Adenoviral-mediated overexpression of catalase inhibits endothelial cell proliferation

MICHELA ZANETTI,1 ZVONIMIR S. KATUSIC,2 AND TIMOTHY O’BRIEN1

Departments of 1Endocrinology and 2Anesthesiology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

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Zanetti, Michela, Zvonimir S. Katusic, and Timothy O’Brien. Adenoviral-mediated overexpression of catalase inhibits endothelial cell proliferation. Am J Physiol Heart Circ Physiol 283: H2620–H2626, 2002; 10.1152/ajpheart.00358.2001.—Although hydrogen peroxide (H2O2) induces proliferation of vascular smooth muscle cells, its role in endothelial cell proliferation is unclear. Our aim was to study the role of hydrogen peroxide in endothelial cell proliferation by overexpressing catalase. Human aortic endothelial cells were transduced with adenoviral vectors encoding β-galactosidase (Adβgal) or catalase (AdCat) or were exposed to diluent alone (control). Transgene expression was demonstrated by β-galactosidase staining, Western analysis, and significantly increased enzyme activity in AdCat-transduced cells. Overexpression of catalase decreased DNA synthesis in AdCat compared with control and Adβgal-transduced cells (536.8 ± 31 vs. 1,875 ± 132.9 vs. 1,347.5 ± 93.7 dpm/well, respectively; P < 0.05 vs. control and Adβgal). Six days after transduction with AdCat (multiplicity of infection = 50), cell numbers were significantly reduced (AdCat: 38 ± 1.8% of cell counts in control, P < 0.05; and 45 ± 2.0% of cell count in Adβgal, P < 0.05). Incubation with aminotriazole 10 mmol/l, an inhibitor of catalase, prevented this effect. The number of apoptotic cells was increased one- and threefold 2 and 4 days, respectively, after transduction with AdCat. Exogenous administration of low concentrations of H2O2 (50 μM) significantly increased cell proliferation, whereas it was inhibited by higher concentrations. These results suggest that H2O2 is an important modulator of endothelial cell proliferation.

Address for reprint requests and other correspondence: T. O’Brien, Clinical Science Institute, University College Hospital, Galway, Ireland (E-mail: timothy.obrien@nuigalway.ie).

HYDROGEN PEROXIDE (H2O2) is generated by the vascular wall (19, 27) and plays both a physiological and pathophysiological role in vascular homeostasis. Extensive evidence shows that in vascular smooth muscle cells (VSMC), H2O2 regulates cell proliferation and survival, because in this cell line low concentrations of H2O2 are mitogenic (23, 27), and scavenging H2O2 by administration of antioxidants or catalase overexpression results in increased apoptosis (5, 28). In both experimental and human atherosclerosis, increased production of oxygen-derived free radicals within the vessel wall has been described (13, 20), which may alter VSMC proliferation. In contrast, the role of oxy-

gen-derived free radicals in endothelial cell proliferation is unclear.

Increasing levels of hydrogen peroxide are known to inhibit cell proliferation and to promote mitochondrial dysfunction and death in endothelial cells (4, 15, 18, 23). In addition, activation of the c-Jun NH2-terminal kinase as well as of the p38 MAPkinase/heat shock protein 27 pathways has recently been identified as a potential mechanism of H2O2-induced stress response in endothelial cells (6, 14). Despite these findings, the role of endogenously generated H2O2 in the regulation of endothelial cell proliferation and survival is still incompletely defined. Desphande et al. (9) recently showed that oxygen-derived free radicals produced by a Rac-1-regulated oxidase protect endothelial cells from apoptosis. However, endothelial cells have previously been shown to be resistant to treatment with the exogenous antioxidants pyrrolidinedithiocarbamate (PDTC), N-acetyl-L-cysteine (NAC), and catalase (27, 28). These studies are affected by intrinsic limitations inherent to the experimental approach. First, antioxidants such as PDTC and NAC have numerous actions, which are not limited to scavenging H2O2. Secondly, exogenous catalase uptake by endothelial cells may be receptor mediated. Lack of this membrane receptor could affect intracellular protein concentration and activity. Thus the precise role of H2O2 in the regulation of endothelial cell proliferation remains to be defined.

