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α_1-Adrenergic Stimulation and β_2-Adrenergic Inhibition of DNA Synthesis in Vascular Smooth Muscle Cells

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SUMMARY

Effects of catecholamines on DNA synthesis in vascular smooth muscle cells (VSMC) were investigated in a chemically defined medium that included insulin, transferrin, and sodium selenite. Smooth muscle-rich preparation was obtained from rat aortic media and VSMC were further purified by cell cloning. A clone that was positive for smooth muscle actin and was negative for the coagulation factor VIII was used in this study. The fetal calf serum-induced proliferation was enhanced by α-adrenergic and inhibited by β-adrenergic stimulation. When cells of low passages were used, dose-response curves for norepinephrine were biphasic; when cells were subconfluent, norepinephrine stimulated DNA synthesis at as low as 1 nM and was apparently ineffective at more than 100 nM. When cells were confluent, the effect of norepinephrine was inhibitory at lower concentrations (<1 nM) and stimulatory at relatively higher concentrations. Cells of higher passages exhibited only inhibitory effects of the amine. Stimulatory and inhibitory effects on DNA synthesis were mediated through α_1- and β_2-adrenergic receptors, respectively. Thus, the α_1-agonist phenylephrine was more potent than the α_2-agonist clonidine in stimulating DNA synthesis. An α_1-adrenergic antagonist, prazosin, was more effective than the α_2-adrenergic antagonist yohimbine in antagonizing the stimulatory effect of norepinephrine. β-Adrenergic agonists inhibited DNA synthesis with IC_{50} values in the nanomolar range; the rank order of potency of agonists was isoproterenol > salbutamol ≥ (−)-epinephrine ≫ (−)-norepinephrine, consistent with β_2-receptor specificity. (+)-Epinephrine or (+)-norepinephrine, the stereoisomers of the catecholamines, were ineffective. The inhibitory effects of norepinephrine were reversed by β-adrenergic antagonists, with the rank order of potency of pindolol > butoxamine > atenolol, consistent with β_2-receptor specificity. The dose-response curves of norepinephrine, therefore, seemed to be determined by a balance between α_1-receptor-mediated stimulation and β_2-receptor-mediated inhibition of DNA synthesis. Minimum time required for exhibiting α_1-adrenergic or β_2-adrenergic effects was between 6 and 15 hr, suggesting that the G_0 or G_1 phase of the cell cycle might be the site of action. These results show that catecholamines dually modulate DNA synthesis in VSMC through specific adrenergic receptors.

Among major risk factors for atherosclerosis in humans, hypertension, psychological stress, and cigarette smoking are related to catecholamines (1–3). In fact, it has been shown experimentally that catecholamines aggravate atherosclerosis in animals and humans (4–6). Circulating catecholamines may affect the functions of many tissues. It is established that abnormal proliferation of VSMC is a key event in early stages of artherosclerosis (7). Therefore, it is possible that catecholamines have direct effects on the proliferation of VSMC.

For this purpose, we have used pure VSMC, because the conventional primary culture of VSMC from rat aorta is often contaminated with endothelial cells whose DNA synthesis is known to be modulated by catecholamines (8), implying that data on the mixed population does not necessarily reflect those for VSMC. More importantly, endogenous substances such as prostacyclin and endothelin released from endothelial cells affect profoundly the DNA synthesis of VSMC (9, 10). We have also employed a chemically defined medium that is free from FCS, because catecholamines may be metabolized by monoamine oxidase present in the serum (11).

We have found that catecholamines have direct and dual effects on DNA synthesis of VSMC through specific adrenergic receptors, stimulation through α_1-adrenergic receptors and inhibition through β_2-adrenergic receptors.

Experimental Procedures

Materials. The chemicals were obtained from the following companies. [^{3}H]TdR (42 Ci/mmol), Amersham, UK; butoxamine, Burroughs Wellcome Co. (Research Triangle Park, NC); isoproterenol, atenolol, salbutamol (albuterol), sodium selenite, insulin, transferrin, superoxide dismutase, and catalase, Sigma (St. Louis, MO); FCS (lot. 1111620), Hyclone (Logan, UT); and trypsin/EDTA, and Earle's M199,

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ABBREVIATIONS: VSMC, vascular smooth muscle cells; RACS, rat aortic clonal smooth; RACE, rat aortic clonal endothelium; TdR, thymidine; FCS, fetal calf serum.

GIBCO (Grand Island, NY). Monoclonal antibodies (IgGl, κ) to chicken gizzard actin were purchased from Biogenex Laboratories (San Ramon, CA) (catalogue no. 291901, lot L090058) and goat anti-human factor VIII antisera from ICN ImmunoBiologicals (Lisie, IL) (catalogue no. 68-112, lot 0013). ImmunoStaining kits, goat universal kit (Histogen PAP, HP000-5G, lot PG028) and mouse universal kit (Stravigen B-SA, HA000-5M, lot AM128), were purchased from Biogenex Laboratories. Other chemicals were obtained as described previously (12, 13). Tissue culture plasticware was purchased from Costar (Cambridge, MA).

Immunohistochemistry. Cells were fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer for 15 min at room temperature. After cells were washed with phosphate-buffered saline, they were processed according to the manual of the staining kits, which consisted of 3% hydrogen peroxide for blocking endogenous peroxidase, normal sera of appropriate animals for control, 3-amino-9-ethylcabazol and *N*, *N*-dimethyl formamide for dye, secondary antibodies of appropriate animals, and peroxidase-labeled antibodies (14, 15).

