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# Original Research Communication

# Mechanism of Hydrogen Peroxide-Induced Cell Cycle Arrest in Vascular Smooth Muscle

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## ABSTRACT

**Reactive oxygen species such as hydrogen peroxide ($H_2O_2$) can positively and negatively modulate vascular smooth muscle cell (VSMC) growth. To investigate these paradoxical effects of $H_2O_2$, we examined its effect on apoptosis, cell cycle progression, and cell cycle proteins. High concentrations of $H_2O_2$ (500 μ*M* to 1 m*M*) induced apoptosis, whereas moderate concentrations (100 μ*M*) caused cell cycle arrest in G1. $H_2O_2$ (100 μ*M*) blocked serum-stimulated cyclin-dependent kinase-2 (CDK2) activity, but not CDK4 activity, suggesting that cell cycle arrest occurred in part by inhibiting CDK2 activity. The serum-induced increase in cyclin A mRNA was also completely suppressed by $H_2O_2$, whereas cyclin D1 mRNA was not affected. In addition, $H_2O_2$ caused a dramatic increase in expression of the cell cycle inhibitor p21 mRNA (9.67 ± 0.94-fold at 2 h) and protein (8.75 ± 0.08-fold at 8 h), but no change in p27 protein. Finally, $H_2O_2$ transiently increased p53 protein levels (3.16 ± 1.2-fold at 2 h). Thus, whereas high levels of $H_2O_2$ induce apoptosis, moderate concentrations of $H_2O_2$ coordinate a set of molecular events leading to arrest of VSMCs at the G1/S checkpoint of the cell cycle. These results provide insight into the mechanisms underlying positive and negative regulation of VSMC growth by $H_2O_2$ in vascular disease.** *Antioxid. Redox Signal.* 4: 845–854.

## INTRODUCTION

THE RESPONSE OF VASCULAR SMOOTH MUSCLE CELLS (VSMCs) to reactive oxygen species (ROS) is variable. Agonist-induced generation of intracellular ROS or exposure of cells to oxidants has been reported to lead to proliferation (21, 29), senescence (8), or apoptosis (6, 15). Although the precise identity of the NAD(P)H oxidase responsible for these effects is not completely known, $H_2O_2$ has emerged as an important mediator of both growth and apoptotic responses of VSMCs (15, 21, 25, 29). The mechanisms by which hydrogen peroxide ($H_2O_2$) regulates these different responses are not entirely clear. It is likely that the dose and duration of exposure, as well as the modulation of specific molecular targets, are critical determinants of the apparent paradoxical effects of ROS on cell growth (5). Because cell proliferation and apoptosis contribute to many vascular diseases, including atherosclerosis and hypertension, understanding the differential responses of VSMCs to $H_2O_2$ is of paramount importance.

Cell cycle proteins comprise the final common pathway by which the cell is directed toward mitosis, growth arrest, or apoptosis. Internal and external signals modulate cyclin-dependent kinases (CDKs 2, 4, and 6) by altering the availability of their positive regulators (cyclins E, A, and D), the levels of CDK inhibitory proteins (CDKIs) such as p21 and p27, and their phosphorylation status (17). An additional important negative regulator of the cell cycle is p53, which regulates both p21 and cyclin A expression (20). The only well characterized substrate of the CDKs is the retinoblastoma gene product, Rb, which mediates progression through the G1, S, and G2 phases of the cell cycle (19). Several studies in

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fibroblasts suggest that these proteins and enzymes may be responsive to ROS (2, 4), but the effect of $H_2O_2$ on their expression and activity in VSMCs has not been studied.

We previously showed that endogenous, low levels of ROS are required for hypertrophy (29) and identified several upstream signaling molecules that are redox-sensitive (13). In this study, we sought to gain insight into the reported paradoxical effects of $H_2O_2$ on VSMC growth, and to define the molecular mechanisms by which $H_2O_2$ modulates cell cycle progression. We found that whereas high concentrations of $H_2O_2$ induce apoptosis, intermediate concentrations of $H_2O_2$ potently inhibit serum-induced progression through the cell cycle, apparently via a decrease in cyclin A expression and CDK2 activity, as well as an up-regulation of p21 and p53. These results provide insight into the potential redox-sensitive molecular targets involved in abnormal VSMC growth in vascular disease.

## MATERIALS AND METHODS

### *Materials*

The following investigators kindly provided the cDNAs used in this study: Dr. Charles Sherr, St. Jude's Children's Research Hospital (cyclin D1); Dr. James Roberts, Hutchinson Cancer Center (p27); Dr. Jonathan Pines, Wellcome/CRC Institute (Cyclin A); and Dr. Elizabeth Nabel, University of Michigan (p21). Antibodies to p21 (sc-756), p27 (sc-1641), p53 (sc-6243), and CDK2 (sc-163), as well as GST-Rb and protein A/G agarose, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

### *Cell culture*

Rat aortic VSMCs were isolated and cultured as described previously (12). VSMCs were synchronized for the cell cycle experiments by replacing Dulbecco's modified Eagle's medium (DMEM)/10% calf serum with DMEM/0.1% calf serum when the cultures were 60% confluent. Cells were maintained in low-serum medium for 48 h before addition of serum with or without $H_2O_2$.

### *Northern blot analysis*

Total RNA was isolated from VSMCs using the one-step TRI reagent lysis (Molecular Research Center) (14). The membranes were subjected to autoradiography using phosphor screens for the PhosphorImager, and results were quantified using the ImageQuant software (Molecular Dynamics).

