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Adrenergic Stimulation of Hepatocyte Growth Factor Expression

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Hepatocyte growth factor (HGF), a potent mitogen, is released into plasma at increased levels following injury to certain tissues, including the liver. Early increases in plasma HGF are not due to a release from the injured liver, but rather from distal organs, particularly the lung. We have investigated the ability of norepinephrine (NE), which rises rapidly in plasma after liver resection, to trigger elevated production of HGF in MRC-5 human embryonic lung fibroblasts. Levels of HGF released to culture media and of HGF mRNA increased when cultures were exposed to NE, or to other adrenergic agonists. While stimulation of either beta- or alpha$_1$-adrenergic receptors increased HGF expression, responses to NE appear to be mediated primarily via beta receptors. Since NE has already been shown to act as a comitogen with HGF, our findings suggest that adrenergic hormones may act both to induce production of HGF at distal sites, and to enhance the response to HGF at target tissues.

Hepatocyte growth factor (HGF) was originally purified from rat plasma and platelets by its ability to stimulate DNA synthesis in isolated adult hepatocytes (1, 2), and has since been shown to be involved in liver regeneration and in growth regulation of other organs. HGF levels in the plasma increase dramatically by 2 h after partial hepatectomy (PHX) or CCl_4-induced liver damage in rats, while liver HGF levels increase by 24 h after CCl_4 administration. These increases are followed by waves of DNA synthesis in the remaining liver (3). Following unilateral nephrectomy, HGF mRNA increases in the remaining intact kidney (4). Following damage by $HgCl_2$ administration, injection of recombinant HGF enhances renal regeneration by stimulating DNA synthesis in tubular epithelial cells (5).

Interestingly, in response to these types of damage, HGF mRNA also increases in the lung. This increase was shown to occur specifically in the lung endothelial cells following PHX (6). Mediators have not been identified in this distal response to injury; a non-proteinaceous factor capable of increasing production of HGF by MRC-5 human embryonic lung fibroblasts has been isolated but not characterized (7).

Several substances have been shown to increase in plasma following PHX, including norepinephrine (NE) (8). NE has already been shown to have a role in hepatocyte growth, synergistically increasing DNA synthesis in combination with either epidermal growth factor (EGF) (9) or HGF (3). In this study, we have demonstrated that NE is capable of increasing both the transcription and secretion of HGF by MRC-5 fibroblasts. Furthermore, we show that NE exerts its regulatory effects primarily through the β-adrenergic receptor, although both α_1 and β agonists were able to mimic the effect of NE. Therefore, NE is likely to be at least partially responsible for the increase in HGF production seen in the lung following liver or kidney damage.

MATERIALS AND METHODS

Except where noted, all chemicals and reagents were from Sigma Chemical Co., St. Louis, MO. Benoxathian, yohimbine, and alprenolol were obtained from Research Biochemicals International, Natick, MA. Ligation reagents and PCR adapter primers were from Ambion, Inc., Austin, TX.

Cell culture. MRC-5 human embryonic lung fibroblasts (ATCC, Rockville, MD), passage 13 to 17, were grown in Minimal Essential Media with Earle's Salts (MEM) (Life Technologies, Gaithersburg, MD) containing 0.2 mM aspartate, 0.2 mM serine, 1.0 mM pyruvate, 1.0 mM proline, and 26 mM $NaHCO_3$ supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B. At the start of each experiment, growth medium of subconfluent cultures was replaced with fresh culture media without FBS. Adrenergic agonists and/or antagonists were added from aqueous stock solutions, and the release of HGF

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into media and the expression of HGF mRNA in these cultures were analyzed.

Determination of HGF concentration in media. Media samples were taken from cultures in six-well plates at 24 h posttreatment, and HGF concentration was determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN).

