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α_1-Adrenergic receptor mRNA level is regulated by norepinephrine in rabbit aortic smooth muscle cells

(down-regulation)

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ABSTRACT **Prolonged agonist exposure results in a decrease in the density of α_1-adrenergic receptors in rabbit aortic smooth muscle cells. A cDNA for the α_1-adrenergic receptor was used to assess the effect of norepinephrine on α_1-adrenergic receptor mRNA level in cultured vascular smooth muscle cells from the rabbit aorta. Norepinephrine caused a transient decrease (81% ± 5%; $n = 9$) in α_1-adrenergic receptor mRNA. The effect was concentration dependent (EC_{50}, ≈0.3 μM; maximal effect, 10 μM). The maximum decrease occurred after 4 hr of exposure to norepinephrine and was followed by a gradual return to control levels by 24 hr. The decrease in mRNA level was blocked by prazosin, but not propranolol, and was mimicked by phenylephrine. These results indicate that the effect is mediated by stimulation of the α_1-adrenergic receptor and suggest that it involves one or more α_1-adrenergic-coupled second messenger pathways. The decrease in α_1-adrenergic receptor mRNA caused by norepinephrine exceeds that caused by actinomycin D, suggesting that norepinephrine may cause a decrease in the stability of α_1-adrenergic receptor mRNA. Actinomycin D also blocked the norepinephrine-induced decrease in mRNA level, further suggesting that the effect of norepinephrine requires induction of transcription, presumably leading to synthesis of a labile factor that is necessary for the effect of norepinephrine on α_1-adrenergic receptor mRNA level.**

The effect of sympathetic nervous system stimulation on vascular tone is mediated through the interaction of catecholamines, primarily norepinephrine, with α- and β-adrenergic receptors on the vascular smooth muscle cell plasma membrane. The adrenergic receptors, therefore, present potentially important sites for regulating the vascular responsiveness to sympathetic stimulation. The major adrenergic receptors on vascular smooth muscle cells are of the α_1 and β_2 subtypes, although α_2 and β_1 subtypes are also present in some vessels. The expression of α- and β-adrenergic receptors in several tissues, including vascular smooth muscle, can be regulated by catecholamines (1–5). Exposure to catecholamines for periods in excess of several hours results in a decrease in the density of α_1- and β_2-adrenergic receptors (1–5).

Regulation of the density of adrenergic receptors on a vascular smooth muscle cell must be a function of the relative rates of receptor synthesis and degradation and also likely involves a cycling of receptors between cell surface and nonsurface accessible sites (2). The availability of a cDNA probe for the β_2-adrenergic receptor has provided insight into the effects of β_2-adrenergic receptor stimulation on the expression of mRNA for that receptor (6–8). Although there has been indirect information regarding the regulation of α_1-adrenergic receptor synthesis based on apparent rates of receptor appearance and disappearance (2), relatively little is known about how catecholamines cause a decrease in α_1-adrenergic receptor density. Recently, Cotecchia *et al.* (9) have cloned the gene for the α_1-adrenergic receptor. Norepinephrine causes a marked decrease in the density of α_1-adrenergic receptors in rabbit aortic smooth muscle cells (RbSMCs) (4, 5). The purpose of these experiments was to determine whether norepinephrine affects the level of α_1-adrenergic receptor mRNA in RbSMCs.

METHODS

Cell Culture. RbSMCs were cultured and passaged as described (10), except that experimental plates were grown in M199 medium (Sigma) supplemented with 5% fetal calf serum (Sigma).

[^{3}H]Prazosin Binding. α_1-Adrenergic receptor density was determined by using a saturating concentration of [^{3}H]-prazosin (0.5 nM) in a crude cellular homogenate as described (10).

Preparation of RNA and Northern Blot Analysis. For Northern blot analyses, RNA from three confluent 100-mm plates was pooled for each experimental condition. Total RNA was extracted from cell cultures by the guanidinium isothiocyanate/cesium chloride method (11). mRNA species were denatured with formaldehyde and formamide and separated by size by electrophoresis on a 1.3% agarose/1.5% formaldehyde gel. RNA was transferred to nitrocellulose membranes by capillary transfer and was fixed by baking in a vacuum oven at 80°C for 2 hr.

