Trophic Effect of Norepinephrine on Arterial Intima-Media and Adventitia Is Augmented by Injury and Mediated by Different α1-Adrenoceptor Subtypes

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Abstract—In vivo studies have suggested that norepinephrine (NE) directly contributes to normal vascular wall growth and worsening of hypertrophy, atherosclerosis, and restenosis. However, it is unknown whether these effects are secondary to hemodynamic changes caused by systemic NE or α-adrenoceptor (AR) antagonists. Herein, we determined if NE directly stimulates growth of medial smooth muscle cells (SMCs) and adventitial fibroblasts (AFBs) that we have shown express α1-ARs in similar abundance. The rat aorta was isolated before injury, 4 days after, or 12 days after balloon injury, and maintained under circumferential tension in organ culture for 48 hours with 1 μmol/L NE. Intima-media and adventitia were separated and DNA content, protein synthesis, and protein content measured. In uninjured aorta, NE increased DNA and protein content similarly in adventitia, and increased only protein content in intima-media, suggesting AFB proliferation and SMC hypertrophy. In vessels isolated 4 or 12 days after injury, NE increased all 3 endpoints in both layers by up to 20-fold greater than in uninjured vessels. These effects were dose-dependent and were unaffected by α2- or β-AR blockade (except increased DNA content in adventitia that was also inhibited by α2-AR blockade). Intima-media growth was blocked by KMD3213 (α1A-AR antagonist) and adventitial growth by AH11110A (α1B-AR antagonist), whereas BMY7378 (α1D-AR antagonist) had no effect. NE decreased SMC marker proteins (eg, α-smooth muscle actin and desmin) and augmented the changes induced by injury. These data suggest that prolonged stimulation of α1A- and α1B-ARs induces growth of SMCs and AFBs, respectively, that is significantly augmented by injury. (Circ Res. 2001;89:815-822.)

Key Words: artery ■ smooth muscle cell ■ adventitial fibroblast ■ growth ■ organ culture

Endothelial injury, eg, by balloon angioplasty, causes smooth muscle cells (SMCs) to begin proliferating within two days and to migrate to the intima where they proliferate and form lumen-narrowing lesions.1 Recent evidence has shown that adventitial fibroblasts (AFBs) can also contribute (by as much as 50%) to neointimal lesion growth.2,3 Wilcox et al2 found that AFB proliferation began within hours after balloon injury and exceeded medial SMC proliferation measured over 4 weeks. Moreover, AFBs modulated to myofibroblasts, ie, α-smooth muscle actin (αSM-actin) expressing cells, by one week after injury, followed by deposition of a collagen-rich adventitial thickening. Similar findings have been reported by others.3 Whether catecholamines directly influence vascular wall growth is unknown because of difficulty in distinguishing a direct action from trophic effects of altered arterial pressure and blood flow velocity that accompany changes in sympathetic activity or plasma catecholamines. Therefore, support for this hypothesis remains indirect: (1) most large arteries are innervated with adrenergic nerves;4 (2) medial SMCs of large arteries express multiple α-adrenoceptor (AR) subtypes, several of which do not mediate constriction5; (3) AFBs from these same vessels surprisingly express multiple α-AR subtypes with a similar total α1-AR abundance as medial SMCs6; and (4) we and others have shown that norepinephrine (NE) causes hypertrophy of growth-arrested and proliferation of nonarrested cultured SMCs (see Faber et al7 for references); hypertrophy is mediated by α1- but not α2- or β-ARs8; norepinephrine induces proliferation of cultured AFBs.9 In vivo studies have reported that (5) local or systemic sympathetic denervation attenuates wall growth during maturation (reviewed in Head7); (6) NE infusion, albeit at hypertensive doses, causes wall hypertrophy and DNA synthesis in injured and uninjured arteries and augments atherosclerosis (see Faber et al9 and Head5); (7) α1-AR antagonists (although at concentrations that cause hypotension) attenuate neointimal lesion growth,7–10 angiotensin II–induced DNA synthesis,11 atherogenesis,12 and hypertensive wall hypertrophy13; and (8) increased sympathetic activity is associated with atherosclerosis in animals and humans.6,14 Unfortunately, these previous in vivo studies were unable to determine whether the effects were secondary to hemody-
namic alterations. Likewise, trophic responses to NE of vascular cells in culture may not mimic their in situ behavior. Therefore, the purpose of this study was to determine whether NE causes direct growth of the intact rat aorta, whether injury modifies any such trophic action, and to identify the adrenergic receptor type(s) involved.