RNA analysis. Total RNA was extracted from cultures 9 h posttreatment, using Tri-Reagent (Molecular Research Center, Inc, Cincinnati, OH). Samples (5 μg) were run in 1.2% agarose and 2.2 M formaldehyde gels and transferred to positively charged nylon membranes (Boehringer-Mannheim, Indianapolis, IN) by overnight capillary elution or using the PosiBlot pressure system (Stratagene Cloning Systems, La Jolla, CA). The membranes were then dried, UV-crosslinked, prehybridized, and hybridized overnight at 55°C with digoxygenin-labeled riboprobe for human HGF. Antisense HGF probe was transcribed from a template created by ligating a T7 promoter to a 230-bp PCR fragment amplified from within the 0.85 kB EcoRI fragment of the human HGF cDNA. Chemiluminescent probe detection employed anti-digoxygenin alkaline phosphatase-conjugated antibody (Boehringer-Mannheim) binding and Lumi-Phos 530 (Lumigen, Inc, Southfield, MI) as substrate. Blots were reprobed with digoxygenin-labeled riboprobe for cyclophilin; riboprobe template for cyclophilin was created as for HGF, using mouse cDNA from Ambion, Inc. NIH Image program version 1.59 was used for analysis of band intensity from scanned X-ray films. Band intensities for HGF and cyclophilin hybridization signals showed a linear relationship to the concentration of target mRNA, over the range of total RNA loaded in these experiments (data not shown). The cyclophilin band intensity was used to correct for total RNA loaded, and HGF levels were expressed as the ratio of HGF signal to cyclophilin signal in each sample.

Adrenergic receptor binding. Saturation binding studies were performed on MRC-5 cells in culture, using ^{125}I-iodocyanopindolol and ^{3}H-prazosin to assess β and α_1 receptors, respectively. Subconfluent cultures were washed three times in ice-cold binding buffer (20 mM Hepes, pH 7.5, 0.2% bovine serum albumin, 0.8 mM ascorbate), before incubation (25°C, 1 h with agitation) in buffer with radioligand (7 pM to 1.0 nM iodocyanopindolol, 4440 dpm/fmol; 40 pM to 0.81 nM prazosin, 200 dpm/fmol). Nonspecific binding was assessed at all concentrations of ligand in the presence of 2 μM propranolol (β receptors) or 10 μM benoxathian (α_1 receptors).

RESULTS

NE increases HGF expression. Subconfluent MRC-5 cultures incubated with 10 μM NE released significantly more HGF into the media then did control cultures that received no hormone. Controls produced 1718 $\pm$ 196 pg/ml HGF after 24 h, and NE-treated cultures released 1.8 $\pm$ 0.3 times control values ($n = 8$ independent experiments, $p < 0.01$ by one-tailed paired *t* test). To determine if this stimulation was due to increased transcription of the HGF gene, total RNA was extracted and analyzed by Northern blotting. By 9 h after addition of NE, HGF transcripts were increased 2.3 $\pm$ 0.3 fold, relative to controls (Fig. 1).

NE's stimulation of HGF expression is mediated primarily through the β-adrenergic receptor. MRC-5 cultures were pre-exposed (5 min) to selective adrenergic blockers (10 μM) before stimulation with NE, and the release of HGF into the media monitored by ELISA. Benoxathian, yohimbine, and alprenolol are antagonists of α_1, α_2, and β adrenergic receptors, respectively.

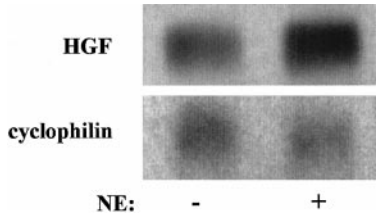

FIG. 1. Effects of norepinephrine on HGF transcription. Subconfluent MRC-5 cultures were incubated with or without 10 μM NE. Total RNA was extracted 9 h posttreatment and analyzed by Northern blotting. Blot shown is representative of 10 independent experiments. Increases in HGF:cyclophilin expression ratios with NE addition were significant; $p < 0.005$ by paired *t* test.

As shown in Fig. 2A, alprenolol, the β receptor antagonist, blocked the NE effect on HGF release. Benoxathian had a smaller inhibitory effect on the NE response, while yohimbine did not reduce the level of HGF secretion.

The blockers had similar effects at the transcriptional level, as determined by Northern analysis of total RNA following 9 h of incubation. Alprenolol had the greatest inhibitory effect, reducing the NE effect to control levels, as shown in Figs. 2B and 2C. Doses of alprenolol as low as 10 nM were able to completely block NE's stimulatory effect on HGF transcription (data not shown). Benoxathian appeared to have a slight inhibitory effect, but this effect was not statistically significant.