### *Cell lysis and western blot analysis*

VSMCs were washed three times with ice cold phosphate-buffered saline and lysed in 50 m*M* Tris HCl, pH 7.4, 250 m*M* NaCl, 0.1% NP-40, 5 m*M* EDTA, 10 m*M* NaF, 0.1 m*M* dithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysate was stored in aliquots at −80°C until use. Protein concentration in the lysate was measured by the Bradford assay (Bio-Rad). Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with either 9% or 12% acrylamide depending on the size of the protein of interest. Following immunoblotting of the proteins onto Hybond membrane (Amersham Life Science), 5 μg of primary antibody was used per miniblot to detect the protein of interest. The immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and the ECL western blot analysis system.

### *Immunoprecipitation of cyclin–CDK complexes and kinase assay*

VSMC lysate (30 μg) was precleared with rabbit IgG. Anti-CDK antibody (1 μg) was added to the cleared fractions, incubated overnight at 4°C, and the immune complexes were precipitated with protein A/G agarose for 1 h at 4°C. The immunoprecipitates were washed and resuspended in 15 μl of kinase buffer (50 m*M* HEPES, pH 7.5, 10 m*M* $MgCl_2$, 10 m*M* β-glycerophosphate, 2.5 m*M* EGTA, 1 m*M* NaF, 100 μ*M* $NaVO_4$ and 1 m*M* dithiothreitol) containing 10 μCi of [γ-$^{32}$P]ATP and GST-Rb (1 μg) for CDK4 or histone H1 (5 μg) for CDK2. The reaction was stopped after incubation at 30°C for 30 min by the addition of 3× SDS loading buffer. Samples were subjected to SDS-PAGE, and labeled substrate was quantified using a PhosphorImager (Molecular Dynamics).

### *FACS analysis of cell cycle*

VSMCs quiesced as described above were exposed to the indicated treatments for 24 h and stained with propidium iodide for FACS (fluorescence-activated cell sorter) analysis of DNA as described previously (26). The fluorescence distribution from 10,000 cells was captured and analyzed using the CELLQuest program (Becton–Dickinson).

### *Measurement of apoptosis*

Apoptosis was assessed by measuring fluorescein isothiocyanate-conjugated annexin V staining, as well as DNA laddering. For annexin V staining, quiescent cells were treated with $H_2O_2$ for 24 h, harvested, and suspended in 10 m*M* HEPES, pH 7.4, 140 m*M* NaCl, 2.5 m*M* $CaCl_2$ buffer at a concentration of $1 \times 10^6$ cells/ml. Aliquots (100 μl) of the cell solution were then stained with 5 μl fluorescein isothiocyanate-conjugated annexin V (BD–Pharmingen) and 10 μl of propidium iodide (50 μg/ml), and analyzed using the FACSort. DNA laddering was performed using the Suicide Kit (Calbiochem no. AM41) per the manufacturer's instructions.

### *Statistical analysis*

Statistical significance was assessed by Student's paired two-tailed *t* test or analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA, U.S.A.).

## RESULTS

### *Role of $H_2O_2$ in cell cycle progression and apoptosis*

We previously showed that endogenous, low levels of ROS (10–100 n*M* $H_2O_2$) are required for growth (29), but the effects of higher concentrations of $H_2O_2$ on VSMC growth have

not been well defined. We first examined the dose-response relationship between $H_2O_2$ and apoptosis. As shown in Fig. 1A and B, concentrations of $H_2O_2$ of ≤200 μM did not increase apoptosis in VSMC. However, concentrations higher than 200 μM dose-dependently increased apoptosis, to a maximum of 64 ± 11% (n = 3) at 1 mM $H_2O_2$. To determine the response of VSMCs to intermediate, nonapoptotic concentrations of $H_2O_2$, we examined the effect of 100 μM $H_2O_2$ on serum-induced progression through the cell cycle. Nuclei were stained with propidium iodide following treatment with $H_2O_2$ in the presence or absence of serum, and cells were analyzed using FACS (Fig. 1B). Upon quiescence, 93.3 ± 2.0% of the cells were in G0/G1, with ~5% in S/G2/M. Addition of $H_2O_2$ alone to 0.1% calf serum had no effect on this distribution (95 ± 1.2% in G0/G1). When quiescent cells were exposed to 10% serum for 24 h, 35.4 ± 3.3% of the cells moved to the S/G2/M phases of the cell cycle, and 63.5 ± 4.1% of the cells remained in G0/G1 phase. However, when $H_2O_2$ was

