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PPARα and PPARγ Coactivation Rapidly Induces Egr-1 in the Nuclei of the Dorsal and Ventral Urinary Bladder and Kidney Pelvis Urothelium of Rats

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Abstract

To facilitate studies of the rat bladder carcinogenicity of dual-acting PPARα+γ agonists, we previously identified the Egr-1 transcription factor as a candidate carcinogenicity biomarker and developed rat models based on coadministration of commercially available specific PPARα and PPARγ agonists. Immunohistochemistry for Egr-1 with a rabbit monoclonal antibody demonstrated that male vehicle-treated rats exhibited minimal urothelial expression and specifically, no nuclear signal. In contrast, Egr-1 was induced in the nuclei of bladder, as well as kidney pelvis, urothelia within one day (2 doses) of oral dosing of rats with a combination of 8 mg/kg rosiglitazone and 200 mg/kg fenofibrate (specific PPARγ and PPARα agonists, respectively). These findings were confirmed by Western blotting using a different Egr-1 antibody. Egr-1 was induced to similar levels in the dorsal and ventral bladder urothelium, arguing against involvement of urinary solids. Egr-1 induction sometimes occurred in a localized fashion, indicating physiological microheterogeneity in the urothelium. The rapid kinetics supported that Egr-1 induction occurred as a result of pharmacological activation of PPARα and PPARγ, which are coexpressed at high levels in the rat urothelium. Finally, our demonstration of a nuclear localization supports that the Egr-1 induced by PPARα and PPARγ coactivation in the rat urothelium may be biologically active.

Keywords: dual-acting PPAR agonists; fenofibrate; rosiglitazone; rat urinary bladder cancer; Egr-1; PPARα; PPARγ; kidney papilla; urothelium; receptor-mediated.

Introduction

Small-molecule agonists of the peroxisome proliferators–activated receptors α and γ (dual-acting PPAR agonists, here termed PPARα+γ agonists) are seen as promising oral agents for type 2 diabetes (Balakumar et al. 2007; Berger, Akiyama, and Meinke 2005; Fiévet, Fruchart, and Staels 2006). Unfortunately, many of these compounds caused cancer of the urinary bladder epithelium (urothelium) during preclinical testing in rats and mice (Balakumar et al. 2007; Dominick et al. 2006; Oleksiewicz et al. 2008; Rubenstrunk et al. 2007). For the PPARα+γ agonist muraglitazar, the urothelial cancers were suggested to result from the formation of uroliths (urinary stones) (Dominick et al. 2006; Van Vleet et al. 2007; Waites et al. 2007). By contrast, for the PPARα+γ agonists ragaglitazar and naveglitazar, evidence could not be found for a urolith-mediated mechanism (Lima et al. 2006; Long et al. 2008). Instead, a receptor-mediated carcinogenicity mechanism was suggested, in which the urothelial cancers are caused by exaggerated coactivation of PPARα and PPARγ (Egerod et al. 2005; Lima et al. 2006; Long et al. 2008; Oleksiewicz et al. 2005; Oleksiewicz et al. 2008), which uniquely are known to be coexpressed in the urothelium (Chopra et al. 2008; Guan et al. 1997; Jain et al. 1998).

Resolving the conflicting hypotheses for a bladder carcinogenicity mechanism for PPARα+γ agonists is of importance for interpreting the human relevance of the rodent findings, and for developing screening assays to allow preclinical development of this class of compounds. To facilitate the study and comparison of carcinogenicity mechanisms, we have described candidate biomarkers for the early effects of rodent carcinogenic PPARα+γ agonists in the rat urothelium (Egerod et al. 2005; Lima et al. 2006; Oleksiewicz et al. 2005, 2008). One of these candidate early biomarkers is the zinc finger transcription factor Egr-1 (early growth response factor 1, synonyms Zif268, NGFI-A, TIS8, Krox-24) (Christy, Lau, and Nathans 1988; Lemaire, Varnum, and Herschman 1988; Lim et al. 1987; Milbrandt 1987), which is induced by the rat bladder carcinogenic PPARα+γ agonist ragaglitazar in the rat urothelium within four days of oral treatment (Egerod et al. 2005). Egr-1 is closely related to the Wilm's tumor (WT) transcription factor, and Egr-1 overexpression has been associated with prostate as well as bladder cancer development in humans and rodents (Abdulkadir et al. 2001; Abdulkadir 2005; Ghanem et al. 2000; Lokeshwar et al. 2008; Ogishima, Shiina, Breault, Tabatabai et al. 2005; Ogishima, Shiina, Breault, Terashima

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et al. 2005; Oleksiewicz et al. 2008; Rauscher III et al. 1990; Scharnhorst et al. 2000). To further facilitate the study of the bladder carcinogenicity mechanisms of PPARα+γ agonists, we have implemented a model system in which rats are coadministered fenofibrate and rosiglitazone (commercially available specific activators of PPARα and PPARγ, respectively). In rats, oral coadministration of fenofibrate and rosiglitazone mimics the effect of oral administration of the carcinogenic PPARα+γ agonist ragaglitazar on the Egr-1 biomarker in the urinary bladder urothelium. Furthermore, in the rat urothelium, PPARα and PPARγ exhibit positive interactions, based on the finding that fenofibrate and rosiglitazone appear to synergize in causing Egr-1 induction (Egerod et al. 2005; Egerod et al. 2009; Oleksiewicz et al. 2008).

The findings that ragaglitazar or coadministration of fenofibrate and rosiglitazone induce Egr-1 expression in the rat urothelium were generated using selective lysis of the urothelial layer and Western blotting for Egr-1 (Egerod et al. 2005; Egerod et al. 2009; Oleksiewicz et al. 2008). However, it is currently unknown whether induction of Egr-1 in the rat urothelium by PPARα+γ activation results in nuclear Egr-1 accumulation, which would be expected to be essential for Egr-1 function. Also, it is currently unknown whether Egr-1 induction occurs throughout the bladder urothelium (as would be expected for a receptor-mediated effect), or whether it favors the ventral part of the bladder urothelium (which would be expected for a urolith-mediated effect). To address these questions, we have in the present study investigated the induction of Egr-1 in the urothelium of rats orally coadministered fenofibrate and rosiglitazone, using immunohistochemistry.

MATERIALS AND METHODS

PPARα and PPARγ Agonist Formulations

Fenofibrate (PPARα agonist, 40 mg/mL) and rosiglitazone (PPARγ agonist, 1.6 mg/mL) suspensions were formulated in vehicle (0.2% w/w sodium carboxymethyl cellulose, 0.4% w/w Tween 80 and 0.8% w/w sodium chloride). Formulation and handling of suspensions was done as described previously (Egerod et al. 2005; Egerod et al. 2009).

Oral Dosing of Rats

Male Sprague-Dawley outbred rats (NTac:SD), six weeks old, were obtained from Taconic Europe (Denmark) and acclimatized for seven to twelve days. Animals were housed in transparent macrolone type IV cages with two to four animals in each cage. The facility was illuminated with a twelve-hour light/dark cycle, and temperature, humidity, and air change were controlled. Animals had free access to water and a complete pelleted rodent diet.

Two separate animal experiments were performed. In the first experiment, animals were randomized and allocated to four groups comprising five animals in each group: two vehicle-dosed groups and two treatment groups dosed with PPARα and PPARγ agonist combination treatment (rosiglitazone + fenofibrate, 8+200 mg/kg/day, 10 mL/kg). Animals were dosed once daily by oral gavage for seven or fourteen days. One vehicle and one treatment group were euthanized at days 7 and 14. In the second experiment, animals were randomized and allocated to four groups comprising ten animals in each group: two vehicle-dosed groups, and two treatment groups dosed with PPARα + PPARγ agonist combination treatment (rosiglitazone + fenofibrate, 8 + 200 mg/kg/day, 10 mL/kg). Animals were dosed once daily by oral gavage for one or seven days. One vehicle and one treatment group were euthanized at days 1 and 7.

In both experiments, the interval between dosing was twenty-four hours, and animals were euthanized four hours after the last oral dose. In both experiments, body weights prior to euthanasia and liver and heart weights following exsanguination were recorded for all animals.

Sampling of Bladder for Immunohistochemistry

For the first animal experiment, animals were anaesthetised with isoflurane/N_2O (0.7 L min^{-1})/O_2 (0.3 L min^{-1}) with 5% isoflurane to deep anaesthesia thereafter reduced to 2% to maintain stable anaesthesia. The bladder was dissected free and a thin catheter was introduced ventrally into the urinary bladder, approximately 3 mm cranial to the bladder neck. The bladder neck was ligated with silk (3-0) to avoid leakage and the bladder was emptied via the catheter using a 1 mL syringe. Using the catheter 0.5 mL 4 % phosphate-buffered formaldehyde was infused into the bladder lumen. The urinary bladder was marked cranio-ventrally with ink, removed in the inflated state, and immersed in the inflated state in 4 % phosphate-buffered formaldehyde. The anaesthetized animals were thereafter euthanized by exsanguination.

