Platelet-Derived Growth Factor Induces Tissue Factor Expression in Vascular Smooth Muscle Cells via Activation of Egr-1

Motohiro Kamimura, Florian Bea, Tadao Akizawa, Hugo A. Katus, Jörg Kreuzer, Christiane Viedt

Abstract—Activation of vascular smooth muscle cells (SMCs) by platelet-derived growth factor (PDGF) is a seminal event in the initiation and progression of the atherosclerotic lesion and may contribute to atherosclerotic plaque instability with plaque rupture and thrombus formation. Tissue factor (TF), a prothrombotic molecule expressed by various cell types within atherosclerotic plaques, is thought to play a major role in thrombus formation after plaque rupture. This study examined intracellular signaling pathways leading to TF expression and Egr-1 activation, a key element in tissue factor transcription, by PDGF-BB in rat SMCs. PDGF-BB induced TF mRNA and protein expression in a time-dependent manner. Early growth response factor-1 (Egr-1) binding activity was also induced by PDGF-BB, as well as phosphorylation of extracellular signal-regulated kinase. PDGF-BB-induced Egr-1 activation was suppressed by inhibitors of 2 upstream activators of Egr-1, extracellular signal-regulated kinase (ERK) and Src family kinases, whereas antioxidants, phosphatidylinositol 3-phosphate kinase, and p38 MAPK inhibitors had no effect. PDGF-BB–stimulated expression of the transcriptional co-repressor NAB2 was time-dependent. Furthermore, transient transfections of SMCs with wild-type and mutated TF promoter constructs showed that the Egr-1 binding region is an important transcriptional regulator of PDGF-BB–induced TF expression. Taken together, the results suggest that PDGF-BB induces TF expression and activity in SMC by a Src family kinases/ERK/Egr-1 signaling pathway and may therefore contribute to thrombus formation in advanced atherosclerosis and restenosis. (Hypertension. 2004;44:944-951.)

Key Words: platelet-derived growth factor ■ atherosclerosis ■ gene regulation ■ signal transduction ■ transcription

Platelet-derived growth factor (PDGF) is a potent growth factor that functions as an important mediator in the pathogenesis of vascular disease. Whereas PDGF is expressed at low levels in arteries from healthy adults, its expression is increased in conjunction with the inflammatory–fibroproliferative response that characterizes atherosclerosis.1 Studies of balloon catheter-injured arterial tissue, naturally occurring atherosclerosis, coronary arteries after percutaneous transluminal coronary angioplasty, and experimentally induced atherosclerosis revealed increased expression of PDGF and PDGF receptors in these lesions.2 The observations suggest that PDGF, produced by activated macrophages, smooth muscle cells (SMCs), endothelial cells, or released from platelets in thrombi, is important for the formation of the lesion. PDGF is a dimeric cytokine comprising α or β chains or both and is known to mediate its effects through activation of its receptor tyrosine kinase.2 PDGF has been proposed to play an important role in atherogenesis because it can stimulate a variety of different proatherogenic genes in SMCs.1 PDGF can exhibit its cellular effects via activation of tyrosine kinase, Src family kinases, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI 3-kinase).2

The promoters of many genes whose products induce proatherogenic processes such as SMC proliferation, migration, and matrix syntheses contain binding sites for the immediate–early gene and transcription factor early growth response factor-1 (Egr-1).3,4 Egr-1 has been localized in endothelial cells, macrophages, and SMCs in human atherosclerotic plaques, as well as in atherosclerotic lesions at hyperlipidemic mice.5 Activation of Egr-1 has been demonstrated by important proatherosclerotic mediators, such as oxidized low-density lipoprotein, CD40, tumor necrosis factor-α, shear stress, and Chlamydia pneumoniae.5,7 Furthermore, hyperlipidemic mice deficient of Egr-1 display decreased atherosclerosis and vascular inflammation.8 Target genes that are activated by Egr-1 include PDGF-A, PDGF-B, transforming growth factor-β1, membrane type 1 matrix metalloproteinase, and the prothrombotic molecule tissue factor (TF).7,9 which is thought to play a central role in thrombus formation after atherosclerotic plaque rupture.10

In the present study, we investigated signaling pathway and transcription factor mediating induction of TF expression in
human SMCs by PDGF-BB. We show that expression of TF is elevated by PDGF-BB, and that this induction was mainly mediated by Egr-1 binding activation. Increased binding activity of Egr-1 by PDGF-BB is mediated by a Src family kinase-dependent and extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent but reactive oxygen species (ROS)-independent, p38 MAPK-independent, and PI 3-kinase–independent pathway.