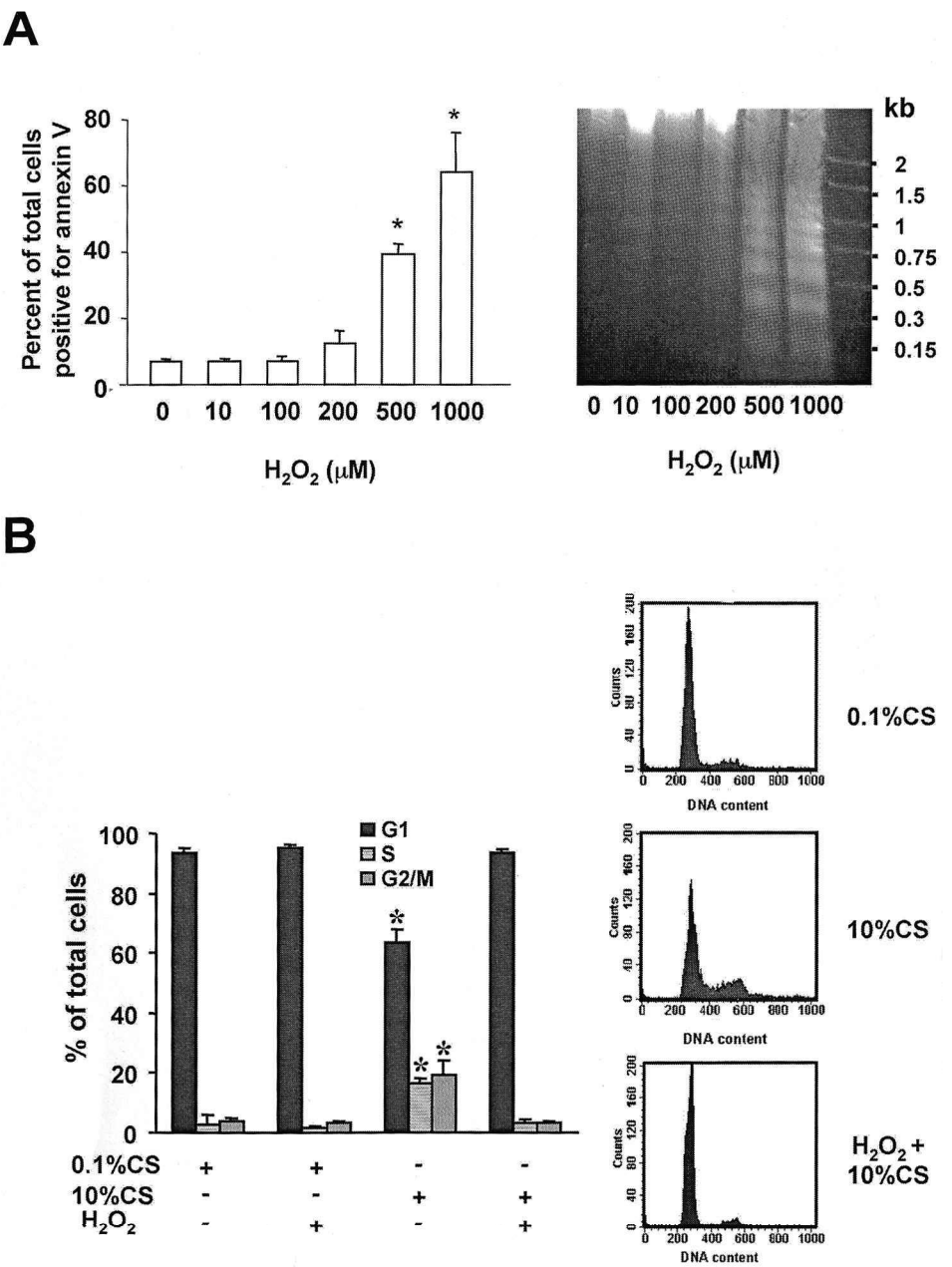


**FIG. 1. Effect of $H_2O_2$ on apoptosis and cell cycle progression.** (**A**) VSMCs were exposed to the indicated concentrations of $H_2O_2$ for 24 h, and apoptosis was assessed by annexin V staining (**left**) or DNA laddering (**right**). Data for annexin V are means ± SE from three experiments. *$p < 0.01$ for control vs. $H_2O_2$. (**B**) VSMCs were quiesced, exposed to 100 μ*M* $H_2O_2$ for 4 h, and incubated with 0.1% or 10% calf serum (CS) for 24 h. **Right** panels show representative histograms of DNA content during the cell cycle. **Left** panel shows the distribution of cells in G1, S, or G2/M expressed as a percentage of total cells. Data are means ± SE from three experiments. *$p < 0.005$ for 10% CS vs. 10% CS + $H_2O_2$.

added 4 h prior to serum, cells were no longer able to progress to S/G2M, but arrested in the G0/G1 phase of the cell cycle (93.2 ± 1.3%). $H_2O_2$ added either concomitantly or 4 h after 10% serum resulted in virtually identical severe cell cycle arrest in G0/G1 (data not shown). These results indicate that treatment of VSMCs with high concentrations of $H_2O_2$ causes apoptosis, whereas intermediate concentrations of $H_2O_2$ prevent progression through the cell cycle, causing arrest in the G0/G1 phase.

### *CDK2, but not CDK4, activity is a target of $H_2O_2$*

Progression through the cell cycle checkpoints (G1/S and serum-sensitive restriction point R in mid-G1) is mainly controlled by the activities of CDK2 and CDK4. To study the mechanisms of $H_2O_2$–induced cell cycle arrest, we first analyzed the effect of $H_2O_2$ on CDK2 and CDK4 activities. Serum treatment potently increased CDK2 activity at 24 h (4.54 ± 0.58-fold over quiescence). However, when 100 μ*M* $H_2O_2$ was added simultaneously with serum, serum failed to activate CDK2 (97 ± 21% of quiescent control) (Fig. 2A). Serum-induced activation of CDK2 requires increased expression of cyclin A. As shown in Fig. 2B, serum elevated cyclin A mRNA expression in a time-dependent manner, with a peak at 12–24 h (8.2 ± 0.6-fold compared with quiescence). However, when cells were exposed to serum in the presence of $H_2O_2$, cyclin A expression at 24 h no longer increased (Fig. 2C). $H_2O_2$ alone (in 0.1% calf serum) had no effect on

