Active Oxygen Species Stimulate Vascular Smooth Muscle Cell Growth and Proto-oncogene Expression

Gadiparthi N. Rao and Bradford C. Berk

Vascular smooth muscle cells (VSMCs) proliferate in response to arterial injury. Recent findings suggest that, in addition to platelet-derived growth factors, growth factors from inflammatory cells and endothelial cells at the site of injury may contribute to VSMC proliferation. We hypothesized that a common mechanism by which endothelial cells and inflammatory cells stimulate VSMC growth could be the active oxygen species (i.e., O$_2^-$, H$_2$O$_2$, and -OH) generated during arterial injury. Using xanthine/xanthine oxidase to generate active oxygen species, we studied the effects of these agents on VSMC growth. Xanthine/xanthine oxidase (100 μM xanthine and 5 micromolar/ml xanthine oxidase) stimulated DNA synthesis in growth-arrested VSMCs by 180% over untreated cells. Administration of the scavenging enzymes superoxide dismutase and catalase demonstrated that H$_2$O$_2$ was primarily responsible for xanthine/xanthine oxidase–induced VSMC DNA synthesis. H$_2$O$_2$ directly increased VSMC DNA synthesis and cell number (maximal at 200 μM) but decreased DNA synthesis of endothelial cells and fibroblasts. This effect was protein kinase C independent: sphingosine, a potent protein kinase C inhibitor, failed to block H$_2$O$_2$-induced VSMC DNA synthesis. H$_2$O$_2$ (200 μM) stimulated c-myc and c-fos mRNA levels by fourfold and 20-fold, respectively, as compared with quiescent levels. In contrast to DNA synthesis, H$_2$O$_2$ induction of c-myc and c-fos mRNA was primarily protein kinase C dependent. These findings show that H$_2$O$_2$ specifically increases VSMC DNA synthesis and suggest a role for this oxidant in intimal proliferation, especially after arterial injury. (Circulation Research 1992;70:593–599)

**KEY WORDS** • aorta • DNA • hydrogen peroxide • vascular injury

All tissues are constantly exposed to exogenous and endogenous oxidants. Vascular endothelial cells exhibit metabolic activities that may produce high concentrations of active oxygen species. In fact, the physiological production of endothelium-derived relaxing factor necessarily involves generation of active oxygen species. Active oxygen species concentrations are increased in blood vessels and myocardium in response to a variety of injury-related conditions such as ischemia, thrombosis and reperfusion, and angioplasty. These same circumstances frequently are associated with intimal hyperplasia and accelerated atherosclerosis. Therefore, there may be a relation between arterial injury, active oxygen species production, and VSMC proliferation. To test this hypothesis, we studied the effects of active oxygen species on VSMC growth and c-myc and c-fos mRNA levels. In this work, we show that H$_2$O$_2$ specifically stimulates VSMC DNA synthesis and proto-oncogene expression.

**Materials and Methods**

**Cell Culture**

VSMCs were isolated from the thoracic aortas of 200–250-g male Sprague-Dawley rats by enzymatic dissociation as described previously. Cells were grown in Dulbecco’s modified Eagle’s medium (DME) supplemented with 10% (vol/vol) heat-inactivated calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained in humidified 95%
air–5% CO₂ at 37°C by passage of 1–3×10⁶ cells/ml on reaching confluence. For all experiments, cells at 70–80% confluence were made quiescent by incubation in fresh DME containing 0.4% calf serum for 72 hours. Throughout the course of these experiments, cells were used at passage numbers 8–15.

**DNA Synthesis**

Quiescent VSMCs were labeled in the presence of agonist or 0.4% serum with 1 μCi/ml [methyl-³H]thymidine (specific activity, 20 Ci/mmol) for 24 hours. To analyze rates of DNA synthesis, quiescent cells were treated with H₂O₂ for various times, and 2 hours before the end of each time point, cells were labeled with 1 μCi/ml [³H]thymidine. After labeling, cells were washed with cold saline, trypsinized, and collected by centrifugation (150g for 5 minutes). The cell pellet was suspended in cold 10% trichloroacetic acid and vortexed vigorously to lyse the cells. The cell lysate was passed through a glass fiber filter. After washing with cold 5% trichloroacetic acid and 70% ethanol, respectively, the filter was dried. Incorporated [³H]thymidine was measured in a liquid scintillation counter (model LS5000 TD, Beckman Instruments, Fullerton, Calif.). A rabbit polyclonal antibody against the PDGF A-chain was kindly provided by Ms. Glenda Bildner, Rorer Pharmaceuticals, King of Prussia, Pa. Experiments were performed three times in duplicate 60-mm dishes. Data are presented as mean±SEM, and treatment effects were analyzed by one-way analysis of variance. Post hoc analysis of significance was performed using a Student-Newman-Keuls comparison.