Materials and Methods
Pertussis toxin (PTX), SB203580, Wortmannin, and PD98059 were from Calbiochem (La Jolla, Calif). GFI09203X was from Biomol (Hamburg, Germany). PDGF-AA, PDGF-BB, N-acetylcysteine (NAC), 4,5-dihydroxy-1,3 benzene-disulfonic acid (tiron), diphenyle isothiourea, Phenylmethylsulfanyl fluoride, and NP-40 were from Sigma (Deisenhofen, Germany). Anti-α actin (monoclonal), Pefabloc, and E-64 were purchased from Roche (Mannheim, Germany). Anti-phospho-ERK1/2 and p38 MAPK were obtained from New England Biolabs (Schwalbach, Germany). Anti-Egr-1 antibody oligonucleotides and control and mutated oligonucleotides were obtained from MWG (Ebersberg, Germany). Cell culture media and supplements were from Gibco BRL (Karlsruhe, Germany). Lipofectamine reagent was from Invitrogen GmbH (Karlsruhe, Germany).

Cell Culture
SMCs were isolated from the thoracic aortas of male Sprague-Dawley rats by enzymatic digestion and cultured as described previously.11 Cells were grown as subconfluent monolayers and used in passages 6 to 9. Before the experiments, the cells were washed with phosphate-buffered saline and grown in serum-free medium (0.1% bovine serum albumin in DMEM) for 24 hours to render SMCs quiescent.

Preparation of Cell Lysates and Western Blot Analysis
Quiescent SMCs were stimulated in either the presence or absence of inhibitors. Cell lysates and immunoblotting were prepared as described previously.11 The following commercially available antibodies were used: anti-TF from American Diagnostica (Greenwich, Conn), and anti-NAB2 from Santa Cruz Biotechnology.

Egr-1 Electrophoretic Mobility Shift Assay: Preparation of Nuclear Extracts
For the electrophoretic mobility shift assay, nuclear protein extracts were prepared according to the method of Schreiber et al,12 as described previously.11

Egr-1 Gel Mobility Shift Assay
Nuclear extracts (2 μg each) were incubated with labeled oligonucleotide probes and 2 μg of poly(deoxyinosine-deoxyctydine)-poly(deoxyuridine-deoxycytidine) in 20 μL of binding buffer (60 mmol/L HEPES, pH 7.9, 50% glycerol, 20 mmol/L Tris-HCl, pH 8.0, 300 mmol/L KCl, 5 mmol/L EDTA, 100 μg/mL bovine serum albumin, 2.5 mg/mL Pefabloc, 25 μg/mL E-64, 5 mmol/L NaF, 1 mmol/L Na3VO3, 5 mmol/L DTT for 5 minutes at RT). The sequence of the double-stranded oligonucleotide used in the present study was as follows: consensus Egr-1, 5′-GGATCCACGGGGGGCGAGCGGGGCGA-3′; and mutated 5′-GGATCCACGGGGGGCGAGCTGCTGGGCGA-3′. The oligonucleotides were labeled with [γ32P]-ATP by using T4 polynucleotide kinase. Binding reactions were resolved on 4% native polyacrylamide gel containing 1× TAE buffer (25 mmol/L Tris, 25 mmol/L boric acid, 0.5 mmol/L EDTA). Gels were run at 150 V in a cold room (4°C) for 2 to 3 hours, dried, and exposed to x-ray film for 12 to 24 hours. In addition, a supershift assay for Egr-1 using rabbit polyclonal antibody against Egr-1 was performed. The specific antibodies were incubated with samples following the initial binding reaction between nuclear protein extracts and 32P-labeled consensus oligonucleotide (1 hour at RT).

