Overexpression of Human Catalase Gene Decreases Oxidized Lipid-Induced Cytotoxicity in Vascular Smooth Muscle Cells

Nalini Santanam, Nathalie Augé, Mimi Zhou, Channa Keshava, Sampath Parthasarathy

Abstract—Reactive oxygen metabolites such as hydrogen peroxide (H$_2$O$_2$) and oxidized fatty acids are proinflammatory and are involved in the pathophysiology of many diseases including atherosclerosis. The effects of these oxidants could be inhibited by the external addition of an antioxidant, suggesting the promotion or propagation of further oxidation. In this study, we describe the stable overexpression of human catalase in smooth muscle cells and the resistance of these cells to cytotoxicity induced not only by the addition of H$_2$O$_2$ but also by the addition of 13-hydroperoxyoctadecadienoic acid (13-HPDOE). The results pose an intriguing possibility of the generation of H$_2$O$_2$. To explain the observed results by which catalase could overcome the effects of 13-HPDOE, we propose that oxidized fatty acids are degraded in the cellular peroxisomes, resulting in the generation of H$_2$O$_2$. 

Key Words: atherosclerosis ■ 13-HPDOE ■ peroxisomes ■ hydrogen peroxide ■ β-oxidation

Disruption of the delicate balance between pro-oxidants and antioxidants has been implicated in the pathophysiology of many chronic diseases, such as atherosclerosis. A plethora of information suggests that oxidants, such as hydrogen peroxide (H$_2$O$_2$) and lipid peroxide (LOOH), induce a number of proatherogenic changes in cell types that are present in the atherosclerotic artery. Most of these effects could be prevented by the addition of extracellular antioxidants, suggesting further propagation of oxidation. Studies that used the manipulation of genes for antioxidant enzymes to test the effects of oxidants on cells also corroborate the assumption that intracellular oxidative stress might play an important role in the transduction of oxidative stress from external sources to intracellular sites. Previously, Shingu et al showed that endothelial cells and smooth muscle cells have very low levels of catalase activity and therefore are more susceptible to damage by H$_2$O$_2$. Several studies have attempted to overexpress catalase enzyme in various cell types and looked for the effect of this enzyme in preventing oxidation-related damage. Yamada et al identified 2 H$_2$O$_2$-resistant cell lines, HP50-2 and HP100-1, which were more resistant to the toxic effects of H$_2$O$_2$ than the parent cell line HL60. They found 3 to 18 times, respectively, higher catalase activity in these cell lines. Transient overexpression of catalase in human umbilical vein endothelial cells and acatalasemic murine fibroblast SV-B2 cells conferred protection on these cells from H$_2$O$_2$-mediated oxidant stress.

Interestingly, many of the effects of peroxidized fatty acids and H$_2$O$_2$ can be mimicked by hydroxy derivatives of fatty acids, such as hydroxy linoleic acids (HODEs) and hydroxy arachidonic acids (HETEs). Considering the finding of Gordon and associates that peroxidized arachidonic acids are degraded in cellular peroxisomes, and on the basis of the fact that peroxisomal degradation of fatty acids, in contrast to their mitochondrial degradation, generates H$_2$O$_2$, we speculated whether the cellular effects of oxidized lipids are mediated via the production of H$_2$O$_2$. To test this hypothesis, we generated smooth muscle cells that overexpressed human catalase in a stable manner. We measured the resistance of these cells to cytotoxicity induced not only by the addition of H$_2$O$_2$ but also by the addition of 13-hydroperoxyoctadecadienoic acid (13-HPDOE). The results are novel as we demonstrate that the cells enriched in catalase are resistant to the damaging effects of an oxidized lipid. A novel scheme is proposed to explain the observed results by which catalase could overcome the effects of 13-HPDOE.

