Molecular Mechanism of Adipogenic Activation of the Angiotensinogen Gene

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Abstract Angiotensinogen gene expression is controlled in a tissue- and development-specific manner. Interestingly, the angiotensinogen gene is abundantly expressed in adipose tissues other than the liver, where it is mainly produced. We investigated the molecular mechanism of angiotensinogen gene expression in a 3T3-L1 preadipocyte-adipocyte system. Although angiotensinogen mRNA was barely detectable in preadipocytes, its levels increased significantly during differentiation. As a whole, the pattern of the change in transcriptional activity of the angiotensinogen promoter was similar to that of the angiotensinogen mRNA levels during adipogenic differentiation, indicating that the activation of the angiotensinogen promoter might be involved in the adipogenic differentiation-coupled gene expression. The proximal promoter region, from -96 to +22 of the transcriptional start site, was sufficient to confer adipogenic activation, and the proximal element from -96 to -52 of the transcriptional start site was necessary for this promoter stimulation. DNA-protein binding experiments showed that this proximal element specifically bound to a nuclear factor induced by adipogenic differentiation. These results suggest that the proximal promoter element from -96 to -52 plays a role in adipogenic activation of the angiotensinogen promoter. (Hypertension. 1994;23:364-368.)

Key Words • genetics • hypertension, obesity-induced • angiotensinogen • adipose tissue

The components of the classic renin-angiotensin system (RAS) are synthesized in various tissues and interact with each other in the circulation to generate the active peptide angiotensin II, which has a variety of actions, such as a vasoconstrictor activity and stimulation of the production and release of aldosterone. Accumulated evidence suggests the existence of a local RAS in various tissues, including those involved in cardiovascular regulation, such as the heart, vascular wall, kidney, adrenal gland, and brain. Angiotensinogen, which is the unique substrate of renin in the RAS in vivo and is produced mainly by the liver, is also abundantly synthesized in fat cells. Although the tissue mRNA content of the genes that compose the RAS is known to change in response to specific stimuli, the functional role of adipogenic expression of the angiotensinogen gene is unclear.

Recent studies showed that 3T3-L1 and 3T3-F442A mouse fibroblast cell lines produced angiotensinogen and that the fibroblast cell lines secreted angiotensinogen increased during the differentiation of these cells into adipocytes. Culture of preadipocytes that can be induced to differentiate into adipocytes provides a suitable system for studying the regulatory mechanisms of angiotensinogen gene expression. We attempted to determine whether the activation of the angiotensinogen promoter would occur in the differentiation-coupled gene expression and to elucidate the significance of adipogenic angiotensinogen using a 3T3-L1 preadipocyte-adipocyte system. Angiotensinogen mRNA accumulation was compared with the level of chloramphenicol acetyltransferase (CAT) activity, an established marker of the transcriptional activity of promoter regions located upstream from the CAT coding gene. We also performed an electrophoretic mobility shift assay (EMSA) to analyze DNA-protein binding activity of nuclear factors to the promoter region. By these experiments, we identified a proximal promoter element from -96 to -52 of the transcriptional start site (angiotensinogen gene activating element; AGE), which may be important for the adipogenic activation of the angiotensinogen gene.

Methods

Cell Culture and Induction of Differentiation

For induction of adipogenic differentiation, 3T3-L1 fibroblasts were grown to confluence (designated as day 0) and cultured in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum, 10 μg/mL insulin, 1 μmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX) for 2 days. At the end of day 2, dexamethasone and IBMX were removed. Cells began to accumulate cytoplasmic triacylglycerol by day 3. Preadipocyte control cells were maintained in DMEM containing 10% calf serum. At no time were lipid droplets observed in time-matched control cells.