Cell culture. VSMC were obtained from one-year-old male Wistar rats basically according to the method of Smith and Brock (16). Preliminary experiments revealed that the above cell population contained a small number of endothelial cells that were identified by cobblestone-like morphology and the presence of anticoagulant factor VIII-related antigen (17). We cloned the cells to eliminate the endothelial cells. Cell cloning was done by use of cloning cylinders (18). Cells were passaged eight times to complete the cloning and were stored in liquid nitrogen. Therefore, the passage number of eight was defined as cells frozen in liquid nitrogen. A VSMC clone used in this study, designated RACS-1, is the same clone as 1YB4 in Ref. 10. Other cloned cells that were not used immediately were stored in liquid nitrogen.

[^{3}H]TdR incorporation into DNA. RACS-1 cells (passages 13–28) were seeded in a 96-well tissue culture cluster (0.32-cm^2/well) at the densities described in the figure legends and were cultured for 3 days in the presence of 10% FCS. The growth medium was then replaced by a chemically defined medium after the cell sheet had been washed three times with the serum-free medium (M199 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite). Cells were incubated in the serum-free medium for 96 hr. The medium was changed to a fresh one every day during this period. Three hours after the last change of the medium, test agents were added to the medium and, 20 hr later, [^{3}H]TdR (1 μCi/well) was added, unless otherwise indicated. The culture was terminated 4 hr after the addition of [^{3}H]TdR. In experiments with catecholamines, we added 25 μg/ml superoxide dismutase and 25 μg/ml catalase in the medium in order to prevent oxidative degradation of the amine, according to the method of Mahax and Insel (19). Those enzymes per se, at the concentration used, did not affect [^{3}H]TdR incorporation to VSMC. At the end of culture, the culture medium was aspirated and replaced by phosphate-buffered saline supplemented with 0.5 mg/ml trypsin and 0.2 mg/ml EDTA, followed by incubation at 37° for 5 min. All cells were harvested with a cell harvester (Model 7020; Skatron, Norway). Trapped cells on a filter mat were treated successively with 3 ml of calcium-free phosphate-buffered saline, 3 ml of 6% (w/v) trichloroacetic acid, and 3 ml of ethanol. The radioactivity of the acid- and ethanol-precipitable DNA large enough (>1.5 μm) to be trapped on the filter mat was counted with 3 ml of scintillation fluid.

Results

Cell morphology. Fig. 1 shows the morphology of two clones isolated from the same preparation of the primary culture of rat aortic media. Fig. 1, a–d, shows a clone (RACS-1) that possesses spindle-like morphology, which is most common among cells cloned from the rat aortic media. However, the clone (RACE-1) shown in Fig. 1, e–h, is of cobblestone-like morphology, which suggests that these cells are endothelial cells, although cobblestone-like VSMC has been also reported (20).

The above contention was strengthened by immunohistochemical findings. The RACS-1 cells were positive for antiactin and were negative for the anti-factor VIII antibodies. The putative endothelial clones (RACE-1) were positive for the anti-factor VIII antibodies. RACE-1 clones were hardly stained by anti-smooth muscle actin antibodies.

Effects of α- and β-adrenergic stimulation on proliferation of VSMC. Fig. 2 shows the effects of α- and β-adrenergic stimulation on the proliferation of RACS-1 cells. We have included 5% FCS, because we noted that the serum-free medium described above did not support the cells for a period longer than 10 days. Norepinephrine alone did not cause apparent effects on the growth induced by 5% FCS. However, norepinephrine together with butoxamine, a β-$_2$-adrenergic blocking agent, stimulated significantly the growth of RACS-1 cells, whereas the amine together with phentolamine, an α_1-adrenergic blocking agent, inhibited the growth of RACS-1 cells at a higher cell population. Butoxamine or phentolamine alone did not change the growth curve induced by 5% FCS.

Effects of norepinephrine on DNA synthesis in VSMC. Different initial densities gave dissimilar shapes of the dose-response curves (Fig. 3, a and b); when cells were seeded at an initial density of 2×10^4 cells/well (subconfluent), the catecholamine showed stimulatory effects on DNA synthesis at concentrations as low as 1 nM, whereas the catecholamine at more than 100 nM apparently had no effects; this is possibly due to totally no effects of the amine or to a balance between a stimulatory and an inhibitory effect on the DNA synthesis (Fig. 3a). The magnitude of the growth-modulatory effects of catecholamines is comparable to that of platelet-derived growth factor[1] and endothelin (10). When cells were seeded at 1×10^5/well (confluent), the shapes of the dose-response curves were apparently quite different; the effect of norepinephrine was slightly inhibitory at low concentrations and stimulatory at relatively high concentrations (Fig. 3b). Cells of higher passages (>18) exhibit only inhibitory effects of the amine. Compared with cells of low passages, they also show greater capacity for [^{3}H]TdR incorporation in the absence of norepinephrine; this might be due to some growth factors secreted by VSMC themselves (21, 22).

Receptor specificity for the stimulatory and inhibitory effects of norepinephrine. The effects of norepinephrine were examined in the presence of α- and β-adrenergic antagonists under the same conditions, as indicated in Fig. 4. Antagonists alone, such as yohimbine, prazosin, phentolamine, or butoxamine, caused no effects on DNA synthesis in VSMC. An α-adrenergic antagonist, phentolamine, blocked the stimulatory effects of norepinephrine (Fig. 4a). An α_1-adrenergic antagonist, prazosin, was more effective than the α_2-adrenergic antagonist yohimbine. Moreover, the α-antagonists reduced [^{3}H]TdR incorporation to a level lower than that of the control group. A β-adrenergic antagonist, butoxamine, added with norepinephrine markedly stimulated DNA synthesis. These data can be interpreted as follows. Norepinephrine has both α- and β-adrenergic effects. Addition of norepinephrine together with α-adrenergic antagonists leaves the β effects intact. Therefore, DNA synthesis was inhibited by β-adrenergic effects. On the

[1] Unpublished data.

