Synthetic Peroxisome Proliferator-Activated Receptor γ Agonists Rosiglitazone and Troglitazone Suppress Transcription by Promoter 3 of the human Thromboxane A_2 Receptor Gene in Human Erythroleukemia cells.

Running title: Effect of thiazolidinedione derivatives on thromboxane A_2 receptor gene expression.

Adrian T. Coyle and B. Therese Kinsella* School of Biomolecular & Biomedical Science, UCD Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland.

***Corresponding author:** B. Therese Kinsella* School of Biomolecular & Biomedical Science, UCD Conway Institute of Biomolecular & Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland. Tel: 353-1-7166727; Fax 353-1-2837211; Email: Therese.Kinsella@ucd.ie

Abbreviations: AP-1, activator protein-1; 15d-PGJ_2, 15-deoxy (grdelta)^{12,14}-prostaglandin J_2; E, exon; EMSA, electromobility shift assay; FBS, foetal bovine serum; HEK, human embryonic kidney; HEL, human erythroleukemia; I, intron; NT, nucleotide; PG, prostaglandin; P, promoter; PMA, phorbol myristic acid; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; Prm, promoter; RLU, relative luciferase units; RXR, retinoic acid X receptor; TP, thromboxane receptor; TI, transcription initiation; TXA_2, thromboxane A_2; UTR, untranslated region; VSM, vascular smooth muscle cell.

Abstract

The human thromboxane (TX)A_2 receptor (TP) gene encodes two TP isoforms, TP(gralpha) and TP(grbeta) that are regulated by distinct promoters designated promoter (Prm) 1 and Prm3, respectively. Previous studies established that 15d-(grdelta)^{12,14}-prostaglandin J_2 (15d-PGJ_2) selectively inhibits Prm3 activity and TP(grbeta) expression through a peroxisome proliferator-activated receptor (PPAR)(grgamma) mechanism without affecting Prm1 activity or TP(gralpha) expression in human megakaryocytic erythroleukemia (HEL) 92.1.7 cells. Herein, we investigated the effect of synthetic thiazolidinedione (TZD) PPAR(grgamma) ligands rosiglitazone and troglitazone on TP gene expression in HEL cells. Like 15d-PGJ_2, both TZDs suppressed Prm3 activity, TP(grbeta) mRNA expression and TP-mediated calcium mobilization without affecting Prm1 or TP(gralpha) mRNA expression. However, unlike 15d-PGJ_2, both TZDs mediated their PPAR(grgamma)-dependent effects through trans-repression of an activator protein-1 (AP-1) element, a site previously found to be critical for basal Prm3 activity. These data provide further evidence for the role of PPAR(grgamma) in regulating the human TP gene; they highlight further differences in TP(gralpha) and TP(grbeta) expression/regulation and point to essential differences between natural and synthetic PPAR(grgamma) agonists in mediating those effects.

Key Words: thromboxane receptor, isoforms, promoter, peroxisome proliferator-activated receptor (grdelta), thiazolidinedione, 15-deoxy -(grdelta)^{12,14}-prostaglandin J_2.

Introduction

Peroxisome proliferator-activated receptor (PPAR)(grgamma) has been implicated in a broad range of cellular functions including adipocyte differentiation [1], glucose homeostasis and insulin sensitization [2, 3], inflammatory responses, carcinogenesis and apoptosis [4, 5]. In addition, there is a significant association between PPAR(grgamma) polymorphism and coronary artery disease [6] and PPAR(grgamma) mRNA expression in human adipose is inversely associated with cardiovascular risk factors [7]. Of the natural PPAR(grgamma) ligands, the prostaglandin derivative 15-deoxy-(grdelta)^{12,14} prostaglandin J_2 (15d-PGJ_2) and 9- or 13-hydroxy-octadecadienoic acid (9-HODE or 13-HODE) mediate potent adipogenic and anti-inflammatory effects [8]. Although 15d-PGJ_2 exerts the majority of it's effects through the activation of PPAR(grgamma), a number of PPAR(grgamma)-independent effects have been reported. These PPAR(grgamma)-independent effects are believed to be due to direct 15d-PGJ_2-covalent modification of target proteins [9].

Several synthetic PPAR(grgamma) agonists such as the thiazolidinedione (TZD) derivatives, rosiglitazone (Avandia®) and pioglitazone (Actos®) are being used in the treatment of type II diabetes mellitus. Both of these drugs are believed to ameliorate type II diabetes *in vivo* by improving the body's sensitivity to insulin [10]. Specifically, TZD-mediated activation of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors has been shown to augment insulin sensitivity through the activation of insulin-responsive genes involved in the control of glucose production, transport, and utilization [11].

PPAR(grgamma) is a member of the nuclear hormone receptor superfamily that can positively or negatively regulate gene expression in response to ligand binding. Typically, PPAR(grgamma) positively regulates gene expression by binding as a heterodimer with the retinoic X receptor (RXR) to PPAR response elements (PPREs) within target genes [12]. When either the PPAR(grgamma) or RXR components of the heterodimer are bound by agonists, their respective ligand binding domains undergo a conformational change that leads to the recruitment of coactivators and consequent transcription of target genes [13]. Liganded PPAR(grgamma) can also inhibit the expression of a number of genes, typically those associated with inflammation [14, 15] and thrombosis [16, 17].

Such inhibitory effects of liganded PPAR(grgamma) are generally mediated by trans-repression, as they do not appear to involve direct binding to the target promoter. This trans-repression reduces the activity of transcription factors key to inflammation and thrombosis, such as NF-(grkappa)B, SP-1 and AP-1, by acting at multiple levels [18]. These include interference with the signalling pathway leading to AP-1 activation through the direct inhibition of cJun [19], the PPAR(grgamma)-dependent sequestration of coactivators including CBP [20] and the impairment of SP-1 binding to DNA [16], as exemplified by PPAR(grgamma)-mediated suppression of the proinflammatory/prothrombotic cyclooxygenase-2, inducible nitric oxide synthase and rat thromboxane A_2 receptor genes, respectively. PPAR(grgamma) is reported to inhibit signalling by NF-(grkappa)B in human aortic smooth muscle cells by direct protein : protein interaction, PPAR(grgamma): NF-(grkappa)B complex formation and trans-repression of NF-(grkappa)B

action and function [18]. Consistent with this, it has recently been discovered that non-pathogenic commensal microflora (e.g *Bacteroides thetaiotaomicron*) attenuate inflammation within the intestinal epithelium through a trans-repression mechanism involving both NF-(grkappa)B and PPAR(grgamma) [21]. In this mechanism, it was established that commensal anaerobic gut bacteroides-induce nuclear association between the RelA (p65) subunit of NF-(grkappa)B and PPAR(grgamma). The subsequent export of the newly formed NF-(grkappa)B :PPAR(grgamma) complex out of the nucleus attenuates NF-(grkappa)B -mediated gene transcription, thereby impeding the inflammatory response [21].

Thromboxane (TX)A_2 is an unstable arachidonate metabolite that plays a key role in haemostasis but is also widely implicated as a mediator in cardiovascular diseases such as thrombosis, atherosclerosis, myocardial infarction, stroke and bronchial asthma [22-26]. Binding of TXA_2 to its receptor TP, a G-protein coupled receptor, induces vasoconstriction and platelet aggregation as well as mitogenic- and hypertrophic growth of vascular smooth muscle [27, 28]. In humans, but not in non-primates, TXA_2 signals through two TP isoforms termed TP(gralpha) and TP(grbeta) that arise through differential splicing [29, 30]. Whilst the biologic significance of two TP isoforms in humans is unclear, there is increasing evidence that they may be physiologically distinct displaying certain differences in their intracellular signalling [31, 32], in their homologous and heterologous desensitization [33-36] and in their patterns of expression [37]. Consistent with this, recent studies have established that TP(gralpha) and TP(grbeta) expression is actually under the transcriptional control of distinct promoters within the single human TP gene located on chromosome 19 [38, 39]. Whilst the originally identified promoter (Prm) 1 directs TP(gralpha) expression, a novel promoter (Prm3) was identified that exclusively directs TP(grbeta) expression [38, 40].

In keeping with the critical role of PPAR(grgamma) within the CV system, as stated, it has been established that both 15d-PGJ_2 and troglitazone suppress expression of the rat TP gene via an interaction of PPAR(grgamma) with SP-1 [16]. However in order to examine the effect of PPAR(grgamma) agonists on human TP expression, their effect on two independently regulated TP isoforms through two distinct promoters, namely TP(gralpha) and TP(grbeta) through Prm1 and Prm3, respectively, must be determined. In a recent study, we established that the endogenous PPAR(grgamma) agonist 15d-PGJ_2 specifically suppressed Prm3-directed gene expression and TP(grbeta) mRNA expression in the megakaryocytic human erythroleukemic (HEL) 92.1.7 cell line while TP(gralpha) mRNA expression and Prm1- directed gene expression was unaffected by 15d-PGJ_2 [41]. Moreover, the effect of 15d-PGJ_2 on TP(grbeta) expression occurred through a novel mechanism involving direct binding of activated PPAR(grgamma)-RXR heterodimers to a PPRE located within the -168 to -141 region of Prm3 [41]. It is, however, now increasingly recognised that PPAR(grgamma) ligands, such as 15d-PGJ_2 and the TZDs, can elicit a range of both shared but also entirely distinct biological effects [42]. For example, although PPAR(grgamma) -mediated repression of the GLUT4 promoter is augmented by 15d-PGJ_2, it is completely alleviated by rosiglitazone [42].

Hence, in view of such distinctions in the biologic actions between endogenous and synthetic PPAR(grgamma) ligands, in the present study we extended our previous investigations by determining the

effect of the synthetic rosiglitazone (Avandia®) and troglitazone (Rezulin®) on Prm1 and Prm3-directed reporter gene expression and on TP(gralpha) and TP(grbeta) mRNA expression. Our data herein data provide further evidence for the role of PPAR(grgamma) in the regulation of the human TP gene; they highlight further differences in the modes of regulation of TP(gralpha) and TP(grbeta) expression but also point to critical differences between natural versus synthetic PPAR(grgamma) ligands in mediating those effects.

