Hepatocyte Growth Factor Regulates Angiotensin Converting Enzyme Expression*

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Hepatocyte growth factor (HGF) is a mitogen, morphogen, and motogen that functions in tissue healing and acts as an anti-fibrotic factor. The mechanism for this is not well understood. Recent studies implicate somatic angiotensin-converting enzyme (ACE) in fibrosis. We examined the effects of HGF on ACE expression in bovine pulmonary artery endothelial cells (BPAEC). Short term treatment of BPAEC with HGF transiently increased both ACE mRNA (3 h) and activity (24 h), as determined by ACE protease assays and reverse transcription-PCR. Incubation of BPAEC with HGF for longer periods suppressed ACE mRNA (6 h) and activity (72 h). In contrast, phorbol ester (PMA) treatment produced sustained increase in ACE mRNA and activity. We examined the short term molecular effects of HGF on ACE using EMSA. HGF and PMA increased transcription from a luciferase reporter with the core ACE promoter, which contains a composite binding site for SP1/3 and Egr-1. Immunocytochemistry and electrophoretic mobility shift assay showed that both HGF and PMA increased Egr-1 binding. HGF also increased SP3 binding, as measured by EMSA. However, HGF and PMA increased the cellular activity of only Egr-1, not SP3, as measured by luciferase reporter assays. Deletion of the Egr-1 site in the reporter construct completely abrogated HGF-induced transcription but only ~50% of PMA-induced activity. Expression of dominant negative Egr-1 and SP3 blocked up-regulation of the ACE promoter by HGF but only reduced up-regulation by PMA. These results show that HGF transiently increases gene transcription of ACE via activation of Egr-1, whereas PMA regulation involves Egr-1 and additional factor(s).

Hepatocyte growth factor (HGF) is produced by cells of mesenchymal origin (e.g. fibroblasts and macrophages), whereas its receptor, c-Met, is expressed primarily by epithelial and endothelial cells (for review, see Ref. 1). HGF has been shown to induce mitogenesis, motogenesis, and morphogenesis in both epithelial and endothelial cell types (1). HGF is required for embryogenesis and organogenesis, and HGF knockouts in mice are lethal in utero (1, 2). The c-Met receptor is a tyrosine kinase receptor with a single transmembrane-spanning region and a conserved tyrosine kinase domain. Signal transduction pathways activated by HGF include Ras, mitogen-activated protein kinases (MAPK) p44/p42 and p38, phosphatidylinositol 3'-kinase, and phospholipase C (1). Through its multifunctional docking region c-Met can bind to proteins containing SH2 (Src homology) domains, including Gab 1, Grb2, SOS, and Src, which appear to be required for downstream signaling events.

HGF functions in a variety of organs as a tissue repair factor. HGF protein is up-regulated in response to tissue damage and inflammation in lung, liver, kidney, and heart (3–6). Maeda et al. (7) and Yanagita et al. (8) show a 3–4-fold elevation of HGF protein in the serum of patients with inflammatory lung disease, interstitial pneumonitis, and bacterial pneumonia. Animal studies show increased levels of HGF in the bronchoalveolar lavage fluid in response to thoracic irradiation or treatment with bleomycin, a chemotherapeutic agent known to induce lung injury (9, 10). The increase of HGF protein in response to tissue damage is thought to function in the repair process in the lung, heart, liver, and kidney (10, 11). In support of this hypothesis, the time course of HGF induction in experimentally induced lung injury in rats correlated with epithelial cell proliferation (8). Also, the use of neutralizing antibodies to HGF drastically reduced DNA synthesis in alveolar epithelial cells after experimentally induced ischemia-reperfusion lung injury in rats (12).