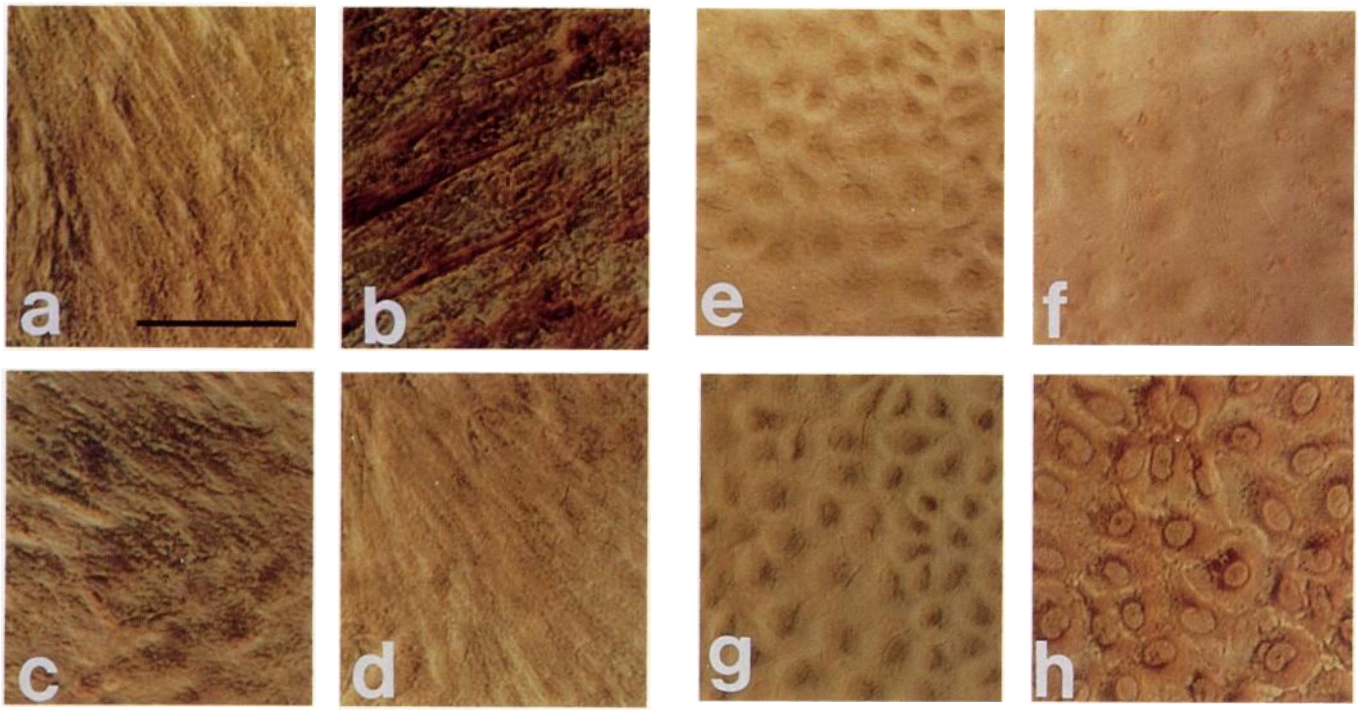

Fig. 1. Morphology and immunohistochemistry of cells cloned from the culture of rat aortic medium. Two clones derived from the same preparation are shown. The photographs were taken when the cloned cells reached confluency. The *black bar* in the *lower right corner* in a represents 100 μm. A clone (RACS-1) that has spindle-like morphology, which is characteristic of VSMC, is shown in a–d. However, another clone (RACE-1), shown in e–h, is of cobblestone-like morphology, indicating that these cells are endothelial cells that contaminated the preparation of the primary culture of VSMC. RACS-1 (a–d) and RACE-1 (e–h) cells were stained with the peroxidase/antiperoxidase system detecting smooth muscle actin (b and f) and factor VIII (d and h). a, c, e, and g, cells stained with control antibodies corresponding to immunized animal species.

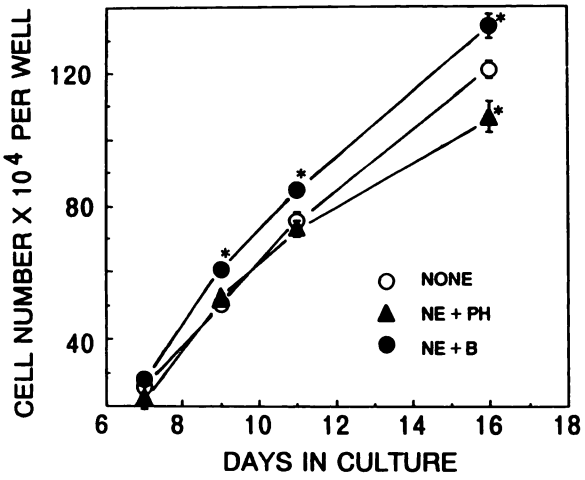

Fig. 2. Effects of α- and β-adrenergic stimulation on proliferation of VSMC. The cloned VSMC (RACS-1, passages 15–17) were seeded at a density of 1.5 × 10^4/dish (2 cm^2) with 0.5 ml of M199 supplemented with 5% FCS. They were allowed to grow for 6 days, and then α- and β-adrenergic agents were added. After the 7th day, the medium supplemented with 5% FCS and added chemicals was changed daily to freshly prepared solutions. The number of cells was counted on the day indicated in the figure. *$p < 0.05$ versus control. *NE*, 1 μM norepinephrine; *PH*, 1 μM phentolamine; *B*, 1 μM butoxamine. Norepinephrine alone or antagonists alone at the concentration used did not produce significant effects on the RACS-1 proliferation.