Decoy Oligonucleotide Technique
Egr-1 decoy and mutated controls were used as described previously.11 Their sequences were as follows: Egr-1 consensus sequence, 5′-GGATCCACGGGGGGCGAGCGGGGCGA-3′; and mutated control 5′-GGATCCACGGGGGGCGAGCTGCTGGGCGA-3′.

Tissue Factor Activity
Seeded in 12-well plates and cultured in standard growth medium were 3×10^4 SMCs. Cells were kept in serum-free medium 24 hours before the stimulation. SMCs were stimulated with PDGF-BB in presence or absence of oligodeoxynucleotides. TF activity was measured with Actochrome TF activity assay kit (American Diagnostica) according to the manufacturer’s protocol. Briefly, after stimulation, cells were washed with ice-cold phosphate-buffered saline 2 times. Assay buffer (300 μL), factor VIIa (25 μL), and factor X (25 μL) were added to each well and rotated orbitally for 15 minutes at 37°C. Then, Spectrozyme factor Xa (25 μL) was added to each well and incubated 15 minutes at 37°C with orbital stirring. TF activity in reaction mixture was measured at 405 nm.

RNA Isolation and Quantitative Polymerase Chain Reaction
Total RNA from SMCs was extracted by Chomczynski method, using TRIZOL (Life Technologies SRL). Specific cDNA was reverse-transcribed from 1 μg RNA. TF DNA was detected and quantified with LightCycler FastStart DNA Master SYBR Green I and Light Cycler system (Roche, Mannheim, Germany) according to manufacturer’s instructions. The primers are as follows: rat TF sense, 5′-GCTCAATGCCTTCTTCAGG-3′; and antisense, 5′-CACCACCTGTAGCTCGGTGA-3′; and rat GAPDH sense, 5′-CCATTGGAAGGCTGGGG-3′; and antisense, 5′-CAAGTGTCATGAGTGACC-3′. The conditions were as follows: initial denaturation 10 minutes at 95°C, followed by 50 cycles of 10 seconds at 95°C, 12 seconds at 55°C, and 18 seconds at 72°C. The reaction, data acquisition, and analysis were all performed by using the Light Cycler instrument.

Plasmids and Transfections
Construction of the wild-type rat TF promoter (−143) and a mutated promoter containing a mutation of the Egr-1 (Egr-1m) site (generously provided by N. Mackmann, The Scripps Research Institute, La Jolla, Calif) have been described elsewhere.7 SMCs (5×10^4 cells per well) were seeded in a 6-well plate 1 day before experiments. SMCs were transfected with 2 μg DNA per well using Lipofectamine reagent according to the manufacturer’s protocol. In all experiments, cells were co-transfected with pRL-CMV, which expresses Renilla luciferase to compensate for variation in transfection efficiencies (<10%). After transfection, cells were incubated in standard growth medium for 48 hours. Then cells were cultured with PDGF-BB for 12 hours. Cell lysates were assayed using Dual Luciferase Reporter System (Promega) according to the manufacturer’s protocol.

Statistics
Multiple comparisons were evaluated by ANOVA, followed by Fisher protected least significant difference method. Data are presented as mean±SEM and values of P<0.05 were considered statistically significant.

Results
Vascular SMC Viability
Almost pure (>98%) rat SMC cultures were confirmed by phase-contrast microscopy and staining for vascular smooth muscle cell (SMC)-specific α-actin. There were no toxic effects at the used concentrations of PDGF-AA, PDGF-BB, inhibitors, plasmids, or oligodeoxynucleotides on cellular
viability, determined by cell morphology, cell number, and measurement of lactate dehydrogenase release into the supernatant (data not shown).

**Activation of Egr-1 by PDGF-AA and PDGF-BB in SMC**

To determine the effects of the growth factors PDGF-AA and PDGF-BB on Egr-1 binding activity in SMCs, cultured cells were stimulated with PDGF-AA (10 ng/L) or PDGF-BB (10 ng/L), respectively. As shown in Figure 1A, PDGF-BB stimulated Egr-1 activity in a time-dependent manner. Increase in PDGF-BB–stimulated Egr-1 binding activation occurred at 15 minutes and peaked by 2 hours, and decreased thereafter to become undetectable after 8 hours. PDGF-AA also stimulated Egr-1 binding activity in the same time-dependent manner, but to a lesser extent than PDGF-BB. Therefore, in the subsequent experiments, we focused on the PDGF-BB effects.

