Lipid peroxides induce expression of catalase in cultured vascular cells

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Abstract **Various forms of oxidized low-density lipoproteins (Ox-LDL) are thought to play a major role in the development of atherosclerosis. The lipid components of Ox-LDL present a plethora of proatherogenic effects in in vitro cell culture systems, suggesting that oxidative stress could be an important risk factor for coronary artery disease. However, buried among these effects are those that could be interpreted as antiatherogenic. The present study demonstrates that various oxidants, including oxidized fatty acids and mildly oxidized forms of LDL (MO-LDL), are able to induce catalase (an antioxidant enzyme) expression in rabbit femoral arterial smooth muscle cells (RFASMC), RAW cells (macrophages), and human umbilical vein endothelial cells (HUVEC). In RFASMC, catalase protein, mRNA, and the enzyme activity are increased in response to oxidized linoleic acid (13-hydroperoxy-9,11-octadecadienoic acid [13-HPODE] and 13-hydroxy-9,11-octadecadienoic acid [13-HODE]), MO-LDL, or hydrogen peroxide (H_2O_2). Such an increase in catalase gene expression cannot totally be attributed to the cellular response to an intracellular generation of H_2O_2 after the addition of 13-HPODE or 13-HODE because these agents induce a further increase of catalase as seen in catalase-transfected RFASMC. Taken together with the induction of heme oxygenase, NO synthase, manganese superoxide dismutase (Mn-SOD), and glutathione synthesis by oxidative stress, our results provide yet more evidence suggesting that a moderate oxidative stress can induce cellular antioxidant response in vascular cells, and thereby could be beneficial for preventing further oxidative stress.**—Meilhac, O., M. Zhou, N. Santanam, and S. Parthasarathy. **Lipid peroxides induce expression of catalase in cultured vascular cells.** ***J. Lipid Res.*** **2000.** 41: **1205–1213.**

Supplementary key words atherosclerosis • 13-HPODE • oxidized linoleic acid • antioxidant defense • mildly oxidized low density lipoprotein • catalase • hydrogen peroxide • smooth muscle cells • macrophages • HUVEC

The role of oxidized low density lipoproteins (Ox-LDL) in atherogenesis has been a topic of great interest for more than a decade. While the initial focus of the oxidation hypothesis centered on the uptake of Ox-LDL by macrophages and the development of foam cells, the cellular effects of oxidized lipids have more recently taken the center stage. It is now well established that oxidized lipids exhibit a wide variety of proatherogenic effects on cultured cells (1). They affect every facet of atherogenesis, cellular accumulation of lipid (2), modulation of gene expression, proliferation of smooth muscle cells, alteration of the endothelial functions, cytotoxicity, fibrinolysis, and plaque disruption (3–5).

The paradoxical beneficial effects of physical activity on cardiovascular disease, despite its potential to induce an oxidative stress, alerted us to the possibility that oxidative stress could, under certain circumstances, trigger an antioxidant response and be possibly antiatherogenic. Accordingly, we demonstrated a reduced susceptibility to oxidation of LDL isolated from chronic conditioned athletes as opposed to the greater propensity of LDL to undergo oxidation isolated from beginning exercisers (6). We proposed that exposure to oxidative stress might lead to the induction of antioxidant defense by the arterial cells.

Catalase is an antioxidant enzyme that is predominantly located in cellular peroxisomes, which catalyzes the dismutation of H_2O_2, forming O_2 and H_2O (7). Previous study from our laboratory has demonstrated that oxidized linoleic acid (13-hydroperoxy-9,11-octadecadienoic acid, [13-HPODE]) could increase intracellular generation of H_2O_2 in smooth muscle cells, mediating cytotoxic effects (8). More importantly, the overexpression of catalase in vascular smooth muscle cells not only abrogated the cytotoxic effects of H_2O_2 but also prevented the cytotoxic effects of 13-HPODE. On the basis of these findings we anticipated an induction of cellular catalase gene expression by oxidants. In the current study, we describe the induction of catalase expression by oxidized linoleic acids (13-HPODE and 13-hydroxy-9,11-octadecadienoic acid [13-HODE]), H_2O_2, and mildly oxidized forms of LDL (MO-LDL) in

Abbreviations: FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; 13-HODE, 13-hydroxy-9,11-octadecadienoic acid; 13-HPODE, 13-hydroperoxy-9,11-octadecadienoic acid; HUVEC, human umbilical vein endothelial cell; MEM, minimal essential medium; MO-LDL, minimally oxidized low density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Ox-LDL, oxidized low density lipoprotein; PBS, phosphate-buffered saline; RFASMC, rabbit femoral arterial smooth muscle cell; TLC, thin-layer chromatography.

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vascular cells. In light of earlier findings by others that the exposure of cultured cells to lipid peroxides results in the induction of heme oxygenase (an antioxidant enzyme) (9), manganese superoxide dismutase (Mn-SOD) (10), nitric oxide synthase (11), and glutathione synthesis (12), it appears likely that a moderate oxidative stress could, in general, induce cellular antioxidant responses and thus could be beneficial in the context of atherosclerosis and other chronic diseases in which oxidative stress has been implicated.

