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Thiazolidinediones inhibit TNFα induction of PAI-1 independent of PPARγ activation

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Abstract

Increased plasminogen activator inhibitor type 1 (PAI-1) levels are observed in endothelial cells stimulated by tumour necrosis factor α (TNFα). Thiazolidinediones (TZDs) may inhibit elevated endothelial cell PAI-1 accounting, in part, for the putative atheroprotective effects of TZDs. In an endothelial cell line, Rosiglitazone (RG) and Pioglitazone (PG) inhibited induction of PAI-1 by TNFα. The specific peroxisome proliferator-activated receptor γ (PPARγ) inhibitor, SR-202, failed to modulate this effect. RG also inhibited the effect of TNFα on a reporter gene construct harbouring the proximal PAI-1 promoter and PAI-1 mRNA in cells co-transfected with a dominant-negative PPARγ construct. RG and PG attenuated TNFα-mediated induction of *trans*-acting factor(s) Nur77/Nurr1 and binding of nuclear proteins (NP) to the *cis*-acting element (NBRE). SR-202 failed to modulate these effects. The observations suggest TZDs inhibit TNFα-mediated PAI-1 induction independent of inducible PPARγ activation and this may involve in the modulation of Nur77/Nurr1 expression and NP binding to the PAI-1 NBRE.

Keywords: Tumour necrosis factor α; Plasminogen activator inhibitor type-1; Thiazolidinedione; Peroxisome proliferator-activated receptor γ

Atherosclerosis is now recognized as a systemic disorder characterized by endothelial cell dysfunction and chronic inflammation within the arterial intima [1]. Increased plasminogen activator inhibitor type 1 (PAI-1) levels are regarded as a marker of endothelial cell dysfunction [2] and are present in patients with type 2 diabetes and those with the metabolic syndrome (MS), a cluster of cardiovascular risk factors including obesity, insulin resistance, hypertension, and dyslipidaemia [3,4]. Diabetes and the MS are characterized by elevated tumour necrosis factor α (TNFα) levels and are associated with accelerated atherosclerosis [3,4]. Mice with a targeted deletion of the PAI-1 gene are protected from the development of biochemical abnormalities associated with the MS and atherosclerosis [5,6]. These observations suggest that TNFα-mediated induction of endothelial cell-derived PAI-1 may contribute significantly to the development and progression of the MS and atherosclerosis.

PAI-1 synthesis is regulated by multiple factors including insulin [7], TNFα, interleukin (IL-1), and transforming growth factor β (TGF-β) [8]. TNFα is secreted by monocytes or macrophages in injured vessel walls and adipose tissue [9] and may contribute to the elevated endothelial cell PAI-1 levels.

Modulation of PAI-1 gene promoter activity involves a number of *cis*-acting elements for several *trans*-acting factors. CAGA elements have been identified in the PAI-1 gene promoter for the SMAD 3 and 4 proteins

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that are responsible for the induction of PAI-1 expression by TGF-β [10]. SP1 elements are thought to be responsible for mediating the response of PAI-1 to glucose [11] and angiotensin II [12]. An activator protein 1 (AP-1)-like binding site mediates the PAI-1 response to protein kinase C (PKC) and protein kinase A (PKA) signals [13,14]. A putative insulin response element has been postulated for position −98/−62 upstream of the transcription initiation site [15]. Recently, binding of the *trans*-acting factor Nur77 to a *cis*-acting element NIGF-B/NBRE has been identified as responsible for mediating induction of PAI-1 expression in response to TNFα stimulation [16]. Whilst the conventionally defined PAI-1 promoter does not contain a binding site for NFκB, a TNFα-responsive NFκB site has recently been identified 15 kb upstream of the transcription initiation site of the PAI-1 gene [17].

TZDs reduce insulin resistance, modify dyslipidaemia [18], improve endothelial function and may be protective against the development of atherosclerosis. TZDs are activating ligands for the peroxisome proliferator-activated receptor γ (PPARγ) [19]. The effects of TZDs on endothelial cell PAI-1 expression in vitro remains inconclusive with reports of decreased [20–22] and increased [23,24] PAI-1 expression using different cell and culture systems.