Incubation with an excess (10-fold and 100-fold, respectively) of an unlabeled Egr-1 consensus sequence served as a control for specific binding by competing with radiolabeled Egr-1 probe (Figure 1B, lane 3 and 4). The specificity was further confirmed by using an antibody to perform supershift assays and by mutated Egr-1 consensus sequence (Figure 1B, lane 5 and 6).

**PDGF-BB–Induced Egr-1 Activation Is Tyrosine Kinase and Src-Family Kinases–Dependent but Gi-Protein and PKC–Independent**

We have previously shown that Gi1,2 plays a role in PDGF-AA but not in PDGF-BB signaling; therefore, we investigated the effect of the G-protein inhibitor PTX.14 Pretreatment of quiescent SMCs with PTX (100 ng/mL, 16 hours) did not show any significant effects on PDGF-BB–induced Egr-1 binding activity. This observation is in line with our previous observation and underscores the differences in PDGF-AA and PDGF-BB signaling. An essential role of the tyrosine kinase in PDGF-BB–induced Egr-1 activation was confirmed using the tyrosine kinase inhibitor genistein (100 μmol/L, 60 minutes), which abolished PDGF-BB–induced Egr-1 activation (Figure 2A). The Src family kinases inhibitor PP2 (1 μmol/L, 30 minutes) was used to demonstrate that the PDGF-BB–induced Egr-1 activation was Src family kinase-dependent. To further define whether the PKC signaling pathway is involved, we used a specific protein kinase (PKC) inhibitor GF109203X (2 μmol/L, 60 minutes). GF109203X had no effect on PDGF-BB–induced Egr-1 binding activity.

**PDGF-BB–Induced Egr-1 Activation Is ERK1/2-Dependent, but ROS, p38 MAPK, and PI 3-Kinase–Independent**

To address the potential involvement of ERK1/2, p38 MAPK, and PI 3-kinase pathway in PDGF-BB–induced activation of Egr-1 in SMCs, we used specific inhibitors. As shown in Figure 2B, SB203580 (10 μmol/L, 30 minutes), a specific inhibitor of p38 MAPK, and Wortmannin (50 μmol/L, 60 minutes), a specific PI 3-kinase inhibitor, had no effect on Egr-1 binding activity in SMCs. In contrast, PD98059 (30 μmol/L, 60 minutes), a selective inhibitor of the MAPK kinase (MEK), inhibited induction of Egr-1 activation.

Recent findings indicate that PDGF plays a role in the generation of ROS in SMCs.14,15 Jin et al16 have shown that hydrogen peroxide increases Egr-1 mRNA levels in SMCs. To investigate the involvement of ROS in PDGF-BB–mediated Egr-1 activation, cells were incubated in the present of the antioxidant NAC (5 mmol/L, 30 minutes) before stimulation with PDGF-BB. As the p38 MAPK inhibitor, NAC shows no effect (Figure 2B) on PDGF-BB–induced Egr-1 activation. The O2− scavenger tiron and the flavoprotein-inhibitor diphenylene iodonium also show no inhibitory effects (data not shown).

**PDGF-BB–Induced NAB2 Activation**

The co-repressor NAB2 can markedly decrease Egr-1 transcriptional activity by binding to an inhibitory domain at the
zinc finger binding domain. Therefore, we examined whether PDGF-BB could activate NAB2. PDGF-BB treatment resulted first in a slight decrease of NAB2 protein within 1 hour, the time period when Egr-1 binding activity is increased. Thereafter, NAB2 protein increased 5.4-fold above the constitutive expression 4 hours after stimulation with PDGF-BB (Figure 3) and returned to baseline levels after 8 hours. NAB2 was expressed at the protein levels, and its time course closely followed Egr-1 binding activity.

