Abstract  The human angiotensin II type 1 (AT₁) receptor gene was isolated and its promoter function analyzed by deletion mutant promoter/luciferase constructs in transfected Cos 7 cells. We found that epidermal growth factor enhanced the human AT₁ promoter activity twofold to threefold. The region between -227 and -366 bp from the 5' end of the cDNA was mapped for a base sequence responsive to the epidermal growth factor stimulation. By computer analysis, PEA3 transcription factor was located in this region and was shown to bind to the promoter by gel shift assay in Cos 7 and HepG2 cells. These results indicated that the human AT₁ receptor enhanced by epidermal growth factor may be due to PEA3 binding to the human AT₁ promoter. (Hypertension. 1994;23[part 2]:1032-1035.)  

Key Words  • angiotensin II  •  epidermal growth factor-urogastrone  •  promoter regions (genetics)

Methods
Cloning of the Genomic Library
A human lymphocyte genomic library in lambda DASH II was obtained from Stratagene. A total of 10⁷ lambda phage plaques were screened on nylon membranes (Hybond-N, Amersham) by using an EcoRI-4ce I fragment of the human AT₁ cDNA as a probe. Hybridization was done in 50% formamide, 6 x SSC (1 x SSC consisted of 0.15 mol/L NaCl and 0.015 mol/L sodium citrate, pH 7.0), 1% sodium dodecyl sulfate (SDS), 2 x Denhardt's solution (0.1% Ficoll, 0.1% bovine serum albumin, and 0.1% polyvinylpyrrolidone), and 10 mmol/L sodium phosphate buffer for 16 to 24 hours at 42°C. Membranes were washed twice in 2 x SSC and 1% SDS at 60°C. A positive clone was subcloned into the Bluescript KS(+) vector for determination of base sequences.

Plasmids
The 5'-flanking region of the human AT₁ gene spanning the first exon was subjected to double restriction enzyme digestion with HindIII and Cla I and subcloned in the Bluescript vector. To construct a human AT₁ receptor promoter/luciferase system, we treated the plasmid containing HindIII and Cla I fragment with exonuclease III by using the Erase-a-Base System (Promega Corp). Nine deletion mutants were generated, inserted into the GL2 basic vector (Promega), and named pLucl through pLuc9.

Expression of Human AT₁ Promoter/Luciferase Constructs
Cos 7 cells were grown in Dulbecco's modified Eagle medium containing 10% calf serum. DNA (10 μg) of each construct was transfected into 1.8 x 10⁵ Cos 7 cells in 300 μL phosphate-buffered saline by electroporation in 0.2-cm gap cuvettes (150 V, 250 microfarads) using a Gene Pulser apparatus (Bio-Rad Laboratories). The cell lysates were prepared 48 hours after transfection by using the Luciferase Assay System (Promega). Luciferase activity of 40-μg protein lysates was measured with a liquid scintillation counter (LS1801, Beckman Instruments). Transfection efficiency was normalized to the levels of pSV-β-galactosidase activity (Promega), which was cotransfected into Cos 7 cells. For experiments with EGF treatment, 100 ng/mL human recombinant EGF (UBI) was added to the culture of the transfected Cos 7 cells 24 hours after electroporation, and luciferase activity was measured as above.

Epidermal Growth Factor-Enhanced Human Angiotensin II Type 1 Receptor

Deng-Fu Guo, Tadashi Inagami

From the Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, Tenn.  
Correspondence to Tadashi Inagami, Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, TN 37232.
Gel Shift Assay

Oligonucleotides were synthesized in a MilliGen/Biosearch Cyclone Plus DNA synthesizer and purified on gels. After 5'-end-labeled (3P) complementary oligonucleotides were annealed, double-stranded oligonucleotides were gel purified. Nuclear extracts were prepared by a standard method13 from Cos 7 and HepG2 cells. Nuclear extracts were mixed with poly d(I-C) and the double-stranded competitor oligonucleotide on ice in 9 uL of (mmol/L) HEPES 10 (pH 7.9), KCl 50, EDTA 0.1, dithiothreitol 0.25, and phenylmethylsulfonyl fluoride 0.25 as well as 10% glycerol. After 10 minutes at 0°C, 10,000 cpm of double-stranded oligonucleotide was added and the incubation continued for 20 minutes. The samples were loaded onto a 5% polyacrylamide gel and electrophoresed in 0.5X Trisborate buffer at 120 V.

