Role of PPARγ and EGFR signalling in the urothelial terminal differentiation programme

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Summary

Recently, considerable interest has focused on the ability of activated peroxisome proliferator-activated receptor γ (PPARγ) to promote cytodifferentiation in adipocytes and some carcinoma cells; however, the role of PPARγ in normal epithelial cytodifferentiation is unknown. Using uroplakin (UP) gene expression as a specific correlate of terminal urothelial cytodifferentiation, we investigated the differentiation-inducing effects of PPARγ activation in normal human urothelial (NHU) cells grown as finite cell lines in monoculture. Two high-affinity activators of PPARγ, troglitazone (TZ) and rosiglitazone (RZ) induced the expression of mRNA for UPII and UPIb and, to a lesser extent, UPIa. The specificity of the effect was shown by pretreating cells with a PPARγ antagonist, GW9662, which attenuated the TZ-induced response in a dose-specific manner. The PPARγ-mediated effect on *UP* gene expression was maximal when there was concurrent inhibition of autocrine-activated epidermal growth factor receptor (EGFR) signalling through either the phosphatidylinositol 3-kinase or extracellular signal-regulated kinase (ERK) pathways. The use of a specific EGFR tyrosine kinase inhibitor, PD153035, correlated with PPARγ dephosphorylation and translocation to the nucleus, indicating a mechanism for regulating the balance between proliferation and differentiation. This is the first identification of specific factors involved in regulating differentiation-associated gene changes in urothelium and the first unambiguous evidence of a role for PPARγ signalling in the terminal differentiation programme of a normal epithelium.

Key words: Differentiation, Kinase, Epidermal growth factor, Peroxisome proliferator activated receptor, Bladder, Uroplakin, Urothelium

Introduction

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily. Activation of the PPAR pathway requires heterodimerisation of ligand-bound PPAR with the retinoid X receptor (RXR) to form a transcription factor that binds specific peroxisome proliferator response elements (PPRE) in the promoters of target genes (reviewed by Blanquart et al., 2003). PPARγ signalling is recognised as having a key role in regulating the programme of gene expression that leads to terminal differentiation of adipocytes (Lowell, 1999). A role for PPARγ signalling has also been proposed in the differentiation programmes of other cell types, including epithelial cells, but there is little unequivocal evidence of a role for PPARγ signalling in normal epithelial differentiation programmes. This paucity of evidence is due to the fact that studies to date have used carcinoma-derived cell lines to investigate the tumour-suppressive effects of PPARγ activation in terms of growth inhibition and/or induction of apoptosis or differentiation (Burgermeister et al., 2003; Chang and Szabo, 2000; Elnemr et al., 2000; Kawa et al., 2002; Lefebvre et al., 1999; Mueller et al., 1998; Sarraf et al., 1998; Xu et al., 2003), and few studies have used objective markers of terminal differentiation. We sought to clarify the role of PPARγ in an epithelium for which there is a well-characterised normal cell culture system and objective markers of the terminal stages of cytodifferentiation.

The urothelium is the highly specialised transitional epithelium that lines the major portion of the urinary tract, including the bladder. It consists of basal, intermediate and superficial cell layers, which function to provide a permeability barrier to urine. The terminally differentiated superficial cell is characterised by the presence of multiple plaques of asymmetric unit membrane (AUM) in the outer leaflet of the apical membrane. The characteristic AUM plaques are formed by the interaction of at least four species-conserved integral transmembrane proteins, known collectively as the uroplakins (UPs). UPIa and UPIb are members of the tetraspanin family of proteins and form plaques by interacting with the unrelated single transmembrane domain UPII and UPIII proteins, respectively (Wu et al., 1995). In normal human urothelium, expression of *UPIa*, *UPII* and *UPIII* genes is restricted to superficial cells, whereas UPIb transcripts are also present in intermediate cells, implying that expression of the *UPIb* gene is less differentiation restricted (Lobban et al., 1998; Olsburgh et al., 2003).

PPARγ expression has been described in the presumptive urothelium of the mouse urogenital sinus and in the mature urothelium of mice, rabbits and man (Guan et al., 1997; Jain et al., 1998; Kawakami et al., 2002), where it has been

described as differentiation associated (Kawakami et al., 2002; Nakashiro et al., 2001). Although activation of PPARγ has been shown to suppress the growth of normal and malignant urothelial cells in vitro (Nakashiro et al., 2001), the effects on differentiation are unknown.

We sought to determine the role of PPARγ activation in normal human urothelial (NHU) cells, exploiting our well-characterised in vitro cell culture model (Southgate et al., 1994; Southgate et al., 2002). In this system, NHU cells are highly proliferative and exhibit a basal/intermediate urothelial cell phenotype that is sustainable over multiple passages, but they do not express markers associated with late/terminal cytodifferentiation, including *UPIa*, *UPII* and *UPIII* genes (Southgate et al., 1994; Southgate et al., 2002). In this report, we show that activation of PPARγ induces the expression of *uroplakin* genes, but that this induction is attenuated by signalling downstream of the epidermal growth factor receptor (EGFR). Our results support a role for PPARγ-mediated signalling in regulating the urothelial differentiation programme and suggest that proliferation will take precedence over differentiation during a regenerative response.

Materials and Methods

Materials

Troglitazone was provided as a gift by Parke-Davis Pharmaceutical Research (Ann Arbor, USA) and rosiglitazone and GW9662 were provided as a gift from GlaxoSmithKline (Worthing, UK). The inhibitors PD153035, PD98059, LY294002, U0126 and SB203580 were obtained from Calbiochem-Novabiochem Biosciences (Nottingham, UK). [^{32}P]CTP was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Tissues

The collection of surgical specimens was approved by the relevant Local Research Ethics Committees and had full patient consent, as required.