Methods

Human catalase cDNA was a generous gift from Dr G.T. Mullenhbach (Chiron Corporation). Minimal essential medium (MEM), penicillin, streptomycin, glutamine, trypsin, HBSS, and FCS were purchased from Cellgro Mediatech Co. Linoleic acid, H$_2$O$_2$, human kidney catalase, 3-(4,5-dimethyl-thiazol-2-yl) 2.5-diphenyl tetrazolium bromide (MTT), 2′-7′-dichlorofluorescein diacetate (DCF-DA), and soybean lipoxigenase were all obtained from Sigma Chemical Co. Rabbit polyclonal anti-human erythrocyte catalase

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org
antibody was obtained from Athens Research and Technology. [3H]thymidine (1 mCi/mmol) was obtained from Amersham. 13-Hydroxyoctadecadienoic acid (13-HODE) was obtained from Cayman Chemicals.

Cell Culture
Rabbit femoral artery smooth muscle cells (RASMCs) (American Tissue Cell Culture) were routinely cultured in MEM supplemented with 10% FCS, 1% l-glutamine, and 1% penicillin/streptomycin. For transfection studies cells were grown in T-75 cell culture flasks until 60% to 80% confluence. For other studies the cells are cultured in 6-well or 24-well dishes. Cells are routinely passaged using trypsin/EDTA method.

Plasmid Construction
An entire 2.3-kilobase (kb) cDNA clone of the human catalase cDNA containing the entire coding region of human catalase was isolated from pBluescript II SK+(pBS-Cat) through digestion of the unique XbaI and SalI site on pBluescript II. This plasmid contains the entire coding sequence of the human catalase cDNA.

Construction of the Expression Vector
The mammalian expression vector used in our study was the pCI-Neo mammalian expression vector (catalog No. E1841, Promega), which carries the human cytomegalovirus (CMV) immediate early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells. The pCI-Neo vector contains the neomycin phosphotransferase gene as the selectable marker for mammalian cells. The XbaI- and SalI-digested 2.3-kb catalase plasmid was inserted into the XbaI and SalI site of the pCI-Neo vector, 3′ to the promoter and 5′ to the poly A signals. The resulting clone of human catalase cDNA in the pCI-Neo vector (pCI-Neo-Cat) was used for transfection studies.

Transfection of Catalase cDNA in RASMCs
RASMCs were plated at a density of 3 to 5×10⁴ cells/100-mm cell culture dish in MEM supplemented with 10% FCS. After 24 hours of incubation, the cells were transfected by the calcium phosphate precipitate method using the Profection Mammalian Transfection Systems kit available from Promega (catalog No. E1200) and used according to the manufacturer’s instructions. The pCI-Neo-Cat vector was transfected into the cells. Transfecting only the pCI-Neo expression vector generated control clones of cells. Approximately 48 hours after transfection, the cells were trypsinized and replated at a 1:4 dilution. The antibiotic G418 (Geneticin; Gibco) was added at 400 μg/mL the following day. G418-resistant colonies were isolated approximately 2 to 3 weeks after transfection by trypsinization. Selected clones were grown to mass culture for further analysis. At least 4 separate clones were used in these studies.

Catalase Enzyme Assay
Cells were cultured to confluence in 100-mm dishes. Cellular lysate was prepared by sonication of suspensions of transfected cells in PBS, pH 7.4, containing 0.05% deoxycholate. This suspension was centrifuged at 40,000g for 20 minutes at 4°C to remove unlysed cells and organelles. Catalase activity in the transfected and untransfected control RASMCs were quantified by the method of Aebi, in which H₂O₂ was reacted with the cell lysates (obtained as described above). The initial rate of disappearance of H₂O₂ (0 to 60 seconds) was recorded spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was defined as the rate constant of the first-order reaction using purified human erythrocyte catalase (Sigma) as a standard. Activity units for catalase were expressed as units per milligram of cell lysate protein. Protein was measured using the Lowry method using BSA as the standard.

DNA Isolation and Southern Blot Analysis
Genomic DNA from the untransfected smooth muscle cells, the vector alone-transfected cells, and the catalase-transfected cells was isolated as described by Sambrook et al. About 10 μg of genomic DNA was digested with restriction endonucleases XhoI and SalI. The digested DNA was then run on a 0.8% agarose gel and transferred onto nylon membrane following the Genius System user’s guide (Boehringer Mannheim). The membrane was then hybridized with digoxigen-labeled catalase cDNA overnight at 62°C. Colorimetric detection with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate were used to detect the hybridized signals.