other hand, addition of norepinephrine together with β-adrenergic antagonist leaves the α effects intact. Therefore, DNA synthesis was stimulated by α-adrenergic effects. When cells were seeded more densely (Fig. 4b), qualitatively similar results were obtained. Cells of higher passages (Fig. 4c) responded only to β-adrenergic stimulation.

Experiments with specific α-adrenergic agonists showed that an α_1-agonist, phenylephrine, was more potent than the α_2-agonist clonidine (Fig. 5), consistent with the above results of antagonist experiments (Fig. 4a). Although clonidine caused some increase in DNA synthesis at 1 μM, this may be explained on the basis of its action on α_1-receptors but with lower efficacy than phenylephrine. These results indicate that the stimulation of DNA synthesis is mediated through α_1-adrenergic receptors.

Onset of α-adrenergic effect on DNA synthesis. [^{3}H] TdR incorporation in VSMC exposed to α_1-adrenergic stimulation for various times was compared with the control (Fig. 6). Enhanced DNA synthesis was observed when α-adrenergic stimulation was present in the medium for 15 hr, whereas 6-hr exposure was not enough to stimulate DNA synthesis.

β_2-Adrenergic receptor-mediated inhibition of DNA synthesis. To substantiate the involvement of β-adrenergic receptors in the inhibition of DNA synthesis, we explored receptor specificity in conditions under which norepinephrine showed only inhibitory effects. Fig. 7 shows dose-response curves of various β-adrenergic agonists. The rank order of potency of effective agonists was isoproterenol > salbutamol > (−)-epinephrine ≫ (−)-norepinephrine, consistent with β_2-receptor specificity (23). (+)-Epinephrine or (+)-norepinephrine, the stereoisomers of the catecholamines, have much lower affinity for β_2-receptors and were ineffective at the highest concentration used (10 μM), implying that the inhibitory effects of the catecholamines are not due to nonspecific toxicity derived from the catechol moiety (24). The effectiveness of sal-

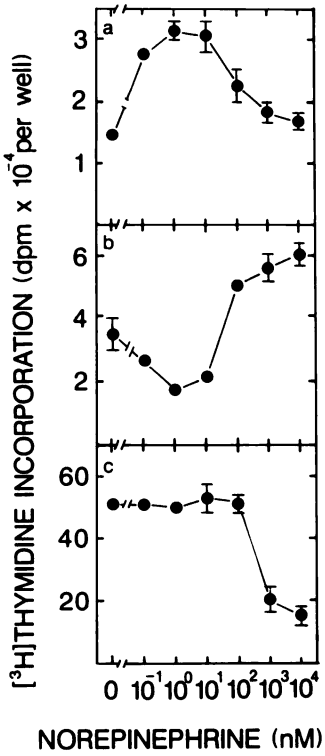

Fig. 3. Effects of norepinephrine on DNA synthesis in VSMC. Cells (a and b, passages between 13 and 18; c, passages between 26 and 28) were seeded at densities of 2×10^4/well (a) or 1×10^5/well (b and c) and were cultured in the presence of 10% FCS. After 3 days, when the cell numbers reached 3×10^4 (a), 1.5×10^5 (b), and 2×10^5 cells per well (c), the growth medium was replaced with a chemically defined medium, which consisted of M199, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite. The medium was changed daily and at 4th day norepinephrine, together with 25 μg/ml superoxide dismutase and 25 μg/ml catalase, was added to the fresh serum-free medium. [^{3}H]TdR (1 μCi/well) was added 20 hr later and the culture was terminated 4 hr after the addition of [^{3}H]TdR. At the end of culture, all cells were harvested and the radioactivity of [^{3}H]TdR incorporated into DNA was measured as described in Experimental Procedures. *Points* and *bars* represent the means and standard errors from six wells. Standard errors at the points without bars were within the *symbols*. Experiments were repeated twice and gave similar results.

butamol, a noncatechol β-agonist, is consistent again with the above contention. Therefore, agonist data show that the inhibitory effects of catecholamines on DNA synthesis are mediated through β_2-adrenergic receptors. (−)-Epinephrine seemed to be somewhat weaker than salbutamol, whereas in other systems epinephrine is considerably more potent than salbutamol (25); some stimulatory effect on α_1-receptors by (−)-epinephrine partly interfered with the inhibitory effects by β_2-receptors. The α-adrenergic agonists methoxamine and clonidine were ineffective (data not shown). Furthermore, the inhibitory effects of norepinephrine were reversed by β_2-adrenergic antagonists (Fig. 8); the rank order of potency was pindolol > butoxamine > atenolol, consistent with β_2-receptor specificity (23, 26).

Onset of β_2-adrenergic effect. [^{3}H]TdR incorporation into VSMC exposed to β_2-adrenergic stimulation for various times was compared with the control (Fig. 9). The inhibition of DNA synthesis by norepinephrine acting on β-receptors was not detectable after 6 hr of exposure but was highly significant after 15 hr of exposure.

