Early stimulation and late inhibition of peroxisome proliferator-activated receptor γ (PPAR γ) gene expression by transforming growth factor β in human aortic smooth muscle cells: role of early growth-response factor-1 (Egr-1), activator protein 1 (AP1) and Smads

Mingui FU*, Jifeng ZHANG*, Yimin LIN*, Xiaojun ZHU*, Luning ZHAO*, Mushtaq AHMAD†, Markus U. EHRENGRUBER‡ and Yuqing E. CHEN*¹

*Cardiovascular Research Institute, Morehouse School of Medicine, 720 Westview Drive SW, Atlanta, GA 30310, U.S.A., †Cardiovascular Division, Emory University School of Medicine, Atlanta, GA 30322, U.S.A., and ‡Brain Research Institute, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Transforming growth factor β (TGF β) and peroxisome proliferator-activated receptor γ (PPAR γ) play major roles in the development of vascular diseases. It has been documented that PPAR γ activation inhibits the TGF β signal pathway in vascular smooth muscle cells (VSMC). Here we examined whether TGF β can regulate PPAR γ expression. Northern blot analyses revealed that both TGF β 1 and 2 exert a biphasic effect (early stimulation and late repression) on PPAR γ gene expression in VSMC. TGF β rapidly and transiently induced early growth-response factor-1 (Egr-1) expression through the mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MEK1)/ERK-mediated pathway. Inhibition of MEK1/ERK by PD98059 not only abrogated the induction of Egr-1 but also abolished the rapid and transient induction of PPAR γ by TGF β . Furthermore, overexpression of NAB2, a repressor of

Egr-1 activation, also blocked the induction of PPAR γ by TGF β in VSMC, suggesting that Egr-1 mediates the rapid and transient induction of PPAR γ by TGF β . With regard to the TGF β repression of PPAR γ expression, activator protein 1 (AP1) and Smad3/4 dramatically inhibited the PPAR γ promoter activity in transient-transfection studies. In contrast, adenovirus-mediated overexpression of a dominant-negative form of c-Jun partially rescued the TGF β -induced PPAR γ repression in VSMC. Taken together, our data demonstrate that Egr-1, AP1 and Smad are part components of the TGF β signal transduction pathway that regulates PPAR γ expression.

Key words: atherosclerosis, peroxisome proliferator-activated receptor γ (PPAR γ), signal transduction, transcriptional factor, transforming growth factor β (TGF β).

INTRODUCTION

Transforming growth factor β (TGF β) molecules form a family of cytokines that have widespread effects on many aspects of growth and development [1]. TGF β isoforms are produced by most cell types and exert a wide range of effects in a context-dependent endocrine, autocrine or paracrine mechanism through interactions with distinct receptors on the cell surface. Studies of human atherosclerotic plaques as well as restenosis lesions after angioplasty have documented TGF β expression within vascular lesions [2]. It has therefore been postulated that TGF β is an important determinant of vessel structure and neointima formation based on its cell-type-specific effects on the regulation of vascular cell growth, migration, differentiation and matrix production [3,4]. We recently demonstrated that stimulation of the peroxisome proliferator-activated receptor γ (PPAR γ) inhibits TGF β -induced connective-tissue growth factor (CTGF) gene expression by interfering with Smad3 signal pathways in vascular smooth muscle cells (VSMC) [5], suggesting that the PPAR γ and TGF β pathways cross-talk to regulate the development of vascular diseases. Interestingly, it was reported that TGF β inhibits a well-defined PPAR γ target gene, CD36, in macrophages by phosphorylating PPAR γ [6]. Taken together, these

data suggest that the TGF β and PPAR γ signal transduction pathways may regulate each other.

PPAR γ is a ligand-activated nuclear transcriptional factor [7,8], which is found predominantly in adipose tissue, where it plays a crucial role in adipocyte differentiation, fat storage and glucose homeostasis [9]. In recent years, the role of PPAR γ in vasculature has been investigated intensively. It was found that PPAR γ plays critical roles in the inhibition of vascular diseases such as atherosclerosis [10–12]. However, the transcriptional regulation of the PPAR γ gene in vasculature remains poorly understood. Our previous studies documented that many growth factors and cytokines, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), angiotensin II (AngII), tumour necrosis factor α (TNF α) and interleukin 1 β (IL-1 β), upregulate PPAR γ gene expression via early growth-response factor-1 (Egr-1) in VSMC [13,14].