After fixation for 1 day, the bladders were sliced longitudinally into ventral and dorsal halves, and each half was further sectioned longitudinally into 4 strips. The 4 strips from either the ventral or dorsal bladder were embedded in the same paraffin block and sectioned at a thickness of 5 μm; that is, each histological section contained either 4 dorsal or 4 ventral strips from the same bladder.

For the second animal study, bladders were removed and processed as described above but without inflating with formaldehyde: that is, in the second study bladders were paraformaldehyde-fixed, paraffin wax-embedded and sectioned in dorsal and ventral parts in a relaxed state.

For both the first and second animal study, 5 rats from each treatment group were used for immunohistochemical analysis of Egr-1 expression in the urinary bladder urothelium (the remaining 5 animals from each group in the second animal study were used for Western blot analysis of Egr-1 expression in the kidney papilla and bladder urothelium).

Sampling of Kidney for Immunohistochemistry

In the second animal experiment (see above), the left kidney was dissected free and removed from the euthanized animals. Kidneys were sectioned into 3 slices by two parallel cuts from

pole to pole along the large curvature. The middle slice containing the kidney papilla was immersed in 4 % phosphate-buffered formaldehyde. After fixation for 1 day, kidney sections were embedded in paraffin and sectioned at a thickness of 5 μm.

In Situ Lysis of the Urinary Bladder Urothelium for Western Immunoblotting

In the second animal experiment (see above), the urinary bladder and neck were dissected free *in situ* in anaesthetised (isoflurane/N_2O/O_2) animals and the bladder urothelium was lysed by inflating with 0.5 mL of a strongly denaturing guanidine isothiocyanate solution into the bladder lumen, as described previously (Egerod et al. 2005; Egerod et al. 2009). The anaesthetized animals were thereafter euthanized by exsanguination.

Sampling of Kidney Papilla for Western Blotting

In the second animal experiment (see above), the right kidney was dissected free and removed from the euthanized animals. Samples of approximately 3x3x3 mm of the kidney papilla tip were homogenized in 1,500 μl polypropylene tubes (Sample Grinding kit, Amersham Biosciences) in approximately 1 mL of a denaturing and reducing loading buffer for SDS-PAGE as previously described (Egerod et al. 2005; Egerod et al. 2009).

Antibodies

The following antibodies were used for Western blotting: Alpha-tubulin mouse monoclonal antibody (Sigma, St. Louis, MO, USA; catalogue number T 6199) raised against purified chick brain tubulin was used at a dilution of 1:6,000. Beta-actin mouse monoclonal antibody (Abcam, Cambridge, UK, catalogue number ab6276) raised against a synthetic peptide correponding to amino acids 1-14 of Xenopus laevis actin was used at a dilution of 1:80,000. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (Abcam, Cambridge, UK, catalogue number ab8245) raised against rabbit muscle GAPDH was used at a dilution of 1:100,000. Egr-1 rabbit affinity purified polyclonal immunoglobulin (Santa Cruz Biotechnology, California, USA, catalogue number C-19, sc-189) raised against a peptide mapping at the carboxy terminus of human Egr-1 was used at a dilution of 1:1,000. HRP-conjugated goat anti-mouse IgG (Cell Signaling Technology, catalogue number 7076) was used at a dilution of 1:20,000. HRP-conjugated horse anti-rabbit IgG (Cell Signaling Technology, catalogue number 7074) was used at a dilution of 1:10,000.

The following antibodies were used for immunohistochemistry: Egr-1 rabbit monoclonal antibody (Cell Signaling Technology, California, USA, catalogue number 4153) raised against a synthetic peptide derived from the amino terminus of human Egr-1 was used at a dilution of 1:750. Biotinylated goat anti-rabbit IgG (DakoCytomation Denmark A/S, Denmark, catalogue number E0432) was used at a dilution of 1:2000.

Immunohistochemistry

Sections (5 μm) of paraffin-embedded tissue were dewaxed in xylene and rehydrated through ethanol. Antigen retrieval was performed by boiling sections for 10 minutes in 10 mM citric acid buffer, pH 6.0 in a microwave oven. Endogenous peroxidase activity was blocked by incubation with 3% (v/v) hydrogen peroxide for 10 minutes. Endogenous avidin binding sites were blocked using an avidin/biotin blocking kit (Vector Laboratories, Inc., California, USA, catalogue number SP-2001). Slides were incubated with avidin for 10 minutes and washed twice in TBST wash buffer (0.05 M Tris-HCl pH 7.6, 0.3 M NaCl and 0.1 % Tween 20) followed by biotin incubation for 10 minutes.

To improve the sensitivity of Egr-1 antigen detection, a tyramide-based catalyzed signal amplification kit was used, essentially according to the manufacturer's recommendations (DakoCytomation Denmark A/S, Denmark, catalogue number K1500). All incubations and washes were done at ambient temperature. Slides were washed twice in TBST and blocked in protein block for 5 minutes, followed by 15 minutes incubation with anti-Egr-1 antibody in antibody diluent (DakoCytomation Denmark A/S, Denmark, catalogue number S3022). The slides were washed 3 times in TBST and underwent 5 sequential incubations with 3x washes in TBST between each step: 15 minutes with biotinylated goat anti-rabbit IgG (diluted in TBST), 15 minutes with streptavidin-biotin complex, 15 minutes with amplification reagent, 15 minutes with streptavidin-peroxidase, and 5 minutes with substrate chromagen solution. Finally, the slides were rinsed in water, counterstained with Mayer's haematoxylin, dehydrated through ethanol into xylene and mounted in DPX (Fisher Scientific, Loughborough, UK, catalogue number D/5319/05).

Blinded Scoring of Egr-1 Immunoreactivity in Immunohistochemical Bladder Specimens

Egr-1 immunolabelling in the nuclei of the bladder urothelium was scored according to predefined scoring criteria, using randomized and blinded specimens, and by a person not involved in the immunohistochemical staining.

Scoring of Egr-1 immunolabelling was based exclusively on immunohistochemical staining in the urothelium, with any staining in the bladder wall not taken into account. However, we found that while bladder wall staining was typically much less intense than the urothelial staining (see Figures 1 and 2), the the two were positively correlated (not shown in detail, but see Figures 1 and 2). The scores were "1" Egr-1 negative, "2" weak Egr-1 positive, "3" moderate Egr-1 positive, and "4" strong Egr-1 positive.

The predefined scoring criteria were as follows: 1, (*Egr-negative*): No nuclear Egr-1 immunolabelling. A granular cytoplasmic staining may be seen. 2, (*weak Egr-1 positive*): Weak Egr-1 immunolabelling in a minority of urothelial nuclei. Occasional urothelial nuclei may exhibit relatively intense Egr-1 immunolabelling, and this can present as rare, relatively intensely staining foci in the urothelial layer. 3, *(moderate*