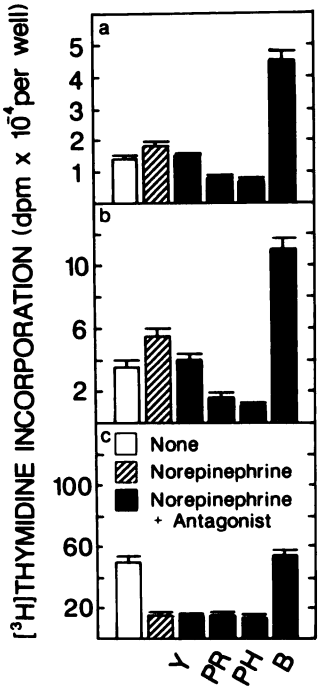

Fig. 4. Adrenergic receptor specificity for the stimulatory and inhibitory effects of norepinephrine on DNA synthesis in VSMC. Norepinephrine (1 μM) alone or in combination with 1 μM yohimbine (*Y*), 1 μM prazosin (*PR*), 1 μM phentolamine (*PH*), and 1 μM butoxamine (*B*) was added once to the medium 24 hr before the harvest. Other experimental conditions in each panel were identical to those indicated in Fig. 3; a, b, and c correspond to Fig. 3 a, b, and c, respectively.

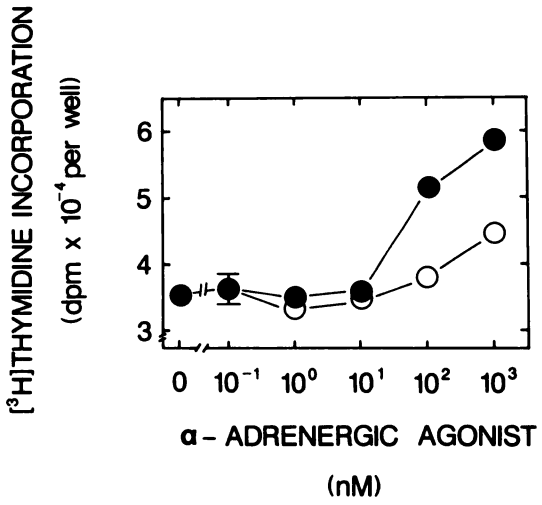

Fig. 5. Effects of α_1- and α_2-adrenergic agonists on DNA synthesis in VSMC. Cells (passages less than 18) were seeded at 2×10^4/well. Phenylephrine (●) or clonidine (○) was added once to the medium 24 hr before the harvest. Other experimental conditions were identical to those in Fig. 3a.

Discussion

Chamley *et al.* (27) showed that antibodies to chicken gizzard actin stained VSMC without staining fibroblasts or endothelial cells. It is well established that factor VIII is a marker of endothelial cells (17). Therefore, it is clear that RACS-1 cells (previously called 1YB4 in Ref. 10) are VSMC and that RACE-1 cells are endothelial cells.

We have shown here that catecholamines modulate DNA

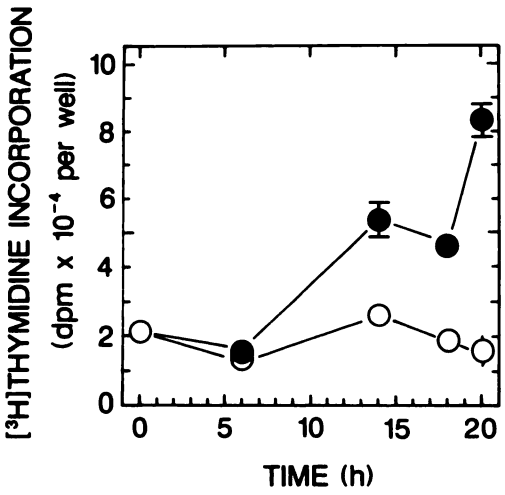

Fig. 6. Onset of α_1-adrenergic effect of DNA synthesis in VSMC. Norepinephrine (1 μM) along with 1 μM butoxamine (●) was added to the medium at indicated hours before the termination of culture. ○, Control. [^{3}H]TdR (1 μCi/well) was always added to the medium 4 hr before the termination of culture. Other experimental conditions were identical to those in Fig. 5.

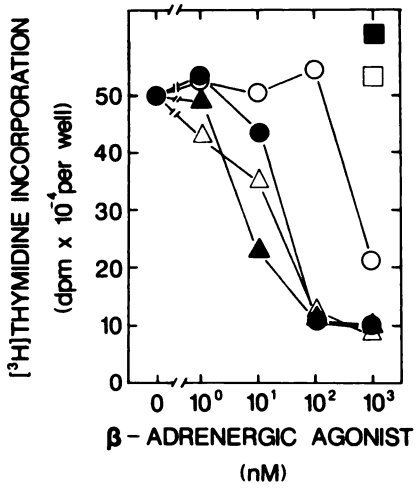

Fig. 7. Effects of β-adrenergic agonists on DNA synthesis in VSMC of high passages. Cells (passages between 26 and 28) were seeded at 1 × 10^5/well and were cultured in the presence of 10% FCS for 3 days. Various compounds tested were added once to the medium 24 hr before the harvest. ▲, (−)-Isoproterenol; △, salbutamol; ●, (−)-epinephrine; ○, (−)-norepinephrine; □, (+)-norepinephrine; ■, (+)-epinephrine. Other experimental conditions were identical to those in Fig. 3.