Surgical specimens of normal urothelium were obtained from patients with no history of urothelial dysplasia or malignancy. Tissues were collected in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES pH 7.6 and 20 KIU aprotinin (Trasylol, Bayer plc, Newbury, UK), as described previously (Southgate et al., 1994; Southgate et al., 2002). Representative pieces of each tissue sample were processed into paraffin wax for histology and immunohistochemistry. The remaining sample was cut into approximately 1 cm^2 pieces, placed into HBSS (Ca^{2+} and Mg^{2+} free) supplemented as above and containing 0.1% (w/v) EDTA, and incubated at 4°C overnight to release pure urothelial cell sheets. The isolated urothelium was used either to establish normal human urothelial (NHU) cell lines (see below) or for RNA or protein extraction.

Cell culture

NHU cell lines were established and maintained in keratinocyte serum-free medium (KSFM) containing bovine pituitary extract and epidermal growth factor at the manufacturer's recommended concentrations (Invitrogen, Paisley, UK) and supplemented with 30 ng/ml cholera toxin (Sigma, Poole, UK). These methods have been described in detail elsewhere (Southgate et al., 1994; Southgate et al., 2002). NHU cell lines were used for these studies between passages 3 and 5. For experiments, cells were seeded at 9×10^5 cells/ml and allowed to attain approximately 70% confluency before treatment with PPARγ ligands and inhibitors. NHU cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

Ribonuclease protection assays

To extract RNA from cell monolayers, cells were solubilised in Trizol™ (Invitrogen, Paisley, UK). Isolation of RNA by chloroform extraction and isopropanol precipitation was performed as recommended by the manufacturer.

Part-length cDNA fragments of the coding region for human *UPIa*, *UPIb*, *UPII*, *UPIII* and *GAPDH* genes were cloned into pGEM-T Easy (Promega, Southampton, UK) as described previously (Lobban et al., 1998). *GAPDH* was used as an internal riboprobe control. ^{32}P-labelled antisense transcripts of the cDNAs of interest were generated from linearised plasmids using the In Vitro Transcription Kit (Promega), according to the manufacturer's protocol. After DNAse treatment, riboprobes were purified by passage through Chromaspin 30-DEPC columns (BD Biosciences Clontech UK, Oxford, UK).

Ribonuclease protection assays were performed using an RPAIII kit in accordance with the manufacturer's protocols (RPAIII kit, Ambion, UK). Approximately 2 fmol of each riboprobe were mixed and hybridised to 5 μg of total RNA. Products were separated on 5% denaturing polyacrylamide gels (Sequagel from Flowgen, Lichfield, UK), visualised by autoradiography and quantified by means of a phosphorimager (BioRad GS-525 Molecular Imager System, Hemel Hempstead, UK). The quantified uroplakin bands were normalised against the GAPDH signal, which was used to correct for loading efficiency. When there was no signal detected a blank was left when quantifying against control.

Immunofluorescence

Cells grown on slides were fixed in a 1:1 mixture of methanol and acetone, air-dried and incubated with titrated PPARγ antibody for 16 hours at 4°C, before washing and incubation in secondary antibody conjugated to Alexa 488 (Molecular Probes, supplied by Cambridge Bioscience, Cambridge, UK). 0.1 μg/ml Hoechst 33258 (Sigma-Aldrich Ltd., Poole, UK) was included in the penultimate wash in order to visualise nuclei. Slides were observed on an Olympus BX60 microscope under epifluorescence illumination.

Immunoprecipitations

Cells were lysed (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml aprotinin and 50 μg/ml leupeptin), extracted and assayed as outlined below. To 200 μg of protein, 10 μg PPARγ-agarose conjugate (Santa Cruz Biotechnology, supplied by Autogen-Bioclear UK, Calne) were added and the samples were rotated overnight at 4ºC. Next day, the samples were centrifuged at 1000 ***g*** for 5 minutes at 4ºC and the pellet retained. The pellet was washed four times in cold PBS, boiled in sample buffer (125 mM Tris, pH 6.8, 4% SDS, 0.01% bromophenol blue and 20% glycerol) before being resolved by SDS-PAGE, as outlined below.

Western blot analysis

Lysates were prepared by treating cells with lysis buffer [25 mM Hepes pH 7.4, 125 mM NaCl, 10 mM NaF, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.2% (w/v) SDS, 0.5% (w/v) sodium deoxycholate acid, 1% (w/v) Triton X-100, 1 μg/ml aprotinin, 10 μg/ml leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride]. Lysates were sheared by passing three times through a 21-gauge needle and left on ice for 30 minutes, before microcentrifugation at 10,000 ***g*** for 30 minutes at 4ºC. The protein concentrations of supernatants were measured by the Bradford assay (Pierce, supplied by Perbio Science UK, Cheshire). Cell extracts were resolved on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies for 16 hours at 4°C. Bound antibody was detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence using the ECL Detection Kit (Amersham Pharmacia).

Antibodies

Monoclonal antibody against PPARγ (clone E-8) and rabbit polyclonal antibody to RXRα (code D20) were obtained from Santa Cruz Biotechnology (supplied by Autogen-Bioclear UK). Rabbit polyclonal antibody to phospho-extracellular signal-regulated kinase (ERK) was obtained from Cell Signalling Technology (supplied by New England Biolabs UK, Hitchin, UK) and monoclonal anti-total ERK (clone 16) was from Transduction Laboratories (supplied by Becton Dickinson, Oxford, UK). Monoclonal anti-phosphoserine (clone PSR-45) was obtained from Sigma-Aldrich, Poole, UK.

