Catecholamine-Induced Vascular Wall Growth Is Dependent on Generation of Reactive Oxygen Species

Tina Bleke, Hua Zhang, Nageswara Madamanchi, Cam Patterson, James E. Faber

Abstract—α1-Adrenoceptor–dependent proliferation of vascular smooth muscle cells (VSMCs) is strongly augmented by vascular injury, and may contribute to intimal growth and lumen loss. Because reactive oxygen species (ROS) are increased by injury and have been implicated as second messengers in proliferation of VSMCs, we investigated the role of ROS in catecholamine-induced VSMC growth. Rat aortae were isolated 4 days after balloon injury, maintained in organ culture under circumferential wall tension, and exposed to agents for 48 hours. The antioxidants N-acetylcysteine (NAC, 10 mmol/L) and Tiron (5 mmol/L) and the flavin-inhibitor diphenylene iodonium (DPI, 20 μmol/L) abolished norepinephrine-induced increases in protein synthesis and DNA content in media. In aortic sections, norepinephrine augmented ROS production (dihydroethidium confocal microscopy), which was dose-dependently inhibited by NAC, Tiron, and DPI. In cultured VSMCs, phenylephrine caused time- and dose-dependent ROS generation (acointase activity), had similar efficacy to thrombin (1 U/mL), and was eliminated by the superoxide dismutase (SOD) mimetic Mn(III)-tetrakis-(4-benzoic-acid)-porphyrin-chloride (200 μmol/L) and Tiron. Phenylephrine-induced ROS production and increases in DNA and protein content were blocked by prazosin (0.3 μmol/L) and abolished in p47phox−/− cells. PEG-SOD (25 U/mL) had little effect, whereas PEG-catalase (50 U/mL) eliminated phenylephrine-induced proliferation in VSMCs. DPI (10 μmol/L) and apocynin (30 μmol/L) abolished phenylephrine-stimulated mitogenesis, whereas inhibitors of other intracellular ROS sources had no effect. Furthermore, PE increased p47phox expression (RT-PCR). These data demonstrate that the trophic effect of catecholamines on vascular wall cells is dependent on a ROS-sensitive step that we hypothesize consists of activation of the NAD(P)H-dependent vascular oxidase. (Circ Res. 2004;94:37-45.)

Key Words: α-adrenergic ■ vascular smooth muscle ■ NAD(P)H oxidase ■ arterial injury ■ proliferation

Vascular smooth muscle cell (VSMC) proliferation, hypertrophy, and migration underlie most hypertrophic vascular diseases, including atherosclerosis, hypertensive wall hypertrophy, and restenosis after vascular injury.1 Catecholamines stimulate cultured VSMCs to undergo these same processes2–5 through stimulation of α1-adrenoceptors (α1-ARs), which are G protein–coupled receptors (GPCRs). Recent studies have shown that catecholamine-induced proliferation is strongly augmented and contributes to hypertrophic growth and restenosis in experimental vascular injury. Thus, treatment of rat aorta in organ culture with norepinephrine (NE) elicited VSMC and adventitial fibroblast proliferation and hypertrophy that were significantly increased after balloon angioplasty.6 Similarly, chronic local elevation of vascular wall NE in vivo worsened neointimal growth and lumen loss after rat carotid injury.7 Moreover, chronic local blockade of α1A-ARs, but not other AR subtypes, reduced neointimal expansion and lumen narrowing in the rat carotid injury model.8 In agreement, chronic systemic administration of an α1A-AR antagonist, at levels without effects on arterial pressure or regional resistance, caused dose-dependent inhibition of neointimal growth and lumen loss.8 These and other studies9 suggest that catecholamines may contribute to excessive growth of vascular wall cells in vascular diseases and injury after surgical procedures. Thus, catecholamines could be direct risk factors for vascular disease, which highlights the need to define the intracellular pathways mediating their trophic activity.

