PPARα and PPARγ are co-expressed, functional and show positive interactions in the rat urinary bladder urothelium

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INTRODUCTION

PPARs (peroxisome proliferator-activated receptors) belong to the nuclear hormone receptor family of transcription factors (Michalik et al., 2006). Three PPARs, produced by three different genes, have been identified: PPAR\(\alpha\), PPAR\(\beta/\delta\) and PPAR\(\gamma\) (Dreyer et al., 1992). PPAR\(\alpha\) and PPAR\(\gamma\) exhibit largely non-overlapping expression patterns: PPAR\(\alpha\) expression is highest in liver, heart, kidney and skeletal muscle (Braissant et al., 1996; Guan et al., 1997; Michalik et al., 2006). PPAR\(\gamma\) expression is highest in adipose tissue, colon, leucocytes and retina (Braissant et al., 1996; Guan et al., 1997; Michalik et al., 2006). PPAR\(\alpha\), also known as PPAR\(\beta/\delta\), is more ubiquitously expressed (Braissant et al., 1996; Escher et al., 2001; Guan et al., 1997; Michalik et al., 2006).

Because of their significance in metabolic regulation, PPARs are important drug targets in, for example, type II diabetes and metabolic syndrome (Berger et al., 2005). Selective PPAR\(\alpha\) agonists (fibrates) normalize blood lipids, and selective PPAR\(\gamma\) agonists (thiazolidinediones) are used as insulin sensitizers (Michalik et al., 2006). Because of the beneficial effects of selective PPAR\(\alpha\) and PPAR\(\gamma\) agonists, dual-acting PPAR\(\alpha + \gamma\) agonists have also been developed, which combine agonist activity for both the PPAR\(\alpha\) and PPAR\(\gamma\) (Brand et al., 2003; Fiévet et al., 2006; Reiffel-Miller et al., 2005). However, the preclinical development of dual-acting PPAR\(\alpha + \gamma\) agonists has been made difficult by carcinogenic effects in rodents, in particular affecting the epithelial lining of the urinary bladder (the urothelium) (Oleksiewicz et al., 2008).

For some dual-acting PPAR agonists, the rat urothelial carcinogenicity has been suggested to be receptor-mediated, i.e. due to exaggerated pharmacology (Oleksiewicz et al., 2008). The urothelium is relatively unique in co-expressing PPAR\(\alpha\) and PPAR\(\gamma\) (Chopra et al., 2008; Guan et al., 1997). However, PPAR\(\alpha\) and PPAR\(\gamma\) expression in bladder urothelium at the protein level has never been examined by quantitative methods; i.e. it is essentially unknown whether PPAR\(\alpha\) and PPAR\(\gamma\) co-expression in the urothelium is of sufficient magnitude for functionality including involvement in carcinogenicity to be plausible. Furthermore, because selective PPAR\(\alpha\) and PPAR\(\gamma\) agonists appear to exhibit a lower propensity for causing bladder cancer in rats than do dual-acting PPAR\(\alpha + \gamma\) agonists (Oleksiewicz et al., 2008), and because...
the selective PPARγ agonist rosiglitazone promotes rather than initiates bladder cancer in rats (Lubet et al., 2008), it appears plausible that the bladder carcinogenic effect of dual-acting PPARα + γ agonists might involve crosstalk between PPARα and PPARγ signalling in the urothelium (Oleksiewicz et al., 2008). It is currently unknown whether functional interactions occur between PPARα and PPARγ in cells coexpressing these two nuclear receptors. To explore these issues, we have in this study compared PPARα and PPARγ expression levels between rat bladder urothelium, and a panel of rat tissue where carcinogenicity by dual-acting PPARα + γ agonists is less common or does not occur (kidney cortex, kidney medulla, kidney papilla, liver, heart, white fat and red skeletal muscle). PPARα and PPARγ functionality and crosstalk were tested by treating rats with specific PPARα and PPARγ agonists, alone or in combination. The main endpoint was expression of the transcription factor Egr-1, which was previously suggested as a candidate biomarker for the carcinogenic effect of dual-acting PPAR agonists in the rat bladder urothelium (Oleksiewicz et al., 2008).

MATERIALS AND METHODS

PPARα and PPARγ Agonist Formulations

Fenofibrate (PPARα agonist, Sigma-Aldrich) and rosiglitazone (PPARγ agonist, kindly supplied by Per Sauerberg and Erik Max Wulff, Novo Nordisk A/S) dry substances were used to make separate suspensions of fenofibrate (40 mg ml−1) and rosiglitazone (1.6 mg ml−1) in a vehicle consisting of 0.2% w/w sodium carboxymethyl cellulose, 0.4% w/w Tween 80 and 0.8% w/w sodium chloride, by sonication and vigorous magnet stirring (concentrations and doses refer to free acid/base).

The suspensions were stored protected from light at 2–8 °C and used within 7 days. Before dosing the suspensions were magnet stirred vigorously, for at least 10 min, and maintained on the stirrer while animals were dosed. A daily record of the weight of each formulation before and after dosing was main-
tained, to verify that the dosages had been administered correctly.

