Published in final edited form as:

Free Radic Biol Med. 2009 June 15; 46(12): 1658–1667. doi:10.1016/j.freeradbiomed.2009.03.018.

Overexpression of Catalase Delays G₀/G₁ to S Phase Transition during Cell Cycle Progression in Mouse Aortic Endothelial Cells

Ogbeyalu E. Onumah¹, George E. Jules², Yanfeng Zhao², LiChun Zhou², Hong Yang², and ZhongMao Guo²

¹ Department of Cancer Biology, Meharry Medical College, Nashville, TN 37208

² Department of Cardiovascular Biology, Meharry Medical College, Nashville, TN 37208

Abstract

While it is understood that hydrogen peroxide (H₂O₂) promotes cellular proliferation, little is known about its role in endothelial cell cycle progression. To assess the regulatory role of endogenously produced H₂O₂ in cell cycle progression; we studied the cell cycle progression in mouse aortic endothelial cells (MAECs) obtained from mice overexpressing a human catalase transgene (*hCatTg*), which destroys H₂O₂. The *hCatTg* MAECs displayed a prolonged doubling time compared to wild-type controls (44.0 ± 4.7 h versus 28.6 ± 0.8 h, *p* < 0.05), consistent with a diminished growth rate and H₂O₂ release. Incubation with aminotriazole, a catalase inhibitor, prevented the observed diminished growth rate in *hCatTg* MAECs. Inhibition of catalase activity with aminotriazole abrogated catalase overexpression-induced antiproliferative action. Flow cytometry analysis indicated that the prolonged doubling time was principally due to an extended G₀/G₁ phase in *hCatTg* MAECs, as compared to the wild-type cells (25.0 ± 0.9 h versus 15.9 ± 1.4 h, *p* < 0.05). The *hCatTg* MAECs also exhibited decreased activities of the cyclin-dependent kinase (Cdk) complexes responsible for G₀/G₁ to S phase transition in the cell cycle, including cyclin D-Cdk4 and cyclin E-Cdk2 complexes. Moreover, the reduction in cyclin-Cdk activities in *hCatTg* MAECs was accompanied by increased protein levels of two Cdk inhibitors, p21 and p27, which inhibit the Cdk activity required for the G₀/G₁ to S phase transition. Knockdown of p21 and/or p27 attenuated the antiproliferative effect of catalase overexpression in MAECs. These results, together with the fact that catalase is an H₂O₂ scavenger; suggest that endogenously produced H₂O₂ mediates MAEC proliferation by fostering the transition from G₀/G₁ to S phase.

Keywords

endothelial cells; cell cycle; catalase; hydrogen peroxide; cyclin-dependent kinase inhibitor

Introduction

Endothelial cell (EC) proliferation contributes to both normal vascular homeostasis and vascular proliferative diseases, such as cancer [1], atherosclerosis, intimal hyperplasia, and

All correspondence should be addressed to: Dr. ZhongMao Guo or Dr. Hong Yang, Department of Cardiovascular Biology, Meharry Medical College, Nashville, TN 37208, Phone: (615) 327-6868, Fax: (615) 321-2949, E-mail: E-mail: Zguo@mmc.edu or E-mail: hyang@mmc.edu.

Conflict of Interests: The authors have no conflicts to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

thrombosis [2,3]. ECs normally exist in the vessel wall in a state of quiescence; however, they can reenter the cell cycle and undergo proliferation in response to physiological and pathological stimuli. Accumulating evidence implicates hydrogen peroxide (H_2O_2), a reactive oxygen species, as a mitogenic stimulus in many cell types, including ECs [4]. It has been shown that exogenous addition of low concentrations of H_2O_2 in culture induces EC proliferation and triggers a number of proliferative signaling pathways [5]. In addition, removal of H_2O_2 by antioxidants, such as catalase [5], diphenyleneiodonium [6] and glutathione peroxidase [7], has been shown to inhibit EC proliferation induced by exogenous H_2O_2 and serum. Moreover, Zanetti et al. [8] reported that aminotriazole, a catalase inhibitor, attenuates catalase-induced suppressive effect on cellular proliferation. These observations suggest that endogenously generated H_2O_2 functions as an intracellular messenger in the proliferative signaling pathways of ECs [9]. Several studies demonstrate that H_2O_2 is produced primarily via the enzyme NADPH oxidase in response to mitogens, such as growth factors [10] and angiotensin II [11]. It is also known that H_2O_2 can be derived from other sources, such as mitochondrial respiration [12], or the enzymes glucose oxidase and xanthine oxidase [5].

Unlike other reactive oxygen species, H_2O_2 is stable, neutral, and freely diffuses across plasma membranes [13], which poises it to participate in a variety of signaling mechanisms, including those associated with cellular proliferation. Specifically, it has been reported that H_2O_2 is capable of activating the signaling pathways downstream of growth factor receptors, including mitogen-activated protein (MAP) kinases [14], and phosphoinositide 3-kinases [15]. In addition, H_2O_2 has been shown to potentiate tyrosine phosphorylation [14,16] and modify conserved cysteine residues in signaling proteins, rendering them active or inactive in signal transduction pathways [17]. Hydrogen peroxide also activates a number of transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells, and activated protein-1 [18], and regulates the expression of growth factors such as vascular endothelial growth factor [19]. Taken together, these observations indicate a crucial role for H_2O_2 in regulating cell proliferative responses, but the exact mechanism remains unknown.

Cellular proliferation is ultimately dependent on the cell cycle progression [20], a nuclear event coupled to the activation of a family of serine/threonine protein kinases called cyclin-dependent kinases (Cdks). These kinases, Cdks 1, 2, 4 and 6, form complexes with cyclin proteins A, B, D and E at specific phases of the cell cycle to promote cell cycle progression. Specifically, the entry of cells from the quiescent phase, G_0 , into the cell cycle is governed by the cyclin D-Cdk4 complex, while the cyclin E-Cdk2 complex regulates the transition from G_1 to S phase, and the S phase, known as the DNA synthesis phase, is regulated by the cyclin A-Cdk2 complex. Finally, transition through the G_2 -M, also known as the mitotic phase, is regulated by the cyclin B-Cdk1 complex. The activity of the cyclin-Cdk complexes is tightly regulated by a number of intracellular and extracellular signals that alter either the cyclin and Cdk availability, the Cdk phosphorylation status, or the levels of Cdk inhibitory proteins (CKIs) [21]. For example, overexpression of CKIs p21 and p27 has been shown to mediate growth arrest, and contribute to G_1 arrest during cell cycle progression [22]. Increasing evidence implies that H_2O_2 regulates cellular proliferation by targeting the progression through the cell cycle. Specifically, it has been reported that the level of H_2O_2 fluctuates along with cell cycle progression [23–25], and removal of endogenous H_2O_2 , by overexpression of catalase and glutathione peroxidase, induces G_0/G_1 arrest [26,27] and decreases DNA synthesis in cells [28]. However, the exact mechanism of this cell cycle regulation by H_2O_2 has not been fully defined.

Catalase is an enzyme that converts H_2O_2 into molecular oxygen and water. To date, investigators have examined the regulatory role of endogenously generated H_2O_2 in EC proliferation through the addition of catalase into cell culture medium [26], or through viral infection of cells to introduce a catalase transgene [8]. These studies do have limitations, as

the administration of catalase to culture medium cannot remove intracellular H_2O_2 , and viral infection cannot introduce equal number of transgenes into each cell. In this report, we took the advantage of an existing transgenic mouse model that overexpresses human catalase (*hCatTg*) approximately 2.5-fold in ECs and vascular smooth muscle cells (VSMCs) [29]. This transgenic mouse model has emerged as a powerful tool in studying the molecular mechanisms of vascular remodeling and vascular related diseases. Specifically, these *hCatTg* mice display reduced pressor response to vasoconstrictor genes [29,30], and show delayed development of atherosclerosis in response to hypercholesterolemia [29].