Western Blot Analysis
Cell lysate at a protein concentration of 10 to 15 μg was separated on a 10% SDS acrylamide electrophoresis. The gel was then blotted onto a nitrocellulose membrane using the Bio-Rad blot system. After blotting, the membrane was first blocked with 5% milk powder in TBST (Tris base, saline, and Tween-20) overnight, and then incubated with the primary antibody (rabbit polyclonal anti-human catalase antibody, 1:250 dilution) for 1 hour, washed, and incubated with secondary antibody anti-rabbit IgG conjugated to horseradish peroxidase (1:1500) for 1 hour. After washing, the signal was detected using the chemiluminescence kit available from Amersham, (ECL kit). The intensity of the band was quantified using a densitometer.

Preparation of 13-HPODE
Stock linoleic acid was prepared in absolute ethanol. The linoleic acid was oxidized to 13-HPODE with immobobilized soybean lipoygenase (100 U/mL) at 37°C for 1 hour. The formation of 13-HPODE was monitored spectrophotometrically by scanning the absorption between 200 and 300 nm (model DB-3500; SLM-AMINCO) using PBS as reference. Under these conditions, the conversion into 13-HPODE is observed as an increase in absorbance at the optical density of 234 nm. Usually, >90% conversion of linoleic acid to 13-HPODE was achieved as determined by the molar extinction coefficient of the conjugated dienes with TLC, HPLC, or the leucumethylene blue (LMB) assay. The LMB assay, which determines the actual peroxide content, usually provided peroxide content of 90% to 94%.

[3H]Thymidine Method
Normally, cells (1×10⁵ cells/well) were grown in a 24-well plate to subconfluence in medium containing 10% FCS for 24 hours. Cells were washed, shifted to medium containing 0.5% serum, and cultured for 24 hours to synchronize growth. H₂O₂ (100 μmol/L) and 13-HPODE (25 μmol/L) were added, and the cells were further incubated in serum-free medium for 24 hours in the presence of 1 μCi of [3H]thymidine. Control cells without any oxidant were also run simultaneously. At the end of 24 hours, cells were washed and the incorporation of [3H]thymidine radioactivity into the cellular DNA was determined. The cells were washed with cold HBSS followed by fixing with 5% trichloroacetic acid to remove unbound radioactivity. The cells were then solubilized in lysis buffer (0.1% sodium carbonate, 1N sodium hydroxide). The radioactivity in the solubilized cells was then quantified after the addition of scintillation cocktail, Ecolume (ICN), in a beta-scintillation counter (Beckman LS 6500).

Cell Viability Assay Using MTT
The MTT assay was used additionally to measure cell viability. The principle of this assay is that the compound MTT (thiazolyl blue) undergoes cellular reduction by the mitochondrial dehydrogenase of viable cells into a blue formazan, which can be measured spectrophotometrically. Briefly, cells were grown in a 24-well plate and incubated with various concentrations of the oxidants (0 to 200 μmol/L H₂O₂ and 1 to 100 μmol/L 13-HPODE) for 24 hours. At the end of incubation, 0.1 mg (50 μL of 2 mg/mL) of MTT was added to each well and incubated at 37°C for an additional 2 hours. After 2 hours, the media were removed carefully, so as not to disturb the formazan crystals formed. Mineral oil or DMSO, which is used to solubilize the formazan crystals, was added to each well, and the plate was allowed to stand overnight at 4°C. The solubilized blue formazan in the mineral oil or DMSO was quantified using a spectrophotometer at a wavelength of 540 nm. There is a linear relationship between the formazan generated and the number of.
Confocal Microscopy for Intracellular H$_2$O$_2$ Measurement

Cells were grown in complete media (medium containing 10% FCS and growth supplements) to 80% confluence in 6-well plates. The media was replaced with serum-free medium and grown for another 24 hours. The quiescent cells were then treated with or without 25 μmol/L 13-HPODE or 13-HODE for 24 hours. The cells were washed thrice with HBSS. Five milliliters of HBSS was added to all the cells followed by 8 mL of 5 μmol/L DCF-DA solution, and cells were incubated for 30 to 40 minutes in the dark. At the end of incubation, the fluorescence intensity produced by the oxidation of DCF-DA by H$_2$O$_2$ or other oxidants was measured by laser confocal microscopy (MRC-1000, Bio-Rad). The excitation wavelength used was 488 nm. The value for fluorescence intensity is obtained from 8 recordings of 4 separate visual fields for each point. The sensitivity of the instrument was calibrated using exogenously added H$_2$O$_2$ and ranged between 10 and 100 nmol/mL.