Oral Treatment of Rats with PPARα and PPARγ Agonists

Male Sprague–Dawley rats, 6 weeks, old, were obtained from Taconic Europe (Denmark), and acclimatized for 11 days before the start of the studies. The rats were housed on a 12:12 h light–dark cycle, in transparent type IV macrolone cages (floor area 1800 cm2, height 31 cm) with two to four animals in each cage. The cages were bedded with aspen wood shavings, and contained wooden blocks (Finn Tapei Oy, Kortteinen, Finland), paper straw material (Enviro-Dri® nesting, Lilllico, Betchworth, UK) and plastic shelters (Bachvent, Seborg, Denmark). Food pellets (complete pelleted rodent diet, Altromin 1320, order 1324, Brogården, Harsholm, Denmark) and water (automated water system) were available ad libitum. Twice weekly the animals were offered a small amount of cereal grain and maize. The animal room temperature was maintained at 18–24 °C, the relative air humidity at 30–70%, and the air change at 8–15 times h−1.

Animals were randomized and allocated to one of eight test groups, as shown.

<table>
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<tr>
<th>Test group</th>
<th>Group size</th>
<th>Dose level (mg kg−1 day−1)</th>
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Rats were randomized and allocated to one of eight test groups, as shown. n.a., Not applicable.

Comparison of PPARα and PPARγ Expression Between Bladder Urothelium and Kidney Cortex, Medulla and Papilla in Male and Female Rats

Five female and five male Sprague–Dawley rats, 6 weeks old, were obtained from Taconic Europe (Denmark), and acclimatized for 7 days. The rats were housed as described above. Lysates of the bladder urothelium, kidney cortex, kidney medulla and kidney papilla for western blotting were prepared as described below.

In Situ Lysis of the Bladder Urothelium for Western Blotting

After 7 days of treatment, animals were anesthetized with isoflurane–N2O (0.7 l min−1)–O2 (0.3 l min−1) with 5% isoflurane to deep anesthesia, thereafter reduced to 2% to maintain stable anesthesia. With animals in deep anesthesia, the bladder urothelium was lysed in situ by injecting 0.5 ml of a strongly denaturing guanidine isothiocyanate solution into the bladder lumen, as described previously (Egerod et al., 2005). The anesthetized animals were then euthanized by exsanguination.
Lysis of Tissue Samples for Western Blotting:

Heart and liver were removed immediately from the euthanized animals, and the weight of the organs was recorded. The apex of the heart (approximately 3 × 3 × 3 mm) and a 3 × 3 × 3 mm piece of the left liver lobe were homogenized in 1500 μl polyporopylene tubes (Sample Grinding kit, Amersham Biosciences), in approximately 1 ml of a denaturing and reducing loading buffer for SDS-PAGE, consisting of 2× LDS sample buffer (Invitrogen, California, USA), 2× reducing agent (Invitrogen), 1:200 vol. protease inhibitors (cocktail III, Calbiochem, Damstadt, Germany), 1:100 vol. phosphatase inhibitors (cocktail II, Calbiochem) and 10 mM EDTA. The LDS buffer was proprietary, but contained bromophenol blue tracking dye, density agent and high detergent levels, probably SDS.

Mesenterial white fat from the abdomen and the superficial (red) part of the gastrocnemius muscle from the right hind leg were homogenized likewise, in denaturing and reducing loading buffer for SDS-PAGE.

Approximately 3 mm-thick slices were prepared of the right kidney by two parallel cuts from pole to pole, along the large curvature. From these slices, samples of the cortex and medulla, as well as the whole papilla, were manually dissected, and homogenized in denaturing and reducing loading buffer for SDS-PAGE.

Tissue lysates were stored at −20 °C.

Antibodies

Alpha-tubulin mouse monoclonal antibody (Sigma, Missouri, USA, catalog number T 6199) raised against purified chick brain tubulin was used at a dilution of 1:6000.

Beta-actin mouse monoclonal antibody (Abcam, Cambridge, UK, catalog number ab6276) raised against a synthetic peptide corresponding to amino acids 1–14 of Xenopus laevis Actin was used at a dilution of 1:80000.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Abcam, Cambridge, UK, catalog number ab8245) raised against rabbit muscle GAPDH was used at a dilution of 1:100000.

Egr-1 rabbit affinity purified polyclonal immunoglobulin (Santa Cruz Biotechnology, California, USA, catalog number sc-19, sc-189) raised against a peptide mapping at the carboxy terminus of human Egr-1 was used at a dilution of 1:1000.

PMP70 (peroxisomal membrane marker) rabbit polyclonal immunoglobulin (Abcam, Cambridge, UK, catalog number ab3421), raised against a synthetic peptide corresponding to amino acid residues 644–659 from rat PMP70 was used at a dilution of 1:5000.

PPARα rabbit affinity purified anti-peptide immunoglobulin (Affinity Bioreagents, catalog number PA1–822) raised against a synthetic peptide corresponding to amino acid residues 1–18 from murine PPARα was used at a dilution of 1:1000.

PPARγ rabbit monoclonal antibody (Cell Signaling Technology, California, USA, catalog number 2443), raised against a synthetic peptide derived from the sequence around His494 of human PPARγ was used at a dilution of 1:1000.

HRP-conjugated goat anti-mouse IgG (Cell Signaling Technology, catalog number 7076) was used at a dilution of 1:10000. HRP-conjugated horse anti-rabbit IgG (Cell Signaling Technology, catalog number 7074) was used at a dilution of 1:10000.