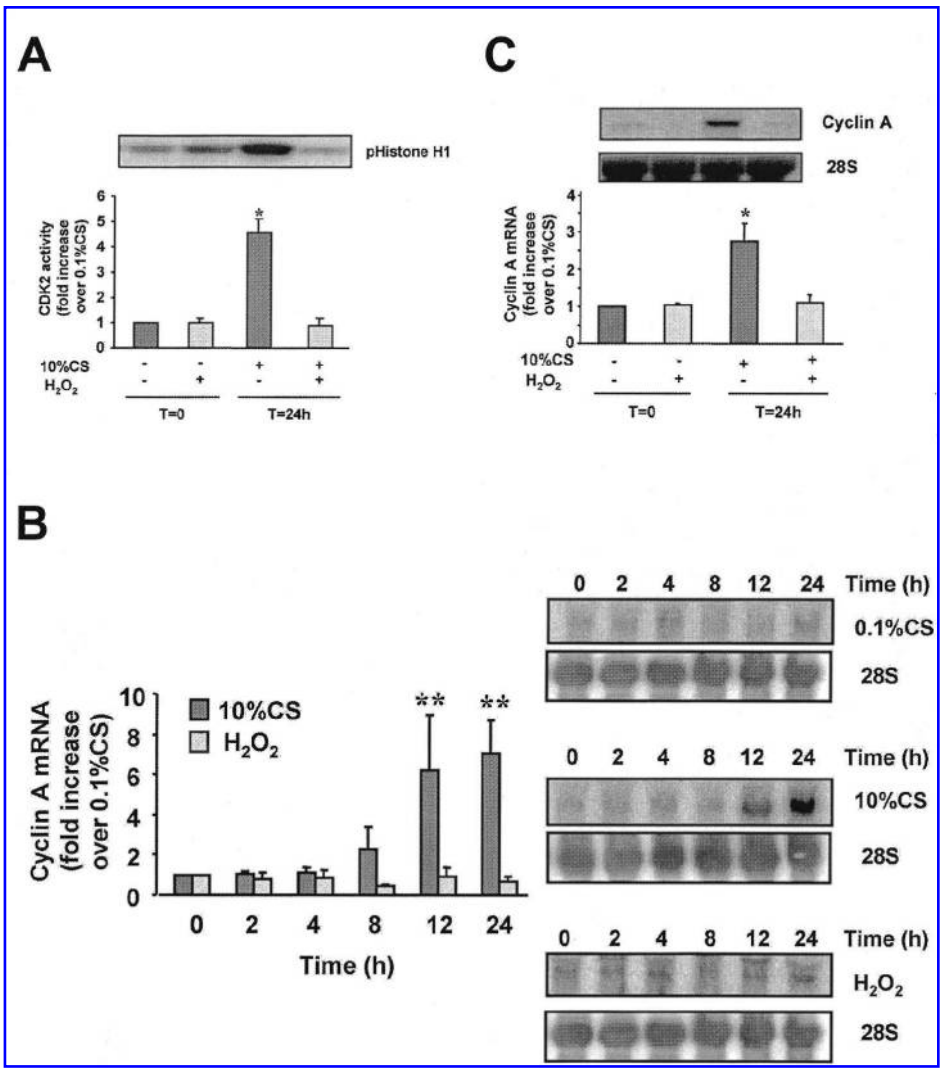


**FIG. 2. Effect of $H_2O_2$ on serum-induced CDK2 activity and cyclin A expression.** (**A**) VSMCs were quiesced in 0.1% serum, pretreated with 100 μ*M* $H_2O_2$ for 4 h as indicated, and assayed for CDK2 activity at 0 and 24 h as described in Materials and Methods ($n = 3$). Histone H1 was used as a substrate for CDK2. (**B**) Time course of cyclin A mRNA expression. $H_2O_2$ was added to quiescent cells in 0.1% serum. Parallel dishes were maintained in 0.1% serum or exposed to 10% calf serum. **Right** panels are representative northern blots. **Left** panel shows means ± SE of three experiments after normalization to 28S rRNA. (**C**) Effect of $H_2O_2$ on cyclin A mRNA expression at 0 and 24 h after addition of 10% serum. $H_2O_2$ was added simultaneously with serum. **Upper** panel is a representative northern blot. **Lower** panel describes means ± SE of three experiments after normalization to 28S rRNA. *$p < 0.005$ for 10% CS vs. 10% CS + $H_2O_2$; **$p < 0.005$ for 10% CS vs. $H_2O_2$.

cyclin A mRNA (Fig. 2B). Because cyclin E also interacts with CDK2 to regulate its activity, we examined the effect of serum and $H_2O_2$ on cyclin E mRNA expression. Neither agonist significantly affected cyclin E levels (data not shown), suggesting that cyclin E is not a major target of $H_2O_2$.

CDK4 activation occurs prior to CDK2 activation, and is required for progression from mid- to late G1. It was conceivable, therefore, that $H_2O_2$ would also inhibit CDK4 activity. As expected, serum increased CDK4 activity at 24 h (Fig. 3A). Surprisingly, $H_2O_2$ alone also increased CDK4 activity in the presence of 0.1% calf serum. However, simultaneous addition of 100 $\mu M$ $H_2O_2$ had no effect on the increase in CDK4 activity due to serum. Consistent with this observation, $H_2O_2$ did not alter expression of cyclin D1, the positive regulator of CDK4 (Fig. 3B). Thus, in the presence of moderate concentrations of $H_2O_2$, serum-stimulated cells progress normally to late G1, but are unable to pass the G1/S checkpoint because $H_2O_2$ inhibits CDK2 activity.

