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Induction of Tissue Factor Expression in Endothelial Cells by Basic Fibroblast Growth Factor and its Modulation by Fenofibric acid

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Abstract

Background: Tissue factor (TF), expressed in endothelial cells (ECs) and enriched in human atherosclerotic lesions, acts as a critical initiator of blood coagulation in acute coronary syndrome. Basic fibroblast growth factor (bFGF) induces the proliferation and migration of ECs and plays a role in angiogenesis and restoration of endothelial integrity. As TF is implicated in angiogenesis, we studied the effect of bFGF on TF gene and protein expression. Methods: Human umbilical vein ECs (HUVECs) were exposed to bFGF. TF mRNA was assessed by Northern blot and TF protein was assessed by Western blot. TF promoter activity was assessed by transient transfection assay and transcription factor was identified by electro mobility shift assay.

Results: bFGF increased TF mRNA and protein expression in HUVECs. Increased TF mRNA was attenuated by inhibition of extracellular signal-regulated kinase kinase in human ECV304 cells. Transient transfection assays of the human TF promoter-luciferase construct (-786/+121 bp) demonstrated that bFGF induced transcription was dependent on the elements within the -197 to -176 bp relative to the transcription start site of the human TF gene. This region contains NF-κB like binding site. Electro mobility shift assay showed that bFGF increased nuclear translocation or DNA binding of NF-κB transcription factor to TF promoter. Nucleotide substitution to disrupt NF-κB like site reduced bFGF stimulated promoter activity. Fenofibric acid, an agonist ligand for the peroxisome proliferator activated receptor-α, reduced basal and bFGF stimulated TF expression.

Conclusions: These results indicate that bFGF may increase TF production in ECs through activation of transcription at NF-κB binding site, and control coagulation in vessel walls. Fibrate can inhibit TF expression and therefore reduce the thrombogenecity of human atherosclerotic lesions.

Background

Tissue factor (TF) is an integral membrane protein, which binds to coagulation factor VII/VIIa and initiates the coagulation cascade [1]. TF expression can be inducible with inflammatory cytokines, lipopolysaccharide (LPS) and oxidized low-density lipoprotein in fibroblast, vascular smooth muscle cells and monocytes in human atherosclerotic lesions [2]. TF expressed on the surface of vascular wall acts as the major procoagulant for thrombus formation [3].

Basic fibroblast growth factor (bFGF) elaborated by vascular cells can induce proliferation, morphological changes and migration of smooth muscle cells and endothelial cells (ECs) and modulate angiogenesis [4]. bFGF promotes re-endothelialization with functional endothelium after balloon injury or induced atherosclerosis and modulates TF expression in monocytes and smooth muscle cells in vivo [5]. In ECs TF expression is regulated through a promoter region, which encompasses potential binding sites for activator protein-1 (AP-1), nuclear factor (NF)-κB and SP-1 transcription factors [6]. LPS-induced TF expression in cells of the monocyte lineage requires functional interaction between these transcription factors [2]. However, the influence, if any, of bFGF on synthesis of TF in ECs has not been elucidated. The potential influence may well contribute to physiologic or pathophysiologic consequences of elaboration of bFGF.

The mechanisms inhibiting TF expression in ECs remain largely unexplored. Fenofibric acid is a synthetic ligand for the peroxisome proliferator activated receptor (PPAR)-α [7]. PPARα, as well as the other members of the PPAR family, are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Certain fatty acids and fibric acid derivative, which activates PPARα, reduces TF expression in human monocyte cells and macrophages in vitro [8]. However, to the best of our knowledge, there has been no detailed documentation on the effect of PPARα on TF expression in ECs under both clinical and pathological settings. Therefore, in the present study we characterized the influence of bFGF and fibric acid on elaboration of TF in vitro by cultured ECs, and sought to delineate potential mechanisms that may underlie increased TF expression.

Methods

Materials

Human umbilical vein ECs (HUVECs) were obtained from Clonetics (Walkersville, MD). Human ECV304 cell line (ECV cells) was obtained from American Type Culture Collection (Manassas, VA). Penicillin-streptomycin solution, medium 199, Dulbecco's modified Eagle's media (DMEM) and trypsin were purchased from Sigma (St. Louis, MO). Calf serum was obtained from Hyclone (Logan, UT). PD98059 was from Research Biochemical International and GF109203X and genistein were from Sigma. All other chemicals were of the highest available commercial grade.

Cell culture procedures

HUVECs and ECV cells were cultured and stimulated with recombinant human bFGF (Genzyme, Minneapolis, MN) as previously described [9]. HUVECs were incubated in DMEM without serum and exposed to bFGF for 24 hr. Cell lysates were prepared as previously described [5] and stored at -80°C. ECV cells were cultured in medium 199 containing 10% calf serum. In some experiments fenofibric acid (Kaken, Tokyo, Japan) was prepared as previously described [9], and ECV cells were preincubated with fenofibric acid for 24 hr before exposure to bFGF. Cell viability was determined by trypan blue exclusion and MTT assay (Sigma).

