Role of NADH/NADPH Oxidase–Derived H_2O_2 in Angiotensin II–Induced Vascular Hypertrophy

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***Abstract*—**Recent evidence suggests that oxidative mechanisms may be involved in vascular smooth muscle cell (VSMC) hypertrophy. We previously showed that angiotensin II (Ang II) increases superoxide production by activating an NADH/NADPH oxidase, which contributes to hypertrophy. In this study, we determined whether Ang II stimulation of this oxidase results in H_2O_2 production by studying the effects of Ang II on intracellular H_2O_2 generation, intracellular superoxide dismutase and catalase activity, and hypertrophy. Ang II (100 nmol/L) significantly increased intracellular H_2O_2 levels at 4 hours. Neither superoxide dismutase activity nor catalase activity was affected by Ang II; the SOD present in VSMCs is sufficient to metabolize Ang II–stimulated superoxide to H_2O_2, which accumulates more rapidly than it is degraded by catalase. This increase in H_2O_2 was inhibited by extracellular catalase, diphenylene iodonium, an inhibitor of the NADH/NADPH oxidase, and the AT_1 receptor blocker losartan. In VSMCs stably transfected with antisense p22phox, a critical component of the NADH/NADPH oxidase in which oxidase activity was markedly reduced, Ang II–induced production of H_2O_2 was almost completely inhibited, confirming that the source of Ang II–induced H_2O_2 was the NADH/NADPH oxidase. Using a novel cell line that stably overexpresses catalase, we showed that this increased H_2O_2 is a critical step in VSMC hypertrophy, a hallmark of many vascular diseases. Inhibition of intracellular superoxide dismutase by diethylthiocarbamate (1 mmol/L) also resulted in attenuation of Ang II–induced hypertrophy (62±2% inhibition). These data indicate that AT_1 receptor–mediated production of superoxide generated by the NADH/NADPH oxidase is followed by an increase in intracellular H_2O_2, suggesting a specific role for these oxygen species and scavenging systems in modifying the intracellular redox state in vascular growth. **(*Hypertension*. 1998;32:488-495.)**

Key Words: vascular smooth muscle ■ angiotensin II ■ NADH ■ NADPH oxidase ■ hydrogen peroxide ■ superoxide dismutase ■ catalase ■ hypertrophy

Hypertension, atherosclerosis, and mechanical injury share many common pathologic effects on the vessel wall, including vascular smooth muscle proliferation, monocyte/macrophage infiltration, dysfunction of regenerated endothelium, and increased deposition of connective tissue.[1] Remarkably, these conditions are all associated with an increased oxidative stress.[2–4] Although the contribution of low-density lipoprotein oxidation and lipoproteins to atherosclerosis has long been established, recent experiments have shown that early inflammatory events are also redox-sensitive.[5] Furthermore, some forms of hypertension, notably those associated with high circulating levels of angiotensin II (Ang II), are accompanied by and consequent on the production of superoxide (O_2^-).[6,7] Reactive oxygen species have also been implicated in the development of restenosis after angioplasty.[4,8] Thus oxidative stress appears to be an important component of vascular pathology.

One potentially significant consequence of oxidative stress is increased vascular smooth muscle cell (VSMC) proliferation.[9] In VSMCs, the combination of xanthine with xanthine oxidoreductase (which yields H_2O_2 and O_2^-), the naphthoquinolinedione LY 83,583 (which is metabolized intracellularly to O_2^-), and H_2O_2 itself all stimulate DNA synthesis and proliferation and induce the expression of growth-related genes, including c-*fos*, c-*myc,* and c-*jun*.[10–12] Furthermore, treatment of VSMCs with antioxidants can induce apoptosis, which implies that reactive oxygen species are necessary for normal proliferation.[13] Taken together, these observations suggest that regulation of the redox state of the cell may be a general mechanism by which growth signals are transduced.

Recent data from our laboratory indicate that Ang II, an important vasoconstrictor and hypertrophic agent, induces oxidative stress in VSMCs.[3,7,14,15] Ang II stimulates O_2^- generation by activating an NADH/NADPH oxidase. Importantly, inhibition of this enzymatic pathway by diphenylene iodonium (DPI) or by antisense transfection of p22phox, a critical component of the NADPH oxidase, inhibits Ang II–induced hypertrophy.[14,15] Because of the participation of the renin-angiotensin system in several forms of vascular disease,[16] the pro-oxidant effects of Ang II on smooth muscle

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cell proliferation are important. Although many signaling mechanisms initiated and used by Ang II to mediate VSMC proliferation have been well characterized,[17–20] those related to oxidative stress have not been clearly defined.

As noted, we have previously shown that the NADH/NADPH oxidase produces O_2^-.[14] Other oxidases (eg, xanthine oxidase and nitric oxide synthase) are capable of performing both 1- and 1- and 2-electron reductions of O_2^-, thus producing O_2^- and H_2O_2.[21] Whether this is also true for the vascular NADH/NADPH oxidase is unknown. Alternatively, H_2O_2 may be solely derived by dismutation of superoxide by superoxide dismutase (SOD). Because H_2O_2 is then converted by catalase to H_2O or scavenged by glutathione, regulation of any of these enzyme systems may modulate H_2O_2 levels. The specific biological roles of O_2^- and H_2O_2 are unclear, but recent evidence suggests that they may differentially affect intracellular signaling pathways. One example is the p42/44 mitogen-activated protein kinases (MAPK), which are activated by O_2^- but not H_2O_2.[22] Activation of this kinase pathway is necessary but not sufficient for growth, indicating that other redox-sensitive pathways may exist. H_2O_2 activates early response genes required for growth (c-*fos*, c-*jun*)[11,12] as well as another member of the MAPK family, p38 MAPK.[23] Thus, although both O_2^- and H_2O_2 have been implicated in cell growth, their relative importance in agonist-mediated signaling and the regulation of the pathways by which they are produced have not been established.