### *$H_2O_2$ induces the cell cycle inhibitors p21 and p53, but not p27*

Because 100 $\mu M$ $H_2O_2$ blocked serum-induced progression through the cell cycle, we postulated that $H_2O_2$ may stimulate expression of CDKIs, negative regulators of the cell cycle that bind to and inhibit the kinase activity of cyclin–CDK complexes, thus preventing progression. Among them,

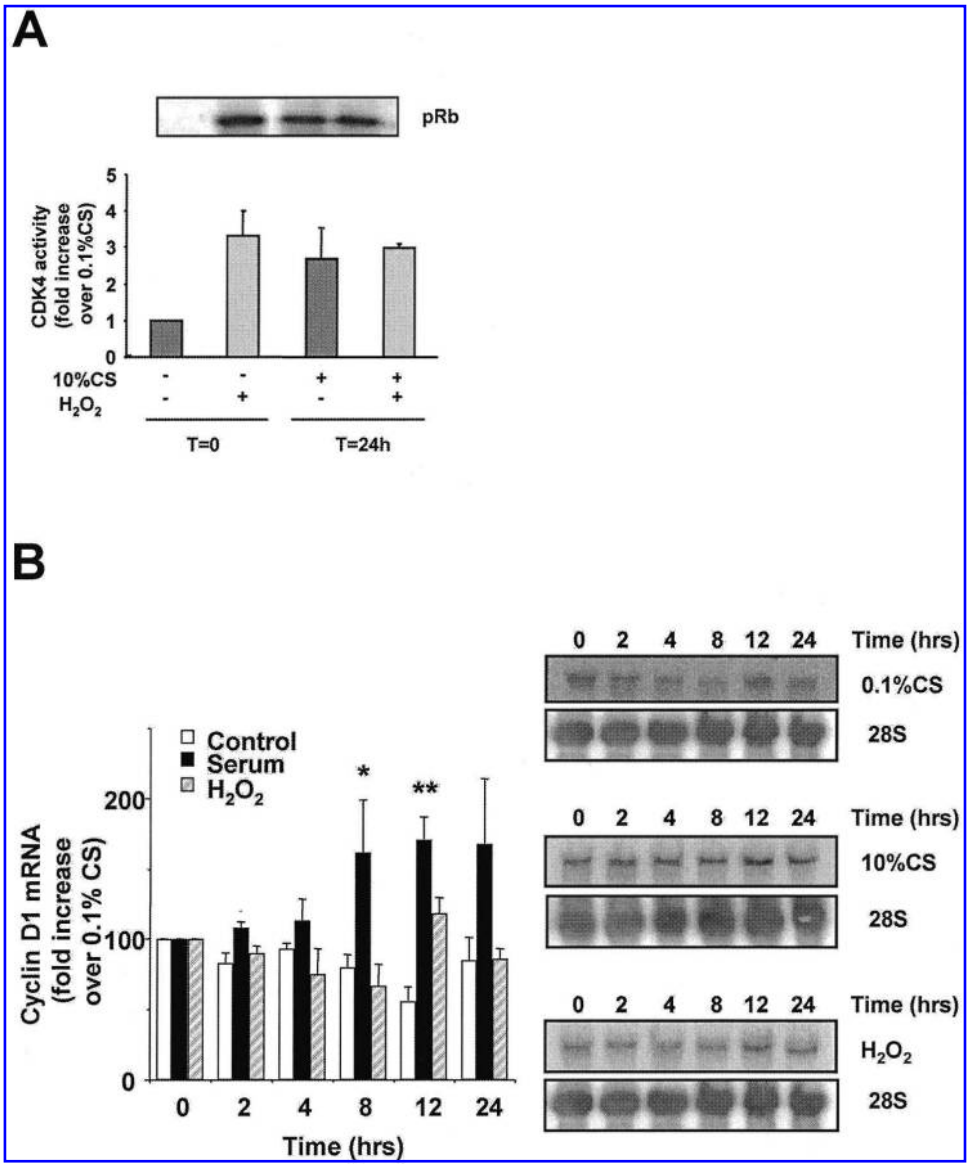


**FIG. 3. Effect of $H_2O_2$ on serum-induced CDK4 activity and cyclin D1 expression.** (**A**) VSMCs were quiesced, pretreated with $H_2O_2$ for 4 h in 0.1% serum, and then exposed to 10% serum alone or in combination with $H_2O_2$ ($n = 2$). $T = 0$ is the point at which serum was added. A 49-kDa fragment of Rb served as a substrate for CDK4. (**B**) Time course of cyclin D1 mRNA expression. $H_2O_2$ was added in 0.1% serum. **Right** panels are representative northern blots. **Left** panel shows means ± SE of three experiments after normalization to 28S rRNA. $*p < 0.05$; $**p < 0.005$ for serum vs. $H_2O_2$.

p21, p27, and p53 are the most important inhibitors in VSMCs (1, 22).

p53 is a transcriptional activator that up-regulates cell cycle inhibitory mechanisms including p21 and GADD45, and inhibits cyclin A expression as well. $H_2O_2$ alone induced a transient increase in p53 mRNA expression, to ~3.14 ± 1.2-fold of the levels in quiescent cells at 2 h (Fig. 4). This is similar to the time course seen in response to $H_2O_2$ in human fibroblasts (7). Neither 0.1% nor 10% serum had any effect on p53 levels.

