A High Level of CCAAT-Enhancer Binding Protein-δ Expression Is a Major Determinant for Markedly Elevated Differential Gene Expression of the Platelet-Derived Growth Factor-α Receptor in Vascular Smooth Muscle Cells of Genetically Hypertensive Rats

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Abstract—Platelet-derived growth factor-α receptor (PDGF-αR) expression is markedly elevated in cultured vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) when compared with normotensive rat strains, Sprague-Dawley, Wistar, and Wistar-Kyoto rats (WKY). This “almost-all-or-none” type of differential expression strongly suggests that PDGF-αR or its transcription-regulating mechanisms or factors are significantly related to genetic hypertension. To evaluate the role of PDGF-αR in vascular remodeling and hypertension, we have investigated the underlying molecular mechanism. We have recently shown that the regulatory domain responsible for this difference is localized to the PDGF-αR promoter region between −246 and −139, which contains an enhancer core sequence specific for CCAAT-enhancer binding proteins (C/EBPs). We defined the roles of this element for hypertensive strain-specific PDGF-αR gene transcription. DNA-protein binding studies by competition in electromobility shift and supershift assays revealed that 2 members, C/EBP-β and C/EBP-δ, are mainly responsible for DNA-protein complex formation; the former acts as a transcriptional repressor and the latter as an activator of the PDGF-αR gene, respectively. Western or Northern blot analyses supported evidence for high expression of C/EBP-δ seen only in SHR-derived VSMCs. Furthermore, forced expression of C/EBP-δ transactivated the transcriptional efficiency of the PDGF-αR gene even in WKY-derived VSMCs, whereas that of C/EBP-β had an opposite effect in SHR-derived VSMCs. These findings indicate that differential expression of members of the C/EBP family, mainly C/EBP-δ and possibly C/EBP-β, are responsible for the strain-specific gene transcription of PDGF-αR in VSMCs. (Circ Res. 1999;84:64-73.)

Key Words: platelet-derived growth factor α-receptor ■ vascular smooth muscle cell ■ promoter activity ■ strain-specific gene transcription ■ CCAAT-enhancer binding protein

Vascular remodeling is considered to play a major role in the genesis and perpetuation of hypertension and cardiovascular degenerative diseases. Several factors should contribute to it, but exact mechanisms of these specific factors are not clear.

Platelet-derived growth factor (PDGF) is one of the major mitogens and is responsible for proliferation and migration of vascular smooth muscle cells (VSMCs). The dimeric ligands (PDGF-AA, -AB, and -BB) exert their biological effects by binding to 2 monomeric units of the receptors, PDGF-α receptor (PDGF-αR) or β-receptor (PDGF-βR). Each subunit of the PDGF dimer binds to 1 receptor molecule; therefore, 2 molecules of receptors are necessary to accommodate the PDGF dimer on binding the ligand. These 2 receptor moieties form a noncovalent dimer. In recent years, the roles of PDGF and its receptor on the growth and differentiation of the VSMCs have been proposed. An overexpression of PDGF or its receptor is observed in the atherosclerotic lesion and restenotic vessel wall after balloon injury, indicating the importance of PDGF control in the etiology of various cardiovascular diseases, including coronary artery disease, atherosclerosis, and diabetic microangiopathy. In response to hemodynamic forces, physical injury, or circulating factors, cells in the vessel wall are activated to release growth modulators, cytokines, proteolytic enzymes, and matrix components, thereby participating in the process of vascular remodeling. In fact, VSMCs are capable of producing and releasing PDGF-A chain (but not PDGF-B chain) in a growth- and development-dependent manner, which may contribute to the autocrine and paracrine growth–stimulating.
mechanism of blood vessels through the PDGF-αR. Although ample expression of PDGF-βR in VSMCs was known, vascular expression of PDGF-αR had been controversial. Previously, we reported a markedly elevated expression of PDGF-αR in the aortic VSMCs of a genetically hypertensive rat strain, spontaneously hypertensive rats (SHR), whereas VSMCs derived from a control strain, Wistar-Kyoto rats (WKY), did not respond to PDGF-AA in DNA or protein synthesis. In these cells, expression of PDGF-αR was not detected. Interestingly, there was no difference in the expression of PDGF-βR. Obviously, there is a strain-specific differential expression of PDGF-αR between the hypertensive and normotensive animals. To gain insight into the molecular mechanism of its differential expression, we studied the regulatory mechanism of the PDGF-αR gene expression in cultured VSMCs and showed that the major regulatory domain responsible for the strain-specific gene transcription resides between –246 and –139 in the promoter region.

We have also found that this domain contains an enhancer core sequence for CCAAT/enhancer-binding proteins (C/EBP), which was shown to interact with nuclear extracts obtained from VSMCs derived from WKY and SHR. These findings strongly suggest that expression of specific members of the transcriptional factors of C/EBP is involved in hypertensive hypertrophy of blood vessels. In the present study, we have demonstrated that specific members of the C/EBP family, mainly C/EBP-δ and possibly C/EBP-β, regulate a strain-specific transcriptional activation of the PDGF-αR gene, and to our knowledge this is the first report that shows a functional importance of C/EBP-δ as a trans-acting nuclear factor in the cardiovascular system.