Reactive oxygen species (ROS) such as superoxide anions (O2−) and hydrogen peroxide (H2O2) are important second messengers in intracellular signal transduction pathways for several functions, including VSMC growth.10 In particular, ROS mediate proliferation by several GPCR agonists and growth factors in VSMCs, including angiotensin II,11 thrombin,12 platelet-derived growth factor (PDGF),13 and endothelin-1.14 The membrane-bound NAD(P)H oxidase is the major source of ROS production in both the intact vessel wall and cultured VSMCs.15–18 The enzyme generates O2− by NAD(P)H-derived one-electron reduction of molecular oxygen, that subsequently may be rapidly dismutated to form...
H₂O₂. 19 This “vascular” oxidase shows similarities with the better characterized “phagocytic” NAD(P)H oxidase. The phagocytic oxidase consists of a membrane-associated flavocytochrome (cytochrome b₅₅₆) that is composed of two glycoproteins, gp91phox (Nox 2) and p22phox, and the cytosolic components, p47phox, p67phox, p40phox, and the G protein Rac1/2. 19 Activation of the phagocytic NAD(P)H oxidase requires association of p47phox and p67phox, followed by their targeting to the plasma membrane by Rac-GTP. 19 p47phox plays a key role in assembly and activation of the oxidase, because in its absence, p67phox fails to assemble with the membrane-bound subunits. 19 In addition, p47phox regulates electron transfer from flavin adenine dinucleotide (FAD) to the heme center of cytochrome b₅₅₆, leading to O₂⁻ generation. 19 Although the vascular NAD(P)H oxidase is believed to be similar to the phagocytic oxidase, isoforms of all the vascular subunits have not been identified and the mechanism of activation is not completely understood. Several other ROS-generating systems, including the mitochondrial electron transport chain, nitric oxide synthase, xanthine oxidase, and cyclooxygenase have also been implicated in intracellular signaling cascades leading to changes in cell structure, function, and proliferation. 20 However, the NAD(P)H oxidase is a much larger source of induced ROS in the vascular wall than these other enzymes. Besides activation by certain extracellular agonists, mechanical and chemical injury of arteries also strongly increase NAD(P)H oxidase–associated ROS generation, VSMC proliferation, and neointimal formation that are inhibited by treatment with antioxidants. 21–23

The responsible pathways mediating the proliferative, hypertrophic, and chemotactic actions of catecholamines in VSMCs are not well understood. Norepinephrine-induced proliferation of VSMCs is mediated by extracellular signal-regulated kinases (ERK) 1/2, 24 These mitogen-activated protein kinases (MAPK) also appear to mediate α₁-AR-stimulated hypertrophy in cultured cardiomyocytes. 25 Although ROS generation is involved in ERK1/2-dependent hypertrophy of cultured cardiomyocytes by α₁-AR stimulation, 26 only one report suggests ROS may be involved in adrenergic proliferation of cultured VSMCs. 27 Furthermore, no studies have examined this question in the intact vessel wall. Therefore, the purpose of the present study was to determine if ROS mediate α₁-AR-induced VSMC growth in vitro and in the vascular wall ex vivo, and whether the NAD(P)H oxidase is the source of catecholamine-stimulated ROS generation.

Materials and Methods

Balloon injury of the rat thoracic aorta was performed according to standard methods. 6 Four days after injury, the aorta was placed into organ culture as described previously for measurement of DNA and protein content. 3,4,28 Protein synthesis was measured during the last 24-hour interval using [³⁵S]methionine incorporation. 4 The oxidative fluorescent probe dihydroethidium (DHE) and confocal microscopy were used to detect in situ levels of ROS in aorta sections. 29 Images were acquired by an observer blinded to the treatment groups. The aconitase assay was used to measure ROS generation in cultured cells. 30 p47phox mRNA expression was measured by reverse transcription-PCR (RT-PCR). 31 Data are expressed as mean±SEM. Statistical analysis was performed using Student’s t test, with significance defined as P<0.05. An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Norepinephrine-Induced Growth of Injured Aorta Media Is Inhibited by Antioxidants

To test the hypothesis that catecholamines’ mitogenic actions are mediated through generation of ROS, we first investigated the effects of antioxidants on NE-induced growth in aortas that had been balloon-injured 4 days earlier in vivo and then placed into organ culture. Consistent with our previous study, 6 NE (1 μmol/L, 48 hours) increased protein synthesis (171±14% of vehicle) and DNA content (135±7%) (Figure 1). Norepinephrine-induced growth was abolished by the
antioxidants Tiron and NAC, as well as with DPI, an inhibitor of NAD(P)H oxidase and other flavin-containing enzymes. NAC alone had no effect. DPI alone lowered baseline protein synthesis, an effect that has been noted elsewhere.32 Protein synthesis was not measured in the Tiron alone group.

Norepinephrine Augments ROS Production in Aorta Media
We next used in situ dihydroethidium (DHE) confocal microscopy to determine if NE augments ROS generation (Figure 2). Norepinephrine enhanced DHE intensity in injured media by 167±13% of vehicle (average response of Figures 2E and 2F, n=14). The increase was abolished by NAC and DPI and dose-dependently inhibited by Tiron. Tiron and NAC alone inhibited baseline activity (Figure 2E), possibly reflecting an effect of injury itself. The increase in DHE intensity induced by NE was not blocked by inhibition of the MAPK (ERK1/2) kinase, MEK1/2, with PD-98059 or UO-126, or by the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG-1478 (Figure 2F). These results suggest that the ROS-dependent signaling step in the adrenergic trophic pathway is upstream of the EGFR-ERK1/2 pathway. PD-98059 and AG-1478 had small inhibitory effects alone, suggesting that injury itself may increase ROS and activate the EGFR-ERK pathway.

