Overexpression of Human Catalase Inhibits Proliferation and Promotes Apoptosis in Vascular Smooth Muscle Cells

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Abstract—The role of reactive oxygen species, such as superoxide anions (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), in modulating vascular smooth muscle cell proliferation and viability is controversial. To investigate the role of endogenously produced H$_2$O$_2$, rat aortic smooth muscle cells were infected with adenoviral vectors containing cDNA for human catalase (AdCat) or a control gene, $\beta$-galactosidase (AdLacZ). Infection with AdCat resulted in dose-dependent increases in intracellular catalase protein, which was predominantly localized to peroxisomes. After infection with 100 multiplicity of infection (MOI) of AdCat, cellular catalase activity was increased by 50- to 100-fold, and intracellular H$_2$O$_2$ concentration was reduced, as compared with control. Infection with AdCat reduced [H]thymidine uptake, an index of DNA synthesis, in cells maintained in medium supplemented with 2% serum (0.37±0.09 disintegrations per minute per cell [AdLacZ] versus 0.22±0.08 disintegrations per minute per cell [AdCat], P<0.05). Five days after infection with 100 MOI of AdCat, cell numbers were reduced as compared with noninfected or AdLacZ-infected cells (157780±8413 [AdCat], P<0.05 versus 233700±3032 [noninfected] or 222410±5332 [AdLacZ]). Furthermore, the number of apoptotic cells was increased 5-fold after infection with 100 MOI of AdCat as compared with control. Infection with AdCat resulted in induction of cyclooxygenase (COX)-2, and treatment with a COX-2 inhibitor overcame the AdCat-induced reduction in cell numbers. These findings indicate that overexpression of catalase inhibited smooth muscle proliferation while increasing the rate of apoptosis, possibly through a COX-2–dependent mechanism. Our results suggest that endogenously produced H$_2$O$_2$ importantly modulates survival and proliferation of vascular smooth muscle cells. (Circ Res. 1999;85:524-533.)

Key Words: catalase ■ apoptosis ■ vascular smooth muscle cell ■ cell proliferation ■ hydrogen peroxide

Proliferation of vascular smooth muscle cells is an important contributing factor in the pathophysiology of hypertension and atherosclerosis, as well as in coronary artery restenosis after angioplasty and stent placement. However, the factors that induce proliferation of vascular smooth muscle cells, which normally exist in the arterial wall in a state of quiescence, are unknown. Recently, it has been reported that reactive oxygen species, such as superoxide anions (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are capable of stimulating vascular smooth muscle cell proliferation. These oxidants were shown to be rapidly produced by smooth muscle cells after exposure to platelet-derived growth factor or angiotensin II, factors that stimulate smooth muscle cell growth. In addition, the production of reactive oxygen species in the blood vessel wall is enhanced in experimental models of hypercholesterolemia, hypertension, diabetes, and balloon injury to the coronary arteries. These oxidants are capable of stimulating vascular smooth muscle cell proliferation.

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induced apoptosis in vascular smooth muscle cells.\textsuperscript{1-3} The findings of the latter 2 studies are most likely not related simply to the amount of reactive oxygen species applied, given that even low concentrations of H\textsubscript{2}O\textsubscript{2} produced apoptosis rather than proliferation. Thus, the precise role of reactive oxygen species in smooth muscle cell proliferation and apoptosis remains to be definitively established.

In this study, we examined the role of endogenously produced H\textsubscript{2}O\textsubscript{2} in smooth muscle cell proliferation and apoptosis in rat aortic smooth muscle cells (RASMCs) by infecting the cells with an adenoviral vector encoding the gene for human catalase. We chose to examine H\textsubscript{2}O\textsubscript{2}, because the studies by Rao and Berk\textsuperscript{4} and Sundaresan et al\textsuperscript{5} specifically implicated this substance as an important factor in smooth muscle cell signal transduction. Adenovirus-mediated gene transfer was used, because this approach results in the overexpression of human catalase within the cells, thereby obviating concerns regarding the purity of the enzyme preparation or fluctuations in intracellular catalase activity caused by variability in the transport of catalase protein into the cells. Our results indicate that overexpression of catalase results in reduction in the intracellular H\textsubscript{2}O\textsubscript{2} concentration, inhibition of smooth muscle cell DNA synthesis and proliferation, and induction of apoptosis. These findings support the hypothesis that endogenously produced H\textsubscript{2}O\textsubscript{2} may play a fundamental role in smooth muscle cell proliferation and survival.

