Norepinephrine-stimulated Hypertrophy of Cultured Rat Myocardial Cells Is an Alpha$_1$ Adrenergic Response

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ABSTRACT We have shown recently that norepinephrine stimulates muscle cell hypertrophy in primary cultures from the neonatal rat ventricle and that this stimulation is not blocked by the beta adrenergic antagonist propranolol. The present study was done to define the adrenergic specificity of the myocyte hypertrophic response to norepinephrine. 90% pure, single-cell cultures of nongrowing myocytes were maintained in serum-free medium 199 with transferrin and insulin. Myocyte size was quantitated 48 h after addition of adrenergic agents, by measuring cell volume, cell surface area, and cell protein. L-norepinephrine increased myocyte size to a maximum 150% of control; half-maximum effect was obtained at a concentration of 0.2 μM. This increase in cell size was inhibited by the nonselective alpha adrenergic antagonist phentolamine and by the alpha$_1$ adrenergic antagonists prazosin and terazosin; it was not inhibited by propranolol or by the alpha$_2$ adrenergic antagonist yohimbine. The beta adrenergic agonist isoproterenol did not increase cell size. Thus, norepinephrine-stimulated hypertrophy of cultured rat myocardial cells is an alpha$_1$ adrenergic response.

INTRODUCTION

We have shown recently that myocyte (MC)[1] hypertrophy and its regulation can be studied in primary cultures obtained from neonatal rat hearts (1). Norepinephrine (NE) stimulates MC hypertrophy, both in serum-supplemented cultures with enlarging MCs and in serum-free cultures (1). MCs in serum-free cultures beat rarely and do not enlarge, but they are viable and responsive to growth stimulation by serum and by NE (1). The hypertrophic effect of NE is not due to a sustained chronotropic action (1) and is not blocked by the beta adrenergic antagonist propranolol (1). In this study, using serum-free cultures and several adrenergic antagonists, we show that the stimulation of MC hypertrophy by NE is an alpha$_1$ adrenergic response.

METHODS

Single-cell cultures were prepared from hearts of day-old rats as described previously (1, 2), except that 5% serum was used during preplating, not 0.5%; this change reduced the

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[1] *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; MC, myocardial cell (heart muscle cell); NE, norepinephrine; NMC, nonmyocardial cell (heart nonmuscle mesenchymal cell).

nonmyocardial cells (NMCs) in the final cultures from 20–25% (1, 2) to 10% (Results). All experiments used serum-free cultures maintained in medium 199 (Hanks salts) with 10 μg/ml human transferrin, 10 μg/ml porcine insulin, 1.5 μM vitamin B_{12}, and 50 U/ml penicillin (1). Unless noted, the medium through culture day 3 also contained 0.1 mM bromodeoxyuridine (BrdU) to prevent NMC proliferation (1, 2). The experimental protocol has been described (1). Small aliquots of freshly prepared solutions of adrenergic agents or their diluent (control) were added to the nongrowing serum-free cultures after a medium change on day 4, and MC size and cell number were determined 48 h later (1). To retard NE degradation, the medium contained 100 μM vitamin C and was kept at pH 7.3; NE concentration (radioenzymatic assay) through 48 h remained at least 75% of that originally present. The dishes from each culture preparation were allocated to separate experiments; each experiment comprised several groups of 3–6 dishes and always included a control group and a group that received NE alone.

Three measures of MC size were used, as previously described and validated: total cell protein, MC surface area, and cell volume (1). Total cell protein was measured in sodium dodecyl sulfate extracts of the washed cells using a modification of the method of Lowry et al. (3) with bovine serum albumin as standard (1). In 250 groups of 3–6 dishes, SDs averaged 6.7% of the mean microgram of protein per dish with a SE of 0.24%. MC surface area was determined by planimetry of MCs on enlarged, calibrated photomicrographs of cells in the dishes (1). Cell volume was calculated from the microscopically measured diameter of rounded MCs removed from the dishes with trypsin; NMCs were not detached (1). The number of MCs and NMCs was counted for at least three dishes in each group, using phase-contrast microscopy; the accuracy and reproducibility of the method have been shown (1, 2). Cell protein concentration in picograms per cell was obtained by dividing the group average microgram of protein per dish by the average total cell number (1).