Materials and Methods

Materials

Mouse recombinant IL-1β was purchased from Boehringer Mannheim Corp (Tokyo, Japan). Cycloheximide (CHX) was purchased from Sigma. Affinity-purified rabbit polyclonal antibodies for C/EBP-α (14AA), C/EBP-β (C-19), and C/EBP-δ (C-22) raised against peptide epitopes corresponding with amino acid sequences of rat C/EBP-α (235–265), rat C/EBP-β (258–276), and rat C/EBP-δ (253–265), were purchased from Santa Cruz Biotechnology. Expression vectors of C/EBP-α, -β, and -δ (MSV/EBP-α, -β and -δ, respectively) were generously provided by Dr. S.L. McKnight (Department of Biochemistry, The University of Texas South Medical Center, Dallas, TX). [α-32P]dCTP (3000 Ci/mmol) and [γ-32P]ATP (6000 Ci/mmol) were obtained from DuPont/NEN.

Cell Culture

Wistar rats, Sprague-Dawley rats, WKY, and SHR were purchased from Charles River Breeding Laboratories (Wilmington, Mass), and VSMCs were isolated from the thoracic aorta of age-matched rats (12 weeks old) described previously.6,7 VSMCs (passages 5 to 12) were seeded in a dish (105 cells/cm2) and maintained in DMEM with 10% heat-inactivated FCS at 37°C in a humidified atmosphere of 95% air-5% CO2. In preparation for experiments, confluent cells, which exhibited a hilly-and-valley pattern that is typical of smooth muscle cells in culture, were made quiescent by placing them in a defined serum-free medium containing insulin (10 μg/mL), transferrin (10 μg/mL), and sodium selenite (10 ng/mL) for 2 days. This medium has been shown to maintain VSMCs in a quiescent and noncatabolic state for an extended period of time.8 All data reported herein were confirmed with multiple pairs of VSMC preparations, and phenotypic changes of VSMCs were consistently monitored by the level of basic calponin (also known as h1 calponin) mRNA. Previously, we confirmed that the level of basic calponin mRNA is downregulated drastically and rapidly when VSMCs are undergoing dedifferentiation, thereby indicating that basic calponin is a very sensitive and reliable phenotypic marker of VSMCs in vitro.9

Synthetic Oligonucleotides and DNA Amplification

Oligonucleotides were synthesized by the solid-phase phosphite triester method with the use of the Applied Biosystems model 380-B DNA synthesizer and purified by electrophoresis on 16% polyacrylamide-8 mol/L urea gels. DNA fragments was generated by polymerase chain reaction (PCR) with a Perkin-Elmer automated thermocycler (model 9600) according to the manufacturer’s specifications. The sequence of DNA fragments and their orientation in final plasmid constructs were determined by the dyeoxy chain termination method on a double-stranded DNA template. The 5'-flanking segment of the PDGF-αR gene spanning –1381 through +68 was prepared by PCR using specific primers, the 5'–end primer (P1), 5’-CCCGAGCTGAAGATACACCG-3', and the 3'–end primer (P2), 5’-CCTCCATCAAGCTCCAACAGT-3'. This 497-bp DNA fragment was ligated onto the promoterless luciferase vector pGL2-Enhancer (Promega, Madison, WI). The resultant plasmid was designated –1381/+68 WT. A mutated construct, designated –1381/+68 MT and containing nucleotide substitution mutations at positions –160 and –156, was generated according to the recombinant PCR technique using the following mutagenic primers: the 5'–end primer (M1), 5’-TGGCCCCAACAAGTACATAAGAGC-3', and the 3'–end primer (M2), 5’-TTGCTCTATGAGCGTGGGGAGGCA-3'. Underlined letters indicate the nucleotide substitution mutation. In an internal deletion mutant, designated –1381/+68 AMT, was also prepared by the same technique by deleting the sequence spanning –165 through –139. Two fragments containing an internal BalI restriction enzyme site were generated by PCR using 2 sets of primers, as follows: (1) the 5'–end primer, P1, and the 3'–end primer, 5’-TGCGGCAATCCCAACCCAGCTT-3', and (2) the 5'–end primer, 5’-TGCCCATGGAGGACTTGAGGCGTC-3', and the 3'–end primer, P2 (underlined letters indicate the internal BalI site). After creating a blunt end on both fragments with BalI digestion, –1381/+68 AMT was finally prepared by the ligation of 2 fragments.