Promoter analysis

The transcriptional start site for each uroplakin gene (NCBI accession numbers: *UPIa*, NM_007000; *UPIb*, NM_006952; *UPII*, AX259986 and *UPIII*, NM_006953) was retrieved from NCBI (www.ncbi.nlm.nih.gov) or by linking to UCSC Genome Browser (http://genome.ucsc.edu). A 2 kb region upstream of the transcriptional start site for each uroplakin was used to determine if there were any PPAR binding sites. Nine high-affinity PPARγ binding sites (Juge-Aubry et al., 1997) were used to construct a PPARγ-defined PPRE matrix in the MatDef programme of Genomatix suite (Quandt et al., 1995; Wolfertstetter et al., 1996) (Table 1). Using the PPARγ-defined PPRE matrix, the 2 kb regions of the uroplakin genes were analysed for PPRE binding sites in the MatInspector programme of Genomatix suite (Quandt et al., 1995). The outcome was that no PPARγ-PPRE binding sites were predicted on the positive strand of the uroplakins.

Statistical analysis

Comparisons between groups were analysed using a two-tailed Student's *t*-test. Differences were considered significant when $P<0.05$.

Results

Expression of PPARγ and RXRα in human urothelial cells

Western blot analysis showed that PPARγ and RXRα proteins were expressed by human urothelium in situ and by all three independent NHU cell lines tested (Fig. 1A).

Response of cultured NHU cells to troglitazone (TZ)

In normal serum-free growth medium, NHU cells grew as monolayers with a typical epithelioid cobblestone morphology (Fig. 1B). The addition of the PPARγ ligand, troglitazone (TZ) (1 μM) resulted in a dramatic change in morphology, with NHU cells forming rosettes of tear-shaped cells that morphologically resembled transitional epithelial cells in situ (Fig. 1B). At higher concentrations of TZ (>5 μM), extensive cell death was observed, with the cytoplasmic blebbing and nuclear fragmentation characteristic of an apoptotic response (Fig. 1B).

Table 1. PPARγ-defined PPRE matrix

Base	Binary matrix																			
A	2	4	5	6	5	1	1	5	0	0	2	1	9	7	9	0	0	0	0	7
C	4	2	1	1	1	7	1	1	0	0	3	6	0	0	0	0	0	0	8	0
G	2	2	2	1	0	1	0	3	9	9	2	0	0	0	0	9	5	0	1	0
T	1	1	1	1	3	0	7	0	0	0	2	2	0	2	0	0	4	9	0	2

The binary matrix was constructed from the high-affinity PPARg binding sites of nine genes (*CYP4A1*, *FABP*, *HMG*, *BIF*, *CYP4A6(Z)*, *Mep*, *PEPCK2*, *PEPCK1*, *ACOA*) (Judge-Aubry et al., 1997), according to the frequency of each base at a particular position.

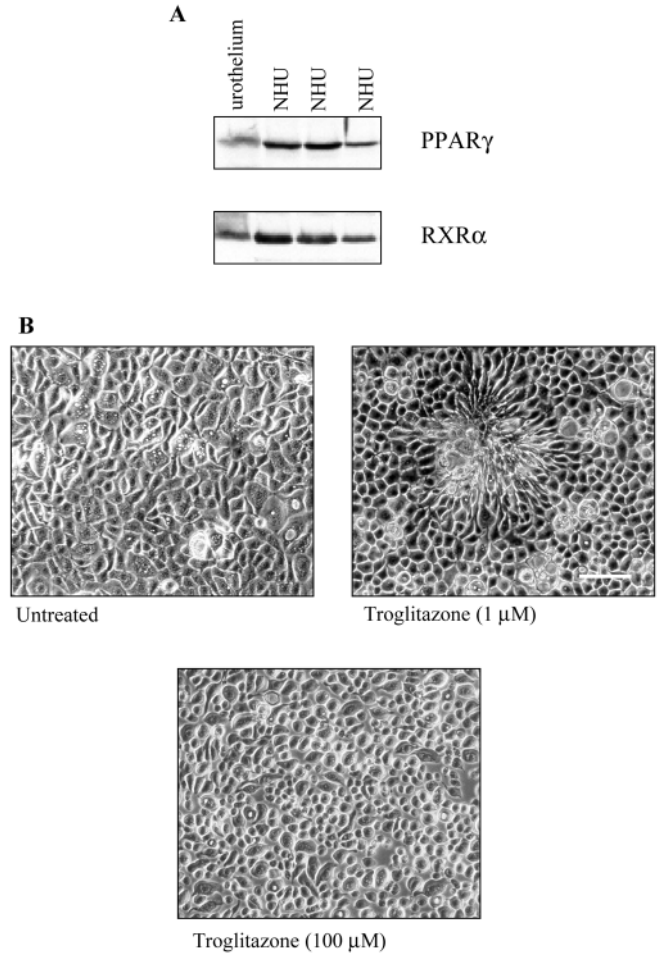


Fig. 1. (A) Western blot analysis of nuclear hormone receptor expression in freshly isolated human urothelium and cultured NHU cell lines. Protein lysates (40 μg/lane) extracted from human urothelium and from three independent NHU cell lines were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and transferred to nitrocellulose membrane. Membranes were probed with specific antibodies against PPARγ and RXRα. (B) Phase contrast morphology of NHU cells grown to 70% confluence and incubated in the absence or presence of TZ (1 μM or 100 μM, as indicated) for 48 hours. Bar, 100 μm.

