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SREBP inhibits VEGF expression in human smooth muscle cells

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Abstract

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that regulate expression of genes encoding enzymes for lipid biosynthesis. SREBPs are activated by HMG-CoA reductase inhibitors (statins). Statins have been also reported to suppress vascular endothelial growth factor (VEGF) expression in vascular smooth muscle cells (VSMCs). Therefore, we hypothesized that SREBPs are involved in statin-mediated regulation of VEGF production in VSMCs. SREBP1 was robustly expressed, and was activated by atorvastatin in VSMCs, as demonstrated by increased levels of the mature nuclear form of SREBP1, and increased promoter activities of a reporter containing sterol regulatory elements by atorvastatin. Moreover, overexpression of SREBP1a dose-dependently suppressed VEGF promoter activity. Site-specific mutation or deletion of the proximal Sp1 sites reduced the inhibitory effects of SREBP1a on VEGF promoter activity. These data demonstrated that SREBP1, activated by atorvastatin, suppressed VEGF expression through the indirect interaction with the proximal tandem Sp1 sites in VSMCs.

Keywords: SREBP; VEGF; Atherosclerosis; Statin; Sp1; Transcription; Vascular cell

Sterol regulatory element-binding proteins (SREBPs) have been established as lipid synthesis transcription factors. SREBPs belong to the basic helix-loop-helix-leucine zipper family and activate the entire program of fatty acid and cholesterol synthesis in liver. There are three members in the SREBP family: SREBP1a and SREBP1c are encoded by the same gene, whereas SREBP2 is from a separate gene [1]. Precursors of SREBPs are localized in the endoplasmic reticulum. When the intracellular free cholesterol level is reduced by sterol starvation or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), a proteolytic cascade cleaves the precursor, releasing the mature form into the cytosol [1]. Mature SREBPs translocate to the nucleus and activate the transcription of the target genes by binding to the sterol regulatory elements (SRE), a nonpalindromic 10-bp motif. SREBPs target genes include the genes involved in cholesterol and fatty acid metabolism in liver and adipose tissue (e.g., genes for HMG-CoA reductase, HMG-CoA synthase, and low-density lipoprotein (LDL) receptor) [1].

SREBPs have been well studied in liver, but not in the cardiovascular system. Recently, several reports revealed that the SREBPs are expressed in vascular cells. Llorente et al. suggested SREBP2 expression in vascular smooth muscle cells (VSMCs) acted as the mediator for VSMC–LDL receptor-related protein (LRP) upregulation [2]. Rodriguez et al. reported the expression of (SREBP)-1 and -2 in porcine aortic endothelial cells (ECs) and in porcine and human VSMCs [3,4]. Thus, SREBPs may also play important roles in vascular cells, but the roles in vascular cells have not been fully clarified yet.

On the other hand, it is well known that statins are useful therapeutic agents for patients with hypercholesterolemia, an important risk factor in atherosclerosis [5]. In addition to the effect of lowering serum cholesterol level, accumulating evidence indicates that statins have pleiotropic effects against progression of atherosclerosis [6,7]. It has

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been reported that statins reduce vascular endothelial growth factor (VEGF) synthesis in ECs and VSMCs in vitro [8–11]. Alber et al. [12] demonstrated that atorvastatin therapy for two months reduced VEGF plasma levels in hypercholesterolemic patients. Since VEGF is reported to be involved in the growth of atherosclerotic plaque, VEGF might be one target of the pleiotropic effects of statins [13–16]. The mechanism by which statins reduce VEGF production remains elusive. Because SREBP is principal mediator for the signaling induced by statins in liver and adipose tissue, we hypothesized that SREBP might also play an important role in VEGF regulation by statins in vascular cells.

This study demonstrated that, in human VSMCs, SREBP1 activation by atorvastatin might be involved in the suppression of VEGF synthesis at transcriptional levels. Furthermore, it demonstrated that SREBPs suppressed VEGF promoter activities through the indirect interaction with the four proximal tandem Sp1 sites.