The protein p21 is a major effector of p53 and binds to and inhibits cyclin E/A–CDK2 and cyclin D–CDK4 complexes, resulting in G1 arrest. This mechanism is mediated through pRb, which sequesters in an inactive form the elongation factor E2F required for DNA synthesis. As shown in Fig. 5A, $H_2O_2$ alone caused a dramatic biphasic increase in p21 mRNA levels, with a peak at 2 h (9.67 ± 0.94-fold increase) and again at 8–12 h (3.52 ± 0.22-fold increase at 12 h). This is strikingly different from the effect of serum, which did not alter p21 mRNA levels. Because CDKIs can be strongly regulated posttranscriptionally (10), we also examined the effect of $H_2O_2$ on p21 protein levels. As shown in Fig. 5B, $H_2O_2$ alone caused a robust increase in the level of p21 protein. By 8 h, p21 protein increased by 8.75 ± 0.08-fold in response to $H_2O_2$, and remained elevated to 4.7 ± 1.3-fold over quiescent levels for as long as 24 h. As expected, serum alone did not increase the level of p21 protein.

To test whether p21 is involved in cell cycle arrest in response to $H_2O_2$ even in the presence of serum, we pretreated the cells with $H_2O_2$ for 4 h and measured p21 protein levels after stimulation with serum. Similar to its effect in quiescent cells, $H_2O_2$ robustly up-regulated p21 protein levels by 2.11 ± 0.47-fold at 6 h and 2.27 ± 0.15-fold at 24 h compared with levels found in serum-stimulated cells (Fig. 5C), suggesting that p21 is a major effector of $H_2O_2$-induced growth arrest.

Another important CDKI, p27, binds to cyclin E–CDK2 and cyclin A–CDK2 complexes, causing a late G1/S phase arrest of the cell cycle. In contrast to the dramatic effect of $H_2O_2$ on p21 expression, $H_2O_2$ had no effect on the down-regulation of p27 mRNA or protein in response to serum (Fig. 6). Thus, p27 does not seem to be an important target of $H_2O_2$ in VSMCs.

## DISCUSSION

In the present study, we provide insight into the molecular mechanisms underlying the paradoxical effects of $H_2O_2$ on VSMC growth. Although high concentrations of $H_2O_2$ induce apoptosis, lower concentrations of $H_2O_2$ induce a coordinated regulation of cell cycle components leading to an arrest of VSMCs in late G1, even when cells are stimulated by a potent mitogenic stimulus such as serum. $H_2O_2$ increases CDK4 activity, which, together with down-regulation of p27 by serum, stimulates progression through mid-G1. However, $H_2O_2$ inhibits CDK2 activity by up-regulating p21 and p53 and down-regulating cyclin A expression. This leads to hypophosphorylation of Rb and prevents dissociation of E2F, thus blocking progression through G1/S (Fig. 7).

Previous reports have indicated that $H_2O_2$ can either promote growth (18) or induce apoptosis (16) of VSMCs. Similar effects have been reported in other cell types (2, 28). In VSMCs, it is clear that some $H_2O_2$ is necessary for growth, because overexpression of catalase (3) or treatment of VSMCs with *N*-acetylcysteine induces apoptosis (24), and endogenously produced $H_2O_2$ (~10–100 n*M*) is required for angiotensin II- and platelet-derived growth factor-stimulated growth (21, 29). Thus, our results indicate that the cellular response to $H_2O_2$ is concentration-dependent: at low, submicromolar concentrations, $H_2O_2$ participates in the mitogenic stimulation (possibly by increasing CDK4 activity; Fig. 3A), at higher concentrations (micromolar) it induces growth arrest, and at millimolar concentrations it triggers apoptosis of VSMCs.

The mechanisms responsible for the growth inhibitory effects of $H_2O_2$ appear to be complex. The present results indicate that $H_2O_2$-induced cell cycle arrest in VSMCs is mediated by several specific redox-sensitive targets, including both positive and negative elements essential for cell cycle progression beyond G1. With regard to positive cell cycle regulators, cyclin A expression in response to serum [essential for progression through G1/S and S phase (11)] and CDK2 activity were completely suppressed by 100 μ*M* $H_2O_2$. In contrast, cyclin D1 and cyclin E (required for progression through mid-late G1) were not affected by $H_2O_2$, indicating that the inhibitory effect of $H_2O_2$ is not the result of general cytotoxicity, but rather arises from modulation of specific

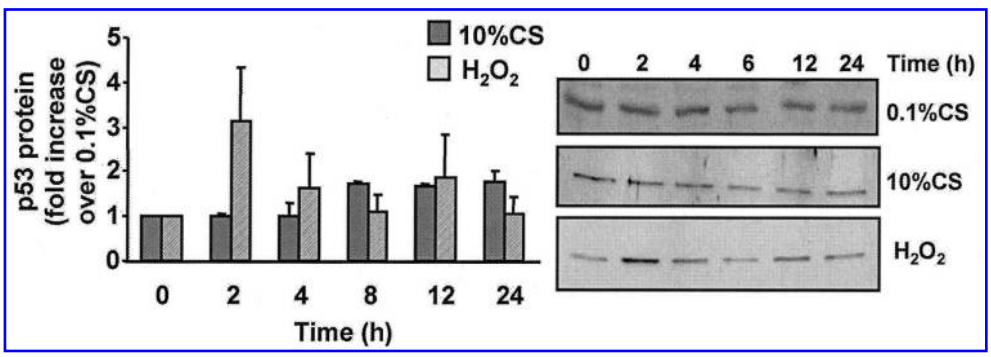


**FIG. 4. Effect of $H_2O_2$ and serum on p53 mRNA expression.** VSMCs were quiesced and exposed to $H_2O_2$ or serum for the indicated times, and cyclin DA mRNA was measured. **Right** panels are representative northern blots. **Left** panel shows means ± SE of three experiments after normalization to 28S rRNA.