DNA Transfection and Luciferase Assay

Plasmids used for transfection experiments were prepared by alkaline lysis of bacterial cultures and purified by precipitation with polyethylene glycol.11 VSMCs were seeded in 60-mm dishes (5×104 cells per dish) 24 hours before transfection. Transfection was performed with cellular DNA by the DEAE-dextran method as described previously.7 For transient transfection experiments, 3 μg of –1381/+68 WT, –1381/+68 MT, or –1381/+68 ΔMT was cotransfected with 6 μg of pSVβ-galactosidase control vector (pSVβ-gal) (Promega) as normalization reference for transfection efficiency. For overexpression experiments, 3 μg of –1381/+68 WT or –1381/+68 MT were cotransfected with 3 μg of a mock vector (MSV) or an expression vector of C/EBP members (MSV/EBP-α, -β, or -δ), in addition to 6 μg of pSVβ-gal. MSV was prepared by deleting an inserted cDNA from the MSV/EBP-δ expression vector. In both cases, 3 μg of pGLA-α-actin promoter in front of a luciferase cDNA,12 was cotransfected with 6 μg of pSVβ-galactosidase control vector (pSVβ-gal) (Promega) as normalization reference for transfection efficiency. For overexpression experiments, 3 μg of –1381/+68 WT or –1381/+68 MT were cotransfected with 3 μg of a mock vector (MSV) or an expression vector of C/EBP members (MSV/EBP-α, -β, or -δ), in addition to 6 μg of pSVβ-gal. MSV was prepared by deleting an inserted cDNA from the MSV/EBP-δ expression vector. In both cases, 3 μg of pGLA, which contains a rat α-actin promoter in front of a luciferase cDNA,12 was cotransfected with 6 μg of pSVβ-gal, and the luciferase activity given by pGLA was used as an internal reference between WKY- and SHR-derived VSMCs. After transfection, cells were incubated for an additional 48 hours in culture medium before enzyme assay. Cells were washed twice with PBS and were incubated for 5 minutes in 250 μL of lysis buffer containing 25 mmol/L Tris-HCl, pH 7.8, 2 mmol/L EDTA, 2 mmol/L DTT, 10% glycerol, and 1% Triton X-100. Cell lysate was scraped with a rubber policeman, transferred to a 1.5-mL microcentrifuge tube, and spun at 12 000 rpm for 10 minutes. Supernatant was transferred to a new tube and directly used for luciferase and β-galactosidase assays. A plastic tube containing 20 μL of supernatant was placed in a luminometer (Optocomp I luminometer, MGM Instruments Inc), 100 μL of 470 μmol/L luciferin was added.
automatically, and integrated peak luminescence was measured over a 45-second window after a 5-second delay. The activity of β-galactosidase was determined by absorbance at 405 nm in a spectrophotometer after a 150-minute incubation of 100 µL of cell lysate with 100 µL of 2× assay buffer (200 mmol/L Na2PO4, 90 mmol/L β-mercaptoethanol, and 8 mg/mL 0.1-nitrophenol-β-D-galactopyranoside). After normalization for transfection efficiency in reference to spectrophotometrically determined β-galactosidase activity, each promoter activity in WKY- or SHR-derived VSMCs was corrected by the value of pGLA observed in the corresponding VSMCs.

**cDNA Probes and Northern Blot Analysis**

A 600-bp fragment of rat PDGF-αR cDNA sequence, a 785-bp fragment of PDGF-βR cDNA sequence, and a 1.3-kb fragment of the rat GAPDH cDNA sequence were used as probes for Northern blot analysis as described previously.7 A 1.1-kb NcoI fragment of C/EBP-α cDNA, a 0.4-kb NcoI fragment of C/EBP-β cDNA, and a 1.0-kb EcoRI–BamHI fragment of C/EBP-δ cDNA were excised from the corresponding C/EBP expression vectors, MSV/EBP-α, -β, and -δ, respectively, and were also used as probes for Northern blot analysis. Each cDNA probe was labeled with [α-32P]dCTP using the random oligonucleotide method. Total cellular RNA used for Northern blot analysis was prepared from VSMCs using the method as described previously.13,14 Electrophoresed in a 1.0% agarose/2.2 mol/L formaldehyde gel, and transferred to a nylon membrane (Hybond-N+; Amersham) after staining with ethidium bromide to verify the relative quantity and quality of the RNA. The membrane was prehybridized and hybridized by standard techniques.15 After high-stringency washing for 1 hour at 60°C, blots were exposed to an x-ray film with an intensifying screen at ~80°C. Developed films were scanned by an image scanner (ES-800C scanner; Epson America, Inc) and analyzed by a computer program (NIH Image 1.49) to measure the relative intensity of each band.

**Electromobility Shift Assay and Supershift Assay**

Nuclear extracts were prepared from VSMCs derived from WKY or SHR according to the method described by Dignam et al.16 After protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories), nuclear extracts were divided into small aliquots, quickly frozen in liquid nitrogen, and stored at ~80°C. For electromobility shift assay and supershift assay, a wild-type C/EBP probe (C/EBP-WT), spanning –165 to –138 of the PDGF-αR promoter sequence, and a mutated C/EBP probe (C/EBP-MT), which was generated by annealing 2 complementary oligonucleotides (M1 and M2), were prepared. Each probe was labeled with [γ-32P]ATP using T4-polynucleotide kinase. Nuclear extracts (2 µg) were incubated with ~1.0 × 10^5 cpm of labeled probes for 30 minutes at room temperature in a 10-µL binding buffer containing 12 mmol/L HEPES-KOH, pH 7.9, 60 mmol/L KCl, 4 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol, and 50 µg/mL of poly(dI-dC)·poly(dI-dC) (Pharmacia LKB Biotechnology, Inc). For competition experiments, a 200-fold molar excess of unlabeled C/EBP-WT probe, Sp-1 probe (Promega), or C/EBP-MT probe was added to nuclear extracts and was incubated on ice for 15 minutes before addition of the labeled C/EBP-WT probe. For supershift assay, reaction mixtures of nuclear extracts and the labeled C/EBP-WT probe were preincubated with 1 µL of antibodies against each subtype of the C/EBP for 4 hours at 4°C before electrophoresis. All reaction mixtures were analyzed by 4% polyacrylamide gel electrophoresis under nondenaturing conditions at 4°C in a low–ionic strength buffer containing 6.7 mmol/L Tris-HCl, pH 7.5, 3.3 mmol/L sodium acetate, and 1 mmol/L EDTA. Finally, the gel was dried and exposed to an x-ray film with an intensifying screen at ~80°C.

