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 electrophoresis 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 peptidic epitopes corresponding with amino acid sequences of rat C/EBP-α (253–265), 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. 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, 10% heat-inactivated FCS at 37°C in a humidified atmosphere of 6000 Ci/mmol) were obtained from DuPont/NEN.

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. VSMCs were seeded in 60-mm dishes (5 x 104 cells/dish) 24 hours before transfection. Transfection was performed with cells at ~70% confluence by the DEAE-dextran method as described previously. For transient transfection experiments, 3 μg of −1381/+68 WT, −1381/+68 MT, or −1368/+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 ΔMT was 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 pGL2, which contains a rat α-actin promoter in front of a luciferase cDNA, was cotransfected with 6 μg of pSVβ-gal, and the luciferase activity given by pGL2, and the luciferase activity given by 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′-TGGCCCCAACAGTACATACAGCAA-3′, and the 3′-end primer (M2), 5′-TGCTCTATATGACTGTC-3′, were ligated into the promoterless luciferase vector pGL2-Enhancer (Promega, Madison, WI). After cutting a blunt end on both fragments with BalI digestion, −1381/+68 ΔMT was finally prepared by the ligation of 2 fragments.

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 were 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′-CCCGAGCTGAAAGATACGCCG-3′, and the 3′-end primer (P2), 5′-CTCCCTCAAGCTGTTGATGAT-3′, was ligated into the promoterless luciferase vector pGL2-Enhancer (Promega, Madison, WI). This produced plasmid was designated −1381/+68 WT. A constructed 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 mutantic primers: the 5′-end primer (M1), 5′-TGGCCCCAACAGTACATACAGCAA-3′, and the 3′-end primer (M2), 5′-TGCTCTATATGACTGTC-3′, were ligated into the promoterless luciferase vector pGL2-Enhancer (Promega, Madison, WI). After cutting a blunt end on both fragments with BalI digestion, −1381/+68 ΔMT was finally prepared by the ligation of 2 fragments.
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 Na3PO4, 90 mmol/L β-mercaptoethanol, and 8 mg/mL 0-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. 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.15,16 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 high-stringency washing for 1 hour at 60°C, blots were exposed to an x-ray film with an intensifying screen at

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 1–10×106 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.) 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 or PDGF-βR 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.7 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, promoter activity of nucleotide substitution mutant (–1381/+68 ΔMT) was significantly reduced to 23% and 18% of wild type in SHR-derived VSMCs, respectively (Figure 3, □). In WKY-derived VSMCs, all 3 constructs (–1381/+68 WT, MT, and ΔMT) showed very low and almost equal levels of promoter activity (10% to 12%) of –1381/+68 WT seen in SHR-derived VSMCs (Figure 3, □).

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). 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.

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. 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
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).

**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 was 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-α 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
Expression 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, 17–26 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. 27 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. 28, 29 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, 30 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, 31 and defective ovulatory functions. 32, 33

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 α.
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 given tissue in vivo. Given that SHR-derived VSMCs largely depend on 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-activator NHE1, to pathogenesis of essential hypertension.

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-β protein were expressed only at minor levels in the nuclear extract from the quiescent cells 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-βR 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.

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