Materials and Methods

Materials:

pGL3Basic, pGL3Enhancer, pRL-Thymidine Kinase (pRL-TK) and Dual Luciferase® Reporter Assay System were obtained from Promega Corporation, Madison, WI, USA. [(grgamma)^{32}P] ATP (6000 Ci/ mmol at 10 mCi/ ml) was from Valeant Pharmaceuticals (ICN), Costa Mesa, USA. The endogenous PPAR(grgamma) ligand15-deoxy-(grdelta)^{12,14}-PGJ_2 was obtained from Calbiochem-Novabiochem, Nottingham, UK. Cicaprost was obtained from Schering AG, Berlin, Germany. The agonist 17 phenyl trinor prostaglandin (PG) E_2 was obtained from Cayman, Chemicals, Michigan, USA and troglitazone from Sigma Aldrich Co, St Louis, USA. Anti-PPAR(grgamma) (sc-7273x) was obtained from Santa Cruz, California. Rosiglitazone was obtained from Dr Stephen Smith, Glaxo-SmithKline, Essex, U.K. All other reagents were molecular biology grade.

Construction of Luciferase-based Genetic Reporter Plasmids

The plasmids pGL3b:Prm1, pGL3b:Prm2 and pGL3b:Prm3 encoding promoter (Prm)1, Prm2 and Prm3 of the human TXA_2 receptor (TP) gene cloned into the pGL3Basic genetic reporter vector have been previously described (35). To identify the site of action of troglitazone and/or rosiglitazone within Prm3, a range of reporter plasmids containing either 5' deletion or mutated subfragments of Prm3 (-1394 to + 1, where the - designation indicates nucleotides 5' of the translational ATG start codon which is designated + 1) were used. These include pGL3b:Prm3 (-1394 to +1), pGL3b:Prm3b (-975 to +1), pGL3b:Prm3a (-404 to +1), pGL3b:Prm3ab (-320 to +1), pGL3b: Prm3aa (-154 to +1), pGL3b:Prm3aab (–106 to +1) and pGL3b:Prm3aaa (-50 to +1), as previously described [40]. The plasmids pGL3b:Prm3$^{AP\text{-}1*}$, pGL3b:Prm3ab$^{AP\text{-}1*}$, pGL3b:Prm3aa$^{AP\text{-}1*}$ and pGL3b:Prm3aaa$^{AP\text{-}1*}$ in which a consensus AP-1 element centred at -27 within Prm3 was mutated have been previously described [40]. Additionally, the role of a previously identified PPAR(grgamma)(b) / retinoic acid X receptor (RXR) IV half sites centred at –159 and -148 within Prm3 in eliciting the effect of troglitazone and/or rosiglitazone was determined using the reporter plasmid pGL3b:Prm3ab^{RXRIV*} [41]. The latter pGL3b:Prm3ab^{RXRIV*} plasmid contains 320 bp of Prm3 sequence (-320 to +1) in which the RXR IV half site was mutated was previously described [41].

Cell Culture

All mammalian cells were grown at 37 ^{o}C in a humid environment with 5 % CO_2. Human erythroleukemic 92.1.7 (HEL) cells and human embryonic kidney (HEK) 293 cells were cultured in RPMI 1640, 10 % foetal bovine serum (FBS) and in Eagle's minimal essential medium (MEM), 10 % FBS, respectively.

Assay of Luciferase Activity

Genetic reporter assays were carried out using the Dual Luciferase Assay System^{TM} (Promega). HEK 293 cells were plated in MEM, 10 % FBS in six well dishes at 1x 10^5 cells/ well. At 70-80 % confluence, cells were co-transfected with recombinant pGL3 Basic control vector, encoding firefly luciferase, or its

recombinant derivatives (0.4 μg/ well) along with pRL TK (50 ng/ well), encoding renilla luciferase, using Effectene (Qiagen) as recommended by the supplier. Approximately 36 hr post transfection, the medium was replaced with fresh MEM, 10 % FBS and was supplemented with troglitazone (10 μM), rosiglitazone (25 nM), 15-deoxy-$^{\Delta 12,\ 14}$-PGJ_2 (15d-PGJ_2; 10 μM) or vehicle (0.1% dimethylsulfoxide). After 16 hr, cells were washed in ice-cold phosphate buffered saline (PBS), were lysed and harvested by scraping in 350 μl Reporter Lysis Buffer (Promega) and centrifuged at 14,000 g for 1 min at room temperature.

HEL cells were transfected using the DMRIE-C transfection reagent, essentially as described by the supplier (Invitrogen Life Technologies). Briefly, 0.5 ml of serum free RPMI 1640 medium was dispensed into a six well dish and 6 μl of DMRIE-C reagent was added. Thereafter, 0.5 ml of serum free RPMI 1640 medium containing 2 μg of recombinant pGL3 Basic vector and 200 ng of pRL-TK was added and DNA / DMRIE-C reagent was complexed by incubation at R.T. for 30 min. Thereafter, 0.2 ml of serum free RPMI 1640 medium containing 2 x 10^6 HEL cells were added and were incubated for 4 hr (37 ^oC in a CO_2 incubator) after which 2 ml of RPMI 1640 medium containing 15 % FBS was added. Approximately 36 hr post-transfection, the medium was replaced with fresh RPMI 1640 medium, 10% FBS and was supplemented with troglitazone (10 μM; or for concentration response studies, with 0 – 20 μM troglitazone), rosiglitazone (25 nM; or for concentration response studies, with 0 – 100 nM rosiglitazone), 15d-PGJ_2 10 μM or vehicle (0.1% dimethylsulfoxide). After 16 hr, cells were washed in ice-cold (PBS) and harvested by centrifugation at 1,200 g for 5 min at 4 ^oC. Cell pellets were resuspended in Reporter Lysis Buffer (100 μl) and were lysed by repeated trituration. Cell lysates were obtained by centrifugation at 14,000g for 1 min at R.T.

To investigate the effect of over-expression of PPAR(grgamma)2, and/or RXR(gralpha) on rosiglitazone and 15d-PGJ_2 - induced inhibition of Prm3 activity, HEL 92.1.7 cells were co-transfected with pGL3b:Prm3ab (1 μg) plus 200 ng of pRL-TK along with either pSG5-hPPAR(grgamma)2 (1 μg) and/or pSG5-mRXR(gralpha) (1 μg). In each case, the total amount of transfected DNA was kept constant (3.2 μg) by using the required amount of empty vector pSG5. Approximately 24 h post-transfection, the medium was replaced with fresh RPMI 1640 medium, 10 % FBS and was supplemented with the vehicle (0.1 % dimethylsulfoxide), 25 nM Rosiglitazone or with 10 μM 15d-PGJ_2. After 16 h, cell lysates were prepared as previously described above.

HEK 293 and HEL cell supernatants were assayed for both firefly and renilla luciferase activity using the Dual Luciferase Assay System™ essentially as previously described [40]. Relative firefly to renilla luciferase activities were calculated as a ratio and were expressed in relative luciferase units (RLU).

Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotides corresponding to the sense and antisense strands of the AP-1 probe (Kin189 and its complement; 0.35 μM of each) were annealed and [^{32}P] end-labelled as previously described [40]. Nuclear extracts were prepared from either vehicle [0.1% v/v dimethylsulfoxide]-, troglitazone (10 μM,

16 h)- or rosiglitazone (25 nM, 16 hr)- treated HEL 92.1.7 cells (1.6 x 10^7 – 2 x 10^7) essentially as previously described [40]. Nuclear extracts (4 μg total protein) were incubated for 15 min at R.T. with/ without a 50-fold molar excess of unlabelled d/s competitor/ non-competitor oligonucleotide (2 μM) in 1X Binding Buffer (20 mM HEPES pH 7.9., 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 4 % Ficoll, 0.5 μg poly dI-dC (Sigma)). The appropriate [^{32}P]-radiolabelled d/s oligonucleotide was then added to each reaction and incubated for 20 min at R.T. Following incubation, binding reactions were subjected to electrophoresis through a 4 % polyacrylamide gel (20 cm x 20 cm) in 89 mM Tris borate and 2 mM EDTA for 3 h at RT, thereafter, gels were dried and analysed by autoradiography.

The sequences of the competitor/ non-competitor oligonucleotides used were as follows: (a) AP-1, (Kin 189; 5'-dGGTGGTGACTGATCCCTCAGGGC-3' corresponding to NTs -32 to -10 of Prm 3). (b) Mutated AP-1*, (Kin 162; 5'-dCGGCC TGATGGGGTGG***ATC***CTGATCCCTCAGGGCTC-3' corresponding to NTs -46 to -7 of Prm 3 where bases mutated from the wild type Prm3 sequence are in bold face italics). (c) Oct 1/ 2, (Kin 195; 5'-dTAATCAC AAGCAAATCTTCTCTC-3' corresponding to NTs -115 to -92 of Prm3). (d) PPAR(grgamma), (Kin 281; 5'-dCATTGAAGGTTGTGTAGGAGTTCACCA-3'; corresponding to NTs -168 to -141 of Prm3). (e) SP-1 consensus element (Promega) with the sequence, 5'-dATTCGATCGGGGCGGGGCG AG-3'. Note, only sequences of the forward oligonucleotides are given and the complementary strands are omitted.

Reverse Transcriptase-Polymerase Chain reaction (RT-PCR) & Southern Blot analysis.

HEL 92.1.7 cells (5 x 10^6 cells approximately) were pre-incubated for 16 hr with 10 μM troglitazone, 25 nM rosiglitazone and 10 μM 15d-PGJ_2 or, as a control, with the vehicle (0.1% dimethylsulfoxide) and total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). DNase 1-treated total RNA was subject to RT-PCR using oligonucleotide primers designed to specifically amplify TP(gralpha)α, TP(grbeta) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences as previously described [38]. Southern blot analysis of the RT-PCR products were carried out using ^{32}P radiolabelled oligonucleotides probes specific for TP(gralpha)/TP(grbeta) and GAPDH mRNA sequences as previously described [38]. Images were captured and quantified by using a Typhoon PhosphorImage Analyser (Amersham).

Measurement of intracellular calcium mobilization

HEL cells were preincubated with rosiglitazone (25 nM), troglitazone (10 μM) or, as controls, with an equivalent volume of the vehicle [0.1% (v/v) dimethylsulfoxide] for 24 or 48 h. Measurement of intracellular calcium ($[Ca^{2+}]_i$) mobilization in FURA2/AM preloaded cells in response to the TP agonist U46619 (1 μM), the IP agonist cicaprost (1 μM) or the EP_1 agonist 17 phenyl trinor PGE_2 (1 μM) was carried out as previously described [43]. The results presented in the figures are representative profiles from at least four independent experiments and are plotted as changes intracellular calcium mobilized ((grdelta)$[Ca^{2+}]_i$ (nM)) as a function of time (s) upon ligand stimulation. Changes in $[Ca^{2+}]_i$ mobilization

were determined by measuring peak rises in intracellular $[Ca^{2+}]_i$ mobilized ((grdelta) $[Ca^{2+}]_i$) and were calculated as mean changes in (grdelta) $[Ca^{2+}]_i \pm$ SEM (nM).