MATERIALS AND METHODS

Materials

Minimal essential medium (MEM), RPMI 1640, Medium 199, Dulbecco's modified Eagle's medium (DMEM)/F12 medium, penicillin, amphotericin, streptomycin, L-glutamine, trypsin–ethylenediaminetetraacetic acid (EDTA), and Hanks' balanced salt solution (HBSS) were purchased from Cellgro Mediatech (Herndon, VA). Fetal calf serum (FCS) was purchased from Atlanta Biologicals (Atlanta, GA). Endothelial mitogen growth factor (EMGF) was obtained from Biomedical Technologies (Stoughton, MA). Linoleic acid, oleic acid, thin-layer chromatography (TLC) plates, hydrogen peroxide, human kidney catalase, soybean lipoxygenase, anti-β-actin antibody, rabbit IgG, and peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse IgG) were all obtained from Sigma (St. Louis, MO). Rabbit polyclonal anti-human erythrocyte catalase antibody was obtained from Athens Research and Technology (Athens, GA). 13-HODE was obtained from Cayman Chemicals (Ann Arbor, MI). [1-^{14}C]Linoleic acid (53 mCi/mmol) was obtained from New England Nuclear (Boston, MA).

Cell culture

Rabbit femoral arterial smooth muscle cells (RFASMC) (American Type Culture Collection [ATCC], Manassas, VA) were routinely cultured in MEM supplemented with 10% FCS, 2 mM l-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL). A stably catalase-transfected RFASM cell line was established in our laboratory as previously described (8). Human umbilical vein endothelial cells (HUVEC) were obtained from the Dermatology Core Cell Service Facility (Emory University, Atlanta, GA) and were grown on 0.1% gelatin-coated plates, in Medium 199 containing 20% heat-inactivated FCS, 2 mM L-glutamine, heparin (100 U/mL), EMGF (0.05 mg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). RAW macrophage cells (RAW 264.7 from the ATCC) were cultured in DMEM/F12 medium, 10% FCS, 2 mM L-glutamine, gentamicin (25 μg/mL). For Western blot and Northern blot studies, cells were grown in 25- and 175-cm^2 cell culture flasks until 90% confluent. For other studies the cells were cultured in 6- or 24-well dishes. Before any experiment, cells were placed in their corresponding serum-free medium for 8–16 h. Cells were passaged with trypsin–EDTA.

Catalase enzyme assay

Cells were cultured to 90% confluence in 6-well dishes. Cellular lysates were prepared by sonication of scraped cells in phosphate-buffered saline (PBS), pH 7.4, containing 0.05% deoxycholate. An aliquot of cell lysate was used for protein estimation, by using a bicinchoninic acid (BCA) kit (Sigma), according to the procedure of Smith et al. (13). Catalase activity was measured by the method of Aebi (7), in which H_2O_2 was used as the substrate. The initial rate of disappearance of H_2O_2 (0 to 60 sec) was recorded spectrophotometrically at a wavelength of 240 nm. The catalase activity was expressed as units per milligram of protein, using a standard curve obtained with commercially available catalase. Catalase activity in control (untreated cells, 32 ± 5 units/mg of protein) is considered as 100% and results are expressed as a percentage of the control.

Preparation of 13-HPODE

Stock linoleic acid (C18:2) was prepared in absolute ethanol. The linoleic acid was oxidized to 13-HPODE, with immobilized soybean lipoxygenase (100 U/mL) at 37°C for 1 h. The formation of 13-HPODE was monitored spectrophotometrically by scanning the absorption between 200 and 300 nm (model DB-3500, SLM-AMINCO; Spectronic Instruments, Rochester, NY) using PBS as reference (14). Under these conditions, the conversion into 13-HPODE is observed as an increase in absorbance at an optical density of 234 nm. Usually, more than 90% conversion of linoleic acid to 13-HPODE was achieved as determined by the molar extinction coefficient of the conjugated dienes, TLC, high-performance liquid chromatography (HPLC), or the leucomethylene blue (LMB) assay. The LMB assay, which is used to determine the actual peroxide content, provided a peroxide content of 90–94% (15).

Isolation of LDL and preparation of minimally oxidized LDL

Blood was collected from healthy donors, and LDL (d 1.019–1.063) was isolated by ultracentrifugation as previously described, using a TL-100 tabletop ultracentrifuge (16). The isolated LDL was dialyzed against PBS, pH 7.4, for 6 h. The concentration of apolipoprotein B (apoB) was determined by standard protein determination, using a BCA kit (Sigma) according to the procedure of Smith et al. (13). MO-LDL was prepared by addition of 5 μM $CuSO_4$ to a 2-g/L LDL solution in PBS at 37°C, and monitoring the formation of conjugated dienes at an optical density of 234 nm for about 1 h. The oxidation was stopped by the addition of 10 μM EDTA. MO-LDL contained 3–5 nmol of thiobarbituric acid-reactive substances (TBARS) per mg of apoB, as determined by the method of Yagi (17).

Northern blot analysis and quantification

Total RNA was isolated from RFASMC after various treatments, using TRI reagent (Sigma). Total RNA (30 μg) was separated on a 1.0% agarose–formaldehyde gel. The gel was stained with ethidium bromide in order to visualize the amount of RNA loaded in each lane. RNA was transferred to a nylon membrane and hybridized with [^{32}P]dCTP random primer-labeled complementary DNA to human catalase, and the hybridization signal was visualized by exposing the membrane to Kodak film (Eastman Kodak, Rochester, NY). Quantification was performed with a densitometer (model GS-700; Bio-Rad, Hercules, CA), and results are expressed in arbitrary units, as the ratio between catalase signal and 18S RNA content for each lane. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not used as internal control because its expression could vary in response to an oxidative stress (18).