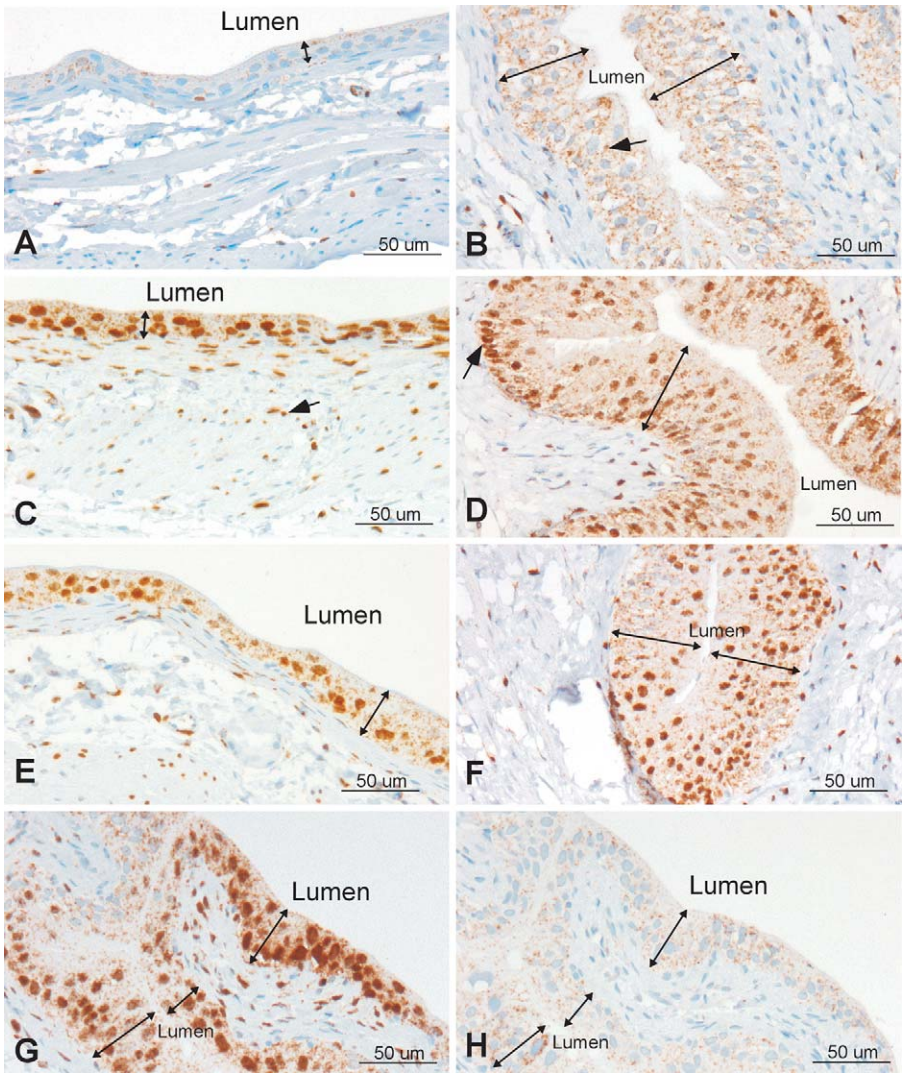

FIGURE 1.—Egr-1 induction in both dorsal and ventral rat urinary bladder by oral treatment with PPARα and PPARγ agonists in vivo. Expression of Egr-1 was examined by immunohistochemistry in extended rat bladder (paraformaldehyde infused)(A, C, E) and in relaxed rat bladder (B, D, F, G, H). All panels represent photomicrographs taken at 250x magnification, with scale bars representing 50 μm. Reddish/brown precipitate indicates positive Egr-1 immunolabelling. (A) Dorsal bladder specimen from animal treated with vehicle for seven days. (B) Dorsal bladder specimen from animal treated with vehicle for 1 day. Arrow, cytoplasmic, granular staining. (C) and (G) Dorsal bladder specimen from animal treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for seven days. Arrow in (C), bladder wall cell nucleus with Egr-1 immunoreactivity. Note that while only occasional bladder cell wall nuclei were Egr-1 immunopositive, essentially all urothelial nuclei were Egr-1 immunopositive. (D) Dorsal bladder specimen from animal treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 1 day. That is, this animal received 2 oral doses over a period of twenty-four hours, and was euthanized four hours after the second dose, as described in materials and methods. Arrow, cluster of basal urothelial cells with strong nuclear Egr-1 immunoreactivity. (E) Ventral bladder specimen from animal treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for seven days. (F) Ventral bladder specimen from animal treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for one day. In other words, this animal received two oral doses over a period of twenty-four hours and was euthanized four hours after the second dose, as described in the Materials and Methods section. (G) and (H) Paired sections, dorsal bladder specimen from animal treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for seven days. (H) Negative (no primary antibody) control. Two-headed arrows, urothelial layer. "Lumen," urinary bladder lumen.

Egr-1 positive): The majority of urothelial nuclei exhibit moderate Egr-1 immunolabelling. Egr-1 immunolabelling in bladder wall may occur, but is not part of the scoring. 4, (*strong Egr-1 positive*): Essentially all urothelial nuclei exhibit strong Egr-1 immunolabelling. Egr-1 immunolabelling in the bladder wall most often parallels the strong urothelial staining, but is not part of the scoring.

The predefined scoring criteria were available to the person performing the scoring in written form, accompanied by high-quality pictures illustrating the 4 scores.

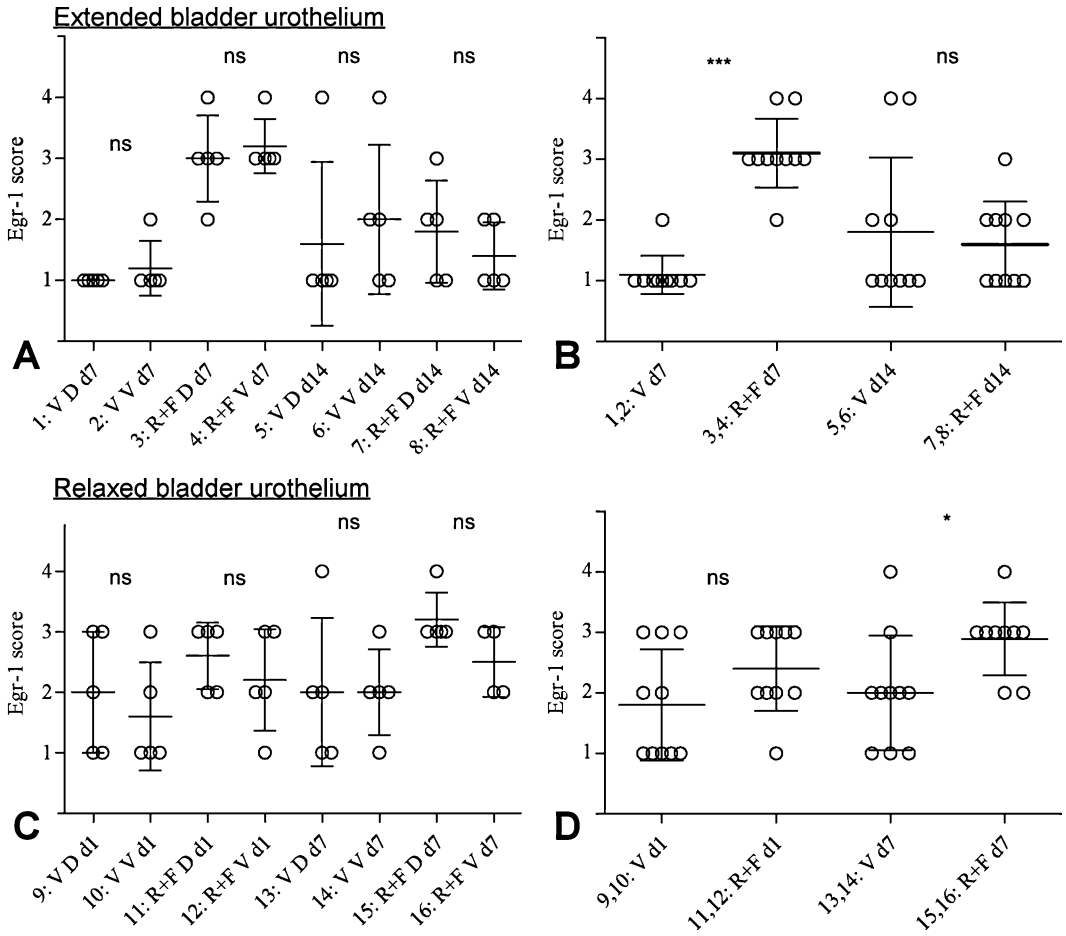

FIGURE 2.—Equal Egr-1 induction in dorsal and ventral rat urinary bladder by oral treatment with a combination of PPARα and PPARγ agonists in vivo. Expression of Egr-1 was examined by immunohistochemistry in extended bladder urothelium (paraformaldehyde infused bladder) (A), (B) and in relaxed rat bladder urothelium (C), (D). Egr-1 immunolabelling was scored according to predefined scoring criteria, with the score 1 indicating no Egr-1 immunoreactivity (Egr-1 negative), and scores 2 through 4 indicating increasing levels of Egr-1 immunoreactivity: "2", weak Egr-1 positive, "3", moderate Egr-1 positive and "4", strong Egr-1 positive. See also methods. (A), Egr-1 scores on dorsal and ventral bladder specimens from animals treated 1 or 7 days, extended bladder urothelium (paraformaldehyde infused bladder). Numbers below plot, treatment groups. 1: Dorsal bladder scores from animals vehicle- treated for 7 days. 2: Ventral bladder scores from animals vehicle-treated for 7 days. 3: Dorsal bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 7 days. 4: Ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 7 days. 5: Dorsal bladder scores from animals vehicle-treated for 14 days. 6: Ventral bladder scores from animals vehicle-treated for 14 days. 7: Dorsal bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 14 days. 8: Ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 14 days. (B) Combined dorsal and ventral bladder scores for the data shown in (A). Numbers below plot, treatment groups. 1,2: Dorsal and ventral bladder scores from animals vehicle-treated for 7 days. 3,4: Dorsal and ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 7 days. 5,6: Dorsal and ventral bladder scores from animals vehicle-treated for 14 days. 7,8: Dorsal and ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 14 days. ***, $P = 0.0001$. ns, not significant (Mann Whitney test). (C) Egr-1 scores on dorsal and ventral bladder tissue specimens from animals treated 7 or 14 days, relaxed rat bladder urothelium. Numbers below plot, treatment groups. 9: Dorsal bladder scores from animals vehicle-treated for 1 day. 10: Ventral bladder scores from animals vehicle-treated for 1 day. 11: Dorsal bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 1 day. 12: Ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 1 day. 13: Dorsal bladder scores from animals vehicle-treated for 7 days. 14: Ventral bladder scores from animals vehicle-treated for 7 days. 15: Dorsal bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 7 days. 16: Ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 7 days. (D) Combined dorsal and ventral bladder scores for the data shown in (C). Numbers below plot, treatment groups. 9,10: Dorsal and ventral bladder scores from animals vehicle-treated for 1 day. 11,12: Dorsal and ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 1 day. 13,14: Dorsal and ventral bladder scores from animals vehicle-treated for 7 days. 15,16: Dorsal and ventral bladder scores from animals treated with rosiglitazone + fenofibrate (8 + 200 mg/kg/day) for 7 days. *, $P = 0.025$. ns, not significant (Mann Whitney test). In all scatter plots, horizontal lines indicate group means, and error bars show +/− one standard deviation.