In addition to the controversy surrounding the effects of TZDs on PAI-1 expression, the molecular mechanisms responsible for these effects remain unclear as no conventional peroxisome proliferator response element (PPRE) has been identified in the PAI-1 gene promoter. The in vitro trans-repression of PAI-1 by TZDs may result, in part, from PPARγ antagonism of other *trans*-activating factors such as AP-1 and NFκB, previously demonstrated in macrophages [25], or could perhaps reflect PPARγ-independent activity similar to trans-repression of endothelial proinflammatory cytokines by TZDs [19]. Such PPARγ-independent effects have also been documented in the setting of macrophage gene expression in lipid metabolism and inflammation [26].

Our study aims to clarify the current controversy surrounding TZD-mediated regulation of TNFα-induced PAI-1 expression in vascular endothelial cells and to identify the molecular mechanisms responsible for this effect.

Materials and methods

Materials. Rosiglitazone (RG) was kindly donated by Glaxo, Smith Kline (GSK), and Pioglitazone (PG) was kindly provided by Takeda Chemical Industries (Japan). The PPARγ inhibitor SR-202, dimethyl-(dimethoxyphosphinyl)-*p*-chlorobenzyl phosphate, was kindly provided by Ilex Onc (Switzerland). TNFα was purchased from Promega (Australia).

Cell culture. The spontaneously transformed human vascular endothelial cell line, C11 STH [27], was cultured to confluence at 37 °C in gelatin-coated Nunclon cell culture dishes in Media-199 supplemented with penicillin/streptomycin, 20% FCS, 20 μg/ml endothelial cell growth factor, and 20 μg/ml heparin. The glucose concentration in cell culture medium was 6.0 mM.

The cells were cultured with or without TNFα (10 ng/ml), with or without RG (10 μM) or PG (10 μM) or SR-202 (100 μM) under serum free conditions for 16 h.

Determination of PAI-1 protein expression. Semiconfluent cultures of C11 STH cells were incubated under serum-free conditions, with or without treatment, for 16 h. The conditioned medium was collected and centrifuged at 10,000 rpm for 1 min, and the PAI-1 protein concentration in the supernatant was quantified using an ELISA assay (TintELIZE PAI-1; Biopool). Experiments were performed a minimum of three times.

Northern blot procedure. Total RNA was extracted by the method of Chomczynski and Sacchi [28]. Ten micrograms of RNA was loaded to each lane and electrophoresed through a 1% agarose gel containing 20% formaldehyde before being transferred to Hybond-N^+ membrane (Amersham, Australia). Filters were hybridized overnight at 42 °C in 50% formamide hybridization buffer [29]. ^{32}P-labelling of DNA was performed by the random priming procedure using the Prima-gene labelling kit (Promega, Australia) and hybridization using the Pst-1 fragments of PAI-1 [29]. After hybridization, the membranes were washed by standard techniques and exposed to Kodak BioMax film (Eastman Kodak, Rochester, NY, USA) at −70 °C with an intensifying screen. Stripping of membranes for rehybridization was performed by heating the membranes at 90 °C for 10 min in 2× SSC containing 0.1% SDS. Experiments were performed a minimum of three times and quantification of the 3.2 kb PAI-1 mRNA transcript (upper band) was performed.

Transient transfection and chloramphenicol acetyl transferase assay. C11 STH cells were transiently transfected with the PPRE-TK-CAT construct [30], the kind gift from Professor Donald Jump (Michigan State University, USA) or the dominant-negative mutant PPARγ expression vector [31,32], the kind gift of Dr. John Wentworth (Royal Melbourne Hospital, Melbourne, Australia), or a PAI-1-CAT promoter construct containing the first 1.4 kb of the human PAI-1 gene promoter sequence [16], using the calcium phosphate precipitation method [33]. Cells were also co-transfected with plasmid pSV-β-galactosidase (Promega, Madison, WI, USA), and β-galactosidase activity was used as an internal control for transfection efficiency. Transfected cells were harvested 2 days later and cellular extracts were then prepared. Chloramphenicol acetyl transferase (CAT) activity assay was performed by incubating 40 μl of protein extracts (25 μg of protein), with 4 μl of 4.4 mm acetyl CoA and 1.0 μl of [^{14}C]chloramphenicol at 37 °C for 4 h. Samples were extracted with 1 ml ethyl acetate, vacuum dried, and finally dissolved in 20 μl of ethyl acetate. The samples were spotted onto TLC plates and processed using standard techniques. Conversion of [^{14}C]chloramphenicol into its acetylated product was assessed by autoradiography. β-Galactosidase activity in cell extracts was determined as previously described [34]. The degree of CAT activity was normalized by adjusting for changes in β-galactosidase activity in the same samples. Experiments were performed a minimum of three times.