Effect of EGF and PPARγ ligands on uroplakin mRNA gene expression in NHU cells

The expression of the four *uroplakin* genes by cultured NHU cells was assessed by ribonuclease protection assay (RPA). In agreement with previous findings (Lobban et al., 1998), mRNA for UPIb was expressed by all NHU cell cultures, UPIa was usually negative, although detected weakly in some post-confluent cultures, and neither *UPII* nor *UPIII* mRNA were ever detected, irrespective of time in culture or degree of confluency. The presence or absence of exogenous EGF in the medium alone had no effect on the expression of uroplakin transcripts by NHU cells (Fig. 2).

The effect of the PPARγ agonist, TZ, was investigated. In the absence of exogenous EGF, expression of *UPII* mRNA was first detected after 3 days treatment with TZ (Fig. 2). In the presence of EGF (5 ng/ml), TZ induced de novo

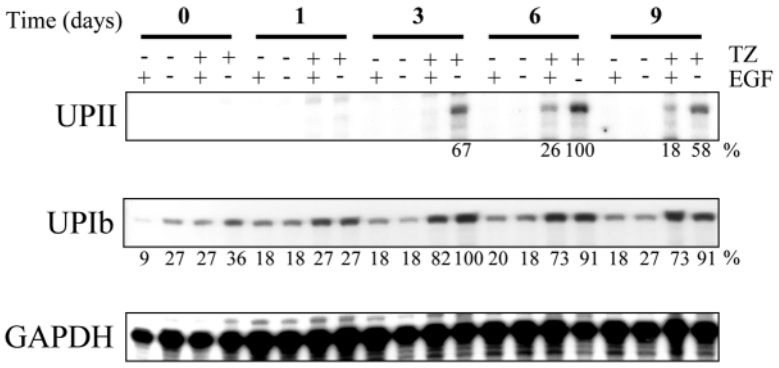


Fig. 2. Ribonuclease protection assay (RPA) to quantify the effect of TZ and EGF on uroplakin mRNA expression in NHU cells. NHU cells were treated in the presence or absence of TZ (1 μM) and/or EGF (5 ng/ml) for the times indicated. Total RNA was extracted and 5 μg were hybridised with ^{32}P-labelled human UPII, UPIb and GAPDH cDNA probes. The samples were electrophoresed and the UP bands were quantified by means of a phosphorimager and normalised against the GAPDH signal, which was used to correct for loading efficiency. Maximum uroplakin expression was taken to be 100%.

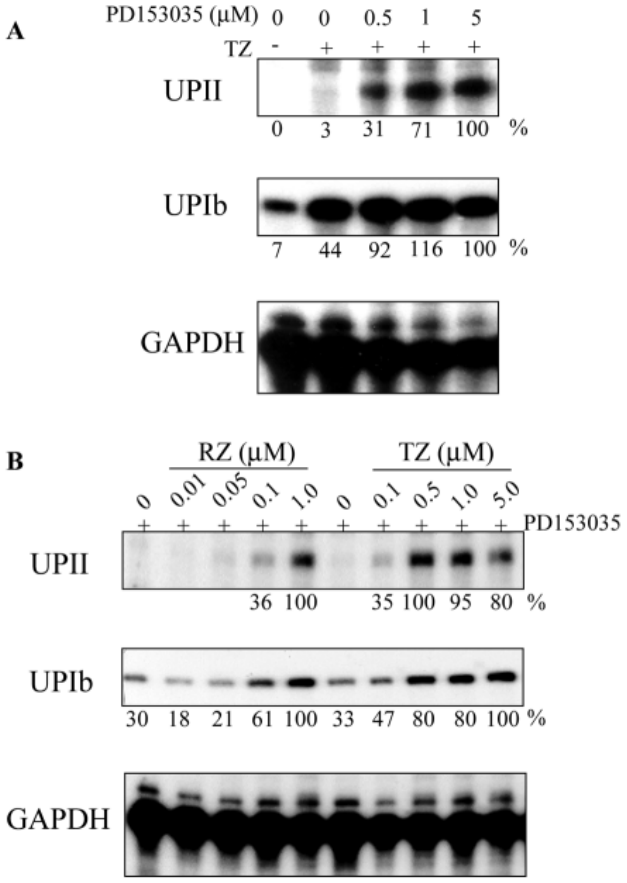


Fig. 3. Ribonuclease protection assay (RPA) to quantify the effect of TZ and PD153035 on uroplakin mRNA expression in NHU cells. (A) NHU cells were treated for 24 hours with or without 1 μM TZ, before incubation for 4 days with the EGFR inhibitor PD153035 at the indicated concentrations. Medium containing appropriate inhibitor was replenished every 2 days. Total RNA was extracted and 5 μg were analysed by RPA to assess the relative uroplakin mRNA expression (maximum uroplakin expression assigned 100%), as described in Fig. 2. (B) Concentration-dependent effects of RZ and TZ on uroplakin mRNA expression in EGFR-inhibited NHU cells. NHU cells were pretreated for 24 hours with the indicated concentrations of RZ or TZ, before being incubated in medium containing PD153035 (1 μM). Total RNA was extracted from samples after 4 days and 5 μg were analysed by RPA to assess relative uroplakin mRNA expression, as described in Fig. 2. Maximum uroplakin expression was taken to be 100%. Note that PD153035 alone had no effect on *UPII* gene expression (B).

expression of *UPII* mRNA, but expression was delayed until 6 days after treatment and the maximum induction was threefold less compared with NHU cells treated with TZ in the absence of EGF (Fig. 2). The effects of TZ and EGF on *UPII* gene expression were mirrored by the induction of the *UPIa* gene, although the magnitude of the response was much weaker (data not shown). TZ also upregulated *UPIb* mRNA expression by up to fourfold above basal by day 3, and expression was enhanced by the absence of EGF (Fig. 2). No *UPIII* expression was detected under any conditions (data not shown).