When two independent scorings were compared, a high degree of agreement was found (Landis and Koch 1977). The weighed kappa-value was 0.858 for relaxed bladder specimens, 0.907 for extended bladder specimens and 0.854 for kidney, confirming the validity of the scoring method outlined above (data not shown).

Western Blotting

The urothelial lysates in guanidine isothiocyanate were dialysed against a urea solution as previously described (Egerod et al. 2005; Egerod et al. 2009). Protein content in urothelial lysates and kidney papilla lysates was determined using NanoOrange staining (NanoOrange® Protein Quantification kit, Molecular Probes, Oregon, USA) and amido black assays (Egerod et al. 2005; Egerod et al. 2009).

Lysates were separated by electrophoresis on fully denaturing and reducing SDS-PAGE using precast 4% to 12% gradient gels. Proteins were electrotransferred to 0.45 μm PVDF membranes and Western blotting was performed as previously described (Egerod et al. 2005; Egerod et al. 2009).

The Western blots were developed using ECL^{Advance} chemiluminescent substrate (GE Healthcare, New Jersey, USA) and a LAS3000 CCD camera (Fujifilm). All exposures were done in the non-saturated (quantitative) range of the CCD camera, as recommended by the manufacturer. For validation of equal protein loading and fine-normalisation for quantitation of the Egr-1 band, membranes were stripped for 1 h at 37 °C (Restore Western blot stripping buffer, Pierce) and reprobed with a load control cocktail of three antibodies (α-Tubulin, β-Actin, and GAPDH).

Quantitative image analysis of the chemiluminescent Western blots was done with MultiGauge software (v2.3, Fujifilm) as previously described (Egerod et al. 2005; Egerod et al. 2009). For quantification of Egr-1 expression levels, at least 3 replicate gels were performed from the same lysates.

Statistical Analysis

GraphPad Prism 5 software (GraphPad Software Inc., CA, USA) was used for statistical analysis.

RESULTS

Oral Coadministration of PPARα and PPARγ Agonists Causes Egr-1 Accumulation in Bladder Urothelial Nuclei

Animals were administered orally vehicle or rosiglitazone + fenofibrate for one, seven, and fourteen days, and Egr-1 expression was examined in the urothelium from dorsal and ventral bladder specimens from both extended (paraformaldehyde infused) and relaxed bladder by immunohistochemistry (Figure 1).

In vehicle-treated animals, specific Egr-1 immunoreactivity was not detected in the urothelium (Figure 1A and B). In some specimens, a weak, diffuse granular cytoplasmic staining was seen (Figure 1B, arrow). This was also seen in specimens where the anti-Egr-1 antibody was omitted (Figure 1H). Also, occasional Egr-1 immunoreactive nuclei were observed in the bladder wall (Figure 1C). The nature of the bladder wall cells expressing Egr-1 immunoreactivity in vehicle-treated animals was not examined further.

By contrast to vehicle-treated animals, rosiglitazone + fenofibrate-treated animals exhibited strong Egr-1 immunoreactivity in urothelial nuclei (Figure 1, C through G). The Egr-1 immunoreactivity induced by rosiglitazone + fenofibrate occurred exclusively in the nuclei of the bladder urothelium. In relaxed bladder specimens, there was a tendency for Egr-1 immunoreactivity to be strongest in the basal compartment of the urothelium (Figure 1D, F, G). However, it is unknown whether this represented a higher sensitivity of the basal urothelium to the action of rosiglitazone + fenofibrate, or a higher density of nuclei towards the basis of the urothelium in relaxed bladder specimens.

The Egr-1 immunoreactivity in the bladder wall nuclei also appeared increased in animals receiving rosiglitazone + fenofibrate. However, while rosiglitazone + fenofibrate strongly induced Egr-1 immunoreactivity in the urothelial nuclei, the induction of Egr-1 immunoreactivity in bladder wall nuclei was mild at best (Figure 1, C through H).

Finally, to monitor treatment efficacy, liver and heart weights were recorded from both experiments. Rosiglitazone + fenofibrate treatment did not affect body weight or heart weight, as expected (not shown, but see Egerod et al. 2009). In contrast, rosiglitazone + fenofibrate treatment significantly increased absolute as well as relative liver weight, the latter measured as liver weight divided by body weight Experiment 1, mean relative liver weights 0.039, 0.064, 0.040 and 0.063 for vehicle day 7, treated day 7, vehicle day 14 and treated day 14 groups, respectively, $P < 0.001$. Experiment 2, mean relative liver weights 0.041, 0.046, 0.040 and 0.063 for vehicle day 1, treated day 1, vehicle day 7 and treated day 7 groups, respectively, $P < 0.001$, two-tailed *t*-test). Fenofibrate is well known to cause strong increases in liver weights in rodents, and we have previously shown that in rats combination-treated with rosiglitazone and fenofibrate, fenofibrate causes the increased liver weight, with rosiglitazone having no or even a protective effect on liver weights (Egerod et al. 2009).

Following Oral Coadministration of PPARα and PPARγ Agonists, Egr-1 Accumulates in Bladder Urothelial Nuclei Within One Day, and Reaches Similar Amounts in Dorsal and Ventral Bladder Urothelium

Egr-1 induction in urothelial nuclei was observed within 1 day of rosiglitazone + fenofibrate dosing (Figure 1, compare B and D). The Egr-1 immunoreactivity in urothelial nuclei was scored on blinded slides by an independent person according to predefined scoring criteria (Figure 2). After 1 day of rosiglitazone + fenofibrate treatment (two oral doses), mean Egr-1 immunoreactivity was increased in rosiglitazone + fenofibrate–treated animals compared to vehicle-treated animals, albeit statistical significance was not reached (Figure 2D). After seven