Effect of NE can be mimicked by selective a_1 and β agonists. To confirm that NE could act on HGF expression through both β and α_1 adrenergic receptors, MRC-5 cultures were incubated with either the β agonist isoproterenol, the α_1 agonist phenylephrine, or a combination of both. The stimulatory effects of the agonists on HGF transcription and on release of HGF into the media were similar. Isoproterenol and phenylephrine were each able to stimulate expression and release of HGF to levels comparable to those induced by NE, as shown in Fig. 3. When both agonists were present, the effect was greater than that of NE or of either single agonist.

It is clear that the β-adrenergic receptor is the primary receptor involved in the mediation of NE's effect on the expression of HGF. The β blocker, alprenolol, was able to reduce the transcriptional effects of NE to control levels, and additional blockade of the α_1 receptor did not further inhibit HGF transcription (data not shown). The importance of the α_1-adrenergic signal is not clear. While the α_1 blocker had a small but significant inhibitory effect on the level of HGF in the media after 24 hr, this inhibition was not significant at the transcriptional level after 9 hr. The α_1 receptor seems unlikely to simply be involved in posttranscriptional regulation, since the α_1 agonist phenylephrine was

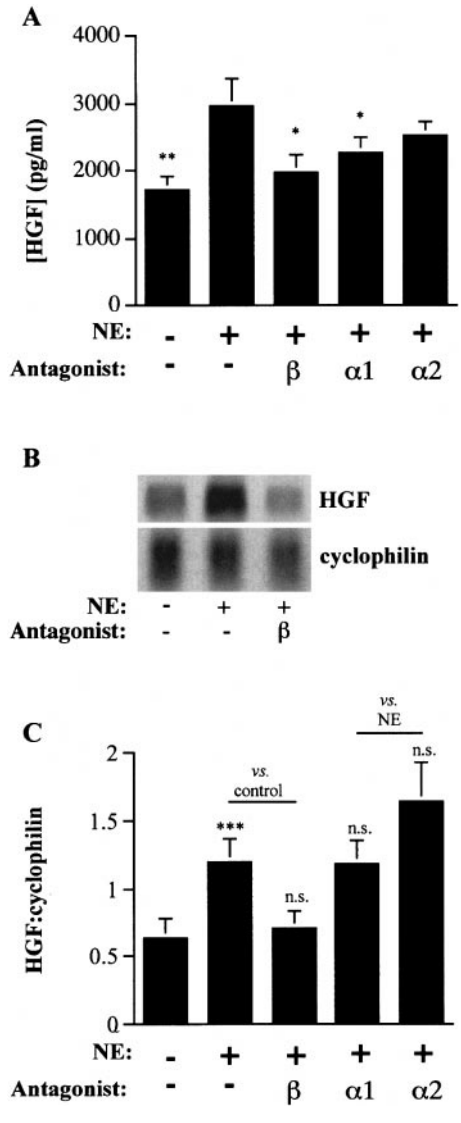

FIG. 2. Effect of adrenergic antagonists on HGF production. Subconfluent MRC-5 cultures were treated with 10 μM benoxathian, yohimbine, or alprenolol (5 min preexposure) and 10 μM NE. (A) Media samples were collected 24 h posttreatment and HGF concentration was determined by ELISA. Data are means of eight experiments; the error bars represent SEM. Significant differences relative to NE-treated groups are indicated; *$p < 0.05$, **$p < 0.005$, by paired *t* test.(B and C) Total RNA was extracted 9 h posttreatment and analyzed by Northern blotting. (B) Blot shown is representative of 9 independent experiments. (C) Data are means of 5–10 experiments; error bars represent SEM. Significant differences between groups are indicated; ***$p < 0.0005$, n.s. = not significant, by paired *t* test.

able to stimulate HGF transcription to the same magnitude as NE.

Adrenergic receptor populations were examined in subconfluent MRC-5 cultures. Estimates of receptor affinity were similar for β and α_1 receptors, but there were more than twice the number of specific β binding sites (150 $\pm$ 46 fmol/mg culture protein for β receptors vs 68 $\pm$ 22 fmol/mg for α_1). Thus it is possible that the preferential use of β receptors simply reflects their greater number at the cell surface.

