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Biomarkers for early effects of carcinogenic dual-acting PPAR agonists in rat urinary bladder urothelium *in vivo*

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Abstract
Small-molecule agonists of the peroxisome proliferator-activated receptor (PPAR) α and γ isoforms (dual-acting PPAR agonists) can cause urothelial cancers in rodents. Rats were dosed orally for 16 days with bladder carcinogenic (ragaglitazar) as well as non-bladder carcinogenic (fenofibrate and rosiglitazone) PPAR agonists and protein changes were assayed in the urinary bladder urothelium by Western blotting. Dose levels reflected 10–20× human exposure, and the ragaglitazar dose was in the carcinogenic range. Ragaglitazar induced expression of the transcription factor Egr-1, phosphorylation of the c-Jun transcription factor and phosphorylation of the ribosomal S6 protein were observed. These changes were also observed in rats dosed with either rosiglitazone or fenofibrate. However, the protein changes were stronger (Egr-1 induction) or of a longer duration (S6 phosphorylation) in ragaglitazar-treated animals. Animals co-administered fenofibrate (a specific PPARα agonist) and rosiglitazone (a specific PPARγ agonist) exhibited Egr-1 and S6 protein changes more similar to those induced by ragaglitazar (a dual-acting PPARα/γ agonist) than either fenofibrate or rosiglitazone alone. The findings suggest that ragaglitazar causes Egr-1, c-Jun and S6 protein changes in the urothelium by a mechanism involving PPARα as well as PPARγ, and that the Egr-1, c-Jun and S6 protein changes might have potential biomarker value.

Keywords: *Peroxisome proliferator-activated receptor (PPAR), rat urothelium, carcinogenesis, biomarker, Egr-1.*

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Introduction

The peroxisome-proliferator-activated receptor (PPAR) agonists currently on the market (thiazolidinediones and fibrates) are orally available antidiabetic and antilipidaemic agents (Kersten et al. 2000). PPARs belong to the nuclear hormone receptor superfamily and function as ligand-activated transcription factors, regulating the expression of genes involved in lipid homeostasis and inflammation (Dreyer et al. 1992, Kersten et al. 2000, Abumrad 2004, Nahle 2004). Three different PPAR isoforms have been identified: α, β/δ and γ (Dreyer et al. 1992). PPARα reaches its highest expression levels in brown adipose tissue, liver, heart, kidney and skeletal

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muscle, while PPARγ is mostly expressed in adipose tissue, colon, leukocytes and retina. Finally, PPARβ/δ is more ubiquitously expressed across tissues (Guan et al. 1997, Kersten et al. 2000, Abumrad 2004, Michalik et al. 2004, Nahle 2004).

Due to the increasing public health importance of diabetes and metabolic syndrome-associated disorders, many new PPAR agonists are under development. In particular, dual-acting PPARα/γ agonists, i.e. agonists with activity against PPARα as well as PPARγ, have shown superiority over specific PPARα and PPARγ agonists for type II diabetes treatment, in animal models as well as in humans (Lohray et al. 2001, Brand et al. 2003, Ebdrup et al. 2003, Larsen et al. 2003, Ye et al. 2003, Saad et al. 2004). Unfortunately, many of these new dual-acting agonists have turned out to be urinary bladder carcinogens in rats (El-Hage 2004).