Materials and Methods
Sprague-Dawley rats (~350 animals, 450 to 500 g; Ziric Laboratories, Pittsburgh, Pa) received standard balloon injury of the descending thoracic aorta. Four or 12 days later, the aorta was placed into organ culture under circumferential tension (0.45 g per mm vessel length). Drugs were added directly to the culture bath. Protein synthesis, protein content, and DNA content were determined for intima-media (IM) and adventitia. Because NE and injury affect cell number, cell size, and matrix content, in Western blots, protein aliquots from cell lysates were loaded as constant amounts of DNA (protein amounts loaded are given in figure legends). Adjacent paraffin-embedded 5-μm serial sections were processed for standard immunohistochemistry.

Data, given as means±SE, were subjected to unpaired 2-tailed t tests, or ANOVA followed by Bonferroni tests for multiple comparisons, unless indicated differently. A value of P<0.05 was considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
Baseline DNA and Protein Content in Normal and Balloon-Injured Aorta
Four days after injury, before neointima formation but when wall proliferation is known to be maximal,1,2 mitotic nuclei were present and media and adventitia exhibited modest thickening (Figure 1). By 12 days a neointima had formed, marking the time generally required to achieve maximum neointimal thickness, for medial proliferation to return to near-quiescence, and neointimal proliferation to decline to 25% of its peak at 7 days.15 To validate DNA content as an index of cell number, preliminary studies demonstrated that DNA content measured from RIPA extracts (predominantly cellular) of cultured aorta SMCs at 2, 4, and 8 days after plating increased in identical proportion to both genomic DNA and cell number measured from replicate plates (N. Yang, C. Erami, J.E. Faber, unpublished data, 1999). To provide baseline data for subsequent experiments, aortae from uninjured rats at 4 or 12 days after injury were placed in organ culture for 48 hours without adrenergic agents present. In uninjured aorta, DNA and protein content in adventitia were 57% and 69% less, respectively, than in IM (Figure 2A), consistent with fewer AFBs than SMCs per vessel length, when measured by hemocytometry of enzymatically dispersed cells (Faber et al; see also Figure 1). The smaller size of AFBs is evident as a 22%-smaller protein/DNA ratio (Figure 2B, uninjured vehicle groups). Four days after injury, DNA and protein content decreased in the IM by 19% and 17%, respectively, and in adventitia by 26% and 28% (Figure 2A). Protein/DNA ratios in day-4 injured vehicle-treated vessels were unchanged (Figure 2B), suggesting, together with Figure 2A data, that cell number declined in IM and adventitia at day 4, due to either cell necrosis and/or apoptosis offsetting the proliferation known to be present in both layers at this time1,2,15 or from possible migration of AFBs to the IM.2,3 Thus, medial and adventitial thickening at day 4 (Figure 1) presumably reflects edema and/or increased extracellular matrix and not increased cell number. In day-12 vessels, protein content in IM returned to control, but DNA increased 37% (Figure 2A). This probably arose from the presence of increased number of smaller cells, as indicated by the ratio reduction (Figure 2B), presumably reflecting recently divided smaller SMCs in the IM and/or migration to the IM of the smaller AFBs. In adventitia of day-12 aorta, DNA returned to uninjured levels but protein remained reduced (Figure 2A), suggesting that recently divided smaller AFBs were present, as supported by the ratio reduction (Figure 2B). These baseline data are consistent with the time-course of proliferation and centripetal migration of SMCs and AFBs reported in balloon-injured rat and porcine arteries.1,3,15

Trophic Effect of Norepinephrine Is Augmented by Injury
In IM of uninjured aorta, 1 μmol/L NE for 48 hours caused a modest 8±3% increase in protein content, but no change in DNA content (Figure 3A). Protein synthesis measured during the last 24-hour interval was unchanged. Adventitia DNA and protein content increased 8±4% and 12±5%, respectively (Figure 3A). For uninjured aorta, NE increased the protein/DNA ratio of IM but not adventitia (Figure 2B). Together with Figure 3A data, this suggests that NE induced hypertrophy of SMCs and hyperplasia of AFBs.

