondary structure and then verifying the unique nature of the sequence using Blast. Finally, selected ODN were screened to ensure that they did not fold or pair, again using Mulfold, and were synthesized using phosphorothioate-modified bases and purified by HPLC. The single-stranded loop depicted in this figure was the area chosen for antisense ODN targeting.

man monocyte p47phox antisense and sense oligonucleotides to down-regulate the expression of p47phox protein and the activity of NADPH oxidase. We chose p47phox as the target for this intervention as natural mutations in p47phox have been shown to block the production of $O_2^{\cdot-}$ by NADPH oxidase. **Figure 1** depicts the predicted 2° structure of a segment of the mRNA for human p47phox showing the single-stranded loop that we chose to target with antisense ODN.

To examine the proficiency of the selected antisense ODN in modulating expression of the target protein, we evaluated the effect of antisense versus sense ODN treatment on p47phox protein expression. We found that the most complete inhibition was obtained when the antisense ODN treatment continued for 3 days as compared to 1 or 2 days (data not shown). For the 72-h treatment, antisense or sense ODN were added 48 h after initial treatment to replenish the ODN. Results of a representative Western blot are shown in **Fig. 2A** where human monocytes were treated with antisense or sense ODN at −72 h and −24 h and then lysed and analyzed for p47phox expression at zero h as described in Methods. Our results show that the expression of p47phox protein was substantially reduced in antisense ODN-treated cells whereas the sense ODN control caused no inhibition of expression of p47phox. Dose-dependent inhibition was observed with maximal inhibition at 10 μm ODN. The blots shown here are representative of three experiments where the inhibition by antisense ODN ranged from 45–80%. In a separate experiment, the p47phox was stripped and reprobed with antibodies to ERK1/2 kinase revealing no inhibition of expression of this unrelated, constitutively produced protein (Fig. 2B).

The next step was to determine the effect of p47phox antisense in functional assays. In $O_2^{\cdot-}$ experiments, cells were plated and cultured with various concentrations of ODN for 3 days, as above. After the incubation period the medium was changed to serum-free RPMI 1640 without phenol red and the $O_2^{\cdot-}$ assay was performed as described. Results of a representative experiment shown in **Fig. 3** reveal that the p47phox antisense ODN was effective at inhibiting $O_2^{\cdot-}$ production in these functional assays at concentrations of 5–10 μm while the sense ODN had no inhibitory effect. This finding indicates that selective inhibition of p47phox expression interferes with $O_2^{\cdot-}$ production, p47phox is essential for respiratory burst activity in intact human monocytes, and that NADPH oxidase is the source of $O_2^{\cdot-}$. The data were subjected to statistical analyses; the results of these analyses are reported in the legend for Fig. 3 and indicate a significant difference between cells treated with activator and those treated with activator and antisense ODN. Significant inhibition was