**Immunoblotting**

Western blot analysis for members of the C/EBP family was performed by the method described previously.6 Briefly, nuclear extracts (2.5 µg) prepared from WKY- and SHR-derived VSMCs were directly subjected to immunoblotting for C/EBP-α, -β, and -δ. After boiling with sample buffer, SDS-polyacrylamide gel electrophoresis was done using a 12.5% gel according to the method of Laemmli,17 and proteins in the gel were transferred to a polyvinylidene difluoride membrane (Trans-Blot transfer medium; Bio-Rad Laboratories) by electroblotting for 1 hour at 100 V. The membrane was treated with diluted antibodies against C/EBP-α, -β, and -δ, and immunoreactive proteins were detected by autoradiography using chemiluminescence detection system (ECL Western Blotting Analysis System; Amersham).

**Statistical Analysis**

Statistical evaluation was performed by ANOVA (m × n factorial design), and multiple comparisons between 2 groups were evaluated by means of Duncan’s new multiple-range test. All data are expressed as mean±SE, and statistical significance is defined as P<0.05.

**Results**

**Differential Expression of PDGF-αR mRNA Between VSMCs of Normotensive and Genetically Hypertensive Rats**

The level of PDGF-αR mRNA was determined by Northern blot analysis using VSMCs derived from 4 different rat strains (Figure 1). PDGF-βR mRNA was expressed highly and almost equally in VSMCs derived from 4 rat strains. In contrast, PDGF-αR mRNA was expressed highly and consistently only in VSMCs derived from SHR (lane 4) but not in those from normotensive strains, Wistar (lane 1), Sprague-Dawley (lane 2), and WKY (lane 3). This differential expression of PDGF-αR mRNA was confirmed with 5 sets of independently prepared VSMC samples. The almost-all-or-none type of strain-specific expression of PDGF-αR mRNA was observed even in presumably differentiated cells after 30 passages (data not shown).
Effect of Mutations Disturbing Consensus Sequence of C/EBP on PDGF-αR Gene Transcription

Figure 2 shows the nucleotide sequence of the 5'-flanking region of the PDGF-αR gene spanning –255 through +45, which is numbered beginning with the transcription start site (Figure 2, adenine residue [#1]). Previously, we have reported that this region contains putative cis-acting elements, including 2 CCAAT motifs, CAAT box-binding transcription factor (CTF)/CCAAT box-binding protein (CBP) and C/EBP, which are localized in tandem upstream of the transcription start site. Using DNase I footprinting and electromobility shift assay, we have also demonstrated that the enhancer core sequence for C/EBP, which overlapped with a binding site for nuclear factor for IL-6 (NF-IL6), interacted with nuclear extracts from both WKY- and SHR-derived VSMCs, suggesting that this CCAAT motif may act as an important cis-acting element in the basal transcription activity of the PDGF-αR gene. To obtain unequivocal evidence for C/EBP binding and promoter-activating function of the CCAAT motif, 2 mutated promoter-luciferase constructs, –1381/+68 MT and –1381/+68 ΔMT, were prepared as shown in Figure 3. Promoter activity of each mutated construct was presented as relative luciferase activity in reference to the activity of wild type (–1381/+68 WT), observed in VSMCs derived from SHR, which was set as 100%. In SHR-derived VSMCs, all 3 constructs (–1381/ +68 WT, –1381/+68 ΔMT) showed very low and almost equal levels of promoter activity (10% to 12%) of –1381/+68 WT seen in SHR-derived VSMCs (lanes 8 and 11). In addition, a 200-fold molar excess of an unrelated Sp-1 probe had no effect on the DNA-protein complex formation (lanes 3 and 6). When a substitution-mutated probe (C/EBP-MT) was used as a labeled probe, the intensity of each band was markedly reduced in nuclear extracts from both WKY- and SHR-derived VSMCs (lanes 8 and 11). In contrast, the closely shifted band, B1, seen only in nuclear extracts from SHR-derived VSMCs, was supershifted only by antibodies against C/EBP-α, -β, and -δ (Figure 5). In WKY-derived VSMCs, band B2 was supershifted only by antibodies against C/EBP-β but not by other antibodies. In contrast, the closely shifted band, B1, seen only in nuclear extracts from SHR-derived VSMCs, was supershifted by antibodies against either C/EBP-β or C/EBP-δ. Antibodies against C/EBP-α did not supershift any bands using nuclear extracts from both WKY- and SHR-derived VSMCs.

Characterization of C/EBP Members That Interact With the PDGF-αR Gene Promoter

Competitive experiments were performed to determine whether wild-type C/EBP probe (C/EBP-WT) was specifically shifted by nuclear extracts from WKY- and SHR-derived VSMCs (Figure 4). Nuclear extracts from WKY-derived VSMCs generated a single major band B2 (lanes 1 and 7), whereas those from SHR-derived VSMCs generated an additional closely shifted band B1 (lanes 4 and 10). Although a 200-fold molar excess of an unlabeled C/EBP probe competed them out completely (lanes 2 and 5), a 200-fold molar excess of an unrelated Sp-1 probe had no effect on the DNA-protein complex formation (lanes 3 and 6). When a substitution-mutated probe (C/EBP-MT) was used as a labeled probe, the intensity of each band was markedly reduced in nuclear extracts from both WKY- and SHR-derived VSMCs (lanes 8 and 11). In addition, a 200-fold molar excess of the unlabeled C/EBP-MT probe did not affect the complex formation of the C/EBP-WT probe and proteins in nuclear extracts from both WKY- and SHR-derived VSMCs (lanes 9 and 12). C/EBP consists of several subtypes, each of which has specific regulatory functions in the transcription of genes regulating distinct physiological functions. To determine the specific subtype of C/EBP that is involved in the transcriptional activity of the PDGF-αR gene in VSMCs, a supershift assay was performed using specific antibodies against 3 major members of the C/EBP family, C/EBP-α, -β, and -δ (Figure 5).