In vessels that remained in vivo 4 days after injury, NE induced a marked trophic effect, increasing DNA content, protein synthesis, and protein content in IM by 37±5%,
76±2%, and 27±4%, respectively, and in adventitia by 12±3%, 49±5%, and 23±5% (Figure 3A). The increased sensitivity of IM and adventitia to NE remained evident in vessels 12 days after injury (Figure 3A). Protein/DNA ratios were unaffected in IM or adventitia of 4-day or 12-day vessels (Figure 2B). Together with Figure 3A data, this suggests that NE augmented the proliferation of SMCs and AFBs induced by injury. Comparison of data in Figures 2A and 3A shows that the increased sensitivity of injured aorta to NE is not a result of expression of data as percentage changes relative to vehicle-treated baseline control values. The trophic effect of NE was dose-dependent when examined in vessels 4 days after injury, with threshold generally between 10 to 100 nmol/L (Figure 3B). However, 10 nmol/L NE reduced DNA and protein content in adventitia, possibly by β-AR inhibitory actions.

Sham balloon-injury groups were not studied because they would have required an additional 44 animals, and it was felt unlikely that the surgical drugs and sterile neck surgery, rather than balloon injury of the thoracic aorta, could have caused the greatly augmented trophic response to NE.

Angiotensin II and Norepinephrine Have Similar Trophic Actions

To compare efficacy, responses were obtained in day-4 injured vessels exposed to either 1 nmol/L angiotensin II (Ang II) or 1 μmol/L NE. These concentrations induce 90% to 100% of their maximal trophic effect in cultured rat aorta SMCs to either agent,16 and give similar near-maximal contractions of arteries in vitro. NE and Ang II produced similar trophic responses (Figure 4A).
Trophic Effect of Norepinephrine Is Mediated by \(\alpha_1\)-Adrenergic Receptors

**Uninjured Aorta**

To determine which AR type(s) mediates the trophic effects of NE, 1 \(\mu\)mol/L NE was tested over 48 hours in the presence of atipamezole (a high-affinity \(\alpha_2\)-AR antagonist with >8000-fold selectivity over \(\alpha_1\)- or \(\beta\)-ARs; see Virtanen\(^6\)), alone or combined with the \(\beta\)-AR antagonist propranolol (both at 1 \(\mu\)mol/L). In uninjured IM, the same increase in protein, but not DNA content induced by NE alone, was obtained in the presence of \(\alpha_2\)- and \(\beta\)-AR blockade (Figure 5A). However, \(\beta\)-AR stimulation opposed \(\alpha_1\)-AR hypertrophy when concomitant \(\alpha_2\)-AR stimulation was blocked. \(\alpha_2\)-AR stimulation opposed an \(\alpha_1\)-AR–mediated increase in protein synthesis, suggesting an inhibitory effect of \(\alpha_2\)-ARs on protein synthesis. However, \(\alpha_2\)-AR blockade did not cause an accompanying increase in protein content, suggesting that protein degradation may have been equally stimulated. In adventitia of uninjured aorta, there was a trend toward similar but smaller effects on protein synthesis and content (Figure 5B) (protein content and synthesis groups were nonsignificant by ANOVA). In addition, the NE-induced increase in DNA content was abolished by \(\alpha_2\)-AR blockade.