PDGF-BB–Induced ERK1/2 and p38 Activation Is Src Family Kinase-Dependent

To demonstrate the activation and phosphorylation of ERK1/2 and p38 MAPK, we performed Western blot analysis with phospho-specific ERK1/2 and p38 MAPK antibodies. PDGF-BB induced a time-dependent ERK1/2 and p38 MAPK activation. The phosphorylation of ERK1/2 and p38 MAPK was rapidly increased by PDGF-BB and reached a maximum at 5 to 15 minutes after stimulation (data not shown). Next, we studied the effect of PP2, PD98059, SB203580, and Wortmannin, respectively, on PDGF-BB–induced ERK1/2 and p38 MAPK activation. As shown in Figure 4, PP2 and PD98059 inhibited the PDGF-BB–induced phosphorylation of ERK1/2, whereas SB203580 or Wortmannin did not have any significant inhibitory effect. As expected, the PDGF-BB–induced p38 MAPK activation was inhibited by PP2 and SB203580 but not by the MEK or PI-3 kinase inhibitor.

Induction of TF Synthesis by PDGF-BB in SMCs

To investigate the effect of PDGF-BB on the expression of TF, SMCs were treated with PDGF-BB (10 ng/mL) and synthesis was measured by Western blot analysis. A 7.2-fold increase above the constitutive expression of TF was observed 12 hours after stimulation. Figure 5A shows that a detectable increase of TF was seen within 8 hours of stimulation, peaking at 12 hours, and was maintained for up to 48 hours. Parallel with the increase in TF expression, PDGF-BB (10 ng/mL) induced a time-dependent induction of TF mRNA, suggesting regulation at the pretranslational level (Figure 5B). The increase in TF mRNA was maximal 2 hours after stimulation with PDGF-BB, ie, a 3.0-fold increase (n=4, P<0.05) compared with control conditions. Actinomycin D (5 μg/mL) completely blocked the increase in PDGF-BB–stimulated TF mRNA, suggesting that the expression of TF is dependent on transcriptional regulation (data not shown).

To investigate the signal transduction pathways leading to PDGF-BB–mediated TF expression, cells were pre-incubated with specific inhibitors. As shown in Figure 5C, pretreatment with genistein, PP2, and PD98059 inhibited TF mRNA expression without affecting basal levels. Preincubation of SMCs with SB203580 or Wortmannin did not inhibit the PDGF-BB–induced TF mRNA synthesis.
Significance of Egr-1 Activation for the PDGF-BB–Induced TF Synthesis

The functional role of Egr-1 in PDGF-BB–mediated TF gene regulation was elucidated further by the use of cis element double-stranded (decoy) oligonucleotides, which scavenge active transcription factors, thereby blocking their binding to the promoter regions in target genes. Gel mobility shift confirmed that decoy oligonucleotides against Egr-1 binding sites specifically competed, whereas control decoy oligonucleotides did not (data not shown). Pretreatment with Egr-1 decoy oligonucleotides (10 μM, 6 hours), but not control decoy oligonucleotides, specifically inhibited TF mRNA and protein expression as TF activity response to PDGF-BB (Figure 6).

Mutation of the Egr-1 Binding Site Inhibits Induction of Reporter Activity by PDGF-BB

PDGF-BB stimulated SMCs that had been transfected with the promoter construct containing the wild-type Egr-1 binding site (TF-143) showed a 2.9-fold increase of luciferase reporter activity compared with control. SMCs that had been transfected with mutated Egr-1 binding site (TF-143 Egr-1m) showed approximately a half luciferase activity compared with wild-type (Figure 7). These results indicate that Egr-1 plays an important role in regulating TF gene expression in response to PDGF-BB stimulation.

Discussion

First, it has been documented that the transcription factor Egr-1 plays an important role in the pathogenesis of atherosclerosis. However, the regulation and upstream signaling mechanisms of Egr-1 binding activity are poorly defined. Secondly, it has been noted that the Egr-1 protein in various cells of the vasculature is rapidly and transiently induced by different growth factors and other extracellular signals. In this study, we could show that the growth factors PDGF-BB and PDGF-AA induce a time-dependent Egr-1 binding activity in SMCs. Furthermore, we demonstrated that the PDGF-BB–induced Egr-1 activation is tyrosine kinase-dependent, Src family kinase-dependent and ERK-dependent. PDGF-BB induces the co-repressor NAB2, thus providing a negative feedback to limit Egr-1 activity. In addition, we demonstrated that the Egr-1 binding is necessary for PDGF-BB–induced TF expression and activity.
Recently, it was shown that Egr-1 and Egr-1 responsive genes are upregulated in atherosclerosis lesions. However, the factors inducing Egr-1 expression in the vasculature are not well-understood.