Statistical Analysis

Statistical analyses were performed using the two-tailed Students' unpaired t-test throughout or using one-way analysis of variance (ANOVA; Figure 5 & 6), employing GRAPHPRISM3 software (Graphpad Software, San Diego, CA, USA). All values are expressed as mean ± standard error of the mean (S.E.M.). *P*-values of less than or equal to (≤) 0.05 were considered to indicate statistically significant differences using both t-tests and ANOVA one-way comparisons.

Results

The effect of Rosiglitazone and Troglitazone on Promoter (Prm) 1, 2 and 3 activity.

Previous studies have established that the PPAR(grgamma) ligands 15d-PGJ_2 and troglitazone suppress expression of the TXA_2 receptor (TP) gene in rat vascular smooth muscle cells [16]. In humans, the TP gene encodes two TP isoforms, termed TP(gralpha) and TP(grbeta), and is under the transcriptional regulation of three distinct promoter (Prm) regions termed Prm1, Prm2 and Prm3 [38]. Recent studies have revealed that 15d-PGJ_2 selectively inhibits Prm3-directed reporter gene expression and TP(grbeta) mRNA expression through a PPAR(grgamma)/RXR(gralpha) -dependent mechanism in the megakaryocytic HEL 92.1.7 cell line without affecting TP(gralpha) expression or Prm1- or Prm2-directed gene expression [41] Herein, we sought to extend those studies by investigating the effect of members of the thiazolidinedione (TZD) class of synthetic PPAR(grgamma) ligands on expression of the human TP gene in HEL cells.

Initially the effect of rosiglitazone and troglitazone on Prm1-, Prm2- and Prm3-directed reporter gene expression in transfected HEL cells and, as a control, in human embryonic kidney (HEK) 293 cells was investigated. Prm1, Prm2 and Prm3 each directed efficient basal luciferase activity in both HEL and HEK 293 cells (Figure 1A & 1B), albeit at significantly different levels relative to each other consistent with previous reports [38, 40]. In addition pre-incubation with 25 nM rosiglitazone or 10 μM troglitazone for 16 hr had no significant effect on Prm1- or Prm2-directed luciferase expression in either cell type (Figure 1A & 1B). On the other hand, rosiglitazone and troglitazone each resulted in an approximately 1.6-fold reduction in Prm3-directed luciferase expression ($p < 0.02$) in HEL cells but neither TZD agent affected Prm3 activity in HEK 293 cells (Figure 1A & 1B). Moreover, both rosiglitazone and troglitazone suppressed Prm3-directed luciferase expression in HEL cells, but not in HEK 293 cells, in a concentration- and time- dependent manner but had no significant effect on Prm1- or Prm2- directed reporter gene expression in either cell type regardless of the concentration or incubation time examined (Data not shown). Neither TZD agent affected the level of luciferase expression in either HEL or HEK 293 cells transfected with the pGL3Control plasmid (Figure 1A & 1B).

Effect of Rosiglitazone and Troglitazone on TP(gralpha) and TP(grbeta) mRNA expression and function in HEL cells

Hence, in view of the finding herein that rosiglitazone and troglitazone selectively suppressed Prm3-, but not Prm1-, directed reporter gene expression, their effect on TP(gralpha) and TP(grbeta) mRNA expression in HEL cells was investigated. Reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blot analysis confirmed expression of TP(gralpha), TP(grbeta) and glyceraldehyde 3'phosphate dehydrogenase (GAPDH) mRNA in HEL cells (Figure 2A & 2B, lanes 1 –3, respectively) with an approximately 2-fold higher level of TP(gralpha) relative to TP(grbeta) mRNA expression in non-stimulated or vehicle-treated cells. Pre-incubation with either troglitazone (Figure 2A & 2B, lanes 7 & 9), rosiglitazone (Figure 2A & 2B, lanes 10 & 12) or, as a control, with 15d-PGJ_2 (Figure 2A & 2B, lanes

4 & 6) did not significantly affect the levels of either TP(gralpha) or GAPDH mRNA expression in HEL cells relative to the vehicle-treated cells (Figure 2A & 2B, lanes 1 & 3). In contrast, pre-incubation with 15d-PGJ_2, troglitazone and rosiglitazone resulted in approximately 1.6 – 1.7-fold reductions in TP(grbeta) mRNA expression in HEL cells compared to vehicle-treated cells (Figure 2A & 2B; lanes 2, 5, 8 & 11).

To determine the effect of rosiglitazone and troglitazone on TP function, HEL cells were pre-incubated with either 25 nM rosiglitazone or 10 μM troglitazone for 24 and 48 h and its effect on TP-mediated intracellular calcium ($[Ca^{2+}]_i$) mobilization in response to the selective TXA_2 mimetic U46619 was investigated. In addition as controls, we also investigated the effect of rosiglitazone and troglitazone and on signalling by unrelated receptors, namely the prostacyclin receptor (IP) and the EP_1 subtype of the prostaglandin (PG)E_2 receptor. The overall level of U46619 mediated $[Ca^{2+}]_i$ mobilization was significantly reduced following pre-incubation with either rosiglitazone or troglitazone, as illustrated in Figure 3A-3C. Specifically, U46619-mediated $[Ca^{2+}]_i$ mobilization was reduced from 23.2 ± 2.7 nM in vehicle treated cells to 14.4 ± 2.4 nM (P = 0.0376) and 15.6 ± 1.5 nM (P = 0.045) following pre-treatment of cells for 24 hr with rosiglitazone or troglitazone, respectively (Figure 3). Similar data was obtained following 48 h incubation with troglitazone or rosiglitazone (data not shown). In contrast neither rosiglitazone (25 nM, 24 h) nor troglitazone (10 μM, 24 h) significantly effected $[Ca^{2+}]_i$ mobilization by the control IP and EP_1 receptors in response to their respective agonists cicaprost (compare (grdelta)$[Ca^{2+}]_i$ = 74.1 . ± 9.4 nM, n = 4 *vs* (grdelta) $[Ca^{2+}]_i$ = 79.6 . ± 4.7 nM, n = 4 *vs* (grdelta)$[Ca^{2+}]_i$ = 73.3 ± 3.7 nM, n = 4) and 17 phenyl-trinor PGE_2 (compare (grdelta)$[Ca^{2+}]_i$ = 163.2 ± 2.0 nM, n= 4 *vs* (grdelta) $[Ca^{2+}]_i$ = 143.5 ± 25.45 nM, n = 3 *vs* (grdelta)$[Ca^{2+}]_i$ = 149.5 ± 9.2 nM, n = 5) in either vehicle-, rosiglitazone- or troglitazone- treated cells, respectively).

Localisation of the site(s) of action Rosiglitazone and Troglitazone site of action within Prm3.

To identify the site(s) of action of rosiglitazone and troglitazone within Prm3, their effect on reporter gene expression directed by a series of recombinant pGL3Basic plasmids containing successive 5' deletion subfragments of Prm3 was investigated (Figure 4). Consistent with previous data [40], following transfection into HEL cells it was evident that under basal /non-stimulated conditions the core Prm3 is composed of three distinct regulatory regions comprising an upstream repressor sequence, located between -404 to -320, and two positive regulatory regions, between -154 to -106 and -50 to + 1, required for efficient basal gene expression (Figure 4). For example, 5' deletion of Prm3 (-1394 to +1, relative to the translational ATG start site) to generate a -404 subfragment did not significantly affect the level of basal luciferase activity in vehicle-treated HEL cells (Figure 4). However, 5' deletion of Prm3 from a -404 bp to a -320 bp fragment yielded an approximately 2-fold increase in basal luciferase activity while 5' deletion of the -320 bp to a -154 bp fragment did not yield a further alteration in basal luciferase expression (Figure 4). These data established that the -404 to -320 region contains negative regulatory element(s), the removal of which results in increased basal Prm3 activity whilst nucleotides located between -320 and -154 do not significantly affect basal Prm3 activity [40]. Moreover, further 5' deletion of the -154 to yield either the -106 and -50 subfragments resulted in the successive removal of two

positive regulatory elements, located between -154 to -106 and -106 to -50, containing consensus Oct-1/2 and AP-1 elements, respectively, that are reported to be required for efficient basal Prm3 activity (Figure 4; [40]).

Pre-incubation with rosiglitazone and troglitazone and, as a control, with 15d-PGJ_2 each resulted in approximately 1.5 – 1.6-fold reductions in luciferase activity directed by Prm3 ($p < 0.05$) and the -404 ($p < 0.05$) and -320 ($p < 0.05$) subfragments (Figure 4). However, whilst 15d-PGJ_2 did not significantly affect the level of luciferase activity directed by the -154 subfragment or by its smaller subfragments, both rosiglitazone and troglitazone significantly suppressed reporter gene expression by the -154 ($p < 0.05$), -106 ($p < 0.05$) and even by the -50 ($p < 0.05$) subfragments resulting in approximately 1.6-fold reductions in luciferase activity in each case (Figure 4).

Hence, it is evident that the inhibitory effects of the TZD derivatives rosiglitazone and troglitazone on reporter gene expression directed by Prm3 is in direct contrast to the effect of the endogenous PPAR (grgamma) agonist 15d-PGJ_2 in HEL 92.1.7 cells (Figure 4; [41]. Specifically, the site of action of 15d-PGJ_2 was identified within the -168/-141 regions of Prm3 [40].

PPARγ response elements are bipartite response elements composed of both PPAR(grgamma) and RXR(gralpha) docking sites. To further investigate whether rosiglitazone and troglitazone are truly acting through site(s) different to that of 15d-PGJ_2, their effect on reporter gene expression by the -320 Prm3 subfragment was compared to the -154 Prm3 subfragment, in which the PPAR(grgamma) component of the 15d-PGJ_2- responsive (PPAR(grgamma)(b); centred at -159) was deleted, or to a -320 Prm3 subfragment in which the RXR component of PPRE(b) PPRE(b) (RXRIV; centred at -148) was disrupted by site directed mutagenesis (Figure 5A). Whilst 15d-PGJ_2 significantly suppressed reporter gene expression by the -320 subfragment of Prm3, it had no effect on luciferase expression directed by the -154 subfragment or by the -320 subfragment in RXR component of PPRE(b) was mutated. On the other hand, both rosiglitazone and troglitazone each significantly suppressed reporter gene expression by the -154 subfragment and by the latter -320 subfragment containing the mutated PPRE(b), again resulting 1.5 – 1.6-fold reductions in luciferase activity (Figure 5A). These data strongly suggest that the effects of rosiglitazone and troglitazone are mediated through different/independent site(s) of action to that of 15d-PGJ_2 within Prm3 and do not involve the PPRE centred at the -168/-141 region of Prm3.