**A: Intima-Media**

**B: Adventitia**

**Figure 4.** In day-4 (d4) injured aorta, angiotensin II (Ang II) and NE, when compared at equi-effective concentrations for trophic effects on cultured SMCs,\(^2\) had similar trophic effects on intima-media and adventitia (panel A) and similarly reduced expression of SMC marker proteins and vinculin in intima-media that are inhibited by the \(\alpha_1\)-A-antagonist KMD3213 (panel B). Protein aliquots were loaded as constant amounts of DNA in all lanes, where mean±SE amounts (\(\mu\)g) were control, 25.0±0.7; Ang II, 27.5±0.5; KMD, 27.0±0.9; and NE+KMD, 25.5±0.7. See Figures 2 and 3 for other abbreviations. Asterixes indicate 2-tailed \(t\) tests.

**Figure 5.** Trophic effect of NE on intima-media (A) and adventitia (B) is mediated by \(\alpha_1\)-adrenoceptors (ARs). See Figures 2 and 3 for abbreviations and text for additional details. Key: NE for stimulation of all AR types; NE plus \(\alpha_2\)-AR antagonist atipamezole for stimulation of \(\alpha_1\)- and \(\beta\)-ARs; and NE plus atipamezole and the \(\beta\)-AR antagonist propranolol for stimulation of \(\alpha_1\)-ARs. All drugs used at 1 \(\mu\)mol/L and present for 48 hours. 3-bar groupings for each parameter were not significant by 1-way ANOVA, except for uninjured intima-media, where for protein synthesis the hatched and black bars were significantly different from the open bar, and for uninjured adventitia, where for DNA content the open bar was significantly different from the hatched and closed bars by Bonferroni test. Asterixes indicate 1-tailed \(t\) tests.
Injured Aorta

In IM (Figure 5A) and adventitia (Figure 5B) of day-4 and day-12 injured vessels, the trophic actions NE were unaffected by blockade of either α2-ARs alone or in combination with β-AR blockade (groups nonsignificant by ANOVA, with the exception of DNA content in day-4 adventitia, where blockade of α2- but not β-ARs, during concomitant α1-AR stimulation, abolished the increase in DNA (Figure 5B). Thus, the augmented trophic effects of NE in both IM and adventitia of injured aorta are mediated by α1-ARs, along with a modulatory β-mediated inhibition of DNA increase in adventitia when α2-ARs are blocked. α1-AR–induced proliferation is inhibited by β2-ARs in cultured SMCs.17

Adrenergic-Induced Growth of Medial SMCs Is Blocked by α1A-AR and Adventitial Fibroblasts by α1B-AR Antagonists

Four days after balloon injury, aortae were treated for 48 hours with 1 μmol/L NE alone, or in the presence of 0.1 μmol/L of KMD3213, AH 11110A, or BMY7378 for blockade of α1A-, α1B-, or α1D-ARs. These are the most selective antagonists available. Reported Ki (nmol/L) for BMY7378 at cloned rat receptors for α1D-, α1B-, and α1A-ARs average 1.2, 320, and 320, respectively (see Faber et al3), demonstrating α1D selectivity of 267-fold. Ki for KMD3213 at cloned rat α1A and submandibular gland membranes averaged 0.28, and showed 56-fold and 583-fold selectivity against α1A- and α1B-ARs, respectively (see Faber et al3, Saussy et al18, and Yamada et al19), and 200-fold selectivity for α1A over α1B in binding and functional studies.20 We confirmed the selectivity of BMY7378 and KMD3213 at 0.1 μmol/L for blockade of α1D- and α1A-ARs in radioligand binding studies of transfected cells and rat aorta SMCs and AFBs.5 At the cloned α1B-AR, the Ki for AH11110A is 79.4 nmol/L, with 32- and 26-fold selectivity over α1A- and α1D- ARs, respectively.18 with a similar 10- to 20-fold selectivity also reported (α1B>α1A>α1D; see Giardina21). NE-induced increases in DNA content, protein content, and protein synthesis were inhibited by KMD3213 in IM but not in adventitia (Figure 6). In contrast, AH11110A inhibited the trophic effects in adventitia but not in IM (protein synthesis was only measured in the KMD experiment). The α1D antagonist BMY7378 had no effect in either layer. Antagonists alone were not tested because of the number of animals (240) that would be required and because the concentrations of the antagonists that were used have not been shown to exhibit partial agonist activity.