Assays for TF antigen by Western blotting

TF antigen was assayed by Western blotting as previously described [10]. In brief, equivalent amounts of cell lysates were electrophoresed and transferred to polyvinylidene difluoride membranes. Membranes were incubated with 0.2 μg/ml mouse anti-human TF IgG (American Diagnostica, Greenwich, NY). Membranes were washed and incubated with alkaline-phosphatase conjugated guinea pig anti-mouse IgG (Sigma) diluted 1:5000. Membranes were incubated with chemiluminescent enhancer system (Immun-Star, Bio-Rad, Hercules, California) and exposed to XAR film (Kodak, Rochester, NY), which was then developed. Bands were quantified with the use of a densitometer and ImageQuant software (Molecular Dynamics, Sunnydale, CA).

Isolation of total RNA and Northern blotting

Total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform method. Northern blot analysis was performed as described previously [9]. Gel-purified polymerase chain reaction (PCR) fragments for TF (-786 to +121 bp) and β-actin, radiolabeled with α-^{32}P-dCTP by random priming, were used as probes. Autoradiography was performed and band intensities were determined by densitometric analysis.

Promoter-luciferase vector and expression plasmid

The human TF promoter 5' flanking region from -786 to +121 (907 bp) was amplified by PCR from human genomic DNA isolated from umbilical vein with the use of forward PCR primer 5'-AGAGGCAAACTGCCAGATGT-3' (-786 to -766) and the reverse primer 5'-TGTCTACCAGTTGGCGGCCG-3' (+101 to +121), respectively [11]. The PCR product was gel-purified and subcloned into the multiple cloning sites of the pT7Blue T-Vector (Novagen, Madison, WI). The T-vector was

digested with EcoR I and Hind III and subcloned into the multiple cloning sites of the promoterless *Renilla* luciferase reporter gene vector pRL-null (Promega, Madison, WI). Basal expression of luciferase activity of the TF promoter vector was detected by the TF Full (-786/+121) luciferase vector, which includes two AP-1, three SP-1 (Egr-1), and two NF-κB binding sites. The 5' deletion mutants were generated by PCR with the use of 5' primers complementary to the TF gene sequence. Deletion mutants of TF promoter vectors were constructed as follows: pTF 1F (-513/+121) luciferase lacks distal AP-1 like binding site, but retains two NF-κB like binding sites, proximal AP-1 like binding site and distal SP-1/Egr-1 like binding site. pTF 2F (-318/+121) luciferase lacks distal NF-κB like binding site, but retains proximal AP-1 like binding site, proximal NF-κB like binding site and distal SP-1/Egr-1 like binding site. pTF 3F (-197/+121) luciferase lacks both AP-1 like binding sites and distal NF-κB like binding site, but retains proximal NF-κB like binding site and distal SP-1/Egr-1 like binding site. pTF 4F (-176/+121) luciferase lacks proximal NF-κB like binding site, but retains distal SP-1/Egr-1 like binding site. pTF 5F (-129/+121) luciferase lacks distal SP-1/Egr-1 like binding site. Point mutation of TF promoter vectors was also constructed [9]. Basal expression of luciferase activity of TF promoter vector was detected by the TF Full luciferase vector, which has normal proximal NF-κB like site (5'-CGGAGTTTCC-3'). One mutation vector has two point mutations in NF-κB like site as underlined (5'-CGGAGTTTGG-3') and another mutation vector has two point mutations in NF-κB like site (5'-CGGAGTTTGG-3') and one point mutation in SP-1/Egr-1 like site (5'-AGGCAGGGCAGGGGTG-3') as underlined.

DNA transfection and the luciferase assay

DNA transfection and the luciferase assay were performed as previously described [9]. ECV cells of approximately 80% confluency were cotransfected with each TF promoter *Renilla* luciferase fusion DNA reporter construct (20 μg of pRL vector) and a *Firefly* luciferase pGL vector to control for transfection efficiency (20 μg) introduced by electroporation (240 V, 960 μFD). These cells were cultured in DMEM with 10% calf serum for 24 hours, stimulated with bFGF (10 ng/ml) in DMEM containing 10% calf serum for 24 hours and harvested. Cell lysate luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega). Normalized luciferase activity was calculated as the ratio of luciferase activity to control vector activity. Results for each reporter construct were expressed as fold induction compared with results in transfected, unstimulated cells.