Influence of EGFR signalling on PPARγ-mediated response

Although the effects of TZ on uroplakin gene expression were reproduced in at least ten independent NHU cell lines, the extent of the response was variable, even in the absence of exogenous EGF. To determine whether the response of NHU cells to TZ was modulated by autocrine activation of the EGFR, experiments were performed in which cells were treated with TZ in the presence of each of the potent EGFR inhibitors, PD153035 and AG1478. A concentration-dependent enhancement of the TZ-induced expression of *UPII* and *UPIb* genes was found with each of the two EGFR inhibitors (Fig. 3A, illustrated with PD153035).

Observations were confirmed using a second PPARγ agonist, rosiglitazone (RZ) (Fig. 3B). The induction of uroplakin mRNA by the PPARγ agonists was dose dependent, with maximum induction of *UPII* mRNA at 0.5 to 1 μM TZ or 1 μM RZ in the presence of PD153035 (Fig. 3B).

Effective period of exposure to TZ

Initial experiments were performed in the constant presence of PPARγ agonist. However, further studies combining PPARγ activation with EGFR inhibition showed that *UPII* mRNA expression could be induced by just 2 hours exposure to TZ (1 μM), although maximal expression was found when cells were treated with TZ for 24 hours (Fig. 4A).

Timecourse studies of NHU cells treated with TZ for 24 hours and maintained for up to 8 days in the presence of PD153035 (1 μM) showed maximal effects on the mRNA expression of *UPII*, *UPIa* and *UPIb* 4 days after treatment with TZ (Fig. 4B).