To confirm that DHE fluorescence reflects ROS levels, sections from aortae previously treated for 48 hours with NE were preincubated for 5 minutes with high levels of DPI (50 μmol/L) or Tiron (20 mmol/L) before incubation with DHE. Both agents strongly reduced the NE signal by 84±3% (n=8) and 60±9% (n=5), respectively.

Norepinephrine Induces ROS-Dependent Proliferation in Adventitia
Like medial VSMCs, stimulation of α1-ARs on adventitial fibroblasts (AFBs) causes their proliferation and adventitial thickening, which are strongly increased after injury in both organ culture6 and in vivo.7 In the present study, results for
adventitia were similar to media for all groups in Figure 1. Norepinephrine tended to increase protein synthesis and DNA content by 140±18% and 118±10% of vehicle (P<0.11 and P<0.14, n=5 per group, respectively), and NAC prevented these increases (84±15% and 99±7%, respectively, n=6 per group). Tiron and DPI also inhibited adventitial growth by NE, and all three inhibitors had no significant effect alone. Norepinephrine caused an increase in DHE fluorescence over vehicle [7±1 (n=10) versus 12±2 (n=14), fluorescence units, P=0.02; Figure 2], and changes similar to those obtained for media were also obtained for adventitia for the other treatment groups shown in Figure 2.

Norepinephrine Effects in Uninjured Aorta
Because the uninjured artery is less sensitive than the injured artery to the trophic effect of NE, we performed a more limited study of uninjured aorta and only examined media. Norepinephrine (1 μmol/L, 48 hours) caused a small nonsignificant increase in protein synthesis (112±11% of vehicle, n=6). Tiron (5 mmol/L) inhibited this response (78±4%, P<0.05, n=6) and had an inhibitory effect alone (59±5%, P<0.001, n=6) as reported in cell culture. Changes in DNA content mimicked these results. Norepinephrine increased DHE fluorescence (71±4) above vehicle (34±4, P<0.001, n=6), and this was partially inhibited by Tiron (51±4, P<0.01, n=6) but unaffected by AG-1478 (1 μmol/L) (62±7, n=6). Tiron and AG-1478 had no effects alone.

Phenylephrine Increases Intracellular ROS Production in Cultured VSMCs
To confirm the DHE results, intracellular levels of ROS were determined by their ability to inhibit aconitase activity in vitro. Stimulation of cultured VSMCs with the selective α1-AR agonist phenylephrine (PE) induced a time-dependent decline in aconitase activity, with a maximum response at 10 minutes (−45.3±8.5%) that remained reduced at 60 minutes (Figure 3A). The 10-fold higher concentration of PE than NE used herein reflects PE’s known 10-fold lower potency for activation of α1-ARs. Maximal trophic concentrations of NE (1 μmol/L) and thrombin (1 U/mL), measured after 20 minutes exposure, were equi-effective: aconitase activity was decreased −37.2±5.5% by NE (n=4) and −34.2±7.6% by thrombin (n=4). Thrombin served as a positive control for generation of ROS in VSMCs. Inhibition of aconitase activity by PE (at 10 minutes) was concentration-dependent, with potent inactivation at 10 μmol/L PE (−61.8±3.9%) (Figure 3B). Reduction in aconitase activity was inhibited by Tiron and the SOD mimetic MnTBAP (Figure 3C). Tiron and MnTBAP had no effect alone, although there was a trend for MnTBAP to increase baseline activity (ie, to lower basal ROS), as reported by others.

Phenylephrine-Induced Growth of VSMCs Is Inhibited by Catalase
To determine the relative roles of O2− and H2O2 in catecholamine-induced VSMC growth, PEG-SOD and PEG-catalase were examined. The increase in DNA and protein content induced by PE was little affected by PEG-SOD, but abolished by PEG-catalase (Figure 4).

Prazosin Blocks Phenylephrine-Induced ROS Production and Growth of VSMCs
To confirm our previous studies showing that catecholamine trophic effects are α1-AR dependent and to determine if their ROS dependence, shown herein, is also α1-AR dependent, the α1-AR antagonist prazosin was examined. Prazosin inhibited both ROS production (aconitase inhibition; Figure 5A) and VSMC growth (Figure 5B).
Phenylephrine-Stimulated ROS Generation and Proliferation Are Abolished in p47<sup>phox</sup><sup>−/−</sup> VSMCs

