PPARγ-independent thiazolidinedione-mediated inhibition of NUR77 expression in vascular endothelial cells

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Abstract

The thiazolidinediones (TZDs) have been reported to reduce atherogenesis in preclinical models and atherosclerosis in clinical trials in pre-diabetic and diabetic patients. Although peroxisome proliferator-activated receptor γ (PPARγ)-mediated effects on gene expression have been thought responsible for this effect, a complete understanding of the molecular mechanisms responsible remains to be fully elucidated. We have previously reported PPARγ-independent modulation of NUR77 (also known as Nr4a1), an orphan nuclear receptor deemed important in the atherogenic process, in association with TZD-mediated inhibition of tumour necrosis factor α (TNFα) induction of plasminogen activator inhibitor type 1 expression. Here, we report NUR77 mRNA expression is increased in human vascular endothelial cells (HUVEC) stimulated by TNFα and that this effect is inhibited by a TZD in a PPARγ-independent manner. TZD treatment of HUVEC also inhibited the stimulatory effects of TNFα on NUR77 promoter activity, again in a PPARγ-independent manner, confirming the transcriptional nature of this effect. TZD treatment also attenuated the binding of nuclear proteins to the nuclear factor kappa B (NF-κB)-binding site of the NUR77 promoter in HUVEC in a PPARγ-independent manner. In addition, TZD treatment also inhibited TNFα-mediated induction of NF-κB1 mRNA expression. Our results suggest a potential PPARγ-independent molecular mechanism for the anti-atherogenic effects of TZDs involving NF-κB1-mediated transcriptional inhibition of cytokine-mediated induction of the orphan nuclear receptor NUR77 in HUVEC.

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Introduction


Thiazolidinediones (TZDs) reduce insulin resistance, modify dyslipidaemia (Mimura et al. 1994), improve endothelial function and are thought to be protective against the development of atherosclerosis (Dormandy et al. 2005, Wöhrle et al. 2008). Some recent studies have suggested adverse cardiovascular effects of TZDs, in particular rosiglitazone (Home et al. 2007, Nissen & Wolski 2010).

TZDs are activating synthetic ligands for the peroxisome proliferator activating receptor family of nuclear receptors (PPAR), particularly the PPARγ receptor, which acts to regulate gene transcription (Bar-Tana 2001). PPARγ is expressed most abundantly in adipose tissue, pancreatic β cells, vascular endothelium and macrophages (Dubois et al. 2000, Willson et al. 2001). Some evidence suggests that the effects of TZDs are mediated independent of PPARγ with previous studies demonstrating PPARγ-independent TZD-mediated inhibition of TNFα-stimulated plasminogen activator inhibitor type 1 (PAI-1, also known as SERPINE1) in human vascular EC cells (Liu et al. 2005).

The molecular mechanisms responsible for the PPARγ-independent regulation of PAI-1 expression by TZDs remain to be fully elucidated although previous studies have demonstrated that the binding of the orphan nuclear receptor NUR77 (also known as Nr4a1) to its cis-acting response element in the proximal part of the PAI-1 promoter is crucial for upregulation of PAI-1 expression in vascular endothelial cells in response to TNFα (Gruber et al. 2003) and that TZDs modulate NUR77 expression in TNF-stimulated vascular endothelial cells in a PPARγ-independent manner (Liu et al. 2005).
NUR77, a member of orphan nuclear receptors NR4A subfamily, was initially identified as a growth-factor-inducible gene (Milbrandt 1988). Orphan nuclear receptors are ligand independent (Baker et al. 2003, Wang et al. 2003) and consist of an N-terminal transactivation domain, a central DNA-binding domain and a C-terminal ‘ligand-binding’ domain (He 2002). NR4A proteins bind to the NGFI-B response element (NRE) sequence (AAAGGTTCA) as monomers (Wilson et al. 1991) and to the palindromic NUR77 response element (NURRE) sequence (TGATT- TTTGGAAATG) as homodimers (Philips et al. 1997) in responsive gene promoters.

NUR77 expression is increased in atherosclerotic vessels and co-localises with PAI-1 (Gruber et al. 2003). A dominant-negative form of NUR77 abolishes the effect of TNFα on PAI-1 expression (Gruber et al. 2003). Pei et al. (2005, 2006) demonstrated that NUR77 is expressed in macrophages within human atherosclerotic lesions and is highly induced by various inflammatory stimuli including TNFα via the nuclear factor kappa B (NF-κB) signalling pathway.

Our current studies aimed to determine the molecular mechanisms responsible for TZD-mediated regulation of NUR77 expression with a view to determining the potential molecular basis for the anti-atherogenic action of this class of agents.