Electrophoretic mobility shift assay

For electrophoretic mobility shift assay (EMSA) ECV cells were preincubated for 24 hours with fenofibrate 100 μmol/L and then stimulated for 2 hours with bFGF 100 ng/ml before nuclear extracts were prepared. The NF-κB oligonucleotide
(ACGCGTCGGAGTTTCCTTGAACGGAGTTTCCGATC)
spanning the 2 tandem NF-κB like sites (as underlined above) in the human TF promoter was end-labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by Klenow fragment of DNA polymerase I (Amersham Life Science, Buckinghamshire, England) and purified with NucTrap column (Stratagene, La Jolla, CA). Nuclear extracts (0.3 μg) were incubated with the labeled NF-κB like oligonucleotide under standard conditions. In the indicated experiments nuclear extracts were incubated with rabbit polyclonal anti-NFκB p50, rabbit polyclonal anti-NFκB p65 or mouse monoclonal anti-c-Rel IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or nonspecific IgG before the addition of radiolabeled NF-κB probes. DNA-protein complexes were electrophoretically separated. Specificity was determined by addition of an excess of unlabeled NF-κB oligonucleotide to the nuclear extracts before formation of DNA-protein complexes.

Statistics

Data are means ± SD. Differences were assessed by analysis of variance with Bonferroni's least significant post hoc tests for comparisons within multiple groups. Significance was defined as $P < 0.05$.

Results

Effects of bFGF on TF expression in ECs

bFGF (100 ng/ml) increased TF mRNA accumulation in HUVECs as assessed by Northern blotting (Figure 1A). An increased response to bFGF was evident by 1 hr with further increases at 2 hr and 4 hr but it decreased after 8 hr. bFGF increased TF protein accumulation in the cell lysate in a concentration-dependent fashion (Figure 1B). Peak effects were seen with 10 ng/ml bFGF. An increase in TF mRNA expression induced by bFGF was seen with ECV cells as well (Figure 1C). bFGF increased TF protein accumulation in the cell lysate in a concentration-dependent fashion (Figure 1D). Peak effects were seen with 10 ng/ml. The response was diminished somewhat with concentrations of 100 ng/ml. Total protein content in the cell lysates was not altered by bFGF (results not shown).

Effects of PD 98059 and GF109203X on TF mRNA

To investigate the intracellular mechanisms involved in the induction of TF mRNA ECV cells were treated for 1 hr with each of the various inhibitors of intracellular signaling pathway before addition of bFGF (Figure 2). Inhibition of extracellular signal-regulated kinase (ERK) kinase with PD98059 blocked basal and bFGF stimulated TF mRNA levels. In contrast, GF109203X, an inhibitor of protein kinase C (PKC) pathway, blocked only bFGF stimulated TF mRNA levels and had no significant effect on