TGF β signalling is initiated upon its binding to two cell-membrane receptors, termed type I (T β RI) and type II (T β RII). Both receptors are serine/threonine kinases, and TGF β binding results in autophosphorylation of T β RI and T β RII. Smads, a novel family of signalling proteins, are the primary downstream effectors of TGF β receptor activation. Activated heteromeric Smad complexes translocate into the nucleus where they act as

Abbreviations used: AngII, angiotensin II; AP1, activator protein 1; bFGF, basic fibroblast growth factor; BMP2, bone morphogenic protein 2; CTGF, connective-tissue growth factor; Egr-1, early growth-response factor-1; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HASMC, human aortic smooth muscle cells; IL-1 β , interleukin 1 β ; MAPK, mitogen-activated protein kinase; MEK1, MAPK/ERK kinase 1; NAB2, nerve-growth-factor-induced A-binding proteins 2; PDGF, platelet-derived growth factor; pERK, phospho-ERK; PPAR γ , peroxisome proliferator-activated receptor γ ; TGF β , transforming growth factor β ; TNF α , tumour necrosis factor α ; VSMC, vascular smooth muscle cells.

¹ To whom correspondence should be addressed (e-mail echen@msm.edu).

transcription factors for TGF β -responsive genes [15]. In addition to Smad proteins, mitogen-activated protein kinases (MAPKs) have also been implicated in mediating downstream TGF β signalling [16].

In the present study, we tested whether TGF β affects the signal pathway that regulates PPAR γ gene expression in VSMC.

MATERIALS AND METHODS

Materials

Human recombinant TGF β 1, TGF β 1, bone morphogenic protein (BMP2), PDGF-BB, bFGF, AngII, TNF α , IL-1 β , PMA, PD98059 and actinomycin D were purchased from Sigma. Lipofectamine 2000 was from Invitrogen. [α - 32 P]dCTP was obtained from Perkin Elmer Life Sciences. Luciferase assay kits were from Promega. Rabbit anti-PPAR γ polyclonal antibody (SC-7196) and mouse anti-phospho-extracellular signal-regulated kinase (pERK) monoclonal antibody (SC-7383) were purchased from Santa Cruz Biotechnology.

Plasmids and adenoviral recombinants

The reporter construct pGL3- γ 1p3000 containing 3000 bp of the human PPAR γ 1 gene promoter was a generous gift of Dr J. Auwerx (Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, Strasbourg, France) [17]. pcDNA3-Egr-1 was constructed in our laboratory as described previously [13]. pcDNA3-FosB and pcDNA3-JunD were obtained from Dr Y. Yamamura (Department of Retroviral Regulation, Tokyo Medical and Dental University Medical Research Division, Tokyo, Japan) [18]. pcDNA3-Smad3 and pcDNA3-Smad4 were kindly provided by Dr S. Cheng (Division of Bone and Mineral Diseases, Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO, U.S.A.) [19]. NAB2 (nerve-growth-factor-induced-A-binding proteins 2)-expressing adenovirus (AdGFP NAB2) and its control, green fluorescent protein (GFP)-expressing adenovirus (AdGFP), were described previously [20]. The adenovirus containing dominant-negative c-Jun (AdTAM67), generated from wild-type c-Jun by deletion of amino acid residues 3–122 in the N-terminal transcriptional activation domain, was kindly provided by Dr M. J. Birrer (Biomarkers and Prevention Research Branch, National Cancer Institute, Rockville, MD, U.S.A.) [21].

Cell culture

Human aortic smooth muscle cells (HASMC) were purchased from Bio-Whittaker (San Diego, CA, U.S.A.) and cultured in smooth muscle cell growth medium-2 containing 5% fetal bovine serum (Invitrogen), 2 ng/ml human bFGF, 0.5 ng/ml human epidermal growth factor, 50 μ g/ml gentamicin, 50 ng/ml amphotericin-B and 5 μ g/ml bovine insulin. For all experiments, early-passage HASMC (passages 5–7) were grown to $\approx$ 90% confluence and rendered quiescent by culturing in Optium-MEM (Invitrogen) for 24–48 h. The HepG2 cell line and the rat VSMC line (A7r5) were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum in a 5% CO $_2$ humidified atmosphere at 37 °C. For the mRNA stability assay, cells were pretreated with TGF β 1 for 2 h before adding actinomycin D to inhibit mRNA transcription. Adenovirus infection was performed at a titre of 5 plaque-forming units/cell as described previously [20].