Recent studies suggest that HGF is also an anti-fibrotic factor. Animal studies show that administration of HGF protein induces proliferation of epithelial and endothelial cells and promotes normal tissue regeneration while at the same time preventing the development of fibrosis (11). The simultaneous or delayed administration of HGF to mice undergoing bleomycin-induced lung injury prevents both endothelial and epithelial cell apoptosis, the appearance of fibroblast foci, and the accumulation of collagen found in pulmonary fibrosis (13, 14). Thus, HGF appears to have the capacity to mediate tissue repair in a manner that circumvents aberrant tissue remodel-

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The abbreviations used are: HGF, hepatocyte growth factor; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; BPAEC, bovine pulmonary artery endothelial cells; DN, dominant negative; Egr-1, early growth response 1; EMSA, electrophoretic mobility shift assay;
ing pathways. However, the mechanism for the anti-fibrotic activity of HGF is not well understood.

Somatic angiotensin-converting enzyme (ACE) is a zinc metalloproteinase expressed primarily on the luminal surface of vascular endothelial cells (15, 16). The primary physiological role of ACE is maintenance of blood pressure homeostasis, but ACE is also up-regulated in response to injury in tissues including heart, lung, and kidney (17–19). Recent findings suggest that increased ACE expression in damaged tissues is associated with remodeling and fibrosis (20–25).

Here we have examined HGF regulation of ACE gene expression in BPAEC and compare the findings with regulation by the phorbol ester, phorbol 12-myristate 13-acetate (PMA), a known positive regulator of ACE gene expression. In contrast with the sustained up-regulation of ACE by PMA, HGF biphasically regulates ACE with only a transient period of up-regulation followed by sustained down-regulation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The MEK1/2 (MAPK kinase1/2) inhibitor U0126 was purchased from New England Biolabs, Beverly, MA. Other reagents are described below. Human HGF protein was the kind gift of Dr. G. vande Woude (Van Andel Research Inst., Grand Rapids, MI).

**Cell Culture and HGF and PMA Treatment**—Primary bovine pulmonary artery endothelial cells (BPAEC) were either obtained from freshly slaughtered calves as described previously (26) or purchased from Cell Applications, Inc. (San Diego, CA). Passage 3–8 cells were used for all experiments. BPAEC were cultured in RPMI 1640 with antibiotics (penicillin and streptomycin), fungisone, and 10% fetal bovine serum; cells were grown in 5% CO2 at 37°C in a humidified atmosphere in a Fisher Scientific Isotemp culture incubator. PMA was dissolved in Me2SO and diluted at least 1:1000 into cell culture. Me2SO was added to control experiments at the same dilution. HGF was diluted in 20 mM Tris, 50 mM H9262 g/ml bovine serum albumin; vehicle alone was used in controls.

**ACE Hippuryl-L-Histidyl-L-Leucine Assay**—BPAEC were washed twice in phosphate-buffered saline (PBS) to remove dead cells and then scraped into PBS and sonicated (3 times for 10 s on ice) to extract cellular protein. ACE catalytic activity was determined by a fluorometric assay with the synthetic substrate hippuryl-L-histidyl-L-leucine as described by Friedland and Silverstein (27). Specificity of substrate hydrolysis was confirmed by complete inhibition of ACE activity by lisinopril, a specific ACE inhibitor. Protein concentrations were determined according to Lowry et al. (28).

**Semi-quantitative PCR**—Total cell RNA was obtained from cultured cells using Trizol (Invitrogen). RNA concentrations were determined spectrophotometrically at 260 nm. 1 μg of RNA from each sample was reverse-transcribed for 1 h at 37°C using 200 units of Moloney murine leukemia virus reverse transcriptase (MLVRT) in 10 μl of reaction buffer containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl2, 1 mM dithiothreitol, 860 pmol of oligo(dT)16, 0.5 mM each deoxynucleoside triphosphate (dNTP), and 1 unit of RNase inhibitor. Samples were heated to 95°C for 5 min to inactivate the MLVRT and stored at −20°C. All RT reagents were purchased from Applied Biosystems, Foster City, CA. Competitive semiquantitative PCR for ACE mRNA