In the present study, we investigated whether Ang II stimulation of the NADH/NADPH oxidase results in production of H_2O_2 and assessed the effect of Ang II on H_2O_2 accumulation as well as SOD and catalase activity. Most importantly, we also investigated specifically the role of intracellularly produced H_2O_2 in agonist-induced hypertrophy. We found that Ang II stimulated an NADH/NADPH oxidase–dependent accumulation of intracellular H_2O_2 through dismutation of O_2^- without altering the activity of SOD or catalase. Intracellular H_2O_2 was absolutely required for Ang II–induced hypertrophy, which suggests that this reactive oxygen species regulates expression or activation of growth-related signaling pathways. The various oxidant and antioxidant mechanisms that are initiated and orchestrated by Ang II support the notion that these mechanisms are an integral part of the growth-promoting effects of Ang II and ultimately contribute to regulation of the redox state of the cell. These observations suggest that reactive oxygen species may mediate the hypertrophic response and thus may influence the pathogenesis of vascular disease.

Methods

Cell Culture

VSMCs were isolated from rat thoracic aorta by enzymatic digestion as described previously.[24] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and were passaged twice per week by harvesting with trypsin:EDTA and seeding into 75-cm^2 flasks. For experiments, cells between passage levels 6 and 20 were seeded into 35-mm and 100-mm dishes, fed every other day, and used at confluence.

In some experiments, we used rat aortic smooth muscle cells stably transfected with antisense p22phox, a critical component of the NADH/NADPH oxidase, as described by Ushio-Fukai et al.[15] In these transfected cells, p22phox expression is completely eliminated.

Intracellular H_2O_2 Measurement

VSMCs were plated at low density, grown for 48 hours in culture medium containing 10% calf serum, and quiesced for an additional 24 hours in culture medium containing 0.1% calf serum. Cells were then stimulated with Ang II (100 nmol/L) for 4 hours to 24 hours. For assays, medium was replaced with Hanks' solution containing the H_2O_2-sensitive fluorophore 2′,7′-dichlorofluorescin diacetate (DCF-DA, 5 μmol/L) at appropriate times after stimulation, as previously described.[25] Although DCF-DA is oxidized by both H_2O_2 and other peroxides, the complete inhibition of fluorescence in Ang II–stimulated cells by addition of catalase (350 U/mL) and in catalase-transfected cells indicates that the fluorescence signal evoked by Ang II was predominantly derived from H_2O_2. Calibration of this signal with exogenously added H_2O_2 indicated that the increase in fluorescence detects 10 to 100 nmol/L H_2O_2 in a linear fashion.

Superoxide Dismutase Assay

VSMCs exposed to Ang II or media (control) for 4 hours were washed 5 times with 5 mL ice-cold phosphate-buffered saline and scraped from the plate in 5 mL of this same solution. Samples were transferred to a 50 mL centrifuge tube, and the plate was washed twice with an additional 5 mL of phosphate-buffered saline to remove any remaining tissue. Cells were then centrifuged at 740*g* at 4°C for 10 minutes. The supernatant was discarded, and the pellet was resuspended (0.5 mL per dish) in lysis buffer containing protease inhibitors (50 mmol/L monobasic potassium phosphate [pH 7.8], 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mmol/L phenylmethylsulfonyl fluoride). The cell suspension was then dounced 100 times on ice, and the homogenate was stored on ice until use. Protein content was measured in an aliquot of the homogenate by the method of Lowry et al.[26] SOD activity was determined spectrophotometrically by the ability of the homogenate (50 or 100 μg total protein) to inhibit the reduction of cytochrome c by O_2^- generated by the addition of 0.118 mmol/L xanthine and 10 mU/mL xanthine oxidase (final volume, 1.0 mL). In each experiment, a parallel determination was performed in the presence of 1 mmol/L KCN. The Cu/Zn SOD activity was calculated as the activity inhibited by KCN, calibrated with known amounts of purified bovine SOD. Values obtained were expressed as units of SOD per milligram of protein.

Catalase Assay

The enzyme activity of catalase in the cell homogenates was assayed by monitoring decomposition of H_2O_2 (10 mmol/L) by the rate of decrease in absorbance at 240 nm, as previously described by Aebi.[27] Calibrations were performed with known amounts of beef liver catalase. Because the activity of catalase is known to be nonlinear, measurements were restricted to optical density values over the initial 30 seconds of the assay. All measurements of catalase activity were obtained from triplicate cultures and expressed as mean±SE.