Materials and Methods

DMEM, MEM nonessential amino acid, MEM vitamin solution, Tween 20, tetramethoxypropane, and catalase were obtained from Sigma. FCS was purchased from HyClone Laboratories, and gentamicin was obtained from Schering Corp. M-199 medium, HEPES, trypsin, and L-glutamine were obtained from Sigma and prepared for use by the University of Iowa Cancer Center. 2'-7'-Dichlorodihydroflurorescin diacetate (DCPH-DA), 5- (and 6)-carboxyl-2'-7'-dichlorodihydroflurorescin diacetate (carboxyl-DCFH-DA), and dihydroethidium (HE) were obtained from Molecular Probes. Rabbit anti-human erythrocyte catalase (IgG fraction) was purchased from Athens Research and Technology, Inc, and rabbit polyclonal anti-cyclooxygenase (COX)-2 antibody was purchased from Cayman Chemical Co. The enhanced chemiluminescence Western blotting analysis system was purchased from Amersham Life Science, and the in situ apoptosis detection kit was purchased from Oncor.

Cell Culture

RASMCs were prepared from 3-month-old Sprague-Dawley rats as previously described.\textsuperscript{13} The cells, which stained positively for \textalpha -actin but did not take up acetylated low-density lipoprotein, were grown in DMEM supplemented with 10% FCS, MEM nonessential amino acids, MEM vitamin solution, 2 mM L-glutamate, 50 \textmu M L-glutamine, and 15 \textmu M L-HEPES, in a humidified atmosphere containing 5% CO\textsubscript{2} at 37°C. Stocks were subcultured at subconfluence by trypsinization. All experiments were performed on cells between passages 8 and 20 grown to 90% to 95% confluence in 12-well plates, T-150 flasks, or 100-mm\textsuperscript{2} dishes.

Infection of Smooth Muscle Cells With Adenoviral Vectors Containing cDNA for Human Catalase

Initially, cDNA constructs coding for human catalase were generated by PCR from pCAT41 (American Type Culture Collection) with catalase-specific primers harboring restriction sites for direct cloning into the adenoviral construct, pAdCMV link, containing the cytomegalovirus promoter/enhancer element and simian virus 40 polyadenylation signal. PCR-generated clones were screened for catalase activity by transient infection before generation of adenoviral constructs. Recombinant viruses were generated by coinfection of NheI-cut pAd plasmid with ClaI-cut Ads.dl7001 (E3 deleted) viral DNA into 293 cells, which provide the E1A gene product necessary for viral replication during transfer. After infection, plates were overlaid with agar, and initial plaques were harvested, amplified, and screened for enzymatic activity. Adenovirus harboring the LacZ gene (AdLacZ) coding for the bacterial enzyme \beta-galactosidase was used as a control.\textsuperscript{16}

Virus possessing catalase enzymatic activity (designated AdCat) was plaque purified 3 times and amplified in 293 cells. Purified high-titer stocks of recombinant adenovirus were generated by 2 sequential rounds of CsCl banding. Viral titers were checked by plaque assays on 293 cells and demonstrated a particle:plaque-forming unit ratio of \( \sim 20:1 \).