In this study, we demonstrate that the mouse aortic endothelial cells (MAECs) obtained from *hCatTg* mice displayed a reduced growth rate as compared to wild-type MAECs. The *hCatTg* MAECs also showed a prolonged G_0/G_1 phase duration, which coincided with decreased activity of cyclin D-Cdk4 and cyclin E-Cdk2 complexes, and increased protein levels of the CKIs p27 and p21. These findings suggest that p27 and p21 protein levels, and subsequently the G_0/G_1 phase of the cell cycle are targets of H_2O_2 in regulating EC proliferation. In addition, these findings contribute to understanding of the mechanism whereby overexpression of catalase inhibits the development of atherosclerotic plaques, a pathogenesis that involves EC proliferation.

Materials and Methods

Reagents

Monoclonal mouse antibodies against p27^{kip1}, protein kinase A (PKA) inhibitory peptide, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal rabbit antibodies against cyclin E, cyclin D2, cyclin D3, Cdk2, p21^{cip1}, mouse monoclonal antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), cyclin D1 and Cdk4, GST-retinoblastoma protein/107p/103p fusion protein substrate, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G plus/protein A-agarose beads were purchased from Calbiochem (San Diego, CA). Histone H1 and adenosine 5'-[γ -thio] triphosphate tetralithium salt(ATP) were purchased from Boehringer Mannheim (Pleasanton, CA). Phosphocellulose P81 filter paper was purchased from Whatman (Florham Park, NJ). ³²P-ATP was purchased from GE Healthcare-Amersham (Piscataway, NJ). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from invitrogen (Carlsbad, California). 3-Aminotriazole-1,2,4-Triazole was purchased from Sigma-Aldrich (St. Louis, MO). Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit was purchased from Molecular Probes (Eugene, OR).

Isolating, culturing and characterizing mouse aortic endothelial cells (MAECs)

Transgenic mice overexpressing human catalase (*hCatTg*) were generated as described previously [31]. Under anesthesia with a rodent cocktail, aortas extending from the aortic arch to the branch point of the renal artery were collected from the *hCatTg* mice and their wild type littermates [32]. All procedures for handling the animals were approved by the Institutional Animal Care and Use Committee of Meharry Medical College. MAECs were obtained from mouse aortas using an outgrowth technique [32], cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and maintained at 37°C in a 5% CO₂ atmosphere. These cells displayed a cobblestone-like monolayer and expressed von Willebrand factor and platelet-endothelial cell molecule-1 (CD31), characteristic of freshly isolated ECs [32]. The 8th and 9th passages of the cells were used for experiments.

Measurement of catalase activity—Catalase activity was determined using a spectrophotometric assay as described by Aeby et al. [33]. Briefly, MAECs grown in 6-well

plates at confluence were cultured in serum-free DMEM or medium supplemented with 10% FBS for 12 h in the presence or absence of 10 μ M aminotriazole. The MAECs were collected in 35 mM potassium phosphate buffer (pH 7.2) and sonicated with a Sonic Dismembrator (model 100, Fisher Scientific). The homogenates were centrifuged at 14,000 rpm to remove cellular debris. Protein concentrations were measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). One ml of extract containing 100 μ g of proteins was mixed with 30 μ l of 1% H₂O₂ (Sigma-Aldrich). The decomposition of H₂O₂ was followed directly by a decrease in absorbance at 240 nm, which was monitored using a DU6400 spectrophotometer (Beckman Instrument Inc., Fullerton, CA). One unit of catalase activity is defined as the amount of catalase required to degrade 1 millimole of H₂O₂ in 1 min.

Measurement of H₂O₂

H₂O₂ release from MAECs was measured using an Amplex red hydrogen peroxide assay kit. MAECs grown in a 96-well plate at confluence were made quiescent in serum-free DMEM for 12 h. The medium was then replaced with 150 μ l phenol red-free culture medium supplemented with 10% FBS and incubated for 12, 24 or 36 h. Thereafter, 100 μ l of Amplex red reagent was added into each well and incubated at 37 °C for 30 min. Fluorescence was read using a fluorometer (Fluoroskan Ascent FL, ThermoLabsystems, Franklin, MA) with the excitation wavelength at 540 nm and the emission wavelength at 590 nm. The cumulative H₂O₂ concentration was determined based on the standard curve obtained by incubation of the Amplex red reagent with H₂O₂. Because this measurement reflects the ability of MAECs to release H₂O₂, we refer to it as H₂O₂ release. At the end of the experiments, MAECs in the 96-well plate were lysed and protein level in the lysate was determined. The release of H₂O₂ was expressed as nM/mg protein/h.

Measurement of cellular proliferation and doubling time

MAEC proliferation was measured using the CellTiter Glo luminescence ATP assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, MAECs were grown to confluence in 100 mm dishes and were made quiescent by culturing in serum-free DMEM for 12 h. The MAECs were trypsinized, counted with the trypan-blue exclusion assay, and plated in 96-well plates at a density of 5×10^3 viable cells per well in phenol red-free DMEM supplemented with 10% FBS. In the experiments where aminotriazole was used, 10 μ M aminotriazole was added into the culture medium supplemented with FBS. At the indicated time periods, luminescence intensities were determined using a luminometer (Thermo Scientific, Waltham, MA). Cell numbers at various time points were extrapolated from a standard curve of luminescence intensities generated by growth of specified numbers of wild-type and *hCatTg* MAECs. Growth curves for each cell line were generated by plotting the number of cells versus time. The cell doubling time was calculated using the equation $N = N_0 e^{kt}$ fit to the growth curve in each experiment, where N = total cell number at a given culture time (t), N_0 = the number of cells initially seeded, k = exponential growth rate constant.

Cell cycle analysis

Flow cytometry analysis of cell cycle was performed as described by Darzynkiewicz *et al.* [34]. Briefly, confluent MAECs were made quiescent by growth in serum-free DMEM for 12 h. Once trypsinized, 5×10^5 cells were seeded into 100 mm dishes and cultured in DMEM supplemented with 10% FBS. At the indicated culture time, cells were harvested, permeabilized, and fixed with 70% ethanol for approximately 2 h. The fixed cells were then stained with propidium iodide (1 mg/mL) in a solution containing 0.1% (v/v) triton X and DNase free RNase (100 μ g/mL) to identify nuclei. The stained samples were then analyzed using a FACStar PLUS flow cytometer (Becton Dickinson, Sydney, Australia). The fraction of cells in G₀/G₁, S and G₂-M phases of the cell cycle were determined based on the DNA

content present in each of these phases using ModFit software (Verify software, Topsham, ME).

The time duration of G₀/G₁, S, G₂-M phases was determined by utilizing the graphic method of calculation as described by Okada [35]. Briefly, the fraction (*f*) of cells in particular phases of the cell cycle obtained from the flow cytometry data were plotted exponentially [*ln* (1+*f*)] against cell doubling time obtained from growth curves. The duration of individual phases of the cell cycle was extrapolated from the respective points of the time coordinate.

Western blot analysis

Serum-starved confluent MAECs were trypsinized and 5×10^5 cells were plated in 100 mm dishes. At indicated time points, MAECs were extracted at 4°C into a lysis buffer containing 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% NP-40, 50 mM NaF, and freshly added protease inhibitor cocktail and phosphatase inhibitors [1 mM Na₃VO₄, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The extracts were subjected to sonication and centrifugation to remove cellular debris. Equal amounts of total protein (40 µg) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel. The proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) for western blot analysis. The blot was incubated in 5% nonfat milk diluted in Tris-Buffered Saline with Tween 20 (TBS-T) at room temperature for 1 h to reduce non-specific binding, then immunoblotted with specific primary antibodies and HRP-conjugated secondary antibodies that were detected using ECL-plus chemiluminescence reagent (GE Healthcare-Amersham) as a chemiluminescence substrate. Autoradiograms were analyzed with a Bio-Rad Model GS-700 Imaging Densitometer (Bio-Rad), correcting for background signal using the Quantity One Software (Bio-Rad).