Western Blotting

The urothelial lysates in guanidine isothiocyanate were dialyzed against 8 M urea, 20 mM Tris (pH 8.0), 5 mM EDTA, 1:200 vol. protease inhibitors (cocktail III, Calbiochem, Damstadt, Germany, catalog number 539134), 1:100 vol. phosphatase inhibitors (cocktail II, Calbiochem, catalog number 524625), as previously described (Egerod et al., 2005).

Western blotting is in our opinion the current gold standard for detection and quantitation of Egr-1 protein in rat organ lysates, and probably the only technique where comparison of Egr-1 protein levels between different rat organs can credibly be attempted, as done in this study. Unfortunately, because of the size of the gradient SDS-PAGE gels employed in our study (17 wells), and the requirement that for most precise quantitation, all samples between which comparison is done should be on a single gel (samples cannot be distributed on individual gels due to e.g. variability in protein transfer), it was not possible in this study to analyze individual samples. Instead, samples were pooled and quantitation based on three to five independent western blot experiments (three to five independent technical assay replicates), as described below and in the figure legends. Analyzing pooled samples is expected to be equivalent to analyzing samples from individual animals and this was supported by a study where samples from individual animals were examined by western blotting as well as immunohistochemistry (Egerod et al., submitted).

For the rat experiment comprising oral treatment with PPAR agonists for 7 days, urothelial lysates were pooled for each treatment group, adjusting the volume used from each animal to provide the same total protein contribution to the pool, and finally adjusting the pool volumes to obtain identical protein concentrations in all pools (1.3 mg ml⁻¹). Thus, one vehicle pool comprising 10 animals and seven treatment pools each comprising five animals were made (Table 1). The heart and liver lysates were likewise pooled into eight heart and eight liver lysate pools. Thus, the whole animal experiment, consisting of 45 animals, was reduced to eight urothelial lysate pools, eight liver lysate pools, and eight heart lysate pools. In preliminary experiments, the protein content in all 3 × 8 = 24 organ pools was comprehensively examined and compared by a combination of methods: NanoOrange staining combined with dilution to reduce detergent levels (NanoOrange® Protein Quantification kit, Molecular Probes, Oregon, USA), SDS-PAGE and Coomassie staining (see below), amido black dot blotting (see below) and western blotting with a cocktail of α-Tubulin, β-Actin and GAPDH antibodies.

For the comparison of PPARα and PPARγ expression between bladder urothelium and kidney cortex, medulla and papilla in male and female rats, urothelial lysate, kidney papilla lysate, kidney medulla lysate and kidney cortex lysate were pooled for the five males and five females; i.e. four lysate pools were made from males and four lysate pools were made from females. Pools were made and protein content between the eight lysate pools was compared as described above.

Fully denaturing and reducing SDS-PAGE of lysate pools on precast 4–12% gradient gels, electrotransfer of proteins to 0.45 μm PVDF membranes and western blotting of PVDF membranes was done as previously described (Egerod et al., 2005).

For quantitation of PPAR agonist-induced Egr-1 expression, five replicate western blots were done of the same lysates. For
quantitation of PPAR and endogenous Egr-1 expression, three replicate western blots were done of the same lysates.

Western blots were developed using ECL Advance chemiluminescent substrate (GE Healthcare, New Jersey, USA) and a LAS3000 CCD camera (Fujifilm). Exposures were done in the quantitative (non-saturated) range of the CCD camera, as recommended by the manufacturer. Quantitative image analysis was done with MultiGauge software (v2.3, Fujifilm) (Egerod et al., 2005).

Finally, membranes were stripped (Restore Western blot stripping buffer, Pierce) for 1 h at 37 °C (Pierce) and reprobed with a load control cocktail of three antibodies (α-tubulin, β-actin and GAPDH).

**Coomassie Staining**

SDS-PAGE of tissue lysate pools was done on precast 4–12% gradient gels, according to the manufacturer’s instructions (NuPAGE™ gels, Invitrogen, California, USA). The gels were incubated for 20 min in 50% methanol, 7% acetic acid, then washed for 3 × 5 min in water, stained with GelCode Coomassie blue (Pierce) for 2 h and destained in water for 1 h before scanning on a standard Hewlett Packard flatbed scanner (HP Scanjet 7400C).

**Protein Quantitation by Amido Black Nitrocellulose Dot Blot Assay**

Determination of total protein concentration in urothelial lysates (in guanidine isothiocyanate, GuSCN) and in liver, heart, fat and muscle lysates (in denaturing and reducing loading buffer for SDS-PAGE) was performed using amido black (Dieckmann-Schuppert and Schnittler, 1997; Henkel and Bieger, 1994). Protein samples were applied as 2 μl spots onto nitrocellulose filters (Hybond-C Extra, Amersham). Standards of known protein concentration were prepared by serial dilutions of BSA, spanning 5 mg ml⁻¹ through 0.19 mg ml⁻¹, in GuSCN lysis buffer and in denaturing and reducing loading buffer for SDS-PAGE sample buffer. BSA diluted in these two buffers produced linear standard curves (r² = 0.999) that were parallel. Thus, the amido black method was highly tolerant to the type of buffer the protein was diluted in, and allowed comparison of protein concentration between rat tissue lysates prepared in SDS-PAGE sample buffer and guanidine isothiocyanate solution, as expected (Dieckmann-Schuppert and Schnittler, 1997; Henkel and Bieger, 1994).