**RNA Blot Analysis**

Total cellular RNA was isolated by the guanidine isothiocyanate–cesium chloride procedure of Chirgwin et al. Equal amounts of RNA (20 μg) from quiescent and agonist-treated cells were size-fractionated by electrophoresis on 1% (wt/vol) agarose gel in 25 mM MOPS buffer (pH 7.8) containing 1 mM EDTA and 2% (wt/vol) formaldehyde. RNA was transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.). The c-npy, c-fos, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were labeled with [α-³²P]dCTP using a BRL random primer labeling kit (Bio-Rad Laboratories, Richmond, Calif.) as per the manufacturer’s protocol. Prehybridization, hybridization with radiolabeled probe, and washing of the membranes were performed as previously described. Membranes were exposed to Kodak X-Omat AR x-ray film with an intensifying screen at −70°C for 4–12 hours. Quantitation of the autoradiograms in the linear range of film exposure was performed using a Pharmacia Ultrascan laser densitometer. Experiments were performed two times in duplicate 100-mm dishes.

**Lactate Dehydrogenase Measurements**

Lactate dehydrogenase present in culture medium was measured by the method of Wroblewski and LaDue. Sonicated cell extracts were used as positive controls.

**Results**

To investigate the effects of active oxygen species on VSMC growth, DNA synthesis was measured in quiescent VSMCs exposed for 24 hours to an active oxygen-generating system composed of xanthine and xanthine oxidase. At constant xanthine concentration (100 μM), 2.5 and 5 microunits xanthine oxidase stimulated [³H]thymidine incorporation, which was maximal (approximately twofold) at 5 microunits xanthine oxidase (Figure 1). At concentrations of xanthine oxidase ≥20 microunits, there was no stimulation of DNA synthesis. The maximal effect of xanthine/xanthine oxidase on DNA synthesis was approximately 40% of that of 10% calf serum and equivalent to 10 ng/ml PDGF (recombinant BB, data not shown). Allopurinol (5 mM), an inhibitor of xanthine oxidase activity, prevented the increase in DNA synthesis induced by xanthine/xanthine oxidase (Figure 1). Allopurinol had no effect on DNA synthesis of growth-arrested VSMCs (0.4% serum) or proliferating VSMCs (10% serum).

Xanthine/xanthine oxidase produces a variety of active oxygen species. We sought to determine which active oxygen species induced VSMC DNA synthesis by using the scavenging enzymes superoxide dismutase (1,000 units/ml) and catalase (2,000 units/ml). As is evident from Figure 2, superoxide dismutase did not prevent xanthine/xanthine oxidase–induced VSMC DNA synthesis, indicating no role for the superoxide anion (O₂⁻) in xanthine/xanthine oxidase–induced VSMC DNA synthesis. Catalase alone stimulated DNA synthesis by unknown mechanisms (possibly other proteins, such as proteases, present in the preparation) and was synergistic with xanthine/xanthine oxidase, confounding attempts to delineate the role of H₂O₂.

To determine the role of H₂O₂ in xanthine/xanthine oxidase–induced VSMC DNA synthesis, we added H₂O₂ directly to growth-arrested VSMCs. As shown in Figure 2, H₂O₂ (200 μM) stimulated [³H]thymidine incorpo-
incorporation in quiescent VSMCs by approximately twofold, indicating a stimulation of DNA synthesis. Because H₂O₂ appeared to be the most likely xanthine/xanthine oxidase–generated active oxygen species to stimulate VSMC growth, a time course for [³H]thymidine incorporation in response to H₂O₂ was performed. As shown in Figure 3, H₂O₂ (200 µM) stimulated [³H]thymidine incorporation with peak DNA synthesis at 24 hours. These data suggest that H₂O₂-stimulated [³H]thymidine incorporation was primarily due to cell progression through S phase.

