Fenofibrate Inhibits Endothelin-1 Expression by Peroxisome Proliferator–Activated Receptor α–Dependent and Independent Mechanisms in Human Endothelial Cells
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Fenofibrate Inhibits Endothelin-1 Expression by Peroxisome Proliferator–Activated Receptor α–Dependent and Independent Mechanisms in Human Endothelial Cells

Corine Glineur, Barbara Gross, Bernadette Neve, Corinne Rommens, Gerard T. Chew, Françoise Martin-Nizard, Fernando Rodríguez-Pascual, Santiago Lamas, Gerald F. Watts, Bart Staels

Objective—Dyslipidemia contributes to endothelial dysfunction in type 2 diabetes mellitus. Fenofibrate (FF), a ligand of the peroxisome proliferator–activated receptor-α (PPARα), has beneficial effects on microvascular complications. FF may act on the endothelium by regulating vasoactive factors, including endothelin-1 (ET-1). In vitro, FF decreases ET-1 expression in human microvascular endothelial cells. We investigated the molecular mechanisms involved in the effect of FF treatment on plasma levels of ET-1 in type 2 diabetes mellitus patients.

Methods and Results—FF impaired the capacity of transforming growth factor-β to induce ET-1 gene expression. PPARα activation by FF increased expression of the transcriptional repressor Krüppel-like factor 11 and its binding to the ET-1 gene promoter. Knockdown of Krüppel-like factor 11 expression potentiated basal and transforming growth factor-β–stimulated ET-1 expression, suggesting that Krüppel-like factor 11 downregulates ET-1 expression. FF, in a PPARα-independent manner, and insulin enhanced glycogen synthase kinase-3β phosphorylation thus reducing glycogen synthase kinase-3 activity that contributes to the FF-mediated reduction of ET-1 gene expression. In type 2 diabetes mellitus, improvement of flow-mediated dilatation of the brachial artery by FF was associated with a decrease in plasma ET-1.

Conclusion—FF decreases ET-1 expression by a PPARα-dependent mechanism, via transcriptional induction of the Krüppel-like factor 11 repressor and by PPARα-independent actions via inhibition of glycogen synthase kinase-3 activity. (Arterioscler Thromb Vasc Biol. 2013;33:621-628.)

Key Words: endothelin-1 ■ endothelium ■ glycogen synthase kinase-3 ■ peroxisome proliferator–activated receptor-α ■ type 2 diabetes mellitus

Dyslipidemia contributes to endothelial dysfunction in type 2 diabetes mellitus (T2D) and involves vasoactive peptides, such as endothelin-1 (ET-1). Clinical trials suggest fenofibrate (FF) improves microvascular complications of T2D. FF is a ligand of the peroxisome proliferator–activated receptor-α (PPARα), which on heterodimerization with the Retinoid X Receptor binds to peroxisome proliferator response elements (PPRE) in target gene promoters and activates the transcription of genes, hence improving diabetic dyslipidemia. FF also displays pleiotropic nonlipid effects such as reducing fibrinogen, C-reactive protein, and uric acid levels and the expression of adhesion molecules, tissue factor, interleukin-6, COX-2, and ET-1 and also improving flow-mediated dilatation via an increase in endothelial nitric oxide synthase expression and activity. In macrovascular endothelial cells (ECs), PPARα activation inhibits signaling pathways, such as activator protein (AP)-1 and nuclear factor-κB, thus collectively decreasing vascular inflammation.

ET-1 is a potent vasoconstrictor expressed both in microvascular and in macrovascular ECs. Plasma ET-1 levels are elevated in patients with diabetes mellitus. Genetic factors relate to diabetic microvascular complications and association of the transforming growth factor-β (TGFβ) T869C gene polymorphism with an increased risk of nephropathy and retinopathy in T2D has been observed. The ET-1 promoter has binding sites for AP-1 and Smad transcription factors, which functionally cooperate through cAMP-response element binding protein/p300 to mediate TGFβ-induced transcriptional activation of the ET-1 gene. TGFβ binding to its transmembrane receptor

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kinases initiates intracellular signaling cascades, including Smad-dependent \(^1\) and also Smad-independent pathways.\(^2\)