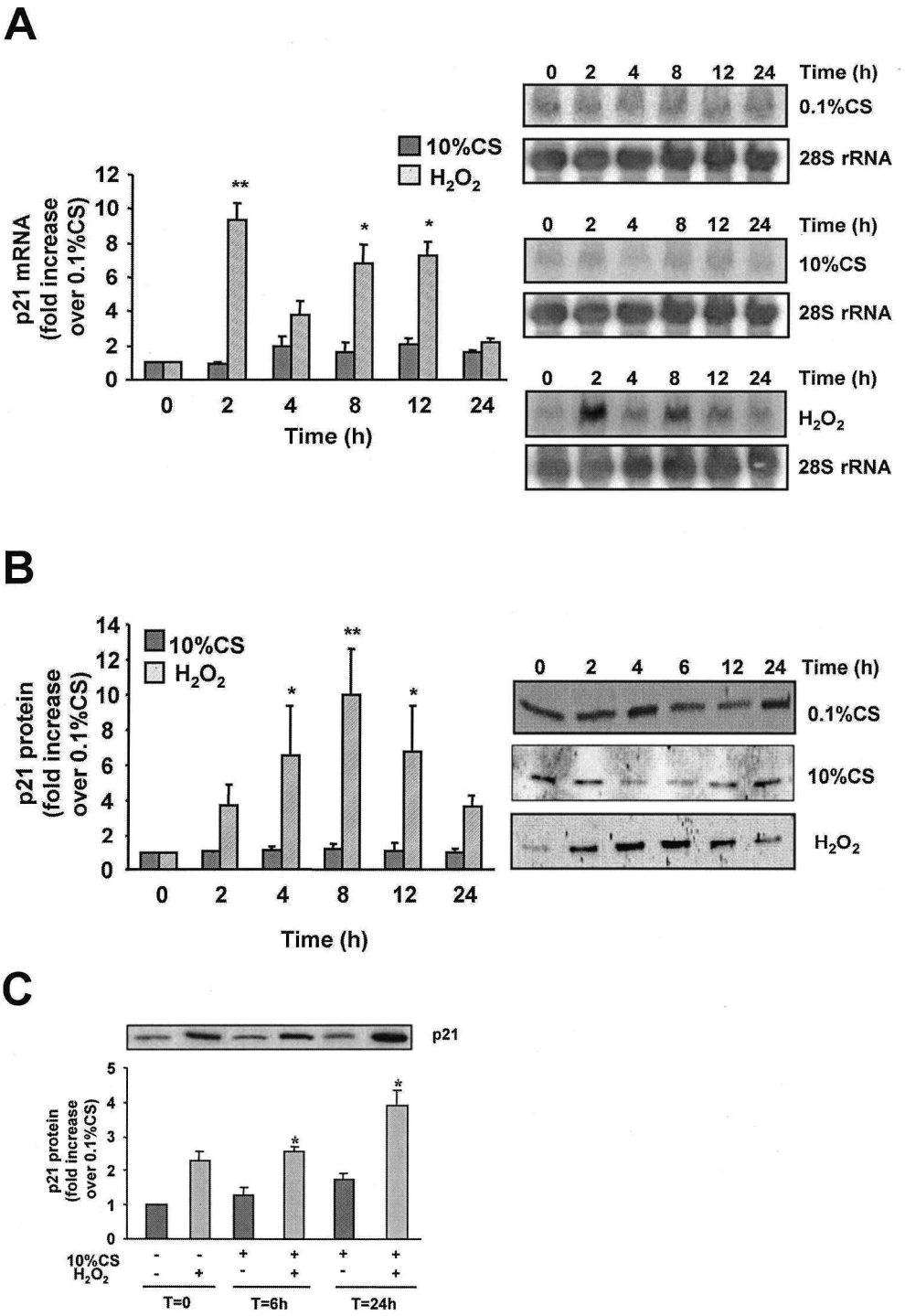


**FIG. 5. Effect of $H_2O_2$ and serum on the regulation of p21.** (**A**) VSMCs were quiesced as described in Materials and Methods, and RNA was isolated from treated cells at the time points indicated. $H_2O_2$ was added in 0.1% serum. **Right** panels show representative northern blots of p21 mRNA regulation by serum and $H_2O_2$. **Left** panel shows means ± SE of four experiments after normalization to 28S rRNA. $*p < 0.05$; $**p < 0.005$ compared with time 0. (**B**) **Right** panels show representative western blots for p21 after exposure of cells to serum or $H_2O_2$ in 0.1% calf serum. **Left** panel shows means ± SE of densitometric data from three experiments. $*p < 0.05$; $**p < 0.005$ compared with time 0. (**C**) VSMCs were quiesced, pretreated with $H_2O_2$ for 4 h as indicated, and exposed to 10% calf serum (CS). p21 protein expression was measured by western analysis at 0, 6, and 24 h. **Upper** panel is a representative western blot. **Lower** panel describes means ± SE of three experiments. $*p < 0.01$ for 10% CS vs. 10% CS + $H_2O_2$.

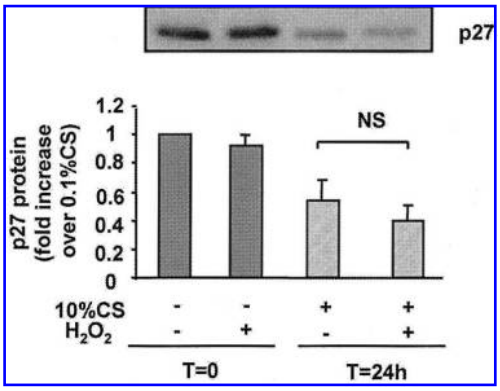


**FIG. 6. Effect of $H_2O_2$ and serum on the regulation of p27.** VSMCs were quiesced, pretreated with $H_2O_2$ for 4 h as indicated, and exposed to 10% calf serum (CS). p27 protein expression was measured by western analysis at 0 and 24 h. **Upper** panel is a representative western blot. **Lower** panel describes means ± SE of three experiments. NS, not significant.

cell cycle targets. The observation that $H_2O_2$ alone increases CDK4 activity as potently as serum suggests that it may stimulate cells to progress from quiescence to mid-late G1, but the subsequent inhibition of cyclin A–CDK2 causes arrest at the G1/S interface.