In vitro kinase assays

The activities of cyclin E-Cdk2 kinase and cyclin D-Cdk4 were measured using an *in vitro* kinase assay [36,37]. Briefly, MAECs were lysed in NP-40 lysis buffer at indicated culture time points. Lysates containing 500 µg of total protein were pre-cleared with 10 µL of protein G plus/protein A-agarose beads for 30 min at 4°C on a rotating rotor. For measurement of cyclin E-Cdk2 activity, the resulting extracts were incubated at 4°C for 12 h with 2 µg of anti-cyclin E antibody, and then for 1 h with 50 µL of protein G plus/protein A-agarose beads. Once the immune complexes were recovered by centrifugation, the beads were washed three times with NP-40 lysis buffer and twice with kinase buffer containing 1 M Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. For measurement of cyclin E-Cdk2 activity, the immunoprecipitated complex was incubated at 37°C for 30 min with 20 µL of histone H1-kinase cocktail (40 µM ATP, 50 µg/mL Histone H1, 20 µM PKA-inhibitory peptide, and 10 µCi/µL of ³²P ATP). For measurement of cyclin D-Cdk4 activity, the extracts were incubated at 4°C for 12 h with 2 µg of anti-Cdk 4 antibody, and then for 1 h with 50 µL of protein G plus/protein A-agarose beads. The immunoprecipitated complex was incubated at 37°C for 30 min with 30 µL of Rb-kinase mix (1 mM DTT, 5 µM ATP, 1 µg of recombinant GST-Rb protein, and 5 µCi/µL of ³²P ATP). The kinase reaction was stopped through the addition of 100 mM EDTA. The reaction products then were spotted on phosphocellulose P81 paper, which were next rinsed in 75 mM phosphoric acid and 96% ethanol, and then air-dried. The radioactivity of reaction products on the paper was determined using a liquid scintillation counter (1209 Rackbeta LKB Wallac, Finland in the Molecular Biology Core Facility (MMC)).

RNA interference to knockdown p21 and p27

Knockdown of p21 and p27 was accomplished by small interfering RNA (siRNA). DNA oligonucleotide encoding siRNAs to target p21 (tgtccaatcctggtgatgt) and p27 (aacggtggaacttgactt) were synthesized by invitrogen (Carlsbad, CA). Negative-control siRNA

was purchased from Clontech (Mountain View, CA). The double strand oligonucleotide was cloned into RNAi-Ready pSIREN-RetroQ-ZsGreen vector (Clontech). The recombinant retroviral constructs were then transfected into MAECs by lipofectamine 2000 (invitrogen). After 6 h of transfection, cells were replenished with fresh medium containing 10% FBS and cultured for additional 48 h. Cells at confluence were made quiescent in serum-free DMEM for 12 h, and then incubated with 10% FBS for indicated time periods. Cell proliferation was determined using the CellTiter Glo luminescence ATP assay, and the p21 and p27 protein levels were determined using Western blot analysis as described above.

Statistical analyses

For the experiments using the 96 well microplate reader, the mean value for each experiment was averaged from triplicate wells in the same plate. The number of experiments was indicated in figure legends. Data are reported as mean $\pm$ standard error of the mean. The differences among wild-type and *hCatTg* MAECs treated with FBS for various time periods were analyzed by multiple-factor analysis of variance and the student's t test. Differences are considered significant at a *P* value less than 0.05. Statistix software was used for statistical analysis of data.

Results

Overexpression of catalase inhibits Mouse Aortic Endothelial Cells (MAEC) proliferation

Previous studies from our laboratory demonstrated endothelial cells and vascular smooth muscle cells (VSMCs) obtained from *hCatTg* mice had approximately a 2.5-fold increase in their catalase activity, and no significant changes in the activities of other antioxidant scavengers, including Mn-SOD, extracellular-SOD, and glutathione peroxidase-1, when compared with properties of cells obtained from wild-type littermates [28]. Data from the current study are consistent with our previous observations, *i.e.*, the catalase activity was about 2.5-fold higher in *hCatTg* MAECs than in wild-type cells in the absence of serum (Fig. 1A). Incubation of MAECs with 10% FBS for 24 h did not significantly alter catalase activity (Fig. 1A). In this study, we also examined the effect of aminotriazole, a catalase inhibitor, on catalase activity in *hCatTg* MAECs. At concentrations less than 10 μ M, aminotriazole reduced catalase activity in a dose-dependent manner. However, it induced cell death at concentrations higher than 50 μ M (data not shown). Data in Fig. 1A show that 10 μ M of aminotriazole reduced catalase activity in *hCatTg* MAECs by 44% and 49%, respectively, in the presence or absence of serum.

To confirm that catalase overexpression indeed decreases endogenous H₂O₂ production in response to cell stimuli, we examined FBS-induced H₂O₂ release from MAECs isolated from wild-type and *hCatTg* mice. The level of H₂O₂ released from wild-type and *hCatTg* MAECs was comparable in the absence of FBS. However, FBS stimulation of H₂O₂ production was significantly decreased in MAECs obtained from *hCatTg* mice. Specifically, incubation of wild-type MAECs with 10% FBS increased H₂O₂ release about 61, 42 and 35% over basal 12, 24 and 36 h respectively, following exposure to FBS (Fig. 1B), whereas no significant increase in H₂O₂ release was observed in *hCatTg* MAECs (Fig. 1B). We also observed that incubation of *hCatTg* MAECs with 10% FBS induced lower levels of intracellular hydroperoxides, as measured by 6-carboxy-2,7-dichlorodihydrofluorescein diacetate (a peroxide-sensitive dye), when compared with wild-type cells (data not shown). These results are consistent with our previous reports that overexpression of catalase in endothelial cells and VSMCs reduced H₂O₂ generation and release induced by epidermal growth factor, oxidized lipoprotein, angiotensin II, and norepinephrine [25,28,33].

In this report, we studied the effect of catalase overexpression on MAEC proliferation. In the presence of 10% FBS, the number of MAECs obtained from *hCatTg* mice and their wild-type littermates increased with time (Fig. 2A). Here, both cell lines displayed standard growth curve characteristics, including lag and exponential growth phases (Fig. 2A), and they reached a stationary growth phase at 72 h (data not shown). However, the growth rate was slower in *hCatTg* in comparison to wild-type MAECs. As the growth curve in Fig. 2A indicates, the number of wild-type MAECs increased approximately 50% after 18 h, moving the cells from the lag growth phase to exponential growth phase. In contrast, the number of *hCatTg* MAECs increased only about 20% after the same culture time, and did not begin their exponential growth phase until 26 h of culturing (Fig. 2A). Consistent with these findings, Fig. 2B indicates that the doubling time of *hCatTg* MAECs was significantly longer than that displayed by the wild-type cells (44.0 ± 4.7 h versus 28.6 ± 0.8 h, $p < 0.05$). In this report, we also studied the effect of aminotriazole on catalase overexpression-induced antiproliferative action. The data in Fig. 2C indicate that addition of 10 μ M aminotriazole into the culture blocked the suppressive effect of catalase overexpression on MAEC proliferation. These observations suggest that increase in catalase activity is responsible for the reduced proliferation in *hCatTg* MAECs.