Northern-blot analysis

Total RNA (20 μ g) isolated from each condition using acid-guanidinium thiocyanate was subjected to electrophoresis through 1% formaldehyde/agarose gels. After transferring to nylon membranes (Bio-Rad), the RNA was cross-linked to the membrane by a UV cross-linker (Stratagene, La Jolla, CA, U.S.A.). 32 P-Labelled cDNA probes were generated by using a random-primer labelling system (Gibco-BRL). Blots were pre-hybridized, hybridized for 1 h and washed once with 1 $\times$ SSC (0.15 M NaCl/0.015 M sodium citrate) at 65 °C, and once with 0.1 $\times$ SSC/1.0% SDS (w/v) at 65 °C for 15 min. The Phosphor-Image Typhoon 9200 was used to detect the 32 P radioactivity of each band, and the density of each band was quantified by ImageQuant Software (Amersham Bioscience, Piscataway, NJ, U.S.A.). Lane-loading differences were normalized using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Analysis of PPAR γ mRNA half-life

PPAR γ mRNA half-life experiments were carried out using HASMC. The cells were exposed to vehicle or TGF β 1 (4 ng/ml) for the indicated periods prior to mRNA stability measurements. Transcription was inhibited by the addition of actinomycin D (5 μ g/ml). Northern-blotting analysis was performed as described above.

Western blot analysis

Total cell lysates (50 μ g) were subjected to SDS/PAGE and electrotransferred to nitrocellulose membrane (Bio-Rad). After blocking in 20 mM Tris/HCl, pH 7.6, containing 150 mM NaCl, 0.1% Tween 20 and 5% (w/v) non-fat dry milk, blots were incubated with specific antibodies against PPAR γ and pERK overnight at 4 °C. The blots were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and immunoactivity was visualized using the enhanced chemiluminescence detection system (ECL; Amersham Bioscience) as per the manufacturer's instructions. The density of each band was quantified by ImageQuant Software.

Transient transfection and luciferase assays

HepG2 and A7r5 cells were grown to 70–80% confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and, by using Lipofectamine 2000, transiently transfected with the pGL3- γ P3000 reporter plasmid [17] and pcDNA3.1 expression plasmids encoding Egr-1, Fos, Jun-D, Smad3 and Smad4. GFP expression plasmids (Clontech, Palo Alto, CA, U.S.A.) were co-transfected as the control for the transfection efficiency. The total amount of transfected DNA was kept constant by adding empty vector. The reporter luciferase assay kit (Promega) was used to measure the luciferase activity from cells after a 48 h transfection with a luminometer (Victor II; Perkin Elmer), according to the manufacturer's instructions. Relative luciferase activity was obtained by dividing the luciferase units by the GFP units in each sample.

RESULTS

Early stimulation and late inhibition of PPAR γ gene expression by TGF β in HASMC

Our previous study demonstrated that PPAR γ activation inhibited TGF β -induced CTGF gene expression by interfering

Figure 1 TGF β exerts a biphasic effect on PPAR γ expression in HASMC

(A–C) Quiescent cells were incubated with TGF β 1 or 2 (both in 4 ng/ml) and BMP2 (100 ng/ml) for different times as indicated. (D and E) Quiescent cells were incubated with increasing concentrations of TGF β 1 (0, 0.1, 0.5, 1, 2 and 4 ng/ml) for 1 h (D) or 24 h (E). Representative Northern blots show the mRNA levels for PPAR γ and GAPDH. Relative PPAR γ mRNA levels normalized by GAPDH mRNA levels are shown in the middle of the panels. (F) Quiescent cells were incubated with TGF β 1 (4 ng/ml) for different times as indicated, and the PPAR γ protein levels determined by Western blot analysis. Equal loading was confirmed by immunoblotting for β -actin. Similar results were obtained in three independent experiments.

Figure 2 TGF β 1 inhibits the PPAR γ expression induced with PDGF, bFGF, AngII, TNF α , IL-1 β and PMA in HASMC

Quiescent cells were pretreated with TGF β 1 (4 ng/ml) for 30 min and then incubated with PDGF-BB (20 ng/ml), bFGF (25 ng/ml), AngII (3×10^{-7} M), TNF α (10 ng/ml), IL-1 β (10 ng/ml) and PMA (100 ng/ml) for 24 h. Representative Northern blots show the mRNA levels for PPAR γ and GAPDH. Relative PPAR γ mRNA levels normalized by GAPDH mRNA levels are shown on the graph.

Figure 3 TGF β 1 does not affect the PPAR γ mRNA stability in HASMC

Quiescent cells were incubated with or without 4 ng/ml TGF β for 2 h, and *de novo* mRNA transcription was then inhibited by the addition of actinomycin D ($5 \mu\text{g/ml}$). Total RNA was extracted at 0, 2 and 4 h after administration of actinomycin D and analysed by Northern blotting. The top panel shows a representative Northern blot; the graph gives for each time point the average values of the PPAR γ signal as a percentage of the initial PPAR γ mRNA level ($n = 3$ independent experiments).