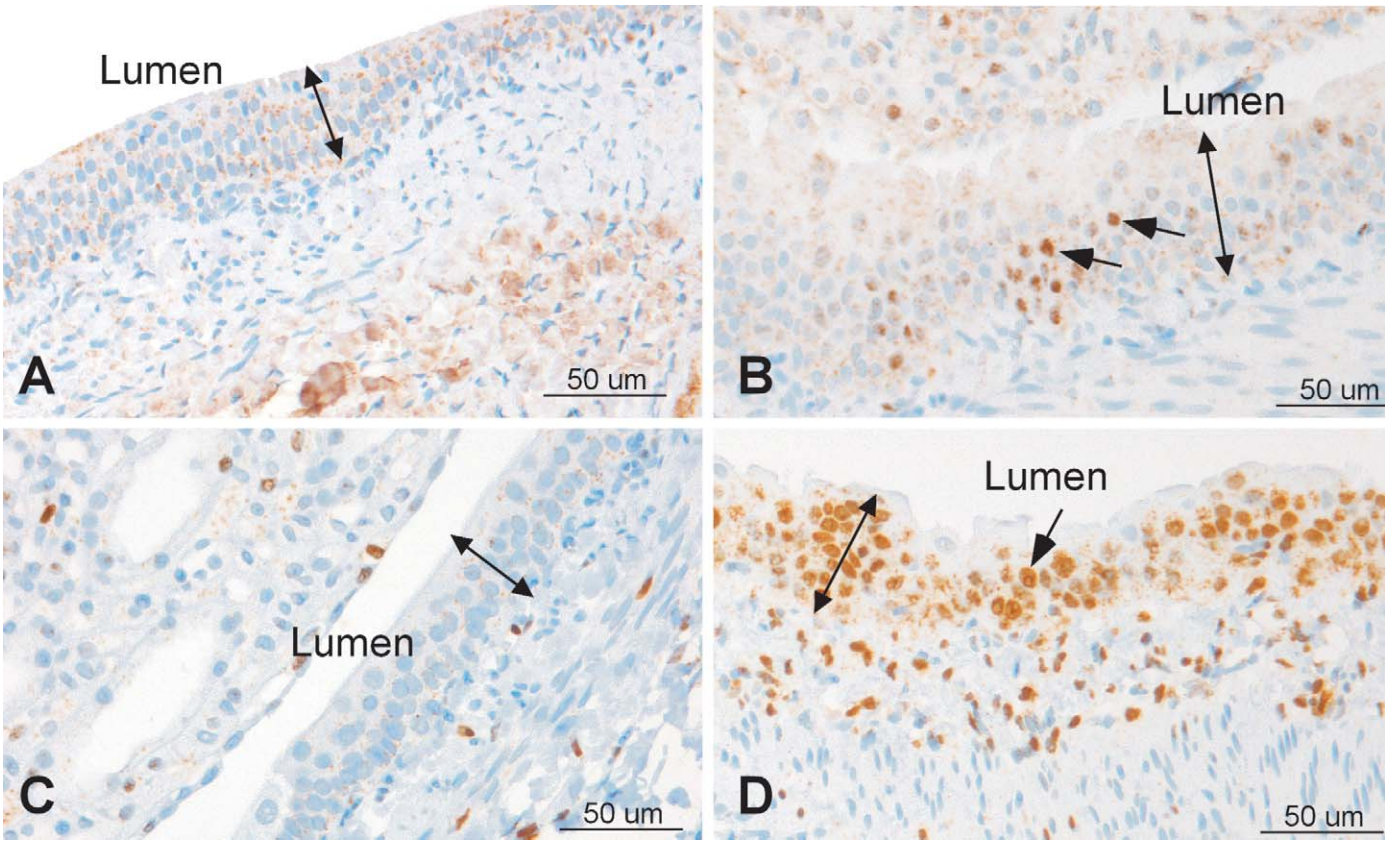

FIGURE 3.—Egr-1 induction in rat kidney papilla urothelium by oral treatment with a combination of PPARα and PPARγ agonists in vivo. (A)-(D) Expression of Egr-1 was examined by immunohistochemistry in the rat kidney papilla urothelium. All panels represent photomicrographs taken at 250x magnification, with scale bars representing 50 μm. Reddish/brown precipitate indicates positive Egr-1 immunolabelling. (A) Kidney papilla urothelium from animal receiving vehicle for 1 day. Ie, this animal received 2 oral doses over a period of 24 hours, and was euthanized 4 hours after the second dose, as described in materials and methods. (B) Kidney papilla urothelium from animal treated with rosiglitazone and fenofibrate (8 + 200 mg/kg/day) for 1 day. Ie, this animal received 2 oral doses over a period of 24 hours, and was euthanized 4 hours after the second dose, as described in materials and methods. Arrow, nuclear Egr-1 immunolabelling in urothelial cells. (C) Kidney papilla urothelium from animal receiving vehicle for 7 days. (D) Kidney papilla urothelium from animal treated with rosiglitazone and fenofibrate (8 + 200 mg/kg/day) for 7 days. Arrow, nuclear Egr-1 immunolabelling in urothelial cell. Two-headed arrows, urothelial layer. ''Lumen'', kidney pelvis lumen. The ragged urothelial morphology eg in panel (B) is due to the antigen retrieval (see materials and methods).

days of rosiglitazone + fenofibrate–treatment (eight oral doses), mean Egr-1 immunoreactivity was also increased in rosiglitazone + fenofibrate–treated animals compared to vehicle-treated animals, and statistical significance was seen in two independent experiments (Figure 2, B and D). After fourteen days of rosiglitazone + fenofibrate treatment (15 oral doses), Egr-1 induction in the urothelium could not be seen by immunohistochemistry (Figure 2B, but is known to occur when examined by the more sensitive Western blotting technique [Egerod et al. 2005] and not shown).

When dorsal and ventral bladder specimens were plotted separately, the same picture could be seen as when dorsal and ventral bladder specimens were plotted together (Figure 2, compare A versus B, and C versus D). In all cases, similar Egr-1 immunoreactivity was observed in dorsal and ventral bladder specimens (Figures 1 and 2), and in no case were the slight differences between dorsal and ventral bladder specimens significant (Figure 2, A and C)

Oral Coadministration of PPARα and PPARγ Agonists Also Causes Egr-1 Accumulation in the Kidney Papilla Urothelium

The urothelium covers not only the urinary bladder, but also the ureters and kidney pelvis. Similarly to the bladder urothelium, the kidney pelvis urothelium of vehicle-treated rats did not exhibit Egr-1 immunoreactivity (Figure 3A and C). Again similarly to the bladder urothelium, nuclear Egr-1 immunoreactivity was induced in the kidney papilla urothelium within 1 day (following two oral doses) of rosiglitazone + fenofibrate (Figure 3B and D). Thus, bladder and kidney papilla urothelia responded identically with rapid Egr-1 expression within 1 day

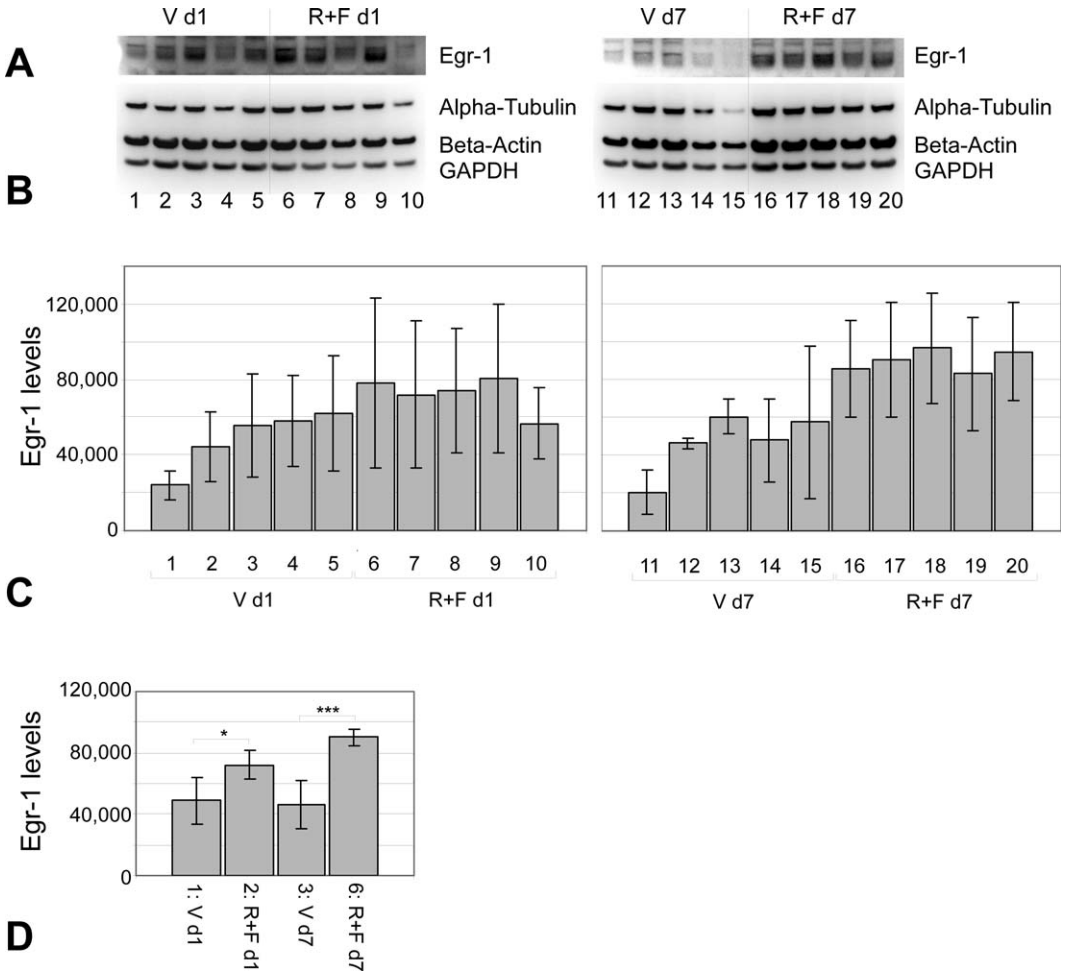

FIGURE 4.—Egr-1 induction in rat kidney papilla by oral coadministration of a PPARα and PPARγ agonist in vivo. (A) Rat kidney papilla lysates from single animals were analysed by Western immunoblotting with an anti-Egr-1 antibody. Numbers below lanes: Rat numbers. Rats 1-5: vehicle day 1. Rats 6-10: rosiglitazone + fenofibrate (R + F) (8 + 200 mg/kg/day) day 1. Rats 11-15: vehicle day 7. 16-20: rosiglitazone + fenofibrate (8 + 200 mg/kg/day) day 7. (B) Western blot with a cocktail of monoclonal antibodies against alpha-tubulin, beta-actin and GAPDH, to confirm equal protein loading. (C) Quantitative analysis of Egr-1 expression for the animals shown in A and B. Numbers below plots: Rat numbers as in A and B. Data is shown as means of 3 replicate Western blots, error bars represent one standard deviation. Egr-1 band intensities were normalized to alpha-tubulin, beta-actin and GAPDH band intensities using MultiGauge software, as described in methods. (D) Means of Egr-1 expression levels for the 4 treatment groups shown in C. Error bars represent one standard deviation. Rats 1-5, treatment group 1: Vehicle day 1. Rats 6-10, treatment group 2: Rosiglitazone + fenofibrate (8 + 200 mg/kg/day) day 1. Rats 11-15, treatment group 3: Vehicle day 7. Rats 16-20, treatment group 4: Rosiglitazone + fenofibrate (8 + 200 mg/kg/day) day 7. *$P = 0.0193$. ***$P = 0.0004$, Student's t-test.