To test this hypothesis further, the effect of co-incubation of HEL cells with TZDs in the absence or presence of 15d-PGJ_2 on Prm3-directed reporter gene expression was examined (Figure 5B). In agreement with previous data, 15d-PGJ_2, rosiglitazone and troglitazone each suppressed Prm3 activity by approximately 1.5-fold (Figure 5B). In addition, using ANOVA one way comparisons, it was shown that the inhibitory effect of 15d-PGJ_2 was significantly augmented by both troglitazone ($p < 0.027$) and rosiglitazone ($p < 0.011$) resulting in 2.3-fold and 2.5-fold suppressions in Prm3-directed reporter gene expression, respectively. Co-incubation of cells with rosiglitazone along with troglitazone did not further suppress Prm3-directed gene expression relative to their sole incubations suggesting that unlike 15d-PGJ_2 both agents are acting through similar mechanisms/sites of action.

Investigation of the role of PPAR (grgamma) and RXR(gralpha) in Thiazolinedione - and 15d-PGJ_2- mediated inhibition of Prm3 activity.

As stated, previous studies have shown that 15d-PGJ_2 selectively inhibits Prm3-directed gene expression and TP(grbeta) mRNA expression through a PPAR(grgamma)/RXR(gralpha) -dependent mechanism [41] and data herein show that both rosiglitazone and troglitazone are acting at site(s) independent of the PPRE located between the -168/-141 region of Prm3. Moreover, both TZD derivatives efficiently suppressed reporter gene expression directed by Prm3 subfragments even as small as 106 bp (-106 subfragment) and 50 bp (-50 subfragment; Figure 4 & 5). In view of this, we sought to investigate the role of PPAR(grgamma) and/or RXR(gralpha), if any, in mediating TZD- suppression of Prm3 activity in HEL cells. To achieve this, the effect of transient over-expression of either PPAR(grgamma)2 or RXR(gralpha) alone or in combination on 15d-PGJ_2 and TZD- regulation of Prm3-directed reporter expression was examined (Figure 6). Basal Prm3 activity was unaffected by PPAR(grgamma), RXR(gralpha) or PPAR(grgamma) plus RXR(gralpha) expression in vehicle treated HEL cells in the absence of PPAR(grgamma) ligand (Figure 6A). Moreover, consistent with previous data, both 15d-PGJ_2- (Figure 6C), rosiglitazone- (Figure 6B) and troglitazone (data not shown) each mediated suppression of Prm3 activity relative to that of vehicle-treated cells, regardless of co-transfection of cells with PPAR(grgamma) or RXR(gralpha) or not (Figure 6B & 6C). Moreover, using ANOVA one way analysis it was established that 15d-PGJ_2- mediated suppression of Prm3 was not affected by the expression of either PPAR(grgamma)2 or RXR(gralpha) alone but was significantly augmented by co-expression of both PPAR(grgamma) and RXR(gralpha) ($P = 0.012$; Figure 6C). These data are consistent with previous findings [41]. In contrast, rosiglitazone-mediated suppression of Prm3-activity was augmented by the expression of either PPAR(grgamma)2 alone ($P = 0.002$) or by its co-expression along with RXR(gralpha) ($P = 0.001$) but was unaffected by the expression of RXR(gralpha) alone (Figure 6B).

Examination of the role of a functional AP-1 element within the -50/ +1 region of Prm3

It has been previously established that an AP-1 element centred at -27 bp, e.g contained within the -50 and all larger subfragments, is critical for basal Prm3 activity in both non-stimulated or vehicle-treated HEL 92.1.7 cells and HEK 293 cells [40]. Moreover, PPAR(grgamma) is known to modulate the activity of AP-1 elements within a number of promoters [14, 20, 44]. Hence, the possible role of the AP-1 element in mediating rosiglitazone and troglitazone suppression of Prm3 activity was investigated by comparing their effects on luciferase reporter gene expression directed by a series of Prm3 subfragments containing an intact AP-1 element relative to the equivalent subfragments in which the AP-1* was disrupted by site-directed mutagenesis (Figure 7). The -320, -154 and -50 subfragments containing the AP-1 element each directed efficient reporter gene expression under basal conditions in HEL cells. Disruption of the AP-1* site within the -320 and -154 resulted in 2.7-fold ($p < 0.0003$) and 3-fold ($p < 0.0008$) reductions in reporter gene expression, respectively, under basal conditions while mutation of the

AP-1 element within the -50 subfragment reduced Prm3- directed gene expression ($p < 0.0001$) to levels that were not substantially greater than those directed by the empty pGL3Basic vector (Figure 7). These data confirm the critical role of the AP-1 element for core Prm3 activity and are entirely consistent with previous reports [40]. Reporter gene expression directed by the -320, -154 and -50 subfragments containing the AP-1 element was suppressed by approximately 1.5 – 1.6-fold following either rosiglitazone or troglitazone treatment (Figure 7A), consistent with previous data (Figure 4). However, neither rosiglitazone nor troglitazone suppressed gene expression directed by either the -320 ($p > 0.67$- 0.73, respectively) or -154 ($p > 0.95$- 0.84, respectively) subfragments containing the mutated AP-1* element and had no affect on the residual Prm3 activity directed by the -50 subfragment ($p > 0.75$- 0.72, respectively).

Activated PPAR(grgamma)s are known to reduce transcription factor binding to AP-1 elements *in vitro* [19, 45]. This is predominantly thought to occur through the formation of a "dead end" complex with c-Jun, a major constituent of the AP-1 complex *in vivo* [45]. Previous experiments have confirmed the presence of a functionally active PPAR(grgamma) receptor in HEL cells [41]. We therefore examined the effect of rosiglitazone and troglitazone on nuclear factor binding to the AP-1 element within Prm3. Electrophoretic mobility shift assays (EMSAs) were carried out using a radiolabelled d.s oligonucleotide probe spanning nucleotides -32 to -10 (AP-1^{WT} probe) of Prm3 and nuclear extracts prepared from vehicle, rosiglitazone and troglitazone treated HEL 92.1.7 cells. A diffuse DNA:protein complex was observed following incubation of the AP-1^{WT} probe with nuclear extract prepared from either vehicle- (Figure 8, lanes 2-5) or rosiglitazone- (Figure 8, lanes 6-9) pre-incubated HEL cells. The level of nuclear factor:DNA complex formation was significantly lower using nuclear extract prepared from rosiglitazone-pretreated HEL cells compared with nuclear extract from vehicle treated cells (Figure 8, compare lanes 2 and 6). Nuclear factor:DNA complex formation was efficiently competed by an excess of the corresponding non-labelled d/s AP-1^{WT} oligonucleotide (Figure 8, lanes 3 & 7) but was not competed by the equivalent d/s oligonucleotide in which the AP-1* site was disrupted by site directed mutagenesis using nuclear extract from either vehicle- (Figure 8, lane 4) or rosiglitazone- (Figure 8, lane 8) pre-incubated HEL cells. The specificity of nuclear factor binding to the latter AP-1 site was also verified by the failure of excess non-competitor d.s oligonucleotides based on the previously identified Oct-1/2 (Oct-$1/2^{WT}$, Kin 195) element located within Prm3 adjacent to the AP-1 site to effectively inhibit nuclear factor- DNA complex formation (Figure 8, lanes 5 & 9). Similar data were generated using nuclear extract prepared from troglitazone-pretreated HEL cells (data not shown).

Discussion

There is growing evidence that along with its classical role in (grbeta)-oxidation and adipogenesis, PPAR(grgamma) also plays an important part in the control of inflammatory responses, cell growth and differentiation [46]. Within the vasculature, PPAR(grgamma) appears to broadly offer a cardioprotective role against inflammatory and thrombotic disorders. Moreover, expression of both the rat TXA_2 synthase and rat TXA_2 receptor/TP are suppressed through PPAR(grgamma) dependent mechanisms [16, 17]. Consistent with this, it has recently been demonstrated that expression of the TP(grbeta), but not the TP(gralpha), isoform of the human TXA_2 receptor (TP) was down-regulated by the COX-derived cyclopentone prostaglandin 15d-PGJ_2 through the direct binding of a PPAR(grgamma):RXR heterodimer to a PPRE composed of adjacent PPAR(grgamma) (b) and RXR IV half site direct repeats separated by 5 bp (DR5) and located between the -168/-141 region of Prm3 [41]. Furthermore, in view of the critical link between the enhanced risk of cardiovascular disease in patients with type II diabetes mellitus and in animal models of diabetes mellitus associated with increased synthesis and action of TXA_2 [47-50], these data point to potential combined therapeutic benefits of PPAR(grgamma) agonists in the treatment of type II diabetes and associated cardiovascular disease.

However, it is becoming increasingly more apparent that the nature of the specific PPAR(grgamma) agonist itself can greatly influence PPAR(grgamma)-mediated regulation of gene expression. Differential activation of PPAR(grgamma) responsive genes has been observed using both naturally occurring and synthetic PPAR(grgamma) ligands [42, 51]. For example, 15d-PGJ_2 and troglitazone similarly inhibit the rat thromboxane synthase gene expression in a dose -dependent manner [17]. On the other hand, although PPAR(grgamma)1- and PPAR(grgamma)2- mediated repression of the GLUT4 promoter is augmented by 15d-PGJ_2, it is completely alleviated by rosiglitazone [42]. Moreover, distinct but differential regulation of overlapping sets of genes were observed using the related TZD compounds rosiglitazone and troglitazone [52]. Therefore, the downstream effects of PPAR(grgamma) activation can be profoundly affected by the choice of agonist and this variation is apparent between synthetic agonists as well as between synthetic and naturally occurring agonists. Therefore, in view of the latter findings coupled to the critical roles of both PPAR(grgamma) and TXA_2 signalling within the cardiovascular system, the aim of the present study was to expand our investigations by examining the effect of the thiazolidinedione (TZD) derivatives of PPAR(grgamma) agonists on the expression and regulation of the human TP gene. The TZD derivatives represent a novel class of PPAR(grgamma) agonists under

of co-factors by

e treatment and management of hyperglycema and related secondary effects including various cardiovascular complications associated with type II diabetes mellitus [4, 53].