Western blot analysis and quantification

Cell lysates were prepared by lysis and sonication in a hypotonic buffer (50 mM TRIS [pH 8], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA, containing 1 mM dithiothreitol, 10 mM β-glycerophosphate, aprotinin [10 μg/mL], trypsin inhibitor [10 μg/mL], leupeptin [2 μg/mL], and 0.1 mM phenylmethylsulfonyl fluoride). Samples were sonicated and centrifuged at 13,000 *g* for 10 min at 4°C. Protein extract

(10–25 μg of RFASMC or HUVEC, or 75 μg of RAW cells) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was transblotted onto a nitrocellulose membrane, blocked with 10% milk powder in TBS-T (TRIS-buffered saline [pH 7.4]–0.1% Tween 20) overnight, and then incubated with rabbit polyclonal anti-human catalase antibody (1:500 dilution) for 90 min, washed with TBS-T, and incubated with secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase, 1:1,500 dilution) for 1 h. After five washes, the signal was detected with a chemiluminescence kit (ECL kit; Amersham, Arlington Heights, IL). The membrane was then stripped in 62.5 mM TRIS (pH 6.7), 2% SDS, and 0.75% 2-mercaptoethanol for 30 min at 50°C. After 3 washes in TBS-T, the membrane was reprobed as described above with an anti-β-actin primary antibody (1:2,000 dilution). Films were analyzed by densitometry (Bio-Rad model GS-700). The OD catalase/OD β-actin ratio was calculated and the fold increase is reported for each figure, considering untreated control cells as 1.

Immunocytochemistry for catalase detection

After various treatments, cells grown on cover slides at 50–60% confluence were washed twice with PBS, then fixed for 15 min with 3.7% paraformaldehyde in PBS. Cells were washed twice and then incubated with the primary antibody against catalase (1:50 dilution in 3% bovine serum albumin, BSA) for 1 h, at room temperature. After 2 washes with PBS, cells were incubated with a horseradish peroxidase-conjugated secondary antibody (1:100 dilution in 3% BSA). Cells were washed three times and then incubated with peroxidase substrate diaminobenzidine (DAB), according to the manufacturer's instructions (Sigma); cover slides were then washed, mounted on an aqueous medium, and observed under a microscope. A negative control (replacing primary antibody with 3% BSA–PBS or with the same concentration of rabbit IgG during the first incubation) was done for each condition, and did not show any nonspecific staining.

Cell viability assay using MTT

The MTT assay was also used to measure cell viability (19). The principle of this assay is that the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) undergoes cellular reduction by the mitochondrial dehydrogenase of viable cells into a blue formazan that can be measured spectrophotometrically. Briefly, cells were grown in a 6-well plate and the medium was replaced overnight by serum-free medium, before incubation with various concentrations of the oxidants for 16 or 24 h. At the end of incubation, MTT at 0.1 mg/mL was added to each well and incubated at 37°C for a further 30 min. After 30 min, the medium was removed carefully. Dimethylsulfoxide (DMSO) was added to each well in order to solubilize the formazan crystals. The solubilized blue formazan in DMSO was quantified with a spectrophotometer at wavelength 540 nm. There is a linear relationship between the formazan generated and the number of viable cells present.

Uptake and incorporation of [1-^{14}C]linoleic acid into cellular lipids

RFASMC were seeded in 6-well plates and grown until they reached 90% confluence. Experiments were performed in the absence of serum by using a 25 μM concentration (5,000 dpm/nmol) of a labeled solution of linoleic acid. After various times of incubation, the medium was removed and the cells were washed with PBS. Radioactivity in aliquots of the medium and washings was determined in order to estimate the unincorporated fraction of linoleic acid. The cells were scraped into 2 mL of 0.05% deoxycholate-containing PBS, and the radioactivity was determined in 100 μl of the cell lysate. After acidification by adding 20 μl of 6 N HCl, 4 mL of chloroform–methanol 1:1 (v/v) was added to the 1.9 mL of cell lysate to extract the cellular lipids (20). After centrifugation (10 min, 3,000 rpm), the lower chloroform phase was gently dried (37°C, under nitrogen), dissolved in 100 μL of chloroform for loading on a TLC silica plate. Neutral lipids were separated with a solvent system containing *n*-hexane–diethyl ether–acetic acid 90:20:1.5 (v/v/v), and identified by iodine in the presence of standards. Spots were scraped off and the radioactivity was determined. Results are expressed as a percentage of total radioactivity.

RESULTS

Catalase activity in RFASMC treated with 13-HPODE or 13-HODE

RFASMC were grown in 6-well dishes until subconfluent. The cells were then transferred to serum-free medium. After 16 h, cells were incubated with 13-HPODE or unoxidized linoleic acid (C18:2) (**Fig. 1A**), 13-HODE (Fig. 1B), MO-LDL or native LDL (Fig. 1C), or H_2O_2 (Fig. 1D) for 16 h. Catalase activity was determined in cell homogenates as described in Materials and Methods, using H_2O_2 as the substrate. Under the conditions used, none of the oxidants used was cytotoxic for RFASMC, except for H_2O_2 used at 50 μM (MTT viability test, data not shown). As seen in Fig. 1A–D, catalase activity increased by 1.5- to 2-fold in cells treated with 13-HPODE, 13-HODE, MO-LDL, or H_2O_2 in a concentration-dependent manner, except when cells were treated with 50 μM H_2O_2 (likely due to the cytotoxicity). Neither C18:2 nor native LDL increased catalase activity (Fig. 1A and C). To test whether the increased activity involved an activation of the catalase gene and synthesis of protein, we first investigated the catalase protein level in RFASMC by both Western blot and immunocytochemistry.