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**Fig. 1.** Regulation of ACE enzyme activity and ACE mRNA by HGF and PMA. **A** and **B**, 2-day postconfluent BPAEC were treated with HGF (10 ng/ml, A) or PMA (10^{-7} or 10^{-9} M, B) for the indicated times. Cell lysates were assayed for ACE activity using the hippuryl-L-histidyl-L-leucine assay, a specific assay for ACE proteolytic activity. Results are expressed in terms of % control for each time point. Data show means ± S.D., n = 3. Representative data are shown. **C** and **D**, 2-day post-confluent BPAEC were treated with 10 ng/ml HGF (C) or 10^{-9} M PMA (D) for the indicated times. RNA was harvested and used for competitive semiquantitative RT-PCR for ACE (upper panels) or α-tubulin (lower panels). Internal standards (IS) were used for both ACE and tubulin to control for PCR efficiency. Representative data are shown. Densitometry results of ACE/tubulin are shown in graphs; data are the means ± S.D., n = 3. The asterisk indicates statistical significance from control for both enzyme activity or mRNA levels, p < 0.05.
manufacturer fluence were transfected with promoter-reporter constructs by Gene- using a mutant internal standard (29, 31). PCR results were normalized to PCR results of tubulin, also determined (reverse), with uppercase bases corresponding to ACE gene sequences.

The primers were 5'-GAAGGCCCTTTCTCCAGCTTCC-3' (forward) and 5'-GCAGTACTGATCCGCCCTCCTCCGCTC-CAGAATC-3' (reverse); reaction buffers and cycles were the same as above.

The minimal Egr-1-responsive reporter plasmid pEB1S1Luc contains four binding sites for Egr-1 and has been previously described (33). The minimal SP1/3-responsive reporter plasmid pAEG1Suc contains four binding sites for Sp1 derived from the aldolase C promoter and was described previously (34). Basal transcription mediated by this GC-rich region of the aldolase C gene is abrogated by dominant negative SP1 and SP3 mutants.2 The expression vector for dominant negative Egr-1, pCMV-FLAG-NLS-Egr-1DN, was described in Zhang et al. (35). As a dominant negative SP3, we designed an expression vector encoding a chimeric protein of the GC-rich region of the mouse protein RE-1-silencing transcription factor (REST) with the DNA binding domain of the SP3 transcription factor. The cDNA encoding human SP3 was purchased from ATCC (ATCC 95505, accession number M97191). To construct the DN SP3 expression vector we first cloned the N-terminal repression of REST into plasmid pkxFLAG-CMV (Sigma), generating plasmid pCMV-FLAG-REST-N. The SP3 cDNA was cut with Asp718 and filled in with the Klenow fragment of DNA polymerase I, and the fragment was inserted into the fill-in BglII site of plasmid pCMV-FLAG-REST-N. The REST/SP3 chimera contains amino acids 1–419 of REST fused to amino acids 485–653 of SP3 (36).

**Dual Luciferase-Assay**—Transfected BPAEC were washed twice with PBS, lysed with Promega passive lysis buffer, and assayed for firefly and Renilla luciferase activities by the dual luciferase assay (Promega) according to the manufacturer's instructions in a Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA was performed as described by Garner and Rezvani (37). To prepare nuclear extracts cells were washed in PBS and incubated in 10 μl Hepes (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM tetrasodium pyrophosphate for 15 min at 4°C. IGEPA-CE-630 (Sigma) was then added at a final concentration of 0.6 μg/ml. Samples were vortexed and centrifuged. Pellet nuclei were resuspended in extraction buffer (50 mM Hepes (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, and 1% (v/v) glycerol) then mixed vigorously for 20 min and centrifuged for 5 min. The supernatants were harvested, and protein concentrations were determined using the BCA protein assay (Pierce). To perform EMSA binding reaction mixtures containing 2 μg protein of nuclear extract, 1 μg of poly(dI-dC)-poly(dI-dC) and 32P-labeled double-stranded oligonucleotide probe containing consensus Egr-1 sequence (Santa Cruz Biotechnology) in 100 μl NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, and 20 mM Tris-HCl (pH 7.5) were incubated for 20 min at room temperature. Electrophoresis of samples through a native 6% polyacrylamide gel (acrylamide:bis, 29:1) was followed by autoradiography. Supershift experiments were performed by incubating 2 μg of antibody (Santa Cruz Biotechnology) in the binding reaction mixture for 1 h at 4°C before the addition of the 32P-labeled oligonucleotide probe to start the binding reaction. All experiments were repeated at least three times.