Figure 4 ERK/Egr-1 signal pathway mediates the early stimulation of PPAR γ gene expression by TGF β in HASMC

(A and C) Quiescent cells were incubated with 4 ng/ml TGF β for the indicated times (A) or pretreated with PD98059 (50 μ M) for 30 min and then incubated with TGF β (4 ng/ml) for 15 min (C). Phosphorylated, i.e. activated, ERK1/2 (pERK1/2) and actin levels were analysed by Western blotting. (B and D) Cells were incubated with 4 ng/ml TGF β for different times as indicated (B), or pretreated with PD98059 (50 μ M) for 30 min and then stimulated with TGF β (4 ng/ml) for 1 h (D). The mRNA levels for Egr-1, NAB2 and PPAR γ were analysed by Northern blot analysis. Equal loading was confirmed by GAPDH. (E) Cells were infected with an adenovirus expressing both NAB2 and GFP (to control for the infection efficiency) or a control adenovirus expressing GFP alone for 2 h, washed and then cultured in growth medium for 24 h. Quiescent cells (culturing in Opti-MEM for 24 h) were incubated with 4 ng/ml TGF β for 1 h. Representative Northern blots show the mRNA levels for PPAR γ and GAPDH. Three independent experiments led to similar results.

with Smad3 signal pathway in VSMC [5], suggesting that the PPAR γ and TGF β signal transduction pathways might cross-talk in their regulation of vascular disease development. However, it remained unknown whether TGF β can affect PPAR γ signalling. Therefore, as the first step, we examined the effect of TGF β on PPAR γ gene expression by Northern blot analyses. Quiescent HASMC were incubated with TGF β 1 or 2 (both at 4 ng/ml) or BMP2 (100 ng/ml) for various times as described in Figure 1. Interestingly, a biphasic effect of both TGF β 1 and 2 on PPAR γ gene expression was found in HASMC (Figures 1A and 1B). A rapid and transient stimulation of PPAR γ mRNA expression by TGF β 1 and 2 occurred, starting as early as 30 min and reaching a peak at 1 h after addition of TGF β 1/2. At longer incubation times (6–12 h), by contrast, PPAR γ mRNA expression was inhibited by TGF β 1 and 2, and became almost undetectable at 24 h. BMP2, a member of another subfamily in the TGF β superfamily, also rapidly and transiently induced PPAR γ expression in HASMC, which peaked at 2 h and returned to the baseline at 12 h (Figure 1C). To further investigate the biphasic effect of TGF β 1/2 on PPAR γ expression, quiescent HASMC were incubated with increasing concentrations (0, 0.5,

1, 2 and 4 ng/ml) of TGF β 1 for 1 and 24 h respectively, and steady-state PPAR γ mRNA levels were assessed by Northern blots. TGF β 1 treatment for 1 h (Figure 1D) in HASMC stimulated PPAR γ mRNA expression in a dose-dependent manner, and incubation with TGF β 1 for 24 h (Figure 1E) markedly inhibited PPAR γ mRNA expression in a dose-dependent fashion. Western blot analysis confirmed the significant repression of PPAR γ expression on the protein level in HASMC by 24 h TGF β 1 treatment (Figure 1F). Taken together, our data document a specific, biphasic effect of TGF β 1/2 on PPAR γ expression in HASMC.

TGF β represses PPAR γ expression induced by other growth factors and cytokines

To determine the effect of TGF β on inducible PPAR γ gene expression in HASMC, cells were treated with or without TGF β 1 (4 ng/ml) for 30 min, and then exposed to PDGF-BB (20 ng/ml), bFGF (25 ng/ml), AngII (3×10^{-7} M), TNF α (10 ng/ml), IL-1 β (10 ng/ml) and PMA (100 ng/ml) for 24 h. Thereafter, PPAR γ

Figure 5 Human PPAR γ 1 promoter activity is transactivated by Egr-1, but inhibited by AP1 and Smad3/4

The top panel gives a schematic representation of the pGL3- γ P3000 luciferase reporter construct used. HepG2 cells were co-transfected with pGL3- γ P3000 and pcDNA3-Egr-1, pcDNA3-c-Fos plus pcDNA3-JunD, or pcDNA3-Smad3 plus pcDNA3-Smad4. The GFP reporter plasmid was co-transfected as a control for the transfection efficiency. Cells were collected for the luciferase activity assay at 48 h after transfection. Luciferase activities normalized by the GFP activities were expressed relative to co-transfection of empty pcDNA3 (means $\pm$ S.E.M., $n = 6$).

mRNA levels were assessed by Northern blot analyses. In consistence with our previous report [14], all factors used in the absence of TGF β 1 significantly induced PPAR γ mRNA expression in HASMC at 24 h. The treatment with TGF β 1, however, blocked the growth factor- and cytokine-induced PPAR γ mRNA expression in HASMC (Figure 2). These results suggest that TGF β 1/2 is a pleiotropic factor that is different from other growth factors and cytokines in regulating PPAR γ gene expression.