Immunodetection of catalase in RFASMC by Western blot after treatment with various oxidants

Western blot analysis of RFASMC with anti-human catalase antibody was performed under denaturing conditions (SDS-PAGE). The 240-kDa tetramer characterizing catalase was recognized by the antibody as a single 60-kDa band, corresponding to the monomer. As seen in **Fig. 2A**, treatment of RFASMC with 13-HPODE and 13-HODE at various concentrations for 16 h was able to induce at least a 1.5-fold increase in catalase protein levels. The membrane was then stripped and reprobed with an anti-β-actin antibody. Ox-LDL is known to play a central role in atherosclerosis. When LDL undergoes mild oxidation (MO-LDL), its lipid peroxide content increases (21, 22). Lipids containing oxidized linoleic acids (e.g., 13-HPODE and 13-HODE) have been observed in LDL extracted from atherosclerotic patients (23). These lipids are suggested to be responsible for some of the proatherogenic effects attributed to Ox-LDL (24, 25). Results presented in Fig. 2B show that MO-LDL and 13-HPODE were able to induce a dose-dependent increase in catalase protein after 16 h of treatment. It may be noted that higher concentrations of either 13-HPODE or MO-LDL were toxic for RFASMC under these conditions (MTT assay, data not shown). Linoleic acid (C18:2,

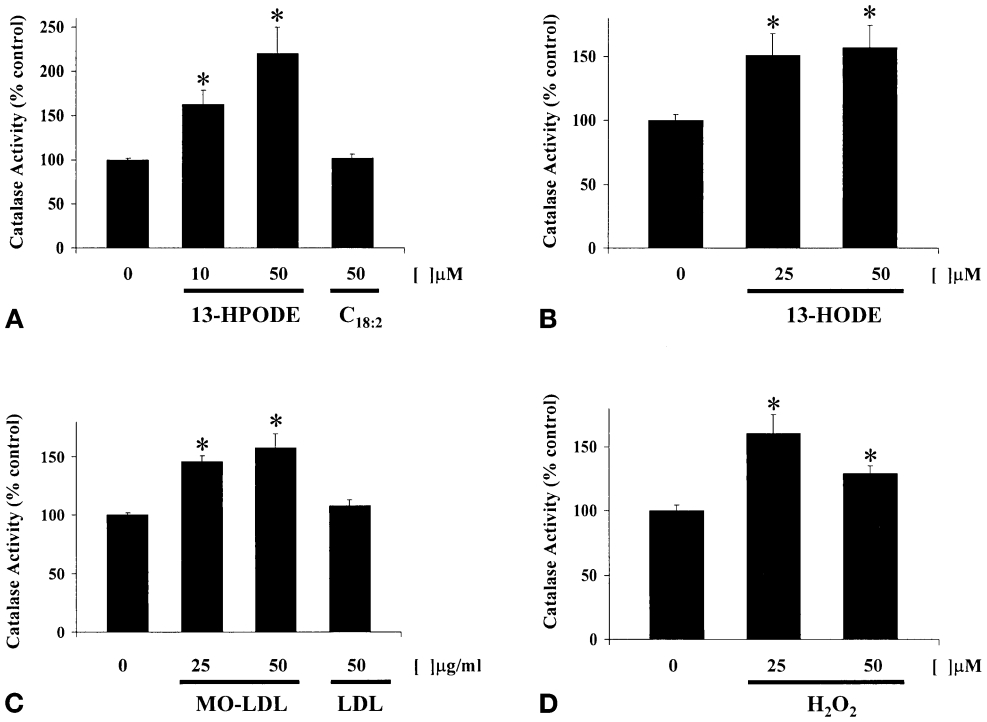

Fig. 1. Catalase enzyme activity in RFASMC. The cells were grown to subconfluence, and after lysis the catalase enzyme activity was measured with H_2O_2 as the substrate at OD_{240}. Catalase activity was expressed as a percentage of control (nontreated cells were considered as 100% activity = 32 ± 5 U/mg protein). Cells were placed in serum-free medium for 16 h and stimulated for an additional 16 h (except for the control) by unoxidized (C18:2, 50 μM) or oxidized linoleic acids (13-HPODE and 13-HODE at 10, 25, or 50 μM) (A and B), minimally oxidized (MO-) or native LDL (25 or 50 μg/ml) (C), and H_2O_2 (25 or 50 μM). Results are expressed as the mean of 3 separate experiments ± SEM. Statistical analysis was performed using Student's *t*-test, and an asterisk (*) indicates when $P < 0.03$, comparing control versus stimulated groups.

50 μM), used as a control, poorly stimulated the expression of catalase protein, suggesting the effect is specific to oxidized products. Native LDL at 50 μg/mL showed a slight increase in catalase protein, probably because of their moderate oxidation during the process of isolation and/or the incubation period with RFASMC.

An increase in catalase protein could be seen after 4 to 16 h of treatment of cells with 13-HPODE (10 μM). However, no significant increase in catalase protein could be noticed after 24 h of stimulation, suggesting a transient activation of the protein (data not shown).