Studies on Smad-independent gene expression have identified a novel family of TGF\(\beta\)-inducible Sp1/Krüppel-like factors (KLF)-like transcription factors contributing to the effects of TGF\(\beta\) on cell growth and differentiation.\(^3\) KLF11 is a member of this family, which mediates TGF\(\beta\)-induced actions, for example, the repression of c-myc by TGF\(\beta\) in epithelial cells. Mechanistically, KLF11 accomplishes this function by promoter binding via specific GC-rich sites and recruiting the Sin3-histone deacetylase chromatin remodeling complex.\(^4\)

Glycogen synthase kinase-3 (GSK3) is a widely expressed and highly conserved serine/threonine protein kinase, which controls TGF\(\beta\) signaling.\(^5\) Thus, GSK3 mediates TGF\(\beta\)-induced phosphorylation of Smad3 at the linker region.\(^6\) GSK3 is different from other kinases because it is generally highly active in resting cells, but inhibited in response to cellular signals,\(^7\) including signals activating receptor tyrosine kinases that activate the phosphatidylinositide 3-kinase (PI3K)/Akt pathway.\(^8\)

The immortalized human microvascular EC line (HMEC-1) was a generous gift of F.J. Candal (Atlanta, GA).\(^9\) The KLF11 expression vector was a gift from R. Urrutia (Rochester, MN).

Materials and Methods

RNA Analysis

HMEC-1 cells were incubated with vehicle or FF (100 \(\mu\)mol/L) in MCDB131 medium containing 0.2% fetal calf serum, with or without TGF\(\beta\) (10 ng/mL), GSK3 inhibitor IX (50 nmol/L), Kenpaullone (2.3 \(\mu\)mol/L), Wortmannin (1 \(\mu\)mol/L), or insulin (100 \(\mu\)mol/L). For RNA quantification, see Methods in the online-only Data Supplement.

KLF11-Luc Promoter Constructs

A 2-\(\times\)kb KLF11 promoter region (\(-1910+144\)) was amplified from genomic DNA as described in Methods in the online-only Data Supplement.

Transfections

HMEC-1 cells were seeded at 40\(\times\)10\(^4\) cells/well in a 24-well plate and transfected with PromoFectin-HUVEC transfection reagent (Promocell GmbH) as described in Methods in the online-only Data Supplement.

Western Blot Analysis

Proteins were resolved by SDS-PAGE electrophoresis followed by western blotting using specific antibodies (antiphospho-GSK-3\(\alpha/\beta\)-Ser-21/9) [Cell Signaling Technology, Ozyme], goat polyclonal antibody anti-KLF11:sc-23162 [Santa Cruz, TEBU-Bio], and monoclonal anti-\(\beta\)-actin:clone AC-15 [Sigma]) and the Immobilon Western HRP chemiluminescent substrate (Millipore).

Figure 1. Fenofibrate (FF) decreases endothelin-1 (ET-1) and increases Krüppel-like factor 11 (KLF11) mRNA and protein expression in human microvascular endothelial cell line-1 (HMEC-1) cells. HMEC-1 cells were treated with vehicle or FF in the presence or absence of transforming growth factor-\(\beta\) (TGF\(\beta\)) and KLF11 mRNA analyzed using real-time quantitative polymerase chain reaction and normalized to cyclophilin mRNA (means\(\pm\)SEM of triplicate determinations; A to C). ET-1 secretion was analyzed by ELISA and KLF11 protein by western blotting (A, D). Band intensity was analyzed using the ImageQuant software (values indicated). Statistical differences were calculated using unpaired \(t\)-test (1-tailed) (vehicle vs FF 100 \(\mu\)mol/L; \(P\)=0.0009 for ET-1 mRNA expression; vehicle vs FF 50 \(\mu\)mol/L; \(P\)=0.013; vehicle vs FF 100 \(\mu\)mol/L; \(P\)=0.0009; TGF\(\beta\) vs TGF\(\beta\)+FF 50 \(\mu\)mol/L; \(P\)=0.016; TGF\(\beta\) vs TGF\(\beta\)+FF 100 \(\mu\)mol/L; \(P\)=0.0009 for ET-1 mRNA expression; vehicle vs FF 50 \(\mu\)mol/L; \(P\)=0.013; vehicle vs FF 100 \(\mu\)mol/L; \(P\)=0.0009; TGF\(\beta\) vs TGF\(\beta\)+FF 50 \(\mu\)mol/L; \(P\)=0.0004 for ET-1 secretion; vehicle vs FF; \(P\)=0.024 for ET-1 at 24 h and TGF\(\beta\) vs TGF\(\beta\)+FF; \(P\)=0.0374 for ET-1 at 8 h; \(P\)=0.00019 for ET-1 at 16 h and vehicle vs FF: \(P\)=0.0005 for KLF11 at 8 h; \(P\)=0.0156 for KLF11 at 16 h; \(P\)=0.0002 for KLF11 at 24 h and TGF\(\beta\) vs TGF\(\beta\)+FF; \(P\)=0.0462 for KLF11 at 8 h; \(P\)=0.0016 for KLF11 at 24 h). ns indicates not significant.