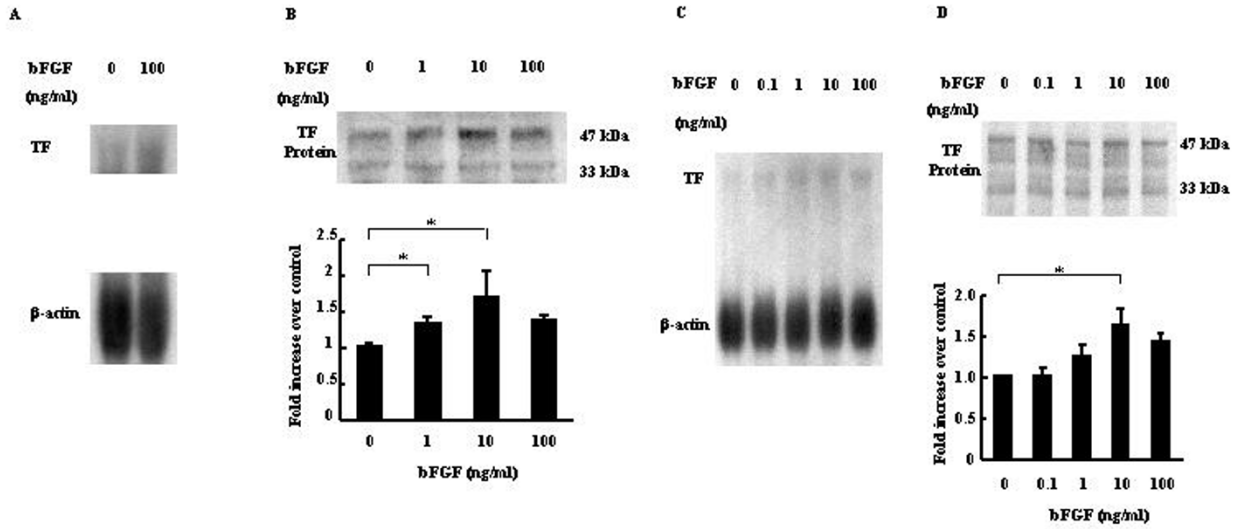

Figure 1
(A) Upregulation of TF mRNA in HUVECs stimulated with bFGF (100 ng/ml) for 4 hr. The expressions of TF mRNA and of β-actin were determined by Northern blot analysis as described in Methods (n = 3). A representative autoradiograph is shown. (B) Effects of bFGF on concentrations of TF in HUVECs. Confluent cells were serum starved for 24 hr and then incubated with fresh serum free media containing bFGF (0 – 100 ng/ml) for 4 hr. Concentrations of TF in cell lysates were assayed by Western blotting as described in Methods (n= 6). The upper panel shows a representative blot from six separate experiments. Values are means ± SD of fold increase over control without bFGF. *P < 0.05 as compared to control. (C) Upregulation of TF mRNA in ECV cells stimulated with bFGF (0–100 ng/ml) for 4 hr. Expressions of TF mRNA and of β-actin were determined by Northern blot analysis as described in Methods (n = 3). A representative autoradiograph is shown. (D) Effects of bFGF on concentrations of TF in ECV cells. Confluent cells were serum starved for 24 hr and then incubated with fresh serum free media containing bFGF (0 – 100 ng/ml) for 4 hr. Concentrations of TF in cell lysates were assayed by Western blotting as described in Methods (n = 6). The upper panel shows a representative blot from six separate experiments. Values are means ± SD of fold increase over control without bFGF. *P < 0.05 as compared to control.

basal TF mRNA levels. Genistein, an inhibitor of tyrosine kinase, had no significant effect, either (result not shown). β-actin mRNA levels did not change in any experimental conditions.

Determination of the DNA regions critical for basal and bFGF inducible TF transcriptional activity

To identify the basal and bFGF responsive elements in the 5' flanking region of the TF gene, transient transfections with several TF promoter-luciferase reporter constructs were performed in ECV cells (Figure 3). With progressive 5' deletion of the TF promoter basal activity was reduced first (TF Full vs pTF1) (Figure 3A). Lower basal promoter activity observed in pTF1 suggests that the region between -786 bp and -513 bp may contain enhancer element(s). Furthermore, upon deletion of the region at -513 to -197 bp basal activity was increased (pTF3 vs pTF1, pTF3 vs pTF2), suggesting that distal NF-κB and proximal AP-1 sites are not so important for basal promoter activity. But deletion of the region at -197 to -129 bp reduced basal

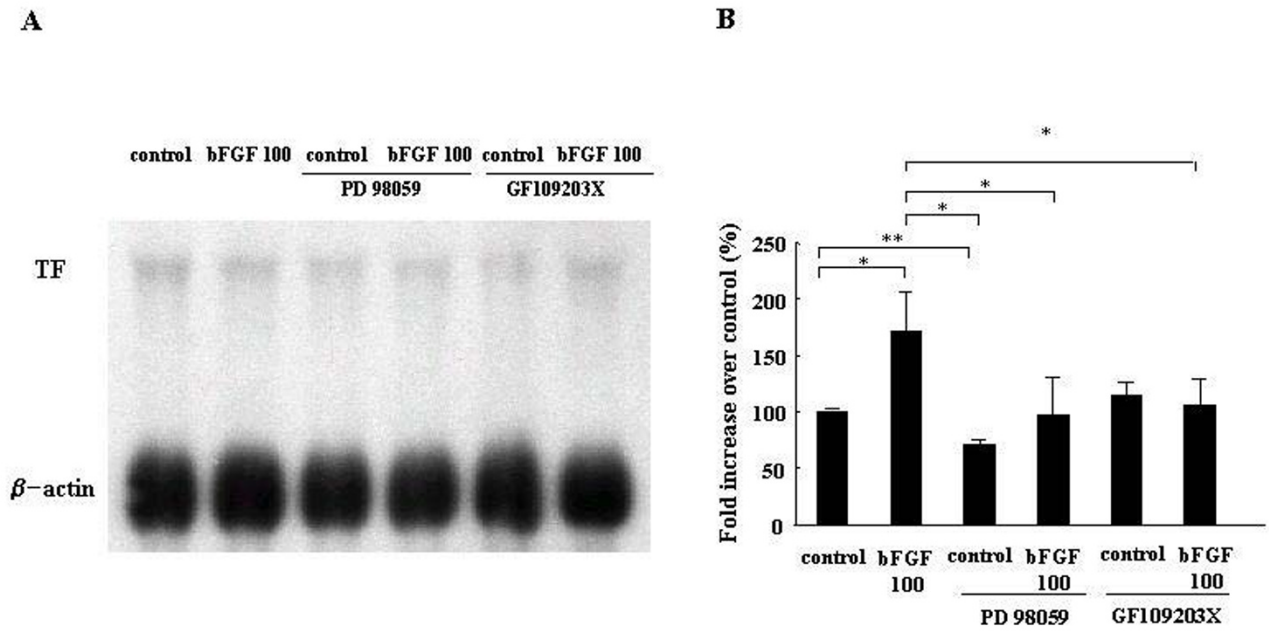

Figure 2
Effects of PD98059 and GF109203X on TF mRNA. (A) A representative autoradiograph from three separate experiments. (B) Effects of PD98059 (30 μM) and GF109203X (2 μM) on TF mRNA levels. ECV cells were incubated with or without indicated inhibitors for 1 hr and then bFGF was added. Cells were incubated for 4 hr. Total RNA was isolated and analyzed by Northern blot. PAI-1 mRNA levels were analyzed by densitometry and normalized to β-actin signals. Values are means ± SD (n = 6) of fold increase over control without bFGF. *$p < 0.05$ and **$p < 0.01$.