To prove that H₂O₂-induced DNA synthesis was associated with cell cycle progression and division, cell number was counted 48 hours after exposure to 200 µM H₂O₂. Cell number increased by 21±5% (n=6, p<0.05) in response to H₂O₂, demonstrating a mitogenic effect. To show that H₂O₂-induced [³H]thymidine incorporation in VSMCs was not due to a cell damage/repair process, we measured lactate dehydrogenase release and trypan blue exclusion as markers of cell viability. Exposure of VSMCs to 200 µM H₂O₂ for 24 hours caused no increase in lactate dehydrogenase release into the medium: 7.2±2×10⁻³ IU/10⁶ cells versus 6.8±1.3×10⁻³ IU/10⁶ cells in H₂O₂-treated and control cells, respectively (p>0.1). In contrast, when cells were lysed by hypotonic medium, lactate dehydrogenase release was 48±3×10⁻³ IU/10⁶ cells (p<0.001). The percentage of cells that excluded trypan blue under these conditions was also not different: 95±4% versus 96±5% in H₂O₂-treated and control cells, respectively (p>0.1).

To determine the specificity of H₂O₂-induced DNA synthesis for vessel wall cells, human dermal fibroblasts and bovine aortic endothelial cells were growth-arrested and exposed to 200 µM H₂O₂ for 24 hours. As shown in Figure 4, H₂O₂ failed to stimulate DNA synthesis in bovine aortic endothelial cells and human dermal fibroblasts as measured by [³H]thymidine incorporation. In fact, there were statistically significant decreases of 59±11% and 85±1%, respectively (p<0.05). Thus, among the cell types tested, H₂O₂-induced [³H]thymidine incorporation was specific for VSMCs.

The role of protein kinase C (PKC) in H₂O₂-induced DNA synthesis was investigated next. For this purpose, quiescent VSMCs were exposed to H₂O₂ (200 µM) for 24 hours in the presence and absence of sphingosine, a potent PKC inhibitor, and [³H]thymidine incorporation was measured. Sphingosine did not prevent H₂O₂-induced VSMC DNA synthesis (Figure 5). This result suggests that H₂O₂-induced VSMC growth was independent of PKC activation. Whereas sphingosine alone had no effect on [³H]thymidine incorporation in VSMCs, it significantly potentiated H₂O₂-induced DNA synthesis (p<0.05 versus H₂O₂ alone), an unexpected finding that further indicates that H₂O₂-induced [³H]thymidine incorporation was not due to DNA re-

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**Figure 2.** Bar graph showing effect of superoxide dismutase (SOD) and catalase (Cat) on xanthine plus xanthine oxidase (XO)–induced vascular smooth muscle cell DNA synthesis. Growth-arrested vascular smooth muscle cells were stimulated with the indicated agonists in the presence of 1 µCi/ml [³H]thymidine for 24 hours. Additions were as follows: xanthine plus XO (100 µM xanthine, 5 microunits/ml XO, columns 2–4) plus SOD (1,000 units/ml, column 3) or Cat (2,000 units/ml, column 4), or SOD alone (column 5), Cat alone (column 6), or H₂O₂ alone (200 µM, column 7). *p<0.05 vs. 0.4% serum.

**Figure 3.** Time course for H₂O₂ induction of vascular smooth muscle cell DNA synthesis. Growth-arrested vascular smooth muscle cells were stimulated with H₂O₂ (200 µM) and pulsed with 1 µCi/ml [³H]thymidine for 2 hours before the end of each time point. Results are the mean of triplicate determinations. *p<0.05 vs. 0.4% serum.

**Figure 4.** Bar graph comparing the effect of H₂O₂ on [³H]thymidine incorporation in vascular smooth muscle cells (RASMs), bovine aortic endothelial cells (BAECs), and human dermal fibroblasts (HDFs). Growth-arrested RASMs, BAECs, and HDFs were stimulated with H₂O₂ (200 µM) or 10% calf serum in the presence of 1 µCi/ml [³H]thymidine for 24 hours. All values were significantly different from 0.4% serum control.
pair, since PKC activation is known to stimulate DNA damage.29,30