Preparation of nuclear extracts. The preparation of nuclear extracts from C11 STH cells was performed as previously described [35]. Cells were added to 0.1 ml of NP40 lysis buffer [0.5% NP40, 10 mM NaCl, 10 mM Tris (pH 7.4), 3 mM $MgCl_2$, 5 mM DTT, and 1 mM PMSF]. After 10 min incubation on ice, samples were briefly centrifuged and washed with 0.2 ml NP40 buffer, and pellets were resuspended in 100 μl of buffer C [420 mM NaCl, 20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 5 mM DTT, and 5 mM PMSF]. After incubation on ice for 20 min and centrifugation at 13,000*g* at 4 °C for 20 min, the supernatants containing nuclear proteins (NPs) were collected and stored at −80 °C.

Electrophoretic mobility shift assay. Gel-purified oligonucleotides (100 ng) were labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP.

Annealing was performed by adding a fourfold molar excess of the complementary strand and gel purified as previously described [35]. After elution from native acrylamide gel, samples were precipitated with ethanol and finally resuspended in NaCl/Tris/EDTA buffer [100 mm NaCl, 100 mm Tris (pH 7.4), and 10 mm EDTA (pH 7.4)] to approximately 100 cp/μl. To perform the electrophoretic mobility shift assays (EMSAs), 4–10 μg of NP extracts in 4 μl of Osborne buffer D [20 mM Hepes (pH 7.9), 2.0 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 5 mM DTT, 5 mM PMSF, and 50 mM KCl] [36] was incubated at 4 °C for 15 min with 1 μl poly[d(I–C)] (1 μg; used as a nonspecific competitor; Boehringer Mannheim, Germany) and 3 μl of SMK buffer (12 mM spermidine, 12 mM $MgCl_2$, and 200 mM KCl). Four microliters of ^{32}P-labelled probe (100 cp) diluted in Osborne buffer D was then added and the mixtures incubated on ice for a further 15 min before being applied to a native 5% polyacrylamide gel [36].

For competition titration experiments, extracts were incubated with 100 ng of unlabelled oligomers of either identical or unrelated sequence for 15 min prior to the addition of the probe. After electrophoresis, gels were fixed, dried, and autoradiographed overnight at −70 °C with an intensifying screen. The sequence of the labelled NBRE oligomer is 5′-AGAGGGCAGAAAGGTCAAGGG-3′ and the sequence of the unrelated competitor is 5′-TCAGCAAGGGA AATGGCTT-3′. Experiments were performed a minimum of three times.

Western blot procedure. Nuclear extracts from C11 STH cells (5.0 μg) were separated by SDS–PAGE under reducing conditions and transferred to nylon membranes. Membranes were hybridized with a polyclonal antibody directed against Nur77/Nurr1 (E20; Santa Cruz). A secondary antibody coupled to horseradish peroxidase was added and the immunocomplexes were assessed by enhanced chemiluminescence. Experiments were performed a minimum of two times.

Statistical methods. The effect of compounds on PAI-1 protein production, mRNA expression, and CAT activity were assessed by analysis of variance (ANOVA). Data were expressed as means ± SEM and $*P < 0.05$ was considered statistically significant.

Results

Effect of PG and RG on constitutive and TNFα inducible PAI-1 mRNA expression and protein secretion

Both PG and RG at 10 μM had no effect on constitutive expression of PAI-1 mRNA and PAI-1 protein secretion (Figs. 1A–C). TNFα-induced PAI-1 mRNA and protein expression, whilst PG and RG, at 10 μM, attenuated TNFα-mediated induction of PAI-1 mRNA and protein expression (Figs. 1D–F). All results reached statistically significant levels and were of a magnitude similar to that previously reported in the literature [21,22,37]. Both the upper (3.2 kb) and lower (2.3 kb) PAI-1 mRNA transcripts were identified as previously described [38]. Modulation of both PAI-1 mRNA transcripts by treatment with TNFα and PG or RG was observed, consistent with previous reports [21–23,37]. Quantification of the upper (3.2 kb) PAI-1 mRNA transcript was performed in all Northern blot experiments to facilitate graphical data presentation.