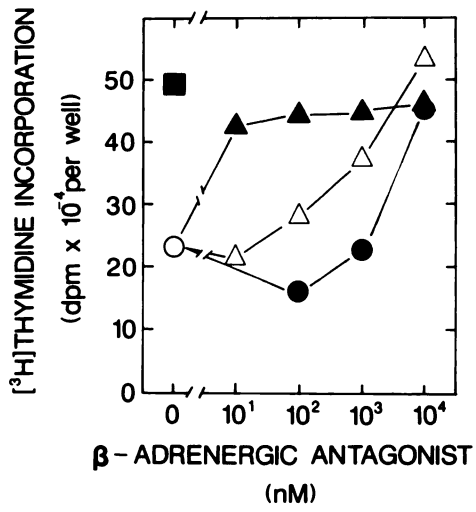

Fig. 8. Reversal by β-adrenergic antagonists of norepinephrine-induced inhibition of DNA synthesis in VSMC of high passages. Pindolol (▲), butoxamine (△), or atenolol (●) was added together with 1 μM norepinephrine (○). ■, None. Experiments were done as described in the legend to Fig. 7.

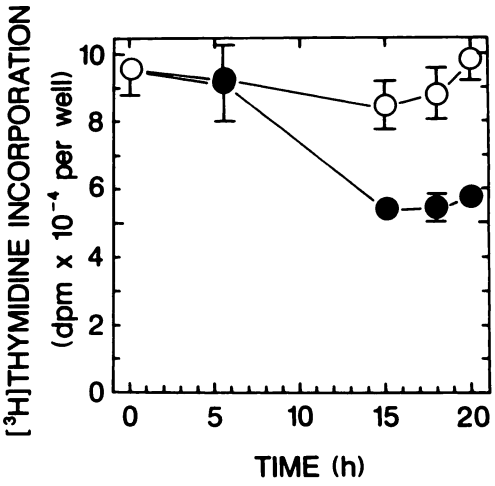

Fig. 9. Onset of β_2-adrenergic effect on DNA synthesis in VSMC of high passages. Cells (passages between 26 and 28) were cultured in the presence of 10% FCS for 3 days. Norepinephrine (1 μM) along with 1 μM phentolamine (●) was added to the medium at indicated hours before the termination of culture. ○, Control. Other experimental conditions were identical to those in Fig. 6.

synthesis by pure VSMC in a chemically defined medium, which contained insulin, transferrin, and sodium selenite without any catecholamine-metabolizing enzymes. In order to circumvent nonenzymatic degradation of catecholamines, we have used catalase and superoxide dismutase (10). It is probable, therefore, that catecholamines modulate DNA synthesis without any enzymatic or nonenzymatic modifications of the catecholamine molecules. There have been two reports on the mitogenic action of catecholamines in VSMC (28, 29), although the authors did not estimate the purity of preparations in which contamination with endothelial cells could make the interpretation difficult. They also claimed that catecholamines require FCS to show their stimulatory effects on VSMC. This phenomenon can be interpreted as requirement of those factors, such as insulin, transferrin, and sodium selenite, that are necessary for maintaining cell growth in general (30). The alternative possibility is that serum provides mitogens for VSMC that initiate growth and that adrenergic stimulation augments rates of growth factor-dependent pathways.

The dose-response curve of norepinephrine was biphasic. However, the stimulatory and inhibitory effects are mediated through different receptors, with stimulation by α_1-receptors and inhibition by β_2-receptors. It is known that phenotype expression of VSMC with low generation number depends upon the cell seeding density (31). We have demonstrated in this study that the expression of adrenergic signal transduction also depends upon the cell density of VSMC. Cells with low density showed somewhat higher affinity for α_1-adrenergic than for β_2-receptors. Cells with high density showed the opposite situation.

Our data are consistent with other reports *in vivo* showing α-receptor-mediated stimulation and β-receptor-mediated inhibition of ornithine decarboxylase activity (32, 33) and methoxamine (α_1-adrenergic agonist)-increased focal but not diffusive growth of subintimal cells (34). The latter finding is intriguing, because it suggests that only the VSMC that possess α_1-receptors could respond to methoxamine. Dashwood and Bagnall (35) reported that α_1-receptor binding sites are confined to the VSMC close to the endothelium. Therefore, it is possible that only VSMC clones obtained from those near the endothelium may express α_1-adrenergic receptors, and it is unlikely that α_1-adrenergic receptors are distributed among all VSMC present in the media. Although this study used a particular VSMC clone from the media, it could be a population of cells that are able to respond to α_1 stimulation *in vivo*.

Although direct enhancement of cell growth by α_1-adrenergic stimulation has been reported with other types of cells (8, 36, 37), to our knowledge this is the first report that DNA synthesis is inhibited through β_2-adrenergic receptors. Cells with high passage numbers in culture became refractory to α-adrenergic stimulation of DNA synthesis and responded only to β stimulation. The affinity for β-receptors also appeared lower than that of cells with low passage number. This could be partly because of loss of α-receptors or because of some other mechanisms, although we cannot specify the reason from this study. Whatever the mechanisms are, the β_2-receptor-mediated inhibition of DNA synthesis after many passages can be inferred as a negative feedback mechanism that intrinsically controls the growth of VSMC. It may be interesting to note that some hormones, including insulin, follicle-stimulating hormones and sex hormones, also show stimulatory and inhibitory effects on DNA synthesis in melanoma cells (38).