A number of studies have identified three promoter regions termed Prm1, Prm2 and Prm3 within the single human TP gene located on chromosome 19 [29, 38, 39, 54]. Hence as a first step in examining the effects of rosiglitazone and troglitazone on TP gene expression, their action on Prm1, Prm2 and Prm3-directed luciferase expression was investigated. Similar to that observed with 15d-

PGJ_2, both rosiglitazone and troglitazone selectively inhibited Prm3 activity but had no effect on reporter gene expression by Prm1, Prm2 or by the pGL2control plasmid in the platelet-like megakaryocytic HEL 92.1.7 cell line. Furthermore, mirroring that seen using 15d-PGJ_2, neither rosiglitazone nor troglitazone had any effect on Prm1, Prm2 or Prm3 activity in HEK 293 cells. HEK 293 cells are reported not to express significant levels of functional PPAR(grgamma) isoforms [55]. Hence, the absence of rosiglitazone or troglitazone responsiveness in HEK 293 cells tentatively suggests that the inhibitory effect of these TZD compounds on Prm3 activity is mediated, at least in part, through PPAR(grgamma) activation *in vivo*.

Thereafter, the effect of rosiglitazone, troglitazone and, as a control, 15d-PGJ_2 on TP(gralpha) and TP(grbeta) mRNA expression in HEL 92.1.7 cells was investigated. Similar to that previously reported for 15d-PGJ_2 [41], rosiglitazone and troglitazone selectively inhibited expression of TP(grbeta) mRNA without affecting TP(gralpha) or GAPDH mRNA expression. Moreover, there was no significant difference between the level of TP(grbeta) inhibition observed using rosiglitazone and troglitazone and that seen using 15d-PGJ_2. In agreement with the inhibitory effect of rosiglitazone and troglitazone on TP(grbeta) expression functional studies demonstrated that these TZD compounds also reduced TP-mediated $[Ca^{2+}]_i$ mobilization in response to the TXA_2 mimetic U46616. Conversely, neither rosiglitazone nor troglitazone had any significant effect on signalling by the control receptors IP and EP_1 in response to cicaprost and 17 phenyl trinor PGE_2, respectively. These findings are consistent with previous studies examining the effect of 15d-PGJ_2 on TP-mediated $[Ca^{2+}]_i$ mobilization [41]. Therefore, the synthetic PPAR(grgamma) agonists rosiglitazone and troglitazone appear to be equally selective at inhibiting Prm3 activity as the endogenous PPAR(grgamma) agonist 15d-PGJ_2. This is in agreement with the observed effect of 15d-PGJ_2 and troglitazone on TP expression in rat vascular smooth muscle cells, which showed that both agonists inhibited rat TP mRNA expression to a similar extent [16].

To determine the site(s) of action of rosiglitazone and troglitazone within Prm3, initially their effect on luciferase reporter gene expression-directed by Prm3 and a range of 5'deletion Prm3 subfragments was determined in HEL cells. Consistent with previous data [40], it was evident that under basal non-stimulated conditions the core Prm3 is composed of three distinct regulatory regions comprising an upstream repressor sequence, located between -404 to -320, and two positive regulatory regions, located between -154 to -106 and -50 to + 1, respectively. Moreover, the extent of rosiglitazone and troglitazone-suppression of Prm3 activity was equivalent and was independent of the size of the Prm3 subfragment investigated, i.e 1394 bp versus 50 bp.

These data suggested that the site of action of rosiglitazone and troglitazone are located between the -50 and +1 within Prm3 at site(s) entirely distinct from the PPRE, composed of the adjacent PPAR(grgamma) (b) and RXR IV half sites and located between the -168/-141 within Prm3, that was previously confirmed to mediate 15d-PGJ_2-suppression of Prm3 [40]. Consistent with this, while 15d-PGJ_2 had no effect on luciferase expression directed by the -154 Prm3 subfragment from which the PPAR(grgamma) component of PPRE(b), centred at -159, was deleted or by a -320 Prm3 subfragment in

which RXR IV* component of PPRE(b), both rosiglitazone and troglitazone efficiently suppressed reporter gene expression by each of the latter Prm3 subfragments. The hypothesis that the effects of rosiglitazone and troglitazone are mediated through a different site(s) of action to that of 15d-PGJ_2 within Prm3 was further tested by examining the cumulative effect of co-incubating 15d-PGJ_2 plus rosiglitazone and 15d-PGJ_2 plus troglitazone on Prm3 activity in HEL cells. It was postulated that if rosiglitazone and troglitazone were indeed inhibiting Prm3 activity through a different site of action to 15d-PGJ_2, then co-incubation with either 15d-PGJ_2 plus rosiglitazone or with 15d-PGJ_2 plus troglitazone might augment the inhibitory effect observed following stimulation with 15d-PGJ_2 alone. In support of this argument, co-incubation with either 15d-PGJ_2 and rosiglitazone or 15d-PGJ_2 and troglitazone resulted in a significantly higher level of Prm3 inhibition than that observed following incubation with 15d-PGJ_2, rosiglitazone or troglitazone stimulation alone. Co-incubation of cells with rosiglitazone along with troglitazone did not further suppress Prm3-directed gene expression relative to their sole incubations suggesting that both TZD agents are acting through the same site within Prm3.

To further investigate the role of PPAR(grgamma) and it's possible interaction with RXR(gralpha) in mediating TZD- suppression of Prm3 activity in HEL cells, the effect of transient over-expression of either PPAR(grgamma)2 or RXR(gralpha) alone or in combination on TZD- and, as a control, on 15d-PGJ_2- regulation of Prm3 activity was investigated. While over-expression of PPAR(grgamma), RXR(gralpha) or PPAR(grgamma) plus RXR(gralpha) had no affect on basal Prm3 activity and, consistent with previous findings [41], expression of both the PPAR(grgamma) and RXR(gralpha) transcription factors were necessary to augment 15d-PGJ_2-mediated inhibition of Prm3, expression of only PPAR(grgamma), but not RXR(gralpha), was necessary to augment rosiglitazone and troglitazone mediated inhibition of Prm3-directed luciferase expression. Hence, these data confirm that the role of RXR(gralpha) differs in TZD- relative to 15d-PGJ_2-mediated inhibition of Prm3 activity, suggesting that alternative mechanisms are involved.

Thus, it appears that rosiglitazone and troglitazone mediate Prm3 inhibition through similar site(s) of action, but at site(s) distinct to the previously described PPRE located between -168 /-141 region of Prm3. Moreover, the TZD derivatives can efficiently suppress gene expression directed by Prm3 subfragments even as small as 50 bp suggesting that the TZD-responsive PPAR(grgamma) site may be located between -50 to +1 within Prm3. Bioinformatic analysis revealed that there was no predictable consensus PPREs within the –50/+1 region of Prm3. However, this sequence contains a consensus AP-1 element, centred at -27 bp, that was previously found to be critical for basal Prm3 activity in both HEL 92.1.7 cells and HEK 293 cells [40]. In the absence of a PPRE, the inhibitory actions of ligand bound PPAR(grgamma) are predominantly mediated by transcriptional repression via DNA binding independent mechanisms or trans-repression [18]. One established mechanism involves impairing the DNA binding ability of positively regulating transcription factors by the formation of a "dead end complex" between activated PPAR(grgamma) and the target transcription factor. For example, a PPAR(grgamma) / SP-1 interaction is believed to underlie the repressive effect of PPAR(grgamma) on rat TP expression [16].

Other reported mechanisms include PPAR(grgamma) agonist-mediated reduction in STAT1 and STAT3 phosphorylation and the sequestration of coactivators recognised by both activated PPAR(grgamma) and positively regulating transcription factors [20, 56]. PPAR(grgamma) agonists are known to reduce AP-1 activation in several experimental systems, by mechanisms that involve direct interaction of PPAR(grgamma) with c-Jun [19] or JunD [45], inhibition of c-Fos expression [57] and the competitive binding of activated PPAR(grgamma) to a composite PPRE/AP-1 site [58].

Hence, in view of the fact that TZDs can inhibit gene expression-directed by the -50 sugfragment of Prm3, the possible role of the AP-1 element in mediating rosiglitazone and troglitazone suppression of Prm3 activity was investigated. Disruption of the AP-1 site within a series of Prm3 deletion subfragments resulted in a dramatic loss in basal Prm3 activity, consistent with the putative role of this site in the formation of the transcriptional pre-initiation complex [40]. However, neither rosiglitazone nor troglitazone suppressed gene expression directed by a range of Prm3 subfragments containing the mutated AP-1* element consistent with a role for the AP-1 element in mediating TZD-suppression of Prm3 activity. Hence, collectively, these data confirmed that the AP-1 site centred at -27 not only forms a part of the core Prm3 required for the basal promoter, but also has an important part to play in rosiglitazone / troglitazone-regulated Prm3 activity in HEL 92.1.7 cells. EMSAs employing nuclear extracts from HEL cells confirmed that nuclear factor binding to the AP-1 element was specifically reduced in TZD- relative to vehicle pretreated cells, suggesting that rosiglitazone or troglitazone activated-PPAR(grgamma) inhibits through the trans-repression of either the Fos or Jun components of the AP-1 complex. Specifically, it is proposed that direct protein-protein interactions between the activated PPAR(grgamma) and either Fos or Jun could inhibit the formation of a transcriptionally active AP-1 complex, thereby inhibiting Prm3 activity. Consistent with this, previous studies have shown that the direct physical interaction of PPAR(grgamma) with other nuclear factors such as CREB-binding protein and RelA inhibit of the pro-inflammatory functions of iNos and NF-(grkappa)B, respectively [20, 21]. Moreover, due to well-characterised interaction between the activated PPAR(grgamma) and Jun family members, these would appear to be the most likely targets for the TZD-mediated inhibition of TP(grbeta) expression [19].

The differential mechanisms of PPAR(grgamma) inhibition of Prm3 observed following either 15d-PGJ_2 or rosiglitazone/ troglitazone binding suggests that different PPAR(grgamma) ligands induce unique conformational changes in the receptor, which affect either its DNA binding or co-factor recruitment properties. Consistent with this, the binding PPAR(grgamma) ligands rosiglitazone and pioglitazone were shown to induce alternative conformations within the coactivators recognition surface of PPAR(grgamma) [13]. Moreover, a related study demonstrated that although 15d-PGJ_2 induced PPAR(grgamma) interaction with a range of coactivators, troglitazone did not induce any PPAR(grgamma)/ coactivators interactions [59]. Furthermore, the cellular functions of PPAR(grgamma) are known to be differentially modulated depending on the choice of PPAR(grgamma) agonist. Camp *et al* showed that the insulin sensitising compounds rosiglitazone and troglitazone elicited partially

overlapping but distinct effects on gene expression in a variety of cell types [52]. The alternative recruitment of co-factors by PPAR(grgamma) following either TZD or 15-PGJ_2 activation outlined above may provide another explanation for the observed augmentation in Prm3 inhibition following co-incubation of HEL cells with TZD or 15d-PGJ_2. Specifically, it is possible that the level of Prm3 inhibition observed using either TZD or 15-PGJ_2 alone is restricted by limiting amounts of either 15-PGJ_2-PPAR(grgamma) and/or TZD-PPAR(grgamma) recruited co-factors and that this is overcome by the activating PPAR(grgamma) with both compounds simultaneously.