activity (pTF4 vs pTF3, pTF5 vs pTF3, TF full vs pTF4, TF full vs pTF5), suggesting that proximal NF-κB and distal SP-1/Egr-1 sites are important for basal promoter activity. To identify the 5' flanking region of the TF gene responsible for the effects of bFGF, transient transfections with several TF promoter-luciferase reporter constructs were performed in ECV cells. bFGF increased promoter driven luciferase activity. Relative to the largest promoter fragment tested, bFGF effect was reduced with deletion of the region at -786 to -513 bp and -513 to 318 bp. Deletion of the region at -197 to -176 bp completely abolished the bFGF effect. These data indicate that the major sequence determinant of responsiveness to bFGF resides between -197 to -176 bp. This region contains the binding site for NF-κB. Deletion of the region at -176 to -129 bp, which contains the binding site for SP-1/Egr-1, resulted in no further reduction. To further characterize the responsible region a mutant construct containing 2-nucleotide substitutions in proximal NF-κB like site was generated. Compared with the bFGF induced increased promoter activity in the wild-type substitutions in the NF-κB like site reduced both basal and bFGF inducible promoter activities (Figure 3B). Substitutions in both NF-κB like site and SP-1/Egr-1 like site further reduced basal promoter activity. These data indicate that proximal NF-κB element at -197 to -176 bp and distal SP-1/Egr-1 element at -176 to -129 bp regulate basal TF promoter activity, and that proximal NF-κB element at -197 to -176 bp regulates bFGF inducible TF promoter activity.