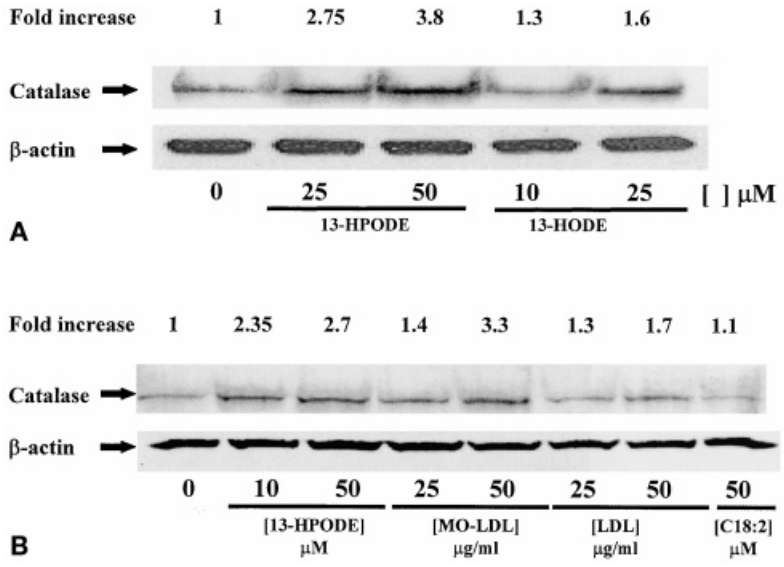

Fig. 2. Western blot analysis of catalase protein level in RFASMC. The cells were grown to subconfluence in a 25-cm^2 flask, placed in serum-free medium for 16 h, stimulated, and then treated as indicated in Materials and Methods. Protein (10–15 μg) was separated on a 10% SDS-polyacrylamide gel. The gel was transblotted onto a nitrocellulose membrane. Western blotting with rabbit polyclonal anti-human catalase antibody (1:500 dilution) identified the catalase protein, using a chemiluminescence kit. Membrane was stripped and reprobed with anti-β-actin antibody (1:2,000 dilution) in order to normalize the catalase level after quantification of both bands. The fold increase is given, considering as 1 the ratio of OD catalase to OD β-actin, after densitometric analysis. (A and B) Cells were treated with 13-HPODE, 13-HODE, MO-LDL, LDL, or linoleic acid (C18:2) at the indicated concentrations for 16 h.

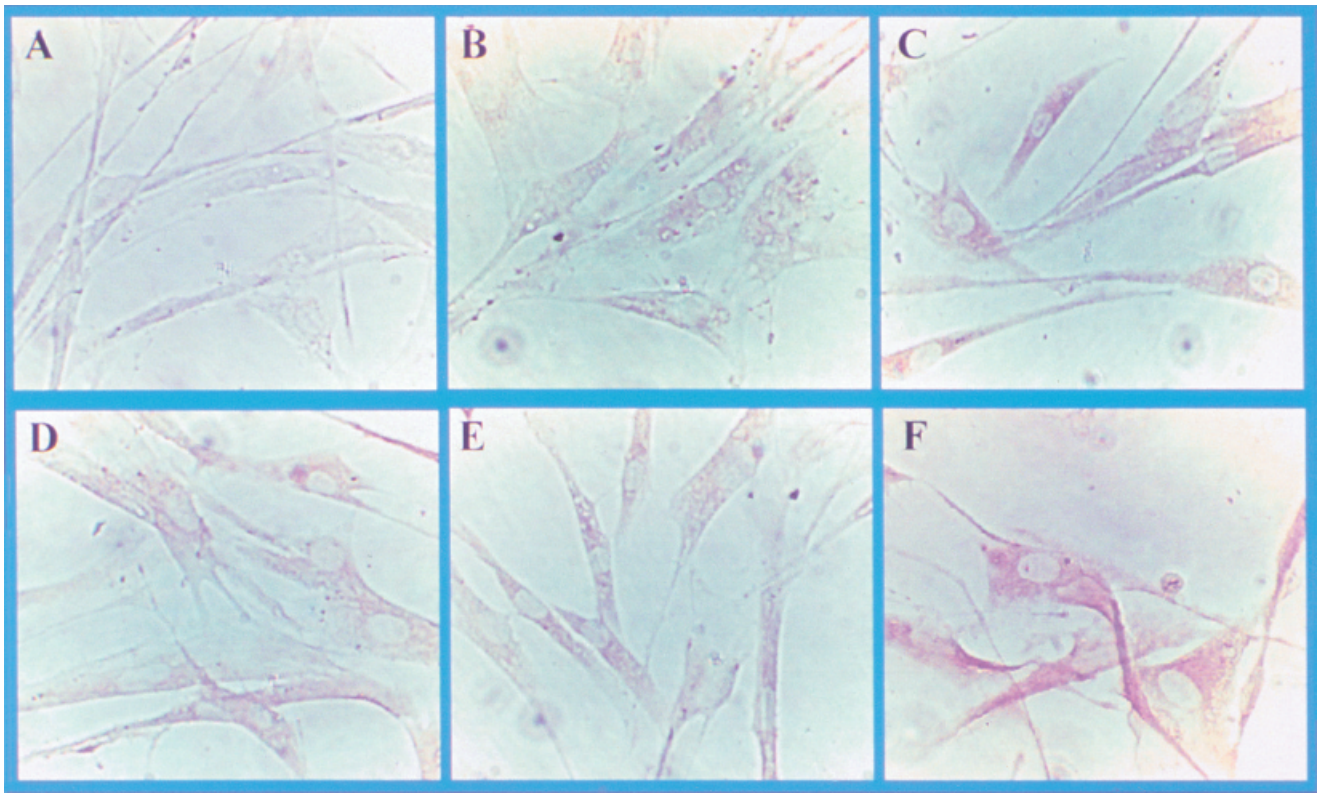

Fig. 3. Immunostaining of catalase in RFASMC. Cells were grown on coverslips until they were 50–60% confluent, washed, and then incubated with the primary antibody against catalase (1:50 dilution). A peroxidase-conjugated secondary antibody–DAB (diaminobenzidine) system was used to visualize catalase in cells. (A) Control untreated cells. (B and C) Cells treated with 13-HPODE at 10 and 50 μM, respectively. (D) Cells treated with 13-HODE at 25 μM. (E) Cells treated with MO-LDL at 25 μg/mL. (F) RFASM cells stably transfected with the catalase gene (8). All treated cells were stimulated for 16 h (B–E).