TGF β does not affect PPAR γ mRNA stability in VSMC

To evaluate whether TGF β regulates PPAR γ gene expression by affecting PPAR γ mRNA stability, we examined the half-life of PPAR γ mRNA in HASMC by Northern blot analyses. Cells were treated with actinomycin D (5 μ g/ml) after 2 h of TGF β (4 ng/ml) stimulation. As shown in Figure 3, the half-life of PPAR γ mRNA was $\approx$ 2.8 h in HASMC, as compared with our previously reported half-life of $\approx$ 4 h [14]. This difference may arise from the different donor cells used in the two studies. Most importantly, however, there was no significant difference between TGF β -treated and untreated cells (Figure 3). These data strongly suggest that regulation of PPAR γ expression by TGF β is independent of altered PPAR γ mRNA stability.

Extracellular signal-regulated kinase (ERK)/Egr-1 signalling pathway mediates the induction of PPAR γ by TGF β

We have previously demonstrated that Egr-1 is a key mediator of PPAR γ expression in HASMC [13]. To examine whether Egr-1 also is involved in the TGF β -induced rapid and transient expression of PPAR γ , we first examined the effect of TGF β on MAPK phosphorylation and Egr-1 expression. As shown in Figure 4(A), TGF β rapidly and transiently induced ERK1/2

Figure 6 Dominant c-Jun protein partially rescues the repression of PPAR γ gene expression by TGF β in HASMC

Cells were infected with AdTAM67 (lanes 2 and 4) or AdGFP control adenovirus (lanes 1 and 3) for 2 h, washed and then cultured in growth medium for 24 h. Quiescent cells, by culturing in Opti-MEM for 24 h, were incubated with or without 4 ng/ml TGF β , as shown, for 24 h. Relative PPAR γ mRNA levels normalized by GAPDH are shown in italics.

phosphorylation, with a peak at 15 min and return to basal levels at 1 h. In addition, TGF β -induced Egr-1 and NAB2 expression in HASMC was time-dependent (Figure 4B). Interestingly, the NAB2 time course was delayed compared with the induction of Egr-1 by TGF β , which is compatible with the negative-feedback role of NAB2 [22]. An inhibitor of MAPK/ERK kinase 1 (MEK1), PD98059, significantly inhibited both the basal and TGF β -induced ERK1/2 phosphorylation (Figure 4C). PD98059 pretreatment blocked the TGF β -induced expression of both Egr-1 and PPAR γ in HASMC (Figure 4D). Finally, recombinant adenovirus-mediated overexpression of NAB2, a selective repressor of Egr-1, also blocked TGF β -induced expression of PPAR γ (Figure 4E). Taken together, these data demonstrate that the rapid and transient expression of PPAR γ induced by TGF β occurs via the ERK/Egr-1 signal transduction pathway.

Activator protein 1 (AP1) and Smad3/4 inhibit human PPAR γ 1 promoter activity

To further explore the mechanisms that may be involved in the regulation of PPAR γ by TGF β , we examined the effects of Egr-1, AP1 and Smad3/4 molecules on the human PPAR γ 1 promoter activity. Expression plasmids for Egr-1, Fos/Jun-D and Smad3/4 were co-transfected together with a plasmid encoding the luciferase reporter downstream from a 3 kb fragment of the PPAR γ 1 promoter (Figure 5, top panel) into HepG2 cells. As shown in Figure 5 (bottom panel), Egr-1 increased the PPAR γ 1 promoter activity by $\approx$ 3.0-fold ($n = 6$), whereas Fos/Jun-D and Smad3/4 significantly decreased the PPAR γ 1 promoter activity by $\approx$ 80 and $\approx$ 60%, respectively. Similar results were obtained with the rat aortic smooth muscle cell line A7r5 (results not shown). In summary, these data indicate that AP1 and Smad3/4 may contribute to the late repression of PPAR γ expression by TGF β .

Dominant-negative AP1 partially reverses the TGF β suppression of PPAR γ expression

To further establish the suppressive role of AP1 factors in TGF β -induced PPAR γ expression, we overexpressed a dominant-negative c-Jun molecule (TAM67) in HASMC by using an adenoviral vector. As shown in Figure 6, overexpression of TAM67 partially prevented the repression of PPAR γ expression

by TGF β , suggesting that other mechanisms (such as Smad3/4 and NAB2) are also contributing to the TGF β -induced repression of PPAR γ .

DISCUSSION

PPAR γ and TGF β play important roles in the pathogenesis of vascular diseases. In this study we found that TGF β has a biphasic effect on PPAR γ expression, exerting a rapid and transient induction and, thereafter, a strong repression. We document that the rapid and transient induction of PPAR γ by TGF β occurs via Egr-1, and that the late repression is mediated by both AP1 and Smad3/4. It has been reported previously that TGF β decreases a PPAR γ target gene, i.e. the surface molecule CD36, in macrophages via phosphorylation of PPAR γ and subsequent reduction in its transcriptional activity [6]. The present study provides a novel mechanism for TGF β decreasing PPAR γ transcriptional activity, namely by repressing transactivation of PPAR γ gene expression.