Materials and methods

Cell culture and materials. Human umbilical artery smooth muscle cells (HUSMCs) and rat clonal smooth muscle cells, A7r5, were cultured to subconfluence in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) (Invitrogen, San Diego, CA) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 mM L-glutamate. Cells were passaged every 2–3 days, and cells within 9 passages were used for the experiments. Atorvastatin was provided to us by Pfizer (New York, NY). Deferoxamine was purchased from Sigma. Mouse IgG monoclonal antibody (2A4) against amino acids 301–407 of human SREBP1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid construct. The expression vectors for human mature SREBP1a, 1c or 2 driven by the CMV promoter were prepared as described [17]. Plasmid pCMV β-gal, encoding a CMV promoter-driven β-galactosidase reference gene, was purchased from Stratagene (La Jolla, CA). Plasmid pSRE-Luc, a luciferase reporter plasmid containing, 5′ to 3′, three tandem copies of repeats 2 and 3 of the LDL receptor promoter has been described [17]. The expression vector phEP-1, which contains the entire coding sequence of human endothelial PAS domain protein 1 (EPAS1), was a gift from Steven L. McKnight (Dallas, TX) [18]. The reporter plasmid pGL2-hVEGF has been previously described and was constructed by inserting the human VEGF promoter from base pairs (bp) −2362 to +61 into pGL2-basic vector (Promega, Madison, WI) containing the firefly luciferase reporter gene [19]. The full-length reporter plasmid of VEGF promoter used here was designated pGL2-VEGF (−2362 +61). Its deletion and site-directed mutation reporters were generated as follows. All constructs were verified by sequencing the inserts and flanking regions of the plasmids. To generate the deletion construct of pGL2-VEGF (−1280 +61), pGL2-VEGF (−2362 +61) was digested with *Sac*I, and the fragment between the *Sac*I site in the 5′-multiple cloning site of pGL2-basic and the internal *Sac*I site of VEGF promoter was deleted. To generate other deletion constructs, we amplified sections of the VEGF promoter by polymerase chain reaction (PCR) with pGL2-VEGF (−2362 +61) as the template, using primers with the *Xho*I site sequence (underlined). The PCR products were digested with *Xho*I and subcloned into the *Xho*I sites of the pGL2-basic vector. The primers for pGL2-VEGF (−1144 +61) were 5′-CCGCTCGAGGTGCTGGCGGGTAGGTTTGA-3′ (sense) and 5′-CCGCTCGAGCTGGTGCTAGCCCCCAGCG-3′ (antisense). The primers for pGL2-VEGF (−713 +61) were 5′-CCGCTCGAGACATGAAGCAACTCCAGTCCC-3′ (sense) and 5′-CCGCTCGAGCTGGTGCTAGCCCCCAGCG-3′ (antisense). The primers for pGL2-VEGF (−2362 −179) were 5′-CCGCTCGAGCTTAAGACACG GGAGTGAGG-3′ (sense) and 5′-CCGCTCGAGCAATGAAGGGGA AGCTCGAC-3′ (antisense). To generate the deletion construct of pGL2-VEGF (−89 +61), pGL2-VEGF (−713 +61) was digested with *Sma*I, and the fragment between the *Sma*I site in the 5′-multiple cloning site of pGL2-basic and the internal *Sma*I site of the VEGF promoter was deleted. To generate site-directed mutations of the Sp1 sites at −74 to −67 (SP1–2) and/or −84 to −78 (SP1–3), the QuickChange mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. The primers (mutations are shown in bold italics and underlined throughout) for the mutation of the Sp1–3 element, designated as mut SP1–3, were 5′-CCGG GG***TT***GGCCGGGGGCGGGGTCCCG-3′ (sense) and 5′-CGGGACC CCGCCCCCGGCC***AA***CCCCGG-3′ (antisense), and the primers for the mutation of both the Sp1–2 and -3 elements, designated as mut SP1-2,3, were 5′-CCGGGG***TT***GGCCGGGGGCG***TT***GTCCCG-3′ (sense) and 5′-CGGGAC***AA***CGCCCCCGGCC***AA***CCCCGG-3′ (antisense).

Western blot analysis. Preparation of whole cell extracts and Western blotting were performed as described [20]. In brief, cell lysates were prepared in a buffer containing 20 mM Tris, pH 8.0, 140 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, dithiothreitol, and protease inhibitors (Complete™, Roche). Cell extracts were clarified by centrifugation. Then, 100 μg of each extract was subjected to SDS–polyacrylamide gel electrophoresis followed by immunoblotting with anti-human SREBP-1 antibody.