Immunocytochemistry of catalase in RFASMC treated with various oxidants

To confirm the results from the Western blot analysis, we stained the treated RFASM cells for the immunodetection of catalase, using a peroxidase–DAB detection system, after 16 h of treatment with various oxidants. **Figure 3** shows a specific cytoplasmic staining for catalase in RFASMC treated with 13-HPODE (Fig. 3B and C, 10 and 50 μM), 13-HODE (Fig. 3D, 25 μm), and MO-LDL (Fig. 3E, 25 μg/mL) in comparison with the control (Fig. 3A, nontreated cells). A positive control of RFASM cells stably transfected with catalase gene (8) showed a strong cytoplasmic staining (Fig. 3F).

Northern blot analysis after stimulation with various oxidants

To determine the effect of oxidants on catalase mRNA, we performed a Northern blot analysis using total RNA isolated from treated cells. Because Western blot analysis showed a sustained overexpression of the protein for up to 16 h of stimulation, we treated the RFASMC for 8 h. **Figure 4** shows that catalase mRNA increased when cells were treated with 13-HODE, 13-HPODE (10, 25, and 50 μM), and MO-LDL (25 and 50 μg/mL) for 8 h. It may be noted that native LDL induced catalase gene expression by almost 2-fold, probably due to a slight oxidation occurring during the process of isolation. Catalase-transfected cells

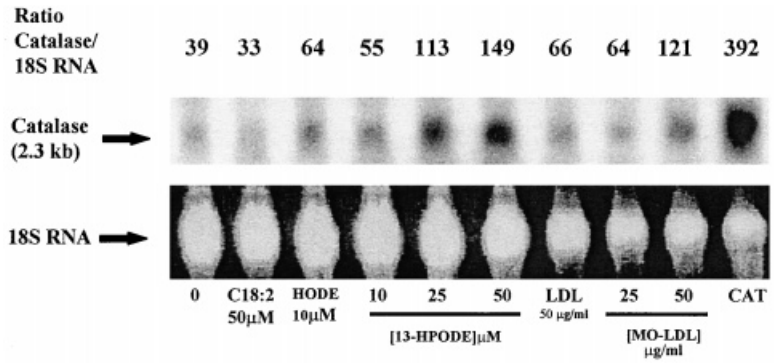

Fig. 4. Northern blot analysis of catalase mRNA. The cells were grown to subconfluence in two 175-cm^2 flasks, placed in serum-free medium for 16 h, and then stimulated as indicated for 8 h. Total RNA (30 μg) was separated on a 1.0% agarose-formaldehyde gel and the RNA was transferred to a nylon membrane. The membrane was hybridized with [^{32}P]dCTP random primer-labeled complementary DNA to human catalase. Bottom lane: Ethidium bromide was used to stain 18S RNA to show the loading in each lane. Top lane: The ratio between catalase signal and 18S RNA (arbitrary units), after quantification by densitometry.

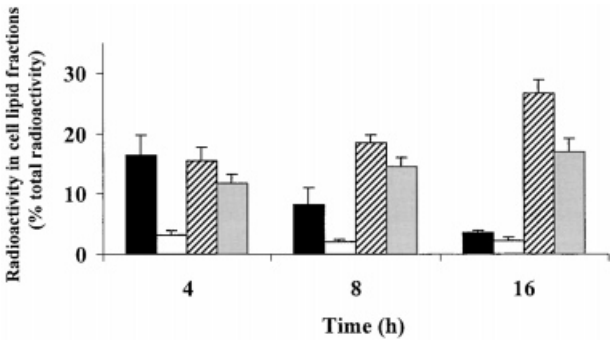

Fig. 5. Cellular distribution of [1-^{14}C]linoleic acid. FASMC were grown in 6-well plates until subconfluent, placed in serum-free medium for 8–16 h, and incubated with [1-^{14}C]linoleic acid for the indicated times. Lipids were extracted from cell lysates and a TLC was run according to the protocol given in Materials and Methods. Radioactivity of each lipid fraction is expressed as a percentage of the total radioactivity added to the cells (solid columns, free fatty acids; open columns, oxidized free fatty acids; hatched columns, triglycerides; gray columns, phospholipids). Results are expressed as means ± SEM of 3 separate experiments performed in duplicate.

are used as a positive control and show a 10-fold increase in mRNA.

These results suggest that the upregulation of catalase by 13-HPODE, HODE, and MO-LDL is at least in part due to transcriptional activation and/or increased catalase mRNA stability.

Uptake and incorporation of [1-^{14}C]linoleic acid into cellular lipids by RFASMC

Smooth muscle cells possess 12-lipoxygenase, which could, under certain circumstances (e.g., on addition of angiotensin II), convert linoleic acid to its oxidized form (26). To determine whether RFASMC could convert C18:2 into 13-HPODE and stimulate catalase gene expression, we determined the conversion of C18:2 in RFASMC after 4, 8, or 16 h of incubation by using [1-^{14}C]linoleic acid. **Figure 5** shows that linoleic acid is readily incorporated into triglycerides (Fig. 5, hatched columns) and phospholipids (Fig. 5, gray columns), and free linoleic acid content decreased during the incubation time (Fig. 5, solid columns). Oxidized linoleic acid has a different migration profile in the TLC system that we used (*n*-hexane–ethyl ether–acetic acid, 90:20:1.5 [v/v/v]) and a small amount of it could be detected in the cells (Fig. 5, open columns) (the stock solution of C18:2 contained about 5% of the oxidized form). No oxidized linoleic acid was released into the medium during the incubation. This was no surprise, as the incubation medium did not contain any stimulation of lipoxygenase pathway that would generate oxidized products.