The sources of the adrenergic agents (4–6) were as follows: L-NE (Sigma Chemical Co., St. Louis, MO), D-NE (Winthrop Laboratories, New York), L-isoproterenol (Sigma Chemical Co.), phentolamine (CIBA-GEIGY Corp., Summit, NJ), prazosin (Pfizer Laboratories Div., Pfizer, Inc., New York), terazosin (2-[4-(tetrahydro-2-furanyl) carbonyl]-1-piperazinyl-6,7-dimethoxy-4-quinazolinamine hydrochloride, an $alpha_1$ adrenergic antagonist (6), Abbott Pharmaceuticals, Inc., North Chicago, IL), yohimbine (Sigma Chemical Co.) and L-propranolol (Ayerst Laboratories, New York). The L-isomer of NE was used except as noted.

The data are presented as the mean±SE, with the number of observations indicated. Analysis of variance and the Student-Newman-Keuls tests were used for comparisons among multiple groups; the unpaired *t* test was used for two groups (7). A *P*-value < 0.05 was taken to indicate a statistically significant difference between means.

RESULTS

Figs. 1–4 and Tables I–III show that NE stimulated MC hypertrophy but isoproterenol did not and that the effect of NE was inhibited by a nonselective alpha adrenergic antagonist and by $alpha_1$ adrenergic antagonists but not by an $alpha_2$ adrenergic antagonist or by propranolol. MCs exposed to NE for 48 h in