Materials and Methods

Cell culture

Human vascular endothelial cells (HUVEC) were cultured to confluence at 37 °C in gelatin-coated cell culture dishes in Media-199 with 20% FCS and 6-0 mM glucose. Human embryo kidney cells (HEK 293) were cultured in DMEM medium with 10% FCS. Both cells were cultured with or without TNFα (10 ng/ml; Promega) and with or without rosiglitazone (10 μM) (kind gift from GlaxoSmithKline) (10 μM) under serum-free conditions for designated times. The specific PPARγ antagonist SR202 dimethyl-(dimethoxyphosphoryl)-p-chlorobenzyl phosphate (Rieux et al. 2002) was kindly provided by Ilex Onc (Geneva, Switzerland) and used at 100 μM in experiments as indicated.

Quantitative RT-PCR

Total RNA was extracted from HUVEC using Trizol reagent (Invitrogen). An aliquot (2 μg) of the total RNA was reverse transcribed in 20 μl reaction mixture at 50 °C for 1 h. cDNA was then amplified by real-time PCR using specific primers. Primers used to amplify human NUR77 were forward: 5′-GCT GCA GAA GTA CTC CACC-3′, reverse: 5′-ACA GCA GCA CTG GGC TTA-3′. Primers used to amplify NF-κB1 were forward: 5′-CAG GAA GAT GTG GTG GAG GAT-3′, reverse: 5′-TGT CTG GCT CCA CAG CCA GGT-3′.

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Transient transfection

The 1.4 kb mouse NUR77 promoter was cloned into pGL3 basic luciferase reporter vector (kind gift from Prof. P Tontonoz, UCLA, USA). The mouse NUR77 promoter construct was transiently transfected into HEK 293 cells by using the calcium precipitate method in triplicate. Luciferase activity was normalised using the co-transfected β-galactosidase. The dominant-negative PPARγ construct (Nugent et al. 2001) was the kind gift from Dr John Wentworth (Royal Melbourne Hospital, Melbourne, Australia).

Preparation of nuclear proteins and electrophoretic mobility shift assay

The nuclear proteins were extracted from HUVEC as described previously (Costa & Medcalf 1996). Cells were added to 0.1 ml NP40 lysis buffer (0-5% NP40, 10 mM NaCl, 10 mM Tris (pH 7-4), 3 mM MgCl2, 5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF)) and incubated on ice for 10 min. Then 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 MgCl2, 0-2 mM EDTA, 25% glycerol and 5 mM PMSF). After incubation on ice for 20 min, samples were centrifuged for 20 min at 4 °C and the supernatants containing nuclear proteins were collected and stored at −80 °C for later use.

Oligonucleotides were labelled with [γ-32P]ATP by using T4 polynucleotide kinase. After annealing with complementary strand, double-stranded oligonucleotides were gel purified as previously described (Costa & Medcalf 1996). Electrophoretic mobility shift assays (EMSAs) were carried out by adding 4–10 μg nuclear proteins to 4 μl Osborne buffer D (20 mM HEPES (pH 7-9), 2-0 mM MgCl2, 0-2 mM EDTA, 20% glycerol, 5 mM DTT, 5 mM PMSF and 50 mM KCl; Osborn et al. 1989) and incubating at 4 °C for 15 min with 1 μl poly[d(1-C)] and 3 μl SMK buffer (12 mM spermidine, 12 mM MgCl2 and 200 mM KCl). In all, 4 μl 32P-labelled probe (100 c.p.m.) diluted in Osborne buffer D were then added and the mixture was incubated on ice for further 10 min before being applied to a native 5% polyacrylamide gel (Osborn et al. 1989). For competition experiments, nuclear proteins were incubated with 200 ng unlabelled oligomers of either identical or unrelated sequence for 15 min prior to the addition of the probe. After electrophoresis, gels were fixed and dried, then autoradiographed over one or two nights at −70 °C with an intensifying screen. The sequence of the labelled NF-κB oligonucleotide was 5′-GGG-GGG-TGT-TCC-TCA-CCT-GAG-GCC-3′, and the sequence of the non-specific, unrelated competitor was 5′-GGA-TCC-GAA-TCC-TCC-4′. All experiments were performed three times.
Statistical analysis

The effects of the agents on NUR77 promoter activity were assessed by ANOVA. Data were expressed as mean ± s.e.m. and P < 0.05 was considered statistically significant.