More than 6 hr are required for the α_1- and β_2-adrenergic modulation of DNA synthesis in VSMC. It is unknown whether the continuous presence of catecholamine is necessary for the effects on DNA synthesis in RACS-1 cells. Although we could not exclude the possibility that α_1-adrenergic stimulation produces some growth factors in VSMC, which in turn stimulate DNA synthesis, the time lag may be interpreted as timing of the cell cycle. The adrenergic modification of DNA synthesis might occur at the G_0 or G_1 phase of the cell cycle. Although it is not known what may be a second messenger for regulation of DNA synthesis upon stimulation of α_1- and β_2-adrenergic receptors, it is possible that inositol trisphosphate, diacylglycerol, and cyclic nucleotides may be important (39); calcium, calmodulin-dependent protein kinase, cAMP-dependent protein kinase, and protein kinase C phosphorylate putative proteins that might directly regulate DNA synthesis. At least two endogenous polypeptides phosphorylated by protein kinase C are shown to be present in VSMC (40).

The increase in plasma catecholamines aggravates the extent of atherosclerosis (4–6). The plasma concentration of catecholamines reaches about 5 nM even in a mild stress state in humans, such as public speaking (41). Therefore, the effects of catecholamines at the concentration ranges of less than 10 nM demonstrated in this study could be physiologically meaningful *in vivo*. Because overall response to catecholamines depends on cell density, it is worthwhile pointing out that individual VSMC in most places are separated from one another by the connective tissue skeleton in rats (42) and in humans (43), suggesting that cell density *in vivo* may be subconfluent. Therefore, catecholamines at concentrations as low as 1 nM are likely to stimulate VSMC through α_1-adrenergic receptors. However, as stated above, VSMC after many generations may respond better to β_2 stimulation, resulting in a decrease in VSMC growth. VSMC without β_2-receptor-mediated inhibition, if any, could proliferate to a greater extent than those cells equipped with such negative control systems.

In summary, circulating catecholamines possibly contribute to modification of pathogenesis of atherosclerosis by α_1- and β_2-adrenergic receptor-mediated modulation of VSMC growth.

Acknowledgments

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References

1. Goldstein, D. S. Plasma norepinephrine in essential hypertension: a study of the studies. *Hypertension (Dallas)* **3**:48–52 (1981).
2. Cryer, P. E., M. W. Haymond, G. V. Santiago, and S. D. Shah. Norepinephrine and epinephrine release and adrenergic mediation of smoking-associated hemodynamic and metabolic events. *N. Engl. J. Med.* **295**:573–577 (1976).
3. Kones, R. J. Emotional stress, plasma catecholamines, cardiac risk factors and atherosclerosis. *Angiology* **30**:327–339 (1979).
4. Helin, P., I. Lorenzen, C. Garbarsch, and N. E. Matthiessen. Arteriosclerosis in rabbit aorta induced by noradrenaline. *Atherosclerosis* **12**:125–132 (1970).
5. Kukreja, R. S., B. N. Datta, and R. N. Chakravarti. Catecholamine-induced aggravation of aortic and coronary atherosclerosis in monkeys. *Atherosclerosis* **40**:291–298 (1981).
6. Bhattacharya, S. K., R. N. Chakravarti, and P. L. Wahl. Noradrenaline-induced myocardial infarction in monkeys given an atherogenic diet. *Atherosclerosis* **20**:241–252 (1974).
7. Ross, R. The pathogenesis of atherosclerosis: an update. *N. Engl. J. Med.* **314**:488–500 (1986).
8. Sherline, P., and R. Mascardo. Catecholamines are mitogenic in 3T3 and bovine aortic endothelial cells. *J. Clin. Invest.* **74**:483–487 (1984).
9. Morisaki, N., T. Kanzaki, N. Motoyama, Y. Saito, and S. Yoshida. Cell-cycle-dependent inhibition of DNA synthesis by prostaglandin I_2 in cultured rabbit aortic smooth muscle cells. *Atherosclerosis* **71**:165–171 (1988).
10. Nakaki, T., M. Nakayama, S. Yamamoto, and R. Kato. Endothelin-mediated stimulation of DNA synthesis in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **158**:880–883 (1989).
11. Tabor, C. W., H. Tabor, and S. M. Rosenthal. Purification of amine oxidase from beef plasma. *J. Biol. Chem.* **208**:645–661 (1954).
12. Nakaki, T., T. Nakadate, K. Ishii, and R. Kato. Postsynaptic *alpha*-2 adrenergic receptors in isolated rat islets of Langerhans: inhibition of insulin release and cyclic 3′:5′-adenosine monophosphate accumulation. *J. Pharmacol. Exp. Ther.* **216**:607–612 (1981).
13. Nakaki, T., T. Nakadate, S. Yamamoto, and R. Kato. *Alpha*$_2$-adrenergic receptor in intestinal epithelial cells: identification by [^{3}H]yohimbine and failure to inhibit cyclic AMP accumulation. *Mol. Pharmacol.* **23**:228–234 (1983).
14. Hsu, S. M., L. Raine, and H. Fanger. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase technique: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **30**:1079–1086 (1982).
15. Sternberger, L. A. *Immunocytochemistry*. John Wiley & Sons, New York (1986).
16. Smith, J. B., and T. A. Brock. Analysis of angiotensin-stimulated sodium transport in cultured smooth muscle cells from rat aorta. *J. Cell. Physiol.* **114**:284–290 (1983).
17. Jaffe, E. A. Endothelial cells and the biology of factor VIII. *N. Engl. J. Med.* **296**:377–383 (1977).
18. Adams, R. L. P. *Cell Culture for Biochemist.* Elsevier, Amsterdam, 98–103 (1980).
19. Mahax, L. C., and P. A. Insel. Use of superoxide dismutase and catalase to protect catecholamines from oxidation in tissue culture studies. *Anal. Biochem.* **136**:208–216 (1984).
20. Gordon, D., L. G. Mohai, and S. M. Schwartz. Induction of polyploidy in cultures of neonatal rat aortic smooth muscle cells. *Circ. Res.* **59**:633–644 (1986).
21. Nilsson, J., M. Sjolund, L. Palmberg, J. Thyberg, and C.H. Heldin. Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein. *Proc. Natl. Acad. Sci. USA* **82**:4418–4422 (1985).
22. Majack, R. A., S. C. Cook, and P. Bornstein. Control of smooth muscle cell growth by components of the extracellular matrix: autocrine role for thrombospondin. *Proc. Natl. Acad. Sci. USA* **83**:9050–9054 (1986).
23. Weiner, N. (A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds.). in *Goodman and Gilman's the Pharmacological Basis of Therapeutics.* Norepinephrine, epinephrine, and the sympathomimetic amines, MacMillan Publishing Co., New York, 145–214 (1985).
24. Graham, D. G., S. M. Tiffany, W. R. Bell, Jr., and W. F. Gutknecht.