In initial studies, subconfluent smooth muscle cells were incubated with 100 multiplicity of infection (MOI) of AdLacZ or vehicle (3% sucrose in PBS solution) in serum-free DMEM. After 3 hours, the medium was replaced with 2% FCS-DMEM, and the incubation was continued for an additional 22 to 45 hours. The efficiency of gene transfer was then examined by histochemical staining for \beta-galactosidase, as described previously.\textsuperscript{16} Under these conditions, only 3\%\% (n=6) of the AdLacZ-infected cells stained positively for \beta-galactosidase, suggesting a low efficiency of gene transfer. In subsequent incubations, the adenovirus was premixed with a cationic polymer (poly-L-lysine, 250 molecules/adenoviral particle), which was recently reported by Fasbender et al\textsuperscript{17} to improve the efficiency of adenovirus-mediated gene transfer. Forty-eight hours after exposure to 100 MOI of AdLacZ premixed with poly-L-lysine, 49\%\% (n=4) of the cells stained positively for \beta-galactosidase, indicating marked improvement in gene transfer efficiency. When cells were infected with 300 MOI of AdLacZ premixed with poly-L-lysine, \textgtrsim 95% of the cells stained positively for \beta-galactosidase. Consequently, all incubations (including vehicle controls) described in this study were conducted in the presence of poly-L-lysine.

Western Blot Analysis

Cells were infected with adenovirus as described above and then sonicated in 0.05 mol/L potassium phosphate buffer (pH 7.8) on ice with three 30-second bursts, using a Vibra Cell sonicator (Sonics and Materials, Inc) at 10% output and 80% duty cycle. Protein concentrations were measured using a Bio-Rad protein assay with BSA as the standard. Samples were denatured with SDS loading buffer at 95°C for 5 minutes and then separated on an SDS-12% polyacrylamide gel with a 4% stacking gel in SDS-Tris-glycine running buffer. The protein was electrophoretically transferred to a nitrocellulose membrane, which was then blocked with 5% (wt/vol) nonfat milk in TTBS buffer (0.02 mol/L Tris:0.15 mol/L NaCl buffer [pH 7.45] and 0.1% Tween 20) for 1 hour at room temperature on an orbital shaker. The membrane was then incubated with specific rabbit anti-human catalase (1:1000) in TTBS buffer overnight at 3°C to 7°C. The use of this antisera, which does not cross-react with other antioxidant enzymes, has been described previously.\textsuperscript{18} The blot was incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:10 000, Boehringer Mannheim) for 1 hour at room temperature. The anti-catalase antibody was then detected using the enhanced chemiluminescence detection system and exposed to x-ray film. Relative areas of each blot were determined by densitometry.

In other experiments, cells were infected with adenoviral vectors as described above, and 48 hours later, the protein was harvested and subjected to Western blotting using an antibody to COX-2 (1:1000), as described previously.\textsuperscript{19}

Immunohistochemical Localization of Catalase Protein

After adenoviral infection, cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.2, for 30 minutes at room temperature. The cells were then incubated with rabbit anti-human catalase (1:200 dilution in PBS/0.5% Triton X-100).
Multiplicity of Infection

Figure 1. Effects of AdCat infection on expression of catalase protein. RASMCs grown to subconfluence in T-75 flasks were infected with 30 to 300 MOI of AdLacZ (left) or AdCat (right) in serum-free DMEM. Uninfected cells (0 MOI) were included as controls. After 3 hours, the medium was removed, 2% FCS-DMEM was added, and the incubation was continued for an additional 45 hours. Medium was then removed, and cell lysates were prepared and subjected to Western blot analysis using an antibody specific to human catalase. The same amount of protein (10 μg) was applied to each lane. Numbers shown at the bottom of each blot are the relative areas (determined by densitometry), and numbers shown beneath the arrows refer to MOI.

Determination of Antioxidant Enzyme Activity

Cell extracts were prepared by sonication, and protein determination was performed as described above. Catalase activity was measured as described previously. Briefly, cell extracts (200 to 400 μg) were added to 3 mL of 10 mmol/L H₂O₂ in 50 mmol/L potassium phosphate buffer (pH 7.8), and disappearance of H₂O₂ was immediately measured at 240 nm for 30 seconds. Catalase activity was expressed in k units.