Results

TZD-mediated regulation of NUR77 mRNA expression

Previous studies have shown that in HUVEC, expression of NUR77 mRNA is highly inducible by TNFα (Gruber et al. 2003) although the effects of TZDs on TNFα-mediated induction of NUR77 expression in this cell type are unknown. HUVECs were treated with TNFα and rosiglitazone for 1, 2 and 4 h. Real-time quantitative PCR (qPCR) was used to identify NUR77 mRNA expression in HUVEC cells. Time-course experiments demonstrated that expression of NUR77 mRNA was significantly induced by TNFα as early as 1 h by up to 18-fold over untreated control cells, and then reduced to 2-fold over untreated cells at 4 h treatment (Fig. 1). Co-treatment with rosiglitazone of TNFα-treated HUVEC cells resulted in a profound inhibition of TNFα induction of NUR77 mRNA expression to approximately fivefold at 1 h (Fig. 1). The inhibitory effect of rosiglitazone on TNFα-mediated induction of NUR77 expression was demonstrated to be independent of PPARγ activity as addition of the specific PPARγ inhibitor SR202 (Rieusset et al. 2002) did not affect the inhibitory action of rosiglitazone on TNFα-mediated induction of NUR77 mRNA expression at 1 h (Fig. 2).

TZD-mediated regulation of NUR77 promoter activity

The mouse 1.4 kb NUR77 promoter was used to examine the effects of rosiglitazone on regulation of TNFα induction of NUR77 promoter activity. TNFα stimulation (10 ng/ml) induced NUR77 promoter activity within 1 h and continued for up to 3 h (Fig. 3a). Rosiglitazone had no significant effect on TNFα-induced NUR77 promoter activity within 3 h (Fig. 3a) although a trend to inhibition was noted at the later time point (e.g. 3 h; Fig. 3a). Specific treatment with TNFα for 4 h resulted in significant upregulation of NUR77 promoter activity and a significant inhibitory effect of rosiglitazone on TNFα-induced NUR77 promoter activity at 4 h (Fig. 3b). Treatment of transfected cells with the specific PPARγ inhibitor SR202 had no effect on suppression of TNFα-stimulated NUR77 promoter activity by rosiglitazone (Fig. 3b), suggesting that this transcriptional effect was PPARγ independent. Co-transfection of a PPARγ dominant-negative construct into the cells transfected with the NUR77 promoter construct also had no effect on rosiglitazone-mediated suppression of TNFα-stimulated NUR77 promoter activity (Fig. 4), confirming the PPARγ-independent nature of this effect.

Nuclear protein binding to the NF-κB-binding site in the NUR77 promoter

The human and mouse Nur77 promoters share highly conserved binding sites for NF-κB and AP-1 transcription factors (Pei et al. 2005). To define the molecular mechanism of TZD-mediated inhibition of NUR77 expression, we performed EMSAs using the NF-κB-binding sites within the NUR77 promoter region (nt −1151 to −1170). EMSA results demonstrate that 32P-labelled oligonucleotides harbouring the NF-κB-binding site within the NUR77 promoter specifically bind nuclear proteins from HUVEC cells (Fig. 5). The intensity of the protein/DNA complex increased with TNFα treatment, which was subsequently downregulated by the concomitant treatment with rosiglitazone (Fig. 5). Co-treatment with the specific PPARγ inhibitor SR202

Figure 1 Time course of NUR77 mRNA expression. HUVEC cells were treated with TNFα (10 ng/ml) with or without rosiglitazone (10 μM) for the indicated times. T, TNFα; R, rosiglitazone; T+R, TNFα+rosiglitazone (n=3).

Figure 2 Effect of SR202 on 1 h TNFα-induced NUR77 mRNA expression. Control, untreated; T, TNFα (10 ng/ml); R, rosiglitazone (10 μM); T+R, TNFα+rosiglitazone; SR202, treated with 100 μM SR202 (n=3).
had minimal effect on rosiglitazone attenuation of specific nuclear protein binding in TNFα-stimulated cells, suggesting that this effect is independent of PPARγ activity (Fig. 5).

**TZD-mediated modulation of NF-κB1 mRNA expression**

Having identified TZD-mediated attenuation of nuclear protein binding to the NF-κB-binding site in the NUR77 promoter, we were interested in determining the effect of TZDs on the expression of NF-κB1 mRNA as a possible molecular mechanism for this observation. Previous studies have demonstrated that the induction of NUR77 expression by lipopolysaccharide (LPS) was severely attenuated in the fibroblasts lacking the three NF-κB subunits, NF-κB1, C-REL and REL A (Pei et al. 2005).

Real-time qPCR was used to identify NF-κB1 mRNA expression in HUVEC cells. Experiments demonstrated that expression of NF-κB mRNA was significantly induced by TNFα at 1 h by up to fourfold over untreated control cells, and then significantly reduced to twofold over untreated cells by co-treatment with rosiglitazone of TNFα-treated HUVEC cells (Fig. 6).