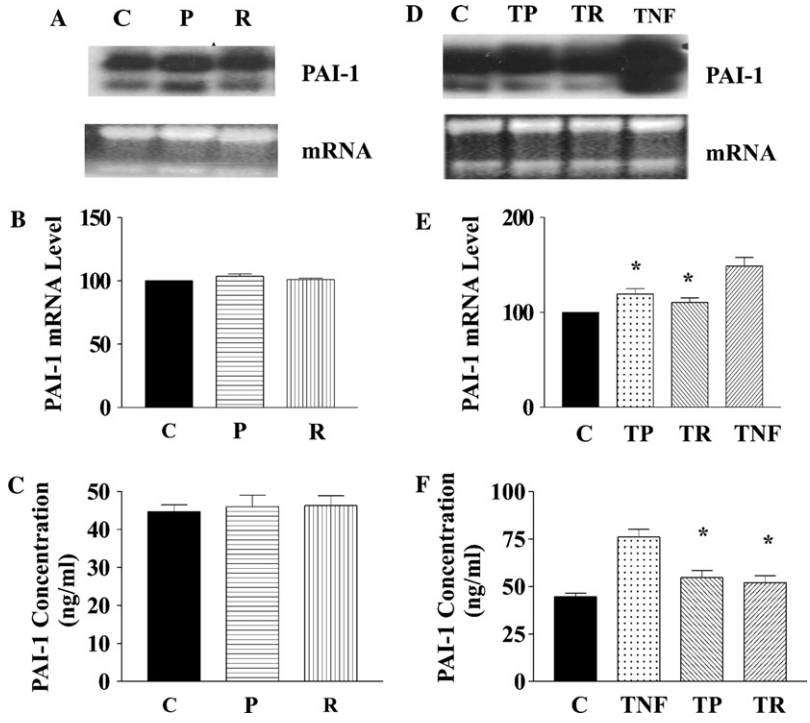


Fig. 1. (A,D) Northern blot of Pioglitazone and Rosiglitazone treatment on PAI-1 mRNA expression. 18S and 28S mRNA expression provides a loading control. (B,E) Quantitation of Pioglitazone and Rosiglitazone treatment on PAI-1 mRNA expression. (C,F) Effects of Pioglitazone and Rosiglitazone on PAI-1 protein secretion. C, control; P, Pioglitazone; R, Rosiglitazone; TNF, TNFα; TP, TNFα + PG; TR, TNFα + RG. [$n = 3$; $*P < 0.05$; versus TNF].

PG and RG attenuation of TNFα-stimulated PAI-1 mRNA and protein expression is independent of inducible PPARγ activation

SR-202, a potent and specific inhibitor of inducible PPARγ activation, did not significantly modify the inhibitory effects of RG and PG on TNFα-stimulated PAI-1 mRNA expression and protein secretion (Figs. 2A–C).

Both PG and RG at 10 μM increased expression of the PPRE-TK-CAT expression vector transiently transfected into C11 STH cells. These results indicate the presence of an inducible PPARγ system in the C11 STH cell system (Fig. 2D). SR-202 treatment significantly reduced CAT-reporter gene expression induced by RG and PG in C11 STH cells to control levels demonstrating that SR-202 is an effective inhibitor and abolishes inducible PPARγ activation (Fig. 2D).

A dominant-negative PPARγ construct identifies RG-mediated inhibition of TNFα-induced PAI-1 expression as independent of inducible PPARγ activation

In C11, STH cells co-transfected with the dominant-negative PPARγ and PPRE-TK-CAT constructs, no significant RG (10 μM)-inducible CAT activity was observed demonstrating successful inhibition of inducible PPARγ activation in this cell system (Fig. 3A).

RG treatment at 10 μM, continued to significantly attenuate TNFα-induced reporter gene activity in C11 STH cells, co-transfected with a 1.4 kb PAI-1 promoter construct [16] and the dominant-negative PPARγ construct (Fig. 3B). As expected, RG treatment alone had no effect on CAT activity in cells co-transfected with the 1.4 kb PAI-1 promoter construct and the dominant-negative PPARγ construct (Fig. 3B).

In addition, RG treatment at 10 μM continued to significantly attenuate TNFα-induction of PAI-1 mRNA in cells transfected with the dominant-negative PPARγ construct (Fig. 3C).