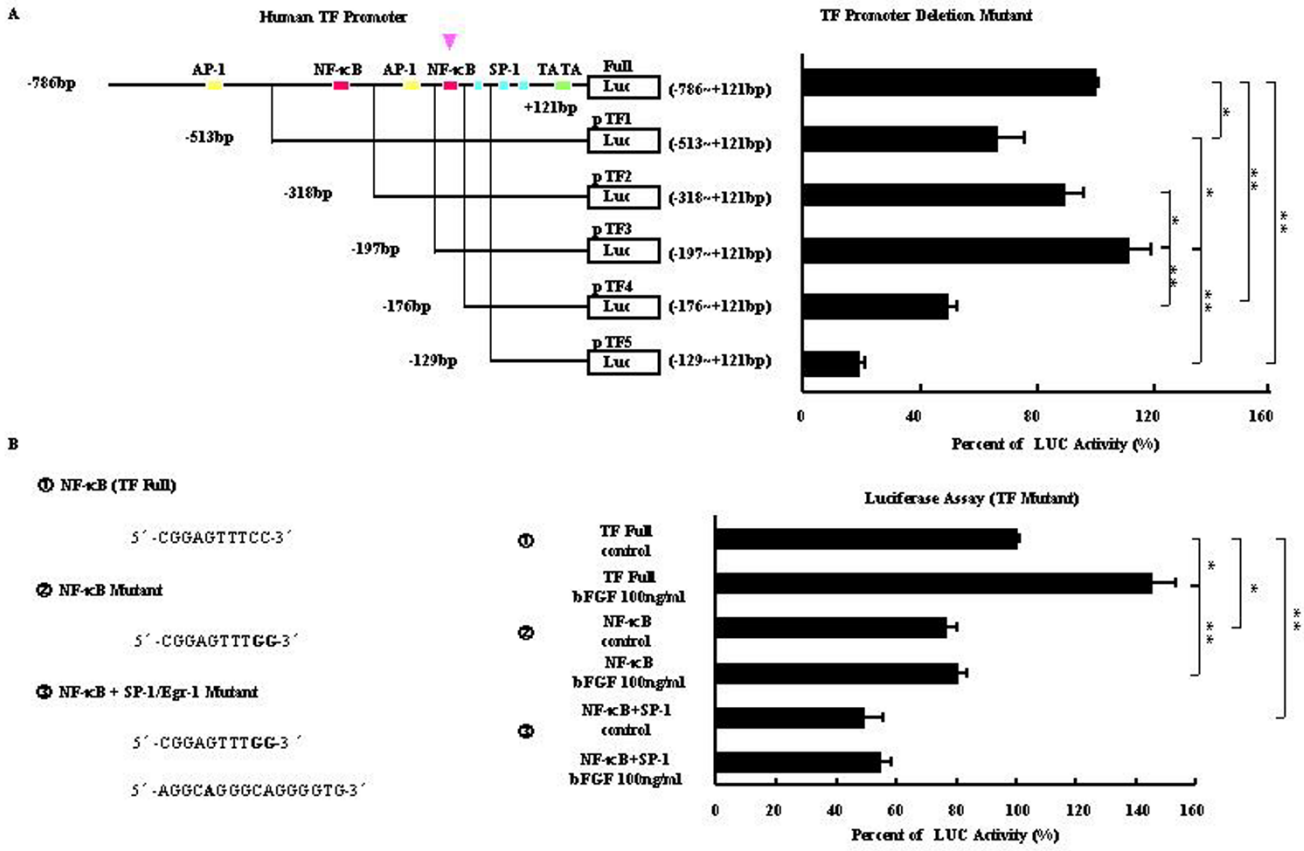

Figure 3

(A) Effects of progressive 5' deletions of the TF gene on basal and bFGF inducible TF transcriptional activity. For determination of promoter activity raw data were tabulated as the ratio of *firefly* luciferase activity to that of *Renilla* luciferase activity (means ± SD, n = 18), and values were reported as relative to the value for the control plasmid (assigned a value of 100). *p < 0.05 and **p < 0.01. Constructs of transfected plasmids are indicated at the left panel. TATA: TATA box. (B) Effects of point mutations in NF-κB like site and SP-1/Egr-1 like site on TF promoter activity (n = 3). Promoter activity was determined and values were reported as relative to the value for the TF full without bFGF stimulation. Basal expression of luciferase activity of TF promoter vector was detected by the TF Full luciferase vector, which has normal NF-κB like site (5'-CGGAGTTTCC-3'). One mutation vector has two point mutations in NF-κB like site as underlined (5'-CGGAGTTTGG-3'). Another mutation vector has two point mutations in NF-κB like site as underlined (5'-CGGAGTTTGG-3') and one point mutation in SP-1/Egr-1 like site (5'-AGGCAGGGCAGGGGTG-3'). *p < 0.05 and **p < 0.01.

Electrophoretic mobility shift assay

To determine whether NF-κB binding protein was responsible for bFGF effect EMSA was performed using oligonucleotides corresponding to the TF NF-κB like site. bFGF induced nuclear translocation and DNA binding of the transcription factor NF-κB to the TF promoter (Figure 4). The addition of an excess of cold probe inhibited DNA-protein complex formation. The addition of the p65 antibody induced a dramatic shift of the band. The addition of the p50 antibody effectively inhibited interaction between NF-κB protein and oligonucleotide containing NF-κB binding site without a dramatic shift of the band. The addition of the c-Rel antibody did not affect the migration of the band. These results show that NF-κB complexes contained p65 predominantly and p50 and c-Rel in only a minor portion of the band.

Effects of fenofibric acid

Fenofibric acid diminished both basal and bFGF inducible TF mRNA expression in ECV cells as assessed by Northern blotting (Figure 5A,5B). When fenofibric acid (100 μM) was present in media of ECV cells transfected with the TF promoter-luciferase reporter construct, both basal TF promoter activity and bFGF (100 ng/ml) stimu-