Autoxidation versus covalent binding of quinones as the mechanisms of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward c1300 neuroblastoma cells *in vitro*. *Mol. Pharmacol.* **14**:644–653 (1978).
25. Minneman, K. P., L. R. Hegstrand, and P. B. Molinoff. Pharmacological specificity of *beta-1* and *beta-2* adrenergic receptors in rat heart and lung *in vitro*. *Mol. Pharmacol.* **16**:21–33 (1979).
26. Bieth, N., B. Rouot, J. Schwartz, and J. Velly. Comparison of pharmacological and binding assays for ten *beta*-adrenoceptor blocking agents and two *beta*-adrenoceptor agonists. *Br. J. Pharmacol.* **68**:563–569 (1980).
27. Chamley, J. H., U. Groschel-Stewart, G. R. Campbell, and G. Burnstock. Distinction between smooth muscle, fibroblasts and endothelial cells in culture by the use of fluoresceinated antibodies against smooth muscle actin. *Cell Tissue Res.* **177**:445–457 (1977).
28. Blaes, N., and J. P. Boissel. Growth-stimulating effect of catecholamines on rat aortic smooth muscle cells in culture. *J. Cell. Physiol.* **116**:167–172 (1983).
29. Bauch, H.-J., J. Grunwald, P. Vischer, U. Gerlach, and W. H. Hauss. A possible role of catecholamines in atherogenesis and subsequent complications of atherosclerosis. *Exp. Pathol.* **31**:193–204 (1987).
30. Barnes, D., and G. Sato. Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.* **102**:255–270 (1980).
31. Chamley-Campbell, J. H., and G. R. Campbell. What controls smooth muscle phenotype? *Atherosclerosis* **40**:347–357 (1981).
32. Thadani, P. V., and S. M. Schanberg. Effect of stress and sympathetic activity on rat cardiac and aortic ornithine decarboxylase activity. *Life Sci.* **25**:1009–1016 (1979).
33. Majesky, M. W., H.-Y. Yang, and M. R. Juchau. Interaction of *alpha* and *beta* adrenergic stimulation on aortic ornithine decarboxylase activity. *Life Sci.* **36**:153–159 (1985).
34. Majesky, M. W., M. A. Reidy, E. P. Benditt, and M. R. Juchau. Focal smooth muscle proliferation in the aortic intima produced by an initiation-promotion sequence. *Proc. Natl. Acad. Sci. USA* **82**:3450–3454 (1985).
35. Dashwood, M., and J. Bagnall. An autographic demonstration of prazosin binding to arterial vessels in the rat. *Eur. J. Pharmacol.* **78**:121–123 (1982).
36. Burch, R. M., A. Luini, D. E. Mais, D. Corda, J. Y. Vanderhoek, L. D. Kohn, and J. Axelrod. $alpha_1$-Adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. *J. Biol. Chem.* **261**:11236–11241 (1988).
37. Cruise, J. L., K. A. Houck, and G. K. Michalopoulos. Induction of DNA synthesis in cultured rat hepatocytes through stimulation of $alpha_1$ adrenoreceptor by norepinephrine. *Science (Wash. D. C.)* **227**:749–751 (1985).
38. Mather, J. P., and G. H. Sato. The growth of mouse melanoma cells in hormone-supplemented, serum-free medium. *Exp. Cell Res.* **120**:191–200 (1979).
39. Rozengurt, E. Early signals in the mitogenic response. *Science (Wash. D. C.)* **234**:161–166 (1986).
40. Nakaki, T., B. C. Wise, and D.-M. Chuang. Substrates for protein kinase C in a cell free preparation of rat aorta smooth muscles. *Life Sci.* **42**:1315–1321 (1988).
41. Dimsdale, J. E., and J. Moss. Plasma catecholamines in stress and exercise. *J. Am. Med. Assoc.* **243**:340–342 (1980).
42. Pease, D. C., and W. J. Paule. Electron microscopy of elastic arteries: the thoracic aorta of the rat. *J. Ultrastruct. Res.* **3**:469–483 (1960).
43. Toda, T., N. Tsuda, I. Nishimori, D. E. Leszczynski, and A. Kummerow. Morphological analysis of the aging process in human arteries and aorta. *Acta Anat.* **106**:35–44 (1980).

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