NADH/NADPH Oxidase Assay

NADH/NADPH oxidase activity was measured as described previously.[14] Briefly, control VSMCs or cells that had been exposed to diethyldithiocarbamate (DETC) or vehicle for 4 hours in the presence or absence of Ang II were washed, and cells were scraped from the plate. Cells were then centrifuged at 740*g* at 4°C for 10 minutes. The supernatant was discarded, and the pellet was resuspended (0.5 to 1.0 mL per dish) in lysis buffer. The cell suspension was then dounced, and the homogenate was stored on ice until use. Protein content was measured in an aliquot of the homogenate by the method of Lowry et al.[26] NADH/NADPH oxidase activity was measured in a luminescence assay with 500 μmol/L lucigenin as the electron acceptor and either 100 μmol/L NADH or 100 μmol/L NADPH as the substrate (final volume, 0.9 mL). The reaction was started by the

addition of 100 μL of homogenate (50 to 300 μg protein). Luminescence was monitored as described previously.[14]

[^{3}H]Leucine Incorporation

To measure hypertrophy of VSMCs, cells were plated at low density, grown to 60% confluence for 2 to 3 days in DMEM containing 10% calf serum, and grown for an additional 48 to 72 hours in DMEM containing 0.1% calf serum. Twenty-four hours before harvest, cells were incubated with [^{3}H]leucine (2 μCi/mL) in the presence or absence of 100 nmol/L Ang II; after washing, [^{3}H]leucine incorporation was assessed as described previously.[14] In some experiments, changes in total protein were measured by the Biorad microassay, based on the method of Bradford.

Stable Transfection of Catalase Expression Plasmid

Human catalase cloned into the eukaryotic expression plasmid pCI-neo was a kind gift of Drs Sampath Parthasarathy and Nalini Santanam. Four micrograms of purified pCI-neo alone or pCI-neo/catalase plasmid in 100 μL H_2O was gently mixed with lipofectin solution (100 μL). The DNA/liposome complex was added directly to 40% to 50% confluent VSMCs plated in 60-mm dishes in Opti-MEM I reduced serum medium and incubated for 18 hours at 37°C. The medium was then changed to DMEM containing 20% fetal bovine serum (FBS). After 48 hours, transfected VSMCs were split 1:3 into 100-mm dishes and incubated in DMEM containing 10% FBS and 400 μg/mL geneticin. Eight days after selection, geneticin-resistant colonies were isolated with the use of cloning cylinders. Transfected cells were maintained in selection medium until they were plated into 35- or 100-mm dishes for experiments.

Measurement of Catalase mRNA

Total RNA was extracted from cells as described previously.[28] Ten-microgram RNA samples were separated by electrophoresis in 1.0% agarose gels containing 6.6% formaldehyde. RNA was transferred onto a nylon membrane and immobilized by UV cross-linking (Stratalinker, Stratagene). The probe, catalase cDNA derived from *Xba*I/*Sal*I digestion of pCI-neo/catalase, was labeled with [α-^{32}P]-dCTP with the use of a random primer labeling kit (Prime-It II). After UV cross-linking, membranes were prehybridized at 68°C for 2 hours in QuikHyb solution (Stratagene). The hybridization was performed for 2 hours at 68°C with ^{32}P-labeled probe in the same solution. Membranes were briefly rinsed and washed twice in 1×SSC+0.1% SDS at 50°C. Staining of the 28S rRNA band by ethidium bromide, after transfer to the membrane, was used for normalization.

Measurement of SOD and Catalase Protein Levels

Confluent untransfected VSMCs, VSMCs transfected with pCI-neo alone, or VSMCs transfected with pCI-neo/catalase plasmid in 100-mm dishes were washed 3 times with 5 mL of ice-cold phosphate-buffered saline (PBS). Cells were scraped in ice-cold sonication buffer, pH 7.4 [(mmol/L) 50 HEPES, 5 EDTA, 50 NaCl], containing protease inhibitors (10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin) and phosphatase inhibitors [(mmol/L) 50 sodium fluoride, 1 sodium orthovanadate, 10 sodium pyrophosphate]. For measurement of catalase, samples were sonicated for 30 seconds ×3 on ice. For measurement of SOD, 1% Triton X-100 was included in the buffer, and the Triton-soluble fraction was collected by centrifugation at 10 000*g* (4°C, 20 minutes). Extracted protein was quantified by the Bradford assay. Proteins were separated with SDS-PAGE and transferred to nitrocellulose membranes (catalase) at 100 V for 1 hour or PVDF membranes (SOD) at 50 V for 2 hours. Membranes were blocked for 1 hour with PBS containing 5% nonfat dry milk and 0.1% Tween 20 and were incubated for 1 hour with primary anti-human erythrocyte catalase antibody (1:500) or SOD antibody (1:100) in PBS containing 1% nonfat dry milk and 0.1% Tween 20 and then incubated with HRP-conjugated secondary antibody for 1 hour. Catalase and SOD protein levels were detected by ECL chemiluminescence.

Ang II Receptor Binding

The Ang II receptor binding assay was performed as described previously.[29] B_{max} (maximum number of binding sites) was determined with single saturation point binding.

Statistical Analysis

Overall statistical significance was assessed by Student's paired 2-tailed *t* test or analysis of variance on untransformed data, followed by comparison of group averages by contrast analysis, with the use of the SuperANOVA statistical program (Abacus Concepts). A value of $P<0.05$ was considered to be statistically significant.