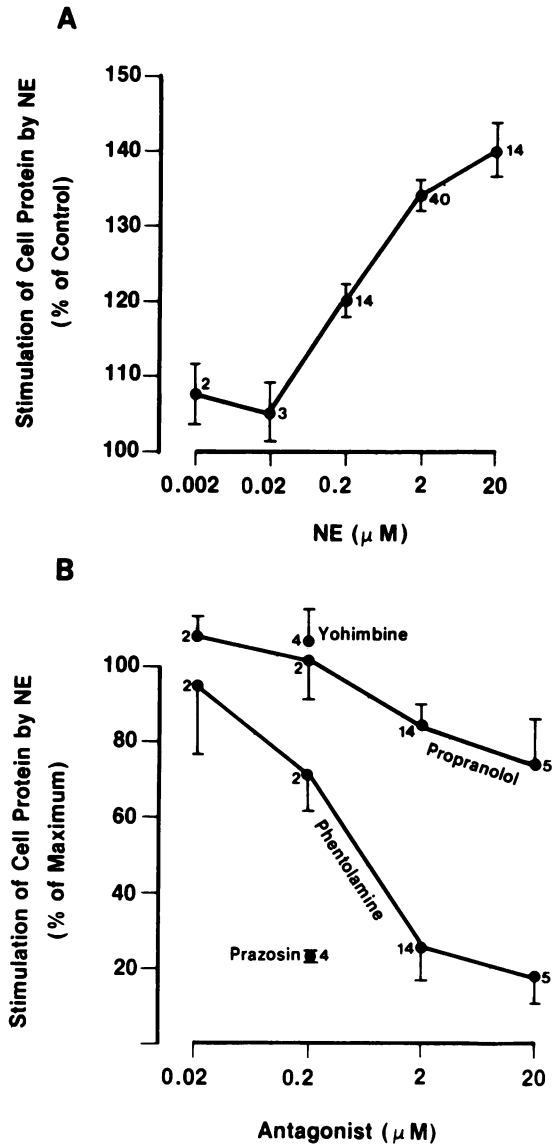

FIGURE 1 (*A* and *B*) The stimulation of cell protein by NE is inhibited by the nonselective alpha adrenergic antagonist phentolamine and by the $alpha_1$ adrenergic antagonist prazosin but not by the $alpha_2$ adrenergic antagonist yohimbine or the beta antagonist propranolol. In *A*, the stimulation of cell protein, as percentage of control, is plotted against the final concentration of NE. In *B*, the stimulation of cell protein by NE plus antagonist, as a percentage of the maximum stimulation by NE alone in the same experiment, is plotted against the final concentration of antagonist. These data are from 13 culture preparations. The points are the mean±SE for the number of experiments indicated. For the data in *B*, the stimulation by 2 μM NE alone was considered maximum and was a mean 134, 135, 137, and 140% of control for the experiments with propranolol, phentolamine, yohimbine, and prazosin, respectively. In a total of 42 experiments, control dishes had 61.4±2.8 μg protein or 465±10 pg/cell.

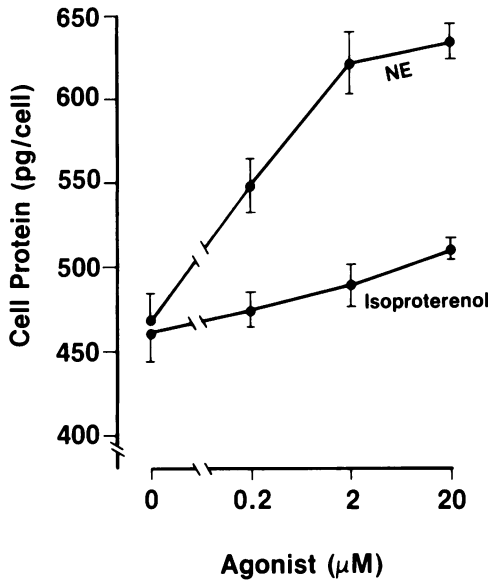

FIGURE 2 NE increases cell protein concentration but isoproterenol does not. Cell protein concentration is plotted against final concentration of agonist. Each point is the mean±SE for eight experiments. All concentrations of NE are significantly different from control ($P < 0.01$). There is a trend for isoproterenol to increase cell protein, but the 10% stimulation by 20 μM isoproterenol is not significantly different from control.

serum-free cultures were a maximum of ~50% larger than control MCs as measured by cell volume (Table I), cell protein (Figs. 1-3 and Table II), and MC surface area (Table III). Taking the single experiment with the greatest NE effect, the stimulation of cell protein was 141.3±4.4% in 13 preparations, and 152.3±4.4% in the seven most recent consecutive preparations. The EC_{50} (concentration giving 50% stimulation) for the hypertrophic effect of NE was ~0.2 μM (Figs. 1-3). The stimulation by NE was inhibited by the nonselective alpha adrenergic antagonist phentolamine (Figs. 1 and 4 and Tables I-III) and by the $alpha_1$ adrenergic antagonists prazosin and terazosin (Figs. 1 and 3 and Table I), but not by the nonselective beta adrenergic antagonist propranolol (Figs. 1, 3, and 4, and Tables I-III) or the $alpha_2$ adrenergic antagonist yohimbine (Fig. 1 and Table I). The nonselective beta agonist isoproterenol did not increase cell protein significantly (Fig. 2). Propranolol did not add to the inhibition of the NE effect by alpha adrenergic antagonists (Tables I and II), even when alpha adrenergic blockade was incomplete (Table II). The alpha adrenergic nature of the NE response was confirmed by all three assays of MC size (all figures and tables) and in cultures not given BrdU (Table I). The NE effect on cell protein was similar by all methods of data examination (Figs. 1-3 and Table II). The stimulation of hypertrophy was stereoselective: In one experiment 20 μM D-NE increased cell protein to 123% of control, whereas 20 μM L-NE induced a 142% increase ($P < 0.025$ for D- vs. L-isomer.)