Effect of 13-HPODE on catalase expression in catalase-transfected RFASMC

Because H_2O_2 induces catalase expression (Figs. 1 and 2) in RFASMC, it is possible that the peroxisomal degradation of HPODE might generate H_2O_2, which might be responsible for the activation of catalase gene. To test this, we incubated catalase-transfected RFASMC (CAT-RFASMC) with 13-HPODE and looked for further activation of catalase. As shown in **Fig. 6**, oxidized linoleic acid stimulates further the expression of catalase in CAT-RFASMC. However, only a 1.5-fold induction was observed, suggesting that the intracellular generation of H_2O_2 was not the only mechanism by which HPODE might activate catalase.

Immunodetection of catalase protein in macrophages and HUVEC by Western blot, after stimulation with oxidized/unoxidized C18:2 and H_2O_2

Induction of catalase by oxidative stress could be of a great importance in the vascular wall and especially in the prevention of atherosclerosis. To investigate if oxidative stress could induce catalase in other cell types present in the arterial wall, we looked for catalase expression in response to HPODE in HUVEC and RAW cells (macrophages).

Figure 7A shows the induction of catalase in RAW cells by 13-HPODE (25 and 50 μM) and H_2O_2 (50 μM). As compared with the basal level, induction of catalase by the same concentration of 13-HPODE (50 μM) is stronger in macrophages than in smooth muscle cells (7-fold induction versus ~3-fold). We included oleic acid (an oxidation-resistant fatty acid) as a control because macrophages can convert linoleic acid to its oxidized form (27); however, neither linoleic acid (C18:2) nor oleic acid (C18:1) was able to induce catalase.

Incubation of a primary culture (passage 2) of HUVEC with 13-HPODE and 13-HODE for 16 h (Fig. 7B) resulted in a 1.3- to 1.7-fold increase in catalase protein. Low passages of HUVEC were used because higher passages did not respond to oxidative stress (passage higher than 3 or an endothelial cell line CRL 1998 [ATCC], data not shown).

DISCUSSION

In this study we demonstrate that exposure of smooth muscle cells, macrophages, and endothelial cells to oxidative stress results in an induction of catalase. The presence

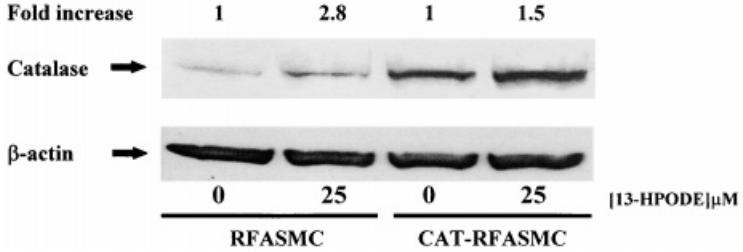

Fig. 6. Catalase protein level in RFASMC and RFASMC transfected with catalase (CAT-RFASMC) after stimulation with 13-HPODE. Subconfluent cells were placed in serum-free medium for 16 h and then stimulated with 25 μM 13-HPODE for another 16 h before Western blot analysis for catalase and β-actin. Densitometric analysis was performed, and the OD catalase/OD β-actin ratio was considered as 1 in nontreated cells.

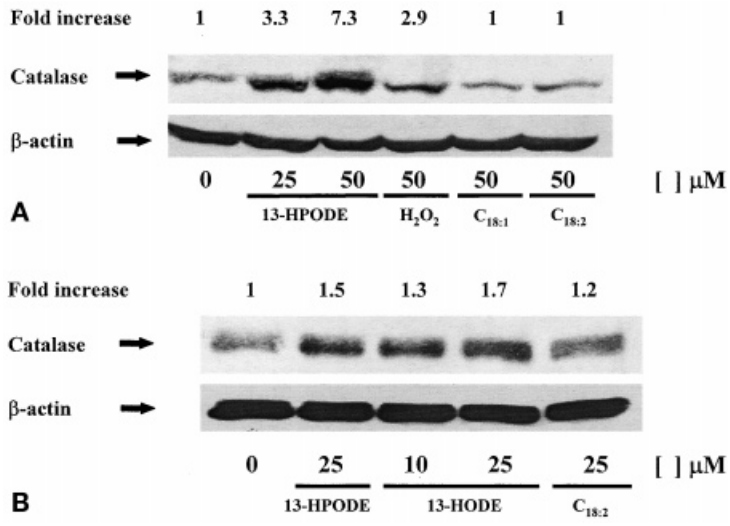

Fig. 7. Catalase protein level in RAW cells and HUVEC after stimulation by various oxidants. Subconfluent cells were placed in serum-free medium for 16 h and then stimulated with various oxidants or control nonoxidized fatty acids for another 16 h. Western blot analysis was performed with 75 μg (RAW) or 10 μg (HUVEC) of total protein from cell lysates. After densitometric analysis, the OD catalase/OD β-actin ratio was considered as 1 in nontreated cells. (A) RAW cells (macrophages) were incubated for 16 h with 13-HPODE, hydrogen peroxide (H_2O_2), oleic acid (C18:1), or linoleic acid (C18:2) at the indicated concentrations. (B) HUVEC (passage 2) were incubated for 16 h with 13-HPODE, 13-HODE, or linoleic acid (C18:2) at the indicated concentrations.

of oxidized lipids in the intimal area of atherosclerotic artery is well documented (28–30), which suggests that endothelial cells, smooth muscle cells, and eventually macrophages are subject to an oxidative stress. Smooth muscle cells can migrate, proliferate, or die in response to oxidative stress (31–33). The transfection of smooth muscle cells by the catalase gene inhibited the oxidative stress-induced production of intracellular H_2O_2 and subsequent cell death (8). Those transfected cells overexpressing catalase also had a reduced angiotensin II-induced hypertrophy (34). In smooth muscle cells, catalase seems to play an important role, and the induction of this gene by a moderate oxidative stress (noncytotoxic conditions) could be atheroprotective in vivo.