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation experiments were performed as described in Methods in the online-only Data Supplement.

Statistical Analyses

Data are expressed as the means\(\pm\)SEM, and differences analyzed for statistical significance by 1-way ANOVA or the Student \(t\)-test (1-tailed).

Human Study

Thirty-five T2D subjects of similar age\(^{10}\) were randomized, double-blind to treatment with FF 200 mg/d or placebo for 12 weeks. Plasma ET-1 was measured at baseline and after treatment using a high-sensitivity radioimmunoassay (Biotrak

Downloaded from http://atvb.ahajournals.org/ at Centro Biologia Molecular Severo Ochoa on April 25, 2014
RPA 545, Amersham Biosciences, UK). Treatment effects (FF and placebo) on plasma ET-1 levels were analyzed using paired t test (within treatment group) and the Mann–Whitney U test (between treatment groups).

**Results**

**FF Decreases ET-1 mRNA While Increasing KLF11 Expression in HMEC-1 Cells**

We previously showed that PPARα activators downregulate human ET-1 promoter activity by reducing AP-1 DNA-binding activity after thrombin stimulation. Because ET-1 expression is also TGFβ-regulated, we evaluated the effect of FF treatment on TGFβ induction of ET-1 in HMEC-1 cells (Figure 1A and 1B). FF inhibited the TGFβ-mediated increase in ET-1 expression in a dose- and time-dependent manner, an effect most pronounced at 100 μmol/L of FF and already observed within 8 hours of treatment. The effects of FF on ET-1 mRNA expression were accompanied by similar changes in ET-1 secretion in the cell culture medium (Figure 1A). Because a transcriptional repressor could be involved in this action of FF, because KLF transcription factors are involved in cardiovascular diseases and mediate TGFβ-induced actions, and because recent transcriptomic analysis proposed KLF11 as a PPARα-regulated gene in human hepatocytes, we measured KLF11 expression in HMEC-1 cells. FF treatment induced KLF11 mRNA (Figure 1C) already within 8 hours. Because KLF11 is a TGFβ-inducible early gene, we also investigated the effect of TGFβ on KLF11 expression in confluent HMEC-1 cells treated or not with FF. Whereas FF potently increased KLF11 expression, no further increase occurred on cotreatment with TGFβ (Figure 1C). FF also increased KLF11 protein expression (Figure 1D).

