Transforming Growth Factor-β Receptor Requirements for the Induction of the Endothelin-1 Gene

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Expression of the endothelin (ET)-1 gene is subject to complex regulation by numerous factors, among which the cytokine transforming growth factor-β (TGF-β) is one of the most important. TGF-β action is based on the activation of the Smad signaling pathway. Smad proteins activate transcription of the gene by cooperation with activator protein-1 (AP-1) at specific sites on the ET-1 promoter. Smad signaling pathway is initiated by binding of the cytokine to a heteromeric complex of type I and type II receptors. Signal is then propagated to the nucleus by specific members of the Smad family. Most cell types contain a type I receptor known as ALK5. However, endothelial cells are unique because they coexpress an additional type I receptor named ALK1. These forms do not constitute redundant receptors with the same function, but they actually activate different Smad-mediated expression programs that lead to specific endothelial phenotypes. TGF-β/ALK5/Smad3 pathway is associated to a mature endothelium because it leads to inhibition of cell migration/proliferation. Conversely, TGF-β/ALK1/Smad5 activates both processes and is more related to the angiogenic state. We have analyzed the TGF-β receptor subtype requirements for the activation of the ET-1 gene. For that purpose, we have overexpressed type I receptor and Smad isoforms in endothelial cells and analyzed the effect on ET-1 expression. Our experiments indicate that TGF-β induces ET-1 expression preferentially through the activation of the ALK5/Smad3 pathway and, therefore, the expression of the vasoconstrictor may be associated to a quiescent and mature endothelial phenotype. Exp Biol Med 231:700–703, 2006

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Introduction

Since its molecular identification, endothelin (ET)-1 has been regarded to play a significant role in the pathophysiology of cardiovascular diseases. ET-1 is synthesized as a precursor molecule of 212 amino acids, the preproendothelin-1, which is proteolytically cleaved along several steps to yield the bioactive form of 21 residues. According to the literature, transcription of the gene is the rate-limiting step of biosynthesis from vascular endothelial cells (8). One of the most potent regulators of ET-1 levels is the cytokine transforming growth factor-β (TGF-β) (5, 6). Recently, we have described the molecular mechanism by which TGF-β induces the expression of ET-1 gene in vascular endothelial cells. TGF-β action is based on the activation of the Smad signaling pathway and the cooperation between Smad and activator protein-1 (AP-1) transcription factors at specific binding sites within the ET-1 promoter (11, 12).

Smad signaling pathway is initiated by binding of TGF-β to a heteromeric complex of type I and type II serine/threonine kinase receptors (7). The type I receptor, also known as activin receptor-like kinase (ALK), acts downstream of the type II receptor and propagates the signal to the nucleus by phosphorylating specific members of the Smad family, receptor-regulated (R)-Smads, at their extreme C-terminal serine residues. Phosphorylated R-Smads form complexes with the common partner (Co)-Smad (i.e., Smad4), which accumulate in the nucleus, where they participate in transcriptional regulation of target genes (2). In most cell types, TGF-β signals via a type I receptor form known as ALK5. However, endothelial cells are unique because they coexpress an additional type I receptor named ALK1. These forms do not constitute redundant receptors with the same function, but they actually activate different Smad-mediated expression programs. Whereas activated

ALK5 induces the phosphorylation of Smad2 and Smad3, activated ALK1 has been shown to induce the phosphorylation of Smad1 and Smad5 (3, 9, 10). Interestingly, the activation of these two distinct type I receptor/Smad signaling pathways results in different physiologic effects. Whereas TGF-β/ALK5/Smad3 leads to inhibition of cell migration and proliferation and is associated to a mature endothelium, TGF-β/ALK1/Smad5 activates both processes and is more related to the angiogenic state (3, 10).

The aim of the present study was to investigate which form of TGF-β type I receptor leads to the activation of the ET-1 gene. Because TGF-β regulates the activation state of the endothelium either via ALK5 (maturation of blood vessels) or via ALK1 (angiogenic process), the knowledge of the form leading to ET-1 expression may allow assignment of the ET-1 gene to a particular endothelial phenotype. Our results indicate that TGF-β induces ET-1 expression preferentially through the activation of the ALK5/Smad3 pathway.