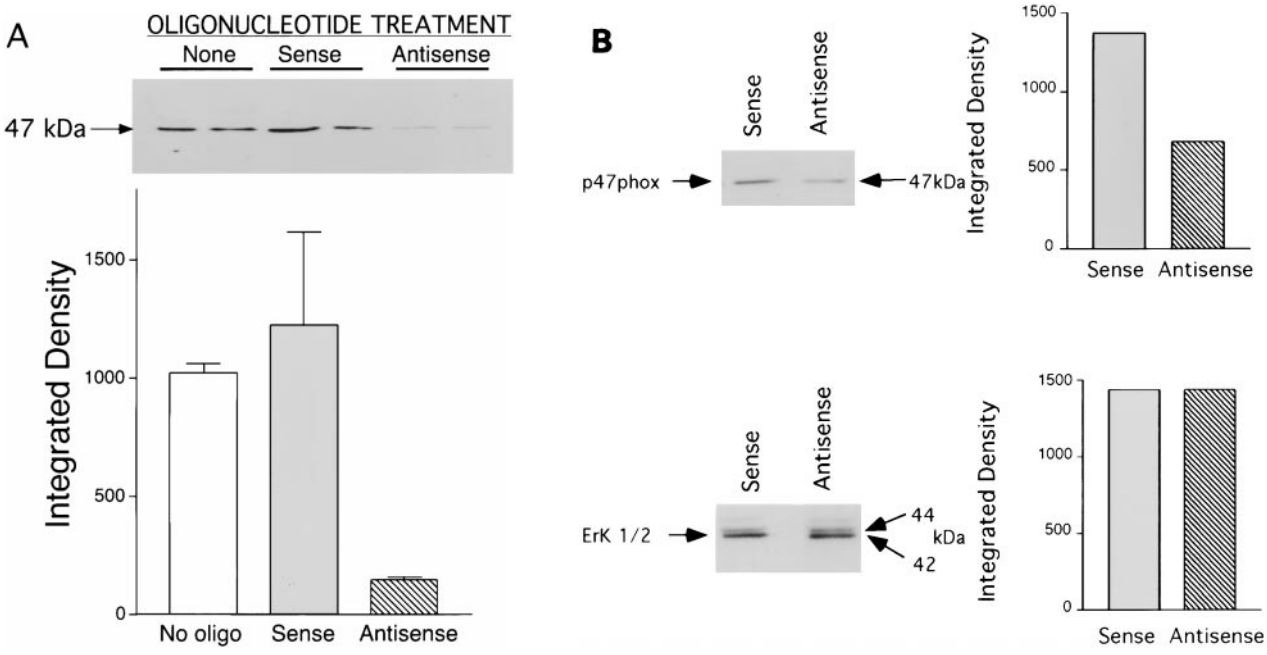

Fig. 2. p47phox antisense ODN treatment inhibits p47phox protein expression in human monocytes. A: Human monocytes (2.5×10^6/ml) were treated with 5 μm antisense or sense ODN for 72 h with a refeeding at 48 h. Cells were lysed in hypotonic lysis buffer and lysates were run on 10% SDS-PAGE gels. Proteins were transferred to a PVDF membrane by the semi-dry method. Human p47phox was detected using a 1:1000 dilution of human p47phox polyclonal antibody followed by incubation with a 1:1000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG. The blot was developed by ECL. Samples were run in duplicate as indicated. The left-hand arrow indicates the migration of p47phox (47 kDa) based on the migration of molecular mass markers that were in adjacent lanes. The samples were loaded in duplicate and the bars represent the average density for two bands per group. The bar graph depicts integrated densities of p47phox bands in the blot as determined by analysis of lightly exposed film by the software program NIH image. Error bars represent the data range of duplicates. B: In a separate experiment monocytes were treated as above with antisense or sense ODN to p47phox and then the blot of the PAGE gel was probed with antibody to p47phox, developed by ECL and exposed to film. The blot was then stripped and reprobed with antibody to Erk1/2 kinase as an unrelated, constitutively produced protein. This blot was similarly developed. Bar graphs represent the integrated density of the relative OD curves derived from NIH image analysis of lightly exposed films of the blots developed by ECL.