Assessment of Intracellular Reactive Oxygen Species

Intracellular generation of reactive oxygen species was detected using the oxidant-sensitive probes DCFH-DA and HE, and the oxidant-insensitive analog of DCFH-DA, carboxyl-DCFH-DA. DCFH-DA is distributed throughout the cell and fluoresces green when oxidized by H₂O₂, whereas HE localizes to the nucleus and fluoresces red when oxidized by O₂⁻. Simultaneous localization of both oxidized dyes within a cell produces an orange to yellow fluorescence. In contrast, the fluorescence of carboxyl-DCFH-DA is unaffected by H₂O₂ or O₂⁻. DCFH-DA and HE are not absolutely specific for a single substrate, but they represent the best available reagents for measuring intracellular reactive oxygen species. Cells were grown to subconfluence in 100-mm² dishes and infected with adenoviral vectors as described previously. Forty-eight hours later, the cells were washed and incubated for 30 minutes with HE (5 μmol/L) and DCFH-DA (10 μmol/L), and after the cells were rinsed, fluorescence was detected by confocal laser scanning microscopy. Excitation and emission wavelengths were 488 and 525 nm, respectively, for DCFH-DA and 488 and 610, respectively, for HE. Images were collected and analyzed using the Confocal Assistant program, as described above. In separate experiments, infected cells were prepared as described above, incubated with 10 μmol/L DCFH-DA or carboxyl-DCFH-DA, and analyzed by confocal laser scanning microscopy, using excitation and emission wavelengths of 488 and 525 nm, respectively.

Assessment of DNA Synthesis and Cell Numbers

To examine cellular DNA synthesis, near-confluent RASMCs were placed in DMEM supplemented with 2% FCS for 24 hours, after which they were infected with various MOI of AdCat or AdLacZ for 3 hours followed by washing and incubation in 2% FCS-DMEM. After 45 hours, the medium was replaced with fresh serum-free medium or medium containing 2% FCS; 24 hours later, [³H]thymidine was added, and the incubation was continued for an additional 5 hours. This medium was removed, and the cells were washed with cold PBS, incubated in 20% trichloracetic acid for 30 minutes, and then washed and incubated in 0.25N NaOH for 12 hours. The cells occupied by fluorescent cells.
were then lysed by vortexing and analyzed for radioactivity by liquid scintillation counting. All experiments were performed at least 2 times in triplicate in 12-well plates, and the thymidine uptake data are expressed as disintegrations per minute per cell.

Cell numbers were obtained in experiments performed as described above, with the exception that after gene transfer, cells were incubated in medium containing 0%, 2%, or 4% FCS, which was replaced with fresh medium every other day. At the indicated times, the cells were harvested by trypsinization and counted in a hemocytometer.

**Determination of Apoptosis**

The terminal deoxyribonucleotidyl transferase–mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay for detecting DNA fragmentation was performed using a commercially available kit (ApopTag Plus, Oncor). Briefly, the samples were preincubated with equilibration buffer for 5 minutes and subsequently incubated with terminal deoxyribonucleotidyl transferase in the presence of digoxigenin-conjugated dUTP for 1 hour at 37°C. The reaction was terminated by incubating the samples in stopping buffer for 30 minutes. After 3 rinses with PBS, the fluorescein-labeled antidigoxigenin antibody was applied for 30 minutes, and the samples were rinsed 3 times with PBS. The samples were then stained, mounted with Oncor propidium iodide/antifade, and examined by laser confocal microscopy.