**FF-Activated PPARα Stimulates KLF11 Transcription via a PPRE in the KLF11 Promoter**

Computer-assisted transcription factor mapping using Genomatix (http://www.genomatix.de/) identified a putative PPRE in the human KLF11 promoter at position −267/−244 (Figure 2A). To assess whether PPARα regulates KLF11 promoter activity, the upstream regulatory sequence (−1903/+153) of the human KLF11 gene was cloned in front of a luciferase reporter vector (PPARα wild-type (WT)) or a PPARα expression (black) or pSG5 control vectors (white) (meansSEM of triplicate determinations). Statistical differences were calculated using unpaired t test (1-tailed) (vehicle vs FF; P<0.0001 for transfection with hPPARα and −2 kb-KLF11-Luc; pSG5 vs hPPARα: P<0.0006 for cells transfected with the construct containing the WT KLF11 promoter). Electrophoretic mobility shift assay was performed with radiolabeled KLF11 PPRE or a DR1 consensus probe and in vitro translated PPARα and Retinoid X Receptor (RXR) proteins. Competition assays used increasing amounts of cold WT or mutant oligonucleotides and supershift used an anti-PPARα antibody (Ab). Chromatin immunoprecipitation (ChIP) assays were performed using anti-PPARα antibody. Confluent HMEC-1 cells were treated with vehicle or FF for 24 hours. Total extracts were used as controls. Samples were analyzed by quantitative polymerase chain reaction (meansSEM of triplicate determinations). Statistical differences were calculated using unpaired t test (1-tailed; vehicle vs FF: P<0.0008). The sequences and positions of oligonucleotides used in the ChIP assays are indicated in (A, horizontal arrows). RLU indicates relative light units.
assay analysis using a radiolabeled oligonucleotide corresponding to the (−267/−244) \( KLF11 \) promoter sequence, containing the PPRE, showed binding of the PPARα/Retinoid X Receptor heterodimer to the probe which is competed by cold wild-type, but not mutated PPRE oligonucleotide (Figure 2D). Finally, chromatin immunoprecipitation assays showed PPARα binding to the \( KLF11 \) promoter increased by FF treatment (Figure 2E).

**FF Increases KLF11 Binding to the \( ET-1 \) Promoter, Which Contributes to the Reduction of Basal and TGFβ-Induced \( ET-1 \) Expression in HMEC-1 Cells**

To test whether PPARα induction of KLF11 accounts for the effect of FF on \( ET-1 \) expression, we investigated whether KLF11 binds to the \( ET-1 \) promoter in cell. In silico analysis identified potential KLF11 binding sites in the −650-bp region of the \( ET-1 \) promoter (Figure 3A). Chromatin immunoprecipitation assays using primer pairs covering these putative KLF binding sites showed specific binding of KLF11 on the \( ET-1 \) promoter in confluent HMEC-1 cells, which was increased by FF (Figure 3A).

siRNA knockdown of KLF11 or PPARα expression to, respectively, 50% and 80% of control (Figure 3C) enhanced TGFβ-induced \( ET-1 \) mRNA expression, suggesting that both PPARα and KLF11 exert inhibitory actions on TGFβ-induced \( ET-1 \) expression (Figure 3B).

**The PI3K/Akt/GSK3 Pathway Is Involved in the \( ET-1 \) mRNA Decrease by FF**

Surprisingly, TGFβ-induced \( ET-1 \) expression was still reduced by FF, albeit to a lesser extent, on siRNA knockdown of PPARα, suggesting PPARα-independent mechanisms (Figure 4A). Because the PI3K/Akt/GSK3 pathway also mediates TGFβ signaling,16 we analyzed the effect of FF on GSK3β phosphorylation in HMEC-1 cells. FF increased the inactivating Ser-9 phosphorylation of GSK3β (Figure 4B). The induction of GSK3β phosphorylation was observed early and remained elevated for 24 hours, suggesting that FF-induced GSK3 phosphorylation could contribute to the regulation of \( ET-1 \) expression. To confirm the implication of the GSK3 pathway in the decrease of \( ET-1 \) mRNA expression by FF, different GSK3 inhibitors were tested on confluent HMEC-1 cells. Treatment for 24 hours with the GSK3 inhibitors IX and Kenpaullone decreased \( ET-1 \), but not KLF11 mRNA expression (Figure 4C and 4D). These results suggest that FF-mediated reduction in
GSK3 activity contributes to the decrease of ET-1 mRNA levels in a KLF11-independent way. Because insulin receptor activation in the endothelium induces the PI3K/Akt pathway and vasodilatation, we investigated also the effect of insulin on ET-1 expression. Insulin decreased ET-1 expression in HMEC-1 cells (Figure 4E), an effect reverted by wortmannin, an inhibitor of Akt. Moreover, insulin strongly increased the inhibitory phosphorylation of GSK3, which was reduced by treatment with wortmannin (Figure 4F). Moreover, wortmannin treatment increased ET-1 expression in line with a role of Akt/GSK3 pathway in ET-1 regulation, even on KLF11 knockdown (Figure 4G). On KLF11 knockdown, FF still inhibited the wortmannin-mediated increase of ET-1 expression, suggesting that FF acts downstream of Akt.