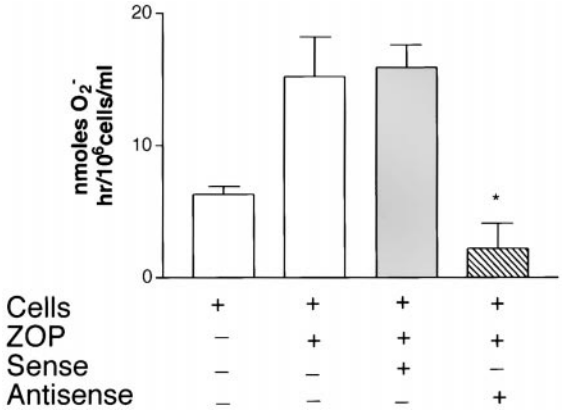

Fig. 3. p47phox antisense ODN treatment inhibits O_2^- production by activated human monocytes. Human monocytes were plated in 96-well tissue culture plates at a concentration of 1.0×10^5/0.1 ml/well. Monocytes were treated with antisense or sense ODN at the indicated concentrations for 3 days (with refeeding after 2 days). After the incubation period the cells were washed with RPMI without phenol red and incubated with 2 mg/ml ZOP, 160 μm cytochrome c, ± SOD 150 U/ml for 1 h at 37°C. After incubation the absorbance was read in a Molecular Devices Thermomax microplate reader at 550 nm. O_2^- production was calculated by determining the SOD-inhibitable O_2^- produced from cells as described in Methods. The results are from a representative experiment of three performed. The data represent the mean ± standard deviation of triplicate determinations. Cells = human monocytes, Activator = ZOP, Antisense = p47phox antisense ODN, Sense = p47phox sense ODN. These data were analyzed using the unpaired, one-tailed Student's *t* test. The asterisk indicates that O_2^- values from cells treated with ZOP and antisense ODN were significantly different from those obtained with cells treated with ZOP alone ($P = 0.01$) or as compared to those obtained with cells treated with ZOP plus sense ODN ($P = 0.003$).

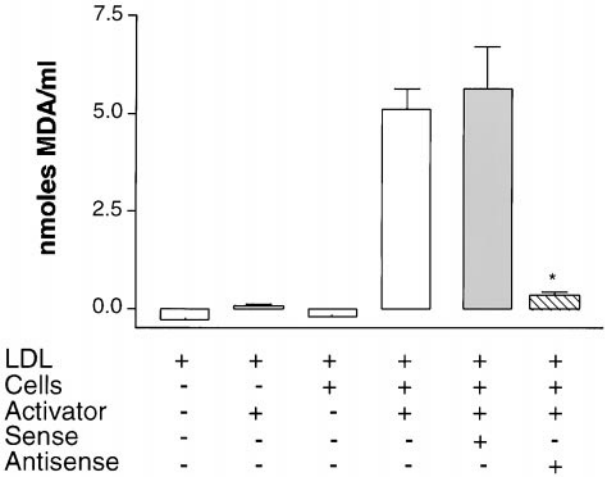

Fig. 4. p47phox antisense ODN treatment inhibits monocyte-mediated LDL lipid oxidation. Human monocytes (1.0×10^5/0.1 ml/well) were cultured in 96-well plates in 10% BCS DMEM. Cells were treated with antisense or sense oligonucleotides at the concentrations indicated for 3 days (with a refeeding after 2 days). After the treatment period, cells were washed with RPMI and incubated in the presence of ZOP (2 mg/ml), ± SOD 150 U/ml, and 0.5 mg cholesterol/ml for 24 h. After the incubation period the TBARS assay was performed as described herein. Plates were read in a Cytofluor II microwell fluorescence reader at 530/590 (excitation/emission. Calculations were made from the standard curve using the Cyto Calc II software to determine nanomole equivalents. Results of a representative TBARS experiment, of four performed, are shown. LDL = low density lipoprotein, Cells = human monocytes, Activator = ZOP, Antisense = p47phox antisense ODN, Sense = p47phox sense ODN. These data were analyzed using the unpaired, one-tailed Student's *t* test. The asterisk indicates that LDL oxidation values from cells treated with ZOP and antisense ODN were significantly different from cells treated with ZOP alone ($P = 0.0001$) or from cells treated with ZOP plus sense ODN ($P = 0.0005$).

also observed when the antisense ODN-treated group was compared with the sense ODN-treated group. A significant difference was not found between cells treated with activator and cells treated with activator and sense ODN.

To determine whether NADPH oxidase-derived O_2^- was indeed required for the oxidation of LDL by human monocytes, we treated monocytes with antisense or sense ODN and then assessed their ability to mediate LDL oxidation by activating them with ZOP in the presence or absence of LDL (0.5 mg cholesterol/ml). After the 18–20 h incubation period the TBARS assay was performed. This assay provides a general measure of the formation of lipid oxidation products. Results of a representative experiment are presented in **Fig. 4**. The data indicate that LDL oxidation by human monocytes is inhibited by treating human monocyte p47phox antisense ODN at a concentrations of 5–10 μm and that the sense ODN control had no effect at these concentrations. Significant inhibition was observed in the antisense ODN-treated group as compared with either the sense ODN-treated group or the untreated group. There was no significant difference between the LDL oxidation mediated by cells treated with activator as compared with those treated with activator and sense ODN. *P* values are reported in the legend for Fig. 4. It should be noted that the results of these experiments are very reproducible regardless of the use of different batches of LDL. This likely relates to the extreme measures taken to protect the LDL from oxidation during isolation.