In this study, we investigated the role of endogenously produced H2O2 in endothelial cell growth by transducing human aortic endothelial cells (HAECs) with an adenoviral vector encoding the gene for human catalase. By doing this, we sought to 1) determine whether gene transfer of catalase to endothelial cells would affect cell proliferation and 2) determine the role of apoptosis in catalase-mediated alterations of endothelial cell proliferation.

MATERIALS AND METHODS

Cell cultures and reagents. HAECs were obtained from Clonetics (San Diego, CA). They were cultured in endothelial basal medium (EBM) medium (Clonetics) containing 2% FBS, 50 μg/ml gentamycin, 12 μg/ml bovine brain extract, 1
µg/ml hydrocortisone, and 10 ng/ml human EGF. HAECs between the third and the seventh subpassages were used for the experiments.

Construction, propagation, and purification of adenoviral vector. Recombinant adenovirus containing the cDNA encoding the human catalase gene driven by a cytomegalovirus promoter was a kind gift of Dr. R. G. Crystal (University of New York, New York, NY). It was generated as described previously (10). Virus was purified by double cesium gradient ultracentrifugation and was dialyzed against 10 mmol/l Tris, 1.0 mmol/l MgCl₂, 1.0 mmol/l HEPES, and 10% glycerol for 4 h at 4°C. Viral titer was determined by plaque assay.

Adenoviral vectors encoding β-galactosidase (Adβgal), used in all experiments as a control, was a kind gift of Dr. James M. Wilson (University of Pennsylvania, Philadelphia, PA). It was propagated, isolated, and quantified as described previously (17). Viral stocks were stored at −70°C.

Transduction with adenoviral vectors. HAECs were plated at the optimal density for each experiment and cultured overnight in EBM medium with 2% FBS. For all the experiments, HAECs were transduced with adenoviral vectors 24 h after plating. Cells were exposed to a solution of PBS with 0.5% albumin containing an adenoviral vector concentration at 15–50 multiplicity of infection (MOI) for 1 h at 37°C. The viral solution was then removed and replaced with regular medium.

X-Gal staining. For histochemical staining of β-galactosidase, HAECs were plated at a density of 2 × 10⁵ in six-well plates (Corning Glass Works) and infected with adenoviral solutions containing increasing concentrations of Adβgal (0, 25, 50 MOI). Twenty-four hours later, cells were washed with PBS and fixed for 5 min in 4% paraformaldehyde-0.4% glutaraldehyde in PBS. A solution (12.5 μl) of 500 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer-Mannheim, Indianapolis, IN) was added to each experimental well, incubated for 4 h at 37°C, and finally rinsed in PBS and immersed in fixative. Each well was examined under a light microscope, and efficiency of gene transfer to the endothelial monolayer was visually assessed.

Western blot analysis for human catalase protein. HAECs were plated at a density of 1 × 10⁵ in 100 mm plates and transduced the next day with adenoviral vectors at 50 MOI. After transduction, cells were cultured in EBM containing 2% FBS for 48 h to allow expression. Soluble proteins were extracted by lysing and sonicating the pellets. After centrifugation, the supernatant was collected, and total protein concentration was determined by the bicinchoninic acid (BCA) assay. Prestained protein markers (Bio-Rad; Hercules, CA) and 20 μg of protein were loaded on 4% stacking/10% separating SDS/PAGE. The resolved proteins were transferred to a 0.2-µm nitrocellulose membrane on a semidry electrophoretic transfer system (Bio-Rad) for Western blot analysis. Blots were blocked overnight at 4°C in 5% dry milk solution and then incubated with a polyclonal anti-catalase antibody (1:1,000; Biodiagense) for 12 h at 4°C. After washing, blots were incubated with a donkey anti-sheep IgG horseradish peroxidase-conjugated secondary antibody (1:5,000; Biodiagense), and then exposed to X-ray film. Chemiluminescence (ECL) was performed using an enhanced ECL Kit (Amersham, Piscataway, NJ).