**Discussion**

Our previous studies showed that FF improves macro- and microcirculatory function. We now investigated the molecular mechanisms underlying the beneficial effect of FF on microcirculation in diabetes mellitus by studying ET-1 regulation. In human microvascular endothelial HMEC-1 cells, FF significantly decreased ET-1 mRNA, an effect most pronounced on TGFβ activation. We thus hypothesized that a transcriptional repressor accounts for this action of FF. KLF11 has been recently identified by Affymetrix GeneChip technology as a PPARα-induced gene in mouse and human hepatocytes. We show that KLF11 is a bona fide PPARα target gene in human ECs. KLF11 induction by FF occurs at the transcriptional level via a PPRE located near its transcription start site. KLF11 regulates ET-1 because reduction of endogenous KLF11 by siRNA increased ET-1 expression. Therefore, we identify a novel molecular pathway regulating ET-1 expression through PPARα activation in the endothelium.
on the PI3K/Akt-GSK3 pathway as it is involved in TGFβ expression. Therefore, we examined the effects of FF

ET-1 expression by interfering with Smad and AP-1 sites. Therefore, KLF11 may repress basal and TGFβ putative KLF11 binding sites in the proximity of Smad and promoter.14 The present study identified, in the factors at specific binding sites within the proximal ET-1 promoter remains to be determined. Wortmannin, which inhibits insulin-activation of Akt,31 induced ET-1 especially on KLF11 knockdown. However, on KLF11 expression reduction, FF still inhibited ET-1-induction by wortmannin illustrating the existence of mechanisms different from KLF11 and downstream the Akt pathway. In line, potential roles of nuclear factor-κB and AP-1 in the effect of FF on ET-1 expression were identified in macrovascular ECs and cardiomyocytes.11,25,32,33

ET-1 expression is also inhibited by insulin, via activation of the Akt/GSK3 pathway suggesting that FF mimics the effect of insulin. In T2D patients, FF treatment was associated with a decrease in plasma ET-1 levels, most relevantly in those whose ET-1 levels were significantly elevated compared with nondiabetic controls (data not shown). Despite random allocation of subjects to treatment groups, the lower plasma ET-1 levels in the placebo group could be owing to sampling variation, and may account for why the between-group changes were less significant after adjustment for baseline values. Nevertheless, these observations provide a partial explanation for our finding that FF improves resistance vessel function and ambulatory blood pressure in diabetes mellitus34,35 and could provide a contributing mechanism explaining the beneficial effects of FF on microvascular complications of diabetes mellitus in the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) and Action to Control Cardiovascular Risk in Type 2 Diabetes (ACCORD) trials, including a significant reduction in the need for laser treatment of retinopathy, as well as a delay in the progression of nephropathy.36–38 There is also a significant reduction in the risk of minor amputations in the absence of large-vessel disease, as observed in the FIELD study.39 Our study supports the hypothesis that the beneficial effects of FF treatment in T2D patients on microvascular circulation may be, in part, mediated by decreased ET-1 levels. FF acts by counteracting effects of TGFβ, which is increased in T2D40 by decreasing AP-1 binding on the ET-1 promoter (Figure IV in the online-only Data Supplement) and GSK3 activity (Figure V in the online-only Data Supplement). TGFβ elevation has been linked to diabetic complications, particularly to nephropathy, retinopathy, and cardiovascular diseases.41,42

In conclusion, we propose a novel molecular mechanism whereby FF may improve the microcirculatory dysfunction in T2D (Figure 5B). FF significantly decreases ET-1 expression by a PPARα-dependent mechanism, via the transcriptional activation of the KLF11 repressor, and by PPARα-independent actions via the inhibition of GSK3 activity.

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Figure 5. A. Diabetic patients divided into 2 groups were randomized double-blind to Fenofibrate (FF) treatment (200 mg/d; n=17) or placebo (n=18) for 12 weeks. Plasma endothelin-1 (ET-1) was measured at baseline and after treatment, and data values are presented as mean (SEM) in units of pg/mL (P=0.032, baseline vs end), in placebo group (P=0.415). FF vs placebo (P=0.010). B. Scheme summarizing the molecular mechanisms of FF on ET-1 expression in human microvascular endothelial cell line-1 (HMEC-1) cells. FF increases Krüppel-like factor 11 (KLF11) expression via activation of peroxisome proliferator-activated receptor (PPARα) resulting in a decrease of ET-1 expression. FF increases the inhibitory phosphorylation of glycogen synthase kinase-3β (GSK3β) decreasing ET-1 expression. These combined effects contribute to a significant time-dependent decrease of ET-1 expression and subsequent endothelial dysfunction and vascular complications.
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Disclosures

None.