NP binding to the PAI-1 NBRE and Nur77/Nurr1 expression is inhibited by RG and PG in TNFα-stimulated C11 STH cells independent of inducible PPARγ activity

NPs extracted from C11 STH cells specifically bound to a ^{32}P-labelled oligonucleotide harbouring the PAI-1 NBRE element as demonstrated by EMSA. Increased binding was observed upon TNFα stimulation and both PG and RG treatments attenuated the TNFα-mediated

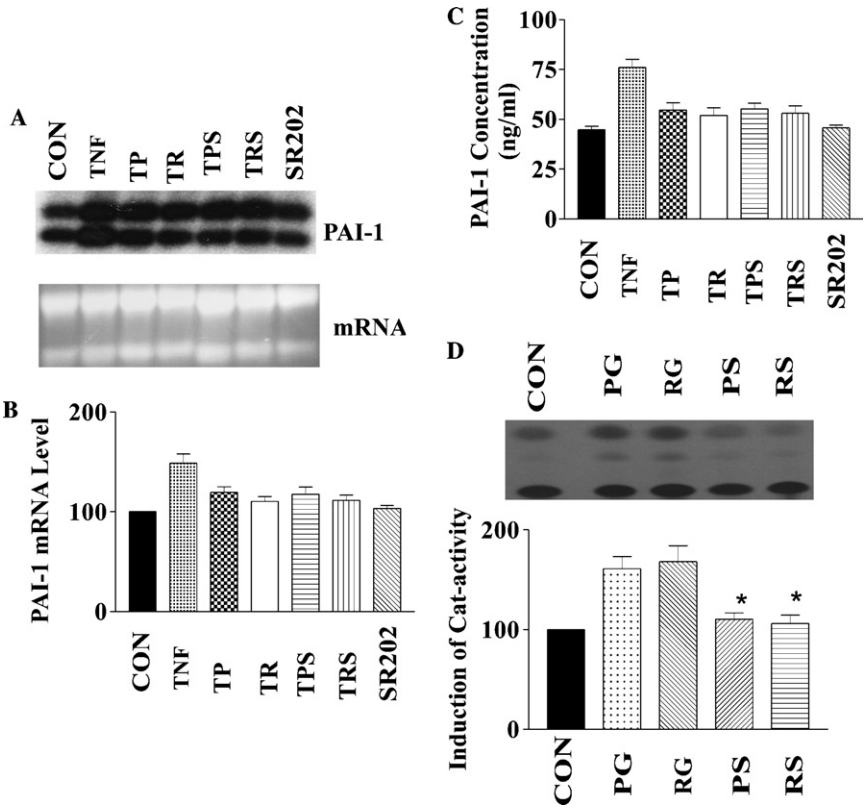


Fig. 2. (A) Northern blot of Pioglitazone, Rosiglitazone, and SR-202 treatment on TNFα-induced PAI-1 mRNA expression. 18S and 28S mRNA expression provides a loading control. (B) Quantitation of Pioglitazone, Rosiglitazone and SR-202 treatment on TNFα-induced PAI-1 mRNA expression. (C) Effects of Pioglitazone, Rosiglitazone, and SR-202 on PAI-1 protein secretion. (D) Effect of Pioglitazone, Rosiglitazone, and SR-202 treatment on PPRE-TK-CAT activity. Con, control; TNF, TNFα; PG, Pioglitazone; RG, Rosiglitazone; TP, TNFα + PG; TR, TNFα + RG; PS, PG + SR-202; RS, RG + SR-202; TPS, TNFα + PG+SR-202; TRS, TNFα + RG + SR-202. [$n = 3$; $^{*}P < 0.05$; versus PG or RG, respectively].