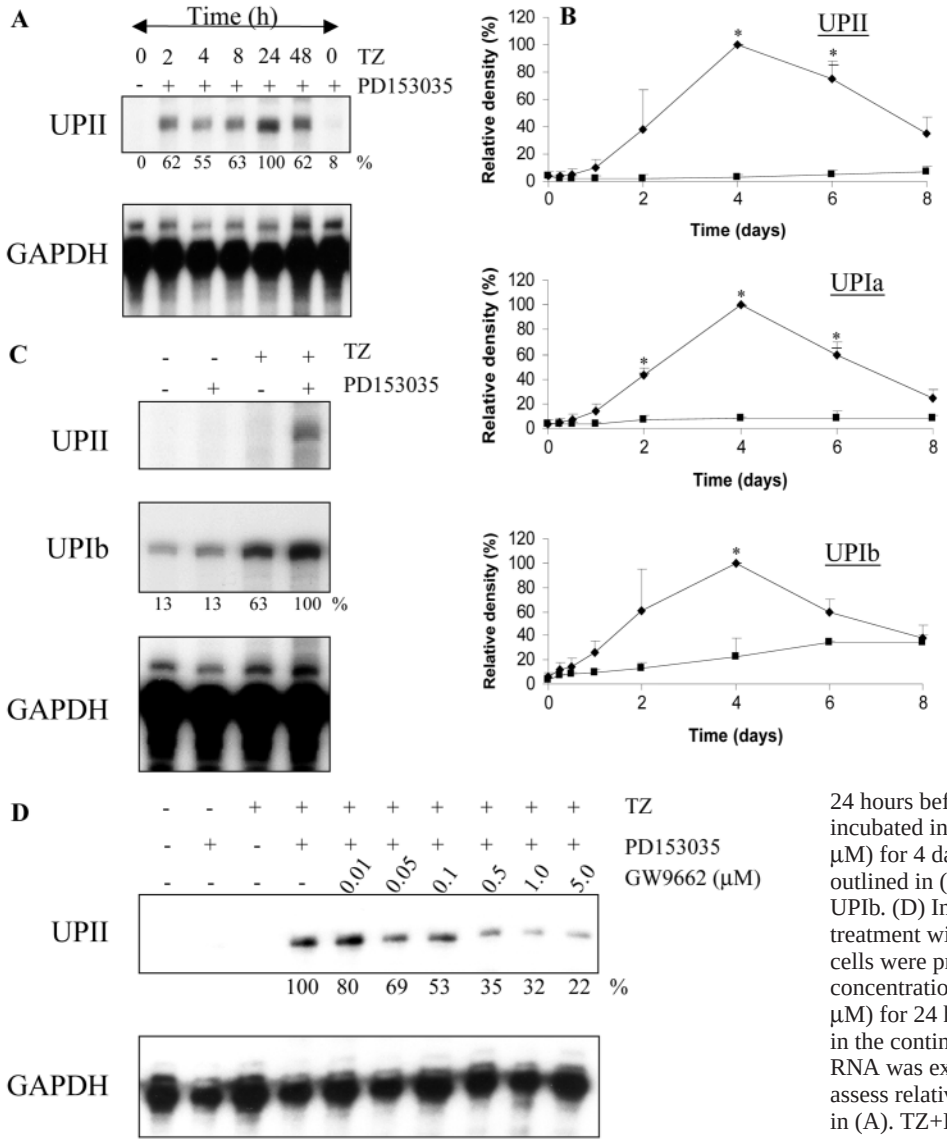


Fig. 4. (A) NHU cells were treated with TZ (1 μM) for the length of time indicated, the medium was changed and the cells were maintained in medium containing PD153035 (1 μM) for 4 days. The RNA was extracted and the RPA was performed and analysed as outlined in Fig. 2. (B) NHU cells were treated for 24 hours with TZ (1 μM) and then maintained in medium in the presence or absence of PD153035 (1 μM) for the times indicated. Uroplakin mRNA expression was quantified by phosphorimager analysis of the RPA hybridisation signal and normalised to the GAPDH signal, to correct for sample loading. Uroplakin expression in TZ-exposed NHU cells treated with PD153035 for 4 days was designated 100%. Diamonds, TZ+PD153035; squares, TZ-PD153035. The data is the mean + s.e.m. of three experiments performed on three independent NHU cell lines.*P<0.005 TZ compared with TZ+ PD153035. (C) NHU cells were treated in the absence or presence of TZ (1 μM) for 24 hours before the medium was changed and cells were incubated in the presence or absence of PD153035 (1 μM) for 4 days. RPA was performed and quantified as outlined in (A). TZ+PD153035 was taken to be 100% for UPIb. (D) Inhibition of PPARγ activation by pre-treatment with the PPARγ antagonist GW9662. NHU cells were pretreated for 3 hours with GW9662 at the concentrations indicated, before being exposed to TZ (1 μM) for 24 hours and thereafter to PD153035 (1 μM), all in the continued presence of GW9662. After 4 days, total RNA was extracted and 5 μg were analysed by RPA to assess relative uroplakin mRNA expression, as described in (A). TZ+PD153035 was taken to be 100%.

Using the optimised treatment regime confirmed that neither PD153035 nor TZ alone induced *UPII* gene transcription, but that NHU cells required both TZ and PD153035 to induce de novo UPII mRNA expression (Fig. 4C). UPIb mRNA expression was upregulated by TZ alone (fivefold), but not by PD153035 alone. UPIb mRNA expression was futher enhanced (twofold above TZ alone) when cells were treated with both TZ and PD153035 (Fig. 4C).

Effect of inhibiting PPARγ on the TZ-induction of *UPII* expression

To verify that the TZ-mediated induction of *UPII* expression was mediated by PPARγ activation, NHU cells were pretreated with the PPARγ antagonist, GW9662 (0-5 μM), before exposure to TZ and PD153035. GW9662 inhibited the TZ-mediated induction of *UPII* mRNA expression in a dose-dependent manner, with 5 μM resulting in 80% inhibition relative to the positive control (Fig. 4D).

Effects of EGFR signalling on PPARγ in NHU cells

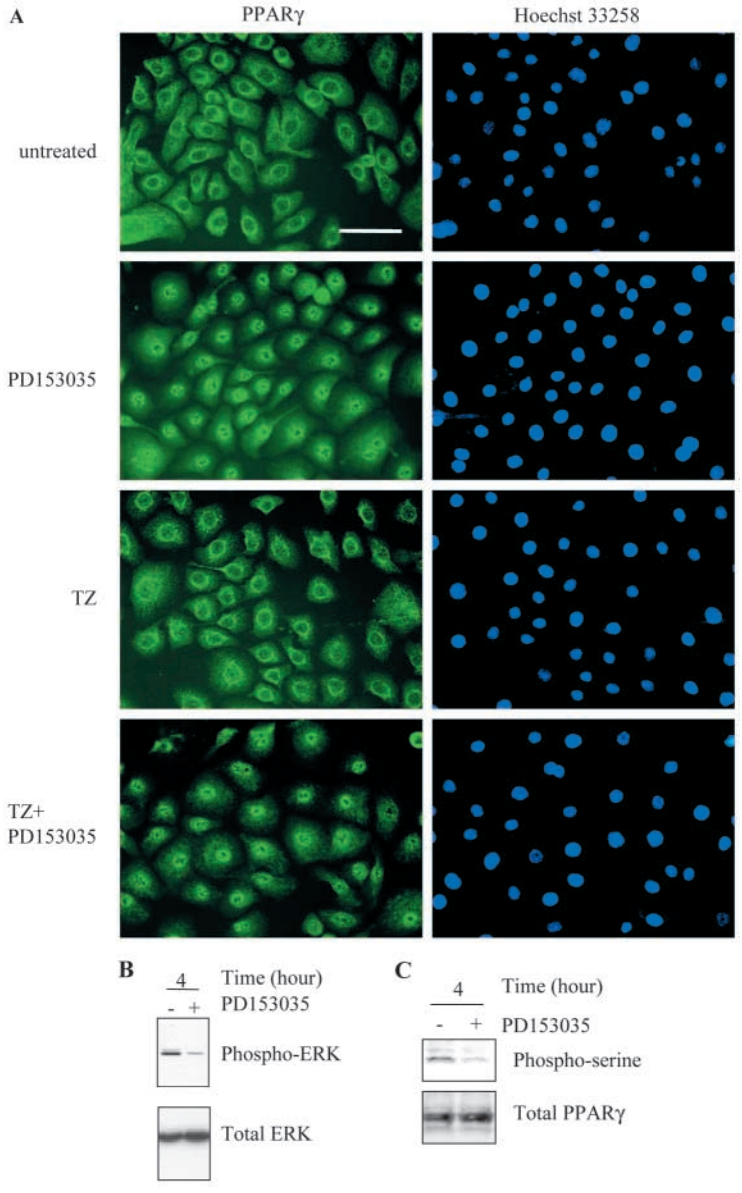
When NHU cells were maintained in the presence of exogenous EGF, localisation of PPARγ was predominantly perinuclear and excluded from the nuclei (Fig. 5A). Treatment of NHU cells with PD153035 resulted in the translocation of PPARγ to the nucleus, irrespective of whether cells were treated with TZ. When cells were treated with TZ, without inhibition of EGFR signalling, the majority of cells showed perinuclear localisation of PPARγ. In cultures not treated with PD153035, occasional cells were observed with nuclear PPARγ localisation; this probably reflected downregulation of EGFR signalling in these cells by other mechanisms (e.g. contact inhibition).