**RESULTS**

**Effect of PPARα and PPARγ Agonists on Organ and Body Weights**

Body weights were obtained at the first and last days of dosing. As expected for young rats, all groups exhibited increases in body weight during the experiment, but no significant differences were seen in bodyweight gains between vehicle and treated groups (not shown).

Neither rosiglitazone nor fenofibrate nor the combination treatments significantly affected heart weights, albeit small, non-significant increases in heart weights were seen in both rosiglitazone dose groups, and the low-dose fenofibrate group (not shown). Likewise, rosiglitazone did not affect liver weights (Fig. 1). In contrast, animals treated with the PPARα agonist fenofibrate, either alone or in combination with rosiglitazone, exhibit-
ated increased liver weights compared with the vehicle group (Fig. 1, see also discussion). Maximal increase in relative liver weight was seen with 50 mg kg

−

1 fenofibrate, and liver weights were not further increased in animals receiving 200 mg kg

−

1 fenofibrate. Finally, coadministration of rosiglitazone appeared to counteract the fenofibrate-induced increase in liver weights (Fig. 1, compare the 50 mg kg

−

1 fenofibrate and 50 mg kg

−

1 fenofibrate + 20 mg kg

−

1 rosiglitazone groups, P = 0.0317 and 0.0079 for absolute and relative liver weights, respectively, Mann–Whitney test).

Urinary Bladder Urothelium Coexpresses PPARα and PPARγ, at Levels above those Found in Liver, Heart, Fat and Skeletal Muscle

Lysates of bladder urothelium, liver, heart, fat and muscle were examined for PPARα and PPARγ expression by western blotting (Fig. 2A). Comparison of PPAR expression levels between tissues was complicated by several factors. First, some tissue such as fat and skeletal muscle yielded low protein levels, estimated by Coomassie staining (Fig. 2B). To improve the sensitivity of PPAR detection, we loaded maximal amount of protein for each tissue type. Consequently, there was up to a 4-fold difference in total protein load applied to the SDS-PAGE gel between tissues (Fig. 2B and C). Second, housekeeping proteins commonly used for normalization, such as α-tubulin, β-actin and GAPDH, exhibited tissue-specific expression (Fig. 2A, bottom panel). Therefore, α-tubulin, β-actin and GAPDH were primarily used to verify equal loading for the same type of tissue (Fig. 2A, bottom panel, compare for example three lanes with bladder lysate). Finally, tissue lysates were prepared in sample buffers containing high detergent levels. Therefore, protein loading was determined by an amido black dot blot assay which is tolerant to detergent and Coomassie-stained SDS-PAGE gels were used as extra control (Fig. 2B and C).

Highest PPARα levels were detected in bladder urothelium, liver, kidney and heart, while fat and muscle did not exhibit PPARα bands (Figs 2A and 3A, top panel, angled arrowheads in Fig. 2A). In the liver as well as kidney, two bands were seen with the PPARα antibody (Figs 2A and 3A, top panels). The affinity-purified anti-PPARα antibody, directed against the 18 first N-terminal amino acids of PPARα, is known to produce a doublet band (abcam technical information). Also, others have reported doublet bands from rat liver with antibodies against the first 101 N-terminal amino acids of PPARα (Lemberger et al., 1996). In bladder urothelium and heart, only one band was seen with the PPARα antibody, which comigrated with the quicker-migrating band in liver and kidney (Figs 2A and 3A, top panels). Therefore, the quicker-migrating, bottom band was used for PPARα expression comparison between tissue (Fig. 2A, top panel, angled arrows). It is possible that the slower-migrating band represented, for example, phosphorylation of PPARα (Passilly et al., 1999; Shalev et al., 1996), i.e. this PPARα quantitation strategy might have underestimated PPARα expression in the liver and kidney by approximately 2-fold. Highest PPARγ levels were detected in bladder urothelium, heart, fat and muscle, while the liver did not exhibit a PPARγ band (Fig. 2A, middle panel, angled arrowheads).

For any given tissue type, PPARα and PPARγ levels differed minimally between vehicle-treated animals and animals

Figure 2. PPARα and PPARγ expression in rat bladder urothelium, liver, heart, fat and muscle. Numbers above lanes: treatment groups. 1, Vehicle; 4, fenofibrate (200 mg kg

−

1 per day); 6, rosiglitazone 1 fenofibrate (8

−

200 mg kg

−

1 per day). Bladder: selectively lysed bladder urothelium. Liver, lysate of whole liver. Heart, lysate of heart apex. Fat, lysate of white mesenterial fat. Muscle, lysate of red, outer, part of gastrocnemius muscle (skeletal muscle). All lysates comprised pools of five rats (treatment groups 4 and 6) or 10 rats (treatment group 1). (A) Western blots. PPARα and PPARγ band location are indicated by arrows. Bottom panel, Western blot with a cocktail of monoclonal antibodies against α-tubulin, β-actin and GAPDH, to compare protein loading. (B) Coomassie-stained SDS-PAGE gel, using the same loading (μl lysate per well) as in panel (A). (C) Total protein load per lane in the western blots (panel A) and Coomassie gel (panel B), determined by amido black dot blot assay.
receiving fenofibrate or the fenofibrate + rosiglitazone combination (Fig. 2A, compare for example PPARα and PPARγ bands between the three lanes with heart lysate). Any effect of the PPAR agonists on PPAR expression levels was not investigated further in this work.