N-Benzoyl LMB Method for Peroxide Detection

This method measures the amount of free lipid peroxides present in the system. It is a very common method to measure peroxides such as H$_2$O$_2$ and other lipid peroxides. Different concentrations of 13-HPODE (5 to 25 μmol/L) were incubated alone or with 100 or 200 μU of human catalase at 37°C for 1 hour. After incubation, 40 μL of the sample was put in each well of a 96-well microtiter plate. The plate was incubated with 100 μL of N-benzoyl LMB color reagent (5 mg LMB in dimethylformamide, 0.05 mol/L potassium phosphate buffer, pH 5.0, 1.4 g Triton X-100, and 5.5 mg hemoglobin). After 5 minutes at room temperature, the sample was read at 660 nm in a microtiter plate reader.

Results

Catalase Protein and Enzyme Activity in Transfected RASMC Cells

Catalase enzyme activity was measured in cell homogenates using H$_2$O$_2$ as the substrate by the method of Aeberi. As seen in Figure 1, the enzyme activity of the transfected cells (pCI-Neo-Cat) was nearly 4- to 6-fold higher as compared with the cells transfected with the vector alone or untransfected cells.

Western blot analysis of the untransfected and transfected cells using rabbit anti-human erythrocyte catalase IgG fraction as the primary antibody was performed. Human catalase is a 240-kDa tetramer protein. As seen in Figure 1 (inset), pCI-Neo-Cat cells had approximately 4-fold higher intensity 60-kDa band (monomer) compared with the untransfected and the vector alone-transfected cells.

Stable Integration of Human Catalase cDNA in the Transfected RASMCs

Genomic DNA was isolated from both untransfected and transfected cells and a Southern blot analysis was performed using a nonradioactive method of detecting the nucleotides. The primers used for probing the genomic DNA were prepared by digesting the pCI-Neo expression vector containing the entire 2.3-kb human catalase cDNA with XbaI and SstI. This digestion results in a 5.5-kb fraction of the pCI-Neo vector and a 2.2-kb fraction of the human catalase cDNA. The Southern blotting (Figure 2) showed both the 5.5-kb and the 2.2-kb fractions, thereby confirming the stable integration of the human catalase cDNA in the pCI-Neo-Cat-transfected cells. Cells transfected with the vector alone showed only the 5.5-kb fraction. There was no detectable band in the untransfected cells even though a faint band corresponding to the endogenous catalase cDNA was seen in all 3 cell types.

Effect of H$_2$O$_2$ on Catalase-Transfected Cells

To establish whether the catalase overexpressors could prevent the injury caused by H$_2$O$_2$, [H]$^3$H]thymidine incorporation was performed. The catalase-transfected and untransfected cells were exposed to 100 μmol/L H$_2$O$_2$ for 24 hours, and cell proliferation was measured as an index of [H]$^3$H]thymidine incorporation. There was at least 36% incorporation in the catalase-transfected cells compared with the vector alone-transfected cells (10%) and untransfected cells (18%) as seen in Figure 3A. To measure whether the decreased cell proliferation was caused by cytotoxicity, the cells were incubated with increasing concentrations of H$_2$O$_2$, and the viability was measured using H$_2$O$_2$ as the substrate at optical density of 240 nm. One unit of catalase activity was defined as the rate constant of the first-order reaction using purified human erythrocyte catalase as a standard. Activity units for catalase were expressed as units per milligram of cell lysate protein. Inset, Ten to fifteen micrograms of the cell lysate protein was separated on a 10% SDS acrylamide electrophoresis. The gel was transblotted onto a nitrocellulose membrane. Western blotting using rabbit polyclonal anti-human catalase antibody (1:250 dilution) identified the catalase protein using chemiluminescence kit. Cont indicates untransfected control cells; Neo, vector alone-transfected cells; and Cat, catalase-transfected cells.