Materials and Methods

Cell Culture. Bovine aortic endothelial cells (BAECs) were obtained and cultured using methods described previously (11). Cells for RNA experiments were seeded on 10-cm diameter culture plates; 6-well plates were used for protein isolation and were cultured on 24-well plates for transfection experiments and for immunofluorescence studies by confocal microscopy.

Reporter Plasmids and Cell Transfection. A luciferase reporter driven by a –650-bp fragment of the human ET-1 (–650 ppET-1-prom-luc) promoter was generated as described (11). ALK5/Smad3-reporter (CAGA-luc) consisted of 12 tandem repeats of the upstream Smad3 binding element from human plasminogen activator inhibitor-1 (PAI-1) promoter linked to a viral minimal promoter and to a luciferase gene and was a generous gift from Dr. Aris Moustakas (Biomedical Center, Uppsala, Sweden) (1). ALK1/Smad1/5/8-reporter (BRE-luc) construct consisted of two tandem repeats of the Smad elements from inhibitor of differentiation-1 (Id-1) linked to a cassette minimal promoter-luciferase and was a generous gift from Dr. Peter ten Dijke (LUMC, Leiden, The Netherlands), as were wild-type and mutant overexpression plasmids (pCMV5-derived) for ALK5 and ALK1 (3, 4). Overexpression plasmids for Smad1, Smad3, and Smad5 (pCMV5-derived) were obtained from Dr. Liliana Attisano (University of Toronto, Ontario, Canada).

Transient transfection experiments were performed with BAECs seeded on gelatin-coated 24-well plates (60%–70% confluency) and luciferase activity was determined as described previously (11). Overexpression of TGF-β type I receptor and Smad forms was followed by Western blot analyses and immunofluorescence microscopy using specific antibodies.

RNA Isolation and RNase Protection Analyses. For RNA experiments, confluent BAEC monolayers were washed with phosphate-buffered saline and processed for RNA isolation by guanidium thiocyanate/phenol/chloroform extraction. To detect and quantify bovine ET-1 and 28S RNA levels, RNase protection experiments were performed as described previously (11).

Statistical Analysis. Experimental data were analyzed by unpaired Student's t test in the case of normal distribution of data or using nonparametric tests as appropriate. The P values obtained are indicated in the text or in the figure legends when statistically significant ($P < 0.05$).

Results

We have described previously that TGF-β increases ET-1 mRNA levels and ET-1 promoter activity in bovine aortic endothelial cells. Two distinct Smad-dependent regulatory pathways can be activated by TGF-β via different TGF-β type I receptor forms. To find out about the specific set of type I receptor/Smad isoforms that TGF-β uses to induce ET-1 expression, we have overexpressed wild-type and mutant (kinase-deficient and constitutively active) forms of type I receptor forms ALK1 and ALK5, as well as Smad isoforms. Overexpression was followed by Western blot and immunofluorescence microscopy using specific antibodies (data not shown). Functional validation of the overexpression was performed by cotransfection of constructs together with ALK5- and ALK1-specific reporters as shown for constitutively active (ca) forms of ALK5 and ALK1 in Figs. 1A and 1B. As an ALK5-specific reporter, we have used a luciferase construct driven by a synthetic tandem repeat based on the upstream Smad3 binding element from the human PAI-1 promoter, whereas a luciferase reporter under the control of the Smad1/5 binding sites present in the mouse Id-1 promoter was employed as specific ALK1 reporter. Figures 1C, 1D, and 1E also show the effect of the overexpression of TGF-β type I receptor and Smad isoforms on ET-1 promoter activity estimated by cotransfection of a –650/+173-bp ET-1 promoter fragment-luciferase construct. In the absence of TGF-β, ALK5 ca was more effective than ALK1 ca in potentiating ET-1 promoter activity (Fig. 1C). Under conditions of stimulation with TGF-β, only ALK5 wild-type was able to potentiate activity, ALK1 wild-type was without effect. In the same way, kinase-deficient ALK5 produced a significant inhibition of TGF-β–induced promoter activity, whereas ALK1 kinase deficient did not (Fig. 1D). When looking at the effect of the overexpression of Smad isoforms, Smad3 was the most potent compared with Smad1 and Smad5 (Fig. 1E). Taken together, these results suggest that both ALK5/Smad3 and ALK1/Smad1 activation are able to potentiate ET-1 promoter, although ALK5/Smad3 signaling seems to be much more effective.