Transient transfection, VEFG assay, and luciferase activity assay. HUSMCs in 6-well plates were transfected with SREBP-1a expression vectors using the Human AoSMC Nucleofector Kit (AMAXA biosystems) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were incubated under serum deprived (FCS 0.2%) conditions for 40 h, and then VEGF protein levels in the medium were determined by ELISA using a human VEGF immunoassay kit (R&D Systems, MN) according to the manufacturer's instructions. For the reporter assay, A7r5 cells were transfected with firefly luciferase reporter plasmids of the SRE promoter, or VEGF promoters together with SREBP-1a and/or EPAS1 expression vectors using LipofectAmine (Invitrogen) according to the manufacturer's protocol. As an internal control vector, phRL-TK (Promega), containing the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide Renilla luciferase expression, was also co-transfected. Twenty-four hours after transfection, cells were washed and lysed. The activity of both firefly and Renilla luciferase was determined using the Dual Luciferase Assay kit (Promega). The firefly luciferase activity in various experiments was normalized to the Renilla luciferase activity to equalize transfection efficiency.

Statistical analysis. Comparisons between groups were made by a factorial analysis of variance followed by unpaired Student's t test when appropriate. Significance was accepted at $p < 0.05$.

Results

Atorvastatin activates SREBP1 in HUSMCs

At first, we confirmed the expression of SREBP1 in HUSMC by Western blotting (Fig. 1A, left lane). The expression of SREBP2 was also confirmed by RT-PCR using the primers described previously (data not shown). HUSMC were incubated with vehicle or 1 μM of atorvastatin for 16 h. While only the membrane bound precursor SREBP1 (115 Da) was found in vehicle treated cells, the mature form of SREBP1 (75 Da), which is cleaved from precursor SREBP1, appeared in atorvastatin-treated cells (Fig. 1A). The appearance of mature SREBP1 suggested SREBP1 activation by atorvastatin. To confirm SREBP activation, A7r5 cells transfected with SRE reporter gene were incubated with atorvastatin for 24 h. Atorvastatin

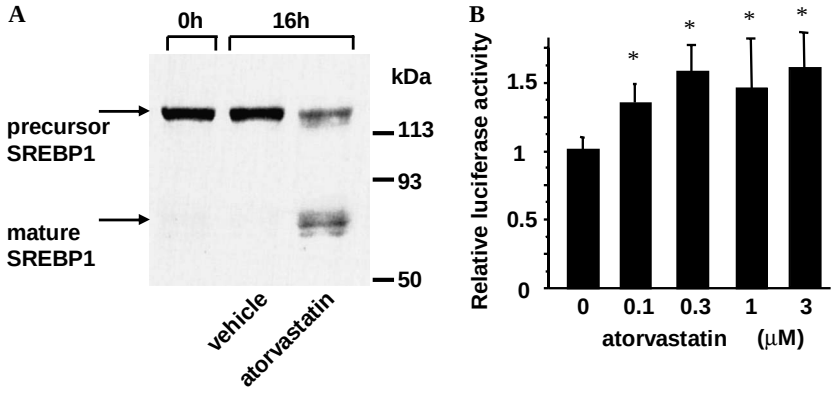

Fig. 1. SREBP1 expression and stimulation by atorvastatin in VSMCs. (A) Release of mature SREBP1 by atorvastatin. HUSMCs were incubated with vehicle (0.1% ethanol) or 1 μM of atorvastatin for 16 h. After the incubation, 100 μg of whole cell extracts was subjected to SDS–polyacrylamide gel electrophoresis followed by immunoblotting with anti-human SREBP-1 antibody. The membrane bound precursor SREBP1 has migrated at 115 kDa and the mature SREBP1 has migrated at 75 kDa. (B) Activation of SRE reporter gene in VSMCs treated with atorvastatin. A7r5 cells were transfected with 0.2 μg of SRE reporter gene and then incubated with 0.1, 0.3, 1, or 3 μM atorvastatin or without atorvastatin (vehicle) for 24 h. Transcription activity mediated through the SRE was assessed by determining the activity of luciferase, and the values were expressed as arbitrary units. The values are means ± SD from three independent experiments. $*P < 0.05$ vs without atorvastatin (vehicle). The experiments were performed in triplicate.

induced SRE driving promoter activities dose-dependently, thereby confirming SREBP activation (Fig. 1B). These experiments revealed that at least SREBP1 was activated by atorvastatin in VSMCs.