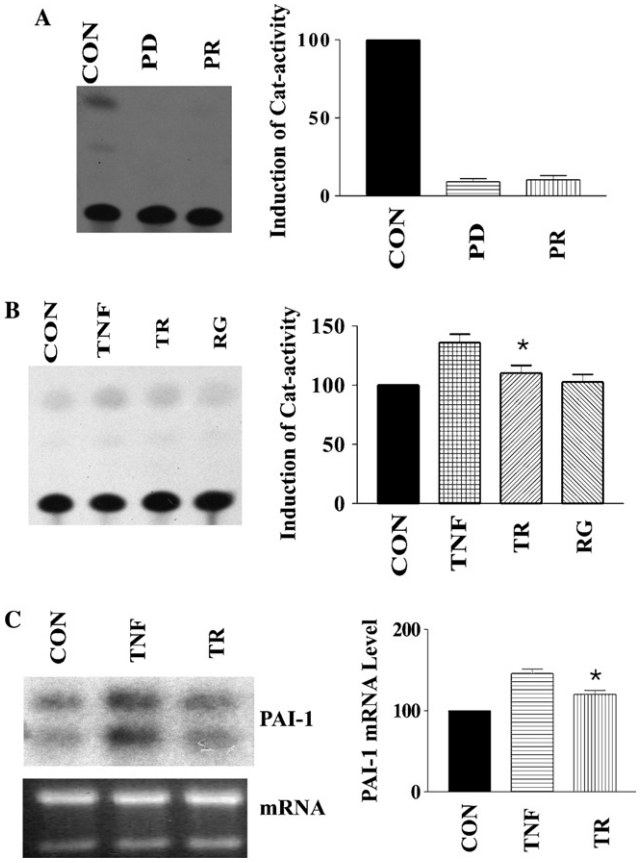


Fig. 3. (A) Effect of Rosiglitazone treatment on PPRE-TK-CAT activity in C11 STH cells co-transfected with the PPRE-TK-CAT, plasmid pSV-β-galactosidase, and dominant-negative PPARγ mutant constructs. CON, C11 STH cells transfected with the PPRE-TK-CAT construct and plasmid pSV-β-galactosidase; PD, C11 STH cells co-transfected with the PPRE-TK-CAT, plasmid pSV-β-galactosidase, and dominant-negative PPARγ mutant construct; PR, PD + RG. The panel on the right represents quantification of the observed effects over a minimum of three experiments. (B) Effect of Rosiglitazone and/or TNFα treatment on PAI-1-CAT activity in C11 STH cells co-transfected with the 1.4 kb PAI-1-TK-CAT, plasmid, pSV-β-galactosidase, and dominant-negative PPARγ mutant constructs. CON, C11 STH cells transfected with the 1.4 kb PAI-1-TK-CAT construct, plasmid pSV-β-galactosidase, and dominant-negative PPARγ mutant construct. TNF, TNFα-stimulated C11 STH cells co-transfected with the 1.4 kb PAI-1-TK-CAT, plasmid pSV-β-galactosidase, and dominant-negative PPARγ mutant construct; RG, Rosiglitazone + CON; TR, TNFα + RG. (C) Northern blot of Rosiglitazone treatment on TNFα-induced PAI-1 mRNA expression in C11 STH cells co-transfected with the plasmid pSV-β-galactosidase and dominant-negative PPARγ mutant construct. 18S and 28S mRNA expression provides a loading control. The right panel shows the quantitation of PAI-1 mRNA expression. CON, C11 STH cells co-transfected with plasmid pSV-β-galactosidase and dominant-negative PPARγ mutant construct; TNF, TNFα stimulated C11 STH cells co-transfected with the plasmid pSV-β-galactosidase and dominant-negative PPARγ mutant construct; TR, TNF + RG [$n = 3$; *$P < 0.05$; versus TNF].

increase in binding of NPs while use of SR-202 had minimal effect on RG and PG attenuation of NP binding (Fig. 4). To determine if the effects of TNFα and PG and RG on NP binding correlated with expression of

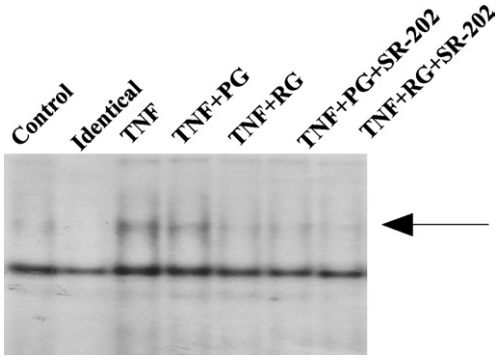


Fig. 4. C11 STH cells were untreated (control), treated with a 100-fold molar excess of unlabelled identical oligonucleotides (identical), TNFα (TNF), TNFα + PG, TNFα + RG, TNFα + PG + SR-202, and TNFα + RG + SR-202. Specific binding is indicated by black arrow.

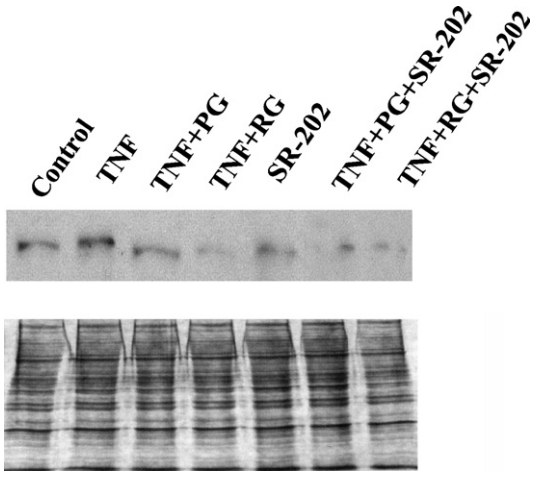


Fig. 5. Detection of Nur77/Nurr1 by Western blot was observed in unstimulated C11 STH (control) cells, and C11 STH cells treated with TNFα (TNF), TNFα + PG, TNFα + RG, TNFα + PG + SR-202, TNFα + RG + SR-202, and SR-202 alone. Coomassie stain of SDS–PAGE gel indicates balanced protein loading.