To study the mechanisms of H2O2-induced growth, the effect of H2O2 on expression of the early growth response genes c-myc and c-fos was analyzed. Quiescent VSMCs were treated with concentrations of H2O2 shown to stimulate maximal DNA synthesis (200 μM). As shown in Figure 6, steady-state levels of c-myc and c-fos transcripts were induced fourfold and 20-fold, respectively, in VSMCs 1 hour after addition of H2O2. H2O2 induced a biphasic increase in c-myc mRNA levels with peaks at 1 and 8 hours; c-fos mRNA levels returned to baseline within 2 hours. H2O2 had no effect on the steady-state levels of the constitutively expressed GAPDH mRNA. The time courses for H2O2-stimulated increases in steady-state c-myc and c-fos mRNA levels were similar to the time course observed with 10% calf serum.22,31

To investigate the roles of different active oxygen species in the induction of early growth signals, c-myc and c-fos mRNA levels were measured in growth-arrested VSMCs after stimulation with xanthine/xanthine oxidase at concentrations that caused maximal DNA synthesis. As shown in Figure 7, the xanthine/xanthine oxidase–induced increase in c-myc and c-fos mRNA levels was similar to the increase seen with H2O2. No significant differences were observed for GAPDH mRNA levels between quiescent and xanthine/xanthine oxidase–treated cells. Superoxide dismutase did not block xanthine/xanthine oxidase–induced c-myc and c-fos mRNA accumulation, indicating no role for superoxide anion in the xanthine/xanthine oxidase–induced mRNA levels of c-myc and c-fos. Catalase alone induced c-myc and c-fos mRNA a result consistent with its effects on [3H]thymidine incorporation (Figure 2). Allopurinol (5 mM) blocked xanthine/xanthine oxidase–induced increases in c-myc and c-fos mRNA. These findings show that, of the active oxygen species generated by the xanthine/xanthine oxidase system, H2O2 is the primary stimulator of c-myc and c-fos mRNA accumulation.

PKC is required for induction of c-fos mRNA in VSMCs by serum and PDGF.21 Exposure to the PKC activator, phorbol 12-myristate 13-acetate (PMA), for 1 hour increased c-myc and c-fos mRNA levels in VSMCs eightfold and 12-fold, respectively, over untreated cells (Figure 8, compare lanes 1 and 3). To demonstrate that this was due to PKC stimulation, PKC was downregulated by exposure to 200 nM phorbol 12,13-dibutyrate (PDBU) for 48 hours. This treatment has been shown to decrease PKC activity by >90% in VSMCs, as measured by PMA-induced phosphorylation of histone III-S and an 80,000-d acidic PKC substrate, and to prevent serum and angiotensin II induction of c-fos mRNA in VSMCs.31 PMA-stimulated increases in c-myc and c-fos mRNA were decreased dramatically by PKC downregulation (Figure 8, compare lanes 3 and 6). PDBU pretreatment inhibited H2O2-induced c-myc and c-fos mRNA expression by approximately 95% and 70%, respectively (Figure 8, compare lanes 1, 2, and 5). Nearly identical results were obtained using 5 μM spingosine (data not shown). Little change was observed in steady-state GAPDH mRNA levels in VSMCs after these treatments (Figure 8). These data indicate that H2O2 induces c-myc and c-fos mRNA primarily by PKC-dependent mechanisms, although a PKC-independent mechanism cannot be excluded, especially for c-fos induction.

Previously, we demonstrated that uric acid, the product of xanthine/xanthine oxidase metabolism, stimulated VSMC DNA synthesis and cell division.32 This was due to induction of an autocrine growth mechanism as shown by expression of PDGF A-chain mRNA and inhibition of growth by anti-PDGF antibody. To determine whether H2O2 stimulated the same pathway, [3H]thymidine incorporation was measured in the pres-
ence of preimmune serum or anti-PDGF antibody. There was no inhibition of DNA synthesis: 300 ± 3% and 475 ± 13% increases over 0.4% serum, respectively (p < 0.1). In contrast, uric acid-stimulated [3H]thymidine incorporation was inhibited by 100 ± 8%. Thus, H2O2-stimulated DNA synthesis in VSMCs is not dependent on release of PDGF.