A full-length cDNA probe for the α_1-adrenergic receptor (supplied by S. Cotecchia, M. G. Caron, and R. J. Lefkowitz, Duke University Medical Center, Durham, NC) (9) was uniformly labeled with ^{32}P to a specific activity of 2–5 × 10^8 cpm/μg by the random hexamer priming method (12) and hybridized to nitrocellulose blots overnight at 42°C as described (13). Hybridized blots were washed twice for 15 min at room temperature with 300 mM NaCl/30 mM trisodium citrate, pH 7.0/0.1% SDS, and four times for 15 min at 60°C with 30 mM NaCl/3 mM trisodium citrate, pH 7.0/0.1% SDS. The amount of α_1-adrenergic receptor mRNA per lane was determined by exposing the washed blots to Kodak XAR film with an intensifying screen at −70°C overnight and measuring the density of the exposed film with a laser densitometer (LKB). The size of the hybridizing species was estimated by using the 18S and 28S ribosomal RNA bands as standards.

After the analysis of α_1-adrenergic receptor mRNA, all filters were probed with a ^{32}P-labeled oligonucleotide complementary to 18S ribosomal RNA, washed, and autoradiographed as described (13). Hybridization of this oligonucleotide was used as a measure of total RNA loaded for each sample. All autoradiographic values for α_1-adrenergic recep-

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Abbreviation: RbSMC, rabbit aortic smooth muscle cell.

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tor mRNA levels were normalized relative to the level of 18S ribosomal RNA to correct for potential differences in the amount of RNA loaded.

[^{3}H]Uridine Incorporation. In preliminary experiments, RbSMCs on 35-mm culture plates were incubated with various concentrations of actinomycin D (0.5–10 μg/ml; Sigma) for 30 min prior to addition of [^{3}H]uridine (1 μCi/ml; 1 Ci = 37 GBq; New England Nuclear). After 4 hr, cells were washed three times with ice-cold phosphate-buffered saline, precipitated with 5% trichloroacetic acid, and counted in a liquid scintillation counter. Actinomycin D, at a concentration of 5 μg/ml, inhibited [^{3}H]uridine incorporation by >95%.

Data Analysis. Data are presented as the mean ± SEM. Statistical analysis was performed by two-tailed nonpaired *t* tests.

RESULTS

Incubation of RbSMCs with norepinephrine (10 μM) caused a time-dependent ($t_{1/2}$, ≈6 hr) decrease in α_1-adrenergic receptor density to 34% ± 8% (n = 6) of the control value at 24 hr (Fig. 1). In the continued presence of norepinephrine, receptor density remained at 35–40% of control for 72 hr (Fig. 1).

The hamster α_1-adrenergic receptor cDNA hybridized with high stringency to a mRNA from RbSMCs of ≈2.1 kilobases (kb) (Fig. 2*A*). Incubation of RbSMCs with norepinephrine (10 μM) caused a time-dependent decrease in the level of α_1-adrenergic receptor mRNA as normalized to 18S ribosomal RNA (Fig. 2). The level of α_1-adrenergic receptor mRNA had already decreased by ≈50% after 2 hr of incubation and continued to decrease for 4 hr, at which time it was 19% ± 5% (n = 9) of the control level.

The level of α_1-adrenergic receptor mRNA began to increase by 8 hr, and by 24 hr it had returned to 103% ± 13% (n = 3) of the basal level (Fig. 2). Addition of norepinephrine (10 μM) to the cells every 4 hr for 24 hr did not prevent the recovery of mRNA levels at 8 or 24 hr. Therefore, the increase in α_1-adrenergic receptor mRNA after 4 hr was not simply due to oxidation or metabolism of norepinephrine in the incubation medium.

To determine the receptor by which norepinephrine exerted its effect on α_1-adrenergic receptor mRNA levels, RbSMCs were treated with various adrenergic agonists and antagonists. The effects of norepinephrine were concentration dependent, with an EC_{50} of ≈0.3 μM (Fig. 3). The α_1-adrenergic agonist phenylephrine (10 μM) mimicked the effects of norepinephrine on α_1-adrenergic receptor mRNA

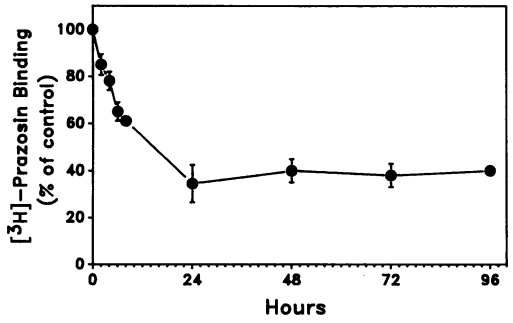

FIG. 1. Time course of the norepinephrine-induced decrease in α_1-adrenergic receptor density. Saturation binding of [^{3}H]prazosin was performed on crude homogenates of RbSMCs treated with norepinephrine (10 μM) for the times indicated. Data depicted are the means ± SEM of four to six experiments, each performed in triplicate. The data are normalized to the control value of α_1-adrenergic receptor density in each experiment (mean control density, 60 ± 6 fmol per mg of protein; n = 6).