Differential Expression of C/EBP Subtypes and Effect of IL-1β on PDGF-αR Gene Expression

The results obtained above in the supershift assay (Figure 5) suggested an abnormality in the expression of C/EBP-δ and possibly of C/EBP-β in SHR-derived VSMCs. Direct evidence for such a differential expression between WKY- and SHR-derived VSMCs was obtained by Northern blot analysis of C/EBP subtype mRNAs, as shown in Figure 6. The results clearly indicate a robust expression of C/EBP-δ mRNA only in SHR-derived VSMCs, but it was negligible in WKY-derived VSMCs, in parallel with a similarly marked difference in PDGF-αR mRNA expression. On the other hand, no clearly recognizable difference was seen in the expression of C/EBP-α and C/EBP-β mRNAs between WKY- and SHR-derived VSMCs.
WKY- and SHR-derived VSMCs. IL-1β is one of the well-known inducers mainly for C/EBP-d and possibly for C/EBP-b.18 To determine whether PDGF-αR expression is dependent on C/EBP-d expression, we used IL-1β as a tool for induction of C/EBP-d expression and investigated the effect of IL-1β on either C/EBP-δ or PDGF-αR expression in the absence or presence of CHX using WKY-derived VSMCs (Figure 7). A high level of PDGF-αR mRNA expression was accompanied by a similarly marked induction of C/EBP-δ mRNA in WKY-derived VSMCs following treatment with IL-1β (10 ng/ml) in the absence of CHX. Time-course studies were conducted following IL-1β stimulation (data not shown). A rapid induction of C/EBP-δ mRNA within 30 minutes, peaking at 3 hours, was followed by slower emergence of PDGF-αR mRNA (3 to 6 hours), which reached a maximum level at 12 to 24 hours and slowly disappeared beyond 48 hours. This time course indicates a causal relationship in which C/EBP-δ induced transcription of the PDGF-αR gene. To see whether C/EBP-δ gene expression is activated by IL-1β without any other de novo protein synthesis, the ability of

Figure 3. Effect of nucleotide substitution mutation and internal deletion disrupting consensus sequence for the C/EBP on PDGF-αR gene transcription. The nucleotide sequence was also numbered beginning with the transcription start site (marked #1 in Figure 2). The sequence spanning –1381 through +68 was fused into the luciferase expression vector pGL2-Enhancer, which was designated –1381/+68 WT, and 2 different mutated constructs for –1381/+68 WT were prepared. One is a nucleotide substitution mutant (–1381/+68 MT), and the other is an internal deletion mutant (–1381/+68 ΔMT). Each construct was cotransfected with pSVβ-gal to WKY-derived (■) and SHR-derived (▲) VSMCs using the DEAE-dextran method. After normalization for transfection efficiency in reference to spectrophotometrically determined β-galactosidase activities, each promoter activity in WKY- or SHR-derived VSMCs was corrected by the value of pGLA observed in the corresponding VSMCs. Finally, promoter activity thus corrected was presented as relative luciferase activity in reference to the activity of wild-type (WT) construct observed in VSMCs derived from SHR that was set as 100%. All data are expressed as mean±SE of 6 separate assays. *P<0.05, significant difference between groups; †P<0.05, significant difference between strains.

Figure 4. Electromobility shift assay for the C/EBP recognition site of PDGF-αR gene promoter. Nuclear extracts (2 μg) obtained from WKY-derived (lanes 1 to 3 and 7 to 9) or SHR-derived (lanes 4 to 6 and 10 to 12) VSMCs were incubated with labeled C/EBP-WT probe (all lanes except 8 and 11) or labeled C/EBP-ΔWT probe (lanes 8 and 11) for 30 minutes at room temperature. For competition experiments, a 200-fold molar excess of unlabeled C/EBP-WT probe (lanes 2 and 5), unlabeled Sp1 probe (lanes 3 and 6), or unlabeled C/EBP-ΔWT probe (lanes 9 and 12) was incubated with nuclear extracts from WKY- or SHR-derived VSMCs on ice for 15 minutes before adding labeled C/EBP-WT probe. Reaction mixtures were resolved on 4% polyacrylamide gel under nondenaturing conditions at 4°C in a low–ionic strength buffer before autoradiography. The positions of specific DNA-protein complexes are indicated as B1 and B2, and that of the free DNA probe as Free.