(two oral doses) of rosiglitazone + fenofibrate treatment (Figures 1 and 3).

Confirmation of Egr-1 Accumulation in Bladder and Kidney Papilla Urothelium by Western Blotting

For the kidney papilla, significant induction of Egr-1 was observed by Western blotting following 1 day (two oral doses) as well as seven days (eight oral doses) of rosiglitazone + fenofibrate treatment (Figure 4, A through E).

Similarly, by Western blotting, significant induction of Egr-1 expression in the bladder urothelium was observed following 1 day (two doses) as well as seven days (eight doses) of oral rosiglitazone + fenofibrate treatment (Figure 5, A through D).

Thus, there was complete concordance between immunohistochemistry (Figures 1, 2, and 3) and Western blot (Figures 4 and 5) analysis of Egr-1 induction by rosiglitazone + fenofibrate treatment in the bladder and kidney pelvis urothelium. However, while Egr-1 induction following 1 day (two oral doses) of rosiglitazone + fenofibrate treatment was observed in the urothelium by immunohistochemistry as well as Western (Figures 1D and 2D, 5D), statistical significance was only seen with the more sensitive Western blotting technique (compare Figures 2D and 5D). Furthermore, the level of Egr-1 induction was larger in the bladder urothelium than in the kidney papilla (compare Figures 4D and 5D). This likely reflected that the bladder urothelium lysate used for Western represented predominantly one cell type, the bladder urothelium, while the

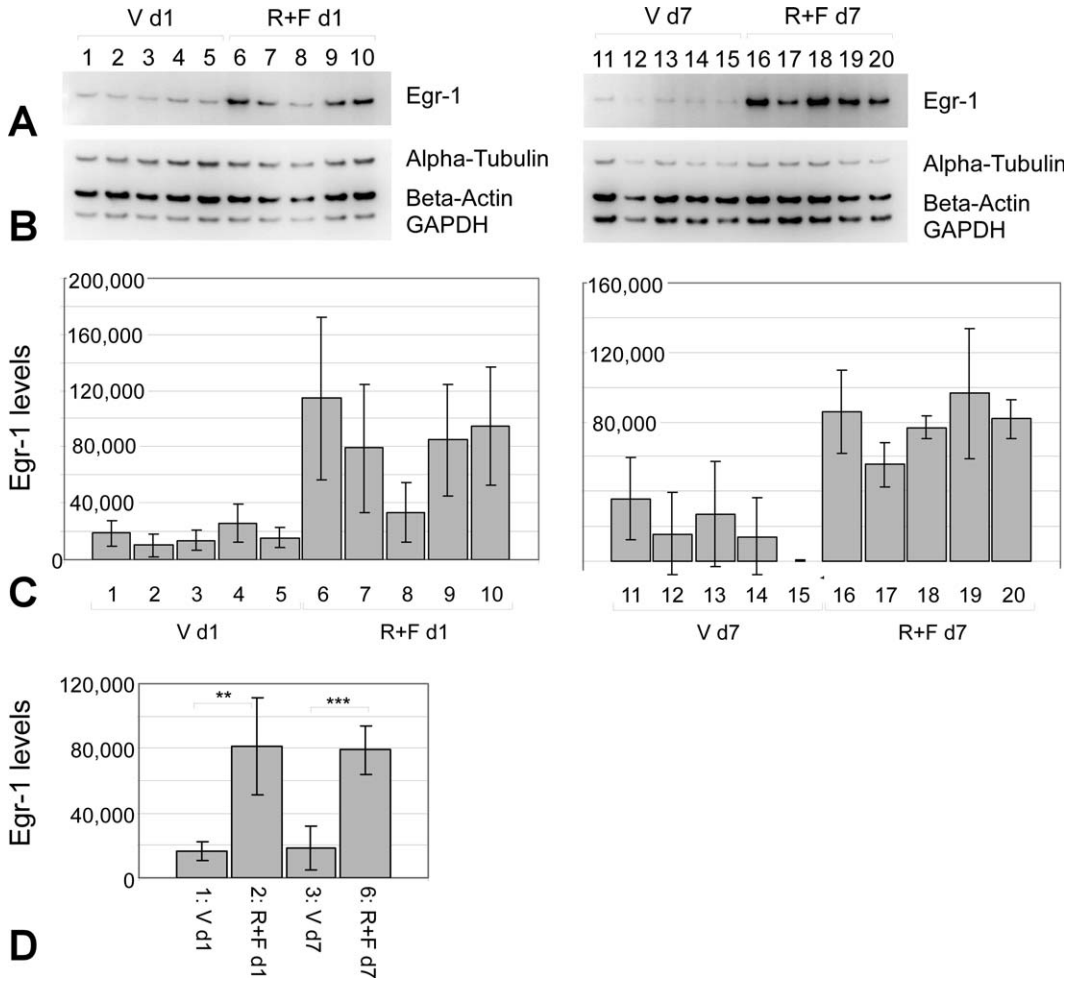

FIGURE 5.—Egr-1 induction in rat urinary bladder urothelium by oral coadministration of a PPARα and PPARγ agonist in vivo: Confirmation of immunohistochemistry data by Western blotting. (A) Individual rat bladder urothelial lysates were analysed by Western immunoblotting with an anti-Egr-1 antibody. Numbers above lanes, rat numbers. Rats 1-5: vehicle day 1. Rats 6-10: rosiglitazone + fenofibrate (R + F) (8 + 200 mg/kg/day) day 1. Rats 11-15: vehicle day 7. 16-20: rosiglitazone + fenofibrate (8 + 200 mg/kg/day) day 7. (B) Western blot with a cocktail of monoclonal antibodies against alpha-tubulin, beta-actin, and GAPDH to confirm equal protein loading. (C) Quantitative analysis of Egr-1 expression in the animals shown in A and B. Numbers below plots: Rat numbers as in A and B. Left panel, mean of 5 replicate Western blots. Right panel, mean of 3 replicate Western blots. Error bars represent one standard deviation. Egr-1 band intensities were normalized to alpha-tubulin band intensities using MultiGauge software, as described in methods. (D) Mean Egr-1 expression levels for the 4 treatment groups shown in (C). Error bars represent one standard deviation. Rats 1-5, treatment group 1: Vehicle day 1. Rats 6-10, treatment group 2: Rosiglitazone + fenofibrate (8 + 200 mg/kg/day) day 1. Rats 11-15, treatment group 3: Vehicle day 7. Rats 16-20, treatment group 4: Rosiglitazone + fenofibrate (8 + 200 mg/kg/day) day 7. $^{**}p = 0.0076$. $^{***}p = 0.0002$, Student's *t*-test.

kidney pelvis lysate represented a mixture of kidney pelvis urothelium and parenchyma (see description of lysate manufacture in methods).

DISCUSSION

The dual-acting PPARα+γ agonist ragaglitazar, as well as a number of other dual-acting PPARα+γ agonists, have caused urothelial carcinomas in rats (Oleksiewicz et al. 2008). By microarray analysis, quantitative RT-PCR as well as Western blotting, ragaglitazar was found to induce the expression of the Egr-1 transcription factor in the bladder urothelium of orally-treated rats (Egerod et al. 2005). Thus, Egr-1 has been suggested as a candidate biomarker for the early rat bladder-carcinogenic effects of ragaglitazar (Egerod et al. 2005; Oleksiewicz et al. 2008; Egerod et al. 2009). As mentioned, rat bladder-carcinogenic effects have been observed also for dual-acting PPARα+γ agonists other than ragaglitazar (Oleksiewicz et al. 2008). Therefore, to facilitate further mechanistic studies, we have implemented a model system utilizing oral treatment of rats with commercially available specific agonists of PPARα (fenofibrate) and PPARγ (rosiglitazone) (Egerod et al. 2005; Lima et al. 2006). Oral coadministration of fenofibrate and rosiglitazone was found to recapitulate the effects of ragaglitazar on Egr-1 expression in the rat bladder urothelium (Egerod et al. 2005; Lima et al. 2006; Oleksiewicz et al. 2008), and

positive interactions between PPARα and PPARγ agonists in inducing Egr-1 expression were found (Egerod et al. 2009).