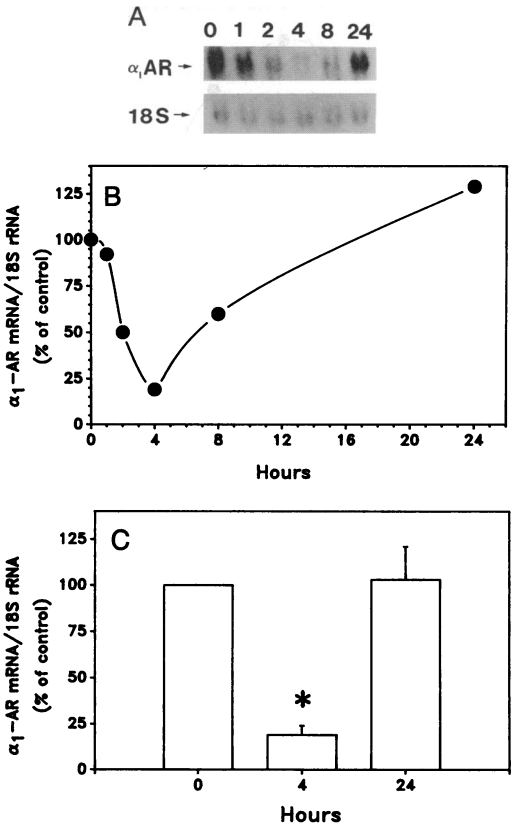

FIG. 2. (*A*) Time course of the effect of norepinephrine on the level of α_1-adrenergic receptor (AR) mRNA. RbSMCs were incubated with norepinephrine (10 μM) for the indicated times. mRNA was extracted, subjected to Northern blot analysis with ^{32}P-labeled cDNAs for the α_1-adrenergic receptor and 18S ribosomal RNA, and autoradiographed as described. (*B*) The intensities of the 2.1-kb α_1-adrenergic receptor bands from the autoradiograph in *A* were quantitated by laser densitometry, normalized to the intensity of the 18S ribosomal RNA band, and are expressed as a percentage of the control levels. (*C*) The intensity of the autoradiographic signal for the α_1-adrenergic receptor mRNA (normalized to 18S ribosomal RNA) from cells incubated with 10 μM norepinephrine for 0, 4, and 24 hr. The data represent the mean ± SEM for nine (0 and 4 hr) or three (24 hr) experiments. *, $P < 0.01$ vs. both 0 and 24 hr.

levels at 4 hr (21% of control; data not shown). The α_1-adrenergic receptor-selective antagonist prazosin (1 μM; 30-min preexposure) prevented the norepinephrine-induced changes in α_1-adrenergic receptor mRNA level, whereas pretreatment with the β-adrenergic antagonist, *dl*-propranolol (1 μM; 30-min preexposure), had no effect (Fig. 3). Treatment of RbSMCs with prazosin or *dl*-propranolol alone had no effect (data not shown). These results indicate that the norepinephrine-induced decrease in α_1-adrenergic receptor mRNA is mediated by activation of the α_1-adrenergic receptor.

As a first approach to assessing the mechanism by which norepinephrine decreases the level of α_1-adrenergic receptor mRNA, the effects of actinomycin D, an inhibitor of transcription, were studied in control cells and in cells exposed to norepinephrine. At a concentration of 5 μg/ml, which inhibited [^{3}H]uridine incorporation by >95% (data not shown), exposure to actinomycin D alone for 4 hr caused a 46% ± 10% decrease in α_1-adrenergic receptor mRNA, which was significantly less than the 81% ± 5% decrease caused by 10 μM norepinephrine (Table 1). This finding indicates that the effect of norepinephrine on α_1-adrenergic receptor mRNA cannot be accounted for entirely by the inhibition of transcription and