SREBP1 suppresses VEGF expression

To investigate the possible involvement of SREBPs in VEGF synthesis, we focused on SREBP1a, because SREBP1a is highly expressed in actively growing cells such as cultured cells [1]. Overexpression of mature SREBP1a suppressed VEGF production dose-dependently in HUSMC (Fig. 2A). Deferoxamine (DFO) is known to activate VEGF production through the hypoxia responsive element (HRE) of the VEGF promoter. 100 μM DFO induced a 6- to 7-fold increase in VEGF concentration in the culture medium in this study (Fig. 2B). SREBP1a overexpression also suppressed DFO-induced VEGF production dose-dependently (Fig. 2B). SREBP1a suppressed both basal and DFO-induced VEGF production. Basal VEGF promoter activities were also suppressed by SREBP1a expression dose-dependently, while overexpression of β-galactosidase, as a control, did not suppress VEGF promoter activity (Fig. 2C). We also investigated the suppressive effects of the other SREBP isoforms on VEGF promoter activity (Fig. 2D). Not only SREBP1a but also SREBP1c and 2 suppressed basal VEGF promoter activities. Among the three isoforms, SREBP2 expression reduced the promoter activities most significantly. These results suggest SREBPs could regulate VEGF production at the transcriptional level.

Proximal Sp1 sites of VEGF promoter are responsible for the suppressive effects of SREBP1

The VEGF promoter has 3 E-box regions, E-box1 from −1568 to −1563, E-box2 from −1169 to −1164, E-box3 from −773 to −768, and the HRE from −974 to −965, where basic helix-loop-helix transcription factors might bind and has four tandem Sp1 sites within the short region from −94 to −52. There are neither classical SRE nor reported SRE-like sequences between −2362 and +61 of this promoter. To locate the putative SREBP1 responsive region, we prepared deletion constructs of the VEGF promoter as shown in Fig. 3A. Using this series of deletion constructs, we measured the suppressive effects of SREBP1a using a dual luciferase reporter assay. There was no significant loss of suppressive effects between pGL2-VEGF (−2362 +61) and pGL2-VEGF (−713 +61), suggesting that the putative SREBP1a responsive region was unlikely to exist between −2362 and −713 in the VEGF promoter (Fig. 3A). pGL2-VEGF (−89 +61) still maintained basal promoter activity, although the percent suppression of promoter activity by SREBP1a was slightly decreased (Fig. 3A). pGL2-VEGF (−89 +61) contains three of the four proximal tandem Sp1 sites (−94 to −52). It is possible that SREBP1a might suppress VEGF promoter activities through the four proximal tandem Sp1 sites, because interaction between Sp1 and SREBPs has been reported in several gene expression studies [1,21–23]. To determine the responsibility of the four proximal tandem Sp1 sites for the suppressive effect of SREBP1a, we prepared the constructs mutated at the Sp1 sites as shown in Fig. 3B (left panel). The percent suppression of basal VEGF promoter activities by SREBP1a overexpression was decreased depending on the number of point mutations or deletions of Sp1 sites (Fig. 3B, upper panel). Because the basal activity of pGL2-VEGF (−2362 −179) was significantly low, the cells were co-transfected with EPAS1 expression vector. EPAS1 has been reported to induce human VEGF promoter activities through HRE (−974 to −965) [19]. EPAS1 induced promoter activities were also suppressed by SREBP1a overexpression in a Sp1 site dependent manner (Fig. 3B, lower panel).