However, so far, all data showing Egr-1 induction in the rat bladder urothelium by PPARα+γ agonists were based on selective lysis of the urothelial layer by infusing a strongly denaturing guanidine buffer in the bladder lumen (Egerod et al. 2005; Egerod et al. 2009). Thus, the localization of the Egr-1 transcription factor in the urothelium of PPARα+γ agonist–treated rats has not been examined.

In the present study, we found that oral coadministration of fenofibrate and rosiglitazone induced Egr-1 immunoreactivity in the nuclei of the urothelial layers of the urinary bladder as well as kidney pelvis (Figures 1, 2, and 3). In both urinary bladder and kidney pelvis, induction of Egr-1 immunoreactivity in the urothelial nuclei occurred very rapidly, after 1 day (2 oral doses) of rosiglitazone and fenofibrate (Figures 1, 2, and 3). When immunohistochemical data were quantitated in a blinded fashion by an independent observer, the induction of Egr-1 immunoreactivity in the nuclei of the bladder urothelium by oral rosiglitazone + fenofibrate treatment was found to reach statistical significance at day 7 (Figure 2). The immunohistochemical data was confirmed by Western blotting, using an independent Egr-1 antibody (Figures 4 and 5). By Western blotting, statistically significant Egr-1 induction was observed following 1 as well as seven days of oral rosiglitazone + fenofibrate treatment (Figures 4 and 5), supporting the immunohistochemistry data (Figures 1, 2, and 3). We currently believe that Western blotting represents the gold standard for detection of Egr-1 in the rat urothelium. In our opinion, immunohistochemistry likely has a lower sensitivity than Western blotting for quantitation of Egr-1 expression in the rat urothelium, due, for example, (a) the masking of nuclear Egr-1 immunostaining by granular cytoplasmic background sometimes observed (Figure 1B) and (b) the fact that by Western blotting, all the Egr-1 protein in the urothelium is assayed, whereas by immunohistochemistry, primarily the local concentration of Egr-1 protein on the section surface is assayed. In short, the immunohistochemistry and Western blot data were fully concordant, and unequivocally showed that oral rosiglitazone + fenofibrate treatment rapidly induced Egr-1 in urothelial nuclei.

Egr-1 induction was observed in the bladder urothelium within 1 day (2 oral doses) of oral rosiglitazone + fenofibrate treatment (Figures 1, 2, and 5). Also, Egr-1 induction occurred to a similar extent in the dorsal and ventral urinary bladder parts (Figures 1 and 2). These observations strongly suggest that the Egr-1 induction was due to pharmacological activation of PPARα and PPARγ in the rat urothelium. In fact, the bladder urothelium is known to be relatively unique in coexpressing PPARα and PPARγ (Guan et al. 1997; Jain et al. 1998; Chopra et al. 2008). We have recently shown by immunohistochemistry that rat as well as human urothelium coexpresses PPARα and PPARγ (Chopra et al. 2008), and that urothelial expression levels of PPARα and PPARγ are very high (Egerod et al. 2009). Specifically, the findings that Egr-1 expression (a) occurred rapidly, (b) occurred to similar levels in dorsal and ventral bladder parts (Figure 2), and (c) also occurred in the kidney pelvis (Figures 3 and 4) rule out that urinary solids were involved.

As mentioned above, Egr-1 has been suggested as a candidate biomarker for the early rat bladder-carcinogenic effects of ragaglitazar (Egerod et al. 2005; Oleksiewicz et al. 2008). The causal relationship between early Egr-1 induction and later bladder cancer development in rats orally treated with ragaglitazar remains to be examined. However, because Egr-1 is a transcription factor, that is, acts by binding to cognate sequences in the genomic DNA and regulating gene expression, an absolute prerequisite for Egr-1 function is nuclear localization (Ogishima, Shiina, Breault, Tabatabai et al. 2005; Ogishima, Shiina, Breault, Terashima et al. 2005; Scharnhorst et al. 2000). Thus, by showing nuclear localization (Figures 1 and 3), our study indirectly supported that the Egr-1 induced by dual activation of PPARα and PPARγ in the rat urothelium may be biologically active. Yet, further studies employing, for example, chromatin immunoprecipitation or reporter gene approaches are required to directly show whether the Egr-1 induced by dual activation of PPARα and PPARγ in the rat urothelium (Figures 1 and 3) is biologically active.

Egr-1 has been shown to regulate the expression of hyaluronidase as well as heparanase in bladder as well as prostate cancer cells (Lokeshwar et al. 2005; Lokeshwar, Cerwinka, and Lokeshwar 2005; Lokeshwar et al. 2008; Ogishima, Shiina, Breault, Tabatabai et al. 2005; Ogishima, Shiina, Breault, Terashima et al. 2005). Heparanase and hyaluronidase play critical roles in the degradation of extracellular matrix and are frequently upregulated in malignant tumors (Lokeshwar et al. 2008; Ogishima, Shiina, Breault, Tabatabai et al. 2005; Ogishima, Shiina, Breault, Terashima et al. 2005). Correspondingly, heparanase as well as hylaluronidase have been shown to promote bladder as well as prostate cancer development (Lokeshwar et al. 2005; Lokeshwar, Cerwinka, and Lokeshwar 2005; Lokeshwar et al. 2008; Lokeshwar and Selzer 2008; Ogishima, Shiina, Breault, Terashima et al. 2005; Ogishima, Shiina, Breault, Tabatabai et al. 2005). In fact, hyaluronidase activity is a recognized urinary biomarker for human bladder cancer (Vrooman and Witjes 2009). Also, Egr-1 is required for prostate tumor development in mice (Abdulkadir et al. 2001; Abdulkadir 2005). Interestingly, while the available literature quoted above indicates that Egr-1 would promote rat bladder cancer, it is also known that Egr-1 may either inhibit or promote hyaluronidase expression depending on hyaluronidase promoter methylation (Lokeshwar et al. 2008). Also, strong overexpression of hyaluronidase may have anti-cancer effect (Lokeshwar and Selzer 2008). In short, there is strong albeit indirect evidence to support that early induction of Egr-1 in the urothelium of the bladder of rats may be relevant to later bladder cancer developent. However, the literature quoted above also suggests that if Egr-1 plays a role in bladder cancer development in rats exposed to dual-acting PPAR agonists, the mechanism is likely complex and may involve different tumor responses at different levels of Egr-1 induction, as well as idiosynchratic responses in individual urothelial cells depending eg

on Egr-1 DNA binding site modifocations (chromatin structure). In fact, Egr-1 immunolabelling was sometimes observed to have a focal pattern in the urothelium (Figure 1D, arrow, Figure 3B, two arrows, and not shown), suggesting microheterogeneity in the sensitivity of the urothelial layer to PPAR activation.

In conclusion, this study examined for the first time the localization of the Egr-1 transcription factor in the urothelium of rats dosed orally with a combination of a specific PPARα agonist (fenofibrate) and a specific PPARγ agonist (rosiglitazone). We found Egr-1 was induced within 1 day (2 oral doses) of treatment. Egr-1 induction occurred to similar extent in dorsal and ventral bladder parts and occurred also in the kidney pelvis urothelium. These findings strongly supported that Egr-1 induction occurred due to pharmacological activation of PPARα and PPARγ, which are in fact known to be coexpressed at high levels in the rat urothelium. Finally, our demonstration of a nuclear localization supports that the Egr-1 induced by PPARα and PPARγ coactivation in the urothelium may be biologically active.

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References

Abdulkadir, S. A. (2005). Mechanisms of prostate tumorigenesis: roles for transcription factors Nkx3.1 and Egr1. *Ann N Y Acad Sci* **1059**, 33–40.

Abdulkadir, S. A., Qu, Z., Garabedian, E., Song, S. K., Peters, T. J., Svaren, J., Carbone, J. M., Naughton, C. K., Catalona, W. J., Ackerman, J. J., Gordon, J. I., Humphrey, P. A., and Milbrandt, J. (2001). Impaired prostate tumorigenesis in Egr1-deficient mice. *Nat Med* **7,** 101–7.