To examine if catecholamine-stimulated ROS production and VSMC growth are mediated by the NAD(P)H oxidase, aortic VSMCs from p47<sup>phox</sup><sup>−/−</sup> mice were studied. As in rat aortic VSMCs, PE caused a reduction in aconitase activity in wild-type cells that was abolished in p47<sup>phox</sup><sup>−/−</sup> cells (Figure 6A). Thrombin served as a positive control for p47<sup>phox</sup>-dependent reduction in aconitase activity. Baseline levels of ROS were not lower in p47<sup>phox</sup><sup>−/−</sup> cells (ie, basal aconitase activity was not greater) than in wild-type cells (0.31 ± 0.01 versus 0.34 ± 0.02 μmol/L NADPH/min per μg protein, respectively), in agreement with one report but not another report. Elimination of PE-induced reduction in aconitase activity in p47<sup>phox</sup><sup>−/−</sup> cells was associated with inhibition of PE-mediated growth (Figure 6B). Furthermore, stimulation of wild-type VSMCs by PE for 1 hour promoted enhanced transcript levels of p47<sup>phox</sup>. The expected absence of expression in p47<sup>phox</sup><sup>−/−</sup> cells served as a negative control (Figure 6C).

Inhibitors of NAD(P)H Oxidase but not of Other Cellular ROS Sources Inhibit PE-Induced Proliferation

Because additional enzymes besides NAD(P)H oxidase are known to generate low levels of ROS, we tested inhibitors of them on PE-induced proliferation. In contrast to DPI and the more specific NAD(P)H-oxidase inhibitor, apocynin, that abolished PE-stimulated proliferation (Figure 7A), inhibitors of nitric oxide synthase (L-NAME), cyclooxygenase (indomethacin), xanthine oxidase (allopurinol), and cytochrome P450 oxidases (17-octadecynoic acid) had no effect on the PE-mediated increase in DNA content (Figure 7B). These inhibitors had no effect alone. At a concentration that reduces oxidative phosphorylation (30 μmol/L), the NADH dehydrogenase inhibitor rotenone also had no effect. However, a higher concentration (100 μmol/L) did reduce the DNA increase by PE (Figure 7C). Data for protein content (not shown) mimicked these DNA results.

Discussion

This is the first study to demonstrate that α<sub>1</sub>-AR–induced VSMC growth in both cell culture and, importantly, the intact vascular wall is mediated by generation of ROS derived from the vascular NAD(P)H oxidase. The ROS-sensitive step in this pathway is upstream of activation of the EGFR and ERK1/2, and appears to be mediated primarily by H<sub>2</sub>O<sub>2</sub>. A previous report in cultured VSMCs suggested catecholamines might increase ROS. In that study, PE increased 2′,7′-dichlorofluorescein diacetate fluorescence and DNA synthesis, and catalase and NAC reduced DNA synthesis by ~40%. These results are consistent with a similar requirement for ROS generation in α<sub>1</sub>-AR–induced ERK1/2-dependent hypertrophy of cultured cardiomyocytes.

We previously found that NE induces growth of medial VSMCs in rat aorta maintained in organ culture, and that this is strongly augmented by balloon injury 4 or 12 days earlier in vivo. This mitogenic effect is mediated by the α<sub>1D</sub>-AR subtype, but not by the α<sub>1A</sub>, α<sub>1B</sub>, or β-ARs that instead are vasoactive in this vessel. Furthermore, α<sub>1</sub>-AR–induced growth contributes significantly to restenosis after rat carotid...
injury-induced hypertrophic remodeling in mice. The results consistent with these observations were obtained in the present study, where NE caused significant proliferation of VSMCs in rat aorta studied in organ culture after receiving balloon injury 4 days earlier in vivo. This was associated with increased ROS generation (detected by DHE fluorescence). Both proliferation and ROS generation were abolished by Tiron, NAC, and DPI (Figures 1 and 2), suggesting that the adrenergic trophic pathway includes a ROS-sensitive step(s). Tiron, NAC, and DPI alone had minimal-to-no inhibitory effects. This likely reflects the effect of injury itself to increase cytokines and growth factors, some of which may activate ROS, together with reduction in the extracellular concentration of these mediators by diffusion into the large (200 mL) tissue culture bath. Although no in vitro system can fully preserve in vivo conditions, the preloaded organ culture model permits VSMCs to be studied in the intact vascular wall.

The source of catecholamine-induced ROS generation was not macrophages or neutrophils that are recruited to the vascular wall after injury. Rather, NE induced a mostly uniform increase in intracellular DHE fluorescence across the media (Figure 2C). We and others have shown that the media is composed almost entirely of cells expressing α-smooth

Figure 6. A and B, Phenylephrine-induced ROS generation and increases in DNA and protein content are abolished in p47phox−/− VSMCs. p47phox−/− (p47−/−) or wild-type (wt) VSMCs were stimulated with PE or thrombin (Thr) for 10 minutes and aconitase activity was measured (A). p47phox−/− or wild-type VSMCs were treated with PE for 48 hours, and DNA and protein content were determined from cell extracts (B). Data are percent of control or vehicle-treated cells and are mean±SEM (n=3 to 9). C, RT-PCR of p47phox and GAPDH in p47phox−/− and wild-type VSMCs after phenylephrine stimulation for 60 minutes.