Materials

All chemicals were of analytical grade or better. Bovine serum albumin, beef liver catalase, and phenylmethylsulfonyl fluoride were from Boehringer Mannheim. Calf serum, lipofectin, geneticin, Opti-MEM, glutamine, penicillin, streptomycin, and trypsin/EDTA were purchased from GIBCO. FBS was from Atlanta Biologicals. Liquiscint was purchased from National Diagnostics. Common buffer salts were obtained from Fisher. DCF-DA was obtained from Acros. The Prime-It II kit and QuikHyb solution were from Stratagene. pCI-neo was from Promega. DETC was purchased from Aldrich. Anti-human Cu/Zn superoxide dismutase IgG was obtained from Biodesign International, and anti-human erythrocyte catalase, IgG-fraction, was obtained from Athens Research Technology. [^{3}H]leucine (140 Ci/mmol) and α^{32}P-dCTP were from DuPont NEN. The ECL Western blotting system was from Amersham. DPI was purchased from Toronto Research Chemicals. All other chemicals and reagents, including DMEM with 25 mmol/L HEPES and 4.5 g/L glucose and calf serum, were from Sigma.

Results

Ang II Stimulation of Intracellular H_2O_2 Production

To determine whether Ang II stimulates intracellular H_2O_2 production, VSMCs treated with Ang II (100 nmol/L, 4 hours) were incubated with DCF-DA, a peroxide-sensitive dye that is incorporated into the cell. Ang II caused a dramatic increase in DCF-DA fluorescence (Figure 1B) compared with that in control cells (Figure 1A). This increase averaged 366±70% of control (Figure 2, n=4, 12 fields for each experiment). Catalase, an enzyme that specifically decomposes H_2O_2 to water and molecular oxygen, completely inhibited the increase in DCF-DA oxidation, which suggests that intracellular H_2O_2 is responsible for the DCF-DA oxidation after Ang II treatment (Figure 1C). The Ang II–stimulated increase in H_2O_2 at all time points was inhibited when cells were preincubated with losartan (10 μmol/L), a specific AT_1 receptor blocker, indicating that this induction was AT_1 receptor specific (Figure 1D).

To determine whether the vascular NADH/NADPH oxidase stimulated by Ang II[14] is the source of H_2O_2, we used 2 approaches. First, VSMCs were preincubated with DPI (10 μmol/L), a molecule that binds to and competitively inhibits flavin-containing enzymes such as the NADH/NADPH oxidase, before Ang II treatment. Preincubation with DPI resulted in complete inhibition of the Ang II–induced increase in H_2O_2 (Figure 3, A through C), which suggests that a flavin-containing enzyme is the source for H_2O_2. Second, to provide more definitive evidence that the ultimate source of H_2O_2 is the NADH/NADPH oxidase, VSMCs transfected with antisense p22phox, which attenuates functional expression of the NADH/NADPH oxidase,[15] were treated with Ang

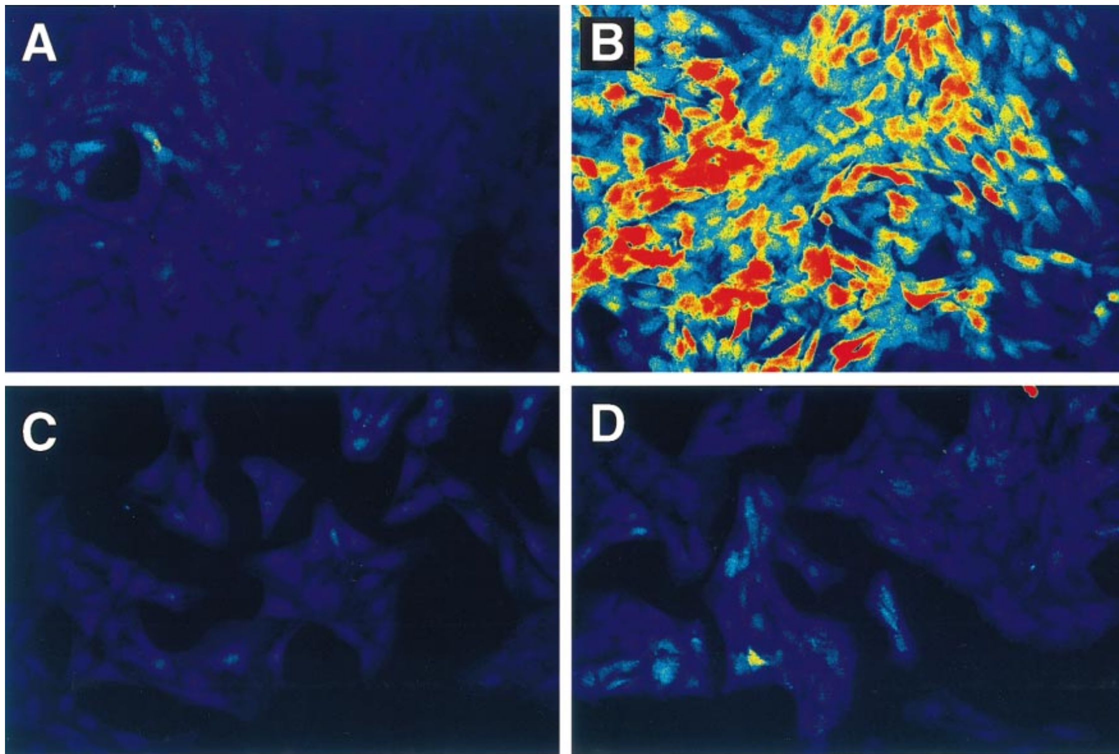

Figure 1. Intracellular H_2O_2 production in VSMCs. VSMCs were stimulated without (A) or with (B) Ang II (100 nmol/L) for 4 hours and incubated with the peroxide-sensitive fluorophore DCF-DA before laser confocal microscopy. VSMCs were stimulated with Ang II in presence of catalase (350 U/mL) (C) or losartan (10 μmol/L) (D) before incubation with DCF-DA and laser confocal microscopy. Fluorescence was visualized at ×20 magnification with laser intensity of 30, iris setting of 3.5, and gain of 1200.