The presence of DNA laddering was determined by agarose gel electrophoresis, as modified according to Rosl. After adenoviral infection of RASMCs, DNA was extracted from the cells by DNAzol (GIBCO-BRL). One microgram of DNA was treated with 5 units of Klenow polymerase using 0.5 μCi of [α-32P]dCTP in the presence of 10 mmol/L Tris:HCl, pH 7.5, and 5 mmol/L MgCl2. The reaction was incubated for 10 minutes at room temperature and terminated by incubating the samples in stopping buffer for 30 minutes. After 3 rinses with PBS, the fluoroscence-labeled antidigoxigenin antibody was applied for 30 minutes, and the samples were rinsed 3 times with PBS. The samples were then stained, mounted with Oncor propidium iodide/antifade, and examined by laser confocal microscopy.

**Statistical Analyses**

All data are expressed as mean±SEM. Differences between mean values of 2 groups were analyzed by Student t tests. Differences between mean values of multiple groups were analyzed by 1-way ANOVA with a Newman-Keuls post hoc analysis. Probability values of 0.05 or less were considered to be statistically significant.
findings suggest that the levels of transduced catalase remained relatively constant for at least 7 days after adenoviral infection.

To confirm that the transduced catalase protein was functional, lysates prepared from control or AdLacZ- or AdCat-infected cells were assayed for catalase activity. Cells infected with 100 MOI of AdCat exhibited 50- to 100-fold increases in total cellular catalase activity as compared with noninfected cells or cells infected with 100 MOI of AdLacZ (42.070 ± 16.119 k/g protein [AdCat]; P < 0.05 versus 318.57 ± 57 k/g protein [noninfected] or 783 ± 17 k/gm protein [AdLacZ]), n = 3 to 4 per group). Infection with 300 MOI of AdCat resulted in profound reductions in cell numbers (see below); consequently, we were unable to harvest sufficient amounts of protein from these cells to perform the catalase activity assay. Taken together, these results suggest that infection with AdCat greatly increased the amount of functional catalase protein contained within RASMC peroxisomes.

Effects of Catalase Overexpression on Intracellular Reactive Oxygen Species

We next investigated whether overexpression of catalase reduced the intracellular concentration of reactive oxygen species. Reactive oxygen species were compared among the 3 groups (noninfected cells and cells infected with either 100 MOI of AdLacZ or AdCat) using the oxidant-sensitive probes DCFH-DA and HE. Figure 4 shows that the green fluorescence of DCFH-DA was markedly reduced by infection with 100 MOI of AdCat (right panel) as compared with 100 MOI of AdLacZ (middle panel) or noninfected cells (left panel), suggesting that overexpression of catalase reduced the intracellular H$_2$O$_2$ concentration (Table). In contrast, infection with AdCat did not affect the red fluorescence of HE, suggesting that catalase transduction did not significantly alter the intracellular O$_2^{-}$ concentration (Table). Moreover, fluorescence of carboxyl–DCFH-DA was similar in AdCat- and AdLacZ-infected cells, suggesting that infection with AdCat did not reduce fluorescence of DCFH-DA by altering uptake, metabolism, or efflux of the probe (data not shown).

Effects of Catalase Overexpression on Cellular Proliferation

To determine whether infection with AdCat inhibited proliferation of RASMCs, cells were infected with 100 MOI of AdLacZ or AdCat and incubated in 2% FCS-DMEM for 48 hours. The medium was then replaced with fresh serum-free medium or medium containing 2% FCS, and [3H]thymidine uptake was determined. Cells infected with AdCat, whether maintained in serum-free DMEM (Figure 5, top) or 2% FCS-DMEM (Figure 5, bottom), exhibited reduced [3H]thymidine uptake as compared with noninfected or AdLacZ-infected cells, indicating a decreased rate of DNA synthesis.

In separate experiments, confluent cells were infected with 100 MOI of AdLacZ or AdCat as described above. These cells, as well as noninfected cells, were then placed in 2% FCS-DMEM for 2 or 5 days, after which they were harvested by trypsinization and counted. Two days after gene transfer, there were no differences in cell numbers among any of the groups (Figure 6, top). In noninfected or in AdLacZ-infected cells, the numbers of cells present 5 days after gene transfer were markedly increased over the corresponding 2-day values, indicating cellular proliferation. In AdCat-infected cells, the number of cells present 5 days after gene transfer was reduced compared with either noninfected or AdLacZ-infected cells.
infected cells, and, in fact, did not differ from the correspond-
ing 2-day value.