**Immunohistochemistry**—BPAEC were grown directly on sterile glass coverslips to 60–80% confluence. Immunohistochemistry was performed at ambient temperature. Cells were washed with PBS for 5 min before fixing in 4% formaldehyde in PBS for 5 min. Cells were washed 3 times with PBS and incubated in 10 μl each and then permeabilized for 2–3 min with Triton buffer (50 mM PIPES, pH 7.0, 1% Triton, 90 mM HEPES, pH 7.0, 0.5 mM MgCl₂, 0.5 mM EDTA, 75 mM KCl). Cells were again washed 3 times with PBS, 3 min each. Primary antibodies (4 μg/ml PBS final) (Santa Cruz Biotechnology) were incubated on cells for 1 h and washed 3 times with PBS 3 min each before the addition of fluorescein isothio- cyanate-conjugated secondary antibody (0.1 μg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. Finally, cells were washed 3 times with PBS and mounted in 90% glycerol, PBS. Cells were visualized, and digital images were made on a Zeiss fluorescent microscope.

**Statistics**—All experiments were repeated at least 3 times. Statistical comparisons were performed using Student's t test for unpaired samples and a two-way analysis of variance for multiple comparisons. Statistical significance was determined p < 0.05. For non-linear regres-

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2 A. Al-Sarray and G. Thiel, submitted for publication.
FIG. 3. PMA and HGF activate the Egr-1 transcription factor in BPAEC. In all experiments BPAEC were placed in serum-free RPMI medium for 18 h and then treated with or \(10^{-6}\) M PMA (A, B, and E) or 50 ng/ml HGF (C, D, and F). Nuclear extracts were prepared, and EMSA was performed using a consensus oligo for Egr-1. A, time course of PMA activation of Egr-1. Cells were treated with PMA for the indicated times before the preparation of nuclear extracts. B, Egr-1 supershift. Cells were treated with PMA for 1 h, and nuclear extracts were incubated with and without antibody directed against Egr-1 before the addition of the Egr-1 consensus oligo. C, time course of HGF activation of Egr-1. Cells were treated with HGF for the indicated times before the preparation of nuclear extracts. D, Egr-1 supershift. Cells were treated with HGF for 1 h, and nuclear extracts were incubated with and without antibody directed against Egr-1 before the addition of the Egr-1 consensus oligo. E, inhibition of PMA activation of Egr-1 by calphostin C (Cal C, a protein kinase C inhibitor) and U0126 (a p42/p44 MAPK inhibitor). Cells were pretreated for 30 min with 100 nM calphostin C, 10 \(\mu\)M U0126, or no inhibitor before the addition of PMA for the indicated times. F, inhibition of HGF activation of Egr-1 by U0126 but not by bisindolylmaleimide I (Bis, a protein kinase C inhibitor), pp2 (an Src and focal adhesion kinase inhibitor) or wortmannin (Wort, a phosphatidylinositol 3-kinase inhibitor). Cells were pretreated for 30 min with 10 \(\mu\)M U0126, 10 \(\mu\)M pp2, 30 \(\mu\)M Wort, 10 \(\mu\)M U0126 bisindolylmaleimide I, or no inhibitor before the addition of HGF for 1 h. In all experiments the Egr-1-containing shifted band is indicated as Egr-1; the supershifted band is indicated as Egr-1 SS. Experiments were repeated at least three times. Representative data are shown for each experiment.
Fig. 4. HGF but not PMA activates the SP3 transcription factor. In all experiments BPAEC were placed in serum-free RPMI medium for 18 h and then treated with 10⁻⁶ M PMA (A and B) or 50 ng/ml HGF (C and D). Nuclear extracts were prepared, and EMSA was performed using a consensus oligo for SP1. A, time course of PMA activation of SP-binding proteins. Cells were treated with PMA for the indicated times before the preparation of nuclear extracts. B, SP1/SP3 supershifts. Cells were treated with PMA for 1 h, and nuclear extracts were incubated with and without antibodies directed against SP1 and/or SP3 before the addition of the SP1 consensus oligo. C, time course of HGF activation of SP-binding proteins. Cells were treated with HGF for the indicated times before the preparation of nuclear extracts. D, SP1/SP3 supershifts. Cells were treated with HGF for 1 h and nuclear extracts were incubated with and without antibodies directed against SP1 and/or SP3 before the addition of the SP1 consensus oligo. The position of shifted bands containing either SP1 or SP3 is indicated by SP. Experiments were repeated at least three times. Representative data are shown.