II for 4 hours. The agonist-stimulated increase in H_2O_2 was dramatically decreased in antisense p22phox-transfected cells (Figure 3D), which indicates that the NADH/NADPH oxidase is the major source for H_2O_2 production in Ang II–stimulated VSMCs.

H_2O_2 production by the NADH/NADPH oxidase could result directly from 1-electron reduction of O_2 or by a 2-step process in which the NADH/NADPH oxidase generates O_2^-, which is then dismuted by SOD to H_2O_2. To distinguish between these possibilities, we measured the effect of DETC,

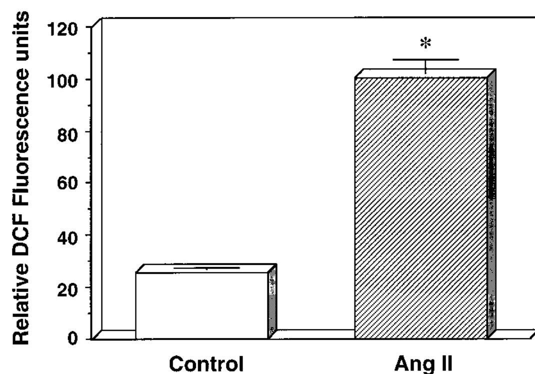

Figure 2. Production of H_2O_2 by Ang II. VSMCs were stimulated without (open bars) or with (hatched bars) Ang II (100 nmol/L) for 4 hours and incubated with the peroxide-sensitive fluorophore DCF-DA before laser confocal microscopy. Values for relative DCF-DA fluorescence intensity (scale 0 to 256 U) are mean±SE obtained from 4 separate experiments in which 12 visual fields were quantified. *$P<0.01$ for increase with Ang II versus without Ang II.

a SOD inhibitor, on Ang II–induced H_2O_2 production. Incubation of VSMCs with DETC (1 mmol/L) before addition of Ang II inhibited H_2O_2 generation by 80±8% (n=4, $P<0.05$). Furthermore, Ang II–induced NADH/NADPH-dependent O_2^- production was higher in DETC-treated cells than in control cells (data not shown), which verifies the efficacy of DETC in inhibiting SOD and indicates that SOD is an obligatory step in H_2O_2 production in VSMCs stimulated with Ang II.

Effect of Ang II on Superoxide Dismutase and Catalase

The above data indicate that the vascular NADH/NADPH oxidase is the predominant source of H_2O_2. However, an increase in H_2O_2 production could also result from an Ang II–induced imbalance between the activity of SOD (which produces H_2O_2) and catalase (which scavenges H_2O_2 in VSMCs). To determine the effect of Ang II on Cu/Zn SOD and catalase, we prepared cell homogenates and assayed enzyme activity. Homogenates of VSMCs treated for 4 hours with Ang II (100 nmol/L) showed no change in SOD activity compared with control cells (Figure 4A). Ang II also had no effect on Cu/Zn SOD protein levels (Figure 4B). Catalase activity was very low in VSMCs and did not change perceptibly with Ang II (data not shown). Together with the observed accumulation of H_2O_2, these data suggest that the SOD present in VSMCs is sufficient to metabolize O_2^- to H_2O_2, which accumulates more rapidly than it is degraded by catalase.