Northern Blot Analysis

Total RNA was extracted from 3T3-L1 preadipocytes and adipocytes by single-step acid guanidinium thiocyanate–phenol–chloroform extraction. Each RNA sample (20 μg) was denatured with 1 mol/L gylcol and 50% dimethyl sulfoxide (DMSO), electrophoresed on a 1.2% agarose gel, and transferred to a Gene Screen Plus membrane.
A genomic 383-bp Aat 1-AatII fragment of the mouse angiotensinogen gene was used as a hybridization probe, and hybridization was carried out at 60°C for 16 hours in a solution containing 1 mol/L sodium chloride, 1% sodium dodecyl sulfate (SDS), 50% dextran sulfate, 100 μg/mL denatured salmon sperm DNA, and 1×10⁶ cpm/mL labeled probe. The filter was washed twice with 2× SSC (1× SSC was 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate) at room temperature for 5 minutes, twice with 2× SSC and 1% SDS at 60°C for 30 minutes, and twice with 0.1× SSC at room temperature. The filter was then subjected to autoradiography at -70°C with an intensifying screen. The mouse skeletal β-actin cDNA probe was provided by Dr Katsuji Tokunaga (Chiba Cancer Center Research Institute, Chiba-ken, Japan).

### Plasmid Construction, Stable DNA Transfection, and CAT Assay

Genomic DNA cloning was performed as described previously. Briefly, the mouse angiotensinogen promoter-CAT chimeric constructs were made as follows: 523-bp (−501 to +22) HinP1, 118-bp (−96 to +22) Sau3A/HinP1, and 73-bp (−51 to +22) Sau3A/HinP1 genomic DNA fragments of the angiotensinogen gene were subcloned into the BglII/HindIII sites of pUCSVOCAT® to generate Ag501, Ag96, and Ag51, respectively. For construction of an internal deletion mutant, an AgΔ501, 405-bp (−501 to −97) HinP1/Sau3A fragment was inserted into the sense orientation to the BglII site of Ag51.

The 3T3-L1 preadipocytes were stably cotransfected with the angiotensinogen promoter-CAT chimeric constructs and pSV2neo DNAs (15 μg per 10-cm dish, 10⁴ logarithmically growing cells) at a ratio of 5 to 1, using calcium phosphate precipitation as previously described. After 12 hours of incubation, the cells were treated with 10% DMSO. Selection of G418 (500 μg/mL) resistance was started 24 hours later. Growing cells (preadipocytes) at a ratio of 5 to 1, using calcium phosphate precipitation as previously described. After 12 hours of incubation, the cells were treated with 10% DMSO. Selection of G418 (500 μg/mL) resistance was started 24 hours later.

### Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay

Crude nuclear extracts from 3T3-L1 preadipocytes and adipocytes were prepared using the modified method of Dignam et al. The angiotensinogen gene activating element (AGE) from −96 to −52 of the transcriptional start site was end-labeled with T4 polynucleotide kinase and [γ-³²P]ATP and used as a probe. Nuclear extracts were preincubated for 15 minutes on ice in a 20-μL reaction mixture containing 12 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 0.1 mmol/L EDTA, 0.5 mmol/L diethiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 12% glycerol, and 500 μg poly(dI-dC). Nonlabeled competitor was included in some of the binding reactions as indicated.

Approximately 0.3 μg of the radiolabeled probe was added and the incubation continued for 30 minutes at room temperature. The incubation mixture was loaded on a 5% polyacrylamide gel in a buffer containing 50 mmol/L Tris base, 50 mmol/L boric acid, and 1 mmol/L EDTA and then electrophoresed at 140 V for 3 hours followed by autoradiography. A double-stranded oligonucleotide that contained a consensus binding motif for the CCAAT/enhancer-binding protein (C/EBP) was synthesized on a Milligen/Bioresearch Cyclone Plus oligonucleotide synthesizer and purified on OPA columns (Applied Biosystems) as described by the manufacturer.

### Statistical Analysis

Values are expressed as mean±SEM of at least three independent experiments. The significance of intergroup differences in parameters was assessed by Student's t test. A value of P<.05 was considered significant.