Norepinephrine Decreases SMC Marker Proteins and Augments Decreases Induced by Injury

Norepinephrine-mediated hypertrophy of uninjured aorta IM was accompanied by decreased αSM-actin, MHC-B, desmin, and vinculin (Figure 7A, Table). Injury alone at 4 days decreased αSM-actin and increased SM2 and at 12 days increased αSM-actin and decreased SM1 and MHC-B (Figures 1 and 7B, Table). This increase in αSM-actin in day-12 IM was evident in the media, whereas a decrease was seen in neointima (Figure 1), in agreement with previous reports.3 Relative to vehicle-treated day-4 injured controls, in day-4 IM, the decrease in αSM-actin was augmented by NE, the NE-induced decreases in desmin and vinculin in uninjured aorta were enhanced, and NE now decreased SM1, whereas its effect to decrease MHC-B in uninjured aorta was abolished (Figure 7C, Table). In day-12 IM, the increases in αSM-actin and decreases in MHC-B were further augmented by NE, whereas the decrease in SM1 was abolished (Figure 7D, Table). In an additional experiment, the similar trophic efficacy of NE and Ang II on day-4 injured IM (Figure 4A) was accompanied by similar greater reductions in SMC marker proteins and vinculin (Figure 4B). Blockade of NE’s trophic effect in IM by KMD3213 was associated with blockade of NE’s reductions in these proteins (Figure 4B).

In uninjured adventitia, only low levels of vinculin and αSM-actin (=6- and 10-fold lower than IM, respectively) were detected (n = 3). Four days after injury, αSM-actin tended to increase (2-fold, P<0.07, n = 4), consistent with evidence for myofibroblast appearance at this time.2,3,15 Vinculin was unchanged, and the other SMC marker proteins remained undetectable. NE had no effect on expression of these proteins in day-4 injured adventitia. Adventitia for
day-12 injured aorta was not assayed. Sham balloon-injury groups were not studied because they would have required 144 adult animals, and it is unlikely that the surgical drugs and sterile neck procedures, rather than balloon injury of the aorta, could have caused the greatly augmented trophic response to NE.

**Discussion**

Our major findings were that 48-hour exposure of the ex vivo aorta to NE increased protein content and reduced SMC marker proteins in IM and increased protein and DNA content by similar amounts in adventitia. This is consistent with the NE-induced SMC hypertrophy and AFB proliferation we measured in these cells cultured from rat aorta (see Faber et al\(^2\)). The trophic and phenotype changes induced by NE were strongly increased by balloon injury. This study is the first demonstration of a direct trophic action for NE on SMCs and AFBs in the intact vascular wall. These results suggest that the attenuation of neointimal growth after balloon injury by systemic \(\alpha\)-AR antagonists\(^7\)–\(^10\) may have resulted from blockade of the direct tropic effect of NE identified herein, rather than from secondary hemodynamic or humoral changes.

The threshold for NE-induced growth was between 10 to 100 nmol/L. This concentration is 5- to 50-fold higher than resting arterial blood levels that average \(\approx\)2 nmol/L in mammals including humans.\(^2,23\) However, it should be emphasized that we only examined NE exposure for 48 hours, that sympathetic activity and plasma NE levels can increase by 10-fold with behavioral or physiological stress, and that plasma levels progressively increase with age and blood pressure in mammals including humans.\(^2\) Moreover, plasma NE predominantly represents spillover from nerves in the vascular wall wherein steady-state estimates range from 1 to 10 000 nmol/L over 1 to 10 Hz stimulation, depending on proximity to nerve varicosities and innervation density.\(^2\) Although possessing advantages over cell culture and in vivo studies where systemic drug administration can cause confounding hemodynamic and neurohumoral changes, our results may not predict in vivo responses. Limitations include absence of normal axial tension, pulsatile pressure, shear stress, blood-borne cells, humoral factors, and injury of cells
at the cut ends of the vessel, although the latter represent a tiny fraction of cells present in these 40-mm-long vessels. However, in contrast to the progressive disturbances in vascular wall cells when arteries are maintained in organ culture without wall tension, the application of tonic circumferential tension to simulate the normal mean arterial pressure, which was done herein, promotes retention of the quiescent contractile SMC phenotype and marker protein expression (see Bardy25). Nevertheless, in vivo pharmacological and gene targeting methods for local stimulation and blockade of AR subtypes are needed to confirm the present findings.