Overexpression of catalase retards cell cycle progression

To further characterize the effects of catalase overexpression on the growth of MAECs, cell cycle analysis was performed using flow cytometry. As shown in the histograms in Fig. 3, most of the quiescent MAECs were arrested in the G_0/G_1 phase of the cell cycle, and a relatively small fraction of cells were in S and G_2 -M phases after 12 h of serum starvation. The fraction of wild-type and *hCatTg* MAECs was comparable when examining those in G_0/G_1 ($88.5\% \pm 1$ vs. $86.7\% \pm 2$), in S ($2.8\% \pm 1$ vs. $2.4\% \pm 0$), and in G_2 -M ($6.8\% \pm 1$ vs. $9.6\% \pm 2$) phases of the cell cycle at 0 h (Fig. 3). Reseeding the cells in medium supplemented with 10% FBS shifted the distribution of cells from G_0/G_1 phase to the S and G_2 -M phases with time. This shift was more profound in wild-type MAECs than in *hCatTg* cells (Fig. 3). Specifically, after 30 h of serum stimulation, approximately 24% of the wild-type cells shifted to the S and G_2 -M phases, respectively, while only approximately 11% of the *hCatTg* MAECs shifted to the S and G_2 -M phases, respectively, at this time. These observations, together with the data shown in Fig. 1, indicate that the time-dependent shift of the fraction of cells transitioning from G_0/G_1 to S and G_2 -M phases mirrors the cell growth rate. Namely, the slower cell growth rate coincided with the lower enrichment of cells in the S and G_2 -M phases over time as observed in *hCatTg* MAECs, as compared with wild-type MAECs. In addition, no sub- G_1 fraction of cells was observed in each of the time points examined, indicating that no dead cells were collected. These results further support our initial findings that catalase overexpression inhibits MAEC proliferation, but does not cause cell death.

To determine the effects of catalase overexpression on the cell cycle kinetics, we used the cell doubling time and the fraction of cells distributed in various cell cycle phases to estimate the phase duration of the cell cycle. As shown in Fig. 4, the G_0/G_1 phase of *hCatTg* MAECs was significantly prolonged compared to that of wild-type cells (26 h vs. 16 h); however, the S and G_2 -M phases in these two lines of cells are comparable. These data suggest that overexpression of catalase retards MAEC proliferation as the result of a prolonged G_0/G_1 phase.

Overexpression of catalase decreases G_0/G_1 -related Cdk activity

To delineate the mechanism for the prolonged G_0/G_1 phase observed in *hCatTg* MAECs in comparison to wild-type MAECs, we measured the activities of Cdk4 and Cdk2, the Cdk necessary for G_0/G_1 progression. Cdk4 activation involves binding of cyclin D to Cdk4, which then phosphorylates the retinoblastoma protein, inducing transition of cells from G_0 to G_1 . Activation of Cdk4 also increases the synthesis of cyclin E, which enhances Cdk2 activity, and eventually the cells will transition from G_1 to S phase. Based on the length of G_0/G_1 phases

observed in wild-type (16 h) and *hCatTg* MAECs (26 h), we assessed the activities of Cdk4 and Cdk2 at 0, 12 and 24 h. As shown in Fig. 5, incubation of MAECs in medium supplemented with 10% FBS for 12 and 24 h induced a time-dependent increase in cyclin D-Cdk4 activity in wild-type, but not *hCatTg* MAECs, leading to a significantly lower cyclin D-Cdk4 activity level in *hCatTg* MAECs compared with the observed levels in wild-type cells at 24 h. Data in Fig. 5 also indicate that the basal level of Cdk2 activity is lower in *hCatTg* MAECs than in wild-type cells. Incubation in medium containing FBS significantly elevated the Cdk2 activity in both *hCatTg* and wild-type MAECs at 12 h and 24 h, however, the increase in magnitude and the pattern of activation differed between the two cell lines. Specifically, the Cdk 2 activity in *hCatTg* MAECs reached its peak level at 12 h, and no further increase was observed from 12 h to 24 h. In contrast, the Cdk 2 activity in wild-type cells was significantly higher at 24 h than 12 h (Fig. 5). Further, the levels of Cdk 2 at each of the time points were lower in *hCatTg* MAECs compared with wild-type cells. Therefore, it is possible that the G₀/G₁ accumulation of *hCatTg* MAECs is due to both an elimination of serum-induced changes in cyclin D-Cdk4 and a reduction in basal cyclin E-Cdk 2 activity, such that, even upon serum stimulation, activity of this complex does not mirror even basal activity that is observed in wild-type MAECs. We next looked to determine if these observed changes in cyclin-Cdk complex activity levels, were due to changes in protein levels of cell cycle regulatory proteins.

The activities of Cdks are known to be regulated by a number of factors, including the protein levels of Cdks and cyclins [20,21]. To determine whether the decrease in cyclins D-Cdk 4 and cyclin E- Cdk 2 activities in *hCatTg* MAECs correlates with changes in the levels of these proteins, western blot analyses were performed, beginning with the D-type cyclins, D1, D2 and D3. As shown in Fig. 6, serum-starved MAECs expressed low levels of D-type cyclins, and there was no significant difference in the levels of these proteins comparing the wild-type and *hCatTg* MAECs; however, in response to FBS, expression levels of cyclin D proteins differed between wild-type and *hCatTg* cells. Specifically, in wild-type MAECs, the cyclin D1 protein levels peaked at 12 h following introduction to FBS, and there was no significant increase from 12 to 24 h. In contrast, cyclin D1 levels in *hCatTg* MAECs significantly increased from 12 to 24 h of culture with medium containing FBS. In addition, the protein level of cyclin D1 in *hCatTg* MAECs was similar to wild-type at 12 h, but was significantly higher than the observed level in wild-type cells following 24 h of incubation in medium containing serum. The pattern of serum-induced changes in cyclin D2 and D3 protein levels was similar for wild-type and *hCatTg* MAECs; the protein level of cyclin D2 and D3 increased with time. However, the increase in magnitude of protein levels was greater in *hCatTg* MAECs than in wild-type cells as we observed significantly higher protein levels of cyclin D2 and D3 in *hCatTg* MAECs than in wild-type cells at 12 h and 24 h of serum culture, respectively. Serum-supplementation did not significantly alter the protein level of Cdk4 in wild-type MAECs as measured at 12 h and 24 h of serum culture, but *hCatTg* MAECs displayed significantly increased levels of Cdk4 at 24 h. Taken together, these data suggest that the observed elimination of cyclin D-Cdk4 activity in *hCatTg* MAECs in response to serum cannot be accounted for by an increase in the steady-state levels of D-type cyclins and Cdk4 protein levels.

As the data in Fig. 7 indicate, the basal protein level of cyclin E was significantly higher in *hCatTg* MAECs than in wild-type cells. Incubation of these cells with serum increased the protein level of cyclin E in both wild-type and *hCatTg* MAECs, but no significant differences were observed between these two cell lines. Data in Fig. 7 also show that the basal level of Cdk2 is lower in *hCatTg* MAECs compared to that in wild-type cells. Here, serum supplementation did not result in a significant change in the protein level of Cdk2 in wild-type MAECs, but did induce a time-dependent increase in *hCatTg* MAECs, with significantly increased Cdk2 protein levels compared with wild-type cells at 12 and 24 h. Again, these changes in steady-state levels of cyclin E and Cdk2 proteins cannot explain the observed dramatic decrease in cyclin E-Cdk2 activity in *hCatTg* MAECs compared with wild-type cells.

Catalase overexpression increases the protein levels of p21 and p27

The activation of G₀/G₁-related cyclin/Cdk complexes and subsequent G₀/G₁ phase progression can be inhibited by the WAF1/Cip1 family of cyclin dependent kinase inhibitors (CKIs), such as p21 WAF1/Cip1 (p21) and p27kip1 (p27) [38]. Thus, seeking to assess the role of these CKIs in the observed altered cell cycle progression of our transgenic MAECs, we measured the protein level of p21 and p27 in MAECs obtained from wild-type and *hCatTg* mice. As data in Fig. 8 indicate, the basal level of p27 was significantly higher in *hCatTg* MAECs than in wild-type cells, and incubation of MAECs in medium containing serum significantly increased the p27 protein level in both wild-type and *hCatTg* MAECs, reaching its peak level at 12 h. However, the abundance of p27 protein at 12 h was significantly higher in *hCatTg* MAECs than in wild-type cells. The basal level of p21 was comparable in wild-type and *hCatTg* MAECs, however, after incubation in medium containing serum, the p21 protein levels in *hCatTg* MAECs was significantly higher than those in wild-type cells at 12 and 24 h of serum culture. These changes in p21 and p27 could, indeed, account for the observed decrease in activity of cyclin D-Cdk4 and cyclinE-Cdk2 complexes, due to either the inhibition and/or the sequestration of these cyclin-Cdk complexes.