**RESULTS**

**HGF Biphatically Enhances ACE Activity and Expression**—ACE activity was measured in BPAEC cell lysates after time courses of treatment with HGF and PMA (Fig. 1, A and B). ACE activity in response to 10 ng/ml HGF increased at 24 h (1.8-fold above control) but then decreased, first back to basal levels at 48 h and then to 50 ± 2% of control levels at 72 h (Fig. 1A). In contrast, treatment of BPAEC with PMA induced increased ACE activity at all time points examined up to 72 h. In response to 10⁻⁹ and 10⁻⁷ M PMA, ACE enzyme activity increased at 24 h (1.3- and 1.6-fold above control, respectively) and at 48 h (1.9- and 3.5-fold above control, respectively) (Fig. 1B).

Studies with a number of biological and pharmacological agents show that changes in ACE activity correlate with changes in ACE mRNA (39, 40). Semiquantitative RT-PCR showed that ACE mRNA levels changed biphatically with HGF, and treatment with PMA positively regulated ACE mRNA at all times examined (Fig. 1, C and D). Within 3 h ACE mRNA increased in BPAEC in response to 50 ng/ml HGF (Fig. 1C). Longer exposure (6–24 h) of BPAEC to HGF resulted in a steady decrease in ACE mRNA, to <50% of control levels at 24 h (Fig. 1C). ACE mRNA increased within 6 h in response to 10⁻⁷ M PMA (Fig. 1D), in agreement with findings by others (41).

**HGF and PMA Activation of Transcription Factors**—We and others previously identified a core region of the ACE promoter from −230 to the +1 transcription start site that is sufficient for activation by several agents (30, 42, 43). This core ACE promoter contains potential binding sites for a number of transcription factors including Egr-1, SP1, and ets-1. Previous studies show a correlation between increased ACE gene expression and up-regulation and activation of Egr-1 (30, 44). HGF has not previously been shown to activate the Egr-1 transcription factor in these cells, and we wished to determine whether Egr-1 could be involved in the early induction of ACE protein and mRNA by HGF. Immunohistochemical experiments with BPAEC showed that PMA increases the nuclear level of Egr-1 within 30 min (Fig. 2A). No change is seen for ets-1, SP1, or SP3, all of which are present in the nuclei of both control and PMA-treated cells. Treatment of cells with HGF also resulted in the increase of nuclear Egr-1 (Fig. 2B). No change was seen for ets-1, SP1, or SP3, which were constitutively nuclear as found for PMA (data not shown). The lack of change in SP1/3 and ets-1 is in agreement with findings by others that show that these factors are constitutively nuclear.