To confirm the preferential role of ALK5 for the TGF-β action on ET-1 expression, we have taken advantage of the use of a commercial and specific ALK5 inhibitor known as SB-431542. Preliminary experiments showed that this substance at 10 μM concentration was able to almost

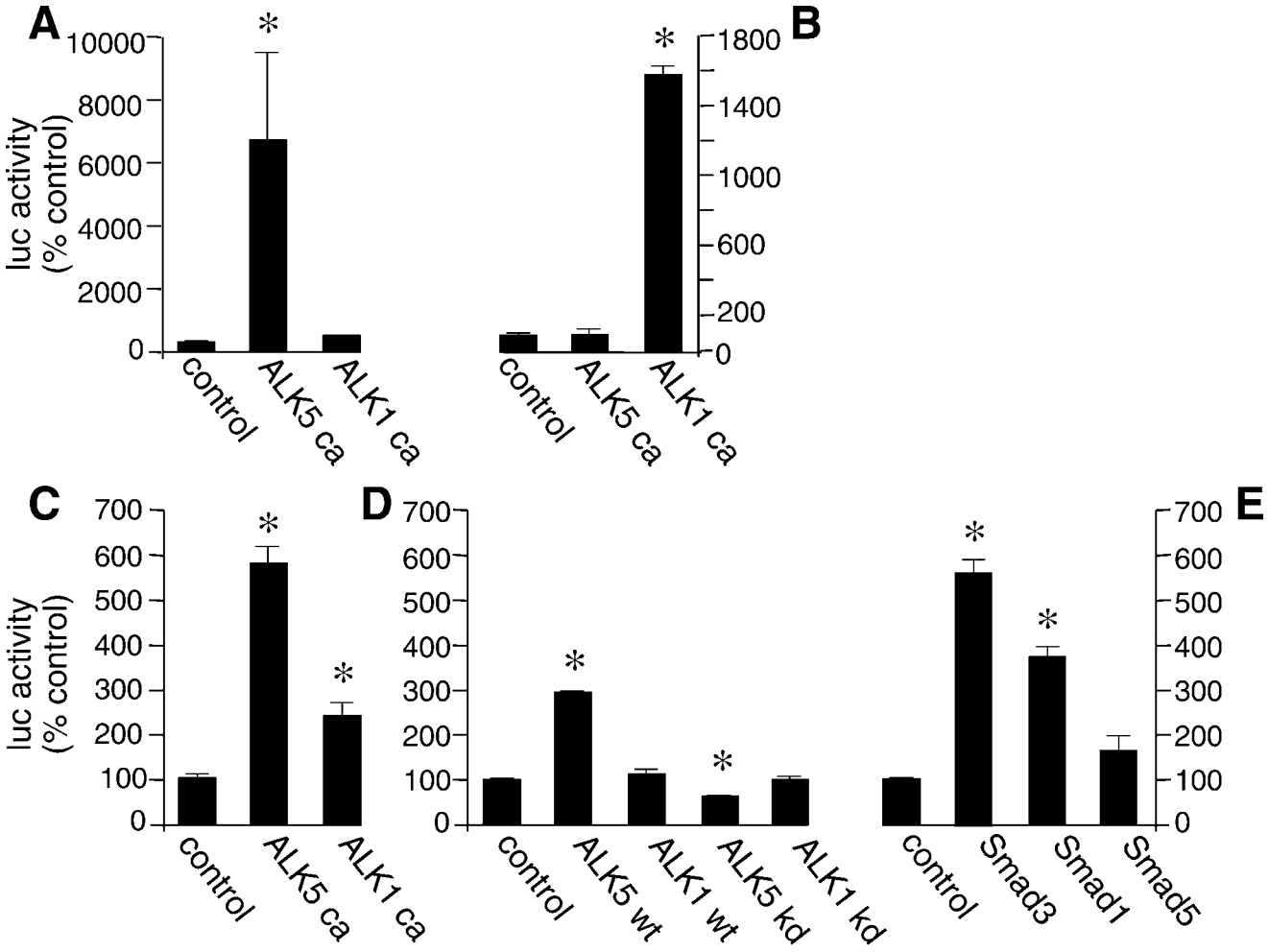

Figure 1. Effect of the overexpression of ALK5, ALK1, and Smad isoforms on ALK5- and ALK1-specific reporters and on human ET-1 promoter. The effect of the overexpression of constitutively active (ca) forms of ALK5 and ALK1 on ALK5- (A) and ALK1-specific (B) luciferase reporters was analyzed by cotransfection experiments in BAECs. Cotransfection of overexpression plasmids together with a –650/+173-bp fragment of the human ET-1 promoter linked to a luciferase gene is shown in panels C through D. (C) Effect of ALK5 and ALK1 ca in the absence of TGF-β. (D) Effect of ALK5 and ALK1 wild-type (wt) and kinase-deficient (kd) forms in the presence of 5 ng/ml TGF-β. (E) Effect of Smad3, Smad1, and Smad5 in the presence of TGF-β. Luciferase activity was measured by luminometry and expressed as percentage of control pCMV5 empty vector (mean ± S.D. $n = 3$, $^*P < 0.05$ vs. control).

completely suppress the observed potentiation of ALK5-specific reporter by ALK5 ca, whereas it has no effect on that of ALK1-specific reporter by ALK1 ca (data not shown). Figure 2A shows that SB-431542 dose dependently inhibited TGF-β–induced ET-1 promoter activity. This inhibitor was also able to reduce ET-1 mRNA levels either under basal conditions or after stimulation with TGF-β (Fig. 2B). In conclusion, these results confirm that an important part of the TGF-β–induced ET-1 gene transcription goes through ALK5 receptor.

Discussion

TGF-β is a multifunctional cytokine that regulates many different biological processes. In the context of the endothelium, it is involved in the development of the vascular system and affects the function of endothelial cells. In contrast to the majority of the cell types, endothelial cells posses two opposing type I receptor/Smad pathways (3, 10). ALK1 induces Smad1/5 phosphorylation leading to the induction of genes such as Id-1 or endoglin, and it is associated to an increase in endothelial cell proliferation and migration. The ubiquitous ALK5 promotes Smad2/3 activation with the concomitant induction of PAI-1, collagen type I α2, or fibronectin genes, which is more related to the maturation of the endothelium through inhibition of cell proliferation/migration (3, 10).

TGF-β is also one of the most potent inducers of the expression of ET-1, and we found interesting to analyze the endothelial cell context in which the TGF-β action takes place by investigating the specific ALK/Smad pathway leading to ET-1 expression. Our results indicate that TGF-β induces ET-1 gene expression preferentially by activation of the ALK5/Smad3 pathway. Hence, as shown in the scheme depicted in Fig. 2C, the ET-1 gene can be mainly assigned to the group of genes activated by ALK5/Smad3 such as PAI-1. Interestingly, this assignment suggests also that the