Knockdown of p21 and p27 attenuates the antiproliferative effect of catalase

To confirm that upregulation of p21 and/or p27 indeed contributes to the antiproliferative effect of catalase overexpression, the expression of p21 and p27 in *hCatTg* MAECs was suppressed by p21 and p27 siRNAs. The knockdown efficiency was confirmed by detection of p21 and p27 proteins. As shown in Fig. 9, the p21 and p27 protein levels in the *hCatTg* MAECs transfected with p21, p27 or both p21 and p27 siRNAs were reduced about 23–50% at 0 and 24 h of serum culture, when compared with those transfected with non-specific control siRNAs. Correspondingly, the p21 and p27 mRNA levels were significantly reduced in *hCatTg* MAECs transfected with p21, p27 or both p21 and p27 siRNAs, as measured by real-time RT-PCR assay (data not shown). We also observed that the FBS-induced cell proliferation was comparable in *hCatTg* MAECs transfected with or without control siRNAs (Figs. 1 and 9), suggesting that transfection with control siRNAs did not alter cell proliferation. However, transfection of *hCatTg* MAECs with p21 and/or p27 siRNAs elevated FBS-induced proliferation. The enhance effect induced by combinational transfection of p21 and p27 siRNAs was greater than those induced by p21 or p27 siRNA alone. For example, incubation with 10% FBS for 36 h increased the cell number about 2.4-, 2.3- and 2.7-fold, respectively, in *hCatTg* MAECs transfected with the p21, p27 or both p21 and p27 siRNAs, but only about 1.8-fold in cells transfected with control siRNAs (Fig. 9). These findings suggest that upregulation of p21 and p27 is a mechanism by which overexpression of catalase inhibits endothelial proliferation.

Discussion

In this report, we observed that mouse aortic endothelial cells (MAECs) obtained from mice overexpressing the human catalase gene displayed a decreased growth rate and a prolonged doubling time, when compared to those obtained from wild-type mice. These findings are in accord with previous reports showing an inhibitory role of catalase overexpression in cellular proliferation [8,26,28,39], but extend these finding in an important way, by examining endogenous H₂O₂ production occurring in cells obtained from transgenic mice, rather than ex vivo in response to virally transduced heterologous catalase overproduction. Data from this report also demonstrated that overexpression of catalase blocked FBS-increased H₂O₂ in MAECs, and that aminotriazole, which inhibited catalase activity in the presence or absence of serum in the culture medium, blocked the suppressive effect of catalase overexpression on MAEC proliferation. These observations support the view that endogenously generated

H₂O₂ contributes to cell proliferation, and that reduction in H₂O₂ bioavailability is a mechanism by which catalase inhibits endothelial cell (EC) proliferation.

Another explanation for the antiproliferative effect of catalase overexpression is that an increased catalase protein level may reduce cellular nitric oxide (NO), which has been shown to function as a mitogen [40]. It has been reported that catalase is able to inhibit NO synthesis [41] and bind NO [42]. These observations suggest that overexpression of catalase may reduce NO bioavailability. However, there are also reports showing that H₂O₂ is able to neutralize NO, and that overexpression of catalase elevates cellular NO due to reducing H₂O₂ bioavailability [43]. We therefore examined the effect of catalase overexpression on NO in previous studies, and observed that overexpression of catalase altered neither the NO level nor NO synthase activity in MAECs [32]. The changes in proliferation we observed in this report cannot be attributed to secondary changes in NO levels.

Cellular proliferation is dependent on the cell cycle progression, in which cells transit through the G₀/G₁ phase to the S-phase, and eventually to the G₂-M phase. Data from this report demonstrate that overexpression of catalase in MAECs leads to a low enrichment of cells in the S and G₂-M phase over time, and prolongs the G₀/G₁ phase duration of their cell cycle. These observations suggest that ECs overexpressing catalase spend more time in the G₀/G₁ phase, thereby delaying the time for DNA synthesis to occur. In accordance with our observations, previous studies have shown that exogenous addition or adenoviral transduction of catalase is able to induce G₀/G₁ cell cycle arrest [39], increase the fraction of G₀/G₁ cells [26], decrease DNA synthesis [28], and inhibition of angiotensin II-induced vascular smooth muscle cell hypertrophy [11]. Similarly, increased expression of glutathione peroxidase, which destroys H₂O₂ using glutathione as a cofactor, has been shown to prolong the G₁ phase of the cell cycle [27] and inhibit cellular proliferation [44]. Taken together these findings indicate that endogenously generated H₂O₂ potentiates cellular proliferation by mediating the G₁-S transition.

The transition of cells through one phase to another in the cell cycle is tightly controlled by a series of cyclins and their cognate cyclin-dependent kinases. An important finding in this report is that the prolonged G₀/G₁ phase in cells overexpressing catalase correlates with a reduced activity of G₀/G₁-related Cdks. We observed that FBS stimulation of *hCatTg* MAECs barely enhanced the activity of the cyclin D-Cdk4 complex, which is required for the reentry of quiescent cells into the cell cycle. In addition, both the basal activity and the serum-induced activity of the cyclin E-Cdk2 complex are markedly reduced in *hCatTg* MAECs, as compared to wild-type cells. These data suggest that the observed decrease in cyclin D-Cdk4 and cyclin E-Cdk2 activities could be a causal mechanism by which overexpression of catalase prolongs the duration of the G₀/G₁ phase, and that endogenously generated H₂O₂ serves as an intracellular messenger used by serum growth factors to activate the G₀/G₁-related Cdks and trigger progression of cells from G₀/G₁ to S-phase.

An unexpected observation in this report is that the decrease in Cdk activity in *hCatTg* MAECs was not associated with a decreased level of cyclin and Cdk proteins. Instead, the *hCatTg* cells demonstrated significantly increased G₀/G₁ phase regulatory proteins, including D-type cyclins, Cdk4, cyclin E and Cdk2. These data contradict the accepted paradigm that increases in the protein level of cyclins and Cdks directly corresponds to increases in the activity of Cdks, and therefore, increases cellular proliferation. However, there are studies that have shown a decrease in cellular proliferation with increased protein levels of cyclin D1 [45], cyclin E [46] and Cdk4 [47]. The mechanism underlying the increased cyclin and Cdk protein levels in cells displaying a reduction in proliferation is unclear. One possible explanation is that the increase in expression of these proteins is a compensatory response of cells to suppressed Cdk activity.

Since the change in steady-state protein levels of the cyclins and Cdks could not explain the decreased activities of G₀/G₁-related Cdks observed in *hCatTg* cells, we examined the effect of catalase overexpression on p21 and p27 expression, as association of these regulatory proteins with cyclin-Cdk complexes inhibits the phosphorylation of Cdk by cyclin dependent kinase-activating kinase, a crucial event for the G₀/G₁ to S transition [38]. Indeed, the serum-stimulated p21, and both basal and serum-stimulated p27 levels were significantly increased in *hCatTg* MAECs as compared to wild-type cells. We then examined the antiproliferative role of p21 and p27 by knocking down the expression of these proteins. Our data demonstrated that siRNA knockdown of p21 and/or p27 increased *hCatTg* MAEC proliferation, and that combinational knockdown of p21 and p27 showed greater enhance effect on cell proliferation than knockdown of either p21 or p27 alone. These observations suggest that an increase in the abundance of p21 and p27 could be, at least in part, a mechanism by which overexpression of catalase and consequent reduction in H₂O₂ inhibits MAEC proliferation. This interpretation is supported by a number of studies showing that delayed cellular proliferation is associated with increased protein levels of p27 and p21 [46,48–50]. Conversely, siRNA-mediated knockdown of p27 or 21 has been shown to promote cancer cell proliferation [51], abrogate the antiproliferative effect induced by chemical compounds [52,53], and restore endothelial cell proliferation impaired by expression of dominant-negative focal adhesion kinase-related nonkinase [54].