Although the TBARS assay is widely used as a measure of LDL lipid oxidation products, we used a second method involving HPLC separation and detection of oxidized cholesteryl esters to test whether treatment with antisense ODN to p47phox inhibited the oxidation of LDL by activated human monocytes. Previous studies in our lab have shown that the major fatty acid oxidation products in monocyte-oxidized LDL are hydroperoxyoctadecanoic acid (HPODE) and hydroxyoctadecadienoic acid (HODE) esterified to cholesterol (5). We therefore examined the formation of these predominant oxidation products in LDL that was incubated with activated monocytes, pretreated with antisense or sense ODN. Samples were analyzed by HPLC and monitored for absorbance at 236 nm to detect conjugated dienes. The profile of a typical chromatogram from three similar experiments is shown in **Fig. 5**. Treatment of monocytes with p47phox antisense ODN inhibited the production of the major LDL lipid oxidation products, cholesteryl-HPODE and cholesteryl-HODE. The sense ODN in these experiments

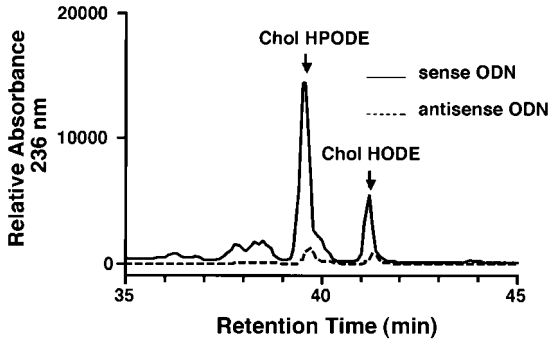

Fig. 5. Reversed-phase HPLC of LDL lipid oxidation products produced by activated human monocytes treated with p47phox antisense or sense ODN. Human monocytes (2.5×10^6 cells/ 2 ml/ well) were treated with 10 μm of p47phox antisense and sense ODN as described in Methods. After ODN treatment, cells were incubated with 0.5 mg LDL cholesterol/ml for 24 h. After the incubation period BHT was added, lipids were extracted, and samples were analyzed by reversed-phase HPLC as described in Methods. Absorbance was monitored at 236 nm. Dashed lines indicate the HPLC profile from antisense ODN-treated samples whereas solid lines indicate results obtained from sense ODN-treated controls. Results with control cultures lacking ODN treatment resembled those obtained with sense ODN (data not shown). Retention times of samples were compared to internal standards of cholesteryl-HPODE and cholesteryl-HODE. The data are representative of similar results obtained in repeat experiments.

had no effect. These data confirm that the oxidation of LDL by activated human monocytes is selectively inhibited by treatment of the monocytes with p47phox antisense ODN and confirm the results obtained in the experiments detecting LDL oxidation with the TBARS assay. Together, these data confirm and extend our prior studies indicating that $O_2^{\cdot-}$ produced by ZOP-activated human monocytes is required for the oxidation of LDL as inactivation of an essential component of the NADPH oxidase complex blocked $O_2^{\cdot-}$ production and effectively inhibited oxidation of LDL.

DISCUSSION

In this study we examined whether $O_2^{\cdot-}$, produced by activated human monocytes, and which we previously reported was involved in the oxidation of LDL, was truly of monocyte derivation and whether it indeed was required for monocyte-mediated LDL oxidation. To address this question we designed an antisense ODN to p47phox, an essential component of the NADPH complex. We showed by Western blot analysis that the expression of p47phox in human monocytes treated with p47phox antisense ODN for 3 days was inhibited in a dose-dependent fashion with maximal inhibition in the 5–10 μm range. We conclude that the antisense to p47phox mRNA selectively inhibited expression of p47phox as levels of an unrelated protein were unaffected as was p47phox in monocytes treated with the control, sense ODN.