The most striking effect of $H_2O_2$ in VSMCs is its induction of the CDKI p21, a major negative cell cycle regulator that binds to and inhibits G1 cyclin/CDK complexes. $H_2O_2$ up-regulated p21 at both the mRNA and protein levels (Fig. 5). Similar results have been reported in human fibroblasts (7). Inactivation of CDKs results in hypophosphorylation of Rb, which keeps E2F inactive (23). E2F is required for further induction of cyclin E and A and other factors essential for S phase progression; thus, maintaining E2F in an inactive state leads to late G1/S arrest. In addition to its effect on CDKs, p21 binds to proliferating cell nuclear antigen, resulting in the latter's inability to activate DNA polymerase δ, thereby affecting DNA replication (27). The increase in p21 levels following $H_2O_2$ treatment of VSMCs, in combination with the decline in the ability of immunoprecipitated cyclin A–CDK2 complexes to phosphorylate Rb, indicates that p21 plays a major role in inhibiting cell cycle progression.

p21 gene transcription has been shown to be regulated in part by the tumor suppressor protein p53 (9). p53 is a transcription factor induced by DNA damage that inhibits cell growth by several potential mechanisms (20). Among these, the induction of p21 (9) and the inhibition of cyclin A promoter activity (20) may be the most relevant to $H_2O_2$-induced growth arrest. $H_2O_2$ induces p53 in VSMCs; however, in contrast to sustained induction of p21 mRNA, this increase is transient (peak 2 h). This suggests that, in addition to p53, alternative mechanisms are involved in $H_2O_2$-mediated induction of p21 in VSMCs, consistent with findings in other systems (7).

The inhibitory effect of $H_2O_2$ on cell cycle progression may have important consequences in the process of human coronary atherosclerosis. In atherosclerosis, the macrophage-derived growth factors and $H_2O_2$ may concomitantly stimulate adjacent cells. VSMCs derived from human atherosclerotic plaques have a lower proliferation rate than those isolated from normal coronary arteries, undergo senescence as evidenced by the lack of proliferation after several passages, and have a higher rate of spontaneous apoptosis (1). Many, if not all, of the targets of $H_2O_2$ identified here have been shown to be involved in atherosclerosis. A recent study documented that p21 is elevated in VSMCs of atherosclerotic human coronary arteries and that this correlates with the severity of the disease (22). Although their level of p53 is similar to that of VSMCs isolated from normal arteries, they are more sensitive to p53-mediated apoptosis (1). The effects of $H_2O_2$ *in vitro* thus seem to recapitulate closely the observations from human atherosclerotic plaque, implying a potential mechanistic link. Our data suggest that high levels of $H_2O_2$ can potentially recruit VSMCs from quiescence via its stimulatory effect on CDK4 activity (Fig. 3A), while preventing full proliferation. Although the growth arresting effect may be favorable, the activation of VSMCs from quiescence may make them more prone to apoptosis or senescence than quiescent cells, thus contributing to plaque rupture or fibrosis.

In summary, the response of VSMCs to $H_2O_2$ suggests an elaborate mechanism with multiple targets for the inhibition of full cell cycle progression. Results from this study lend further support to the involvement of ROS in the regulation of vascular cell growth, important in many vascular diseases. These experiments define the molecular mechanisms by which $H_2O_2$ controls VSMC growth, and provide insight into the regulation of redox-sensitive genes and proteins involved in vascular pathophysiology.

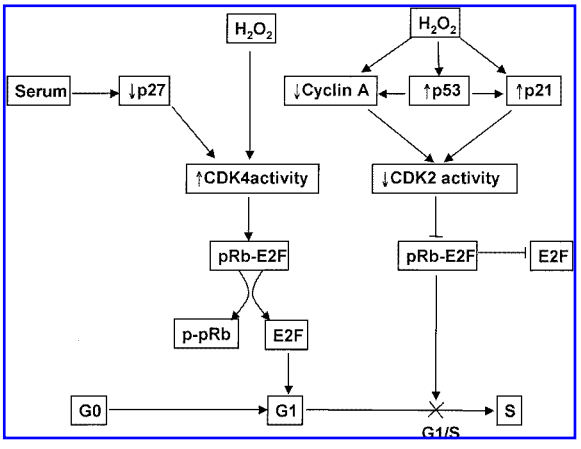


**FIG. 7. Model of molecular mechanisms responsible for $H_2O_2$-induced cell cycle arrest.** Moderate concentrations of $H_2O_2$ increase CDK4 activity, which together with down-regulation of p27 by serum stimulates progression through mid-G1. $H_2O_2$ inhibits CDK2 activity by up-regulating p21 and p53 and down-regulating cyclin A expression. This leads to hypophosphorylation of Rb and prevents dissociation of E2F, thus blocking progression through G1/S.

## ACKNOWLEDGMENTS

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## ABBREVIATIONS

CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitory protein; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; $H_2O_2$, hydrogen peroxide; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; VSMC, vascular smooth muscle cell.

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