RAPID COMMUNICATION

PPAR γ -independent thiazolidinedione-mediated inhibition of *NUR77* expression in vascular endothelial cellsYunshan Hu^{1,2}, Hong Bin Liu^{1,2}, Richard W Simpson^{2,3} and Anthony E Dear^{1,2}¹Australian Centre for Blood Diseases and ²Eastern Clinical Research Unit, Biotechnology Division, Monash University, 6th Floor Burnett Tower, 89 Commercial Road, Prahran, Melbourne, Victoria, Australia 3181³Department of Diabetes and Endocrinology, Box Hill Hospital, 4th Floor Clive Ward Centre, Arnold Street, Box Hill, Melbourne, Victoria, Australia 3128

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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 (PPAR γ)-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 PPAR γ -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 (TNF α) induction of plasminogen activator inhibitor type 1 expression. Here, we report *NUR77* mRNA expression is increased in human vascular endothelial cells (HUVEC) stimulated by TNF α and that this effect is

inhibited by a TZD in a PPAR γ -independent manner. TZD treatment of HUVEC also inhibited the stimulatory effects of TNF α on *NUR77* promoter activity, again in a PPAR γ -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- κ B)-binding site of the *NUR77* promoter in HUVEC in a PPAR γ -independent manner. In addition, TZD treatment also inhibited TNF α -mediated induction of *NF- κ B1* mRNA expression. Our results suggest a potential PPAR γ -independent molecular mechanism for the anti-atherogenic effects of TZDs involving NF- κ B-mediated transcriptional inhibition of cytokine-mediated induction of the orphan nuclear receptor *NUR77* in HUVEC.

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Introduction

Type 2 diabetes is characterised by accelerated atherosclerosis (Hobb 2006). Elevated tumour necrosis factor α (TNF α) levels and hyperglycaemia are implicated in diabetes-associated endothelial cell dysfunction and may be causal in premature atherosclerosis (Morigi *et al.* 1998, Liu *et al.* 2005, 2008, Norata *et al.* 2006, Kumar *et al.* 2007, Iwasaki *et al.* 2008).

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 PPAR γ receptor, which acts to regulate gene transcription (Bar-Tana 2001). PPAR γ 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 PPAR γ with previous studies demonstrating PPAR γ -independent TZD-mediated inhibition of TNF α -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 PPAR γ -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 TNF α (Gruber *et al.* 2003) and that TZDs modulate *NUR77* expression in TNF-stimulated vascular endothelial cells in a PPAR γ -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 (NBRE) sequence (AAAGGTCA) as monomers (Wilson *et al.* 1991) and to the palindromic NUR77 response element (NURRE) sequence (TGATA-TTTX₆AAATGCCA) 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 TNF α 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 TNF α via the nuclear factor kappa B (NF- κ B) 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 TNF α (10 ng/ml; Promega) and with or without rosiglitazone (10 μ M) (kind gift from GlaxoSmithKline) (10 μ M) under serum-free conditions for designated times. The specific PPAR γ antagonist SR202 dimethyl-(dimethoxyphosphinyl)-*p*-chlorobenzyl phosphate (Rieusset *et al.* 2002) was kindly provided by Ilex Onc (Geneva, Switzerland) and used at 100 μ M in experiments as indicated.

Quantitative RT-PCR

Total RNA was extracted from HUVEC using Trizol reagent (Invitrogen). An aliquot (2 μ g) of the total RNA was reverse transcribed in 20 μ l 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- κ B1 were forward: 5'-CAG GAA GAT GTG GTG GAG GAT-3', reverse: 5'-TGT CTG GCT CCA CAG CCA GGT-3'.

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 PPAR γ 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 μ l 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 μ g nuclear proteins to 4 μ l 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 μ l poly[d(1-C)] and 3 μ l SMK buffer (12 mM spermidine, 12 mM MgCl₂ and 200 mM KCl). In all, 4 μ l 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- κ B oligonucleotide was 5'-GCG-GGC-TGT-TCC-TCA-CTC-CA-3', and sequence of the non-specific, unrelated competitor was 5'-CTT-GAT-CCA-TCC-ACT-GCC-GG-3'. 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 $\pm$ 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 TNF α (Gruber *et al.* 2003) although the effects of TZDs on TNF α -mediated induction of *NUR77* expression in this cell type are unknown. HUVECs were treated with TNF α 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 TNF α 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 TNF α -treated HUVEC cells resulted in a profound inhibition of TNF α induction of *NUR77* mRNA expression to approximately fivefold at 1 h (Fig. 1). The inhibitory effect of rosiglitazone on TNF α -mediated induction of *NUR77* expression was demonstrated to be independent of PPAR γ activity as addition of the specific PPAR γ inhibitor SR202 (Rieusset *et al.* 2002) did not affect the inhibitory action of rosiglitazone on TNF α -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 TNF α induction of *NUR77* promoter activity. TNF α stimulation (10 ng/ml)

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

Figure 2 Effect of SR202 on 1 h TNF α -induced *NUR77* mRNA expression. Control, untreated; T, TNF α (10 ng/ml); R, rosiglitazone (10 μ M); T+R, TNF α +rosiglitazone; SR202, treated with 100 μ M SR202 ($n=3$).

induced *NUR77* promoter activity within 1 h and continued for up to 3 h (Fig. 3a). Rosiglitazone had no significant effect on TNF α -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 TNF α for 4 h resulted in significant upregulation of *NUR77* promoter activity and a significant inhibitory effect of rosiglitazone on TNF α -induced *NUR77* promoter activity at 4 h (Fig. 3b). Treatment of transfected cells with the specific PPAR γ inhibitor SR202 had no effect on suppression of TNF α -stimulated *NUR77* promoter activity by rosiglitazone (Fig. 3b), suggesting that this transcriptional effect was PPAR γ independent. Co-transfection of a PPAR γ dominant-negative construct into the cells transfected with the *NUR77* promoter construct also had no effect on rosiglitazone-mediated suppression of TNF α -stimulated *NUR77* promoter activity (Fig. 4), confirming the PPAR γ -independent nature of this effect.