In additional experiments, cells were infected with 30 to
300 MOI of AdCat or, as a control, AdLacZ. After gene
transfer, the cells were maintained in 4% FCS-DMEM for 2
or 6 days, after which they were harvested and counted. Once
again, 2 days after gene transfer, there were no significant
differences in cell number among any of the groups (data not
shown). In the AdLacZ group, the number of cells present 6
days after gene transfer was more than twice the 2-day value
(Figure 6, bottom). At this time, cells infected with AdCat
exhibited dose-dependent reductions in cell numbers, with
only the 30-MOI value exceeding that of the 2-day AdLacZ
value. Taken together, these results suggest that overexpres-
sion of catalase inhibited RASMC proliferation.

Effects of Catalase Overexpression on Apoptosis

To test whether overexpression of catalase increased the
rate of apoptotic cell death in RASMCs, near-confluent
cells were infected with 100 MOI of either AdLacZ or
AdCat, or treated with vehicle, and then maintained in 2% FCS-
DMEM as described previously. After 48 hours, the
TUNEL assay was performed. The nuclei of noninfected
RASMCs (Figure 7A) and AdLacZ-infected RASMCs (Figure 7B)
typically were elliptically shaped and stained by propidium
iodine (red) but were not concomitantly labeled by TUNEL. However, condensed nuclei labeled by
TUNEL (green) were frequently observed in AdCat-
infected RASMCs (Figure 7C), consistent with enhanced
apoptosis. The number of TUNEL-labeled nuclei in

AdCat-infected cells was 5-fold greater than in AdLacZ-
infected or noninfected cells (Figure 7D).

In separate experiments, cells were infected with 300 MOI of
AdLacZ or AdCat and maintained in 2% FCS-DMEM for
48 hours, after which the TUNEL assay was performed. Under
these conditions, ~13% of the AdCat-infected cells were
TUNEL positive, as compared with 3% of the AdLacZ-
infected cells (Figure 8, top). To confirm the enhanced
apoptosis, agarose gel electrophoresis was used to examine
for DNA laddering, a sign of fragmentation of nuclear DNA
into oligonucleosomal subunits. Substantial DNA laddering
was detected in cells infected with AdCat, whereas little or no
laddering was detected in noninfected or AdLacZ-infected
cells (Figure 8, bottom).

Taken together, these results suggest that overexpression of
catalase resulted in an increased frequency of apoptotic cells
in populations of smooth muscle cells.

Effects of Gene Transfer of Catalase on
Expression of COX-2

Chen et al reported that incubation of RASMCs with
catalase protein resulted in rapid and sustained induction
of COX-2. To investigate whether COX-2 was also induced in
the RASMCs after gene transfer of catalase, noninfected cells

Figure 5. Effects of AdCat infection on RASMC DNA synthesis. RASMCs were grown to confluence in 6-well plates, infected
with 100 MOI of AdCat or AdLacZ, and then placed in 2% FCS-
DMEM as described in Figure 1. The medium was then replaced
with fresh serum-free medium (top) or medium containing 2% FCS (bottom), and [3H]thymidine uptake was determined by liq-
uid scintillation counting. Values are expressed as disintegra-
tions per minute per cell; n=6 per group. *P<0.05 vs nonin-
fected or AdLacZ-infected cells.