Discussion

The present study demonstrates that the active oxygen species H2O2 stimulates VSMC proliferation in association with induction of c-myc and c-fos mRNA. Data supporting H2O2 as a novel VSMC growth factor include increases in cell number, DNA synthesis, and proto-oncogene expression. Further, among cells present in the vessel wall, H2O2 was mitogenic only for VSMCs and not for endothelial cells or fibroblasts. Although active oxygen species can cause DNA damage and stimulate DNA repair mechanisms, this is unlikely to explain H2O2-stimulated [3H]thymidine incorporation in VSMCs. First, H2O2 caused no increase in lactate dehydrogenase release or in the number of cells that failed to exclude trypan blue. Second, H2O2 has been shown to be a short patch DNA repair inducer and, unlike agents such as N-acetoxy-2-acetylaminofluorene, causes only small increases (<10% of those shown here) in unscheduled DNA synthesis.33 Third, although H2O2 has been shown to increase unscheduled DNA synthesis (e.g., in hepatocytes), this did not occur until 1.9 mM H2O2 (nearly 10 times the concentration used here) and was marked by increased DNA synthesis within 3 hours.34 Thus, H2O2-stimulated [3H]thymidine incorporation is primarily due to VSMC growth and not to cell damage.

Recent studies indicate that oxidants activate cellular growth and play a significant role in tumor promotion and degenerative diseases.35,36 In fact, the results obtained in these studies of mammalian cells as well as VSMCs may reflect evolution of an oxidant-induced program of gene expression. This is demonstrated by findings that H2O2 activates OxyR protein, a transcriptional factor for oxidative stress-inducible genes in Escherichia coli.37 More recently, oxidant stress has been shown to activate AP-1-mediated transcription directly and NF-kB-mediated transcription by unknown mechanisms. H2O2-induced c-fos and c-myc expression may therefore represent part of an oxidant-induced growth program. Although proto-oncogene induction was dependent on PKC activation, VSMC DNA synthesis was not decreased by the PKC inhibitor, sphingosine. This suggests that other signal events are involved in H2O2-stimulated VSMC growth. Recently, it has been shown that vanadate may be converted into both V(4+)-OO and V(4+)-OOH by superoxide or H2O2 in the presence of NADPH.40 These vanadate peroxides are potent inhibitors of phosphotyrosine phosphatases. By increasing tyrosine phosphorylation, such active oxygen species by-products may stimulate growth factor–like events.

The importance of oxidant-induced growth in the vessel wall is underscored by the increasing evidence for monocyte/macrophage involvement in atherogenesis and acute arterial injury syndromes.4 In models of acute arterial injury such as occur with unstable angina, plaque rupture, or balloon angioplasty, there is even more compelling data for inflammatory cells and their products as the cause of VSMC proliferation. In patients with unstable angina, circulating levels of markers for leukocyte activation (such as C-reactive protein41 and fibrinogen degradation peptide B342) are increased, inflammatory cells are present in the adventitia of the vessel wall,43 and VSMCs express new antigens (such as Ia and HLA-DR) induced by activated T lymphocytes.44 Endothelial cells can also generate active oxygen species.18,19 In fact, inflammatory cell-derived peptides such as tumor necrosis factor and N-formyl-Met-Leu-Ph e have been shown to activate endothelial cell xanthine oxidase by conversion of xanthine dehydrogenase to xanthine oxidase.18 Thus, endothelial cells exposed to inflammatory products in vivo may further contribute to formation of active oxygen species.
It is now clear that xanthine/xanthine oxidase products including active oxygen species are important growth factors for the blood vessel, based on the present study, our previous work showing that uric acid is a VSMC mitogen, as well as other studies. Uric acid failed to induce early growth events, such as PKC activation or proto-oncogene expression, but stimulated an autocrine growth mechanism involving the PDGF A-chain. The present findings that both xanthine/xanthine oxidase and H$_2$O$_2$ stimulated early growth signals suggest that a synergistic effect among active oxygen species may promote VSMC growth. During arterial injury, the differential effects of H$_2$O$_2$ on VSMCs, endothelial cells, and fibroblasts may lead to accelerated VSMC growth in this manner: H$_2$O$_2$ selectively damages endothelial cells, causing release of VSMC growth factors (e.g., fibroblast growth factor), while H$_2$O$_2$ also selectively stimulates VSMC cell cycle progression. Future in vivo studies will determine the importance of active oxygen species for VSMC proliferation in atherosclerosis and restenosis.

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