When band intensity (Fig. 2A) was normalized to protein content determined by the amido black dot blot assay (Fig. 2C), the bladder urothelium was found to express approximately 11-fold higher levels of PPARα than the liver, which is generally held to have high PPARα expression (Fig. 2, not shown in detail). Also, bladder urothelium expressed approximately 12-fold higher levels of PPARγ than fat, the archetypal PPARγ-regulated tissue (Fig. 2, not shown in detail). Urothelium and heart were the only tissues to co-express PPARα and PPARγ, and urothelium was unique in the high level of coexpressed PPARα and PPARγ (Fig. 2A and B, compare bladder and heart).

Finally, it should be mentioned that the western blotting technique is very well suited to comparison of PPAR protein levels between various tissues (Fig. 2), but due to limited sensitivity, the western blotting results in Fig. 2 do not contradict PPARα expression in skeletal muscle (Braissant et al., 1996; Escher et al., 2001; Guan et al., 1997), nor rule out PPARγ expression in liver.

Inverse Gradients of PPARα and PPARγ Expression through the Kidney, but Unique and Sex-specific PPARγ/PPARα Ratio in the Urothelium

In the kidney, PPARα expression increased from the papilla to the medulla and cortex (Fig. 3A). In contrast, PPARγ expression decreased from the papilla, through the medulla, to the cortex (Fig. 3A). Similarly to the liver (Fig. 2A), the PPARα antibody produced double bands on kidney lysates (Fig. 3A).

Co-expression of PPARα and PPARγ was thus found in all kidney samples (Fig. 3A). As the kidney cortex, medulla and pelvis lysates represented homogenized samples of whole tissue (see methods), it could not be determined whether the co-expression of PPARα and PPARγ occurred at the single-cell level. In contrast, because bladder urothelium was selectively lysed (see Materials and Methods), the results in Figs 3A and 2A unequivocally showed co-expression of high levels of PPARα and PPARγ by a single cell type, the bladder urothelium. These results are in agreement with a recent immunohistochemical study (Chopra et al., 2008).

Because of these inverse gradients of PPARα and PPARγ expression through the kidney, the ratio of co-expression of PPARα and

Figure 3. Comparison of PPARα and PPARγ expression at different sites in urogenital tract, and between male and female rats. Bladder, selectively lysed bladder urothelium. Cortex, lysate of dissected kidney cortex. Medulla, lysate of dissected kidney medulla. Papilla, lysate of dissected kidney papilla. M, male rats. F, female rats. The rats were not treated with PPAR agonists, and all lysates comprised pools of five rats. (A) representative western blot. PPAR bands are indicated by arrows. All lanes were loaded with 3 μg total protein, determined by amido black dot blot assay. Bottom panel, western blot with a cocktail of monoclonal antibodies against α-tubulin, β-actin and GAPDH, to compare protein loading. (B) Quantitative analysis of PPARα and PPARγ expression in bladder urothelium, mean of three western blots. PPARα and PPARγ band intensities were quantitated using MultiGauge software, and normalized to actin band intensities. ***P = 0.03 compared with PPARγ/PPARα ratio in male rats, Student’s t-test.
PPARγ differed between kidney cortex, medulla and papilla (Fig. 3A). In the papilla, the PPARγ/PPARα ratio was higher than in the cortex and medulla, and similar to the bladder urothelium (Fig. 3A, not shown in detail). This suggested that the kidney pelvis urothelium might be a major contributor to the PPARα and PPARγ expression seen in the kidney papilla.

Finally, the PPARα and PPARγ expression levels in kidney cortex and medulla appeared similar between male and female rats (Fig. 3A, not shown in detail). In contrast, in the bladder urothelium, females exhibited lower PPARα expression and higher PPARγ expression levels than males (Fig. 3B), leading to significantly higher PPARγ/PPARα ratios in the urothelium of female rats than in the urothelium of males (Fig. 3B).

Endogenous Levels of the Egr-1 Transcription Factor in Rat Urothelium, Liver and Heart

Quantitation of expression of the zinc finger transcription factor Egr-1 (synonyms Zif268, NGFI-A, TIS8, Krox-24; Christy et al., 1988; Lemaire et al., 1988; Lim et al., 1987; Milbrandt 1987) was done by chemiluminescent western blotting, using the commercially available C-19 antipeptide rabbit immunoglobulin (see Materials and Methods). In preliminary experiments, six different commercially available Egr-1 antibodies were tested in western blotting on rat mammary gland adenocarcinoma cells (SMT/2A LNM) and human breast adenocarcinoma cells (MCF-7) stimulated with phorbol myristate acetate to induce Egr-1 expression, as well as liver lysates from rats treated with PPAR agonists to induce Egr-1 expression. The C-19 antipeptide rabbit immunoglobulin proved the best reagent for western blot analysis of Egr-1 expression. C-19 provided clear bands of the expected size in human as well as rat cell lines and rat organ lysates (Fig. 4A). The C-19 immunoreactivity could be abolished by preadsorption with immunogen peptide (not shown). However, as we found extensive lot variation for C-19, all experiments were done with a single, pre-verified C-19 lot. Furthermore, the Egr-1 expression data reported in this study was verified by immunohistochemistry, using a different and monoclonal Egr-1 antibody (FLE, in preparation).