Figure 6. Effects of overexpression of catalase on cell numbers. Confluent RASMCs in 6-well plates were infected with vehicle or
100 MOI of AdLacZ or AdCat (top), or with 30 to 300 MOI of
AdLacZ or AdCat (bottom). Cells were incubated in medium
supplemented with 2% (top) or 4% serum (bottom), and cell
counts were obtained after 2 and 5 to 6 days after treatment, as
depicted. Values are expressed as mean±SEM; n=3 for each
group. AdLacZ values shown in the lower panel are from cells
infected with 300 MOI. Top, *P<0.05 vs corresponding 2-day
value; +P<0.05 vs 5-day AdCat value. Bottom, *P<0.05 vs
AdLacZ 2-day value.
or cells infected 100 MOI of AdLacZ or AdCat were maintained in 2% FCS-DMEM for 48 hours. Cell lysates were then prepared, and Western blotting was performed using a specific antibody for COX-2. In cells infected with AdCat, the expression of COX-2 protein was markedly increased as compared with noninfected cells or cells infected with AdLacZ (Figure 9, top).

To address whether induction of COX-2 might be involved in the suppression of cell proliferation after gene transfer of catalase, cells were pretreated with vehicle or a selective inhibitor of COX-2, NS-398,30 and then infected with 100 MOI of AdLacZ or AdCat. Cells were maintained in 2% FCS-DMEM in the continuous presence of vehicle or NS-398 for 7 days, after which they were harvested and counted. In AdLacZ-infected cells, treatment with NS-398 did not significantly alter cell numbers as compared with vehicle (Figure 9, bottom). However, treatment with NS-398 overcame the reduction in cell numbers subsequent to gene transfer of catalase. Similar results were obtained in a separate experiment performed as described above but continued for 5 days after gene transfer (AdLacZ, 194070±13950 [vehicle] versus 218 700±6160 [NS-398] cells/mm³, P>0.05; AdCat, 100 930±9530 [vehicle] versus 154 070±3910 [NS-398] cells/mm³, P<0.05).

**Discussion**

We have shown that infection of RASMCs with an adenoviral vector containing the cDNA for human catalase resulted in overexpression of catalase within cellular peroxisomes, reduction in the intracellular concentration of prooxidants (presumably H₂O₂), suppression of DNA synthesis and cell proliferation, and induction of apoptotic cell death. These findings suggest an important role for endogenously produced H₂O₂ in regulating vascular smooth muscle cell growth and apoptosis.

Reactive oxygen species participate in numerous intracellular signaling processes and have been suggested to promote tumor cell proliferation.31,32 Studies also indicate that O₂⁻- and H₂O₂ can stimulate proliferation of vascular smooth muscle cells.4 Furthermore, Sundaresan et al3 reported that in RASMCs, platelet-derived growth factor stimulated H₂O₂ production and DNA synthesis, and that both of these responses were blocked by preincubating the cells with catalase, which markedly increased intracellular catalase activity. This suggests that the levels of endogenous antioxidant enzymes could importantly influence the rate of vascular smooth muscle cell proliferation.

Using adenovirus-mediated gene transfer, we were able to express high levels of catalase within the peroxisomes, the site where intracellular catalase normally is located within cells.33 Moreover, immunofluorescence studies indicated that the levels of transduced catalase remained relatively constant for up to 7 days after adenoviral infection. The expressed catalase was functional, as demonstrated by the 50- to 100-fold increase in cellular catalase activity after infection with 100 MOI of AdCat. This magnitude of increase in catalase activity might, on first consideration, appear to be nonphysiological. However, catalase activity in cultured cells has been reported to be up to 50-fold less than in freshly harvested tissue.34,35 Thus, the levels of intracellular catalase...
achieved in the present study likely represent moderate, physiologically relevant increases compared with the levels ordinarily present in vivo.

Experiments with oxidant-sensitive probes suggested that overexpression of catalase led to a reduction in the intracellular H$_2$O$_2$ concentration but did not affect the concentration of O$_2^-$. The rate of cellular proliferation, determined by assessing [3H]thymidine incorporation and cell numbers, was reduced after gene transfer of catalase. These latter experiments were performed in confluent, unstimulated smooth muscle cells, as well as in cells maintained in 2% or 4% FCS-DMEM. Under these conditions, the rate of cell proliferation is low, similar to that which occurs in the arterial wall in vivo. Thus, our results support the hypothesis that intracellular H$_2$O$_2$ may play a fundamental role in regulating vascular smooth muscle cell proliferation.