Figure 5. Supershift assay for the C/EBP recognition site using specific antibodies against C/EBP family. The labeled C/EBP-WT probe was added to nuclear extracts (2 μg) from WKY- or SHR-derived VSMCs and was incubated with 1 μL of specific antibodies against C/EBP-α (α), C/EBP-β (β), C/EBP-δ (δ), or preimmune serum (PI) for 4 hours at 4°C before electrophoresis. The DNA binding and gel mobility shift assay were performed as described in Figure 4 and in Materials and Methods. The positions of specific DNA-protein complexes are indicated as B1 and B2, and that of the free DNA probe as Free.
IL-1β to induce C/EBP-δ gene expression was determined in the presence of CHX (10 μg/mL). CHX alone induced C/EBP-δ mRNA expression, but PDGF-αR mRNA expression was not induced. In addition, CHX with IL-1β allowed C/EBP-δ mRNA induction, but again PDGF-αR mRNA expression was not induced. These results suggest that C/EBP-δ directly activates the endogenous PDGF-αR gene expression. To determine protein levels of 3 C/EBP subtypes expressed in the nuclear extracts obtained from WKY- and SHR-derived VSMCs, immunoblotting analysis using specific antibodies was also carried out (Figure 8). In quiescent VSMCs derived from WKY, protein levels of C/EBP-α, -β, and -δ were very low or almost negligible (lane 1). In contrast, C/EBP-α proteins (42 and 30 kDa) were slightly induced, and both C/EBP-β and C/EBP-δ proteins (C/EBP-β, 36 and 20 kDa; C/EBP-δ, 33 kDa) were markedly induced in the nuclear extracts from WKY-derived VSMCs after treatment with IL-1β for 12 hours (lane 2), as well as in those from quiescent VSMCs derived from SHR-derived VSMCs (lane 3).

Figure 6. Comparisons of mRNA levels for PDGF-αR, C/EBP-α, and C/EBP-δ in quiescent VSMCs derived from WKY or SHR. Total RNA (20 μg) obtained from quiescent VSMCs derived from WKY or SHR was analyzed by Northern blotting for PDGF-αR, C/EBP-α, C/EBP-β, and C/EBP-δ mRNAs. Expression levels of GAPDH mRNA were used as internal control to standardize the amount of total RNA actually blotted onto a membrane.

Figure 7. Effects of IL-1β on the mRNA expression of PDGF-αR and C/EBP-δ in VSMCs derived from WKY in the absence or presence of CHX. Total RNA (20 μg) was prepared from quiescent or IL-1β-treated VSMCs derived from WKY in the absence or presence of CHX and analyzed by Northern blotting for PDGF-αR and C/EBP-δ mRNAs. IL-1β-treated cells were stimulated by treatment with IL-1β (10 ng/mL) for 3 hours. IL-1β (–) and (+) indicate quiescent and IL-1β-treated VSMCs, respectively. CHX (–) and (+) indicate VSMCs cultured in the absence and in the presence of CHX (10 μg/mL), respectively.

Ability of the C/EBP Family to Transactivate PDGF-αR Gene Promoter

To further test the role of the C/EBP family in regulating the transcription of the rat PDGF-αR gene, we evaluated the ability of the C/EBP family to transactivate the promoter of PDGF-αR (–1381/+68) in a luciferase fusion construct. The wild-type PDGF-αR promoter/luciferase construct, –1381/+68 WT, was cotransfected with an expression vector for C/EBP-α, -β or -δ, or a mock vector, MSV. Each promoter activity is presented as relative luciferase activity in reference to the activity cotransfected with a mock vector observed in VSMCs derived from SHR, which was set as 100% (Figure 9). Overexpression of C/EBP-δ significantly transactivated the promoter activity of –1381/+68 WT in both WKY- and SHR-derived VSMCs, the extent of stimulation being on the order of 4.8- and 1.5-fold, respectively. On the other hand, overexpression of C/EBP-β significantly suppressed (by 56%) the promoter activity of –1381/+68 WT in SHR-derived VSMCs but had no significant effect on the basal efficiency in WKY-derived VSMCs. No synergic effects on the promoter activity were observed by overexpression of C/EBP-δ and C/EBP-β together (3 μg each) in either WKY- or SHR-derived VSMCs compared with overexpression of C/EBP-δ alone (data not shown). Overexpression of C/EBP-α did not affect the basal activity of –1381/+68 WT in either WKY- or SHR-derived VSMCs. In addition, a mutated PDGF-αR promoter/luciferase construct, –1381/+68 MT, was also cotransfected with each C/EBP expression vector to show the direct evidence that the C/EBP-δ transactivates the PDGF-αR promoter activity via the identified C/EBP binding site. Using –1381/+68 MT, the ability of the C/EBP-δ to transactivate the PDGF-αR promoter activity was completely abolished in WKY-derived VSMCs and was significantly decreased (by
PDGF-α with earlier observations that PDGF-AA activates C/EBP-β in nuclear extracts from quiescent or IL-1β-stimulated SHR-derived VSMCs. Nuclear extracts obtained and quiescent SHR-derived VSMCs. Nuclear extracts (2.5 μg) were obtained from quiescent (lane 1) or IL-1β-treated (lane 2) VSMCs derived from WKY and quiescent VSMCs derived from SHR (lane 3) and were directly subjected to immunoblotting for C/EBP-α, -β, and -δ. IL-1β-treated cells were stimulated by treatment with IL-1β (10 ng/mL) for 8 hours. Specific antibodies against each C/EBP member recognized 42-kDa and 30-kDa proteins for C/EBP-α, 36-kDa (LAP) and 20-kDa (LIP) proteins for C/EBP-β, or 33-kDa single protein for C/EBP-δ.

29%) in SHR-derived VSMCs compared with using -1381/+68 WT in the corresponding VSMCs.