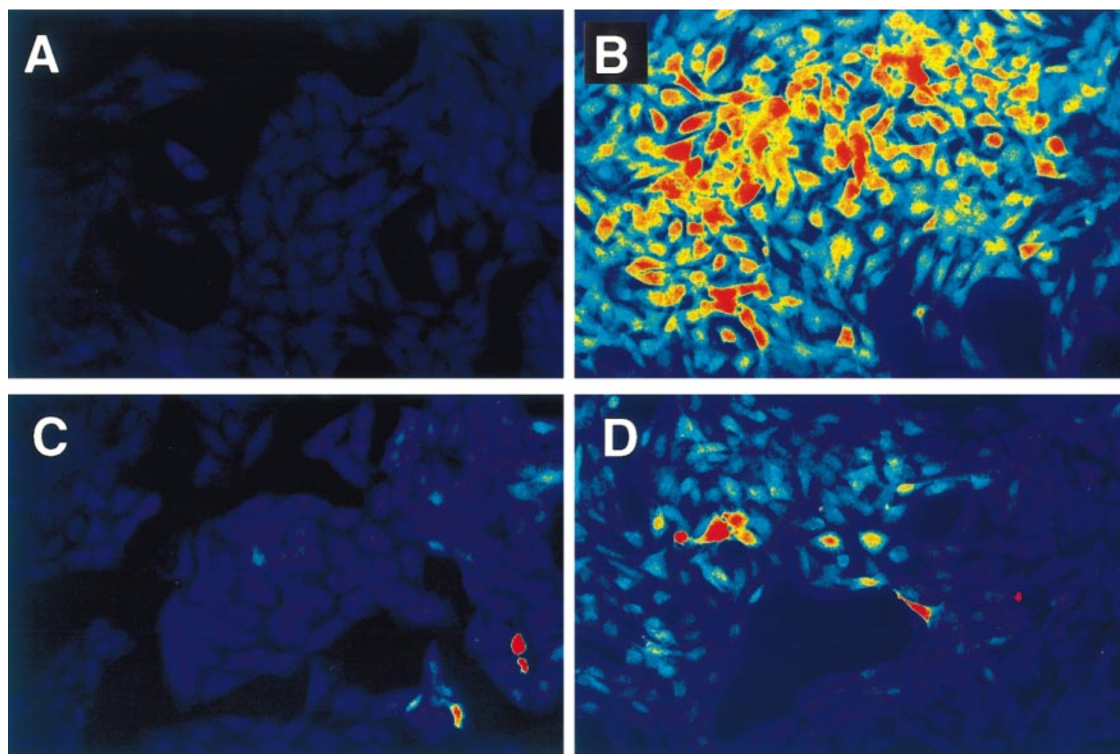

Figure 3. NADH/NADPH oxidase is a major source for intracellular H_2O_2 generation. VSMCs were untreated (A), stimulated with Ang II (100 nmol/L) alone for 4 hours (B), or treated with Ang II in presence of DPI (10 μmol/L) and incubated with the peroxide-sensitive fluorophore DCF-DA (C). VSMCs transfected with antisense p22phox were treated with Ang II for 4 hours before incubation with DCF-DA (D). Images were obtained by laser confocal microscopy. Fluorescence was visualized at $\times$20 magnification with laser intensity of 30, iris setting of 3.5, and gain of 1200.

Role of H_2O_2 in Ang II–Induced Hypertrophy

We have previously shown that NADH/NADPH oxidase activity is required for hypertrophy.[14,15] Because ambient SOD levels appear to be adequate to handle the O_2^- produced, inhibition of SOD should also attenuate hypertrophy if conversion of O_2^- to H_2O_2 by SOD is required for hypertrophic signaling. As shown in Figure 5, Ang II (100 nmol/L) increased [^{3}H]leucine incorporation to 162±3% of control. DETC (1 mmol/L) attenuated this increase by 62±2% (n=4, $P<0.01$).

As a second approach to defining the role of H_2O_2 in Ang II–induced hypertrophy, we used stable transfection to overexpress catalase in VSMCs. We initially isolated 39 clones of geneticin-resistant cells; however, only 2 of these significantly overexpressed catalase mRNA and protein (Figure 6A). Those expressing the highest level of catalase protein were selected for further study. Overexpression of catalase inhibited Ang II–induced H_2O_2 production by 62±6%, which indicates that the catalase was functionally effective and incidentally confirms that DCF-DA oxidation in fact reflects H_2O_2 levels in these cells. As shown in Figure 6B, overexpression of catalase significantly inhibited Ang II–stimulated hypertrophy, as measured by [^{3}H]leucine incorporation, at every dose of Ang II tested. Similar results were obtained with the second line of catalase-overexpressing cells (maximal hypertrophy in response to Ang II, 100 nmol/L: 106±4% control). This effect was not due to differences in AT_1 receptor expression in catalase-overexpressing cells because vector- and catalase-transfected cells were matched for receptor number (B_{max}: 919 fmol/mg protein and 1237 fmol/mg protein in vector- and catalase-transfected cells, respectively). Furthermore, catalase overexpression did not affect phospholipase C activation by Ang II (data not shown), which indicates that the decrease in hypertrophy is not a result of nonspecific inhibition of signaling pathways. To confirm that [^{3}H]leucine incorporation faithfully reflected increased protein synthesis, we measured total protein after 24 hours of Ang II (100 nmol/L) treatment in control and catalase-transfected cells. Ang II caused a 22±4% increase (n=3) in total protein in vector-transfected cells, and this increase was significantly inhibited in catalase-transfected cells (13±3, n=3, $P<0.03$ versus vector). These data suggest that H_2O_2 is a necessary mediator of Ang II–induced hypertrophy.

Discussion

The data presented here provide evidence defining the molecular pathways that lead to agonist-induced regulation of intracellular redox state in VSMCs and establish a sequential link between Ang II–induced NADH/NADPH oxidase activity, an increase in intracellular H_2O_2, and hypertrophy. When oxidase activity is decreased either with DPI or by transfection with antisense p22phox, the Ang II–induced increase in H_2O_2 is eliminated, establishing the NADH/NADPH oxidase as the primary source of H_2O_2 in Ang II–stimulated VSMCs. H_2O_2 appears to be formed by dismutation of O_2^- because DETC inhibited H_2O_2 production. Because SOD and catalase activities are unaffected by Ang II, the ambient level of SOD may be sufficient to metabolize O_2^- to H_2O_2, which accumu-