Measurement of catalase activity. Catalase activity in endogenous cells was quantified spectrophotometrically by the method of Aebi (1). Briefly, 48 h after transduction, cell extracts were prepared by scraping the cells in 50 mM phosphate buffer pH 7.0 containing 0.01% Triton X. After three cycles of freeze thawing, cell lysates were centrifuged 4,000 rpm at 4°C for 15 min. To measure catalase activity, cell extracts were added to 10 mM H₂O₂ in 50 mmol/l phosphate buffer and disappearance of H₂O₂ was recorded at 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. Total protein concentration was determined by the BCA assay. Activity units for catalase were expressed as milliunits relative to milligram of cell lysate protein.

DNA synthesis. DNA synthesis in HAEC was measured by [³H]thymidine incorporation. After transduction, cells were kept quiescent for 24 h with EBM containing 0.1% FBS, and then stimulated for 44 h with fresh medium containing 2% FBS and growth supplements. [³H]thymidine incorporation was determined by adding 1 µCi of [³H]thymidine for 4 h at 37°C. Then cells were washed, DNA was extracted with 0.5 N NaOH, and radioactivity was counted by scintillation spectroscopy.

In some experiments, after quiescence, nontransduced cells were incubated in the presence of H₂O₂ (10–200 µM) for 24 h and [³H]thymidine incorporation was determined as described above.

Cell proliferation. Cells were plated at the same density and infected with adenoviral vectors encoding β-catalase (AdCat) or Adβgal as described. Additional control cells were exposed to diluent alone. Culture medium was changed every 48 h. Cells were counted in a Coulter Counter (model ZM, Coulter Electronics) on day 6 after transduction. In a separate set of experiments, immediately after transduction, amnityrazone (10, 50, or 100 mmol/l) was added to the medium of cells. Cell counts were performed on day 6.

Determination of apoptosis. After transduction with adenoviral vectors, cells were grown in a regular medium for 2 or 4 days. Then, 7.5 µl of a solution containing 100 µg/ml of Hoechst 33342 (Molecular Probes, Eugene, OR) was added to each 500-µl well. Cells were incubated for 5 min at 37°C in the dark. Samples were analyzed by confocal microscopy and quantification of apoptotic bodies was performed both visually and by the aid of computer-assisted software (Uthasca).

Statistical analysis. Results are expressed as means ± SE. Statistical evaluation was performed by the use of ANOVA followed by Fisher test. A value of P < 0.05 was considered statistically significant.

RESULTS

X-Gal staining. Expression of recombinant β-galactosidase in transduced HAECs was confirmed by X-Gal staining (Fig. 1). In the presence of X-Gal, cells expressing the transgene appeared blue. Dose-dependent transgene expression was observed (Fig. 1A). In contrast, control cells did not stain (Fig. 1A).

Western blot analysis for catalase. Catalase expression was sought by Western analysis in AdCat, Adβgal, and control cells. Constitutive catalase expression was detected in control and Adβgal-transduced cells. Expression of catalase was markedly increased in cells transduced with AdCat (Fig. 2A). This experiment was performed in duplicate and repeated on three different occasions with similar findings. Densitometric analysis revealed a significant increase in expression of catalase in AdCat- versus Adβgal-transduced and control cells (Fig. 2B).

Catalase activity. Catalase activity in control, non-transduced, or Adβgal-transduced cells was similar (control: 3.9 ± 0.9 mU/mg protein vs. Adβgal: 3.9 ± 0.7 mU/mg protein).
mU/mg protein, Fig. 3). This finding is in accordance with previous data reported in endothelial cells (12). In contrast, cells transduced with 50 MOI of AdCat exhibited a 10-fold increase in total cellular catalase activity compared with control or Adβgal-transduced cells (37.8 ± 13.9 mU/mg protein; P ≤ 0.03 vs. control and Adβgal, Fig. 3).

DNA synthesis. Endothelial cell proliferation was slightly decreased in Adβgal-transduced cells compared with control cells. At 50 MOI, [3H]thymidine incorporation was markedly (P ≤ 0.05) inhibited in AdCat transduced (536.8 ± 31 dpm/well) compared with Adβgal and control cells after 6 days in culture with EBM containing 2% FBS (Fig. 6, A and B).