Egr-1 has previously been suggested as an early biomarker for the carcinogenic effect of dual-acting PPARα + γ agonists in the rat urothelium (Egerod et al., 2005; Oleksiewicz et al., 2008), but endogenous Egr-1 levels in the urothelium have not been compared with levels in other rat tissue, where dual-acting PPARα + γ agonists do not exhibit carcinogenic effects. In the liver of healthy young rats not exposed to PPAR agonists, we found that Egr-1 was highly expressed (Fig. 4B and C), with approximately 14-fold lower levels in the urothelium, and approximately 60-fold lower levels in the heart (Fig. 4B and C).

High-level Induction of Egr-1 Occurs in the Liver by PPARα but not PPARγ Agonists, and is not Recapitulated in other PPARα Expressing Tissues

The specific PPARα agonist fenofibrate induced significant Egr-1 expression in the liver and heart (Figs 6A and 7A, P < 0.001). While 50 mg kg⁻¹ fenofibrate caused maximal Egr-1 induction in the liver (Fig. 6D), Egr-1 induction in the heart by 50 mg kg⁻¹ fenofibrate was not significant (Fig. 7C). Also, fold-induction levels of Egr-1 by fenofibrate were above 10-fold in the liver (Fig. 6D), but only 2-fold in the heart (Fig. 7C). Thus, liver was more sensitive than heart to Egr-1 induction by fenofibrate (Figs 6D and 7C). This was in agreement with higher PPARα expression in the liver (Fig. 2A). In the urinary bladder urothelium, mild Egr-1 induction by fenofibrate was also apparent, albeit this did not reach statistical significance (Fig. 5A).

The PPARγ agonist rosiglitazone did not induce Egr-1 expression in liver (Fig. 6A) or heart (Fig. 7A). In bladder urothelium, mild
Egr-1 induction appeared to occur at 20 mg kg\(^{-1}\) but not 8 mg kg\(^{-1}\) rosiglitazone, albeit without statistical significance (Fig. 5A). Egr-1 induction could not be detected in fat and muscle lysates from treated or vehicle animals (not shown).

PPAR\(\alpha\) Agonist Treatment Induces the PMP70 Marker of Peroxisomal Proliferation in some (Liver, Urothelium) but not all (Heart) PPAR\(\alpha\)-expressing Tissue

The peroxisome membrane protein 70 (PMP70) has been described as a specific marker for PPAR\(\alpha\) activation and peroxisome proliferation (Colton et al., 2004; Yang et al., 2008). Accordingly, in the liver, maximal PMP70 induction was observed...
**Egr-1 Induction in Urinary Bladder Urothelium, but not Heart or Liver, through Positive Interaction between PPARα and PPARγ Agonists**

As mentioned above, in urinary bladder urothelium, treatment with fenofibrate or rosiglitazone alone induced only mild Egr-1 expression, which did not reach statistical significance (Fig. 5A and D). However, combination treatment with rosiglitazone and fenofibrate did induce significant Egr-1 expression (Fig. 5A and D, *P* < 0.001). The effect of the rosiglitazone and fenofibrate combination was significantly higher than the effect of either drug alone, i.e. rosiglitazone and fenofibrate exhibited synergy with regards to Egr-1 induction in the bladder urothelium (Fig. 5A and D). Compared with animals receiving a combination of 8 mg kg⁻¹ rosiglitazone and 200 mg kg⁻¹ fenofibrate, Egr-1 induction appeared slightly lessened by increasing rosiglitazone to 20 mg kg⁻¹ while maintaining fenofibrate at 200 mg kg⁻¹, or increasing rosiglitazone to 50 mg kg⁻¹ while reducing fenofibrate to 50 mg kg⁻¹ (Fig. 5A and D). In contrast to Egr-1, synergy between rosiglitazone and fenofibrate was not seen for PMP70 induction in the urinary bladder (Fig. 5B).

Finally, in contrast to the bladder, synergy between rosiglitazone and fenofibrate with regards to Egr-1 expression was not seen in the liver or heart (Figs 6 and 7).

**DISCUSSION**

We showed for the first time that PPARα and PPARγ expression levels in the rat urinary bladder urothelium are unexpectedly high (Figs 2 and 3). Urothelial expression of PPARγ was approximately 10-fold higher than in reference organs such as fat, heart and muscle (Fig. 2). Urothelial expression of PPARα was approximately 10-fold higher than in liver (Fig. 2), and comparable to kidney cortex and medulla (Fig. 3). Thus, the urothelium appeared unique in combining PPARα and PPARγ co-expression with high PPARα and PPARγ expression levels (Figs 2 and 3). We speculate that this could contribute to the apparent sensitivity of the rat urothelium to carcinogenesis by dual-acting PPARα + γ agonists (Oleksiewicz et al., 2008). Additionally, while the balance between PPARα and PPARγ expression in the kidney cortex and medulla was similar between male and female rats, it differed between sexes in the urothelium, with females exhibiting significantly higher PPARγ/PPARα ratios than males (Fig. 3). Thus, PPAR expression levels may also explain sex differences in susceptibility to the bladder-carcinogenic effects of dual-acting PPARα+ γ agonists in rats.