Induction of catalase gene expression by hydrogen peroxide, a known substrate, would be expected. Several studies have demonstrated that H_2O_2 could mediate the activation of catalase gene in myocytes (after stimulation by glucose oxidase) (35), and in retinal pigment epithelial cells (after direct stimulation) (36). On the other hand, the basis for the induction of catalase by oxidized fatty acids poses two intriguing possibilities.

i) Such an effect could be a random cellular response to oxidative stress, independent of its metabolic degradation to H_2O_2. Genes unrelated to oxidized fatty acid metabolism such as heme oxygenase (9), Mn-SOD (10), nitric oxide synthase (11), and catalase (current study), as well as glutathione synthesis (12), have been reported to be induced by oxidized lipids. None of these enzymes are constituents of peroxisomes and the only common aspect of their induction is their relationship to their antioxidant potential. An examination of the regulatory sequence of the genes for these enzymes also failed to show a common regulatory sequence.

ii) The induction of catalase also could reflect the adaptive response of cells to oxidative challenge specific to oxidized fatty acids mediated by the generation of H_2O_2. Studies from our laboratory have suggested that oxidized fatty acids (HPODE and HODE) might be degraded in the peroxisomes, resulting in the generation of H_2O_2 (8). However, the intracellular generation of H_2O_2 seems to occur after longer incubation with oxidized linoleic acid. A different mechanism might be involved in the induction of catalase because it occurs much earlier, after 4 to 16 h of incubation with oxidants. The use of catalase-transfected RFASMC, which would deplete cellular H_2O_2, allowed us to show that HPODE induces a further 1.5-fold increase in catalase protein (Fig. 6). This result suggests that the induction of catalase is not totally mediated by H_2O_2 and/or that despite its high expression in these cells, catalase is not able to dismute the intracellular H_2O_2 generated by HPODE. The intracellular compartmentalization of catalase (supposedly in peroxisomes) could prevent its action on H_2O_2 generated, for example, in the region of the plasma membrane or in the cytosol. Furthermore, if the peroxisomal generation of H_2O_2 were involved in the signal transduction leading to the induction of catalase, the nonoxidized linoleic acid should have an effect similar to that of HPODE or HODE (postulating that it is degraded in the same way). Earlier studies from our group have shown that 13-HPODE is poorly taken up by RFASMC even after prolonged incubations (37), suggesting that membrane events could trigger the cellular responses to HPODE. A study by Suc et al. (38) has shown that oxidized lipid end products such as 4-hydroxynonenal could induce direct activation of the epidermal growth factor (EGF) receptor. We can speculate that 13-HPODE could act through a plasma membrane target (which remains to be identified) to trigger a transductional signal leading to the induction of catalase.

The induction of catalase by an oxidative stress could be an important finding if it is applicable to vascular cells in vivo because H_2O_2 mediates various cellular responses, potentially atherogenic. Previous studies have shown that a direct stimulation by H_2O_2 or indirectly by its intracellular production could activate various cellular pathways, including calcium release, tyrosine kinases, mitogen-activated protein kinases (MAPK), or NF-κB (39–42), and a subsequent increase in some gene products: Fas, macrophage colony-stimulating factor, and intercellular adhesion molecule 1 (40, 42, 43). Thus the generation of H_2O_2 could play a key role in the cellular proatherogenic effects of ox-

idized lipids. Our previous studies have shown that cells overexpressing catalase gene are resistant not only to the actions of H_2O_2 but also to those of lipid peroxides (8).

In vivo evidence also suggests that antioxidant enzymes including catalase could be regulated in the arterial wall under pathologic circumstances. For example, increased glutathione peroxidase, Cu-Zn SOD, and catalase have been noted in experimental atherosclerosis (44), suggesting that oxidative stress occurring in the subintimal area could induce an antioxidant response from vascular cells. Whether it is myeloperoxidase- or metal-catalyzed oxidation that is responsible for the oxidation of LDL in the arterial intima, H_2O_2 is an essential component, and thus, the induction of catalase by lipid peroxides before the development of the lesions could be an important protective mechanism preventing intimal oxidation of LDL.

The mechanism of induction of catalase gene expression by oxidized lipids can only be speculated at present. Studies have indicated that oxidized lipids interact with the receptors for peroxisomal proliferators (25). This could suggest that oxidized lipids might specifically influence components associated with the proliferation of peroxisomes. Catalase is a peroxisomal enzyme and innumerable studies have documented its activation by peroxisomal proliferators, such as clofibrate and other fibrates (45, 46). It remains to be established whether other enzymes associated with the peroxisomes are also activated by oxidized lipids. On the other hand, lipid peroxides also induce the expression of nitric oxide synthase, heme oxygenase, Mn-SOD, and so on (9–11). These enzymes are not associated with peroxisomes and it is not currently known whether peroxisome proliferator-activated receptor (PPAR) activators induce those enzymes. More importantly, no PPAR response element has been reported in the promoting region of the catalase gene.

In summary, catalase could be a key enzyme in the protection of cells against oxidative injury and induction of catalase by oxidative stress could be an important cellular defense. While it is premature to suggest that the mechanisms of action of fibrates could also include the activation of catalase, our results suggest that catalase could certainly be an important cellular defense against oxidative stress. The protective role of catalase against atherosclerosis remains to be established.

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