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and catalase-transfected smooth muscle cells were grown in 24-well plates. H$_2$O$_2$ (100 μmol/L) was added, and the cells were further incubated for 24 hours in serum-free medium in the presence of 1 μCi of $[^3$H]thymidine. Control cells without any oxidant were also run simultaneously. At the end of 24 hours, the incorporation of $[^3$H]thymidine radioactivity into the cellular DNA was determined. The results are expressed in percentage of the radioactivity of thymidine incorporated in untransfected cells grown in media containing 10% FCS. Each data point represents the mean±SD (n=5). B, Effect of H$_2$O$_2$ on the viability of catalase-transfected cells as measured by MTT assay. Sixty to eighty percent confluent untransfected and catalase-transfected smooth muscle cells were grown in 24-well plates. H$_2$O$_2$ (0 to 200 μmol/L) was added, and the cells were further incubated for 24 hours in serum-free medium. The cell viability was measured using MTT as described in the Methods section. The results are expressed in percentage of cells viable in untransfected cells grown in media containing 10% FCS. Each data point represents the mean±SD (n=5). Curve 1 is untransfected control cells; curve 2, vector alone-transfected cells; and curve 3, catalase-transfected cells. Abbreviations are as in Figure 1.

cellular DNA was determined. The results are expressed in percentage of the radioactivity of thymidine incorporated in untransfected cells grown in media containing 10% FCS. Each data point represents the mean±SD (n=5). B, Effect of H$_2$O$_2$ on the viability of catalase-transfected cells as measured by MTT assay. Sixty to eighty percent confluent untransfected and catalase-transfected smooth muscle cells were grown in 24-well plates. H$_2$O$_2$ (0 to 200 μmol/L) was added, and the cells were further incubated for 24 hours in serum-free medium. The cell viability was measured using MTT as described in the Methods section. The results are expressed in percentage of cells viable in untransfected cells grown in media containing 10% FCS. Each data point represents the mean±SD (n=5). Curve 1 is untransfected control cells; curve 2, vector alone-transfected cells; and curve 3, catalase-transfected cells. Abbreviations are as in Figure 1.

Effect of In Vitro Addition of Catalase to 13-HPODE

To test whether the results obtained above can be because catalase could directly react with 13-HPODE and inactivate it, 13-HPODE at different concentrations was incubated with either 100 or 200 U of catalase and the amount of hydroperoxide produced was measured using the N-benzyol LMB method$^{27}$ at 660 nm. Figure 5 shows that there was no reduction of reactivity between 13-HPODE and the dye in the presence of catalase, suggesting that catalase does not directly interact with 13-HPODE. When HPODE was reduced to HODE (by pretreatment with Ebselen), as expected, there was no reaction with the LMB reagent (results not shown).

Varying concentrations of 13-HPODE (5 to 25 μmol/L) were incubated with 100 or 200 U of human catalase for 1 hour at 37°C in the presence of N-benzyol LMB reagent (see Methods) in vitro. The color developed was measured spectrophotometrically at 660 nm. Each data point represents the average (n=3). Open bars are control 13-HPODE; striped bars, 13-HPODE plus 100 U catalase; and solid bars, 13-HPODE plus 200 U catalase.
Catalase-transfected Cells

A number of different cell types respond to these agents. Cells scavenged the H$_2$O$_2$ produced. A number of different cell types respond to these agents.

13-HPODE. This suggests that catalase in the transfected clones indeed affords protection against H$_2$O$_2$-induced cytotoxicity. However, to our surprise, these cells were also resistant to the effects of 13-HPODE, a peroxidized fatty acid.

How do we explain these results? First, we considered the possibility that catalase might some how react with and inactivate 13-HPODE. Stoichiometric interaction is unlikely, as it is not possible that such large amounts (μmol/L levels) of catalase protein are generated and secreted outside the cell or are present in the cell cytoplasm. However, our recent studies have shown that only as little as 3% to 10% of the added 13-HPODE is transported into the cells under these conditions, suggesting that the intracellular concentrations of 13-HPODE could have been substantially lower. Therefore, to test whether 13-HPODE could be directly inactivated by catalase, we tested the ability of 13-HPODE to react with N-benzoyl LMB$_{27}$ in the presence of catalase. The results shown in Figure 5 demonstrate that there was absolutely no reduction in the reactivity of the peroxides with the reagent regardless of the presence of catalase.