### RNA Analysis

Angiotensinogen mRNA began to accumulate within 24 hours (P<.01, n=3) after induction of differentiation (day 1, Fig 1). Levels of mRNA increased by day 3, remaining constant thereafter. Extremely low levels of angiotensinogen mRNA were detected in negative control preadipocytes. There was no apparent change in the intensity of β-actin mRNA signals during the course of differentiation (data not shown).

### Stable DNA Transfection Assay

The chimeric construct Ag501 has a 523-bp fragment containing the mouse angiotensinogen promoter region (nucleotide positions −501 to +22) in the sense orientation with respect to the CAT gene. The TK-CAT plasmid that contained herpes simplex virus–thymidine kinase (TK) promoter linked upstream from the CAT coding gene was used as a control, and all results were corrected for variations in transfection efficiency by reference to TK-CAT. TK-CAT did not show any apparent changes in CAT expression during the course of differentiation (data not shown). CAT activity in the Ag501 construct increased markedly (P<.01, n=3) within 24 hours of the induction of differentiation (day 1, Fig 2A). Although CAT activity reached a maximum on day 3, it remained elevated throughout the course of differentiation compared with the level in time-matched control preadipocytes (P<.01, n=3). The mode of the change in CAT expression was similar to that of the accumulation of endogenous angiotensinogen mRNA,
although the increase in mRNA levels seemed to be more stable during differentiation than the increase in CAT expression. There was no increase in CAT activity in the negative control preadipocytes.

The increase in CAT expression in the Ag96 construct resembled that in the Ag501 construct during the course of differentiation (Fig 2B), indicating that only 118 bp (−96 to +22, Ag96) of the angiotensinogen proximal promoter sequences was needed to mediate adipogenic expression of the CAT gene. However, no increase in CAT activity was observed with the Ag51 construct during the course of differentiation (Fig 2C). In addition, internal deletion of a proximal promoter element from −96 to −52 (AGE) dramatically decreased the adipogenic activation of CAT expression (AgΔ501, Fig 3).

**DNA-Protein Binding Analysis**

Although no detectable DNA-protein binding activity to the AGE was found with the nuclear extracts from preadipocytes (lanes 1 and 2, Fig 4), incubation of the AGE with adipocyte-nuclear extracts resulted in formation of a DNA-protein complex, which existed as a slowly migrating band compared with the free probes (designated by the solid arrowheads in lanes 3 and 4, Fig 4). This retarded band represented a sequence-specific interaction between the AGE and proteins in the extracts, as this could be competed out with an excess of the same unlabeled AGE (lane 5, Fig 4). Other additional bands observed in EMSA were due to nonspecific binding, because these bands could not be competed with the unlabeled AGE. Oligonucleotide containing the consensus binding sequences for C/EBP failed to compete with this binding (lane 6, Fig 4). No AGE-binding activity was detectable with the extracts from nonadipocyte cell lines, NIH3T3 and HeLa cells (lanes 7 and 8, Fig 4). The adipogenic differentiation–coupled AGE-binding activity was detectable on the induction

**Fig 2.** Line graphs show time course of transcriptional activity of adipocytes and preadipocytes. On different days of differentiation, cell extracts (40 μg) were prepared from 3T3-L1 adipocytes and preadipocytes, which were stably transfected with the Ag501 (A), Ag96 (B), or Ag51 (C) construct. Relative chloramphenicol acetyltransferase (CAT) activity was determined by comparing the average of three independent experiments with the basal level activity in cell extracts obtained from preadipocytes on day 0. Values are mean±SEM (n=3). *P<.01 vs day 0; +P<.01 vs corresponding preadipocytes.