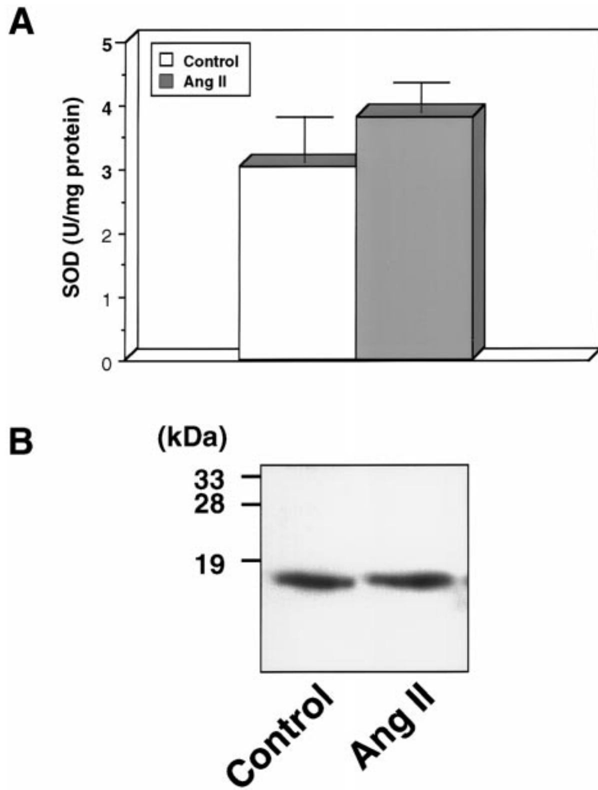

Figure 4. Effect of Ang II on SOD activity and protein levels. VSMCs were stimulated with or without Ang II (100 nmol/L) for 4 hours. A, Cells were homogenized and SOD activity was determined by measuring the ability of the homogenate to inhibit xanthine/xanthine oxidase–induced superoxide production. Each bar represents mean±SEM of 4 experiments. B, SOD protein expression was assessed with Western analysis. This blot is representative of 3 similar experiments.

lates more rapidly than it is degraded by catalase. These data further demonstrate that intracellular NADH/NADPH oxidase-dependent H_2O_2 production is necessary for Ang II–induced protein synthesis, since inhibition of SOD activity or overexpression of catalase profoundly inhibited hypertrophy.

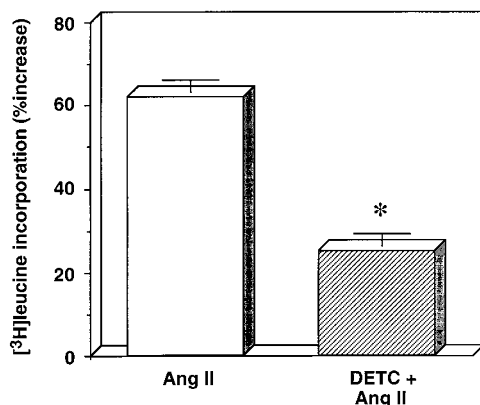

Figure 5. Attenuation of Ang II–induced hypertrophy by inhibition of SOD. [^{3}H]leucine-labeled VSMCs were exposed to Ang II (100 nmol/L) in the presence or absence of DETC (1 mmol/L) for 24 hours. [^{3}H]leucine incorporation was measured as described in "Methods." Data are expressed as percent increase in [^{3}H]leucine incorporation induced by Ang II over the appropriate control. Each bar represents mean of 4 experiments performed in triplicate. *$P<0.01$ for increase in the presence of inhibitor versus increase with Ang II alone.

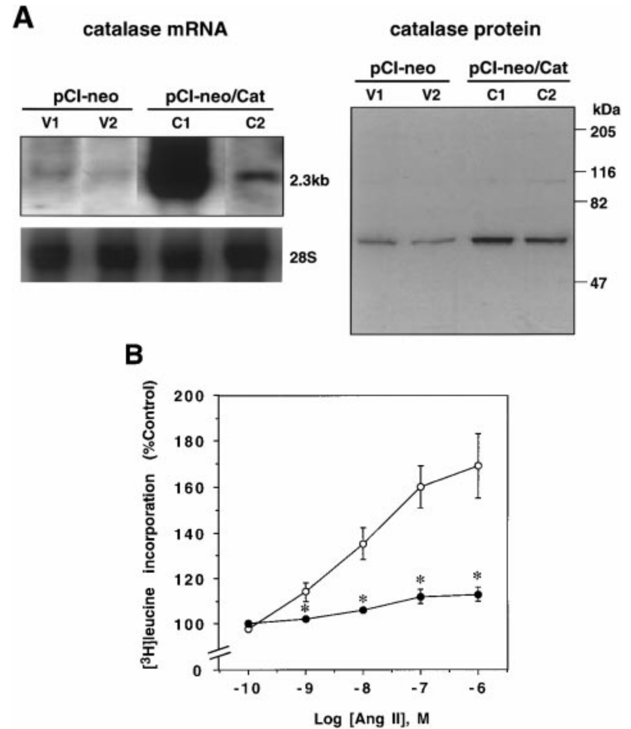

Figure 6. Attenuation of Ang II–stimulated hypertrophy by overexpression of catalase. VSMCs were transfected with vector alone (pCI-neo) or with vector-containing catalase (pCI-neo/Cat) as described in "Methods." A, Representative blots of catalase mRNA (A) and protein (B) levels in vector-transfected clones (V1 and V2) and selected catalase-transfected clones with high mRNA expression (C1 and C2). B, Vector-transfected (○) and catalase-transfected (●) [^{3}H]leucine-labeled VSMCs were exposed to indicated concentrations of Ang II for 24 hours. [^{3}H]leucine incorporation was measured as described in "Methods." Data are expressed as percent increase in [^{3}H]leucine incorporation induced by Ang II over appropriate control. Each bar represents mean of 3 experiments performed in triplicate. *$P<0.05$ for increase in catalase-transfected cells compared with vector-transfected cells.