It is well known that specific PPARα agonists are liver carcinogens in rodents, and the mechanisms behind the hepatocarcinogenic effects have received attention in a large number of studies (Ashby et al. 1994). In contrast, no data are currently available regarding the mechanisms by which dual-acting PPARα/γ agonists induce urinary bladder cancer in rats. The lack of mechanistic knowledge poses an obstacle to the continued development of this class of drugs (Kersten et al. 2000). The present study investigated the changes in the expression and phosphorylation levels of key regulatory proteins in the bladder urothelium of rats orally treated with different PPAR agonists. Because it has been reported that PPAR agonists can have opposite effects *in vivo* (in animals) and *in vitro* (in cell culture) (Burstein et al. 2003, Debrock et al. 2003, Michalik et al. 2004, Nahle 2004), we considered it essential to generate reference data *in vivo*. To allow preliminary evaluation of the biomarker value of the observed protein changes, we included bladder-carcinogenic as well as non-bladder-carcinogenic PPAR agonist drugs. Finally, the bladder urothelium of various species, including rat, is relatively unique in co-expressing both the PPARα and PPARγ isoforms (Guan et al. 1997; B. Chopra, personal communication), and it cannot be ruled out that the bladder cancers in rats treated with dual-acting PPARα/γ agonists arise as a result of receptor-mediated mechanisms (El-Hage 2004). To test this hypothesis, we included animals treated with specific PPARα agonists, animals treated with specific PPARγ agonists, and animals co-administered the specific PPARα and PPARγ agonists.

Materials and methods

Oral treatment of rats with PPAR agonist drug

Male Sprague–Dawley rats, 4–5-weeks-old, were obtained from Charles River Wiga GMbH (Sutzfeld, Germany). They were acclimatized for 8 days before the start of the studies. The rats were housed in transparent type IV macrolone cages (floor area 1800 cm^2, height 31 cm) with three to four animals in each cage. Food pellets and water were given *ad libitum*; the animal rooms were kept on a 12/12-h light/dark cycle.

Animals were randomized and allocated to one of five test groups: a negative control (vehicle) group and four treatment groups — ragaglitazar, rosiglitazone, fenofibrate and rosiglitazone + fenofibrate combination treatment (Table I). Each test group consisted of 21 animals, giving a total of 105 animals. Animals were dosed once daily by oral gavage. The gastric gavage volume was the same for all test groups (5 ml kg^{-1}), except for the rosiglitazone + fenofibrate group (10 ml kg^{-1}). Dose levels were chosen to reflect approximately 10–20× expected human exposure.

Table I. Dose level, PPAR selectivity and bladder carcinogenic effect of the different PPAR agonists.

Test group		Group size	Dose level ($mg\,kg^{-1}\,day^{-1}$)	PPAR agonism	Bladder carcinogenic effect
1	Vehicle	21	n.a.	n.a.	n.a.
2	Ragaglitazar	21	50	dual (α/γ)	+
3	Rosiglitazone	21	8	γ	−
4	Fenofibrate	21	200	α	−
5	Rosiglitazone + Fenofibrate	21	8 + 200	dual (α/γ)	?

Rats were randomized and allocated to one of five test groups, as shown. Dose levels were chosen to reflect approximately 10× human exposure. The ragaglitazar dose was in the carcinogenic range based on previous 2-year carcinogenicity studies.
n.a., Not applicable.

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In situ *lysis of the inner bladder surface*

At days 1, 2, 4, 7, 8, 10 and 15, three animals from each group were anaesthetized 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 urinary bladder and neck were dissected free, but left *in situ* in the anaesthetized animal, and a thin needle (27G) was inserted into the urinary bladder, approximately 3 mm distal to the bladder neck. The bladder neck was ligated with a silk suture to prevent escape of the fluid from the bladder. Any urine present in the bladder was removed and discarded via the needle. Approximately 0.5 ml lysing solution (4 M guanidine isothicyanate, 0.5% N-lauroyl sarcosine, 25 mM citrate pH 5.5) was injected into the bladder and left in place for 2 min, after which the lysate was withdrawn. Histopathological examination confirmed that this procedure selectively lysed the urothelial layer, leaving deeper bladder layers intact (data not shown).

The anaesthetized animals were euthanized by exsanguination. Lysates from three animals (one treatment group) were pooled and stored at $-20^{\circ}C$.