Northern Analysis

RNA was isolated from VSMCs of Sprague-Dawley rats by the acid guanidium thiocyanate-phenol-chloroform extraction method.14 Total cellular RNA (20 ug) was separated on a 1% agarose formaldehyde gel and transferred onto a nylon membrane. 3P-labeled random primer probes were used for the Northern blots: rat AT1A and glyceraldehyde-3-phosphate dehydrogenase. -"P-labeled random primer probes were used for the Northern blots: rat AT1A and glyceraldehyde-3-phosphate dehydrogenase. -"P-labeled random primer probes were used for the

Results

The 5'-Flanking Region of the Human AT1 Receptor Gene

For characterization of the nucleotide sequence of the region upstream of exon 1 in the human AT1 receptor gene, approximately 1.2 kb DNA sequence upstream of exon 1 was completely sequenced. For identification of the sequence of elements that might be involved in the control of human AT1 receptor gene expression, the 5'-flanking sequence of the promoter, Cos 7 was transfected with each of the nine human AT1 promoter/luciferase chimeric constructs were treated with 100 ng/mL EGF for 24 hours, and the luciferase activity was measured. The luciferase activity increased 2.5- to 2.9-fold in pLuc through pLuc5 but not in pLuc6. The transfectant with pLuc8 that contains a cyclic AMP-induced responsive element (CRE) sequence, HNF5 sequence, AP2-responsive element (HNF5; consensus sequence, GT-AGGTATGT) was present at -177 to -181 bp. Two transcription factor AP2 binding sites (consensus sequence, CCA/NGCG/CCGC) were present at -255 to -263 and -1025 to -1032 bp. A glucocorticoid-responsive element (GCGCGG; consensus sequence, GT-ATAATT) was found at -923 to -929 bp. A PE3A binding site (consensus sequence, AGGAAG/A) was present at -291 to -296 bp. These results are shown in Fig 1.

Detection Analysis of the Human AT1 Promoter, by Transient Transfection

All the human AT1 promoter/luciferase chimeric constructs were introduced into Cos 7 cells by electroporation. The construct pLuc4 gave the highest luciferase activity; therefore, its luciferase level was used as a reference (set as 100%) in each series of experiments. The promoter/luciferase constructs and relative activity of luciferase of each construct are shown in Fig 2. Compared with the highest luciferase expression in pLuc4, pLuc5 gave only 65% of pLuc4. A CAAT box sequence was found in the region of pLuc4 beyond that of pLuc5. The transfectant with pLuc8 that contains an overlapping double Sp1 binding sites gave only 7% luciferase activity. The transfectant with pLuc9 gave a background level of luciferase activity comparable to that with the basic plasmid (pGL2) alone. For mapping of the EGF-responsive region of the promoter, Cos 7 cells transfected with each of the nine human AT1 promoter/luciferase chimeric constructs were treated with 100 ng/mL EGF for 24 hours, and the luciferase activity was measured. The luciferase activity increased 2.5- to 2.9-fold in pLuc through pLuc5 but not in pLuc6. Therefore, the EGF-responsive region should be located between the 5' ends of pLuc5 and pLuc6, that is, between -227 and -366 bp. By
Fig 2. Expression chimeric constructs of 5' deleted human angiotensin type 1 (AT₁) receptor promoters and luciferase in transiently transfected Cos 7 cells. Left, Schematic representation of the human AT₁ promoter (black bar), first exon (open box), and luciferase gene (black box). Three TATA boxes and three CAAT boxes, two AP2 and two SP1 binding sites, a GC box, and a cyclic AMP-induced responsive element (CRE) are shown, their positions indicated in parentheses. Nucleotide positions are shown with respect to the 5' end of cDNA. Chimeric human promoter/luciferase constructs are presented with a line, and names of constructs are given. Right, Luciferase activity of each construct was assayed as described in "Methods." Values are expressed as percentages of activity obtained with the construct pLuc4 (100%), which gave the highest activity, and presented as means of four different experiments. Hatched column indicates without epidermal growth factor treatment; black column, with epidermal growth factor treatment.

computer search, a PEA3 transcription factor (−291 to −296 bp), an AP2 active protein (−255 to −263 bp), and a GC box (−245 to −255 bp) were found in this region. Because PEA3 can be activated by EGF, we investigated by gel shift assay whether PEA3 can bind to the human AT₁ receptor.