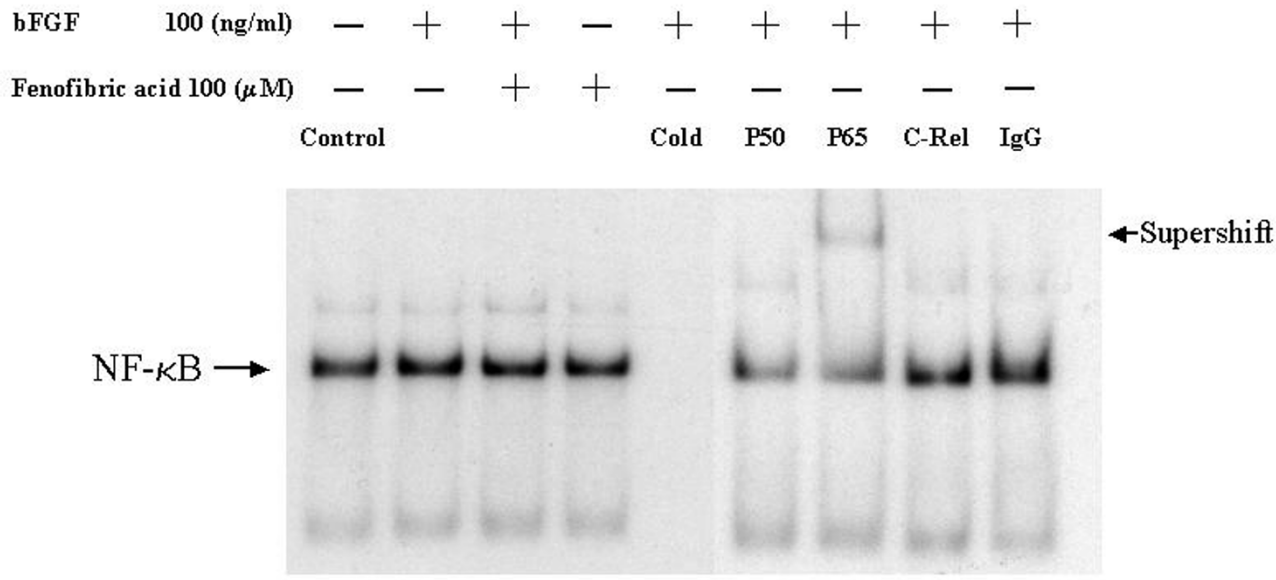

Figure 4
EMSA of ECV cells stimulated with bFGF for 2 hr. Some cells were pretreated with fenofibric acid for 24 hr before bFGF stimulation. EMSA was performed using probes from NF-κB like site of TF promoter and anti-human p50, p65 and C-Rel IgGs or control rabbit IgG. The panel shows a representative autoradiograph from three separate experiments.

lated TF promoter activity were diminished (Figure 5C). Thus, fenofibric acid inhibits TF expression at least partly at the level of transcription. Fenofibric acid did not inhibit bFGF induced nuclear translocation or DNA binding of NF-κB like transcription factor to the TF promoter (Figure 4). Fenofibric acid markedly diminished but did not totally abolish TF protein accumulation in the lysate of ECV cells exposed to bFGF (100 ng/ml) (Figure 5D). At a concentration of 100 μM it suppressed both basal and bFGF inducible TF protein accumulation. Cell viability was unaffected by fenofibric acid at the concentrations used (results not shown).

Discussion

In this study bFGF was shown to influence production of TF in ECs. Increased TF protein correlated with increased mRNA and activity of the promoter. Fenofibric acid, an agonist ligand for PPARα, reduced basal and bFGF stimulated TF expression.

The binding of bFGF to its specific receptor triggers a cascade of events leading to signal transductions including ERK, mitogen-activated protein kinase, Ras and PKC pathways [12,13]. The results in this study suggest that the induction mechanism of TF expression by bFGF may involve ERK kinase. These results are consistent with the recent study, which demonstrated that bFGF induced I-κB degradation and NF-κB activation through ERK1/2 pathway in smooth muscle cells [13]. In addition, platelet-derived growth factor induces TF expression in smooth muscle cells by an ERK pathway-dependent mechanism and, in part, by Ras and PKC pathway-dependent mechanisms [14] and ERK activity is required for NF-κB activation by IL-1β [15]. Our study demonstrated that bFGF