Western blot analysis of urothelial lysates

The urothelial lysates in guanidine isothiocyanate were dialysed against 8 M urea-20 mM Tris (pH 8.0)-5 mM EDTA-1/200 vol. protease inhibitors (cocktail III, Calbiochem, Darmstadt, Germany)-1/100 vol. phosphatase inhibitors (cocktail II, Calbiochem). Dialysis was performed for 4 h at room temperature, using Slide-A-Lyzer mini dialysis units (3500 MWCO, Pierce, Rockford, USA), and changing to fresh dialysis buffer after the first 2 h. After dialysis, protein concentrations were determined with the NanoOrange® Protein Quantification kit (Molecular Probes, Oregon, USA) according to the manufacturer's instructions.

Lysates were pooled for each treatment group into an 'early' (days 1, 2 and 4) and 'late' group (days 7, 8, 10 and 15), adjusting the volumes to obtain identical protein concentrations in all pools ($1.3\,mg\,ml^{-1}$). Thus, the whole animal experiment, consisting of 105 animals, was condensed to ten urothelial lysate pools: five 'early'

pools, consisting of three time points × three animals = nine animals per pool, and five 'late' pools, consisting of four time points × three animals = 12 animals per pool.

SDS-PAGE of urothelial lysate pools was done on precast 4–12% gradient gels, according to the manufacturer's instructions (NuPAGE™ gels, Invitrogen, California, USA). Identical total protein amounts were loaded for each pool, based on NanoOrange® protein determinations. Proteins were transferred to 0.45-μm PVDF membranes (Invitrogen) by electrophoresis for 1 h in a tank transfer unit (Hoefer™ TE22, Amersham Biosciences, San Francisco, USA), in 25 mM Tris base-192 mM glycine, at 4°C and 80 V. The membranes were blocked for 1 h at room temperature in 5 wt% fat-free skim milk powder in 50 mM Tris (pH 7.6)-136 mM NaCl-0.1% Tween 20 (TBS-T). Membranes were then washed three times, for 5 min each time, in TBS-T and incubated with primary antibody diluted in 5 wt% bovine serum albumin in TBS-T overnight at 4°C. The next day the membranes were washed for 3 × 5 min in TBS-T and incubated in horseradish peroxidase-conjugated secondary antibody diluted in 5 wt% fat-free skim milk powder in TBS-T for 1 h at room temperature. Finally, the membranes were washed for 3 × 5 min in TBS-T and protein bands were visualized using ECL^{Advance} chemiluminescent substrate (GE Healthcare, New Jersey, USA) and a LAS3000 CCD camera (Fujifilm). The membranes were subsequently stripped with 'Restore' Western blot stripping buffer for 1 h at 37°C (Pierce), before being reprobed with the loading control β-actin monoclonal antibody. The same procedure as above was followed, except that phosphate-buffered saline with 0.1% Tween 20 (PBS-T) was used instead of TBS-T, the primary antibody was diluted in 5% skimmed milk in PBS-T and membranes were incubated in primary antibody for 1 h at room temperature.

The following antibodies were used for Western blot analysis: Egr-1 rabbit polyclonal antibody was diluted 1:1000 (Santa Cruz Biotechnology, California, USA, C-19, sc-189). Phospho-c-Jun (Ser73) rabbit polyclonal IgG was diluted 1:1000 (Cell Signaling Technology, Massachussets, USA, 9260). c-Jun rabbit polyclonal IgG was diluted 1:1000 (Cell Signaling Technology, 9262). Phospho-S6 ribosomal protein (Ser235/236) rabbit polyclonal IgG was diluted 1:10 000 (Cell Signaling Technology, 2211). S6 ribosomal protein rabbit polyclonal IgG, was diluted 1:1000 (Cell Signaling Technology, 2212). β-Actin mouse monoclonal antibody was diluted 1:80 000 (Abcam, Cambridge, UK, ab6276). HRP-conjugated goat anti-mouse IgG was diluted 1:20 000 (Cell Signaling Technology, 7076). HRP-conjugated horse anti-rabbit IgG was diluted 1:10 000 (Cell Signaling Technology, 7074).