The various antagonists alone had no significant effect on MC size (Fig. 4 and Tables I and III; data for cell protein not shown). Absence of antagonist toxicity was also shown by the fact that inhibition of the NE effect was at least partly reversible. Cells were exposed to 2 μM phentolamine or terazosin for 24 h, then given fresh medium and tested for response to 2 μM NE; NE increased cell protein by 15-32% ($P < 0.05$ vs. control cells). Antagonism was specific for the effect of NE; MC hypertrophy induced by addition of serum (1) was not inhibited by the alpha adrenergic antagonists (data not shown). Cell numbers were equal in control and treated groups. In 20 separate preparations, there were 132,030±8,989 total cells/dish with 10.4±0.4% NMCs in control cultures and 129,772±8,705 total cells/dish

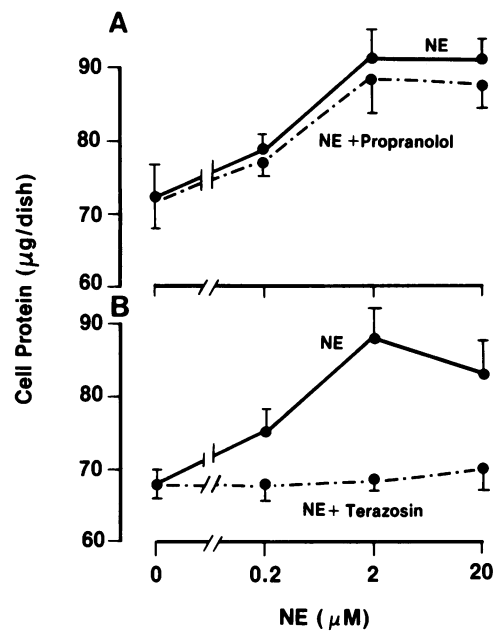

FIGURE 3 The stimulation of cell protein by NE is inhibited by the $alpha_1$ adrenergic antagonist terazosin but not by the beta adrenergic antagonist propranolol. Cell protein per dish is plotted against the final concentration of NE, without (solid lines) or with (broken lines) 2 μM propranolol (*A*) or 2 μM terazosin (*B*). Each point is the mean±SE for six dishes in a single experiment. Mean protein values with 2 μM and 20 μM NE, alone or with propranolol, are significantly different from control ($P < 0.05$). NE plus terazosin is not different from control.