EMSA experiments were performed for all three of the transcription factors used in immunohistochemistry. EMSA with an ets-1 consensus oligo failed to show any activity in control, PMA-treated, or HGF-treated BPAEC (data not shown). EMSA using Egr-1 consensus oligo showed that 10⁻⁷ M PMA increases binding within 30 min, with a peak between 60 and 90 min in BPAEC (Fig. 3A). Supershift was performed to confirm the identity of the EMSA band as Egr-1 (Fig. 3B). A similar time course was found for 50 ng/ml HGF-induced Egr-1 binding in BPAEC (Fig. 3C), which was also confirmed by supershift (Fig. 3D). PMA activation of Egr-1 was inhibited by the protein kinase C inhibitor calcinulin C, as previously shown (41), and by the MAPK p42/p44 inhibitor U0126 (Fig. 3E). PMA-induced Egr-1 binding was also inhibited by the protein kinase C in-
Constitutive binding to an SP1 family consensus oligo was found to be present in control BPAEC (Fig. 4A). This binding was not increased in the presence of \(10^{-7} \text{M PMA}\) at any time point examined including 24 h (Fig. 4A and data not shown). Supershift analysis was performed to determine the isoforms of SP1 present in BPAEC; these experiments resulted in interference patterns rather than supershifted bands (Fig. 4B). A small amount of the binding was disrupted by an antibody specific for the SP1 isoform, but most of the binding was disrupted by an antibody against the SP3 isoform. Examination of the SP1 family consensus oligo using nuclear extracts from BPAEC treated with 50 ng/ml HGF showed that HGF induced an increase in SP1 binding with a time course peaking at 60 min, similar to the time course found for increased Egr-1 binding (Fig. 4C). Supershift analysis revealed that the primary species in this band was the SP3 isoform (Fig. 4D).

**HGF and PMA Regulation of Wild-type and Egr-1/SP Mutant ACE Promoters**—Previous studies by our laboratory and others show that the core ACE promoter (−230 to +1 bp transcription start site, Fig. 5A) is sufficient for basal activity in primary endothelial cells and is regulated by PMA, glucocorticoids, and bleomycin (30, 42, 43). We used the core ACE promoter in a luciferase reporter construct to determine whether this region of the ACE promoter was responsive to regulation by HGF. Within 24–48 h, HGF treatment increased expression from the ACE promoter (Fig. 5B); the level of expression increased over time for as long as 5 days (data not shown). At no time was negative regulation of the ACE promoter detected in our experiments.

Mutagenesis of the ACE promoter was performed to replace the entire region (10 bp) containing the predicted Egr-1 and overlapping SP binding sites (Fig. 5A). The mutant promoter upstream of the luciferase reporter gene was then transfected into BPAEC, and cells were treated for 48 h with HGF. As shown in Fig. 5C, the wild type promoter is positively regulated by HGF at this time point. However, the mutant promoter is no longer up-regulated by HGF compared with basal levels of expression of the mutant promoter.

Phorbol ester regulation of ACE expression has been correlated with the protein kinase C-dependent increase in expression of the Egr-1 transcription factor (41). Bleomycin-induced up-regulation of ACE mRNA also correlates with increased nuclear levels and activation of the Egr-1 transcription factor, which occurs in a p42/p44 MAPK-dependent manner (30). We wished to determine the effect of the Egr-1/SP mutation on PMA-induced activity of the promoter. Experiments showed that although replacement of the Egr-1 site abrogated HGF-induced activation of the ACE promoter, PMA-induced activation was only somewhat reduced compared with basal levels of the mutant promoter (Fig. 5D).

Because our experiments using the core ACE promoter resulted only in up-regulation of the reporter gene by HGF, we also tested the effect of HGF on our largest ACE promoter construct (−4728 bp to +1 bp). This construct also showed only an increased expression in response to HGF, at time points varying from 24 h to 5 days (data not shown).