Quantitation of Western blot data

Images of chemiluminescent Western blots were captured with a LAS3000 CCD camera, as mentioned above. Multiple exposures were made of each PVDF membrane, within the non-saturated response range of the CCD camera. Quantitative image analysis was performed with MultiGauge software (v2.3, Fujifilm).

The following approach was taken to compare phosphorylation of c-Jun and S6 protein between urothelial lysates: replicate gels and membranes were made of the same urothelial lysates. Gel loading of all urothelial lysates was normalized based on NanoOrange® protein determinations. One PVDF membrane was probed with phospho-specific antibody (phospho-c-Jun or phospho-S6), while the other PVDF membrane was probed with antibody that reacts equally well with phosporylated and

non-phosphorylated protein isoforms (c-Jun or S6). For each PVDF membrane, background correction was first performed with the 'spline' option of the MultiGauge software. The ratios between the band intensities of drug- and vehicle-treated animals were then calculated. This yielded:

$$P = \text{band intensity}^{\text{treated}} / \text{band intensity}^{\text{vehicle}}$$

for the membrane probed with the phospho-specific antibody, and:

$$T = \text{band intensity}^{\text{treated}} / \text{band intensity}^{\text{vehicle}}$$

for the membrane probed with the non-discriminatory antibody. The specific, relative amount of phosphorylation (corrected for total protein levels) was then calculated as P/T. The P/T values were thus always 1 for vehicle-treated animals, with values above 1 indicating a fold increase in phosphorylation in drug-treated animals.

The following approach was taken to compare expression of Egr-1 between urothelial lysates. Because we did not detect Egr-1 expression in the urothelium of vehicle-treated animals, a relative measure such as used for phosphoprotein analysis could not be used. Instead, a single gel and membrane was made of the urothelial lysates. Gel loading of all urothelial lysates was normalized based on NanoOrange® protein determinations. Background correction was performed with the 'area' function of the MultiGauge software. The luminescence intensity (arbitrary units, AU) in the area corresponding to the expected localization of the Egr-1 band was then determined for vehicle-treated animals, yielding AU^{vehicle} values. Finally, AU^{vehicle} values were subtracted from the luminescence intensity of the Egr-1 band observed in drug-treated animals, yielding $AU^{\text{treated}} - AU^{\text{vehicle}}$ values. Thus, Egr-1 expression levels were always 0 for vehicle-treated animals, with positive AU values indicating increased Egr-1 expression.

Results

Ragaglitazar induces the Egr-1 transcription factor in rat bladder urothelium

Initially, to explore the mechanisms behind ragaglitazar-induced bladder cancers in rats, DNA microarray studies were performed. The microarray studies identified a number of genes that were up- or down-regulated in the bladder urothelium of ragaglitazar-treated rats. The most consistently upregulated gene was Egr-1 (data not shown). To validate the upregulation of Egr-1 mRNA at the protein level, Western blot was performed (Figure 1).

In vehicle-treated animals, Egr-1 expression could not be detected in the bladder urothelium throughout the 15-day experimental period (Figure 1, lanes 1E and 1L). However, animals treated with the dual-acting PPARα/γ agonists ragaglitazar showed a robust induction of Egr-1 protein (Figure 1, lanes 2E and 2L), confirming the microarray data. The induction of Egr-1 expression in rat bladder urothelium by ragaglitazar was observed in three independent animal experiments (data not shown). Furthermore, the data shown in Figure 1 were confirmed with another anti-Egr-1 antibody (Cell Signaling; data not shown). Finally, when individual samples were analysed, Egr-1 induction was observed as early as 1–2 days after ragaglitazar dosing (data not shown).