In summary, this report demonstrates that catalase overexpression inhibits serum-induced MAEC proliferation by prolonging the G₀/G₁ phase, and occurs in parallel with increased protein levels of p21 and p27 and decreased activities of cyclin E-Cdk2 and cyclin D-Cdk4 complexes. Knockdown of p21 and/or p27 by siRNAs attenuated the antiproliferative effect of catalase overexpression. These observations, together with the fact that catalase scavenges H₂O₂, suggest that endogenously produced H₂O₂ mediates EC proliferation by down-regulating the expression of CKIs, such as p21 and p27, and therefore, enhances the activities of G₀/G₁-related Cdks, which in turn trigger the transition of cells from G₀/G₁ to S phase. Ongoing studies in our laboratory are focused on learning to what extent a decrease in H₂O₂ production also contributes to reduced rates of tubule formation in *hCatTg* MAECs as cell proliferation is only one aspect of this dynamic and highly coordinated process.

Acknowledgments

This study is supported by NIH grants F31HL083921 (Ogbeyalu Onumah), G12RR003032 and K01HL-076623 (Hong Yang), and R01ES014471 (ZhongMao Guo). Ogbeyalu Onumah was partially supported by NHLBI T32 training grant (T32HL07735) and U-54 Cancer Biology training grant (5U54CA091408-07) to Dr. Samuel E Adunyah. Core facilities used for this study include the Molecular Biology Core Facility at Meharry Medical College (G12RR03032), the Flow Cytometry Core Facility at Vanderbilt University, and the Flow Cytometry Core Facility at Veterans Hospital, Nashville, TN. We thank Dr. Lee Limbird for critical reading of the manuscript and Dr. Evangeline Motley for technical support.

List of Abbreviations

Cdk	Cyclin dependent kinase
CKIs	Cyclin dependent kinase inhibitors
ECs	Endothelial Cells
FBS	Fetal Bovine Serum

G₀/G₁ phase

Gap 1 phase of the cell cycle

G₂-M phase

Gap 2 phase and mitotic phases of the cell cycle

GAPDH

Glyceraldehyde 3-phosphate dehydrogenase

HRP

Horseradish Peroxidase

H₂O₂

Hydrogen Peroxide

MAECs

Mouse Aortic Endothelial Cells

hCatTg

Mice heterozygously overexpressing catalase

PKA

Protein kinase A

S phase

DNA Synthesis phase of the cell cycle

wild-type mice

Wild type control littermate mice

References

1. Ushio-Fukai M, Nakamura Y. Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. *Cancer Lett* 2008;266:37–52. [PubMed: 18406051]
2. Sueishi K, Yonemitsu Y, Nakagawa K, Kaneda Y, Kumamoto M, Nakashima Y. Atherosclerosis and angiogenesis. Its pathophysiological significance in humans as well as in an animal model induced by the gene transfer of vascular endothelial growth factor. *Ann N Y Acad Sci* 1997;811:311–322. [PubMed: 9186607]
3. Spyridopoulos I, Andres V. Control of vascular smooth muscle and endothelial cell proliferation and its implication in cardiovascular disease. *Front Biosci* 1998;3:269–287.
4. Yasuda M, Ohzeki Y, Shimizu S, Naito S, Ohtsuru A, Yamamoto T, Kuroiwa Y. Stimulation of in vitro angiogenesis by hydrogen peroxide and the relation with Ets-1 in endothelial cells. *Life Sci* 1998;64:249–258. [PubMed: 10027759]
5. Ruiz-Gines JA, Lopez-Ongil S, Gonzalez-Rubio M, Gonzalez-Santiago L, Rodriguez-Puyol M, Rodriguez-Puyol D. Reactive oxygen species induce proliferation of bovine aortic endothelial cells. *J Cardiovasc Pharmacol* 2000;35:109–113. [PubMed: 10630740]
6. Milovanova T, Chatterjee S, Manevich Y, Kotelnikova I, DeBolt K, Madesh M, Moore JS, Fisher AB. Lung endothelial cell proliferation with decreased shear stress is mediated by reactive oxygen species. *Am J Physiol Cell Physiol* 2006;290:C66–C76. [PubMed: 16107509]
7. Faucher K, Rabinovitch-Chable H, Barrière G, Cook-Moreau J, Rigaud M. Overexpression of cytosolic glutathione peroxidase (GPX1) delays endothelial cell growth and increases resistance to toxic challenges. *Biochimie* 2003;85:611–617. [PubMed: 12829378]
8. Zanetti M, Katusic ZS, O'Brien T. Adenoviral-mediated overexpression of catalase inhibits endothelial cell proliferation. *Am J Physiol Heart Circ Physiol* 2002;283:H2620–H2626. [PubMed: 12427601]
9. Schreck R, Baeuerle PA. A role for oxygen radicals as second messengers. *Trends Cell Biol* 1991;1:39–42. [PubMed: 14731549]

10. Chua CC, Hamdy RC, Chua BHL. Upregulation of vascular endothelial growth factor by H₂O₂ in rat heart endothelial cells. *Free Radic Biol Med* 1998;25:891–897. [PubMed: 9840733]
11. Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, Taylor WR, Griendling KK. Role of NADH/NADPH Oxidase-Derived H₂O₂ in Angiotensin II-Induced Vascular Hypertrophy. *Hypertension* 1998;32:488–495. [PubMed: 9740615]
12. Forman, HJ.; Boveris, A. Superoxide radical and hydrogen peroxide in mitochondria. In: Pryor, WA., editor. *Free Radicals in Biology*. New York: Academic; 1982. p. 65-90.
13. Ohno Y, Gallin JI. Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J Biol Chem* 1985;260:8438–8446. [PubMed: 2989289]
14. Vepa S, Scribner WM, Parinandi NL, English D, Garcia JG, Natarajan V. Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. *Am J Physiol* 1999;277:L150–L158. [PubMed: 10409242]
15. Finkel T. Redox-dependent signal transduction. *FEBS Lett* 2000;476:52–54. [PubMed: 10878249]
16. Simon F, Stutzin A. Protein kinase C-mediated phosphorylation of p47 phox modulates platelet-derived growth factor-induced H₂O₂ generation and cell proliferation in human umbilical vein endothelial cells. *Endothelium* 2008;15:175–188. [PubMed: 18663621]
17. Kappert K, Sparwel J, Sandin A, Seiler A, Siebolts U, Leppanen O, Rosenkranz S, Ostman A. Antioxidants Relieve Phosphatase Inhibition and Reduce PDGF Signaling in Cultured VSMCs and in Restenosis. *Arterioscler Thromb Vasc Biol* 2006;26:2644–2651. [PubMed: 16990553]
18. Shono T, Ono M, Izumi H, Jimi SI, Matsushima K, Okamoto T, Kohno K, Kuwano M. Involvement of the transcription factor NF-kappaB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. *Mol Cell Biol* 1996;16:4231–4239. [PubMed: 8754823]
19. Zhu JW, Yu BM, Ji YB, Zheng MH, Li DH. Upregulation of vascular endothelial growth factor by hydrogen peroxide in human colon cancer. *World J Gastroenterol* 2002;8:153–157. [PubMed: 11833093]
20. Norbury C, Nurse P. Animal Cell Cycles and Their Control. *Annu Rev Biochem* 1992;61:441–468. [PubMed: 1497317]
21. Morgan DO. Principles of CDK regulation. *Nature* 1995;374:131–134. [PubMed: 7877684]
22. Besson A, Dowdy SF, Roberts JM. CDK Inhibitors: Cell Cycle Regulators and Beyond. *Dev Cell* 2008;14:159–169. [PubMed: 18267085]
23. Gao F, Yi J, Yuan JQ, Shi GY, Tang XM. The cell cycle related apoptotic susceptibility to arsenic trioxide is associated with the level of reactive oxygen species. *Cell Res* 2004;14:81–85. [PubMed: 15040894]
24. Menon SG, Sarsour EH, Spitz DR, Higashikubo R, Sturm M, Zhang H, Goswami PC. Redox Regulation of the G1 to S Phase Transition in the Mouse Embryo Fibroblast Cell Cycle. *Cancer Res* 2003;63:2109–2117. [PubMed: 12727827]
25. Goswami PC, Sheren J, Albee LD, Parsian A, Sim JE, Ridnour LA, Higashikubo R, Gius D, Hunt CR, Spitz DR. Cell Cycle-coupled Variation in Topoisomerase IIalpha mRNA Is Regulated by the 3'-Untranslated Region. POSSIBLE ROLE OF REDOX-SENSITIVE PROTEIN BINDING IN mRNA ACCUMULATION. *J Biol Chem* 2000;275:38384–38392. [PubMed: 10986283]
26. Ding Q, Jin T, Wang Z, Chen Y. Catalase potentiates retinoic acid-induced THP-1 monocyte differentiation into macrophage through inhibition of peroxisome proliferator-activated receptor {gamma}. *J Leukoc Biol* 2007;81:1568–1576. [PubMed: 17369494]
27. Wang HP, Qian SY, Schafer FQ, Domann FE, Oberley LW, Buettner GR. Phospholipid hydroperoxide glutathione peroxidase protects against singlet oxygen-induced cell damage of photodynamic therapy. *Free Radic Biol Med* 2001;30:825–835. [PubMed: 11295525]
28. Felty Q, Singh KP, Roy D. Estrogen-induced G1/S transition of G0-arrested estrogen-dependent breast cancer cells is regulated by mitochondrial oxidant signaling. *Oncogene* 2005;24:4883–4893. [PubMed: 15897899]
29. Yang H, Roberts LJ, Shi MJ, Zhou LC, Ballard BR, Richardson A, Guo ZM. Retardation of Atherosclerosis by Overexpression of Catalase or Both Cu/Zn-Superoxide Dismutase and Catalase in Mice Lacking Apolipoprotein E. *Circ Res* 2004;95:1075–1081. [PubMed: 15528470]