The medial hypertrophy induced by NE in uninjured aorta was associated with decreases (although constant amounts of DNA, but more protein, were loaded; see Figure 7 legend) in αSM-actin, MHC-B, and desmin — proteins expressed by the quiescent contractile SMC phenotype of the normal vascular wall. These changes are consistent with the induction of the synthetic proliferative phenotype of SMCs in culture.1 However, they differ from the expression pattern induced at either time-point by injury, per se. Norepinephrine augmented the reduction in αSM-actin evident 4 days after injury, and injury increased the effect of NE alone to decrease MHC-B and vinculin. Similar effects were produced by Ang II in day-4 injured vessels. A limitation of these data for the injured vessels, however, is that changes secondary to SMC phenotypic alterations or to migration of AFBs into the intima-media cannot be distinguished. There is evidence1,2 that 2 to 7 days after balloon injury, AFBs migrate into the media and intima, and thereafter express a myofibroblast phenotype characterized in the expanding neointima by lower levels of expression αSM-actin and desmin. In the present studies, adventitia did not express SMC marker proteins in detectable levels in uninjured or in day-4 or day-12 injured vessels, although αSM-actin doubled to almost significance. This is consistent with evidence that myofibroblasts are only transiently detected in adventitia, generally between 7 and 14 days after balloon injury, presumably because AFBs remaining in adventitia have delayed expression of the myofibroblast phenotype that is either transient or removed by apoptosis.2,3 Thus, possible migration of AFBs after injury could have contributed to the reduced αSM-actin in media of day-4 vessels. Likewise, migration of myofibroblasts into the media could have contributed to the increased levels of αSM-actin evident at day 12. Stimulation by NE of AFB proliferation in intima-media in day-4 vessels could underlie its effect to further reduce SMC marker proteins at this time (Table), as could an effect of NE to stimulate AFB migration. It is not known if NE stimulates AFB migration. An effect of NE to augment migration of myofibroblasts into intima-media in day-12 vessels, or to augment the already increased αSM-actin expression by medial cells at this point during injury repair, could underlie the effect of NE at this stage (Table). The observed injury-induced increase in NE trophic sensitivity is congruent with the doubling of contractile sensitivity to nerve stimulation and 3-fold increase in wall NE content 4 weeks after balloon injury of rabbit aorta.26

Norepinephrine-induced growth appeared to be mediated by the α1A-AR in media and α1B-AR in adventitia of injured aorta, whereas the abundance of α1-AR subtypes is α1D>α1B>α1A in media and the reverse of this in adventitia of, albeit, uninjured aorta whose contraction is mediated by α1D-ARs (see Faber et al2). However, coupling of αARs to trophic pathways may be altered, as evidenced by our previous studies in cultured rat aorta SMCs where the hypertrophic effect of NE appeared to be mediated by α1D-ARs but not the also-present α1B-ARs.27 In that study, although cell culture did not alter expression of mRNA or density of these two receptor types, α1A-AR (and α2D-AR) expression was greatly reduced. In addition, NE induced with similar potency dose-dependent growth of Rat-1 fibroblasts overexpressing each of the cloned α1-AR subtypes,27 demonstrating that all 3 subtypes can couple in an artificial system to trophic pathways.27,28