**Discussion**

Upregulation of PAI-1 is recognised as a marker of endothelial cell dysfunction (Devaraj et al. 2003) and is found in patients with metabolic syndrome (MS) and diabetes. Previous studies have demonstrated inhibition of MS and atherosclerosis in PAI-1-deficient mice (Eitzman et al. 2000, Mao et al. 2004), and therefore inhibition of TNFα-induced endothelial cell PAI-1 expression may reduce the risk of atherosclerosis in diabetes and in patients with MS.

Expression of the orphan nuclear receptor NUR77 is increased in atherosclerotic vessels and co-localises with PAI-1 (Gruber et al. 2003). Previous studies have demonstrated that NUR77 binding to the proximal part of the PAI-1 promoter is crucial for upregulation of PAI-1 expression in vascular endothelial cells in response to TNFα stimulation (Gruber et al. 2003). Our previous results have demonstrated that rosiglitazone attenuates the TNFα-mediated increase in NUR77 expression and binding of nuclear proteins to the PAI-1 NBRE-binding site in a PPARγ-independent manner (Liu et al. 2005). We have been interested in the molecular mechanism responsible for PPARγ-independent regulation of NUR77 expression by TZDs in vascular endothelial cells.

Our current studies demonstrate that rosiglitazone significantly inhibited TNFα-induced NUR77 mRNA expression after 1 h treatment (Fig. 1) and that this effect was PPARγ-dependent.
independent (Fig. 2). Rosiglitazone treatment also reduced TNFα-stimulated NUR77 promoter activity significantly at 4 h (Fig. 3), again in a PPARγ-independent manner (Figs 3 and 4), suggesting the effect of rosiglitazone on NUR77 mRNA expression was transcriptional. The difference in the timing of the effects of rosiglitazone on NUR77 mRNA expression and promoter activity (1 h in the case of NUR77 mRNA and 4 h for inhibition of NUR77 promoter activity) is likely to reflect the translational component of the transfection assay with translation of luciferase protein from mRNA.

The NUR77 gene promoter has no classical PPRE response element, and as our observations suggested a PPARγ-independent effect of rosiglitazone on NUR77 promoter activity, we were interested in identifying the molecular mechanism responsible for this effect.

Previously Pei et al. (2005) have suggested a role for NUR77 in atherogenesis with the rapid induction of NUR77 expression by inflammatory stimuli, such as LPS, a process requiring the action of NF-κB proteins on the NUR77 promoter. Our EMSA results demonstrated that TNFα treatment increased the binding of nuclear proteins from HUVEC cells to the NF-κB-binding site of NUR77 promoter (Fig. 5). Rosiglitazone treatment attenuated the TNFα-mediated increase in binding of nuclear proteins to this binding site. The specific PPARγ inhibitor SR202 failed to inhibit this effect significantly (Fig. 5). In addition, rosiglitazone also inhibited TNFα-mediated induction of NF-κB1 mRNA expression, offering a possible molecular explanation for the effects of rosiglitazone on NUR77 expression (Fig. 6).

Taken together, rosiglitazone-mediated inhibition of TNFα-induced NUR77 mRNA expression correlates with inhibition of NUR77 promoter activity, binding of nuclear proteins from HUVEC cells to the NUR77 NF-κB-binding site and reduced NF-κB1 mRNA expression, independent of inducible PPARγ activation. These findings suggest a novel PPARγ-independent molecular mechanism responsible for TZD modulation of NUR77 expression, which may in turn be responsible for modulation of PAI-1.

The identification of a potentially novel PPARγ-independent molecular mechanism responsible for TZD modulation of NUR77 expression may in part explain the identified effects of TZDs on inhibition of PAI-1 expression and subsequent atheroprotection of TZDs in in vivo models and clinical studies. However, and perhaps more importantly, these observations may afford the potential for design of novel ‘PPARγ-independent’ atheroprotective agents in the future.

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**Figure 5** Effect of TNFα, rosiglitazone and SR202 on binding of nuclear proteins to the NF-κB-binding site in NUR77 promoter in HUVEC cells. Control, untreated; T, TNFα (10 ng/ml); T + R, TNFα + rosiglitazone (10 μM); T + R + SR202, T + R + treated with 100 μM SR202; NS, non-specific (unrelated) oligonucleotide; SPE, identical oligonucleotide (n = 3). Arrow indicates specific binding.

**Figure 6** Effect of TNFα and/or rosiglitazone for 1 h on NF-κB1 mRNA expression in HUVEC cells. Control, untreated; T, TNFα (10 ng/ml); R, rosiglitazone (10 μM); T + R, TNFα + rosiglitazone (n = 3).
Declarations of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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