In summary, these experiments show that similar to the endogenous PPAR(grgamma) ligand 15d-PGJ_2, the TZD compounds rosiglitazone and troglitazone selectively inhibit Prm3 activity but had no effect on the activity of either Prm1 or Prm2. Moreover, this suppression was mirrored by the effect of these compounds on either Prm3 directed-TP(gralpha) or Prm3 directed TP(grbeta) expression. However, the mechanism of action evoked by the either rosiglitazone or troglitazone bound PPAR(grgamma) differed to that evoked by 15d-PGJ_2 bound PPAR(grgamma). Specifically these data suggest that, whereas 15d-PGJ_2 mediates repression through direct binding to a PPRE located within the -168 / -141 region of Prm3, rosiglitazone and troglitazone mediated inhibition of Prm3 occurs by the trans-repression of nuclear factor binding to the AP-1 site at -27.

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FIGURES

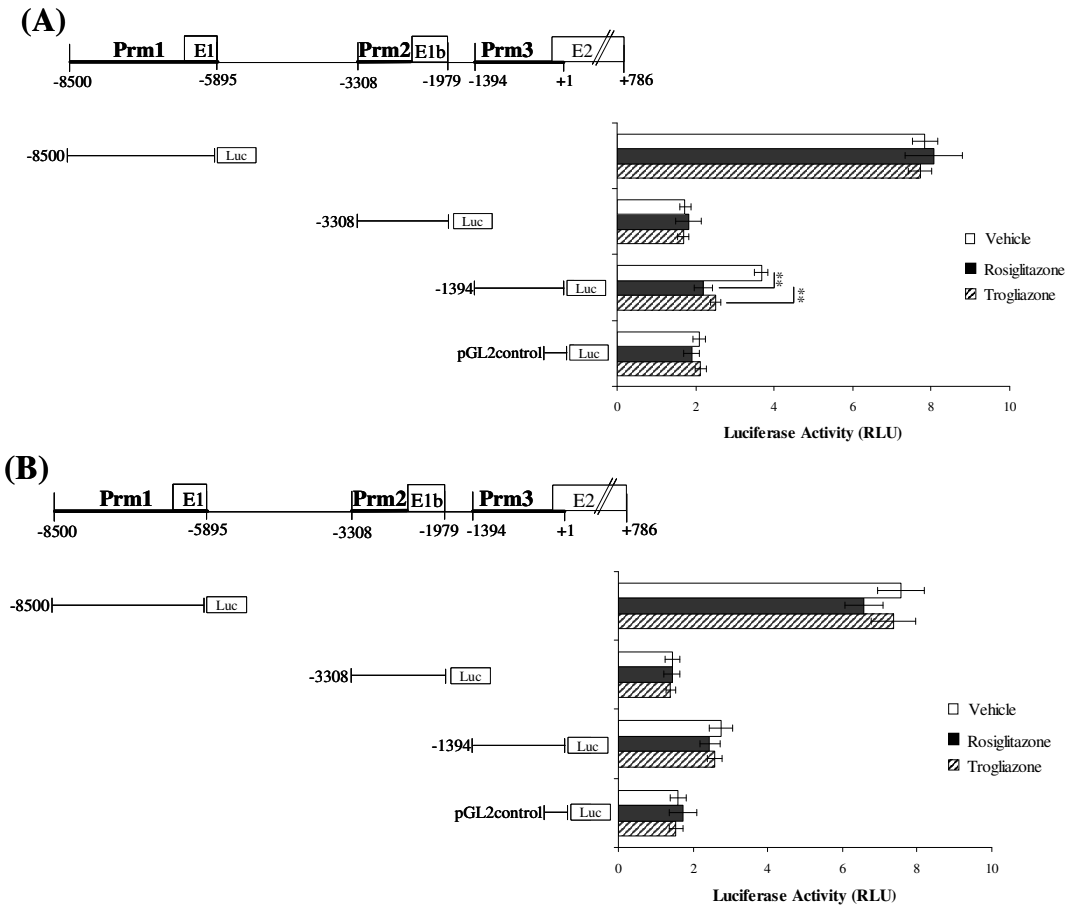

Figure 1. Effect of Rosiglitazone and Troglitazone on Prm1, Prm2 and Prm3-directed Luciferase Expression.

Panels A & B: A schematic of the human TXA_2 receptor (TP) genomic region spanning nucleotides -8500 to +786 encoding Prm1, Prm2 and Prm3 in addition to exon (E) 1, E1b and E2 are illustrated above each panel where nucleotide +1 corresponds to the translational start site (ATG) and nucleotides 5' of that site are given a -designation. Recombinant pGL3Basic plasmids encoding Prm1 (-8500 to –5895), Prm2 (-3308 to -1979), Prm3 (-1394 to +1) or, as a control, pGL3Control were transiently co-transfected along pRL-TK into HEL 92.1.7 (Panel A) and HEK 293 (Panel B) cells. Thirty six hr post-transfection, cells were incubated for 16 hr with either 25 nM rosiglitazone or 10 μM troglitazone (Panels A & B) where vehicle (0.1% (v/v dimethylsulfoxide)-treated cells served as controls. Mean firefly relative to renilla luciferase activity is expressed in arbitrary relative luciferase units (RLU ± SEM; n = 5). The asterisks (*) indicate that Prm3-directed luciferase activity in HEL cells was significantly reduced in rosiglitazone and troglitazone (Panel A) treated HEL cells relative to vehicle treated cells, where ** indicate $P \leq 0.02$.

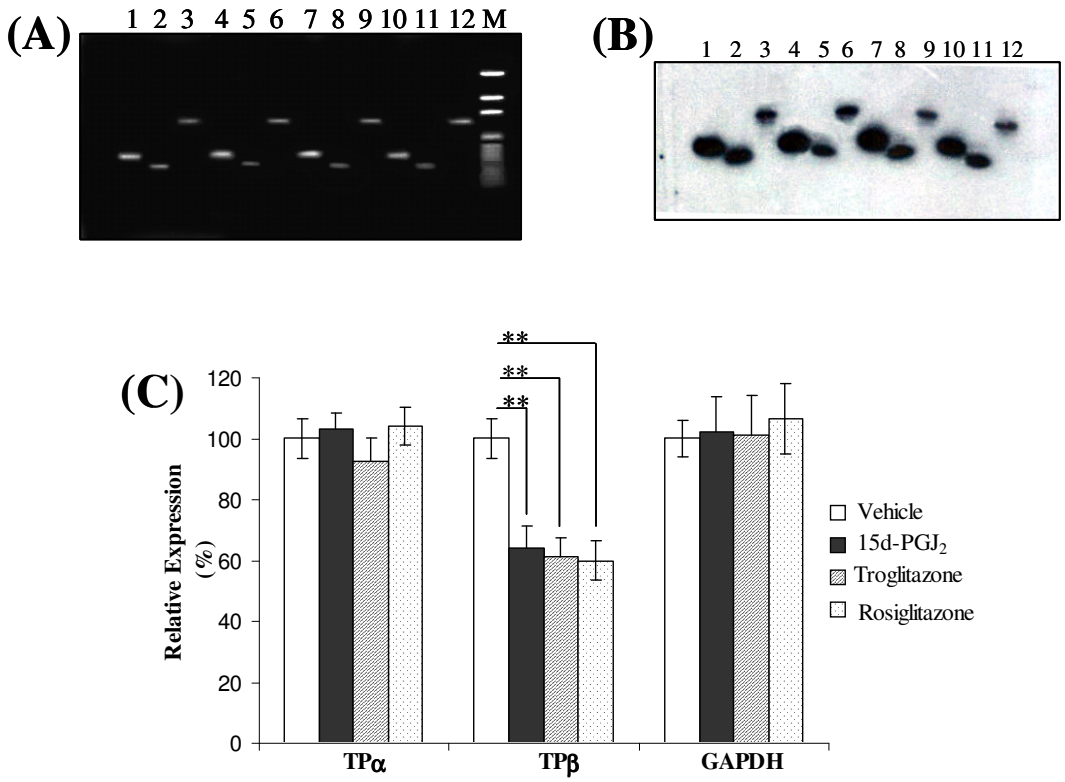

Figure 2. Effect of Rosiglitazone and Troglitazone on TP(gralpha) and TP(grbeta) mRNA expression.

Panels A & B: RT-PCR analysis of RNA isolated from HEL cells pre-incubated for 16 hr with the vehicle 0.1% dimethylsulfoxide (lanes 1-3), 10 μM 15d-PGJ_2 (lanes 4-6), 10 μM troglitazone (lanes 7-9) or 25 nM rosiglitazone (lanes 10-12) using primers designed to specifically amplify TP(gralpha) (Panels A & B, lanes 1, 4, 7 & 10), TP(grbeta) (Panels A & B, lanes 2, 5, 8 & 11) and GAPDH (Panels A & B, lanes 3, 6, 9 & 12) mRNA sequences. Panel A: Agarose gel electrophoresis of RT-PCR products (lanes 1-12; 7 μl / lane) where lane M corresponds to pGEM DNA markers (Promega). Panel B: Southern blot analysis of the RT-PCR products (lanes 1-12) co-screened using ^{32}P radiolabelled oligonucleotide probes specific for TP(gralpha)/TP(grbeta) mRNA and GAPDH mRNA sequences. Panel C: Mean levels of TP(gralpha), TP(grbeta) and GAPDH mRNA expression in 15d-PGJ_2-, troglitazone-, rosiglitazone-treated HEL cells were each represented as a percentage of their expression in vehicle-treated cells (Relative Expression, % ± SEM, n = 4). The asterisks (*) indicate that the level of TP(grbeta) mRNA expression in HEL cells was significantly reduced in 15d-PGJ_2-, troglitazone-, rosiglitazone-treated HEL cells relative to vehicle treated cells, where ** indicates $P \leq 0.02$.