A limitation of this study is that, although there are no better α1B-AR antagonists than AH11110A, it only possesses 10- to 30-fold affinity (α1B>α1A>α1D).18,21 Accordingly, ≈80% of α1B-ARs plus 10% to 20% of α1D- and α1A-ARs should be blocked at the 0.1 μmol/L concentration used herein. However, the KMD data suggests that any low-level of α1A-AR blockade by AH was not sufficient to affect the trophic effect of NE on the intima-media. Also, the completely different effect in media and adventitia of the three antagonists provides a strong confirmation of selectivity. However, in contractile studies of rat aorta (α1D-dependent), rabbit aorta (α1B-dependent), and rat vas deferens (α1A-dependent), Eltze et al29 found 12-fold selectivity of AH for α1B- over α1D-ARs, but no selectivity over α1A-ARs. Additional studies are required to determine if this failure to differentiate in their study, which is inconsistent with binding data18,21 and the trophic results herein, is related to differences in potency of α1-AR-subtype antagonists between vascular and vas deferens, measurement of contractile versus growth responses, or to some other factor. It is also possible that in the day-4 injured aorta, the proportions of α1AR subtypes and/or the fidelity of their intracellular pathways and interaction with other pathways whose activities have been altered by injury-repair, could be different and contribute to the clear outcome of the data in Figures 4B and 6. KMD3213 almost completely abolished the increase of NE in DNA and protein synthesis in media, with no effect on adventitia. In contrast, AH11110A abolished the increase of NE in DNA and inhibited the increase in protein content in adventitia, with no effect on media. BMY7378, however, had no effect on either layer. It is also noteworthy that both media and adventitia were studied while intact and exposed to the same ligands at the same time. Despite the clear conclusion from these results, confirmation is needed by genetic approaches or the development of more selective antagonists.

In the present study, we examined whether the trophic effects of α1-AR stimulation are influenced by concomitant α2D-AR and β-AR stimulation, because these receptors are also present.5,30 Whereas all three classes of ARs participate in the trophic action of NE in uninjured aorta media and adventitia (Figure 5), α1-ARs mediate virtually all of the enhanced effect of NE after injury. The only exception was the NE-induced increase in DNA content in 4-day injured adventitia, where blockade of α2-ARs, but not β-ARs, during
concomitant α-AR stimulation abolished the increase in DNA. As in uninjured vessels, this suggests that concomitant α2-AR stimulation (possibly in association with a reduction in cAMP) might be permissive for α1-AR growth, or itself be stimulatory, and β-AR inhibitory (possibly in association with an increase in cAMP) in the presence of the predominant α1-AR trophic action. The current studies agree with our previous report wherein growth-arrested rat aorta SMCs responded to NE with hypertrophy that was blocked by α1-AR but not α2-AR or β-AR antagonists.27

In conclusion, maintained adrenergic stimulation induced hypertrophy of SMCs and proliferation of AFBs. This may have adaptive structural and mechanical significance by inducing wall thickening to normalize wall stress and responsiveness to myogenic and neurohumoral vasoconstrictors in the presence of elevated arterial pressure in chronic sympathoexcitatory states. It is also well known that sympathetic activity increases with age in humans, in association with wall thickening and stiffening.31 The trophic effects of NE were greatly augmented after injury and appear to be mediated by different α1-AR subtypes on SMCs (α1A) and AFBs (α1B), neither of which contributes significantly to contraction of rat aorta (see Faber et al3 and Saussy et al18). The results provide the first direct evidence that elevated catecholamines might augment restenosis by a direct trophic effect on vascular wall cells. This same trophic action may worsen intimal lesion growth induced by other types of surgical, atherogenic, or hemodynamic injuries. Selective blockade of these receptors may provide a strategy to lessen vascular wall lesion growth.

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References

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Materials and Methods:

**Arterial Injury Model.** Sprague-Dawley rats (~350 animals, 450-500 g) were anesthetized with ketamine plus acepromazine and received atropine (54 μg/kg, sc) and cephalizin (50 mg/kg, im). The left thyroid, occipital, and distal external carotid arteries were steriley exposed and ligated. After heparin administration (125 U/kg im and 125 U/kg sc), 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 wound was treated with nitrofurazone, and pentazocine (10 mg/kg, im) was given for analgesia. All procedures were conducted per NIH guidelines.