In similar vein, it should be mentioned that lysates of bladder urothelial cells were compared with organ lysates containing mixed cell populations from liver, heart, fat and skeletal muscle. Thus, while our study unequivocally showed that urothelial cells co-express PPARα and PPARγ, inferences about cellular coexpression of PPARα and PPARγ in liver, heart, fat and skeletal muscle cannot be made based on our study.

Crosstalk between PPARα and PPARγ in the rat bladder urothelium was explored by comparing rats treated with fenofibrate, rosiglitazone or fenofibrate + rosiglitazone combinations (Fig. 5). Rosiglitazone and fenofibrate are recognized model compounds for specific activation of PPARα and PPARγ, respectively, in rats as well as humans (Bajaj et al., 2007; De et al., 2007; Iglarz et al., 2003; Rose et al., 2007; Wagner et al., 2005). Furthermore, in both rats and humans, rosiglitazone and fenofibrate combination is a...
recognized approach for simultaneous activation of PPARα and PPARγ (Bajaj et al., 2007; De et al., 2007; Iglarz et al., 2003; Rose et al., 2007; Wagner et al., 2005). The doses in our study (Table 1) corresponded to high pharmacological doses (Besson et al., 2005; Chakrabarti et al., 2003; De Souza et al., 2006; De et al., 2007; Iglarz et al., 2003; Rose et al., 2007), to ensure saturation of PPARs across organ systems, and minimize the risk of drug distribution phenomena confounding comparison of PPAR activation effects between organs. Our main endpoint to evaluate PPAR crosstalk was the transcription factor Egr-1. Egr-1 was found to be induced at mRNA as well as protein level in the bladder urothelium of rats following ragaglitazar treatment, and was suggested to be a candidate biomarker for the carcinogenic effect of dual-acting PPAR agonists in the rat bladder urothelium (Egerod et al., 2005; Oleksiewicz et al., 2008).

In the urothelium, Egr-1 expression was not induced by 8 mg kg\(^{-1}\) rosiglitazone, but was mildly and nonsignificantly induced by 20 mg kg\(^{-1}\) rosiglitazone, as well as by fenofibrate at both dose levels (Fig. 5). Strikingly, in animals receiving a combination of 8 mg kg\(^{-1}\) rosiglitazone and 200 mg kg\(^{-1}\) fenofibrate, a strong Egr-1 expression was seen, significantly above the effect of either drug alone (Fig. 5). Thus, fenofibrate and rosiglitazone exhibited positive interaction (in this case synergy) as regards induction of Egr-1 expression in the rat bladder urothelium (Fig. 5). Some PPARα (Wy-14,643) as well as PPARγ agonists (troglitazone) are known to increase Egr-1 expression by nonspecific mechanisms not mediated by PPAR activation (Baek et al., 2003, 2004; Chintharlapalli et al., 2005; Pauley et al., 2002). We consider it highly unlikely that the induction of Egr-1 expression observed in the urothelium of animals co-administered rosiglitazone and fenofibrate represented unspecific (off target, PPAR-independent) effects of rosiglitazone and fenofibrate, for the following reasons: first, while troglitazone caused increased Egr-1 expression by PPARγ-independent mechanisms in HCT-116 human colon cancer cells, rosiglitazone did not (Baek et al., 2003). Second, in animals receiving 200 mg kg\(^{-1}\) fenofibrate, co-administration of 8 mg kg\(^{-1}\) rosiglitazone caused synergistic induction of Egr-1 in the urothelium (Fig. 5), but increasing rosiglitazone to 20 mg kg\(^{-1}\) did not increase, and in fact slightly decreased, Egr-1 induction (Fig. 5). This observed saturation at 8 mg kg\(^{-1}\) rosiglitazone would be expected for PPARγ-dependent effects, but not for nonspecific effects. Third, in those organs where fenofibrate caused significant Egr-1 induction on its own (Figs 6 and 7, heart and liver), the organ with high PPARγ expression (liver, Figs 2 and 6) exhibited higher fold-induction of Egr-1 in the urothelium (Fig. 5), but increasing rosiglitazone to 20 mg kg\(^{-1}\) did not increase, and in fact slightly decreased, Egr-1 induction (Fig. 5). This observed saturation at 8 mg kg\(^{-1}\) rosiglitazone would be expected for PPARγ-dependent effects, but not for nonspecific effects. Third, in those organs where fenofibrate caused significant Egr-1 induction on its own (Figs 6 and 7, heart and liver), the organ with high PPARγ expression (liver, Figs 2 and 6) exhibited higher fold-induction of Egr-1, and at lower fenofibrate doses, than the organ with low PPARγ expression (heart, Figs 2 and 7), supporting a specific, PPARγ-dependent effect of fenofibrate in the liver. Fourth, in tissue where co-expression of PPARα and PPARγ was detected (Fig. 2, urothelium and heart), synergy between fenofibrate and rosiglitazone was detected in the tissue with high PPARα and PPARγ expression (urothelium, Figs 2 and 5), but not the tissue with low PPARα and PPARγ expression (heart, Figs 2 and 7), arguing against the Egr-1 induction representing PPAR-independent effects of rosiglitazone and fenofibrate.