TGF β is a pleiotropic cytokine/growth factor that is thought to play a major role in the development of vascular diseases [1]. Both endothelial cells and vascular smooth muscle cells produce TGF β 1 as an autocrine and paracrine growth factor. It is well documented that TGF β 1 can modulate cellular processes involved in lesion formation, such as cell growth, differentiation, migration and matrix production. Despite the pleiotropic effects of TGF β on VSMC, only a few genes have been characterized as direct targets of TGF β in VSMC. PPAR γ is a ligand-activated nuclear transcription factor [7,8] that is found predominantly in adipose tissue, where it plays a crucial role in adipocyte differentiation, fat storage and glucose homeostasis [9]. It was recently found that PPAR γ also plays critical roles in the development and severity of vascular disease including atherosclerosis and restenosis [10–12]. Although the mechanisms involved in these effects are not understood in detail, inhibitory effects of PPAR γ on VSMC proliferation and migration [23], foam cell development [24], vascular inflammatory response [25] and extracellular matrix accumulation [5] have been suggested. Our previous work has demonstrated that PPAR γ activation inhibits TGF β -induced CTGF gene expression by interfering with Smad3 signalling in VSMC [5], suggesting that the TGF β and PPAR γ signalling pathways may interact. In agreement with this hypothesis, we document in the present study that TGF β affects the PPAR γ signalling pathway by regulating PPAR γ gene expression in VSMC. Our detailed study of the cross-talk between these two signal pathways in VSMC thus provides new insights into the pathogenesis of vascular diseases.

Recently, we have documented that growth factors and cytokines such as PDGF, bFGF, AngII, TNF α and IL-1 β induce Egr-1 expression prior to PPAR γ 1 up-regulation in HASMC [13]. These data suggested for the first time that Egr-1 is both necessary and sufficient to activate human PPAR γ 1 gene expression in VSMC. In the present study, we found that TGF β is different from the previously mentioned growth factors and cytokines, as it exerts a biphasic effect on PPAR γ expression (early stimulation and late repression). Although we reported that PDGF also has a more complex effect on PPAR γ expression [14], it is obviously different from that of TGF β . The levels of PPAR γ mRNA induced by PDGF in HASMC increased at 2 h, but declined to some extent at 6 h, and rose again at 12 h to achieve a second peak at 24 h that was sustained for 72 h. We are now studying the molecular mechanisms of PDGF's effects on PPAR γ gene expression.

With regard to the rapid and transient induction of PPAR γ by TGF β , we observed that the effect of TGF β occurs via Egr-1

induction. Similar to other growth factors and cytokines, TGF β can rapidly and transiently induce Egr-1 expression through the MEK1/ERK pathway. Blocking the MEK1/ERK signal pathway by PD98059 not only abrogated the induction of Egr-1 but also abolished the rapid and transient induction of PPAR γ expression by TGF β . We verified these results by using an adenoviral vector expressing NAB2, a selective repressor of Egr-1. The agreement between the data using the pharmacological probe and the NAB2 adenoviral construct provides the first definitive evidence that Egr-1 mediates the rapid and transient induction of PPAR γ by TGF β .

What are the mechanisms of the late repression of PPAR γ expression by TGF β in VSMC? The suppression is independent of accelerated degradation of PPAR γ mRNA, because TGF β did not interfere with the mRNA half-life of PPAR γ . Although we found that TGF β rapidly and transiently induces NAB2 expression, we believe that this cannot fully explain the long and robust repression of PPAR γ by TGF β since the time course of TGF β -induced NAB2 expression does not match well with PPAR γ repression by TGF β . Our finding that the overexpression of AP1 and Smad3/4 in HepG2 and A7r5 cells repressed PPAR γ promoter activity provided additional possible mechanisms for the suppression of endogenous PPAR γ . In agreement with these data, infection of HASMC with an adenovirus containing a dominant-negative c-Jun molecule partially rescued the TGF β -induced repression of PPAR γ expression. Taken together, our data suggest that the late inhibition of PPAR γ expression by TGF β is mediated via NAB2, AP1 and Smad3.

It has been well documented that p300/cAMP-response-element-binding protein-binding protein (CBP) is a co-activator for Egr-1, AP1 and Smad3/4 [26–28]. AP1 and Smad3/4 may compete with Egr-1 for a limited amount of p300/CBP and thus repress PPAR γ gene expression in VSMC. Alternatively, AP1 and Smad3/4 may bind to their elements located in the PPAR γ gene promoter and recruit transcriptional co-repressors to inhibit PPAR γ gene expression in VSMC. Systematic deletion mapping of the PPAR γ promoter will be required to fully understand this mechanism.