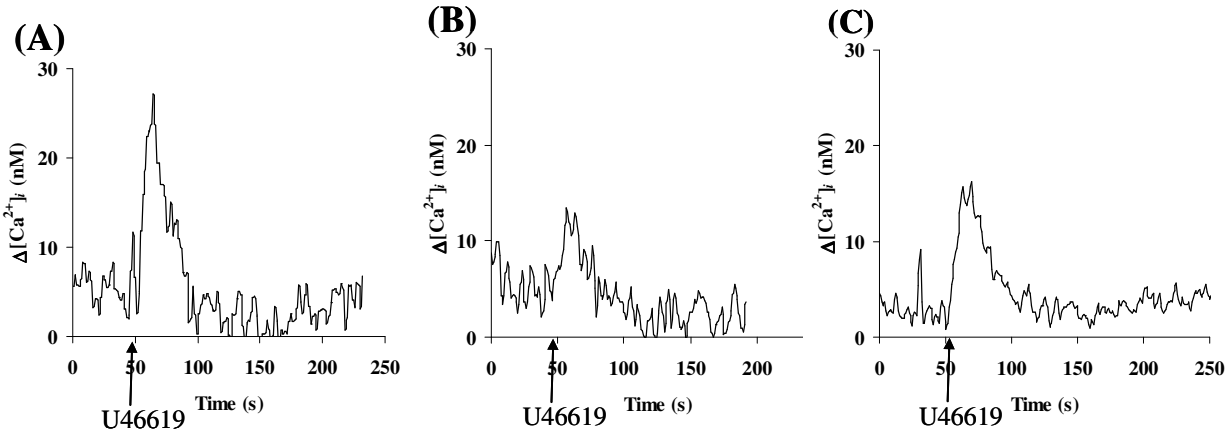

Figure 3. Effect of Rosiglitazone and Troglitazone on TP-mediated intracellular Ca^{2+} mobilization. HEL 92.1.3 cells were pre-incubated for 24 hr with the vehicle 0.1% DMSO (Panel A), 25 nM rosiglitazone (Panel B) or 10 μM troglitazone (Panel C) prior to stimulation with 1 μM U46619, added at the times indicated by the arrows. Data presented are representative profiles from 6-8 independent experiments and are plotted as changes in intracellular Ca^{2+} mobilization ((grdelta)$[Ca^{2+}]_i$, nM) as a function of time (second, s). Actual mean changes in U46619-mediated $[Ca^{2+}]_i$ mobilization (nM ± S.E.M) were as follows: (grdelta)$[Ca^{2+}]_i$ = 23.2 ± 2.7 nM for vehicle treated cells (n = 8); (grdelta)$[Ca^{2+}]_i$ = 14.4 ± 1.5 nM for rosiglitazone - treated cells (n = 6); (grdelta)$[Ca^{2+}]_i$ = 15.6 ± 1.5 nM for troglitazone - treated cells (n = 6).

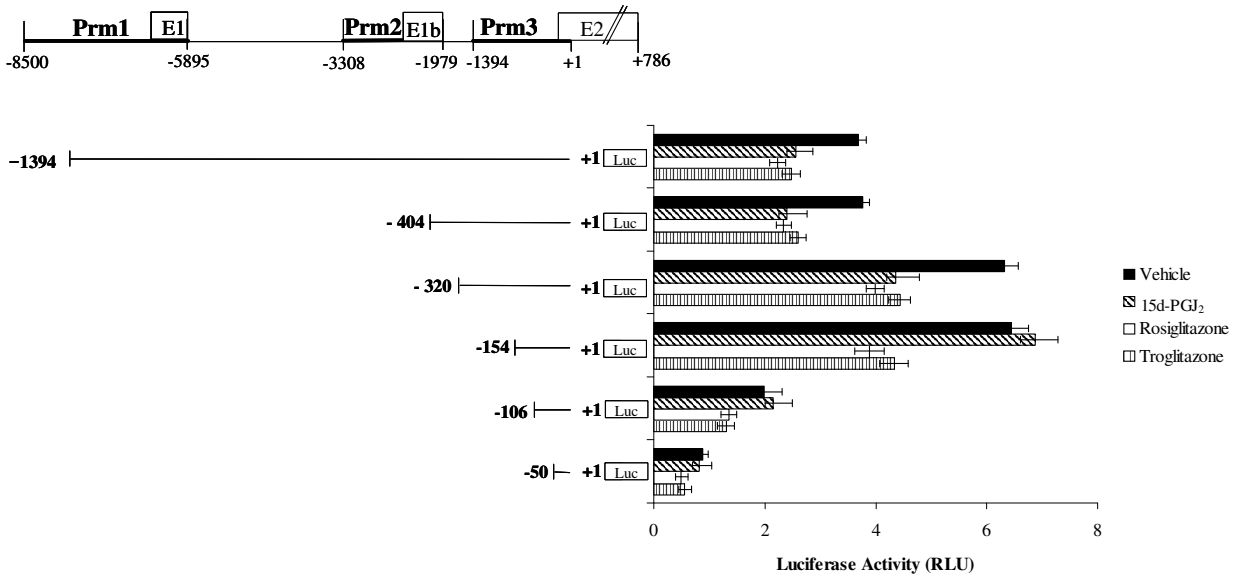

Figure 4. Localisation of the site of action Rosiglitazone and Troglitazone of action within Prm3. Recombinant pGL3Basic plasmids encoding Prm3 (-1394 to +1), Prm3a (-404 to +1), Prm3ab (-320 to +1), Prm3aa (-154 to +1), Prm3aab (-106 to +1) and Prm3aaa (-50 to +1) were transiently co-transfected along with pRL-TK plasmid into HEL 92.1.7 cells. Thirty six hr post-transfection, cells were incubated for 16 hr with either 10 μM 15d-PGJ_2, 25 nM rosiglitazone or 10 μM troglitazone where vehicle (0.1% dimethylsulfoxide)-treated cells served as controls. Mean firefly relative to renilla luciferase activity is expressed in arbitrary relative luciferase units (RLU ± SEM; n = 5).

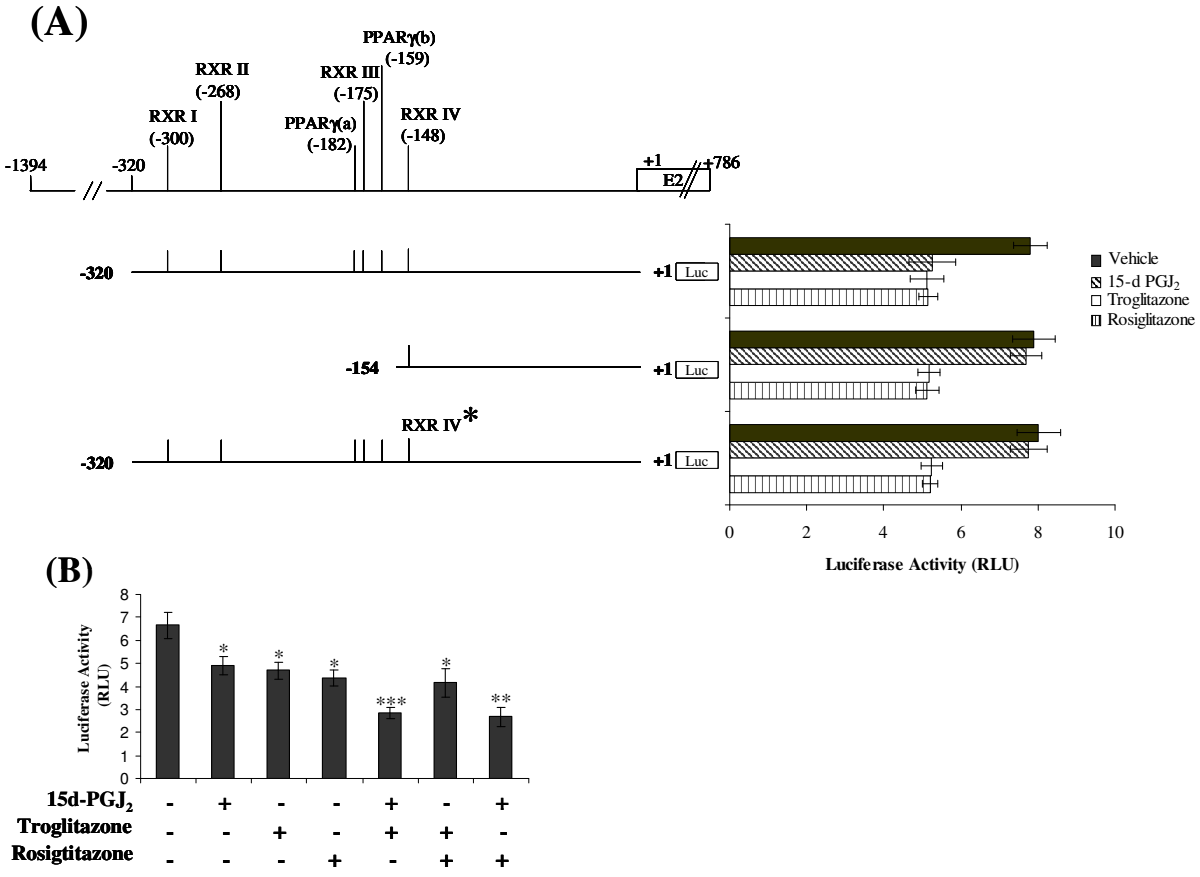

Figure 5. Rosiglitazone- and Troglitazone- mediated inhibition of Prm3 occurs at a site distinct to that of 15d-PGJ_2.

Panel A: A schematic of the TP genomic region encoding Prm3 (-1394 to +1) in addition to exon (E) 2 spanning nucleotides -1394 to +786 are illustrated. In addition, the relative positions of two putative PPAR(grgamma)-responsive elements (PPRE), designated $PPRE^{PPAR\ (grgamma)(a)}$ and $PPRE^{PPAR\ (grgamma)\ (b)}$, respectively, and four putative retinoic acid X receptor responsive elements (RXR), designated RXR I – RXR IV, respectively, within Prm3 are also illustrated. Recombinant pGL3Basic plasmids encoding Prm3ab (-320 to +1), Prm3aa (-154 to +1) or $Prm3ab^{RXRIV*}$, where the RXR IV half site within Prm3ab was mutated, were transiently co-transfected along with pRL-TK plasmid into HEL 92.1.7 cells. Thirty six hr post-transfection, cells were incubated for 16 hr with either 10 μM 15d-PGJ_2, 25 nM rosiglitazone, 10 μM troglitazone where vehicle treated cells served as controls. Mean firefly relative to renilla luciferase activity is expressed in arbitrary relative luciferase units (RLU ± SEM; n = 5). Panel B: HEL 92.1.7 cells were transiently co-transfected with pGL3b:Prm3ab and pRL-TK. Thirty six hr post-transfection, cells were incubated for 16 hr with either 10 μM 15d-PGJ_2, 25 nM rosiglitazone, 10 μM troglitazone, as indicated, where vehicle (0.1% dimethylsulfoxide)-treated cells served as controls. Mean firefly relative to renilla luciferase activity is expressed in arbitrary relative luciferase units (RLU ± SEM; n = 5). The asterisks (*) indicate that Prm3-directed luciferase activity was significantly reduced in HEL cells pre-treated with either 15d-PGJ_2, troglitazone, or rosiglitazone alone or with both rosiglitazone plus troglitazone, rosiglitazone plus 15d-PGJ_2 or with troglitazone plus 15d-PGJ_2 relative to cells pre-treated with the vehicle, where *, **, *** indicate $P \leq 0.05$, $P \leq 0.02$, $P \leq 0.001$, respectively, and statistical analysis shown was performed using the Students' t-test. Results of ANOVA analyses are not shown.