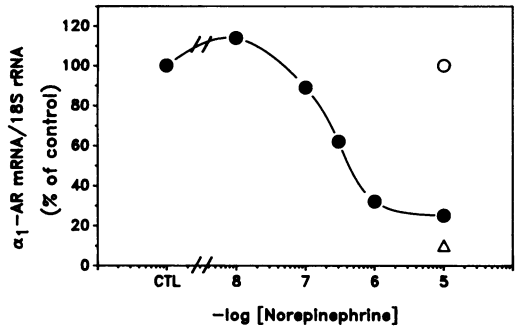

FIG. 3. Concentration dependence and pharmacologic specificity of the norepinephrine-induced decrease in α_1-adrenergic receptor (AR) mRNA level. RbSMCs were incubated for 4 hr with various concentrations of norepinephrine (●), 1 μM prazosin + 10 μM norepinephrine (○), or 1 μM *dl*-propranolol + 10 μM norepinephrine (△). The mRNA was extracted and Northern blots were hybridized with the ^{32}P-labeled cDNA for the α_1-adrenergic receptor. The intensities of the 2.1-kb bands from the autoradiographs were normalized for the level of 18S ribosomal RNA and are expressed as a percentage of control values. Prazosin (1 μM) and *dl*-propranolol (1 μM) alone had no effect on α_1-adrenergic receptor mRNA level (data not shown).

further suggests that norepinephrine may act, at least in part, by decreasing the stability of α_1-adrenergic receptor mRNA.

To determine whether transcription is required for the effect of norepinephrine to occur, cells were exposed to norepinephrine (10 μM) for 4 hr in the presence or absence of actinomycin D (5 μg/ml) added 30 min prior to norepinephrine. Under these conditions, norepinephrine caused no decrease in α_1-adrenergic receptor mRNA over that caused by actinomycin D alone (Table 1). This result suggests that the effect of norepinephrine on α_1-adrenergic receptor mRNA level may be dependent, at some level, on transcription.

DISCUSSION

These experiments show that exposure of vascular smooth muscle cells to norepinephrine results in a decrease in the level of mRNA for the α_1-adrenergic receptor. This effect is blocked by the highly α_1-selective adrenergic antagonist prazosin, but not by the β-adrenergic antagonist *dl*-propranolol, and is mimicked by the α_1-selective agonist phenylephrine, indicating that the effect of norepinephrine on α_1-adrenergic receptor mRNA is mediated by activation of the α_1-adrenergic receptor.

The α_1-adrenergic receptor is known to be coupled to a variety of second messenger pathways. In the RbSMCs used for these experiments, α_1-adrenergic receptor stimulation causes Ca^{2+} mobilization (10) and increases inositol trisphosphates, diacylglycerol, and prostaglandin levels (W.S.C., unpublished observation). In RbSMCs, prostaglandin E_2 causes activation of adenylate cyclase (14). In the present studies, the concentration of norepinephrine that causes a half-maximal decrease in α_1-adrenergic receptor mRNA level ($\approx$0.3 μM) lies within the range of concentrations of norepinephrine (0.1–1.0 μM) that causes a half-maximal stimulation of Ca^{2+} mobilization (10) and generation of inositol trisphosphates and diacylglycerol (W.S.C., unpublished observations) in these cells. Thus, there are several potential second messengers that might be involved in modulating the level of α_1-adrenergic receptor mRNA. Of note, phorbol esters and cAMP cause a decrease in the density of α_1-adrenergic receptors in these cells (14, 15), presumably due to activation of protein kinase C and protein kinase A, respectively. Further studies will be necessary to determine the role of these second messengers in the effects of norepinephrine on α_1-adrenergic receptor mRNA levels.

Table 1. Effect of actinomycin D on α_1-adrenergic receptor mRNA

	n	% of control
Norepinephrine (10 μM)	9	19 ± 5
Actinomycin D (5 μg/ml)	5	54 ± 10*
Norepinephrine + actinomycin D	3	51 ± 8*

α_1-Adrenergic receptor mRNA level after a 4-hr exposure to norepinephrine, actinomycin D, or both. The intensity of the autoradiographic signal for the α_1-adrenergic receptor was normalized for the level of 18S ribosomal mRNA as described. Data are presented as % of control values. Actinomycin D was added 30 min before norepinephrine.
*$P < 0.02$ vs. norepinephrine.

The decrease in α_1-adrenergic receptor mRNA level caused by a maximal concentration of norepinephrine was evident in 1 hr, the earliest time point examined, and was maximal by 4 hr. Over the next 18 hr, the α_1-adrenergic receptor mRNA level returned to basal values. Under similar conditions, the density of α_1-adrenergic receptors in these cells decreased gradually over 24 hr, with only an $\approx$20% decrease at 4 hr and a subsequent maximal decrease of $\approx$65% by 24 hr. In the continued presence of norepinephrine, the density of α_1-adrenergic receptors remained depressed for at least 3 days, whereas removal of norepinephrine was followed by a recovery of α_1-adrenergic receptor number to 85% of control values in the next 24 hr (16).