Nur77/Nurr1, Western blot analysis of NP extracts from C11 STH cells was performed. An increase in Nur77/Nurr1 expression in response to TNFα stimulation with minimal effect of SR-202 treatment on attenuation of TNFα-mediated induction of Nur77/Nurr1 expression by RG and PG (Fig. 5) was observed.

Discussion

Increased circulating levels of PAI-1 are thought to contribute to the risk of accelerated atherosclerosis [3,4]. As previous studies have demonstrated that TNFα can stimulate PAI-1 expression in endothelial cells [8], inhibition of TNFα-induced endothelial cell PAI-1 expression may be beneficial in the prevention and treatment of atherosclerosis in conditions associated with elevated TNFα including diabetes and the MS.

The TZDs are a recently introduced class of compounds identified as activators of the PPARγ nuclear receptors. These agents are thought to exert their therapeutic effect, in part, by modifying transcriptional expression. The present study was undertaken to clarify current anomalies in the literature surrounding modulation of PAI-1 expression by the TZDs in endothelial cells and dissect out the molecular mechanisms responsible for these effects.

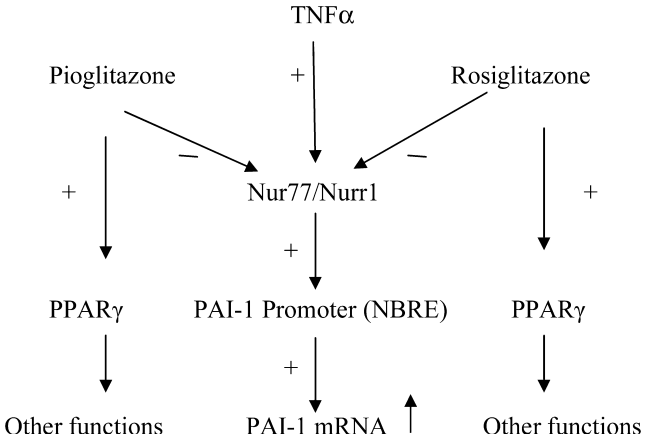
Previously, Kato et al. [21] suggested that TZDs inhibit TNF α-induced PAI-1 mRNA expression, possibly through a PPARγ-mediated mechanism, at low micromolar concentrations (10–20 μM). Studies by Hong et al. [22] supported these findings suggesting that PPARγ activation inhibits PAI-1 production in vascular endothelial cells via direct transcriptional repression, although no PAI-1 promoter *cis*-acting elements or molecular mechanism(s) for this effect are described. In contradistinction to these findings, other reports describe that PPARγ activation in unstimulated or moderately stimulated endothelial cells results in the induction of PAI-1 expression at concentrations similar to those utilized by Kato et al. [21,23,24]. We have clearly demonstrated that PG and RG, widely used TZDs in clinical practice, suppress PAI-1 expression in endothelial cells stimulated by TNFα to levels, and at concentrations, previously reported in the literature [21,22,37].