30. Yang H, Shi M, VanRemmen H, Chen X, Vijg J, Richardson A, Guo Z. Reduction of pressor response to vasoconstrictor agents by overexpression of catalase in mice. *American Journal of Hypertension* 2003;16:1–5. [PubMed: 12517675]
31. Guo Z, Van Remmen H, Yang H, Chen X, Mele J, Vijg J, Epstein C, Ho Y-S, Richardson A. Changes in expression of antioxidant enzymes affect cell-mediated LDL oxidation and oxLDL-induced apoptosis in mouse aorta cells. *Arterioscler Thromb Vasc Biol* 2001;21:1131–1138. [PubMed: 11451741]
32. Yang H, Shi M, Richardson A, Vijg J, Guo Z. Attenuation of leukocyte-endothelium interaction by antioxidant enzymes. *Free Radic Biol Med* 2003;35:266–276. [PubMed: 12885588]
33. Aebi H, Suter H, Feinstein RN. Activity and stability of catalase in blood and tissues of normal and acatalasemic mice. *Biochem Genet* 1968;2:245–251. [PubMed: 5715189]
34. Darzynkiewicz, Z.; Gloria, J. DNA Content Measurement for DNA Ploidy and Cell Cycle Analysis. In: Robinson, F.; Darzynkiewicz, Z.; Dobrucki, J.; Hyun, W.; Orfao, A.; Rabinovitch, P.; Philip, D.; Stewart, C.; Wheelless, L., editors. *Current Protocols in Cytometry*. Mississauga: John Wiley & Sons Inc.; 1997. p. 7.5.1-7.5.24.
35. Watanabe, Okada S. Effects of temperature on growth rate of cultured mammalian cells (L5178Y). *J Cell Biol* 1967;32:301–321.
36. Phelps DE, Xiong Y. Assay for activity of mammalian cyclin D-dependent kinases CDK4 and CDK6. *Methods Enzymol* 1997;283:194–205. [PubMed: 9251020]
37. Jonathon, Pines; Mark, Jackman; Karen, Simpson. Assays for CDK activity and DNA Replication in the Cell Cycle. In: Bonifacino, Juan S.; Harford, Joe B.; Lippincott-Schwartz, Jennifer; Yamada, Kenneth M., editors. *Current Protocols in Cell Biology*. Hoboken: John Wiley & Sons Inc.; 1998. p. 8.2.1-8.2.11.
38. Vidal A, Koff A. Cell-cycle inhibitors: three families united by a common cause. *Gene* 2000;247:1–15. [PubMed: 10773440]
39. Lin SJ, Shyue SK, Shih MC, Chu TH, Chen YH, Ku HH, Chen JW, Tam KB, Chen YL. Superoxide dismutase and catalase inhibit oxidized low-density lipoprotein-induced human aortic smooth muscle cell proliferation: Role of cell-cycle regulation, mitogen-activated protein kinases, and transcription factors. *Atherosclerosis* 2007;190:124–134. [PubMed: 16600249]
40. Zheng J, Wen Y, Austin JL, Chen DB. Exogenous Nitric Oxide Stimulates Cell Proliferation via Activation of a Mitogen-Activated Protein Kinase Pathway in Ovine Fetoplacental Artery Endothelial Cells. *Biol Reprod* 2006;74:375–382. [PubMed: 16251502]
41. Li Y, Severn A, Rogers MV, Palmer RM, Moncada S, Liew FY. Catalase inhibits nitric oxide synthesis and the killing of intracellular *Leishmania major* in murine macrophages. *Eur J Immunol*. 1992
42. Mohazzab H, Fayngersh RP, Wolin MS. Nitric oxide inhibits pulmonary artery catalase and H₂O₂-associated relaxation. *Am J Physiol Heart Circ Physiol* 1996;271:H1900–H1906.
43. Nappi AJ, Vass E. Hydroxyl radical formation resulting from the interaction of nitric oxide and hydrogen peroxide. *Biochim Biophys Acta* 1998;1380:55–63. [PubMed: 9545532]
44. Oberley TD, Schultz JL, Li N, Oberley LW. Antioxidant enzyme levels as a function of growth state in cell culture. *Free Radic Biol Med* 1995;19:53–65. [PubMed: 7635359]
45. Fombonne J, Charrier C, Goddard I, Moyse E, Krantic S. Leptin-Mediated Decrease of Cyclin A2 and Increase of Cyclin D1 Expression: Relevance for the Control of Prepubertal Rat Leydig Cell Division and Differentiation. *Endocrinology* 2007;148:2126–2137. [PubMed: 17303663]
46. Covacci V, Bruzzese N, Sgambato A, Di FA, Russo MA, Wolf FI, Cittadini A. Magnesium restriction induces granulocytic differentiation and expression of p27Kip1 in human leukemic HL-60 cells. *J Cell Biochem* 1998;70:313–322. [PubMed: 9706868]
47. Frost SJ, Simpson DJ, Farrell WE. Decreased proliferation and cell cycle arrest in neoplastic rat pituitary cells is associated with transforming growth factor-[beta]1-induced expression of p15/INK4B. *Mol Cell Endocrinol* 2001;176:29–37. [PubMed: 11369440]
48. Harvat BL, Seth P, Jetten AM. The role of p27Kip1 in gamma interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. *Oncogene* 1997;14:2111–2122. [PubMed: 9160891]