Very recently, Chen et al. reported that incubation of smooth muscle cells with catalase resulted in rapid induction of COX-2 mRNA and protein expression and increased production of prostaglandins. In the present study, gene transfer of catalase also resulted in induction of COX-2 protein. There are a number of potential mechanisms by which the expression and/or activity of COX-2 could be enhanced by catalase. For example, by reducing ROS levels, catalase may increase the activity of adenosine-uridine binding factor, which, in turn, may promote stabilization of COX-2 mRNA. COX is prone to autoinactivation caused by formation of ROS during enzymatic activity, a process that could be abrogated by antioxidant enzymes. Moreover, catalase could enhance COX activity by supplying O$_2$, a substrate for COX-mediated endoperoxide synthesis. Enhanced induction and/or activity of COX-2 could modulate cell growth, perhaps by increasing the formation of PGE$_2$, a potent inhibitor of smooth muscle proliferation. In keeping with this possibility, treatment with a COX-2 inhibitor overcame the reduction in cell numbers subsequent to infection with AdCat. Further studies are required to establish the precise role of COX-2 in catalase-induced suppression of smooth muscle cell proliferation.

Besides reducing the rate of cell proliferation, infection with AdCat increased the rate of RASMC apoptosis.
cently, Tsai et al.\textsuperscript{12} reported that incubation of vascular smooth muscle cells with the antioxidant PDTC or NAC resulted in dose-dependent reductions in cell viability and enhancement in the rate of apoptosis, suggesting that endogenous reactive oxygen species may modulate survival of smooth muscle cells. Our study supports this notion and suggests a particularly important role for H\textsubscript{2}O\textsubscript{2}, given that the enhanced rate of apoptosis after catalase overexpression was observed in conjunction with a reduction in the intracellular concentration of H\textsubscript{2}O\textsubscript{2} but not O\textsubscript{2}\textsuperscript{•−}. The molecular mechanisms whereby H\textsubscript{2}O\textsubscript{2} prevents vascular smooth muscle cells from becoming apoptotic remain to be determined.

Interestingly, Rao and Berk\textsuperscript{4} reported that H\textsubscript{2}O\textsubscript{2} did not stimulate proliferation of endothelial cells, and Tsai et al.\textsuperscript{12} reported that the antioxidants PDTC and NAC did not reduce endothelial cell viability. Thus, in contrast to its actions in smooth muscle cells, H\textsubscript{2}O\textsubscript{2} does not appear to fundamentally regulate endothelial cell proliferation or survival. Antioxidant therapy might therefore be expected to selectively inhibit vascular smooth muscle cell proliferation without adversely affecting the endothelium. This would help to explain the beneficial effects of antioxidant treatment in preventing coronary restenosis after balloon angioplasty, a condition in which smooth muscle cell proliferation has been suggested to play an integral role.\textsuperscript{2,39}

While the findings of this study, as well as those by Rao and Berk,\textsuperscript{4} indicate that H\textsubscript{2}O\textsubscript{2} stimulates smooth muscle cell proliferation, reports by other groups indicate that H\textsubscript{2}O\textsubscript{2} can also induce apoptosis and/or promote vascular smooth muscle cell death.\textsuperscript{13,14,40} These apparently conflicting results could be related to differences in cell types, cell culture conditions, the amount or method of application of H\textsubscript{2}O\textsubscript{2}, the levels of endogenous antioxidant enzymes, or the rates of production of reactive oxygen species within the cells. Nevertheless, these studies collectively suggest that under certain conditions, H\textsubscript{2}O\textsubscript{2} can promote either vascular smooth muscle cell proliferation or cell death, a paradox that underscores the importance of this reactive oxygen species to smooth muscle cell function.

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