It is well known that EGF binding to its cognate receptors activates several signalling cascades, including the mitogen-activated protein kinase (MAPK), ERK. There was an 80% inhibition of phosphorylated ERK after 4 hours treatment of NHU cells with PD153035 (Fig. 5B). Immunoprecipitation showed that PPARγ was dephosphorylated by 70% within 4 hours of treatment with PD153035 (Fig. 5C).

Downstream EGFR signalling pathways

We used a range of kinase inhibitors to identify the EGFR signalling pathways involved in inhibiting PPARγ-mediated expression of the *uroplakin* genes (Fig. 6). In the presence of the MAPK kinase inhibitors PD98059 and U0126, or the phosphoinositide 3-kinase inhibitor LY294002, TZ induced *UPII* mRNA to a similar extent as the induction caused by TZ treated with PD153035. By contrast, there was no TZ-induced *UPII* mRNA expression in the presence of the p38 kinase inhibitor, SB203580. No additive effect was found on the induction of UPII mRNA levels when the cells were treated with TZ together with both PD98059 and LY294002, suggesting that both pathways acted on the same target phosphoprotein. These results suggest that the ERK and phosphoinositide 3-kinase pathways inhibit the ability of TZ to induce *UPII* gene expression (Fig. 6).

Other inhibitors, including those against protein kinase C (bisindolylmaleimide I) and protein kinase A (H-89) had no effect on *UPII* mRNA induction by TZ (data not shown).



Discussion

This is the first report that the expression of *uroplakin* genes can be induced de novo in monolayer NHU cell cultures. This is a significant finding, as expression of these genes provides objective evidence of terminal differentiation in urothelium (Lobban et al., 1998; Olsburgh et al., 2003). Our studies show a role for PPARγ signalling in regulating the urothelial differentiation programme and show that this is conditional on inhibition of downstream EGFR signalling. This has important implications for the regulation of the balance between proliferation and differentiation in the urothelium.

The PPARγ induction of *UPII* transcripts was first detected after 3 days (Fig. 2), implying that the effect on uroplakin gene expression is indirect. This is supported by the fact that we have not identified any potential PPRE binding sites in the upstream region of any of the uroplakin promoters (Table 1). Thus, our data suggest that activation of the PPARγ signalling pathway induces a programme of gene expression, resulting in urothelial differentiation. Nevertheless, our findings are wholly consistent with this being a PPARγ-mediated response, as *uroplakin* gene expression was induced by two independent well-characterised PPARγ agonists and it is blocked by a specific PPARγ antagonist. In effect, the involvement of PPARγ signalling in urothelial cytodifferentiation has similarities to its role in adipocytes, where receptor activation initiates a programme of transcriptional changes leading directly or indirectly (via other transcription factors) to the expression of the mature adipocyte phenotype (Stephens et al., 1999; Subbaramaiah et al., 2001), and thus accounts for an apparent delayed response in the expression of some genes implicated in the differentiation programme.

TZ and RZ are synthetic compounds from the thiazolidinedione class of drugs. Both have been well characterised in terms of their binding specificities and affinities for PPARγ (Willson et al., 2000) and both compounds invoked the same response in terms of inducing *UPII* gene expression. However, our results suggest that TZ was more active than RZ at inducing *uroplakin* gene expression in NHU cells, whereas IC_{50} data from transactivation studies implies that RZ is

Fig. 5. (A) Effect of PD153035 and TZ on the localisation of PPARγ. NHU cells were seeded at 2×10^5 cells/ml onto glass slides, allowed to attach and treated for 4 hours with or without PD153035 (1 μM) in the presence or absence of TZ (1 μM). The slides were fixed and immunofluorescence was performed for PPARγ, with nuclei counterstained using Hoechst 33258. Bar, 100 μm. Western blot analysis was used to show the effect of EGFR inhibition on the phosphorylation of ERK (B) or PPARγ (C). NHU cells were treated with (+) or without (–) PD153035 (1 μM) for 4 hours. (B) Protein lysate (40 μg) from each sample was used to analyse phospho- and total ERK, as described in Materials and Methods. The data are representative of three separate experiments. (C) Protein lysate (200 μg) was used to immunoprecipitate with PPARγ-agarose conjugate (10 μg), as outlined in the Materials and Methods, before being resolved on an 10% SDS-PAGE and transferred to nitrocellulose; phospho-serine or PPARγ was detected using specific antibodies and enhanced chemiluminescence.

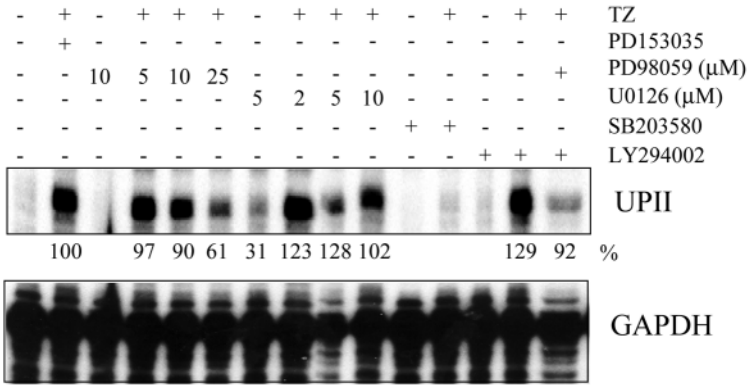


Fig. 6. Effect of kinase inhibitors on *UPII* mRNA expression. NHU cells were pretreated for 1 hour with an inhibitor: PD153035 (1 μM), PD98059 (5, 10, 25 μM), U0126 (2, 5, 10 μM), SB203580 (10 μM) or LY294002 (1 μM) and then for a further 24 hours with or without TZ (1 μM) and inhibitors, as indicated. The medium was replaced with inhibitors alone and replenished every 2 days. After 4 days, total RNA was extracted and 5 μg were analysed by RPA to assess relative *UPII* mRNA expression, as described in Fig. 2. The data are representative of three independent experiments. TZ+PD153035 was taken to be 100%.