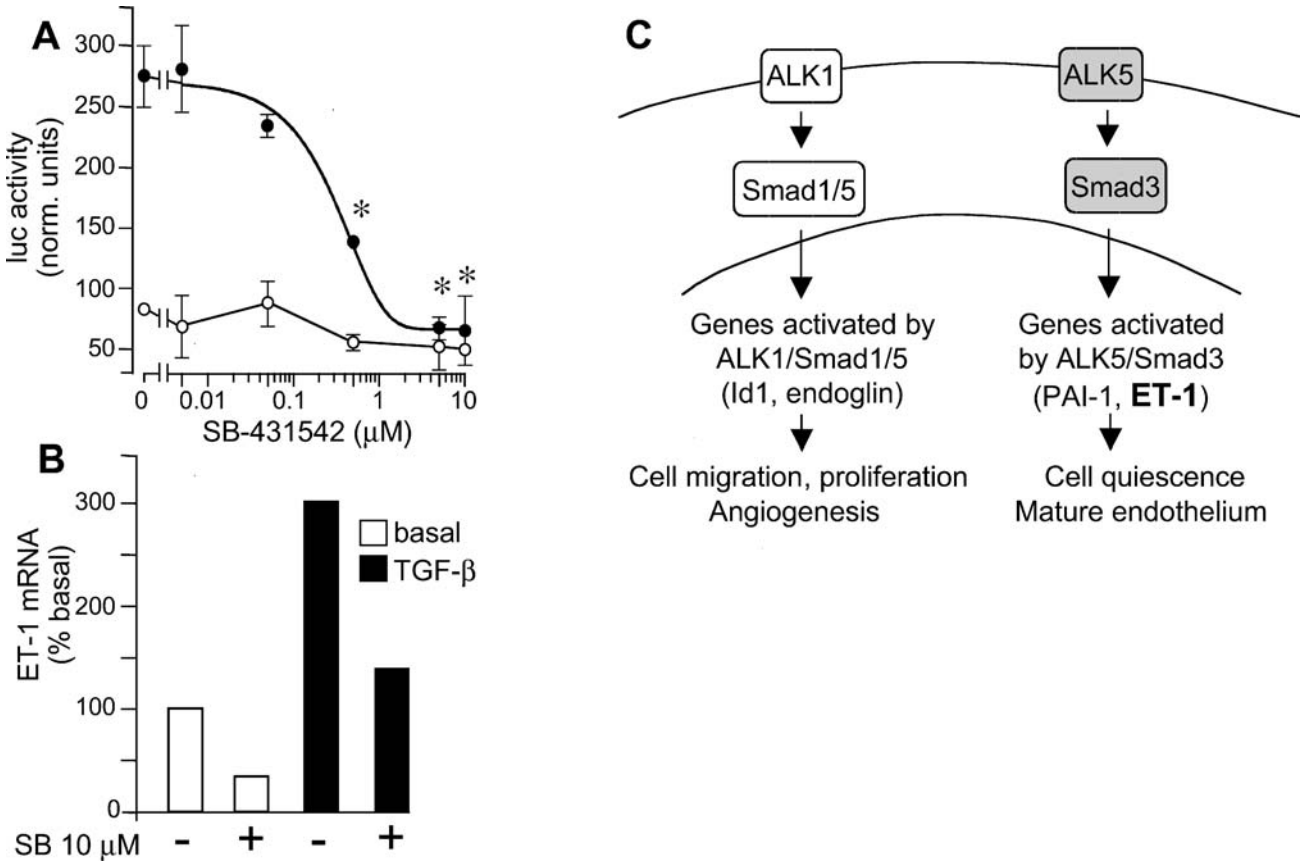

Figure 2. Effect of the ALK5-specific inhibitor SB-431542 on TGF-β induction of the ET-1 promoter and mRNA expression. (A) Cells transiently transfected with the ET-1 promoter construct were preincubated for 30 minutes with increasing concentrations of SB-431542 before the addition of 5 ng/ml TGF-β (solid circles) or medium alone (open circles). Luciferase activity was measured as before and results are shown as normalized units (mean ± S.D. $n = 3$, $^*P < 0.05$ vs. control without the inhibitor in the presence of TGF-β, values in the absence of TGF-β were nonsignificant compared with control without the inhibitor). (B) Cells were preincubated with or without 10 μ*M* SB-431542 before the addition of TGF-β or basal. After 6 hours total RNA was extracted from cells and used in RNase protection experiments to detect and quantify bovine ET-1. Results are expressed as percentage of basal from one of two representative experiments. (C) Scheme showing the TGF-β–activated Smad-dependent regulatory pathways present in endothelial cells. TGF-β–induced ET-1 expression preferentially occurs through activation of the ALK5/Smad3 pathway.

capacity of TGF-β to induce ET-1 expression may be a feature of a mature and quiescent endothelium.

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