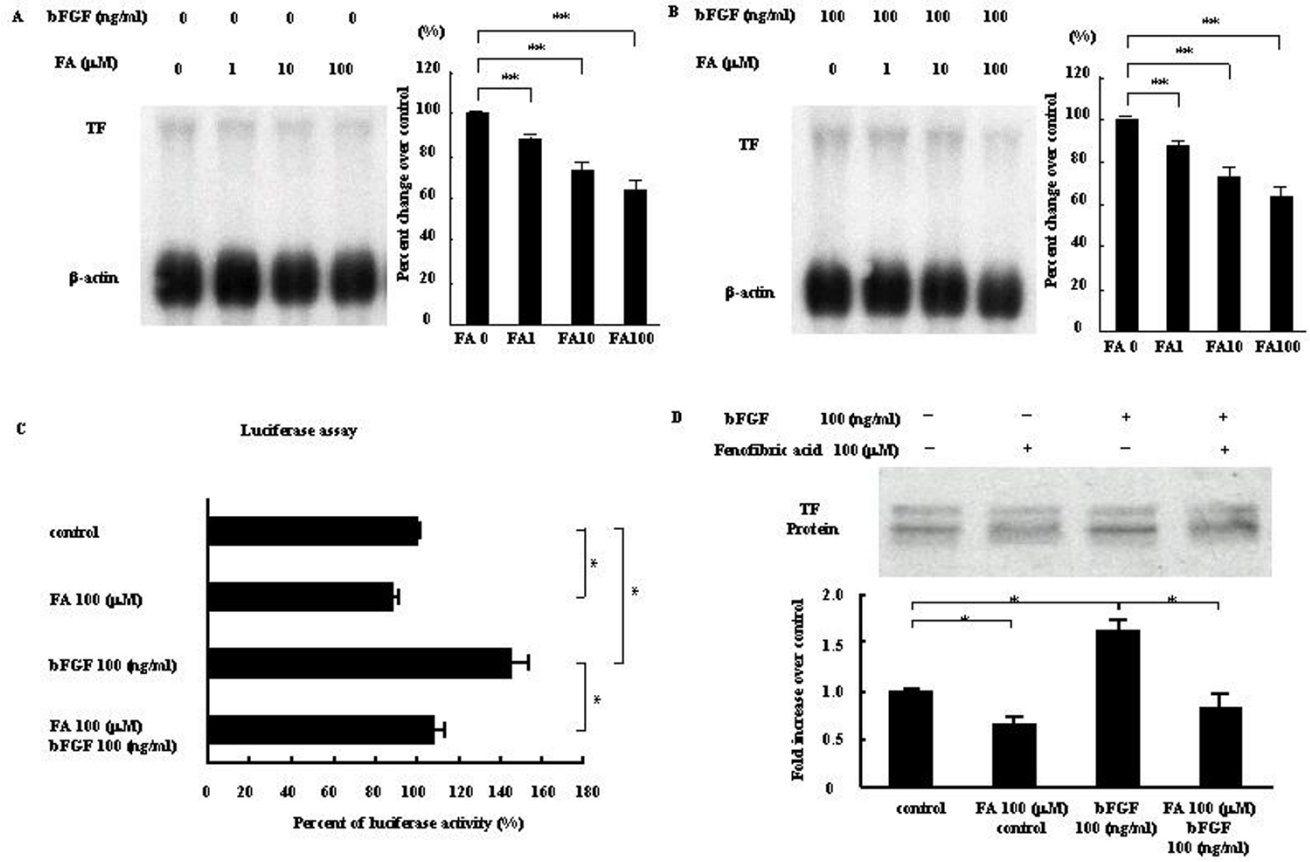

Figure 5

Downregulation of TF by fenofibric acid. (A) Confluent ECV304 cells were serum starved for 24 hr and incubated with fresh serum free media containing fenofibric acid (100 μM) for 20 hr. The magnitudes of expression of TF mRNA and of β-actin were determined by Northern blot analysis as described in Methods (n = 3). A representative autoradiograph is shown on the left. Values are means ± SD (n = 6) of percent change over control without bFGF. **p < 0.01 compared to control. (B) Confluent ECV304 cells were serum starved for 24 hr and incubated with fresh serum free media containing fenofibric acid (100 μM) for 20 hr, and exposed to bFGF (10–100 ng/ml) for 4 hr. The magnitudes of expression of TF mRNA and of β-actin were determined by Northern blot analysis as described in Methods (n = 3). A representative autoradiograph is shown on the left. Values are means ± SD (n = 6) of percent change over control without bFGF. **p < 0.01 compared to control. (C) Effects of fenofibric acid (100 μM) on basal and bFGF inducible TF transcriptional activity. For determination of promoter activity raw data were tabulated as the ratio of *firefly* luciferase activity to that of *Renilla* luciferase activity (means ± SD, n = 4), and values were reported as relative to the value for the control plasmid (assigned a value of 100). FA: fenofibric acid. *p < 0.05 (D) Effects of fenofibric acid on the concentration of TF in ECV cell lysates. Confluent cells were serum starved for 24 hr, incubated with fresh serum free media with or without fenofibric acid (100 μM) for 20 hr, and exposed to bFGF (100 ng/ml) for 24 hr. Concentrations of TF in cell lysates were assayed by Western blotting. Values are means ± SD (n = 6) of fold increase over control without bFGF. FA: fenofibric acid. *p < 0.05

could act in the same manner on TF expression in human ECs.

Deletion analysis of TF promoter revealed that a region between -197 bp and -176 bp contains the elements mediating the bFGF response. A search for the putative transcription factor binding sites within this region showed it to contain an NF-κB like site (CGGAGTTTCC) at -188 to -178 bp. Furthermore, basal promoter activity was regulated by this NF-κB like site and by SP-1/Egr-1 like site (GAGGCGGGGCAGGGGT) present at -169 to -154 bp. NF-κB, AP-1 and SP-1 elements in human TF promoter are involved in the activation of TF gene in ECs [6,16]. Our results suggest that NF-κB element is impor-

tant in TF gene activation after bFGF stimulation in ECs. In addition, SP-1/Egr-1 element is likely required for basal transcription from the TF promoter. The role of transcription repressor of the TF gene in ECs [17] needs further investigation.

PPARα negatively regulates vascular inflammatory gene response by negative cross-talk with NF-κB and AP-1 [18]. PPARα is expressed in ECV cells [19]. PPARα ligand, fenofibric acid, diminished both basal and bFGF inducible expression of TF, potentially reducing the thrombogenicity of atherosclerotic lesions. Deletion of the promoter region containing the NF-κB site inhibited the effect of bFGF and EMSA suggested that fenofibric acid did not inhibit nuclear translocation or DNA binding of the NF-κB like transcriptional factor to the TF promoter. Thus, inhibition of TF synthesis by fenofibric acid may occur transcriptionally as a result of PPARα repression of NF-κB signaling. Further studies are in progress to elucidate the mechanism of fenofibric acid effect using cells from arterial sources. These data indicate that activation of PPARα results in the downregulation of the TF gene and provide new insight into how PPARα-activating fibric acid derivatives might influence atherothrombosis in patients with vascular disease. The present study tested a novel role for PPARα in the control of atherosclerotic plaque thrombogenicity through its effects on TF expression in ECs and suggested that fibric acid may reduce thrombotic complications of atherosclerosis.

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