tenfold more potent than TZ (Willson et al., 2000). Similar inconsistencies have been noted in other cell systems (Davies et al., 2002; Knock et al., 1999; Walker et al., 1998), and this reflects that fact that the degree of PPARγ activation by TZ and RZ is highly dependent on the experimental setting, possibly reflecting variations in the recruitment of specific cofactors to interact with activated PPAR receptors in different cell types (Camp et al., 2000). As pharmacological PPARγ ligands had such a specific effect on urothelial cytodifferentiation, it is interesting to speculate how this might relate to the situation in vivo. It has been shown that candidates for natural PPARγ ligands include the products of cyclooxygenase and lipoxygenase metabolism of long chain fatty acids, such as prostaglandins. The superficial cells of urothelium, by virtue of being bathed in urine, are directly exposed to urinary prostaglandins (Thevenon et al., 2001), and hence this may be a mechanism for inducing urothelial differentiation in superficially positioned cells. Such a hypothesis is compelling, as it would provide a mechanism for differentiation and development of urinary barrier function in intermediate urothelial cells exposed to urine following exfoliation of superficial cells.

Inactivation of EGFR signalling by specific inhibition of the EGFR receptor resulted in a far more consistent and positive PPARγ-mediated effect on uroplakin gene expression in NHU cells than just omitting exogenous EGF from the medium. This implies that urothelial cells show autocrine stimulation of the EGFR and is supported by the demonstration that urothelial cells can produce EGFR ligands, including HB-EGF (Freeman et al., 1997). Inhibition of the EGFR pathway, in the absence of PPARγ activation, had no effect on *uroplakin* gene expression, implying that inhibition of the EGFR pathway is required, but not sufficient, for urothelial cytodifferentiation.

We have shown that direct blocking of EGFR tyrosine kinase activity can be substituted by the inhibition of downstream signalling through the mitogen-activated protein kinase pathway via ERK1 and ERK2 or phosphoinositide 3-kinase. No additive effect was found from the simultaneous inhibition of both pathways, suggesting a common target. PPARγ has been shown to be phosphorylated on Ser84 by ERK2 and other MAP kinases (Adams et al., 1997), resulting in inhibition of PPARγ transcriptional activity (Camp and Tafuri, 1997). This is in keeping with our findings that blocking the EGFR signalling pathway resulted in the dephosphorylation of PPARγ and its translocation to the nucleus.

Unlike epithelia with high turnover rates and renewal from defined proliferative cell pools, such as the epidermis and intestinal epithelium, the terminally differentiated superficial cell of urothelium is long lived, which is consistent with its primary role of providing a robust urinary barrier. As a consequence, the urothelium has no constitutive cell turnover programme and is generally regarded as quiescent, with proliferation occurring in response to injury, damage or infection (Hicks, 1975). Thus, urothelium shows a responsive, rather than constitutive, programme of cell renewal. Where present, mitotic figures have been observed in all cell layers, including superficial cells (Hicks, 1975), implying that there is no irreversible distinction between renewal and differentiated cell pools. On the basis of our observations, we suggest that a switch between proliferation and differentiation exists in urothelial cells regulated at the level of the EGFR, with activation of the signalling pathway triggering proliferation at the expense of differentiation.

In common with *UPII* and *UPIa* genes, expression of UPIb mRNA was upregulated through an EGFR-inhibited PPARγ-mediated pathway, but unlike *UPII* and *UPIa*, NHU cells showed a basal expression of *UPIb* mRNA that was independent of EGFR activity. In situ, the pattern of *UPIb* gene expression also differs significantly from the other uroplakins: in normal urothelium, expression of *UPIb* mRNA is not confined to superficial cells, and is overexpressed in 50% of superficial and invasive transitional cell carcinomas (TCCs) (Lobban et al., 1998; Olsburgh et al., 2003). These observations, which lend further support to *UPIb* gene expression being expressed independently of EGFR status, are intriguing, given the putative role of UPIb in the AUM plaque. They suggest that UPIb may have a further cytoregulatory function, perhaps akin to other tetraspanin family members, in facilitating specific interactions between integrins and other cell surface molecules.

In conclusion, PPARγ activation induces changes in urothelial cells that lead to the expression of specific markers of terminal urothelial differentiation. The data indicate an intimate balance in favour of proliferation over differentiation, regulated at the level of an autocrine-regulated EGFR signalling pathway. In adipogenesis, where preadipocyte cell lines such as 3T3-L1 and F-442A have been invaluable for elucidating mechanisms of adipocyte differentiation, it is now evident that the differentiation programme is controlled through the coordinate regulation and activation of CCAAT enhancer binding protein (C/EBPα) and PPARγ transcription factors (Shao and Lazar, 1997). Our study implicates PPARγ

signalling in urothelial cytodifferentiation, but there are clearly unanswered questions with respect to the precise mechanisms. Normal human urothelium provides a robust cell culture model with objective indicators of terminal urothelial cytodifferentiation that will provide the basis for further study.

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