To evaluate the effects of exogenous H2O2 on endothelial cell proliferation, growth-arrested HAECs were incubated in the presence of increasing concentrations (10–200 μM) of H2O2 for 24 h. The addition of 10 μM of H2O2 did not affect [3H]thymidine incorporation in endothelial cells (Fig. 5). In contrast, incubation of cells in 50 μM of H2O2 caused a significant (P < 0.05) increase in DNA synthesis (Fig. 5). Treatment with a higher amount of H2O2 (200 μM) resulted in inhibition of [3H]thymidine incorporation, suggesting a cytotoxic effect of H2O2 at this concentration (Fig. 5).

Cell proliferation. HAECs transduced with 50 or 25 MOI of AdCat showed a significant (P < 0.05) decrease in cell counts (by ~55 and 33% respectively) compared with Adβgal and control cells after 6 days in culture with EBM containing 2% FBS (Fig. 6, A and B).

To confirm that decreased cell proliferation in AdCat-transduced HAECs was because of a functional effect of catalase expression, in a separate set of experiments, cells were transduced with 50 MOI of AdCat or Adβgal and then incubated in the presence of aminotriazole 10 mmol/l for 6 days. Aminotriazole (10 mmol/l) completely prevented the decrease in cell counts in AdCat transduced HAECs (Fig. 7).

Apoptosis. Forty-eight hours after transduction, the number of apoptotic bodies was significantly (P < 0.0001) increased in HAECs transduced with 50 MOI of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of adenoviral vectors encoding β-galactosidase (Adβgal)-transduced human aortic endothelial cells (HAECs). A: 0 multiplicity of infection (MOI); B: 25 MOI; C: 50 MOI; note viral titer-dependent increase in expression of β-galactosidase in cells exposed to increasing titer of Adβgal.

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of AdCat (7.7 ± 0.6%) compared with Adβgal-transduced and control cells (2.1 ± 0.5 and 3.3 ± 0.7%, respectively) (Fig. 8A).

Four days after gene transfer, the number of apoptotic bodies was almost tripled in the same group [AdCat (9.5 ± 1.2%, P < 0.0001) vs. Adβgal (2.2 ± 0.4) and control (1.7 ± 0.2%)] (Fig. 8B).

**DISCUSSION**

Hydrogen peroxide is involved in the regulation of cell proliferation and survival in a number of cell lines by acting as an essential intracellular signal for several growth factors and cytokines (2, 3, 5, 23, 25, 27, 30). However, the data on the role of hydrogen peroxide in endothelial cell proliferation are sporadic and contradictory. By using adenoviral-mediated gene transfer, we show that catalase overexpression inhibits cell proliferation in HAECs, which suggests that hydrogen peroxide plays a crucial role in intracellular signaling during the induction of endothelial cell proliferation. When cells were treated with aminotriazole, an irre-
versible catalase inhibitor (7, 21, 22), cell proliferation was not affected by catalase overexpression, suggesting a specific role of catalase in mediating reduced cell growth in this cell line. In addition, our data indicate that catalase overexpression induces apoptosis suggesting that hydrogen peroxide prevents apoptosis in endothelial cells.

Although a number of investigators have demonstrated a potential role for H2O2 as a growth-promoting factor in VSMC (5, 23, 27), its effect on endothelial cell proliferation is unclear. In a previous report (28), the addition of antioxidants PDTC and NAC to the culture medium of HAEC did not affect cell viability. In contrast, other studies demonstrated that PDTC dose dependently reduced the viability of endothelial cells and induced cell death (11, 16). However, these effects appeared to depend on the chelating activity of PDTC rather than on its antioxidant properties, because the presence of Cu2+ and Zn2+ was required to affect total cell number in these experiments. In another report, Sundaresan et al. (27) showed that the addition of catalase does not result in increased intracellular protein activity in human umbilical vein endothelial cells and hypothesized that this effect is because of the lack of a specific receptor for catalase on the membrane of this cell line. These contradictory findings prompted us to further investigate potential consequences of scavenging endogenous H2O2 in endothelial cells. By using adenovirus-mediated gene transfer, we were able to overcome limitations connected with intracellular bioavailability of catalase and to overexpress the protein within the cell.