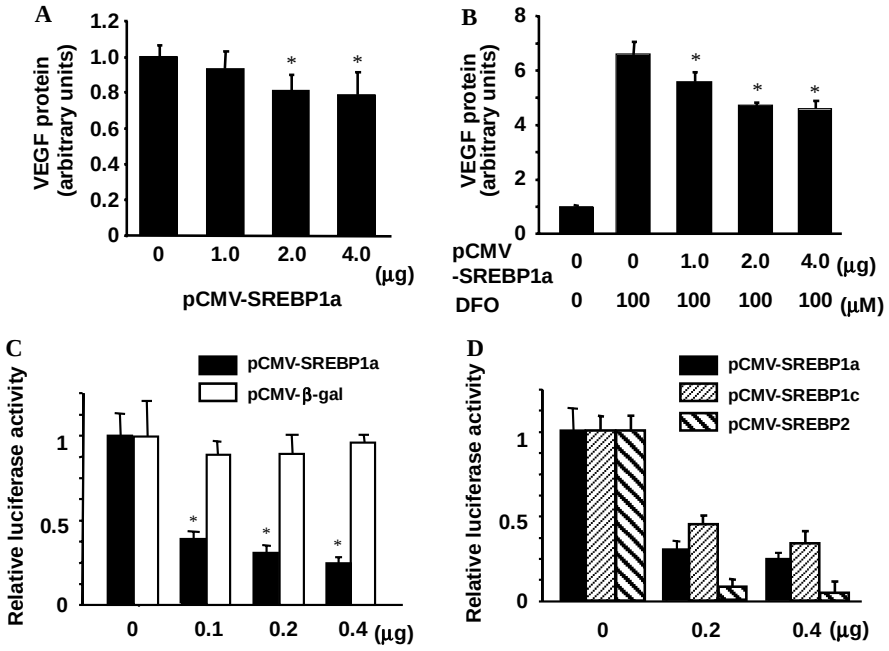

Fig. 2. VEGF suppression by SREBP1a in VSMCs. (A) Suppression of VEGF production by SREBP1a. HUSMC were transfected with 0, 1, 2 or 4 μg of SREBP1a expression vector (pCMV-SREBP1a), the total amount of genes transfected was equalized using empty vector. Twenty-four hour after transfection, cells were incubated under serum-deprived (FCS 0.2%) conditions for 40 h and then VEGF protein levels in the medium were determined by ELISA. VEGF protein levels were normalized to the total protein of the cell extract. Cell viability was confirmed by cell numbers and trypan blue exclusion. The values were expressed as arbitrary units. The values are means ± SD from three independent experiments. $*P < 0.05$ vs empty vector alone. (B) SREBP1a suppressed VEGF production induced by deferoxamine. HUSMC were transfected with 0, 1, 2 or 4 μg of SREBP1a expression vector (pCMV-SREBP1a), the total amount of genes transfected was equalized using empty vector. Twenty-four hour after transfection, cells were incubated under serum-deprived (FCS 0.2%) conditions with vehicle or with 100 μM of deferoxamine (DFO) for 40 h and then VEGF protein levels in the medium were determined by ELISA. The values were expressed as arbitrary units. The values are means ± SD from three independent experiments. $*P < 0.05$ vs empty vector alone with DFO treatment. (C) Suppression of VEGF promoter activity by SREBP1a. A7r5 cells were co-transfected with 0.2 μg of pGL2-VEGF (−2362 +61 of the human VEGF gene promoter) and 0, 0.1, 0.2 or 0.4 μg of SREBP1a expression vector (pCMV-SREBP1a) or β-galactosidase expression vector (pCMV-β-gal). The total amount of genes transfected was equalized using empty vector. Transcription activity of the VEGF promoter was assessed by determining the activity of luciferase. The values were expressed as relative luciferase activity. The values are means ± SD from three independent experiments. $* P < 0.05$ vs the same amount of pCMV-β-gal. (D) Suppression of VEGF promoter activity by each SREBP isoform. A7r5 cells were co-transfected with 0.2 μg of pGL2-VEGF and 0, 0.2 or 0.4 μg of SREBP1a, 1c or 2 expression vector (pCMV-SREBP1a, pCMV-SREBP1c, and pCMV-SREBP2). Transcription activity of the VEGF promoter was assessed by determining the activity of luciferase. The values were expressed as relative luciferase activity. The values are means ± SD from three independent experiments. Each experiment was performed in triplicate.

These results indicated that the four proximal tandem Sp1 sites were responsible for the suppressive effect of SREBP1a on VEGF promoter activity.