49. Musgrove EA, Lee CSL, Cornish AL, Swarbrick A, Sutherland RL. Antiprogesterone Inhibition of Cell Cycle Progression in T-47D Breast Cancer Cells Is Accompanied by Induction of the Cyclin-Dependent Kinase Inhibitor p21. *Mol Endocrinol* 1997;11:54–66. [PubMed: 8994188]
50. Zancai P, Cariati R, Rizzo S, Boiocchi M, Dolcetti R. Retinoic acid-mediated growth arrest of EBV-immortalized B lymphocytes is associated with multiple changes in G1 regulatory proteins: p27Kip1 up-regulation is a relevant early event. *Oncogene* 1998;17:1827–1836. [PubMed: 9778049]
51. Wu FY, Wang SE, Sanders ME, Shin I, Rojo F, Baselga J, Arteaga CL. Reduction of Cytosolic p27Kip1 Inhibits Cancer Cell Motility, Survival, and Tumorigenicity. *Cancer Res* 2006;66:2162–2172. [PubMed: 16489017]
52. Rao A, Coan A, Welsh JE, Barclay WW, Koumenis C, Cramer SD. Vitamin D Receptor and p21/WAF1 Are Targets of Genistein and 1,25-Dihydroxyvitamin D3 in Human Prostate Cancer Cells. *Cancer Res* 2004;64:2143–2147. [PubMed: 15026355]
53. Ling YH, Li T, Yuan Z, Haigentz M Jr, Weber TK, Perez-Soler R. Erlotinib, an Effective Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, Induces p27KIP1 Up-Regulation and Nuclear Translocation in Association with Cell Growth Inhibition and G1/S Phase Arrest in Human Non-Small-Cell Lung Cancer Cell Lines. *Mol Pharmacol* 2007;72:248–258. [PubMed: 17456787]
54. Bryant P, Zheng Q, Pumiglia K. Focal Adhesion Kinase Controls Cellular Levels of p27/Kip1 and p21/Cip1 through Skp2-Dependent and -Independent Mechanisms. *Mol Cell Biol* 2006;26:4201–4213. [PubMed: 16705171]

Fig. 1. The effect of catalase overexpression on MAEC catalase activity and H₂O₂ release
A. Catalase activity. Wild-type and *hCatTg* MAECs were incubated in the culture medium in the presence or absence of 10% FBS and 10 μ M aminotriazole (AT). Catalase activity was determined by measuring the decomposition of H₂O₂ using a spectrophotometric assay. Values represent the mean $\pm$ SEM of 5 separate experiments in which MAECs were pooled from 4 mice. * $p < 0.05$ compared with wild-type cells. † $p < 0.05$ compared with *hCatTg* cells without aminotriazole treatment. **B.** H₂O₂ release. Quiescent wild-type and *hCatTg* MAECs were seeded in medium supplemented with 10% FBS for the time periods indicated. The release of H₂O₂ from MAECs was measured using an Amplex red hydrogen peroxide assay kit. Values represent the mean $\pm$ SEM of 6 separate experiments in which MAECs were pooled from 4

mice. $*p < 0.05$ compared with wild-type cells without FBS incubation (0 h). $^{\dagger}p < 0.05$ compared with wild-type cells at the same culture time period.

Fig. 2. The effect of catalase overexpression on MAEC proliferation

Quiescent, wild-type and *hCatTg* MAECs were cultured with 10% FBS for the time periods indicated in the presence or absence of 10 μ M aminotriazole (AT). Cellular proliferation was determined based on ATP quantitation assays as described in the Materials and Methods. **A.** Cell growth curves. Cellular proliferation is expressed as the percentage increase from the number of cells initially seeded. **B.** Cell doubling times. The proliferation doubling time was calculated using an equation fit to the growth curve as described in the Materials and Methods. **C.** The effect of aminotriazole on catalase overexpression-induced antiproliferative action. Data represent the mean $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * $p < 0.05$ compared with wild-type cells under the same culture conditions.

Fig. 3. The effect of catalase overexpression on the distribution of MAECs in cell cycle phases
 Quiescent wild-type and *hCatTg* MAECs were seeded in medium supplemented with 10% FBS at a density of 5×10^5 cells per 100 mm dish. At the indicated time points, cells were trypsinized, fixed, stained with propidium iodide, and analyzed by flow cytometry. The right panel shows representative DNA histograms for MAECs. The left panel shows fraction (%) of cells distributed in various cell cycle phases at indicated timepoint. Data represent the mean $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * $P < 0.05$ as compared with wild-type cells under the same culture condition.

Fig. 4. The effect of catalase overexpression on the duration of cell cycle phases

Quiescent wild-type and *hCatTg* MAECs were seeded in medium supplemented with 10% FBS and harvested at 6 h intervals over a period of 48 h and analyzed using flow cytometry assays. We used the fraction of cells in each phase of the cell cycle, and the cell doubling time to calculate the duration of the cell cycle phases as described in the Materials and Methods. Data represent the mean $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * $p < 0.05$ compared with wild-type cells under the same culture condition.

Fig. 5. The effect of catalase overexpression on cyclin D-Cdk4 and cyclin E-Cdk2 kinase activities Quiescent wild-type and *hCatTg* MAECs were seeded in medium containing 10% FBS and lysed at indicated time points. Activity of the CyclinD-Cdk4 and cyclinE-Cdk2 complexes were determined by *in vitro* kinase assays as described in the Material and Methods. The kinase activity is expressed as the radioactivity incorporated into the kinase substrates. Data represent the mean $\pm$ SEM of five separate experiments in which MAECs were pooled from four mice. * $p < 0.05$ compared with 0 h of the same genotype cells. † $p < 0.05$ compared with wild-type cells under the same culture conditions.

Fig. 6. The effect of catalase overexpression on the protein levels of cyclin D and Cdk4
 Quiescent wild-type and *hCatTg* MAECs were seeded into dishes with medium containing 10% FBS and lysed at indicated time points. The levels of cyclin D1, cyclin D2, cyclin D3 and Cdk4 were measured using western blot analysis and are expressed as the percentage of their immunoblot intensity relative to GAPDH levels. The values represent mean $\pm$ SEM of five separate experiments. * $p < 0.05$ compared with 0 h of the same genotype cells, † $p < 0.05$ compared with wild-type cells at the same culture time points.

Fig. 7. The effect of catalase overexpression on the protein levels of cyclins and Cdks
 Quiescent wild-type and *hCatTg* MAECs were seeded into dishes with medium containing 10% FBS and lysed at indicated time points. The levels of cyclin E and Cdk2 were measured using western blot analyses and are expressed as the percentage of their immunoblot intensity relative to GAPDH levels. The values represent mean $\pm$ SEM of five separate experiments. * $p < 0.05$ compared with 0 h of the same genotype cells, † $p < 0.05$ compared with wild-type cells at the same culture time points.

Fig. 8. The effect of catalase overexpression on the protein levels of Cdk Inhibitors

Quiescent wild-type and *hCatTg* MAECs were seeded into dishes with medium containing 10% FBS and lysed at indicated time points. The protein levels of p27 and p21 were measured using western blot analyses and are expressed as the percentage of their immunoblot intensity relative to GAPDH levels. The values represent mean $\pm$ SEM of five separate experiments.

* $p < 0.05$ compared with 0 h of the same genotype cells, [†] $p < 0.05$ compared with wild-type cells at the same culture time points.

Fig. 9. Knockdown of p21 and/or p27 increases cell proliferation in hCatTg MAECs

The hCatTg MAECs were transfected with p21 and/or p27 siRNA or control siRNA as described in the Materials and Methods. The transfected cells at confluence were made quiescent in serum-free DMEM for 12 h, and then incubated with 10% FBS for indicated time periods. The p21 and p27 levels were determined by western blot analysis, and expressed relative to GAPDH. Cell proliferation was determined by ATP quantitation assays, and expressed relative to the number of cells initially seeded. Values represent the mean $\pm$ SEM of five separate experiments in which MAECs were pooled from 4 mice * $P < 0.05$ compared with control siRNA-transfected cells, $^{\dagger}p < 0.05$ compared with 0 h of the same genotype cells.