Finally, it should be mentioned that while a synergistic effect was seen in this study between fenofibrate and rosiglitazone in causing Egr-1 induction in the rat urothelium (Fig. 5), in other studies, the effect appeared additive (Egerod et al., 2005 and not shown). Thus, all available data supports a positive interaction between PPARα and PPARγ in terms of Egr-1 induction in the rat urothelium, with additive as well as synergistic effects being observed. The molecular mechanisms responsible for the positive interaction between PPARα and PPARγ signaling in the rat bladder urothelium remain to be determined. However, it has been proposed that crosstalk between nuclear transcription factor receptors such as the PPARs, the estrogen receptor and the thyroid hormone receptor is the rule rather than the exception, and in at least some cases, sharing of transcriptome protein binding partners or binding to the same DNA response sequence is involved (Carey 1998; Schumman et al., 1998).

Egr-1 induction by fenofibrate and rosiglitazone was not observed in fat and skeletal muscle (not shown), but did occur in urothelium, liver and heart (Figs 5–7). Liver, fat and urothelium, but not heart and skeletal muscle, are targets for the carcinogenic effects of specific PPARα agonists, specific PPARγ agonists, and dual-acting PPARα+γ agonists, respectively, in the rat (Oleksiewicz et al., 2008). Thus, Egr-1 induction was seen in two of three carcinogenicity target tissues, and one of two tissues where cancer is not expected in PPAR agonist-treated rats; i.e. Egr-1 expression per se is expected to be neither sufficient, nor required, for the carcinogenic effect of PPARα and γ agonists. This is fully in accordance with the known involvement of Egr-1 in normal physiological processes in for example the heart (Gupta et al., 1991; Jin et al., 2000; Shamim et al., 1999), as well as with the known cancer type-specificity of transcription factor upregulation; i.e. even though overexpression of a given transcription factor may be important for certain cancer forms in some organs, the same transcription factor is not thereby expected to be involved in all cancers forms across different organs. We found that in rats not exposed to PPAR agonists, endogenous Egr-1 expression was highest in liver, lower in urothelium, and lowest in heart (Fig. 4B and C). Thus, it seems plausible that the functional roles of Egr-1 may differ between liver, urothelium and heart. Also, compared with the vehicle group, PPAR agonist-induced Egr-1 expression was above 10-fold in liver and urothelium, but only 2-fold in the heart (Figs 5–7). Thus, endogenous Egr-1 expression levels, as well as the magnitude of Egr-1 induction following dual PPARα+γ activation, differentiated between carcinogenicity target tissue (liver, urothelium) and a tissue where PPAR agonist toxicity, but not cancer, is seen (heart) (Figs 5–7).

The PMP70 peroxisomal membrane protein is a marker for peroxisome proliferation in rats and primates (Colton et al., 2004; Kwanyuen et al., 2006; Wilcke and Alexson, 2001). Peroxisome proliferation and PMP70 expression is induced in the livers of rodents treated with PPARα agonists, and oxidative stress by peroxisomes has been suggested to be involved in the hepatocarcinogenesis by PPARα agonists in rodents (Yeldandi et al., 2000). In agreement with this, we observed upregulation of PMP70 expression in the liver of rats treated with 50 mg kg\(^{-1}\) fenofibrate (Fig. 6). Interestingly, PMP70 was also upregulated by fenofibrate in the urothelium, albeit this required a higher fenofibrate dose than PMP70 induction in the liver, 200 mg kg\(^{-1}\) (Figs 5 and 6). Similarly, peroxisomal β oxidation was found to be induced in the kidney and heart by the PPARα agonist ciprofibrate, but to a lesser extent than in the liver (Cook et al., 2000). To our knowledge, this is the first indication that PPARα activation may cause peroxisome proliferation in the rat urothelium. As PMP70 but not Egr-1 were induced by fenofibrate, this underscored that PPARα activation was not sufficient for Egr-1 induction in the urothelium (Fig. 5), and that Egr-1 induction in animals co-administered fenofibrate and rosiglitazone was a true positive crosstalk effect (Fig. 5), as discussed above. Also, these data open the possibility that oxidative stress may contribute to the carcinogenic effect of peroxisome activation.
dual-acting PPARα + γ agonists in the rat urothelium. This hypothesis is indirectly supported by the observation that male rats in some cases appeared more sensitive than females to the bladder carcinogenic effect of dual-acting PPARα + γ agonists (Lima et al., 2006), and male rats expressed significantly more PPARα (which mediates PMP70 induction) relative to PPARγ than did female rats (Fig. 4). Also, the relevance of PPARα activation for urothelial cancer initiation (by for example free radical production) is indirectly supported by the finding that rosiglitazone (which mediates PMP70 induction) relative to PPARγ agonist, supports cancer promotion rather than initiation (Lubet et al., 2008).

Interestingly, the magnitude of Egr-1 induction, and the presence of PMP70 induction, differed between urothelium, liver and heart of rats co-administered rosiglitazone and fenofibrate (Figs 5–7). Thus, PMP70 represents a new candidate biomarker for the carcinogenic effect of dual-acting PPARα + γ agonists in the rat urothelium, and the combination of the Egr-1 and PMP70 candidate biomarkers may improve the predictive value as regards carcinogenic effect of dual-acting PPARα + γ agonists in the rat urothelium.

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