Whilst PPARγ activation has principally been associated with induction of gene expression in numerous cell systems, it has recently been demonstrated that PPARγ agonists are also capable of inhibiting gene expression, in part, by antagonizing the activities of other activating transcription factors including AP-1, signal transducers and activators of transcription 1 (STAT-1), and NFκB [25]. TZD-mediated antagonism of NFκB activity has been postulated to contribute to the inhibition of TNFα-mediated induction of PAI-1 expression in endothelial cells [37]. In addition, it has also been demonstrated that endogenous ligands of PPARγ may have actions independent of PPARγ activation in endothelial cells [39]. Recent studies, utilizing a dominant-negative approach to assess the effects of PPARγ in regulating glucose uptake into adipocytes in the presence of TZDs also suggest that these agents may act independent of PPARγ activation [31]. Although no classical PPRE response element has been identified in PAI-1 promoter, our results demonstrate that TZDs inhibit TNFα-mediated induction of PAI-1 expression in C11 STH cells, suggesting the possibility of TZDs acting independently of inducible PPARγ activation, perhaps via another *cis*-acting element. In order to determine if these effects were mediated independent of inducible PPARγ activation, we utilized the recently described specific PPARγ inhibitor SR-202 [40] and transfection of a dominant-negative PPARγ construct [31,32]. SR-202 treatment had no significant effect on TZD-mediated modulation of TNFα stimulation of PAI-1 secretion (Figs. 2A–C), suggesting that these effects are mediated independent of inducible PPARγ activation. In addition, RG treatment resulted in persistent inhibition of TNFα-mediated induction of a reporter gene construct harbouring 1.4 kb of the PAI-1 promoter, previously demonstrated to be TNFα responsive [16] and of PAI-1 mRNA in C11 STH cells co-transfected with a dominant-negative PPARγ construct (Figs. 3B and C). This is further strong evidence supporting the hypothesis that TZDs modify TNFα-stimulated secretion of PAI-1 independent of inducible PPARγ activation. The presence of an active, inducible PPARγ system in the C11 STH cell system was confirmed with the activation of a PPRE-TK-CAT construct transfected into C11 STH cells and stimulated with RG (Fig. 2D). The efficacy of SR 202 as an inhibitor of inducible PPARγ activation was also demonstrated using this system (Fig. 2D). Co-transfection of the PPRE-TK-CAT and PPARγ dominant-negative constructs demonstrated effective silencing of inducible PPARγ activation in the C11 STH cell system when treated with RG (Fig. 3A). Whilst addressing the need for inducible PPARγ activation, these experiments do not determine whether our current observations are entirely PPARγ independent. Additional studies, currently being performed in the laboratory, utilizing PPARγ−/− embryonic stem cells will enable the determination of the absolute requirement for PPARγ in TZD-mediated inhibition of TNFα-induced PAI-1 expression. Interestingly, previous reports in the literature suggest that PPARγ receptor is required for the positive effects of its ligands (TZDs) on gene regulation, although the inhibitory effects on gene regulation by TZDs may not require the presence of receptor [26]. In addition, evidence suggests TZDs are also able to mediate regulation of other *trans*-acting factor (e.g., NFκB) binding and activity in the absence of PPARγ [41].

To investigate alternative molecular mechanisms for TZD-mediated inhibition of TNFα-induced PAI-1 expression, in the light of evidence to suggest this effect was independent of inducible PPARγ, we explored the effects of TZDs and TNFα on the binding of NPs to the PAI-1 (NBRE) *cis*-acting response element, previously identified to be important in TNFα-mediated regulation of PAI-1 expression [16]. Our results demonstrated increased binding of NPs to the *cis*-acting element NBRE upon TNFα stimulation. In addition, both PG and RG treatments attenuated the TNFα-mediated increase in the binding of NPs to this element (Fig. 4). The specific PPARγ inhibitor SR-202 failed to modulate this effect significantly. Whilst it has been previously demonstrated that several members of the orphan nuclear receptor subfamily, NGFI-B, including Nur77, Nurr1, and NOR-1, bind in a monomeric and specific manner to the NBRE consensus sequence [42] western blot analysis of NP extracts from C11 STH cells utilizing

the E20 antibody (Santa Cruz), known to recognize the C-terminus of Nur77 or Nurr1, had a similar expression profile to that observed in EMSA studies in response to TNFα, RG, PG, and SR-202 treatment (Fig. 5), suggesting that either Nur77 or Nurr1 binds the PAI-1 NBRE in C11 STH cells. Taken together, these findings suggest that (a) PG- and/or RG-mediated inhibition of TNFα-induced PAI-1 production correlates with the inhibition of binding of NPs of the NGFI-B family to the PAI-1 NBRE-binding site and expression of either Nur77 or Nurr1. (b) These effects are likely to be independent of inducible PPARγ activation. (c) This may represent a novel molecular mechanism to explain the inhibitory effects of TZDs on TNFα stimulated PAI-1 expression in the absence of inducible PPARγ activation.

In conclusion, our findings demonstrate that the TZDs RG and PG inhibit TNFα-mediated induction of PAI-1 expression in C11 STH vascular endothelial cells and that inducible PPARγ activation is unlikely to be required for this effect. Furthermore, we have identified the regulation of TNFα-mediated induction of Nur77/Nurr1 expression and NGFI-B family member NP binding to the PAI-1 gene promoter (NBRE) by TZDs as potentially involved in mediating these effects, also in the absence of inducible PPARγ activation (see model).



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