Discussion

In pursuit of a pivotal molecular mechanism that may underlie spontaneous vascular medial hypertrophy and remodeling in the development of genetic (spontaneous) hypertension, we report here that a marked difference exists between VSMCs derived from SHR and WKY in response to the ability to transcribe PDGF-αR, which is driven by the transcription factor C/EBP-δ. By DNase I footprint, we previously showed that the 5'-flanking region of PDGF-αR has a domain containing 2 CCAAT motifs, CTF/CBP and C/EBP, in tandem. By using mutated expression constructs in electromobility shift and supershift assays, we have now demonstrated that C/EBP-δ is functional in driving the expression of PDGF-αR. Importantly, cultured VSMCs from the hypertensive strain showed a robust expression of C/EBP-δ, whereas the vascular cells from 3 normotensive rat strains did not express a recognizable amount of C/EBP-δ. Taken together with earlier observations that PDGF-AA activates PDGF-αR resulting in hypertrophy of VSMCs, we submit that a high level of the transcriptional factor C/EBP-δ expression is of pivotal importance in the transactivation of the PDGF-αR gene and vascular remodeling, which may be mediated by autocrine PDGF-AA. In support of this hypothesis, direct stimulation of C/EBP-δ gene expression by IL-1β in nonexpressing VSMCs from the normotensive rat strain WKY resulted in a prompt expression of C/EBP-δ and ensuing PDGF-αR gene transcription.

Since C/EBP has been originally identified as a family of "liver-enriched" transcription factors that belongs to the so-called basic region-leucine zipper-class DNA binding proteins, 3 major members of the C/EBP family, α, β, and δ, have been identified and their roles have been studied mainly in the cellular differentiation of hepatocytes or adipocytes in vitro. C/EBP-α and -β are expressed at high levels in terminally differentiated hepatocytes, indicating that these 2 subtypes play a pivotal role in the establishment and maintenance of hepatocellular differentiation. Recently, mice carrying null mutations in C/EBP members have been generated by homologous recombination so that their in vivo functions may be understood. Whereas C/EBP-α-null mice revealed major defects in the glycogen metabolism in the liver and died within 8 hours after birth because of hypoglycemia, C/EBP-β-null mice have been reported to show 2 major defects: a defect in the immune system, especially in the function of macrophages, and defective ovulatory functions.

In contrast, physiological or pathological functions of C/EBP-δ have remained unknown. Cultured hepatocytes or adipocytes express C/EBP-δ at an undetectable or minor level in normal tissues, but its expression is induced rapidly and markedly by lipopolysaccharide or inflammatory cytokines such as IL-1, IL-6, and tumor necrosis.
factor. Therefore, C/EBP-δ has been considered to be involved in the transcriptional regulation of acute-phase reactive proteins such as the third component of complement (C3) gene, α1-acid glycoprotein gene, and thiostatin gene. Since there is a similarity shared by C/EBP-δ and C/EBP-β (also known as NF-IL6) in their responses to inflammatory cytokines, C/EBP-δ is also called NF-IL6β. Very recently, Tanaka et al. reported that C/EBP-δ-null mice reveal no significant defects in organ development. These findings led us to postulate that C/EBP-δ transcription is usually suppressed in normal tissues, whereas its marked induction is involved in pathogenic conditions of a given tissue in vivo. Given that SHR-derived VSMCs exhibit abnormal growth behavior and hypertrophic and hyperplastic responses to serum and that growth factors of these cells provide an attractive material for dissecting the unique regulatory mechanism of C/EBP-δ, such studies should also be relevant in vivo conditions in which the remodeling of arterial wall by angiotensin II activates autocrine production of PDGF-AA.

Nucleotide substitution mutation or global disruption of the C/EBP recognition site in the regulatory sequence of the PDGF-αR gene promoter led to a marked decrease in transcriptional activity of the reporter gene in SHR-derived VSMCs (Figure 3). Evidently, the important role of the regulatory sequence for transcription of the PDGF-αR gene in SHR-derived VSMCs largely depends on the C/EBP cognate nucleotide sequence. Electromobility shift analysis using the C/EBP probe has shown that nuclear extracts from WKY-derived VSMCs generate a single band (B2, Figure 4), whereas those from SHR-derived VSMCs, which express a high level of PDGF-αR mRNA even in a quiescent state, generate an additional closely shifted band (B1). To further clarify the nature of these DNA-protein complexes, we carried out a supershift assay using specific antibodies against 3 major members of the C/EBP family and obtained results indicating that C/EBP-β is mainly involved in generating B2, and both C/EBP-β and -δ are involved in generating B1 (Figure 5). This indicates that 2 members of the C/EBP family, C/EBP-β and -δ, potentially control the basal transcriptional activity of the PDGF-αR gene in VSMCs. Kolyada et al. have recently reported that the C/EBP family is involved in the transcriptional regulation of the Na+/H+ exchanger gene (NHE1) in hepatocytes. Of particular interest is evidence derived from models of genetic hypertension that linked a hyperactive Na+/H+ exchanger in VSMCs, presumably the growth factor-activatable NHE1, to pathogenesis of essential hypertension. They have also demonstrated that the human NHE1 proximal promoter has an exactly matched consensus sequence for C/EBP found at −230 to −222 upstream of the transcription start site, and its basal transcriptional activity is mainly regulated by this cis-acting element. In addition, cotransfection experiments of NHE1 promoter–chloramphenicol acetyltransferase constructs and C/EBP expression vectors showed that C/EBP-α or C/EBP-δ acts as a transcriptional activator of the NHE1 gene in rat hepatocytes. Hohaus et al. have reported that the c-fms gene, which belongs to the class III receptor tyrosine kinase family together with PDGF-αR and PDGF-βR, also has a C/EBP recognition site in the promoter region, and either PU.1 (Spi-1) or C/EBP-α mainly regulates the cell type–specific gene expression in hematopoietic cells.