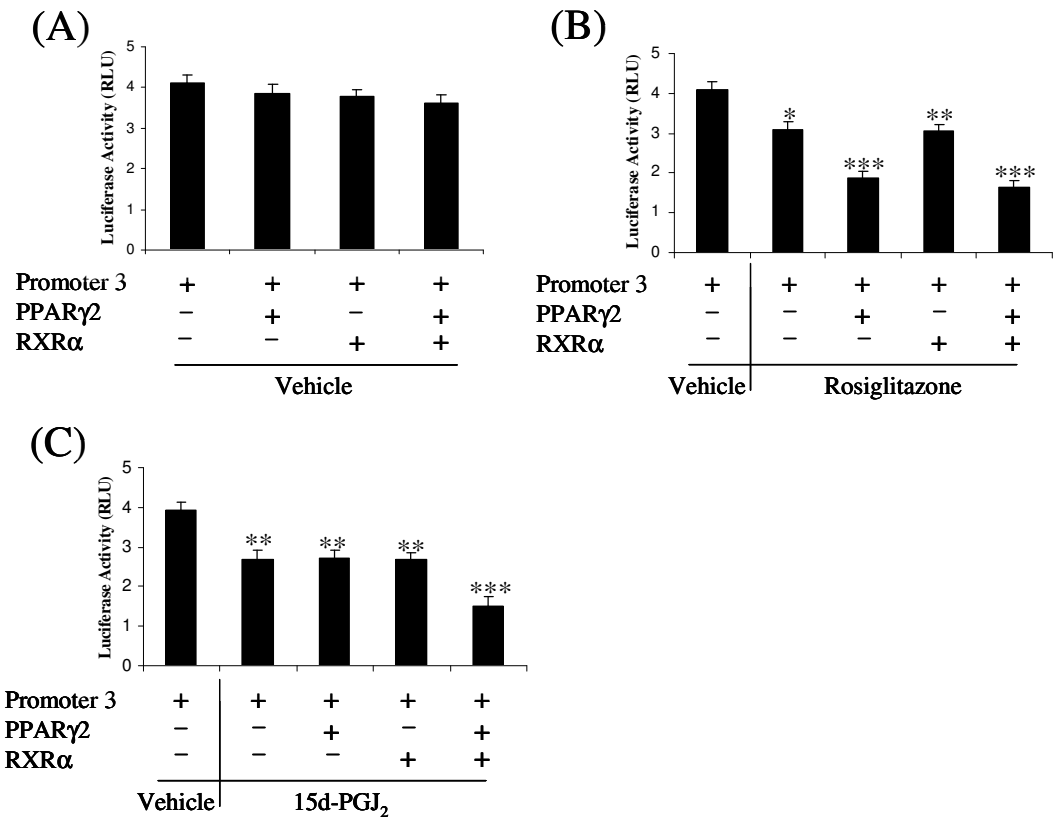

Figure 6. Effect of RXR(gralpha) and hPPAR(grgamma) over-expression on 15d-PGJ$_2$- and Rosiglitazone- mediated inhibition of Prm3-directed Luciferase Expression. Panels A-C: HEL 92.1.7 cells were co-transfected with pGL3b:Prm3ab (1μg) plus pRL-TK (200 ng) together with either pSG5-hPPAR(grgamma)2 (1 μg), pSG5-RXR(gralpha) (1 μg), pSG5-hPPAR(grgamma)2 (1 μg) plus pSG5-RXR(gralpha) (1 μg), or with the required amount of the vector pSG5 to adjust the total amount of DNA to 3.2 μg. Thirty-six hr post-transfection, cells were incubated either with the vehicle (0.1% dimethylsulfoxide; Panel A), rosiglitazone (25 nM; Panel B) or 15d-PGJ$_2$ (10 μM, Panel C) for 16 hr. Thereafter, cells were harvested and firefly and renilla luciferase activity was assayed; results are presented as mean firefly relative to renilla luciferase activity, expressed in arbitrary relative luciferase units (RLU ± SEM; n = 5). The asterisks (*) indicate that Prm3 directed luciferase expression in HEL 92.1.7 cells was significantly reduced in rosiglitazone and 15d-PGJ$_2$ incubated cells relative to vehicle incubated cells, where *, **, *** indicate $P \leq 0.05$, $P \leq 0.02$ and $P \leq 0.001$, respectively and statistical analysis shown was performed using the Students' t-test. Results of ANOVA analyses are not shown.

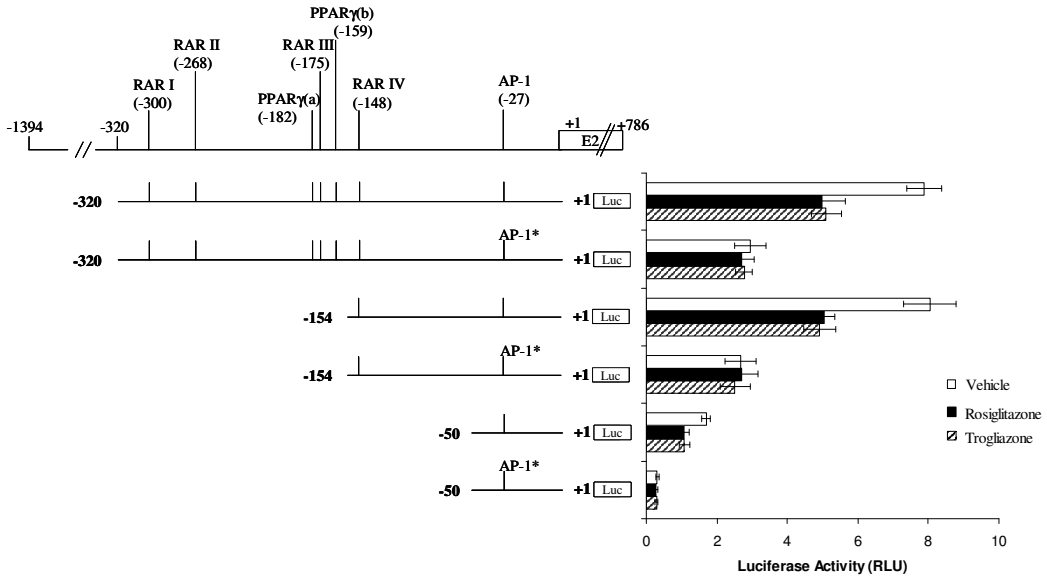

Figure 7. Localisation of the site of action of Rosiglitazone- and Troglitazone- within Prm3.

A schematic of Prm3 (-1394 to +1) and the relative positions of two putative PPAR (grgamma)-responsive elements (PPRE), designated PPAR(grgamma)(a) and PPAR(grgamma) (b), respectively, and four putative retinoic acid X receptor (RXR) responsive elements, designated RXR I – RXR IV, respectively, and an AP-1 element within Prm3 are illustrated. Recombinant pGL3Basic plasmids encoding Prm3ab (-320 to +1), Prm3aa (-154 to +1), Prm3aaa (-50 to +1) or their site-directed variants Prm3ab$^{AP\text{-}1*}$, Prm3aa$^{AP\text{-}1*}$ and Prm3aaa$^{AP\text{-}1*}$, where the Ap-1 element was disrupted, were transiently co-transfected along with pRL-TK plasmid into HEL 92.1.7. Thirty six hr post-transfection, cells were incubated for 16 hr with either 25 nM rosiglitazone or 10 μM troglitazone where vehicle (0.1% dimethylsulfoxide)-treated cells served as controls. Mean firefly relative to renilla luciferase activity is expressed in arbitrary relative luciferase units (RLU ± SEM; n = 5).

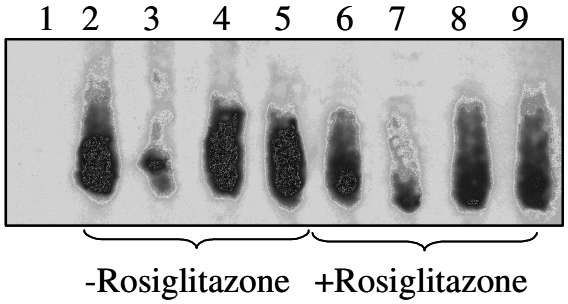

Figure 8. Examination of the effect of Rosiglitazone on AP-1 binding within the –32 to –9 region of Prm3.

EMSAs were carried out using a [(grgamma)]^{32} P-radiolabelled d/s AP-1^{WT} DNA probe (Kin189 and its complement corresponding to nucleotides -32 to -10 of Prm3) and nuclear extract (4μg) prepared from vehicle- (0.1 % dimethylsulfoxide; lanes 2-5) or rosiglitazone - (25 nM; lanes 6-9) pre-incubated HEL 92.1.7 cells as described in the Experimental Procedures. The [(grgamma)]^{32} P-radiolabelled probe was incubated: without nuclear extract (lane 1); with nuclear extract (lanes 2 & 6); with nuclear extract in the presence of a 50- fold excess of non-labelled d/s specific competitor AP-1^{WT} oligonucleotide (Kin 189 and its complement, lanes 3 & 7); with nuclear extract in the presence of a 50-fold excess of non-labelled d/s non-competitor AP-1* oligonucleotide (Kin162 and its complement, where the putative AP-1 element at -27 was mutated, lanes 4 & 8); with nuclear extract in the presence of a 50-fold excess of non-labelled d/s non-competitor Oct-1/2 oligonucleotide (Kin195 and its complement, lanes 5 & 9). DNA/ nuclear factor complexes were subject to polyacrylamide gel electrophoresis followed by autoradiography, as outlined in Experimental Procedures.