References


SUPPLEMENTAL MATERIAL

METHODS

KLF11-Luc promoter constructs

A 2kb KLF11 promoter region (-1910/+144) was amplified from genomic DNA using the GeneAmp PCR system (Applied Biosystems) with a forward primer (5’-AGAACGCGTCTGGTGAGACAGATGAT-3’) containing a MluI site and reverse primer (5’-TGCAAGCTTGGGGAGCAACAAAG-3’) with a HindIII site. The amplified fragment was cloned into the pGL3-basic luciferase vector (Promega, Charbonnières, France). The PPRE mutation was introduced using the QuickChange site-directed mutagenesis kit (Stratagene) and the primers 5’-CGCCGCTGCGAAAATTCCCTTACTTTCCAG-3’ and 5’-CTGGAAAGTAAGGAAATTTTCGACGGCAG-3’. The sequences of all constructs were confirmed by automated sequencing (Applied Biosystems).

Transfection procedure

HMEC-1 cells were seeded at 40x10^3 cells/well in a 24-wells plate and transfected with PromoFectin-HUVEC transfection reagent (Promocell GmbH). After 24 h, the cells were lysed in the reporter lysis buffer (Promega) and luciferase gene expression was monitored. β-galactosidase activity was measured as a control of the transfection. Each transfection experiment was done in triplicate and the results expressed as mean relative light units normalized to β-galactosidase activity. For siRNA studies, HMEC-1 cells were transfected with siRNA at a concentration of 50nM using the Dharmafect transfection reagent (Dharmacon, Thermo-Fisher Scientific) and RNA levels were measured by real-time quantitative PCR. ON-TARGETplus SMARTpool KLF11 and PPARα siRNAs from Dharmacon were used. SiRNA TCF7L2 (sense 5’-CCCACCCUCUUCAGAUGGAAGCUUA-3’; antisense 5’-UAAGCUUCCAUACUGAAGGGUGGG-3’) (TCF7L2HSS110547) were used.

RNA analysis

HMEC-1 cells were incubated with vehicle or FF (100µM) in MCDB131 medium containing 0.2% FCS in the presence or absence of TGFβ (10ng/ml), for the indicated times. HMEC-1 cells were incubated with vehicle, GSK3 inhibitor IX (50nM),
Kenpaullone (2.3µM), Wortmannin (1µM) or insulin (100nM) in MCDB131 medium containing 0.2% FCS, for the indicated times. RNA extraction was performed using TRIzol reagent (Invitrogen) and reverse transcription of 1µg RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR analyses were performed using the Brilliant SYBR Green QPCR Master Mix on the Mx4000 detection system (Stratagene). The primers were human ET-1 5’-CCACCTGGACATCATTTGGGTCA-3’ and 5’-CCCTGAGTTCTTTTCTGGCTTG-3’; human KLF11 5’-AGCATCTGGAGGCAGACA-3’ and 5’-TGCACAGTGGGTGGAACA-3’; cyclophilin 5’-GCATACAGGTCTGGCATCCTGTCC-3’ and 5’-ATGCTGATCTTCTTGCTGGTGTC-3’

Samples were analyzed in triplicate in two independent runs. Ct values were determined for ET-1, KLF11 and normalized to the Ct of cyclophilin using the following equation: Relative values = 2^(-ΔCt target gene/ΔCt cyclophilin).