Figure 7. Effect of inhibitors of ROS-generating enzymes on phenylephrine-stimulated proliferation. A, DPI and apocynin (Apo) blocked PE-stimulated proliferation. B and C, Inhibition of nitric oxide synthase (L-NAME), cyclooxygenase (indomethacin; Indo), xanthine oxidase (allopurinol; Allo), cytochrome P450 (17-octadecynoic acid; ODYA), and NADH dehydrogenase (rotenone; Rot) had no effect on PE-induced increase in DNA content. VSMCs were pretreated with the above inhibitors for 1 hour and stimulated with PE for 48 hours. DNA and protein content were determined from cell lysates. Data are percent of vehicle-treated cells and are mean±SEM (n=4).
muscle actin, when examined 4 days after balloon injury. In addition, PE also caused cultured VSMCs to generate ROS, and with an efficacy comparable to thrombin (Figures 3 and 6A).

To examine the source of ROS, we tested DPI and a more specific inhibitor of NAD(P)H oxidase, apocynin, on catecholamine-stimulated proliferation of cultured VSMCs. Both abolished PE-induced increases in DNA and protein content (Figure 7A).

Because DPI inhibits several flavin-containing enzymes that generate ROS, besides NAD(P)H oxidase, because apocynin may also have other actions, and because few specific inhibitors of the oxidase are available (p22phox-antisense, gp91ds-tat and dominant-negative p67phox), we examined primary cultures of aortic VSMCs obtained from mice genetically deficient in p47phox, a key regulatory subunit of NAD(P)H oxidase. ROS generation and proliferation induced by PE in wild-type cells were both abolished in p47phox−/− cells (Figure 6). Consistent with increase in ROS production, PE caused increased p47phox transcription levels at 1 hour (Figure 6C).

Although these findings suggest that the NAD(P)H oxidase mediates the trophic action of catecholamines on VSMCs, we examined whether induction of other DPI-sensitive ROS-generating enzymes might participate. Phenylephrine-induced proliferation was unaffected by inhibition of nitric oxide synthase (NOS), cyclooxygenase, xanthine oxidase, or cytochrome P450 oxidases (Figure 7B). Although inhibition of NOS with L-NAME can inhibit VSMC proliferation by PDGF, our studies suggest that the pathway mediating α1-AR induced proliferation does not involve nitric oxide or the above oxidases. Interestingly, L-NAME also had no effect on α1-AR–mediated hypertrophy of cardiomyocytes. The NADH dehydrogenase inhibitor, rotenone (30 μmol/L), also did not inhibit PE-induced proliferation. However, a higher concentration of rotenone (100 μmol/L) caused a decrease in PE proliferation. This may reflect a reduction in mitochondrial oxidative phosphorylation and stimulation of apoptosis, an effect that is suggested by the decrease in baseline DNA content caused by this concentration (Figure 7C).

Generation of ROS by PE was rapid, persisted for at least 60 minutes, was dose-dependent, and extended over the same dose range observed for NE-evoked contraction (Figure 3). We have shown in organ culture that α1-AR–mediated proliferation also extends over this same range. Importantly, α1-adrenergic constriction is not associated with altered ROS activity and is not affected by inhibition of the NAD(P)H oxidase or ROS. These correlations strengthen the concept that the signaling pathway used by catecholamines to stimulate VSMCs growth in vivo involves the ROS-sensitive pathway identified in the present study. In addition, the maximum inactivation of aconitase after 10 minutes exposure to PE (40% to 60%), which we used to measure ROS production, is similar to that reported for thrombin and other studies using this assay. To validate this assay, we demonstrated that PE inhibition of aconitase activity was abolished by concomitant treatment with Tiron or MnTBAP (Figure 3C). We also found that NE showed the same maximal efficacy as the α1-AR agonist PE, indicating that simultaneous stimulation of α2- and β-ARs that are also present does not influence ROS generation. This is in agreement with the effect of prazosin to inhibit ROS production and VSMC growth (Figure 5) and our previous results showing no involvement of α2- and β-ARs in catecholamine-induced growth of the intact aorta and carotid artery studied in organ culture and in vivo. The maintained ROS generation by PE (Figure 3A) is similar to the action of certain other ROS-generating mitogenic factors. For example, angiotensin II induces biphasic production of ROS in VSMCs, involving a rapid early peak at 30 seconds, followed by sustained gener-

Figure 8. Proposed model of the adrenergic trophic signaling pathway in VSMCs. α1-Adrenoceptor (AR) mediates the trophic effect of catecholamines in the media of rat aorta and carotid. Subunits of the NAD(P)H oxidase are shown. EGFR indicates epidermal growth factor receptor; O2−, superoxide anion. Compounds depicted in red inhibited ROS production and/or growth of VSMCs in intact aorta media and in cell culture. See text for additional details.
ation for at least 6 hours. Likewise, induction of ROS by thrombin was evident at 15 minutes, elevated further at 1 hour, and remained above baseline for at least 6 hours.