PDGF-BB is present in atherosclerotic lesions, and its involvement in the atherosclerotic process has been confirmed experimentally using different models. The intimal thickening after endothelial denudation was inhibited by administration of neutralizing PDGF antibodies. In addition, infusion of PDGF-BB into rats after carotid injury caused increased intimal thickening. A possible role of PDGF in the atherosclerotic lesions may be to stimulate smooth muscle cells to migrate from the media of the vessel to the intima layer, which then proliferate and produce matrix and prothrombotic molecules at this site. However, PDGF-AA appears to be less important, because no appreciable inhibition of intimal hyperplasia was observed after administration of PDGF-AA antiserum.

Analyzing signaling mechanisms induced by PDGF in SMCs, we found that PDGF-BB--induced Egr-1 binding activity was Src family kinase-dependent and tyrosine kinase-dependent. Src family kinase members are nonreceptor kinases and are activated in response to a number of external stimuli. Src family kinases associate with and are activated by many receptor tyrosine kinases and G protein-coupled receptors. Src is activated by stimulation of cells with PDGF and associated with the activated PDGF receptors. Barone and Courtneidge have demonstrated that the kinases of the Src family regulate myc activation, and that myc is necessary for PDGF-dependent DNA synthesis.

We have previously described a G protein-dependent mechanism by which PDGF-AA, but not PDGF-BB, signaling is transduced through direct coupling of the Go1,2 subunit of the trimeric G proteins to the PDGF tyrosine kinase receptor. On the basis of this finding, we analyzed the effect of PTX on PDGF-BB--induced Egr-1 activation. The results showed that PTX had no inhibitory effects and underscored our recent observation. A further explanation could be that PDGF-BB activates different signaling pathways. Connway et al have shown that the PDGF receptor activates c-Src by different ways: one involving direct binding to the PDGFR (PTX-insensitive) and one involving Gi (PTX-sensitive). Downstream targets of Src include p21ras, which activates MAPK kinase (MEK), which in turn phosphorylates ERK. The involvement of c-Src in PDGF-BB--dependent ERK activation was demonstrated by Connway et al.

Figure 6. Egr-1 decoy oligodeoxynucleotides technique. A, SMCs were pre-incubated with Egr-1 decoy oligodeoxynucleotides (10 μmol/L) or control decoy oligodeoxynucleotides (10 μmol/L) for 6 hours, followed by stimulation with PDGF-BB (10 ng/mL) for 2 hours. TF mRNA expression was quantified as described. Values are shown as mean±SD, n=6. *P<0.05 vs unstimulated control, #P<0.05 vs PDGF-BB. B, Effect of Egr-1 decoy oligodeoxynucleotides on TF protein expression. Immunoblot analysis of cellular extracts from SMCs 12 hours after stimulation with PDGF-BB. Representative blot is shown (top panel). The intensity of each band on the blot was quantitated. *P<0.05 vs unstimulated control, #P<0.05 vs PDGF-BB, n=6. C, Effect of Egr-1 decoy oligodeoxynucleotides on TF activity. TF activity was quantified as described. *P<0.05 vs unstimulated control, #P<0.05 vs PDGF-BB.