In contrast to the strong Egr-1 induction by the dual-acting ragaglitazar, the PPARγ-agonist rosiglitazone (Figure 1, lanes 3E and 3L) and the PPARα-agonist

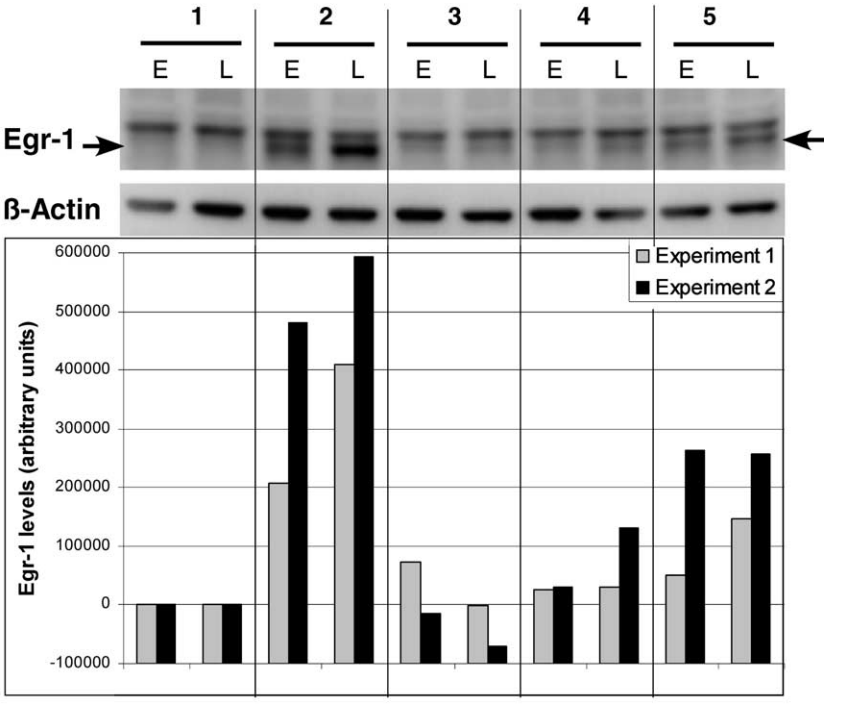

Figure 1. Induction of the Egr-1 transcription factor in rat urothelium after PPAR agonist treatment *in vivo*. Rat bladder urothelial lysates were analysed by Western immunoblotting with an anti-Egr-1 antibody. Animals were treated with: 1, vehicle; 2, ragaglitazar; 3, rosiglitazone; 4, fenofibrate; and 5, rosiglitazone + fenofibrate. See Table I for drug and dosage descriptions. E, early pool (urothelial lysates from treatment days 1, 2 and 4, total of nine animals); L, late pool (urothelial lysates from treatment days 7, 8, 10 and 15, total of 12 animals). The Egr-1 band location is indicated by arrows. β-Actin was used as the loading control. Data from two independent Western blotting experiments are shown in the bar diagram. Values above 0 indicate that Egr-1 protein levels were increased compared with vehicle-treated animals.

fenofibrate (Figure 1, lanes 4E and 4L) caused a much milder Egr-1 induction. Intriguingly, a combination treatment with fenofibrate and rosiglitazone caused a stronger Egr-1 induction than either drug on its own (Figure 1, lanes 5E and 5L). Unfortunately, the study design did not allow examination of the statistical significance of these differences.

Ragaglitazar induces phosphorylation of the ribosomal S6 protein and the c-Jun transcription factor in the rat urothelium

As a complementary approach to the DNA microarray studies mentioned above, we screened a number of proteins known to be involved in cell cycle regulation, apoptosis and cancer, using commercially available activation-state antibodies (antibodies that detect biologically active protein forms, i.e. phosphorylated or cleaved protein forms). The screening was done on bladder urothelium from ragaglitazar-treated rats in a Western blot format. Based on this screening, oral ragaglitazar dosing was found to activate a number of proteins in the rat urothelium, with the S6 ribosomal protein and the c-Jun transcription factor amongst the most strongly affected (data not shown).