Expression of catalase was demonstrated by Western analysis. In an additional set of experiments, functionality of transgenes was confirmed by a 10-fold increase in cellular catalase activity after transduction with 50 MOI of AdCat. Thus adenoviral-mediated gene transfer of catalase resulted in a significant increase in catalase activity in AdCat-transduced compared with Adβgal-transduced and control cells.

We used [3H]thymidine incorporation and cell counts to measure cell proliferation after adenoviral-mediated gene transfer of catalase. As we have described previously, adenovirus-mediated gene transfer per se resulted in an inhibition of proliferation of HAEC line used in these experiments (31). Adenoviral-mediated gene transfer of catalase decreased HAEC proliferation in a dose-dependent manner. Transduction with 50 MOI resulted in a 57% reduction in cell counts, whereas an MOI of 25 reduced cell counts by 33%. Inhibition of endothelial cell proliferation was due to a specific effect of catalase; because treatment with 10 μM aminotriazole completely prevented this effect. The concentrations of aminotriazole that protected endothelial cell proliferation from catalase overexpression in these experiments have been shown to inactivate completely cellular catalase activity, at least in astroglial cells (10).

Currently, the molecular mechanisms by which low concentrations of H2O2 modulate endothelial cell proliferation are poorly understood. Exogenous administration of low concentrations of H2O2 is known to stimulate angiogenesis and migration of endothelial cells (29). These effects are associated with the activation of the tyrosine phosphorylation cascade (24). In addition, Stone and Collins (26) recently reported that H2O2 concentrations 10 μM regulate the phosphorylation status of a pre-mRNA binding protein in endothelial cells. Consequences of this phosphorylation, however, are still unknown. In contrast, the effects of higher amounts of hydrogen peroxide on cell replica-
tion are better defined. H$_2$O$_2$ concentrations $>$100 $\mu$M are cytotoxic, causing cells in culture to undergo growth arrest and apoptosis (8). Our data are in accordance with these findings.

Although experimental evidence supporting the role of H$_2$O$_2$ in the regulation of cell survival in other vascular cell lines is strong (5, 28), direct proof of the involvement of this molecule in regulating endothelial cell survival is lacking. Consequently, we attempted to test the role of H$_2$O$_2$ on endothelial cell apoptosis by transducing HAECs with the adenosine vector encoding human catalase and then staining the cells with Hoechst 33342. In our experiments, we found that catalase overexpression for 2 days did indeed result in increased apoptotic rate, by 100% in HAEC. Furthermore, 4 days after transduction with AdCat, the number of apoptotic bodies was further increased. The mechanism by which endogenous H$_2$O$_2$ protects endothelial cells from apoptosis is still speculative. In a recent study (9), a specific role for reactive oxygen species produced by a Rac-1 regulated oxidase in preventing endothelial cell apoptosis after stimulation with TNF-α has been demonstrated. Other findings support the hypothesis that mitochondrial matrix is important in protecting endothelial cells against H$_2$O$_2$-induced apoptosis (15). However, the mechanism involved in the regulation of the apoptotic process, further studies will be needed to address the protective role of endogenous H$_2$O$_2$ produced in endothelial cells under physiological conditions.

We (31) have previously shown that overexpression of CuZn-SOD resulting in decreased superoxide anion generation resulted in inhibition of endothelial cell proliferation. This observation suggested a role for superoxide anions in endothelial cell proliferation. In the present report, we have extended these findings and shown that overexpression of catalase, a known scavenger of hydrogen peroxide, also inhibits endothelial cell proliferation. Therefore, superoxide and hydrogen peroxide are necessary for endothelial cell proliferation. Furthermore, catalase overexpression was associated with induction of apoptosis, suggesting that hydrogen peroxide is antiapoptotic in endothelial cells. Thus hydrogen peroxide, although injurious when produced in excessive quantities in pathological states, plays crucial roles in endothelial cell proliferation and protection against apoptosis.

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