Figure 7. Reporter construct analysis of the contribution of the Egr-1 binding site to TF expression. SMCs were transfected with plasmids containing TF promoter containing either a wild-type or a mutated Egr-1 binding site (Egr-1m). After transfection, cells were stimulated with PDGF-BB for 12 hours. Luciferase assay of cell lysates was performed. *P<0.05 vs PDGF-BB--stimulated wild-type promoter, (n=3).
al.\textsuperscript{25} Results in the present study demonstrate that PDGF-BB induces a time-dependent ERK activation and that MEK1/2 inhibition by PD98059 reduces ERK activation and Egr-1 binding activity. In contrast, PDGF-BB also induces p38 MAPK activation, but inhibition of p38 MAPK by SB203580 could not inhibit PDGF-BB–mediated Egr-1 binding activity. Consistent with our results, Schwachtgen et al.\textsuperscript{27} have shown that EKRK1/2, but not JNK, signaling pathway is implicated in the induction of Egr-1 activation by shear stress. PI 3'-kinase has a central role in intracellular signal transduction. It can be activated by several different signals including PDGF, it has a number of downstream effector molecules, and it mediates many different cellular responses.\textsuperscript{28} In our study, the PDGF-BB–induced Egr-1 induction was PI 3'-kinase–independent. This is in line with a study from Jones and Agani.\textsuperscript{29} They have demonstrated that neither p38 MAPK nor PI 3-kinase pathway is involved in Egr-1 upregulation by hypoxia. Our recent studies demonstrated that ROS are induced by PDGF and consequently act as second messengers to stimulate the activation of transcription factors, including AP-1.\textsuperscript{30} Jin et al.\textsuperscript{16} have shown that Egr-1 is activated by ROS. Therefore, we studied the effect of antioxidants in our experiments. The results indicate that PDGF-BB–mediated Egr-1 activation could not be inhibited by antioxidants. These studies suggested that PDGF-BB induce different pathways in SMCs. One of these pathways involves Src family kinases, ERK, and Egr-1, whereas another ROS-dependent pathway involves G-protein, PI 3-kinase, p38 MAPK, and AP-1.

Compared to some other transactivators, Egr-1 associates with co-repressor proteins that can modulate transcription of Egr-1–dependent genes. Two co-repressors of Egr-1, NAB1 and NAB2, have been identified. Whereas NAB1 is constitutively expressed in most tissues and appears to be a general transcriptional regulator,\textsuperscript{31} NAB2 may function as an important inducible regulator of gene expression.\textsuperscript{32} Lucerna et al.\textsuperscript{33} have shown that NAB2 overexpression inhibits signals involved in the early phase of angiogenesis. In this study, we demonstrate that PDGF-BB induced the co-repressor NAB2 in a time-dependent manner, thus providing a negative feedback to limit Egr-1 activity. The time course of PDGF-BB–induced NAB2 protein expression is closely followed by the time course of PDGF-BB–mediated Egr-1 binding activity. Consistent with our results, Silverman et al.\textsuperscript{14} have shown a similar time course for NAB2 protein expression in SMCs after PMA stimulation.

The formation of an occlusive thrombus after rupture of an unstable atherosclerotic plaque leads to acute coronary events. Exposure of TF within the atherosclerotic lesions by SMC and macrophages contributes to coagulation and thrombus formation. Egr-1 binds to the TF promoter and is critically involved in TF gene regulation by inflammatory cytokines, serum, and vascular endothelial growth factor.\textsuperscript{10} PDGF is released in large amounts by activated macrophages and platelets in advanced atherosclerosis and restenosis. Therefore, we demonstrated that PDGF increased TF expression and stimulated Egr-1 binding activity. We reasoned that PDGF-BB stimulates TF expression through the Egr-1 activation. To prove this hypothesis, we used the decoy approach against Egr-1 binding sites. Our results show that Egr-1 decoy effectively inhibits TF mRNA and protein expression, as well as TF activity. Furthermore, transient transfections of SMCs with wild-type and mutated TF promoter constructs showed that the Egr-1 binding region is an important transcriptional regulator of PDGF-BB–induced TF expression. These results support the hypothesis that Egr-1 plays a functional role in the regulation of PDGF-BB–induced TF expression in SMCs.

In conclusion, the present data demonstrate that PDGF-BB induces the expression of TF via Egr-1–dependent and ERK-dependent pathway. These data provide for the first time to our knowledge a mechanistic link between PDGF-activated SMC and thrombus formation in atherosclerotic and restenosis lesions.

Acknowledgments
We thank Anne Sterzer for excellent technical assistance. The study was supported by a grant from the Deutsche Forschungsgemeinschaft Bonn/Bad Godesberg, Germany.

References


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Hypertension. 2004;44:944-951; originally published online October 18, 2004;
doi: 10.1161/01.HYP.0000146908.75091.99

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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