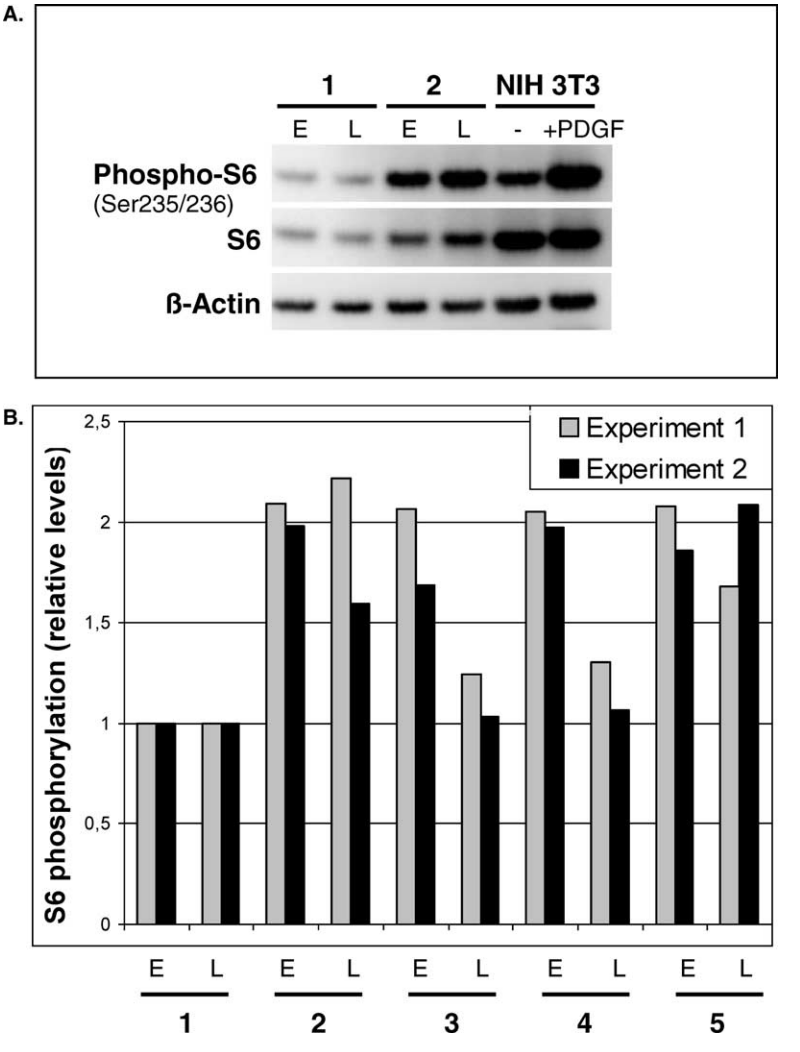

Figure 2. Induction of the ribosomal S6 protein phosphorylation after PPAR agonist treatment *in vivo*. Rat bladder urothelial lysates were analysed by Western immunoblotting for S6 protein abundance (S6) and phosphorylation (phospho-S6, Ser235/236). β-Actin was used as loading control. (A) Representative gel image showing S6 phosphorylation induced by ragaglitazar. For a positive S6 phosphorylation control, commercially available extracts of NIH 3T3 cells with or without platelet-derived growth factor (PDGF) treatment were used (cell signaling). (B) Quantitative analysis of S6 protein phosphorylation in all treatment groups: 1, vehicle; 2, ragaglitazar; 3, rosiglitazone; 4, fenofibrate; and 5, rosiglitazone+fenofibrate. See Table I for drug and dosage descriptions. E, early pool (urothelial lysates from treatment days 1, 2 and 4, total of nine animals); L, late pool (urothelial lysates from treatment days 7, 8, 10 and 15, total of 12 animals). Data from two independent Western blotting experiments are shown in the bar diagram. S6 phosphorylation was quantitated relative to total S6 levels, as described in the Materials and methods. Values above 1 indicate increased S6 phosphorylation, relative to vehicle-treated animals.