Second, we considered the possibility that cells could generate H$_2$O$_2$ from 13-HPODE. Pioneering studies by Gordon and coworkers have demonstrated that oxidized arachidonic acid derivatives are targets of peroxisomal degradation. Degradation of fatty acids in the peroxisomes would result in the generation of H$_2$O$_2$. To test this possibility, we incubated cells with 13-HPODE. Results presented in Figure 6 show that, indeed, cells incubated with 13-HPODE generated more fluorescence, relative intensity was calculated by random quantification of 4 different areas of each picture.

Confocal microscopy for detection of intracellular H$_2$O$_2$. Catalase-transfected and untransfected cells were incubated with 25 μmol/L 13-HPODE or 13-HODE for 24 hours. At the end of the incubation, cells were treated with DCF-DA, and the fluorescence was observed under a confocal microscope as described in the Methods. A, control cell; B, control cell + 25 μmol/L 13-HPODE; C, control + 25 μmol/L 13-HODE; D, vector alone-transfected cell + 25 μmol/L 13-HPODE; and E, catalase-transfected cell + 25 μmol/L 13-HPODE.

The differences in the vector only controls could be because of the sensitivity of the CMV promoter to oxidative stress, which could be reflected in the activities of endogenous catalase in these controls. The cells that were used were from the same batch as the cells that were used for measuring catalase protein and activities. We observed that clones that contained very high levels of catalase failed to grow.

**Decreased Production of Endogenous H$_2$O$_2$ in Catalase-Transfected Cells**

To establish whether the effect caused by 13-HPODE on smooth muscle cells was related to the production of intracellular H$_2$O$_2$, we incubated both transfected and untransfected cells with 25 μmol/L 13-HPODE and measured intracellular H$_2$O$_2$ using confocal microscopy. As seen in Figure 6A, control untransfected cells without any addition had very little signal. However, the untransfected (Figure 6B) and vector alone-transfected cells (Figure 6D) had a 2- to 4-fold increased fluorescence intensity (Table) compared with catalase-transfected cells (Figure 6E) in the presence of 13-HPODE. This suggests that catalase in the transfected cells scavenged the H$_2$O$_2$ produced.

13-HPODE may further get reduced to 13-HODE. We observed that when untransfected cells were incubated with 25 μmol/L 13-HODE, there was an increased production of H$_2$O$_2$ (Figure 6C) as seen under confocal microscope. This observation suggests that oxidized lipids can generate H$_2$O$_2$ intracellularly and catalase could scavenge this.

**Discussion**

Oxidized lipids and H$_2$O$_2$ profoundly affect cellular properties. A number of different cell types respond to these agents.
attributed to the formation of H$_2$O$_2$, and that H$_2$O$_2$ accumulated poorly in cells stably transfected with catalase. As pointed out in the preceding paragraph, it is unlikely that catalase directly inactivated 13-HPODE. These results, together with the finding that the transport of 13-HPODE into cells is markedly reduced, also suggest that 13-HPODE alone could not be responsible for the generation of intracellular fluorescence.

These results go on to explain a puzzling paradox. How do the hydroxy fatty acids such as the HODEs and HETEs affect cells and why would antioxidants prevent their effects? These lipids do not propagate oxidation even in the presence of metal ions. We speculated that their cellular effects also are likely to be mediated by H$_2$O$_2$, generated during their degradation in the peroxisomes. Accordingly, when we incubated cells with 13-HODE (a product that does not react with DCF-DA or LMB), intense fluorescence could be seen (Figure 6C), suggesting the formation of H$_2$O$_2$.

Our results provide a novel approach by which peroxidized lipid-induced cellular toxicity could be prevented. These results also suggest that cellular peroxisomes are an important target for gene induction and the prevention of metabolic toxicity.

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