Balakumar, P., Rose, M., Ganti, S. S., Krishan, P., and Singh, M. (2007). PPAR dual agonists: Are they opening Pandora's Box? *Pharmacol Res* **56,** 91–98.

Berger, J. P., Akiyama, T. E., and Meinke, P. T. (2005). PPARs: Therapeutic targets for metabolic disease. *Trends Pharmacol Sci* **26,** 244–51.

Chopra, B., Hinley, J., Oleksiewicz, M. B., and Southgate, J. (2008). Trans-species comparison of PPAR and RXR expression by rat and human urothelial tissues. *Toxicol Pathol* **36,** 485–95.

Christy, B. A., Lau, L. F., and Nathans, D. (1988). A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. *Proc Natl Acad Sci U S A* **85,** 7857–61.

Dominick, M. A., White, M. R., Sanderson, T. P., Van Vleet, T., Cohen, S. M., Arnold L. E., Cano, M., Tannehill-Gregg, S., Moehlenkamp, J. D., Waites, C. R., and Schilling, B. E. (2006). Urothelial carcinogenesis in the urinary bladder of male rats treated with muraglitazar, a PPAR alpha/gamma agonist: Evidence for urolithiasis as the inciting event in the mode of action. *Toxicol Pathol* **34,** 903–20.

Egerod, F. L., Brünner, N., Svendsen, J. E., and Oleksiewicz, M. B. (2009). PPAR alpha and PPAR gamma are co-expressed, functional and show positive interactions in the rat urinary bladder urothelium. *J Appl Toxicol* Sept 15, Epub ahead of print.

Egerod, F. L., Nielsen, H. S., Iversen, L., Thorup, I., Storgaard, T., and Oleksiewicz, M. B. (2005). Biomarkers for early effects of carcinogenic dual-acting PPAR agonists in rat urinary bladder urothelium in vivo. *Biomarkers* **10,** 295–309.

Fiévet, C., Fruchart, J. C., and Staels, B. (2006). PPARalpha and PPARgamma dual agonists for the treatment of type 2 diabetes and the metabolic syndrome. *Curr Opin Pharmacol* **6,** 606–14.

Ghanem, M. A., Van der Kwast, T. H., Den Hollander, J. C., Sudaryo, M. K., Oomen, M. H., Noordzij, M. A., Van den Heuvel, M. M., Nassef, S. M., Nijman, R. M., and van Steenbrugge, G. J. (2000). Expression and prognostic value of Wilms' tumor 1 and early growth response 1 proteins in nephroblastoma. *Clin Cancer Res* **6,** 4265–71.

Guan, Y., Zhang, Y., Davis, L., and Breyer, M. D. (1997). Expression of peroxisome proliferator-activated receptors in urinary tract of rabbits and humans. *Am J Physiol* **273,** F1013–22.

Jain, S., Pulikuri, S., Zhu, Y., Qi, C., Kanwar, Y. S., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1998). Differential expression of the peroxisome proliferator-activated receptor gamma (PPARgamma) and its coactivators steroid receptor coactivator-1 and PPAR-binding protein PBP in the brown fat, urinary bladder, colon, and breast of the mouse. *Am J Pathol* **153,** 349–54.

Landis, J. R., and Koch, G. G. (1977). The measurement of observer agreement for categorical data. *Biometrics* **33,** 159–74.

Lemaire, P., Revelant, O., Bravo, R., and Charnay, P. (1988). Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. *Proc Natl Acad Sci U S A* **85,** 4691–95.

Lim, R. W., Varnum, B. C., and Herschman, H. R. (1987). Cloning of tetradecanoyl phorbol ester-induced "primary response" sequences and their expression in density-arrested Swiss 3T3 cells and a TPA non-proliferative variant. *Oncogene* **1,** 263–70.

Lima, B. S., Dominick, M., Iversen, L., and Oleksiewicz, M. B. (2006). Peroxisome proliferators activated receptors (PPARs) agonists and rodent tumorigenesis: updating the discussions. Paper presented at DIA 42nd annual meeting, June 18–22, 2006, Philadelphia. Available at.: http://www. softconference.com/260618 (BT Biotechnology/NC Nonclinical Laboratory Safety 299D).

Lokeshwar, V. B., Cerwinka, W. H., Isoyama, T., and Lokeshwar, B. L. (2005). HYAL1 hyaluronidase in prostate cancer: A tumor promoter and suppressor. *Cancer Res* **65,** 7782–89.

Lokeshwar, V. B., Cerwinka, W. H., and Lokeshwar, B. L. (2005). HYAL1 hyaluronidase: A molecular determinant of bladder tumor growth and invasion. *Cancer Res* **65,** 2243–50.

Lokeshwar, V. B., Gomez, P., Kramer, M., Knapp, J., McCornack, M. A., Lopez, L. E., Fregien, N., Dhir, N., Scherer, S., Klumpp, D. J., Manoharan, M., Soloway, M. S., and Lokeshwar, B. L. (2008). Epigenetic regulation of HYAL-1 hyaluronidase expression. Identification of HYAL-1 promoter. *J Biol Chem* **283,** 29215–27.

Lokeshwar, V. B., and Selzer, M. G. (2008). Hyalurondiase: Both a tumor promoter and suppressor. *Seminars in Cancer Biology* **18,** 281–87.

Long, G. G., Reynolds, V. L., Lopez-Martinez, A., Ryan, T. E., White, S. L., and Eldridge, S. R. (2008). Urothelial carcinogenesis in the urinary bladder of rats treated with naveglitazar, a gamma-dominant PPAR alpha/gamma agonist: Lack of evidence for urolithiasis as an inciting event. *Toxicol Pathol* **36,** 218–31.

Milbrandt, J. (1987). A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science* **238,** 797–99.

Ogishima, T., Shiina, H., Breault, J. E., Tabatabai, L., Bassett, W. W., Enokida, H., Li, L. C., Kawakami, T., Urakami, S., Ribeiro-Filho, L. A., Terashima, M., Fujime, M., Igawa, M., and Dahiya R. (2005). Increased heparanase expression is caused by promoter hypomethylation and up-regulation of transcriptional factor early growth response-1 in human prostate cancer. *Clin Cancer Res* **11,** 1028–36.

Ogishima, T., Shiina, H., Breault, J. E., Terashima, M., Honda, S., Enokida, H., Urakami, S., Tokizane, T., Kawakami, T., Ribeiro-Filho, L. A.,

Fujime, M., Kane, C. J., Carroll, P. R., Igawa, M., and Dahiya, R. (2005). Promoter CpG hypomethylation and transcription factor EGR1 hyperactivate heparanase expression in bladder cancer. *Oncogene* **24,** 6765–72.

Oleksiewicz, M. B., Southgate, J., Iversen, L., and Egerod, F. L. (2008). Rat urinary bladder carcinogenesis by dual-acting PPAR alpha + gamma agonists. *PPAR Res* **2008**, 103167.

Oleksiewicz, M. B., Thorup, I., Nielsen, H. S., Andersen, H. V., Hegelund, A. C., Iversen, L., Guldberg, T. S., Brinck, P. R., Sjogren, I., Thinggaard, U. K., Jorgensen, L., and Jensen, M. B. (2005). Generalized cellular hypertrophy is induced by a dual-acting PPAR agonist in rat urinary bladder urothelium in vivo. *Toxicol Pathol* **33,** 552–60.

Rauscher, F. J. III, Morris, J. F., Tournay, O. E., Cook, D. M., and Curran, T. (1990). Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science (New York, N Y)* **250,** 1259–62.

Rubenstrunk, A., Hanf, R., Hum, D. W., Fruchart, J. C., and Staels, B. (2007). Safety issues and prospects for future generations of PPAR modulators. *Biochim Biophys Acta* **1771,** 1065–81.

Scharnhorst, V., Menke, A. L., Attema, J., Haneveld, J. K., Riteco, N., van Steenbrugge, G. J., van der Eb, A. J., and Jochemsen, A. G. (2000). EGR-1 enhances tumor growth and modulates the effect of the Wilms' tumor 1 gene products on tumorigenicity. *Oncogene* **19,** 791–800.

Van Vleet, T. R., White, M. R., Sanderson, T. P., Cohen, S. M., Cano, M., Arnold, L. L., Waites, C. R., Schilling, B. E., Mitroka, J., and Dominick, M. A. (2007). Subchronic urinary bladder effects of muraglitazar in male rats. *Toxicol Sci* **96,** 58–71.

Vrooman, O. P., and Witjes, J. A. (2009). Molecular markers for detection, surveillance and prognostication of bladder cancer. *Int J Urol* **16,** 234–43.

Waites, C. R., Dominick, M. A., Sanderson, T. P., and Schilling, B. E. (2007). Nonclinical safety evaluation of muraglitazar, a novel PPARalpha/gamma agonist. *Toxicol Sci* **100,** 248–58.