Chromatin immunoprecipitation assays

ChIP experiments were performed as described in¹. Immunoprecipitation was performed with the goat polyclonal antibody anti-KLF11 (sc-23162) from Santa Cruz. Final DNA extractions were PCR-amplified using primer pairs that cover the three proximal KLF sites in the ET-1 promoter. The primers were 5’-AGGGAGAGCATCCTGGTT-3’ and 5’-GTCGGAGCTGTTTACCCCA-3’. The PCR products were also analyzed by real-time quantitative PCR using the same primers pair.
Suppl. Figure I: PPARα expression is increased in confluent cells and this is correlated with an increase in KLF11 mRNA and protein expression. (A) PPARα- and KLF-11 mRNA expression was measured in non-confluent (NC) and confluent (C) cells using QPCR and normalized to the cyclophilin mRNA (mean value ± SEM of triplicate determinations). KLF11 protein expression was analyzed by western blotting using anti-KLF11 antibodies (B).
Suppl. Figure II: The pathway β-catenin and LEF/TCF is not involved in the FF-mediated reduction of ET-1 expression. It has been published that GSK3β down-regulation increases TCF7L2 expression. Therefore, we tested the implication of this pathway in FF-mediated reduction of ET-1 expression. (A) HMEC-1 cells were transfected with TCF7L2- or ctrl-siRNA and cell lysates analyzed for ET-1 expression by QPCR and normalized to cyclophilin mRNA (mean value ± SEM of triplicate determinations). Statistical differences were calculated using unpaired t-test (one-tailed) (DMSO versus FF: p-value=0.0114 for ctrl siRNA; TGFβ versus TGFβ+FF: p-value=0.0221 for ctrl siRNA; DMSO versus FF: p-value=0.0084 for TCF7L2 siRNA; TGFβ versus TGFβ+FF: p-value=0.0280 for TCF7L2 siRNA; ctrl- versus TCF7L2-siRNA: p-value=0.0182 for DMSO). (B) HMEC cells transfected with TCF7L2- or ctrl-siRNA were analyzed by QPCR for TCF7L2 and normalized to the cyclophilin mRNA.
Suppl. FigureIII: The Smad proteins control ET-1 transcription via a mechanism involving GSK3. We have tested Smad as a potential target of this pathway because it has been published that GSK3β negatively regulates gene expression through interaction with Smad3. HMEC-1 cells were transfected with a specific Smad3 reporter plasmid (CAGA-luc) and 24h after transfection, treated with FF, GSK3 inhibitor IX (IX) or Kenpaullone (K) with or without TGFβ for 24h. The cell lysates were analyzed for the luciferase gene expression and β-galactosidase activity was measured as a control of the transfection. Each bar is the mean value ± SEM of triplicate determinations. Statistical differences were calculated using unpaired t-test (one-tailed) (TGFβ vs TGFβ+FF: p-value=0.0116; TGFβ vs TGFβ+IX: p-value=0.0112; TGFβ vs TGFβ+K: p-value=0.0111).
**Suppl. Figure IV:** FF decreases the binding of AP-1 on the ET-1 promoter in the presence of TGFβ probably contributing to the stronger effect of FF observed when cells are treated with TGFβ. Confluent HMEC-1 cells were treated with vehicle, FF, TGFβ or both compounds. ChIP assays were performed using anti-c-Jun antibodies (sc-44x) from Santa Cruz. Total extracts (input) were used as controls. The samples were analyzed by semi-QPCR using the oligonucleotides pair 5’-GGGCGTCTGCCTCTGAAGTTAGCAG-3’ and 5’-GACTTGGACAGCTCTCTGCC-3’ surrounding the AP-1 binding site located in the proximal part of the ET-1 promoter. The oligonucleotides pair 5’-GGTCAAAGTTGCCAAAAGGT-3’ and 5’-ACTGAGCCGGAAGCCGAGCCAG-3’ located in the distal part of the ET-1 promoter was used as a negative control.
Suppl. Figure V: Full GSK3 activity is necessary for the ET-1 expression induction by TGFβ. Confluent HMEC-1 cells were treated with vehicle, TGFβ, TGFβ+GSK3-inhibitor IX (IX) or TGFβ+Kenpaullone (K). Cells were analyzed for ET-1 mRNA expression by QPCR and normalized to cyclophilin mRNA (mean value ± SEM of triplicate determinations). Statistical differences were calculated using unpaired t-test (one-tailed) (ctrl vs TGF: p-value=0.0385; TGFb vs TGF+IX: p-value=0.0033; TGFb vs TGFb+K: p-value=0.0083).
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