DISCUSSION

We have shown that NE is able to stimulate the transcription of HGF in MRC-5 fibroblasts. Several other substances have previously been shown to either stimulate (10) or inhibit (11) HGF expression. These findings suggest complex regulation of HGF, which is further supported by molecular analysis of the HGF promoter region. Both the mouse and human HGF promoters have been demonstrated to have several positive and negative regulatory elements (12,13). The rat HGF promoter has multiple transcription start sites and contains both an IL6 response element and a TGF-β1-inhibitory element (14). A Sp binding site is also present, and overexpression of Sp transcription factors has been shown to stimulate HGF promoter activity (15).

Both rat and human HGF promoters have been shown to contain binding sites for the CCAAT/enhancer binding protein (C/EBP) family of transcriptional regulators (14, 16, 17). Of the transcription factors known to regulate HGF promoter activity, C/EBP suggests an attractive hypothesis to explain NE's stimulatory effect on HGF transcription. NE, through interaction with β-adrenergic receptors, would activate protein kinase A (PKA). Phosphorylation by PKA on Ser^{299} allows C/EBPβ to translocate to the nucleus (18)

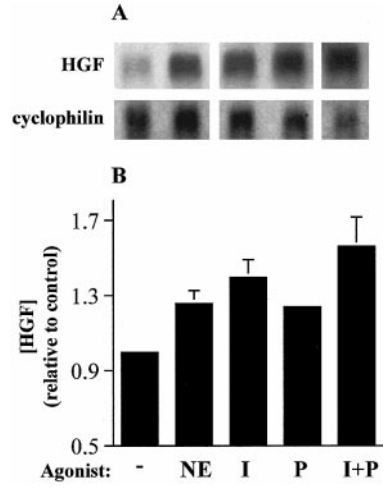

FIG. 3. Effect of adrenergic agonists on HGF production. Subconfluent MRC-5 cultures were treated with NE or with isoproterenol (β agonist), phenylephrine (α_1 agonist), or a combination of α and β agonists. All agonists were 10 μM. (A) Total RNA was extracted 9 h posttreatment and analyzed by Northern blotting. Blot shown is representative of two experiments. (B) Media samples were collected 24 h posttreatment and HGF concentration was determined by ELISA; mean HGF concentration in control cultures was 2526 $\pm$ 330 pg/ml. Data are means of two experiments; the error bars represent SEM.

and to increase the expression of c-*fos* (19); PKA activity has also been shown to increase transcription of the C/EBPβ gene via activation of the cAMP response element binding protein (CREB) (20). In mouse cortical astrocytes, NE has been shown to stimulate C/EBPβ and C/EBPδ production through a cAMP-dependent mechanism, increasing mRNA and protein to maximal levels after 4 h (21). The consequences of specific phosphorylation events *in vivo* remain to be fully understood. *In vitro,* PKA is capable of phosphorylating several serine residues other than Ser^{299} which modulate C/EBPβ binding to DNA in an inhibitory manner (22).

Both CREB and C/EBPβ could provide opportunities for α_1 receptor regulation of HGF expression, as well. Calcium-calmodulin-dependent kinases phosphorylate and activate both CREB (23) and C/EBPβ (24), and protein kinase C-promoted phosphorylation of C/EBPβ (which may be indirect) can also enhance its translocation to the nucleus (25). We found that either β or α_1 receptor agonists could enhance HGF transcription in MRC-5 cultures, and that maximal responses were obtained by stimulating both populations. Similarly, the addition of either dibutyryl cAMP or active phorbol ester has been reported to stimulate HGF production in these cells, and the combination of these two agents produced a synergistic response (26).

The relationship between NE and HGF closely parallels the interplay of NE and EGF. At target tissues, NE acts as a potent co-mitogen, enhancing DNA synthesis in response to either HGF or EGF. We have now shown that NE also stimulates the transcription and secretion of HGF; it is already known to stimulate the release of EGF from salivary glands and from Brunner's glands in the duodenum (27, 28). By modulating both the production of and response to these growth factors, adrenergic signaling can have profound effects on proliferative responses to injury.

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