In most cell types, \( \text{O}_2^- \) generated by NAD(P)H oxidase is rapidly converted by SOD to H\(_2\)O\(_2\). In cultured VSMCs, H\(_2\)O\(_2\) mediates the mitogenic effects of angiotensin II,\(^2\) thrombin,\(^2\) and PDGF,\(^1\) and is itself mitogenic. In agreement with these studies, we found that proliferation of VSMCs by PE was abolished by membrane permeant PEG-catalase (Figure 4). In contrast, PEG-SOD had minimal if any effect. This suggests that SOD is not limiting in the presence of PE stimulation, and also that O\(_2^-\) itself or other ROS arising upstream of SOD (e.g., peroxynitrite) have little or no direct role in mediating adrenergic proliferation. Similar results were reported for thrombin.\(^1\)

We have evidence that activation of the intrinsic tyrosine kinase activity of EGFR and its known downstream activation of the rafl-MEK1/2-ERK1/2 pathway are required for \( \alpha_1\)-AR-induced growth of VSMCs in rat aorta studied in organ culture (unpublished data, H. Zhang, J.E. Faber, 2003). The EGFR tyrosine kinase inhibitor, AG-1478, and the MEK1/2 inhibitors, PD-98059 and UO-126, each abolished catecholamine-induced protein synthesis and DNA accumulation (while having no effects alone). This was associated with augmented phosphorylation of EGFR and ERK1/2 as assessed by immunoblot that was sustained for the duration of PE exposure. To begin to examine whether the ROS-sensitive step in the adrenergic trophic pathway is upstream or downstream of EGFR activation, in the present study, we determined the effect of these above-mentioned agents on ROS generation by DHE fluorescence (Figure 2F). In contrast to their inhibitory effects on adrenergic growth, none had a significant effect on NE-induced ROS activity. This suggests that the ROS-sensitive step resides upstream of EGFR and ERK activation. Norepinephrine-induced ERK activation in cardiomyocytes has also been shown to be dependent on a ROS-sensitive step. In contrast, endothelin-1\(^4\) and angiotensin II\(^5\) activation of ERK in VSMCs is not ROS-dependent; although there is controversy on this point for angiotensin II.\(^6\)

Figure 8 is a proposed signaling pathway for catecholamine-mediated VSMC growth, based on our present results. Stimulation of \( \alpha_1\)-ARs by blood-borne NE or by increased release of NE from vascular nerves induced by injury\(^7\) is proposed to activate NAD(P)H oxidase–dependent generation of O\(_2^-\) and in turn H\(_2\)O\(_2\). Hydrogen peroxide causes activation of the EGFR-ERK1/2 pathway and subsequent induction of protein synthesis and proliferation. Although identification of the steps that are proximal and distal to the indicated mediators in this pathway await investigation, the pathway suggests a direct link or risk factor status may exist between catecholamines, injury, and the progression of vascular disease. It is well known that O\(_2^-\) inactivates nitric oxide that serves as a tonically active, antiproliferative signal. It is also well known that promoters of vascular injury and disease augment ROS activity. This effect was recently underscored by the identification of an apparently crucial role for NAD(P)H oxidase in neointimal response to rat carotid balloon injury.\(^8\) The evidence we present herein for depen-

dence of adrenergic growth on a ROS second messenger, together with the effects of injury on ROS and ROS on nitric oxide availability, may explain why injury so strongly amplifies the mitogenic action of catecholamines: an effect that our recent in vivo evidence suggests contributes to neointimal growth and hypertrophic remodeling in animal models of vascular disease.\(^7,8\) As such, therapeutic agents that reduce oxidative stress or interfere with generation of intracellular ROS may derive their salutary effect, in part, by amelioration of the hypertrophic effects of catecholamines induced by vascular injury and disease.

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References
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Materials and Methods

Materials. Mn-(III)-tetrakis-(4-benzoic-acid)-porphyrin-chloride (MnTBAP) was obtained from Biomol Inc., Plymouth Meeting, PA, Thrombin from Calbiochem, San Diego, CA. Dihydroethidium (DHE) from Molecular Probes Inc., Eugene, OR, and Cell culture materials and SDS from GibcoBRL, Grand Island, NY. All other chemicals and reagents were from Sigma Chemical Co., St. Louis, MO.