Gel Shift Assay Analysis

To demonstrate the presence of PEA3 activity in Cos 7 and HepG2 cell nuclear extracts, we used a PEA3-specific oligonucleotide. Protein binding to the 32P-labeled PEA3 oligonucleotide effectively competed with the specific unlabeled oligonucleotide but not with the AP2 oligonucleotide as shown in Fig 3. This demonstrates that PEA3 activity is present in the cells and presumably interacts with the appropriate sequence in the human promoter.

Discussion

In the present study we sequenced and analyzed the human AT₁ receptor promoter region and also demonstrated that EGF upregulated the human AT₁ receptor. The sequence of the human AT₁ receptor promoter is unusual because it contains the characteristics of promoters of both a housekeeping gene and a regulated gene. Most eukaryotic promoters have a TATA box, an upstream regulatory sequence such as the CAAT box, and tissue-specific or hormonal-responsive elements. Conversely, a typical housekeeping gene promoter does not contain a TATA box but instead has GC-rich regions. The human AT₁ receptor promoter contains not only three TATA boxes and three CAAT boxes but also two Sp1 binding sites, a GC box, and a PEA3 site. These are features of a class of promoters that has complex regulatory processes. The present study of functional features of the promoter region using deletion-mutated promoter/luciferase constructs demonstrates that the maximal expression of the human AT₁ gene requires the sequence from −642 bp. Comparison of the promoter activity of pLuc4 with that of pLuc5 suggests that the region between pLuc4 and pLuc5 was the most critical for the promoter function.

We were not able to detect human AT₁ receptor expression in long passaged human cell lines such as HepG2 and HeLa cells by Northern hybridization.

Fig 3. Bandshift analysis with a PEA3 binding oligonucleotide. The PEA3 oligonucleotide sequence was used in this study: 5'-TCTCCGAGGAAAATGATACCT-3'. Whole-cell extracts (10 mg protein) of Cos 7 and HepG2 cells were preincubated with the indicated molar excess (0, 20, 100×10⁻¹² mol/L) of PEA3 competitor oligonucleotide and AP2 oligonucleotide (1.5 pmol, 5'-GATCGAACTGACGCGGCCCGCT-GT-3') for 10 minutes at 0°C before the addition of 32P-labeled PEA3 oligonucleotide. After another 15 minutes of incubation, protein-DNA complexes were resolved on 5% acrylamide gels. Specific complexes are shown.
Thus, EGF may exert its hypertrophic effects through a double mechanism, its direct effect mediated by the urokinase plasminogen promoter. The present study has shown to upregulate the collagenase promoter and transcription in human cells may be positively regulated by EGF. However, we observed that rat AT1A receptor is actively expressed in rat aortic VSMCs. Moreover, the PEA3 transcription factor-responsive site exerts in the 5'-flanking region (unpublished observation). Therefore, we investigated how EGF may affect the rat AT1A receptor in VSMCs. As shown in Fig 4 the mRNA level of rat AT1A was increased 2.5-fold with EGF treatment compared with untreated cells. This result supported the finding that we obtained with the human AT1 gene promoter function.

EGF was initially identified as a stimulator of eyelid opening in newborn animals and was purified from the male mouse submaxillary gland. It is a potent modulator of cell growth of a variety of cultured cells. EGF has also been reported to be a vasoconstrictor and mitogen expressed in rat aortic VSMCs. Moreover, the PEA3 transcription factor, a single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. It has been reported that the EGF-increased promoter function was positively regulated by EGF.

EGF receptor and its indirect positive effect on AT1 receptor expression.

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D F Guo and T Inagami

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