Discussion

SREBPs might mediate VEGF suppression by atorvastatin in VSMCs

VEGF is reported to accelerate atherosclerotic progression [13–16]. Recently, statins were reported to suppress VEGF production by the vascular wall [11]. VEGF reduction by statins might be one mechanism to suppress atherosclerotic progression. SREBP is known to be the principal mediator activated by statins in liver. Therefore, SREBPs could also mediate the important signals induced by statins in VSMCs. In this study, we demonstrated both SREBP1 activation by atorvastatin in VSMCs and the suppressive role of SREBP1a against VEGF production at the transcriptional level in these cells. We also revealed that SREBP1c and 2 suppressed VEGF promoter activities to different degrees compared with SREBP1a (Fig. 2D). Thus, SREBPs might play a role in the VEGF suppression by statins in VSMCs. However, it is still unclear which isoform is most important for VEGF regulation in VSMCs in vivo. In cultured cells including HepG2 line of human hepatocytes and the 3T3-L1 line of mouse preadipocytes, SREBP1a and 2 are activated in parallel when cells are deprived of sterols [24]. In contrast, SREBP1 and 2 are paradoxically regulated in livers of hamsters fed a diet containing statin [25]. In that study, the amount of both the precursor and mature nuclear forms of SREBP2 is increased by statin, while the mRNA levels of SREBP1c, predominant form of SREBP1 in liver, are selectively down-regulated by statin without alteration of the mRNA levels of SREBP1a, resulting in a profound fall in the amount of both the precursor and mature nuclear forms of SREBP1. Thus, SREBP2 may be activated by statin in any cell type producing this isoform. On the other hand, SREBP1 regulation by statin might depend on relative