On the other hand, our results obtained from Northern blot and supershift assay revealed that although both VSMCs derived from WKY and SHR expressed a high level of C/EBP-α mRNA (Figure 6), antibodies against C/EBP-α did not supershift either band B1 or B2 (Figure 5). Immunoblotting analysis showed that the quiescent VSMCs derived from WKY did not express C/EBP-α proteins (both 44- and 30-kDa proteins), and either IL-1β-treated WKY-derived cells or quiescent SHR-derived cells expressed a minor level of C/EBP-α proteins (Figure 8). These results suggest that C/EBP-α mRNA is not translated in a quiescent state of VSMCs from normotensive rats, and even if it is translated in the IL-1β-treated WKY-derived cells or quiescent SHR-derived cells, C/EBP-α proteins have a defect in the binding activity to the C/EBP cognate nucleotide sequence identified in the promoter region of the PDGF-αR gene. In contrast, both C/EBP-β and -δ proteins were detectable in the nuclear extracts from the quiescent cells derived from WKY. Furthermore, these subtype proteins were markedly increased in VSMCs from WKY after treatment with IL-1β to a level almost equal that of those derived from SHR (Figure 8, lanes 2 and 3), strongly suggesting that C/EBP-β and/or C/EBP-δ are mainly involved in the basal transcriptional activity of the PDGF-αR gene in VSMCs.

To distinguish possible roles of C/EBP-β and -δ, the effects of overexpression of C/EBP-α, -β, and -δ on PDGF-αR gene expression were compared. As shown in Figure 9, only C/EBP-δ acted as the major transcriptional activator and C/EBP-β as the major suppressor of the PDGF-αR gene expression in rat cultured VSMCs, whereas C/EBP-α did not affect transcriptional efficiency of the PDGF-αR gene. Previous reports have shown that C/EBP-β mRNA is translated into 2 molecular forms, as follows: the full-size protein (36 kDa) LAP, a liver-enriched transcriptional activator, and the truncated protein (20 kDa) LIP, a liver-enriched transcriptional repressor that inhibits transcriptional stimulation by LAP. Taken together with this report, we expect that the quiescent VSMCs derived from WKY express mainly LIP protein, which negatively regulates transcriptional stimulation by other members, especially LAP or C/EBP-δ. However, immunoblotting analysis has revealed that only minor levels of LAP protein (not LIP protein) and C/EBP-δ protein were expressed only at minor levels in the nuclear extracts from the quiescent VSMCs derived from WKY. This unexpected result may be due to differences in the cell-type specificity of the transcriptional regulation mechanism by the C/EBP family between VSMCs and hepatocytes. As anticipated, Northern blot analysis (Figure 6) revealed that C/EBP-δ was highly expressed only in VSMCs from SHR, in good accord with results obtained from the supershift assay and immunoblotting analysis. Furthermore, we have demonstrated that IL-1β treatment
of VSMCs derived from WKY can induce PDGF-αR mRNA expression, presumably through the induction of C/EBP-δ protein synthesis without any other de novo protein synthesis (Figure 7, IL-1β/CHX (+/+) lane). This mechanism is supported by a very rapid C/EBP-δ induction by IL-1β occurring within 30 minutes and peaking at 3 hours, which was followed by a slower (3- to 6-hour) emergence (peaking at 12 hours) of PDGF-αR mRNA, indicating that C/EBP-δ expression is directly related to the transactivation of the PDGF-αR gene in VSMCs. Further support of this contention is found in overexpression experiments that showed that only C/EBP-δ can transactivate the expression of the PDGF-αR reporter gene, –1381/+68 WT, in WKY-derived VSMCs as well as SHR-derived VSMCs, suggesting that C/EBP-δ is a key player in differential gene expression or strain-specific gene transactivation of PDGF-αR in VSMCs.

In conclusion, the present studies are aimed at delineating the mechanisms involved in the differential gene expression of PDGF-αR in WKY- and SHR-derived VSMCs. We have produced several findings of importance, which include the following: (1) the identification of an upstream regulatory region spanning –165 through –139 that plays pivotal roles in the control of strain-specific PDGF-αR expression; (2) the identification of an enhancer core sequence for C/EBP that is essential for PDGF-αR promoter function; (3) the determination of members of the C/EBP family that are expressed in a strain-specific manner; and (4) the recognition that C/EBP-δ positively regulates PDGF-αR gene expression in the VSMCs derived from SHR, and possibly negative regulation by C/EBP-β, especially in the quiescent VSMCs derived from WKY. The results obtained herein show evidence for new roles of the C/EBP family on the cellular functions of VSMCs and also provide important information to understanding underlying molecular mechanisms of vascular remodeling and resultant hypertension.

Acknowledgments
This work was supported in part by United States Public Health Service research grants HL-14192, HL-35323, HL-58205, and DK-20593 from the National Institutes of Health and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (Nos. 08457210, 08670797, and 09670723). We especially thank Dr S.L. McKnight (Department of Biochemistry, The University of Texas South Medical Center, Dallas, Texas) for the generous gift of the C/EBP expression vectors MSV/EBP-α, MSV/EBP-β, and MSV/EBP-δ.

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A High Level of CCAAT-Enhancer Binding Protein-δ Expression Is a Major Determinant for Markedly Elevated Differential Gene Expression of the Platelet-Derived Growth Factor-α Receptor in Vascular Smooth Muscle Cells of Genetically Hypertensive Rats

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Circ Res. 1999;84:64-73
doi: 10.1161/01.RES.84.1.64

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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