Whereas both O_2^- and H_2O_2 are established products of the respiratory burst in phagocytic cells when the plasma membrane NADPH oxidase is activated, it is now becoming clear that both are also normally released by a variety of noninflammatory cells, including the different layers of the vessel wall.[3,14,30] Previous studies have suggested that in tumor cells H_2O_2 is likely to be derived from O_2^- released by an NADPH-dependent system, as it is inhibited by DPI.[31] Other intracellular sources of O_2^- generation have also been demonstrated, including mitochondria and peroxisomes.[3,32,33] We show here that the Ang II–induced increase of H_2O_2 in VSMCs is NADH/NADPH oxidase-dependent because it is inhibited in cells treated with DPI or transfected with antisense p22phox. H_2O_2 does not appear to be formed directly from the NADH/NADPH oxidase because the SOD inhibitor DETC attenuated H_2O_2 production, although the relative nonspecificity of DETC permits alternative interpretations. This is the first direct demonstration of the fate of the NADH/NADPH-derived O_2^-.

Exogenous O_2^- and H_2O_2 can each stimulate growth and growth responses in a variety of cultured cell types by functioning as mitogenic stimuli through biochemical processes common to natural growth factors.[9] Both H_2O_2 and O_2^- generation by the naphthoquinolinedione LY 83,583 stimulate mitogenesis in VSMCs; however, only O_2^- activates p42/44 MAPK.[10,22] In contrast, H_2O_2 stimulates p38 MAPK activity in VSMCs, and both p38 MAPK and the p42/44 MAPK are required for Ang II–induced hypertrophy.[34] In the case of exogenously added O_2^-, certain of its effects related to growth stimulation are extremely rapid and appear to be distinct from those of H_2O_2, like early changes in pH and Ca^{2+} concentration in human amnion cells.[35] It has been suggested that adjustment of the redox states of proteins involved in growth pathways is a prerequisite for optimal functioning; at present distinctive effects of O_2^- compared with H_2O_2 are difficult to assess.

In this study, the inhibition of hypertrophy in catalase-overexpressing cells suggests that H_2O_2 may be the biologically important reactive oxygen species in Ang II–induced growth responses. The use of cells transfected with catalase permits targeting catalase intracellularly, overcoming the difficulties of previous studies in which catalase exerted its effect by hydrolyzing H_2O_2 as it diffused out of the cell. Sundaresan et al[36] showed that the mitogenic agent PDGF increased H_2O_2 in VSMCs and that application of high levels of catalase exogenously inhibited PDGF-induced signaling and proliferation. Together with our results, these data suggest that H_2O_2 appears to be important in both hyperplasia and hypertrophy, although the molecular targets of H_2O_2 in the growth program remain unclear.

Although many studies report pro-proliferative effects of reactive oxygen species on VSMCs, some conflicting results have been reported. Fiorani et al[37] found that H_2O_2 induces cell death despite the fact that it increases DNA synthesis. Furthermore, when VSMCs are exposed to glucose/glucose oxidase or diethylmaleate, the resulting H_2O_2 induces apoptosis through the formation of hydroxyl radicals.[38] The explanation for these apparently disparate results may be the magnitude of alterations in redox state. Treatment of VSMCs with antioxidants such as pyrrolidinedithiocarbamate or *N*-acetylcysteine leads to apoptosis, which suggests that some level of oxidant stress is required for normal growth.[13] Thus, although a certain level of oxidant stress appears to be growth promoting, more severe stress may lead to cell death. Because Ang II causes hypertrophy of VSMCs, it appears that the H_2O_2 produced by Ang II is of a magnitude consistent with overall activation of the growth program.

Ang II is a crucial hypertrophic/hyperplastic effector or proinflammatory mediator in hypertension, restenosis after angioplasty, and atherosclerosis.[1] The long-term nature of the NADH/NADPH oxidase-dependent O_2^- generation by Ang II, combined with the apparent association of H_2O_2 with growth, suggests that these oxygen species and their generating enzymes may be an integral part of the intracellular redox system, priming the smooth muscle for hypertrophy and growth. Interestingly, both protein tyrosine kinases and protein tyrosine phosphatases, signaling pathways intimately involved in the growth response in many cell types, are regulated by reactive oxygen species.[22,39] In addition, because the redox state of transcription factors and of protein kinases appears relevant to their general level of activity,[40] it is likely that the overall cellular redox state may be critical. Superoxide and H_2O_2 may induce a growth response by modulating the efficiency of the overall process of signal transduction at various intracellular locations rather than by interacting with sensors by analogy with growth factor–growth factor receptor interaction. This may occur through oxidation of signal transduction proteins or transcription factors through their ability to modulate intracellular scavenging systems like catalase or glutathione peroxidase. In view of the critical balance between the degree of oxidative stress and the antioxidant capacity of scavenging systems in relation to cell growth on one hand and lipid peroxidation and apoptosis on the other, it is important to assess how these may be modified in normal vascular physiology as well as in pathophysiological states.

In summary, we have shown that Ang II increases intracellular H_2O_2 in VSMCs and that this increase is required for hypertrophy. This H_2O_2 is predominantly derived from O_2^- produced by the NADH/NADPH oxidase and the subsequent dismutation by SOD, which supports the notion that both O_2^- and H_2O_2 are growth promoting in Ang II–induced hypertrophy because their existence is mutually dependent. Using a novel cell line that stably overexpresses catalase, we show that this increased hydrogen peroxide is a critical step in VSMC hypertrophy, a hallmark of many vascular diseases. The various oxidant and antioxidant mechanisms that are initiated and orchestrated by Ang II are integral parts of the growth promoting effects of Ang II and ultimately contribute to regulation of the redox state of the cell, which in turn mediates the growth response and contributes to the pathogenesis of vascular disease.

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