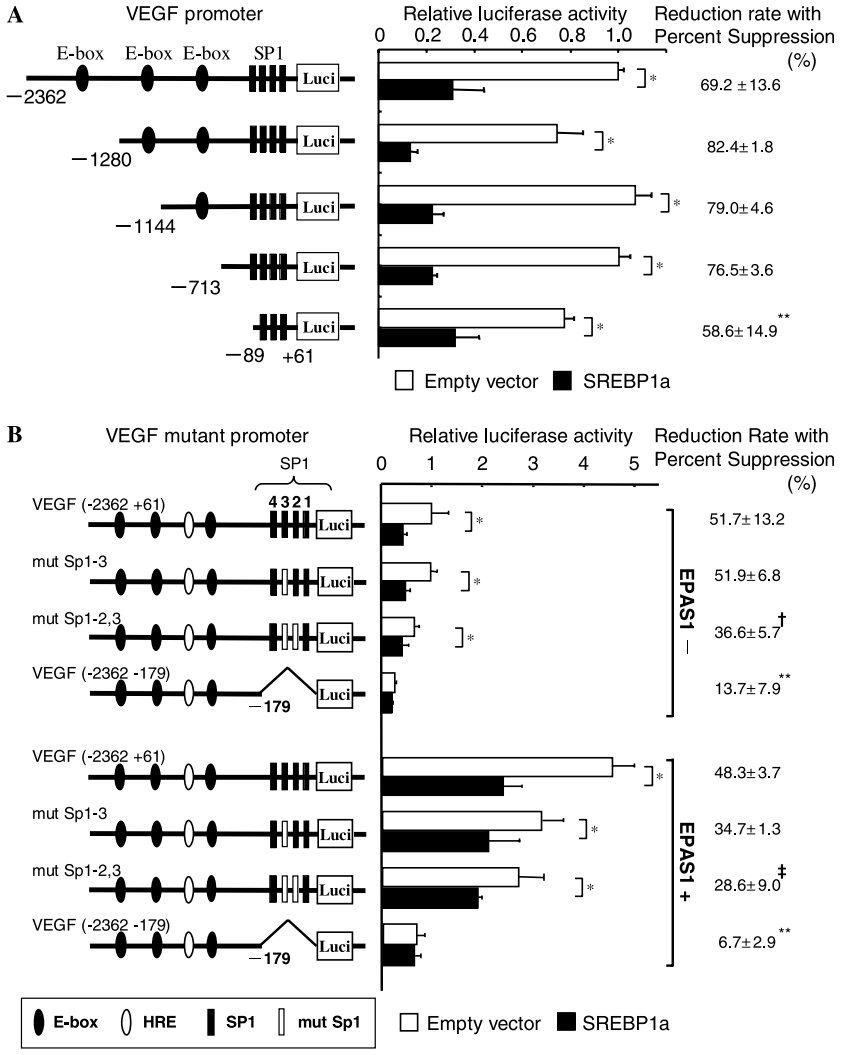

Fig. 3. Identification of an SREBP response region in VEGF promoter. (A) Progressive 5′-deletion analysis of VEGF gene promoter. A series of deletion constructs of the human VEGF promoter containing a luciferase reporter are shown in the left panel. A7r5 cells were transfected with the indicated reporter plasmid and SREBP1a expression vector (pCMV-SREBP1a) or empty vector (pCMV). Relative luciferase activities 24 h after transfection are indicated in the middle panel. Suppression of VEGF promoter activity by SREBP was expressed as the percent luciferase activity in VEGF transfected cells compared with the luciferase level in the cells transfected with empty vector (pCMV). The values are means ± SD from three independent experiments. Experiments were performed in triplicate. *$P < 0.05$ vs empty vector. **$P < 0.05$ vs the other deletion luciferase constructs. (B) Effects of site-specific mutation or deletion of the proximal 4 Sp1 sites within the VEGF promoter on SREBP1a mediated suppression of reporter genes. A7r5 cells were transfected with the indicated reporter plasmid, and SREBP1a expression vector (pCMV-SREBP1a) or empty vector (pCMV), without (upper panel) or with (lower panel) EPAS1 expression vector (phEPAS1). E-box is indicated as a closed circle. EPAS1 response element (HRE) is indicated as an open circle. Sp1 binding sites (Sp1) are indicated in closed boxes. Site-specific mutations of the Sp1 binding site (mut Sp1) are indicated as open boxes. Suppression of VEGF promoter activity by SREBP was expressed as the percent suppression of luciferase activity in VEGF transfected cells compared with the luciferase level in the cells transfected with empty vector (pCMV). The values are means ± SD from three independent experiments. Experiments were performed in triplicate. *$P < 0.05$ vs empty vector, **$P < 0.05$ vs the other deletion luciferase constructs, $^{\dagger}P < 0.05$ vs VEGF (−2362 +61) and mut Sp1-3, and $^{\ddagger}P < 0.05$ vs VEGF (−2362 +61).

distribution of the SREBP1 isoforms in each cell type. In vascular system, mRNA expressions of SREBP1 and 2 have been observed in vivo in aortic samples from pigs [2], although relative distribution of the two SREBP1 isoforms is presently unknown. SREBP1a is reported to be highly expressed in actively growing cells regulating a wide range of genes involved in lipid synthesis and cell growth, while SREBP1c is produced when cells require a lower rate of transcriptional regulation [1,26]. Because VSMCs in neointima of atherosclerotic vascular wall are actively growing, SREBP1a might play an important role in the VEGF regulation in atherosclerotic lesions. Further studies are needed to evaluate the expression and regulation of each SREBP isoform in normal and atherosclerotic regions of the vascular wall.

SREBP1 negatively regulates VEGF promoter activity through four proximal tandem Sp1 sites

Our study revealed that the four proximal tandem Sp1 sites (−94 to −52) are important for SREBP1 to suppress transcriptional activities of the VEGF promoter in VSMCs

(Figs. 3A and B). This site of the VEGF promoter has been reported to be important for basal and growth factor induced transcription [27–29]. However, the mechanism by which SREBP interacts with this region was not fully clarified in this study. SREBPs are thought to activate their target genes, although there are some reports that SREBPs play a role in repression of genes such as caveolin [30], microsomal triglyceride transfer protein [31], phosphoenolpyruvate carboxykinase (GTP) (PEPCK-C) [22], and insulin receptor substrate 2 [32]. In these genes, binding sites for SREBP and some transcription factors are adjacent, or on opposite strands, of the DNA. Binding to the SREBP site and displacing positive regulators specific to these genes could be a possible mechanism for SREBP repression [1]. In the case of the PEPCK-C gene promoter, the SRE and Sp1 binding sites are on opposite strands of the DNA so that the binding of one transcription factor to its site on the DNA precludes the binding of the other [22]. Although neither SRE nor known SRE-like sequences exist around the four proximal tandem Sp1 sites of human VEGF promoter, it is possible that a novel SREBP binding sequence might exist in this region.

Another possibility is that the interaction between SREBP and Sp1 protein leads to suppression of VEGF promoter activity. Bennett demonstrated SREBP–Sp1 interaction in the absence of DNA to form a complex through the ''buttonhead'' domain of Sp1 and the DNA binding domain of SREBP1 [23]. RNA polymerase II is known to bind to the promoter adjacent to specific transcription factor binding sites, and this binding is dependent on the recognition of the protein bound at that site, not on any specific DNA sequence [33]. It is possible that SREBP interaction with Sp1 protein bound to the Sp1 site occludes the recognition domain of the transcriptional apparatus, leading to the subsequent inhibition of transcription. Further study is needed to investigate the SREBP binding to the four proximal tandem Sp1 sites of the VEGF promoter, with or without Sp1 protein.

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