Nuclear protein binding to the NF- κ B-binding site in the *NUR77* promoter

The human and mouse *Nur77* promoters share highly conserved binding sites for NF- κ B 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- κ B-binding sites within the *NUR77* promoter region (nt -1151 to -1170). EMSA results demonstrate that 32 P-labelled oligonucleotides harbouring the NF- κ B-binding site within the *NUR77* promoter specifically bind nuclear proteins from HUVEC cells (Fig. 5). The intensity of the protein/DNA complex increased with TNF α treatment, which was subsequently downregulated by the concomitant treatment with rosiglitazone (Fig. 5). Co-treatment with the specific PPAR γ inhibitor SR202

had minimal effect on rosiglitazone attenuation of specific nuclear protein binding in TNF α -stimulated cells, suggesting that this effect is independent of PPAR γ activity (Fig. 5).

TZD-mediated modulation of NF- κ B1 mRNA expression

Having identified TZD-mediated attenuation of nuclear protein binding to the NF- κ B-binding site in the *NUR77* promoter, we were interested in determining the effect of TZDs on the expression of *NF- κ B1* 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- κ B subunits, NF- κ B1, C-REL and REL A (Pei *et al.* 2005).

Figure 3 (a) Time course of effect of rosiglitazone treatment on TNF α -stimulated *NUR77* promoter activity as assessed by luciferase activity normalised by β -galactosidase activity in transfected HEK 293 cells. Control, untreated; T, TNF α (10 ng/ml); R, rosiglitazone (10 μ M); T+R, TNF α + rosiglitazone ($n=3$). (b) Effect of rosiglitazone and SR202 treatment on mouse *Nur77* promoter construct for 4 h. Control, untreated; T, TNF α (10 ng/ml); R, rosiglitazone (10 μ M); T+R, TNF α + rosiglitazone; SR202, treated with 100 μ M SR202 ($n=3$), * $P<0.05$, T versus control (* $P<0.05$); ** $P<0.05$, T versus T+R and T+R+SR202.

Figure 4 Effect of rosiglitazone and/or TNF α treatment for 4 h on *NUR77* promoter activity in HEK 293 cells co-transfected with a dominant-negative PPAR γ mutant construct. Control, untreated; T, TNF α (10 ng/ml); R, rosiglitazone (10 μ M); T+R, TNF α + rosiglitazone; D, dominant-negative PPAR γ mutant construct ($n=3$), * $P<0.05$, T versus control; ** $P<0.05$, T versus T+R.

Real-time qPCR was used to identify *NF- κ B1* mRNA expression in HUVEC cells. Experiments demonstrated that expression of *NF- κ B* mRNA was significantly induced by TNF α 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 TNF α -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 TNF α -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 TNF α stimulation (Gruber *et al.* 2003). Our previous results have demonstrated that rosiglitazone attenuates the TNF α -mediated increase in *NUR77* expression and binding of nuclear proteins to the PAI-1 NBRE-binding site in a PPAR γ -independent manner (Liu *et al.* 2005). We have been interested in the molecular mechanism responsible for PPAR γ -independent regulation of *NUR77* expression by TZDs in vascular endothelial cells.

Our current studies demonstrate that rosiglitazone significantly inhibited TNF α -induced *NUR77* mRNA expression after 1 h treatment (Fig. 1) and that this effect was PPAR γ

independent (Fig. 2). Rosiglitazone treatment also reduced TNF α -stimulated *NUR77* promoter activity significantly at 4 h (Fig. 3), again in a PPAR γ -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

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

Figure 6 Effect of TNF α and/or rosiglitazone for 1 h on NF- κ B mRNA expression in HUVEC cells. Control, untreated; T, TNF α (10 ng/ml); R, rosiglitazone (10 μ M); T+R, TNF α + rosiglitazone ($n=3$).

PPAR γ -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- κ B proteins on the *NUR77* promoter. Our EMSA results demonstrated that TNF α treatment increased the binding of nuclear proteins from HUVEC cells to the NF- κ B-binding site of *NUR77* promoter (Fig. 5). Rosiglitazone treatment attenuated the TNF α -mediated increase in binding of nuclear proteins to this binding site. The specific PPAR γ inhibitor SR202 failed to inhibit this effect significantly (Fig. 5). In addition, rosiglitazone also inhibited TNF α -mediated induction of NF- κ B1 mRNA expression, offering a possible molecular explanation for the effects of rosiglitazone on *NUR77* expression (Fig. 6).

Taken together, rosiglitazone-mediated inhibition of TNF α -induced *NUR77* mRNA expression correlates with inhibition of *NUR77* promoter activity, binding of nuclear proteins from HUVEC cells to the *NUR77* NF- κ B-binding site and reduced NF- κ B1 mRNA expression, independent of inducible PPAR γ activation. These findings suggest a novel PPAR γ -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 PPAR γ -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 'PPAR γ -independent' atheroprotective agents in the future.

Declaration 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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