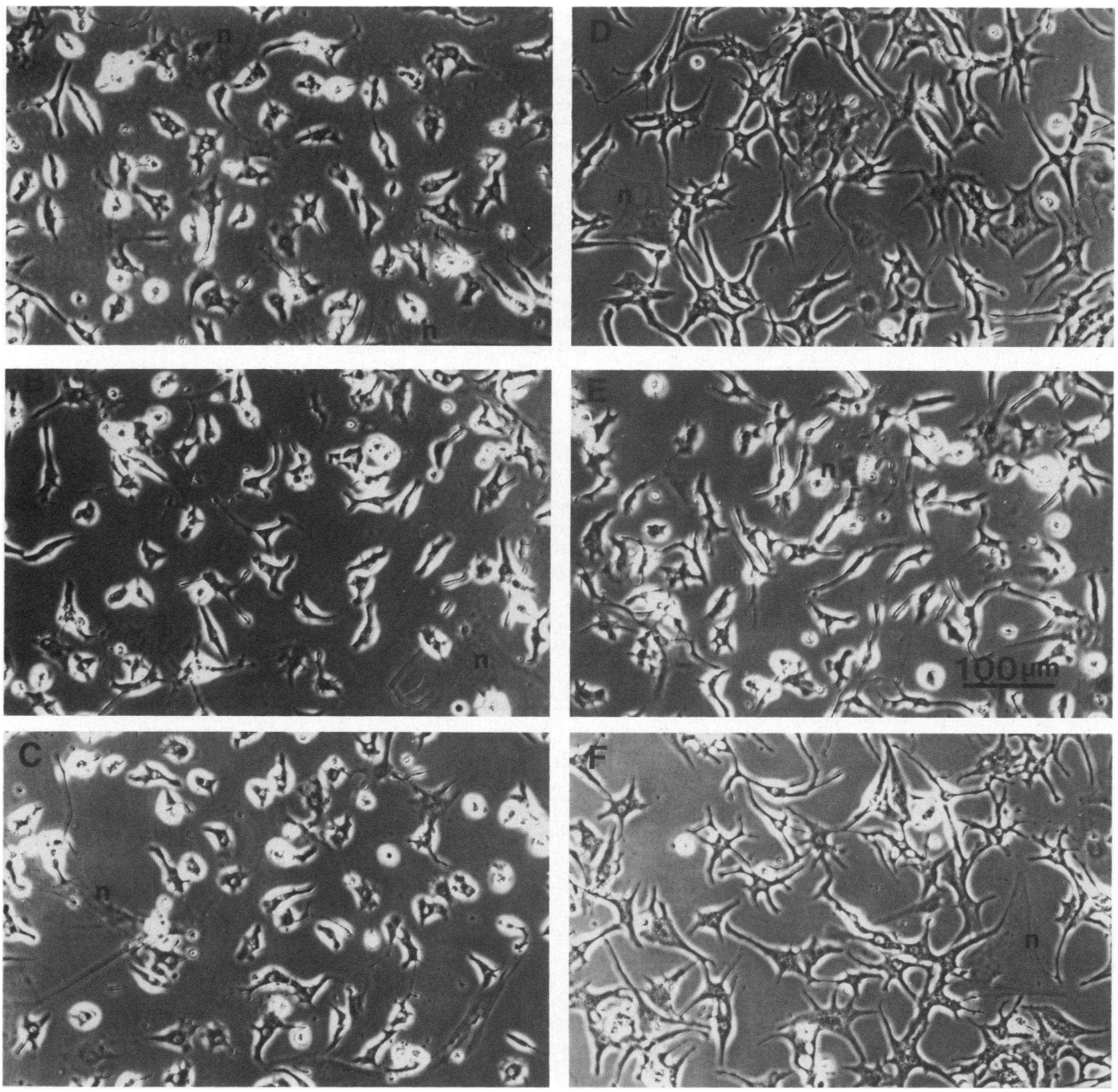

FIGURE 4 NE-stimulated MC size increase is blocked by phentolamine but not by propranolol. These living cells, all from the experiment, were photographed 48 h after the following additions (all 2 μM final concentration): *A*, control; *B*, phentolamine; *C*, propranolol; *D*, NE; *E*, NE plus phentolamine; and *F*, NE plus propranolol. The surface area data in Table III are from a different culture preparation but are representative of the MC size difference shown in these pictures. A few large, phase-lucent NMCs (*n*) can be seen. Original magnification is 51; calibration is in *E*.

with 9.5±0.4% NMCs in NE-treated cultures (P = NS). Isoproterenol and the various antagonists did not change total cell numbers or percent NMCs (data not shown).

DISCUSSION

These results confirm that NE stimulates MC hypertrophy in defined, serum-free cultures without chang-

TABLE I

Alpha$_1$ Adrenergic Regulation of Myocyte Size As Assayed by Cell Volume, in the Presence or Absence of BrdU

Culture	Addition	Myocyte volume	Percent of control	*P* vs. control
		μm^3/cell		
A	Diluent (control)	1,035±18	—	—
	Phentolamine	1,010±37	98	NS
	Propranolol	997±29	96	NS
	Prazosin	985±32	95	NS
	Yohimbine	900±33	87	NS
	NE	1,568±41	152	<0.01
	NE + Phentolamine	1,122±26	108	NS
	NE + Propranolol	1,644±42	159	<0.01
	NE + Prazosin	970±26	94	NS
	NE + Yohimbine	1,408±56	136	<0.01
B	Diluent (control)	983±30	—	—
	Phentolamine	944±36	96	NS
	Propranolol	918±42	93	NS
	Terazosin	909±42	92	NS
	NE	1,668±52	170	<0.01
	NE + Propranolol	1,591±60	162	<0.01
	NE + Phentolamine	1,006±49	102	NS
	NE + Phentolamine + Propranolol	1,011±40	103	NS
	NE + Terazosin	1,002±42	102	NS
	NE + Terazosin + Propranolol	1,013±48	103	NS