While we were preparing this manuscript, two groups also reported the effect of TGF β on PPAR γ expression in other cell lines. They found that TGF β induced both PPAR γ 1 and PPAR γ 2 expression in macrophages [29] and inhibited PPAR γ expression in bone marrow stromal cells [30]. This reflected the pleiotropic effect of TGF β in different cell types.

In summary, the present study reveals that early stimulation of PPAR γ expression by TGF β is mediated via the ERK/Egr-1 signalling pathway, whereas TGF β -mediated late inhibition of PPAR γ expression occurs via AP1 and Smad3, and possibly NAB2. The revealed cross-talk between the PPAR γ and TGF β signalling pathways in VSMC provides important information for the understanding of their roles in vasculature. As both PPAR γ and TGF β also play important roles in other systems such as cancer biology [31–33], it would be very interesting to test whether this cross-talk also exists in cancer cells.

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REFERENCES

- McCaffrey, T. A. (2000) TGF- β s and TGF- β receptors in atherosclerosis. *Cytokine Growth Factor Rev.* **11**, 103–114

- 2 Nikol, S., Isner, J. M., Pickering, J. G., Kearney, M., Leclerc, G. and Weir, L. (1992) Expression of transforming growth factor- β 1 is increased in human vascular restenosis lesions. *J. Clin. Invest.* **90**, 1582–1592
- 3 Nabel, E. G., Shum, L., Pompili, V. J., Yang, Z. Y., San, H., Shu, H. B., Liptay, S., Gold, L., Gordon, D., Derynck, R. et al. (1993) Direct transfer of transforming growth factor β 1 gene into arteries stimulates fibrocellular hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10759–10763
- 4 Wolf, Y. G., Rasmussen, L. M. and Ruoslahti, E. (1994) Antibodies against transforming growth factor- β 1 suppress intimal hyperplasia in a rat model. *J. Clin. Invest.* **93**, 1172–1178
- 5 Fu, M., Zhang, J., Zhu, X., Myles, D. E., Willson, T. M., Liu, X. and Chen, Y. E. (2001) Peroxisome proliferator-activated receptor γ inhibits transforming growth factor β -induced connective tissue growth factor expression in human aortic smooth muscle cells by interfering with Smad3. *J. Biol. Chem.* **276**, 45888–45894
- 6 Han, J., Hajjar, D. P., Tauras, J. M., Feng, J., Gotto, Jr, A. M. and Nicholson, A. C. (2000) Transforming growth factor- β 1 (TGF- β 1) and TGF- β 2 decrease expression of CD36, the type B scavenger receptor, through mitogen-activated protein kinase phosphorylation of peroxisome proliferator-activated receptor- γ . *J. Biol. Chem.* **275**, 1241–1246
- 7 Jiang, C., Ting, A. T. and Seed, B. (1998) PPAR- γ agonists inhibit production of monocyte inflammatory cytokines. *Nature (London)* **391**, 82–86
- 8 Klier, S. A., Lehmann, J. M. and Willson, T. M. (1999) Orphan nuclear receptors: shifting endocrinology into reverse. *Science* **284**, 757–760
- 9 Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* **79**, 1147–1156
- 10 Law, R. E., Goetze, S., Xi, X. P., Jackson, S., Kawano, Y., Demer, L., Fishbein, M. C., Meehan, W. P. and Hsueh, W. A. (2000) Expression and function of PPAR γ in rat and human vascular smooth muscle cells. *Circulation* **101**, 1311–1318
- 11 Law, R. E., Meehan, W. P., Xi, X. P., Graf, K., Wuthrich, D. A., Coats, W., Faxon, D. and Hsueh, W. A. (1996) Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. *J. Clin. Invest.* **98**, 1897–1905
- 12 Li, A. C., Brown, K. K., Silvestre, M. J., Willson, T. M., Palinski, W. and Glass, C. K. (2000) Peroxisome proliferator-activated receptor gamma ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest.* **106**, 523–531
- 13 Fu, M., Zhang, J., Lin, Y., Zhu, X., Ehrenguber, M. U. and Chen, Y. E. (2002) Early growth response factor-1 is a critical transcriptional mediator of peroxisome proliferator-activated receptor- γ 1 gene expression in human aortic smooth muscle cells. *J. Biol. Chem.* **277**, 26808–26814
- 14 Fu, M., Zhu, X., Wang, Q., Zhang, J., Song, Q., Zheng, H., Ogawa, W., Du, J. and Chen, Y. E. (2001) Platelet-derived growth factor promotes the expression of peroxisome proliferator-activated receptor gamma in vascular smooth muscle cells by a phosphatidylinositol 3-kinase/Akt signaling pathway. *Circ. Res.* **89**, 1058–1064