**Role of Egr-1 and SP3 in the HGF-induced Transcriptional Regulation of the ACE Promoter**—To determine whether regulation of the ACE promoter by HGF involved activation of the Egr-1 and SP3 transcription factors, we examined HGF activation of minimal responsive promoters to each factor. HGF treatment of BPAEC transfected with the Egr-1-responsive promoter (pEBS1luc) resulted in an −2-fold increase in luciferase at 16 h (Fig. 6A), indicating that transcriptionally active Egr-1 is synthesized as a result of HGF treatment. In contrast, HGF treatment of BPAEC transfected with the SP1/3-respond-
FIG. 6. Regulation of the minimal Egr-1- and SP3-responsive promoters by HGF and regulation of the ACE promoter by DN Egr-1 and DN SP3. A and B, BPAEC were transfected with the minimal Egr-1- and SP3-responsive promoters (pEBS1 luc and pEBS6 luc, respectively) with a ratio of luciferase:Renilla of 6:1. 24 h after transfection cells were placed in RPMI, 0.01% fetal bovine serum for 24 h before treatment with HGF (50 ng/ml) for 16 h.
HGF-induced ACE Expression

Although ACE expression is regulated by a number of growth factors and pharmacological agents, the mechanism of ACE regulation is not well understood. The core ACE promoter contains consensus transcription factor binding sites, including Egr-1, the SP family, and ets-1. Our data show that both PMA and HGF induce the synthesis of transcriptionally active Egr-1 transcription factor. In contrast, HGF enhanced the DNA binding activity of SP3 without showing an enhanced transcriptional response of an SP1/SP3-responsive model promoter. These data indicate that the increase of SP3 activity found in the EMSA is not biologically significant, perhaps due to the high basal level of activity of this transcription factor.

The predicted Egr-1 binding site in the ACE promoter overlaps with an SP family binding site. Such overlapping Egr-1/SP sites have been identified in several other gene promoters (47–49). The regulation of transcription by these two transcription factors has been shown to be complex; in some genes the two factors are synergistic, whereas in others the factors appear to compete (48, 50, 51). The Egr-1/SP consensus site was mutated in the ACE promoter to determine whether this site is required for either HGF or PMA activation. This mutation reduced basal activity of the promoter, suggesting that the Egr-1/SP site plays a role in the basal activity of the promoter. Also, overexpression of either DN Egr-1 or DN SP3 resulted in reduced basal expression of the 230-bp ACE promoter. Because unstimulated BPAEC contain constitutively active SP3 and the core ACE promoter contains a number of SP1/3 binding sites, it is possible that SP3 contributes to basal activity of the promoter. The mechanism(s) of basal regulation of the ACE promoter is currently being investigated in our laboratory.

We used dominant-negative (DN) mutants of SP3 and Egr-1 in cotransfection experiments with the ACE promoter to examine their effects on expression. These mutants contain intact DN binding regions of SP3 and Egr-1, respectively, but lack any transcriptional activation function. Thus, SP3 or Egr-1 DNA binding sites are blocked by these mutant factors. Basal expression of the ACE gene is regulated at least in part by SP3, and we therefore expected that DN-SP3 would impair basal ACE promoter activity. Likewise, Egr-1 can also bind to the composite element in the ACE promoter; thus, a DN Egr-1 should compete with SP3 and impair basal ACE promoter activity. The results show that in fact expression of either DN Egr-1 or DN SP3 resulted in reduced basal expression from the 230-bp ACE promoter (Fig. 6C). These data indicate that both Egr-1 and SP3 bind in vivo to the ACE promoter.