We then turned our attention to functional studies to determine whether the p47phox antisense ODN treatment inhibited $O_2^{\cdot-}$ production and/or the oxidation of LDL by activated human monocytes. The results of these studies indicated that p47phox antisense ODN inhibited the production of $O_2^{\cdot-}$ whereas, in contrast, treatment with sense ODN caused no inhibition of $O_2^{\cdot-}$ production. We used two different assays to assess LDL lipid oxidation. In the first approach, the TBARS assay, which measures the formation of certain lipid oxidation products, was used as it has been routinely used to monitor LDL oxidation. We found that p47phox antisense ODN effectively inhibited LDL oxidation in ZOP-activated human monocytes. Similar inhibition by antisense ODN treatment was observed using the more specific assay measuring the formation of cholesteryl-HPODE/HODE, the major oxidation products detected on monocyte-oxidized LDL (5). As for $O_2^{\cdot-}$ production, treatment with sense ODN was without effect.

Our previous studies with intact human monocytes showed that ZOP-activated human monocytes produce $O_2^{\cdot-}$ and cause substantial oxidation of LDL lipids. The addition of SOD blocked the oxidation of LDL (12, 14). LDL oxidation was entirely dependent on the activation of the monocytes as one might expect in immunologic responses. Similar studies have been conducted by other groups but in a medium with added free metal ions. This metal ion-dependent, monocyte-mediated oxidation of LDL was less reliant on $O_2^{\cdot-}$ (15, 32). We have found that the dependence of LDL oxidation on activation of the monocytes was circumvented when free metal ions were added, suggesting alternative lipid oxidative pathways in the presence of monocytes and free metal ions (11). Thus, the main reason for the controversy regarding the requisite role for $O_2^{\cdot-}$ in monocyte-mediated LDL oxidation appears to be related to the presence or absence of added pro-oxidant, free metal ions during the monocyte/LDL coculture. The presence of free metal ions in physiologic systems is doubtful, thus the relevance of the activation-independent, free metal ion-dependent oxidation of LDL is unclear (1).

Another case in point is provided by the conflicting data published by two laboratories investigating the ability of monocytes or mononuclear blood cells from patients with CGD to oxidize LDL. Wilkins, Segal, and Leake (17) observed that NADPH oxidase activity was not required for monocyte LDL oxidation; however, the tissue culture medium for these studies was Hams F10 (containing 10 nm $CuSO_4$ and 3 μm $FeSO_4$) to which they added additional $FeSO_4$ (3 μm). When similar studies were conducted in the absence of added free metal ions (in RPMI-1640), cells from CGD patients were notably defective in their ability to oxidize LDL (18).

We must stress that we do not believe that the oxidation of LDL by activated monocytes, in medium lacking added free metal ions, is entirely metal ion-independent. Indeed, metal ion centers of enzymes and likely metal ion complexed in the yeast cell walls of the activator zymosan appear to contribute to the process, but it is the addition of significant amounts of free metal ions that alters the reliance of this oxidation of LDL on monocyte activation and lessens the requirement for $O_2^{\cdot-}$ (7, 10). This topic is reviewed and put in context in our recent JBC minireview (1).

Our lab has been studying the signal transduction pathways involved in the activation of the monocyte NADPH oxidase. In these studies we have found that at least one isoform of PKC, and probably more, are involved $O_2^{\cdot-}$ production as antisense ODN to the cPKC isoforms, and more particularly antisense ODN specific for the PKCα isoform of PKC, substantially inhibit the production of $O_2^{\cdot-}$ (2, 9). We have also reported that $cPLA_2$-specific inhibitors as well as $cPLA_2$-specific antisense ODN inhibited both LDL oxidation and $O_2^{\cdot-}$ production (8). Together these data suggest that both PKC and $cPLA_2$ play significant roles in regulating $O_2^{\cdot-}$ production by human monocytes. Further investigation of the regulatory pathways leading to the activation of the NADPH oxidase complex in monocytes is needed to suggest novel ways to selectively intervene in the activation of this complex by certain stimuli without totally blocking NADPH oxidase activity in an indiscriminate manner.

In conclusion, our studies show that an antisense ODN specific for the mRNA of p47phox, a key component of the phagocytic NADPH oxidase complex, selectively inhibits p47phox protein expression. This antisense ODN also inhibited $O_2^{\cdot-}$ production as well as monocyte-mediated LDL oxidation. These results indicate that $O_2^{\cdot-}$ is generated by the NADPH oxidase enzyme complex of these activated human monocytes and that monocyte-derived $O_2^{\cdot-}$ production is required for monocyte-mediated oxidative modification of LDL.

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