- 15 Massague, J. (1998) TGF- β signal transduction. *Annu. Rev. Biochem.* **67**, 753–791
- 16 Massague, J. and Wotton, D. (2000) Transcriptional control by the TGF- β /Smad signaling system. *EMBO J.* **19**, 1745–1754
- 17 Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R., Najib, J., Laville, M., Fruchart, J. C., Deeb, S. et al. (1997) The organization, promoter analysis, and expression of the human PPAR γ gene. *J. Biol. Chem.* **272**, 18779–18789
- 18 Yamamura, Y., Hua, X., Bergelson, S. and Lodish, H. F. (2000) Critical role of Smads and AP-1 complex in transforming growth factor- β -dependent apoptosis. *J. Biol. Chem.* **275**, 36295–36302
- 19 Nishimura, R., Kato, Y., Chen, D., Harris, S. E., Mundy, G. R. and Yoneda, T. (1998) Smad5 and DPC4 are key molecules in mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12. *J. Biol. Chem.* **273**, 1872–1879
- 20 Ehrenguber, M. U., Muhlebach, S. G., Sohrman, S., Leutenegger, C. M., Lester, H. A. and Davidson, N. (2000) Modulation of early growth response (EGR) transcription factor-dependent gene expression by using recombinant adenovirus. *Gene* **258**, 63–69
- 21 Brown, P. H., Chen, T. K. and Birrer, M. J. (1994) Mechanism of action of a dominant-negative mutant of c-Jun. *Oncogene* **9**, 791–799
- 22 Miano, J. M. and Berk, B. C. (1999) NAB2: a transcriptional brake for activated gene expression in the vessel wall? *Am. J. Pathol.* **155**, 1009–1012
- 23 Marx, N., Schonbeck, U., Lazar, M. A., Libby, P. and Plutzky, J. (1998) Peroxisome proliferator-activated receptor gamma activators inhibit gene expression and migration in human vascular smooth muscle cells. *Circ. Res.* **83**, 1097–1103
- 24 Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J. and Glass, C. K. (1998) The peroxisome proliferator-activated receptor- γ is a negative regulator of macrophage activation. *Nature (London)* **391**, 79–82
- 25 Pasceri, V., Wu, H. D., Willerson, J. T. and Yeh, E. T. (2000) Modulation of vascular inflammation *in vitro* and *in vivo* by peroxisome proliferator-activated receptor- γ activators. *Circulation* **101**, 235–238
- 26 Pouponnot, C., Jayaraman, L. and Massague, J. (1998) Physical and functional interaction of SMADs and p300/CBP. *J. Biol. Chem.* **273**, 22865–22868
- 27 Silverman, E. S., Du, J., Williams, A. J., Wadgaonkar, R., Drazen, J. M. and Collins, T. (1998) cAMP-response-element-binding-protein-binding protein (CBP) and p300 are transcriptional co-activators of early growth response factor-1 (Egr-1). *Biochem. J.* **336**, 183–189
- 28 Vo, N. and Goodman, R. H. (2001) CREB-binding protein and p300 in transcriptional regulation. *J. Biol. Chem.* **276**, 13505–13508
- 29 Kintscher, U., Wakino, S., Bruemmer, D., Goetze, S., Graf, K., Hsueh, W. A. and Law, R. W. (2002) TGF β 1 induces peroxisome proliferator-activated receptor γ 1 and γ 2 expression in human THP-1 monocytes. *Biochem. Biophys. Commun. Res.* **297**, 794–799
- 30 Ahdjoudj, S., Lasmoles, F., Holy, X., Zerath, E. and Marie, P. J. (2002) Transforming growth factor β 2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma. *J. Bone Miner. Res.* **17**, 668–677
- 31 Itami, A., Watanabe, G., Shimada, Y., Hashimoto, Y., Kawamura, J., Kato, M., Hosotani, R. and Imamura, M. (2001) Ligands for peroxisome proliferator-activated receptor gamma inhibit growth of pancreatic cancers both *in vitro* and *in vivo*. *Int. J. Cancer* **94**, 370–376
- 32 Sasaki, H., Tanahashi, M., Yukiue, H., Moiriyama, S., Kobayashi, Y., Nakashima, Y., Kaji, M., Kiriya, M., Fukai, I., Yamakawa, Y. and Fujii, Y. (2002) Decreased peroxisome proliferator-activated receptor gamma gene expression was correlated with poor prognosis in patients with lung cancer. *Lung Cancer* **36**, 71–76
- 33 Wakefield, L. M. and Roberts, A. B. (2002) TGF- β signaling: positive and negative effects on tumorigenesis. *Curr. Opin. Genet. Dev.* **12**, 22–29