The dissociation between the time courses for down-regulation of the α_1-adrenergic receptor and its mRNA is not surprising. It is anticipated that the decrease in receptor number will lag behind the decrease in mRNA level by a period of time that is determined, in part, by the half-life of the receptor. Since the rate of decrease in receptor number observed in response to norepinephrine ($\approx$65% decrease in 24 hr) exceeds the rate at which receptors decrease after inhibition of protein synthesis by cycloheximide ($\approx$9% in 24 hr; ref. 16), these data suggest that, in addition to the effect on mRNA, norepinephrine may accelerate the rate of receptor degradation. Hughes and Insel (17) concluded that agonists caused an increased rate of degradation of α_1-adrenergic receptors in BC_3H-1 cells.

In RbSMCs, exposure to phorbol esters or norepinephrine causes a rapid (minutes) uncoupling of the receptor from inositol phospholipid turnover and Ca^{2+} mobilization (5, 15, 18). Therefore, since it is likely that one or more of the second messenger pathways associated with α_1-adrenergic receptor activation plays a key role in the norepinephrine-mediated decrease in mRNA level, the transient nature of the effect of norepinephrine on mRNA may be related to the uncoupling of the α_1-adrenergic receptor from a second messenger pathway.

Since α_1-adrenergic receptor number remains depressed after 24 hr of norepinephrine exposure, despite the return of mRNA levels to basal values, other mechanisms in addition to the decrease in mRNA must participate in this down-regulation. Further regulation of receptor synthesis might occur at the level of translation, during posttranslational modifications of the receptor protein or during cellular translocation of receptors to the plasma membrane. In addition, an increased rate of receptor degradation may play a key role in both the initial decrease in receptor level and the maintenance of a reduced receptor number at a time when the receptor mRNA has returned to control levels. The decrease in receptor synthesis most likely contributes to the initial norepinephrine-induced decrease in receptor number.

The effects of actinomycin D on α_1-adrenergic receptor mRNA levels, along with the kinetics of the norepinephrine-

induced decrease, may provide some insight into the transcriptional versus posttranscriptional nature of the mechanism by which norepinephrine regulates the expression of α_1-adrenergic receptor mRNA. Since the α_1-adrenergic receptor mRNA is apparently more stable in the presence of actinomycin D (46% decrease in 4 hr) than in the presence of norepinephrine (81% decrease in 4 hr), attenuation of transcription alone is not sufficient to explain the effect of norepinephrine on mRNA level. It is likely that at least some of the effect of norepinephrine is due to a norepinephrine-stimulated decrease in the stability of α_1-adrenergic receptor mRNA. The observation that actinomycin D prevents the norepinephrine-induced decrease in mRNA raises the possibility that norepinephrine induces a labile factor(s) that promotes the effect of norepinephrine on mRNA levels. However, since we cannot exclude the possibility that actinomycin D by itself enhances the basal stability of α_1-adrenergic receptor mRNA by removing a factor necessary for the degradation of the mRNA (19, 20), further studies of the effect of norepinephrine on the rate of α_1-adrenergic receptor mRNA transcription are needed.

Two subtypes of α_1-adrenergic receptors, termed α_{1a} and α_{1b}, have been proposed based on pharmacologic and physiologic characteristics, including affinities for phentolamine and WB4101, inactivation by chloroethylclonidine, and coupling to different signal transduction mechanisms (21–23). Greater than 95% of the α_1-adrenergic receptors in the RbSMCs used for these studies are of the α_{1b} subtype (24). Likewise, the cDNA probe used in these studies was cloned from DDT_1MF-2 cells, which express α_1-adrenergic receptors whose properties resemble those of the α_{1b} subtype (9). Therefore, the present observations likely reflect events involved in the regulation of the α_{1b}-adrenergic receptor subtype.

Recent studies of the β_2-adrenergic receptor, another member of this superfamily of receptors, have shown that β-adrenergic receptor stimulation causes both a rapid (minutes) cAMP-mediated increase in β_2-adrenergic receptor mRNA transcription (6) and a slower (hours) decrease in mRNA level (6–8). A cAMP-dependent element that stimulates transcription initiation has been identified upstream of the β_2-adrenergic receptor gene (6), whereas there is evidence that prolonged agonist stimulation results in a decrease in the stability of β_2-adrenergic receptor mRNA without a decrease in transcription rate (8, 24). It will be of interest to compare the mechanisms by which α_1- and β_2-adrenergic receptor mRNAs are regulated by agonists.

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