We further tested the ability of DN Egr-1 and DN SP3 to block HGF- and PMA-induced activation of the ACE promoter (Fig. 6D). Our data show that both the DN transcription factors blocked the ability of HGF to activate the promoter, with DN Egr-1 the more potent inhibitor. This result is in agreement with our hypothesis that, because of the overlapping sequences in the Egr-1/SP3 composite site, either DN factor is able to block the site. In this case DN Egr-1 is the stronger inhibitor since it directly blocks binding of HGF-induced Egr-1; SP3 also prevents the binding of the activated Egr-1. However, PMA activation of the promoter was only partially reduced by either DN transcription factor, indicating that unlike HGF, regulation of the ACE promoter by PMA must involve additional factor(s). This is in agreement with our findings from the mutated ACE promoter that indicated that the Egr-1/SP3 site was not required for all activation of the promoter by PMA.

DISCUSSION

In the present study we demonstrate that HGF influences ACE expression. HGF causes a transient increase in both ACE enzyme activity and ACE mRNA followed by sustained suppression of both. We also demonstrate in our system that PMA causes sustained up-regulation of ACE activity and mRNA, in agreement with findings by others. Our results also show that the transient increase in ACE expression is likely the result of increased expression of the ACE promoter by HGF. The mechanism(s) of the sustained decrease in ACE mRNA and protein are still under investigation.

(A) or PMA (10−8 M) for 16 h (B). Cell lysates were used in dual luciferase assays normalized to Renilla to control for transfection efficiency. Data are the means ± S.D., n = 3. The asterisk indicates statistical significance from control, p < 0.05. C, BPAEC were transfected with pACE230lac and cotransfected with either a control pCMV vector with no insert (CMV), pCMV-FLAG-NLS-Egr-1DN (DN Egr-1), or pCMV-FLAG-REST/SP3 (DN SP3) and Renilla; transfection ratios were ACEluc:CMV:Renilla 6:3:1. 24 h after transfection cells were placed in RPMI, 0.1% fetal bovine serum for 48 h before harvesting cell lysates for dual luciferase assays. Data are the means ± S.D., n = 4. The asterisks indicate statistical significance from control, p < 0.05. Relative light units.
induced expression of the ACE promoter must, therefore, require other as yet unidentified site(s) and factor(s).

The primary physiological role of ACE is maintenance of blood pressure homeostasis by converting angiotensin I to angiotensin II (Ang II), a potent vasoconstrictor, and by proteolytically inactivating bradykinin, a vasodilator (17–19). ACE is also up-regulated in response to tissue injury; ACE protein and mRNA are induced in experimental heart failure, pressure-overloaded left ventricle hypertrophy, and vascular injury as well as in the pulmonary injury model using bleomycin (21, 40, 52, 53). Basic fibroblast growth factor and vascular endothelial growth factor increase ACE mRNA expression in cell culture, and the temporal sequence of basic fibroblast growth factor and vascular endothelial growth factor expression in tissue injury suggests that these factors may in part mediate increased ACE expression after tissue injury (39, 40, 54).

Physiologically, ACE may function early in tissue repair via the activation of Ang II, which promotes vascular inflammation and smooth muscle cell and fibroblast growth, and increases the deposition of extracellular matrix proteins (40, 55). However, ACE expression and increased Ang II production in damaged tissues are also associated with remodeling and fibrosis (20–25). Beyond its immediate function after injury, ACE is believed to play a role in detrimental long term tissue remodeling. The role of ACE in congestive heart disease is well established, and increasing evidence suggests that elevated levels of ACE activity and, subsequently, Ang II may have a critical function in fibrotic diseases of organs including the heart, lung, and kidney (20–25). Studies of fibrotic tissue in culture show that ACE inhibitors slow myofibroblast growth, possibly reducing Ang II production (23, 56, 57). In animal studies ACE inhibitors, such as captopril and lisinopril, attenuate fibrosis in the heart, kidney, and lung both by reducing fibroblast proliferation and preventing apoptosis of epithelial and endothelial cells (22, 25, 58–62).

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