Rat Aorta Balloon Injury Model. As described previously¹, male Sprague-Dawley rats (450-500 g; Zivic-Miller Laboratories, Pittsburgh, PA) were anesthetized with ketamine (125 mg/kg i.m.) plus acepromazine (1.25 mg/kg i.m.) and received atropine (54 µg/kg, s.c.) and cephalzin (50 mg/kg, i.m.). The left thyroid, occipital, and distal external carotid arteries were isolated steriley and ligated. After heparin administration (125 U/kg i.m. and 125 U/kg s.c.), a 2F embolectomy catheter (Baxter Healthcare, Irvine, CA) was advanced via an external carotid arteriotomy. The balloon was inflated with 40 µl of saline and rotated while withdrawing it the length of the descending thoracic aorta 3 times. The surgical wound was treated with nitrofurazone. Pentazocine (10 mg/kg, i.m.) was given for analgesia. All procedures were conducted per NIH guidelines.

Aorta Organ Culture. Four days after balloon injury, anesthetized rats were perfused transcardially with sterile 4°C phosphate-buffered saline (PBS) at 100 mmHg. The descending thoracic aorta was viewed (100x) under a pool of 4°C PBS, and the periadventitial connective tissue and intercostal arteries were removed aseptically. An organ culture system described in detail elsewhere¹-⁴ was used to maintain the aorta under circumferential wall tension (0.45 g per mm vessel length) in serum-free media consisting of 50% Dulbecco’s modified Eagle’s medium (DMEM), 50% F-12 media, supplemented with 5 mg/L transferrin, 100 µmol/L ascorbic acid, 6 ng/ml selenium, 100 U/ml penicillin and 100 µg/ml streptomycin, in a 37°C, 5% CO₂ incubator. Maintenance of circumferential wall tension favors the quiescent VSMC phenotype²-⁵, whereas
a neointima forms spontaneously and VSMC marker proteins that characterize
the contractile phenotype decline when vessels are placed into organ culture in
the absence of load.\textsuperscript{5} Aortae were pretreated with N-acetylcysteine (NAC, 10
mmol/L), Tiron (2-5 mmol/L), diphenylene iodonium (DPI, 20 µmol/L), PD-98059
(20 µmol/L), AG-1478 (1 µmol/L) or UO-126 (2 µmol/L) for 1 hour before addition
of vehicle or NE (1 µmol/L; dissolved in 100 µmol/L ascorbate in PBS). Media
was changed and drugs re-added at 24 hours. After 48 hours in culture, vessel
length was measured microscopically in 4°C PBS, and intima-media was
separated from adventitia as described previously.\textsuperscript{2-4}

**Protein Synthesis, Protein Content, DNA Content.** Protein synthesis
was measured during the last 24-hour interval using $^{[35]}$S methionine
incorporation (1 µCi/ml; Amersham, Arlington Heights, IL).\textsuperscript{3} Frozen intima-media
was pulverized at -80°C, added to 0.1% RIPA buffer composed of PBS
containing 0.1% Nonidet P40, 0.05% sodium deoxycholate, 0.1% sodium
dodecyl sulfate (SDS) and proteinase inhibitors, and homogenized (model TH,
Omni, Atlanta, GA) on ice at maximal speed for 15 seconds. All subsequent
procedures were conducted on ice. Homogenates were passed three times
through a 21g needle to shear DNA, and cells were allowed to lyse for 30
minutes. After centrifugation for 20 minutes at 14,000 rpm, protein content of the
supernatant (Bradford assay, Beckman Coulter DU 7500) and DNA content
(Hoefer DyNA Quant 200; Amersham Pharmacia, San Francisco) were
determined.

Cultured quiescent VSMCs were pretreated with polyethylene glycol-
superoxide dismutase (PEG-SOD, 25 U/ml), polyethylene glycol-catalase (PEG-
catalase, 50 U/ml), prazosin (0.3 µmol/L), DPI (10 µmol/L), apocynin (30 µmol/L),
Nω-Nitro-L-arginine methyl ester (L-NAME, 100 µmol/L), indomethacin (10
µmol/L), allopurinol (100 µmol/L), 17-octadecynoic acid (ODYA, 5 µmol/L) or
rotenone (30-100 µmol/L) for 1 hour before stimulation with PE (10 µmol/L;
dissolved in 100 µmol/L ascorbated PBS) for 48 hours. Media was changed and
drugs re-added at 24 hours. Cells were then rinsed in ice-cold PBS, lysed in 0.6
ml 0.1% RIPA-buffer and scraped. After passage three times through a 21g needle, lysis for 30 minutes and centrifugation at 14,000 rpm for 20 minutes, protein and DNA content were obtained.