**Fig 3.** Schematic representation of mouse angiotensinogen promoter (A) and chloramphenicol acetyltransferase (CAT) activity of the angiotensinogen gene activating element (AGE)–deleted construct, AgΔ501 (B). A, Restriction endonuclease cleavage sites are indicated as follows: Hi, HpaI; Pv, PvuII; Ha, HaelII; Sa, Sau3AI; and Rs, RsaI. The TATA box is located at nucleotide positions −30 to −25, and the transcription start site is indicated by +1. The AGE is shown by the solid box. B, Basal and differentiation-induced expression of mouse angiotensinogen promoter–CAT hybrid genes in stably transfected 3T3-L1 cells. The AgΔ501 construct has an internal deletion between −96 and −52 of the Ag501 construct. Stable transfection was performed with these constructs as described in "Methods." Cells were harvested either before the induction of differentiation (Pre, 3T3-L1 preadipocytes) or 1 day after the induction (Adp, 3T3-L1 adipocytes), and aliquots of cell extract containing equal amounts of total protein (40 μg) were used in the CAT assay. Relative CAT activities were determined by averaging three independent experiments and comparing them with basal level activity obtained from preadipocytes stably transfected with Ag501 DNA and are expressed as mean±SEM (n=3). Arrows indicate the acetylated forms of 14C-labeled chloramphenicol.
of differentiation (day 1) and did not change markedly during the course of differentiation (data not shown).

Discussion

Our results demonstrate that the activation of the angiotensinogen promoter might be involved in the accumulation of angiotensinogen mRNA in 3T3-L1 adipocytes. Angiotensinogen mRNA increased during the course of adipogenic differentiation, and stable transfection of the 3T3-L1 cells with the angiotensinogen promoter (-501 to +22)-CAT chimeric construct resulted in differentiation-coupled activation of CAT expression. These findings suggest that the 5'-flanking region of the angiotensinogen gene contains information necessary to trigger its adipogenic expression.

Interestingly, the proximal promoter region from -96 to +22 could mediate the differentiation-coupled activation of CAT expression, and deletion of the AGE (-96 to -52) greatly reduced this activation, whereas the basal promoter activity was still retained. Furthermore, adipocyte nuclear extracts, but not preadipocyte nuclear extracts, exhibited a specific AGE-binding activity. These results suggest that the proximal element AGE plays a role in the adipogenic activation of the angiotensinogen promoter rather than in the maintenance of the basal promoter activity and that the AGE-binding factor may be adipogenic differentiation-specific in its binding activity. Previous experiments using adipocyte cell lines showed that the transcription factors C/EBP, activator protein-1 (AP-1), and nuclear factor I (NF I) participate in adipogenic differentiation-induced expression of several genes. The nucleotide sequences of AGE do not have any apparent homology with the consensus binding motifs for C/EBP, AP-1, or NF I. Thus, the AGE-binding protein appears to be distinct from the C/EBP, AP-1, and NF I family of nuclear activators.

In our experiments, oligonucleotides with binding sites for AP-1 and NF I did not compete either (data not shown). Further study is necessary, however, to determine whether the AGE-binding factor is a novel tissue-specific transcriptional modulator of the angiotensinogen gene.

Although the nature of the significant relation between obesity and hypertension is obscure, the RAS, in addition to insulin resistance and the sympathetic nervous system, is proposed to play an important role in obesity-related hypertension. A recent study demonstrated that angiotensinogen gene expression in adipocytes in rats was nutritionally regulated and that fasting and refeeding affected blood pressure and adipocyte angiotensinogen mRNA level in a parallel manner. Linkage analysis and transgenic studies suggest that abnormally elevated expression of the angiotensinogen gene may contribute to the development of hypertension. Further molecular and clinical studies on the regulation of the adipogenic angiotensinogen are needed to provide insight into the pathophysiological nature of obesity-related hypertension.

In conclusion, our study demonstrates an increase in promoter activity of the angiotensinogen gene during adipogenic differentiation and suggests that this change is involved in the accumulation of angiotensinogen mRNA in adipocytes. Our findings also suggest that the proximal promoter element AGE plays a role in the molecular process of adipogenic activation of the angiotensinogen promoter.

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