These data are from two culture preparations. The cells in A were treated with BrdU for the first three culture days; the cells in B were never exposed to BrdU. Final NE concentration was 2 μM. The antagonists were 2 μM, except in *A* prazosin and yohimbine were 0.2 μM. The amount of diluent (control) was equivalent for all dishes. MCs were detached with trypsin 48 h after the compounds were added, and cell volume was determined microscopically. Volumes are the mean±SE of 200–400 cells in *A* and 100 cells in *B*.

ing cell numbers (1), and they further show that this hypertrophic effect of NE is an alpha$_1$ adrenergic response. The evidence is inhibition of the hypertrophic response to NE by phentolamine, terazosin, and prazosin but not by propranolol or yohimbine and failure of isoproterenol to elicit a hypertrophic response. We

TABLE II

Stimulation of Cell Protein by NE Is Inhibited by Phentolamine but Not by Propranolol

Addition	Cell protein	Percent of control	*P* vs. control
	μg/dish		
Diluent (control)	81.56±1.97	—	—
NE	126.09±9.75	155	<0.01
NE + Propranolol	120.24±2.60	147	<0.01
NE + Phentolamine	98.18±3.57	120	NS
NE + Phentolamine + Propranolol	99.36±1.58	122	NS

These data are from a single experiment with four dishes in each group. Final concentration was 2 μM for all additions.

TABLE III

Stimulation of MC Surface Area by NE Is Inhibited by Phentolamine but Not by Propranolol

Addition	MC Surface area	Percent of control	*P* vs. control
	μm^2/cell		
Diluent (control)	1,112±70	—	—
NE	1,761±100	158	<0.01
NE + Propranolol	1,652±77	149	<0.01
NE + Phentolamine	1,256±84	113	NS
Propranolol	987±55	89	NS
Phentolamine	961±64	86	NS

These data are from a single experiment with 50 cells measured in each group. Final concentration was 2 μM for all additions.

previously reported that propranolol did not block the hypertrophic effect of NE or isoproterenol in MC cultures (1). Isoproterenol stimulated hypertrophy less than did NE, and only with prolonged exposure in serum-supplemented medium (1); this effect of isoproterenol (1) may have been explained by the alpha adrenergic agonist activity of high concentrations of this agent (4).

There are no other studies of the regulation of MC hypertrophy in culture by catecholamines. There is evidence that NE stimulates myocardial hypertrophy in vivo independent of hemodynamic changes (8), but whether this response to NE is alpha or beta adrenergic has not been defined (8). Hypertrophy induced by NE injections in vivo is not blocked by the nonselective beta adrenergic antagonist alprenolol (9), and this is consistent with an alpha adrenergic mediated response. In contrast, isoproterenol induces myocardial hypertrophy in vivo, and propranolol antagonizes this effect (10). However, NE release from adrenergic nerve endings and tyrosine hydroxylase activity in cervical sympathetic ganglia are also stimulated by isoproterenol and inhibited by propranolol (11, 12). Our observation that isoproterenol does not stimulate hypertrophy in neuron-free heart cell cultures raises the possibility that isoproterenol produces hypertrophy in vivo indirectly, by increasing NE release.

Alpha$_1$ adrenergic receptors have been identified in cultured rat heart cells by radioligand binding (13). The number of alpha$_1$ adrenergic receptors in the intact heart is similar to the number of beta adrenergic receptors (5), but their function on myocardial cells has been unclear (4, 14). Our results are consistent with the hypothesis that one function of alpha$_1$ adrenergic receptors is to mediate a myocardial hypertrophic response to NE. The peak concentration of NE in an autonomic nerve-smooth muscle synapse ranges from 0.3 to 10.0 μM (15); the approximate EC_{50} for the alpha$_1$ adrenergic-mediated hypertrophic response we observed in culture is within this range. Myocardial glucose transport (16) and phosphofructokinase activity (17) may be under alpha adrenergic control. Activation of glucose transport and phosphofructokinase are early events when growth factors stimulate cell hyperplasia (18). Since cells enlarge before division, these same events may be important when cells grow by hypertrophy. Other evidence besides our study for alpha adrenergic involvement in growth regulation is the inhibition by phentolamine of liver cell regeneration after partial hepatectomy (19).

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