**Detection of In Situ Generation of ROS.** The oxidative fluorescent probe dihydroethidium (DHE) was used to detect in situ levels of ROS in rat aortae. DHE is cell permeable and converted to ethidium by O$_2^••$, which then intercalates with DNA and is detected by fluorescence (488 nm excitation, 610 nm emission). Aortae were cut into 3 mm rings, embedded in OTC (Sakura Finetek USA Inc., Torrance, CA) and frozen in liquid nitrogen. Thirty-micron thick cryostat sections were cut in the dark and placed on glass slides maintained in a humidified ice-cold light-protected chamber filled with nitrogen. Ten µmol/L DHE in HBSS was applied topically to each slide and incubated in the light-protected chamber for 30 minutes (37°C, N$_2$). To test for specificity of the ROS signal, Tiron or DPI, in addition to DHE, was directly added to a given vessel section. After incubation, sections were maintained humidified and light protected at 4°C and immediately imaged in random sequence on a Leica confocal microscope (488 nm argon laser; 590 nm long pass filter). Confocal and laser settings were maintained identical in each experiment and images were acquired by an observer blinded to the treatment groups. Results were pixilated and quantified using Scion Image®.

**Cell Culture.** VSMCs were isolated from male Sprague-Dawley rat thoracic aortae by enzymatic digestion as described previously. Cells were grown in M199 supplemented with 10% fetal bovine serum (FBS), 200 mg/L L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and passaged at ~95% confluence with 0.1% trypsin/ EDTA twice a week. For experiments, cells were used at passages 3 to 4, carried 2 days beyond confluence, and growth-arrested for 2 days in serum-free defined medium, consisting of 50% DMEM, 50% F-12 media, 5 mg/ml transferrin, 100 µmol/L ascorbic acid, 100 U/ml penicillin and 100 µg/ml streptomycin. VSMCs derived from p47phox$^{-/-}$ and wild-type (C57BL/6 background) mice (for primary culture see Ref$^8$) were used.
between passages 6-9, grown in DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.025 µg/ml amphotericin B, carried 2 days beyond confluence, and growth-arrested for 2 days in DMEM plus 0.1% FBS.

**Aconitase Assay.** Aconitase is a tricarboxylic acid cycle enzyme that is highly sensitive to inactivation by $O_2^-$ and has been used to measure ROS generation in cultured cells. Post confluent cells were made quiescent for 2 days and then treated with PE (0.1-10 µmol/L), thrombin (1 U/ml) or NE (1 µmol/L) for 10 minutes, unless otherwise indicated. In some experiments, cells were pretreated with MnTBAP (200 µmol/L), Tiron (5 mmol/L) or prazosin (0.3 µmol/L) for 1 hour prior to PE stimulation. Cells were rinsed with ice-cold PBS, scraped, pelleted and transferred to 500 µl cold homogenization buffer consisting of 50 mM Tris-HCl (pH 7.6), 0.2% Triton-X-100, 1 mmol/L cysteine, 5 mmol/L citrate and 100 mmol/L DTPA. The pellet was dispersed by vortexing and lysed by sonication. Fifty µl of lysate was added to 200 µl of assay buffer [50 mmol/L Tris-HCl (pH 7.6), 30 mmol/L citrate, 0.6 mmol/L MnCl$_2$, 0.2 mmol/L NADP$^+$ and 2 U/ml isocitrate dihydrogenase] in a single cuvette. Production of NADPH was determined by reading absorbance at 340 nm every 10 seconds for 2 minutes (Beckman Coulter DU 7500), and normalized to protein per sample.

**RT-PCR for p47phox.** Total RNA from wild-type and p47phox$^{-/-}$ VSMCs treated with or without PE (10 µmol/L, 1 hour) was isolated by cesium chloride ultracentrifugation. cDNA was obtained by reverse transcription of RNA (0.5 µg) with Superscript II reverse transcriptase (Stratagene Inc., La Jolla, CA) using a poly T primer. PCR amplification was performed using Taq DNA polymerase (Roche, Indianapolis, IN). Specific primers to p47phox (5'-ACATCACAGGCCCCATCATCCTTC-3' and 5'-ATGGATTGTCCTTTGTGCC-3') were used to amplify a 612-bp fragment. Simultaneous amplification was performed with primers specific to the GAPDH gene as an internal control. PCR products were separated by electrophoresis on 1% agarose gels containing ethidium bromide and visualized by UV light.
**Statistical Analysis.** Data are expressed as means ± SEM. Statistical analysis was performed using Student's $t$ test, with significance defined as $p<0.05$. 
References