**Aorta Organ Culture.** Four or 12 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. An organ culture system described in detail elsewhere (1-3) was used to maintain the aorta under circumferential 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 5mg/L transferrin (Sigma), 17.6 mg/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 tension favors the quiescent SMC phenotype (1,4,5), whereas a neointima forms spontaneously and SMC marker proteins that characterize the contractile phenotype decline when vessels are placed into organ culture in the absence of load (4,6). The following drugs were tested (stocks in PBS): Norepinephrine (NE, 0.01-1 μmol/L, Sigma, St. Louis, MO; in 100 μmol/L ascorbate in PBS), α2-AR antagonist atipamazole (Orion-Farmos Pharmaceutical, 1 μmol/L), β-AR antagonist propranolol (Sigma; 1 μmol/L), α1D-AR antagonist BMY 7378 (BMY, RBI Biochemical, Natick, MA; 100 nmol/L), α1A-AR antagonist KMD 3213 (KMD, a generous gift from Dr. Y. Kurashina, Kissei Pharmaceutical, Matsumoto-City, Japan; 100 nmol/L), α1B-AR antagonist AH 11110A (AH, Tocris, Ballwin, MO; 100 nmol/L),
angiotensin II (AngII, Sigma, 1 nmol/L). Media was changed and drugs re-added at 24h. After 48h in culture, vessel length was measured microscopically in 4°C PBS, and intima-media (IM) was separated from adventitia (1-3).

**Protein Synthesis, Protein Content, and DNA Content.** Protein synthesis was measured during the last 24h interval (2). Frozen IM or adventitia were pulverized at -80°C, added to buffer composed of PBS containing 1% nonidet p40 (Sigma), 0.5% sodium deoxycholate (Sigma), 1% sodium dodecyl sulfate (SDS, GibcoBRL, Grand Island, NY) (RIPA), and proteinase inhibitors, and homogenized on ice at maximal speed for 15 sec. All subsequent procedures were conducted on ice. Homogenates were passed 3 times through a 21ga needle to shear DNA, and cells were allowed to lyse for 30 min. After centrifugation for 20 min at 14,000 rpm, protein content of the supernatant was determined in duplicate. DNA content was determined in triplicate (Hoefer DyNA Quant 200; Amersham Pharmacia, San Francisco).

**Western Blot.** Cell lysates were diluted with 6x sample buffer (10% SDS, 38% glycerol, 9.3% dithiothreitol and 125 mmol/L dissolved in 4x Tris-HCl, 0.4% SDS buffer, pH 6.8) and boiled for 5 min. Because NE and injury affect cell number, cell size and matrix content, protein aliquots were loaded as constant amounts of DNA (protein amounts loaded are given in figure legends). After electrophoresis through 7-10% SDS-polyacrylamide gels, electo-transfer to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia), and blocking with Superblock (Pierce, Rochford, IL) for 1h at 25°C, followed by washing with TBST buffer (20 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.2% Tween-20, pH 7.6), separate membranes were probed with primary antibodies to the following proteins: αSM-actin (1A4, DAKO, Denmark; 1:2000), desmin (D1033, Sigma; 1:1500), vinculin (V9131, Sigma; 1:2000), smooth muscle myosins SM1 and SM2 (a generous gift of RS Adelstein, NIH, Bethesda; 1:20,000 and 1:10,000, respectively) and nonmuscle myosin heavy chain-B (MHC-B, PRB-445P, Covance, Richmond, CA; 1:2000). After washing 5 times with TBST, membranes were incubated with a 1:10,000 dilution of the species-specific secondary antibody conjugated to horseradish peroxidase (Sigma) for 1h at RT. After washing 5 times with TBST over 30 min, immunocomplex was visualized (ECL, Amersham Pharmacia
Biotech) on film (X-OMAT, Eastman Kodak, Rochester, NY) and quantified (Kodak Imagestation 440CF).

**Immunohistochemistry.** Aortae were removed after perfusion fixation at 100 mmHg with 4% paraformaldehyde in PBS. Adjacent paraffin-embedded 5 um serial sections from the midpoint of the thoracic aorta were stained or processed for standard immunohistochemistry using biotinylated anti-mouse αSM-actin antibody (DAKO 1A4, 1:50 dilution) or non-immune mouse IgG, and diaminobenzidine visualization.

Data are given as means ± S.E. were subjected to unpaired 2-tailed t-tests, or ANOVA followed by Bonferroni tests for multiple comparisons, unless indicated differently. A value of p < 0.05 was considered significant.

**References:**