A representative gel image of ragaglitazar-induced S6 phosphorylation is shown in Figure 2A. Quantitative analysis showed that ragaglitazar caused a two-fold increase in S6 phosphorylation (Figure 2A and B). Similar findings were done with an antibody against phospho-serine 240/244, i.e. we found that in response to ragaglitazar

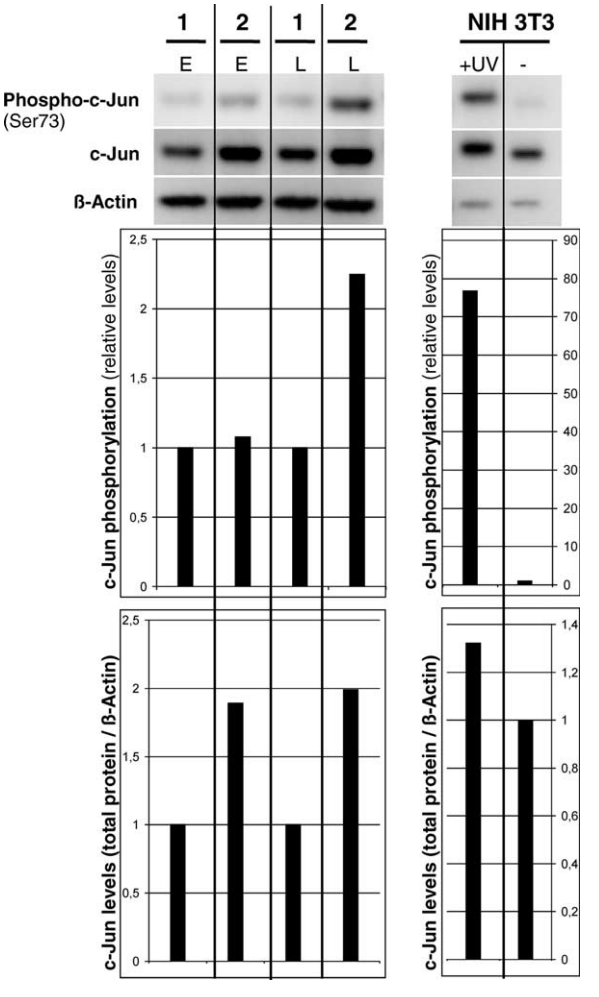

Figure 3. Induction of c-Jun transcription factor phosphorylation after ragaglitazar treatment *in vivo*. Rat bladder urothelial lysates from ragaglitazar-treated animals were analysed by Western immunoblotting for c-Jun protein abundance (c-Jun) and phosphorylation (phospho-c-Jun, Ser73). β-Actin was used as loading control. E, early pool (urothelial lysates from treatment days 1, 2 and 4, total of nine animals); L, late pool (urothelial lysates from treatment days 7, 8, 10 and 15, total of 12 animals). For a positive c-Jun phosphorylation control, commercially available extracts of NIH 3T3 cells with or without ultraviolet light treatment were used (cell signaling). c-Jun phosphorylation was quantitated relative to total c-Jun abundance (top bar diagram); c-Jun abundance was quantitated relative to β-actin levels (bottom bar diagram). Values above 1 indicate increases relative to vehicle-treated animals.

treatment, S6 became phosphorylated on serine 235/236 as well as serine 240/244 (Figure 2 and data not shown). The S6 phosphorylation induced by ragaglitazar was stable, being observed throughout the 15-day experimental period (Figure 2). In contrast, while rosiglitazone and fenofibrate also induced a two-fold increase in S6 phosphorylation, the response was transient and absent through treatment days 7–15 (Figure 2B). Thus, the S6 phosphorylation induced by the dual-acting PPARα/γ agonist ragaglitazar appeared to be qualitatively different from the S6 phosphorylation induced by the specific PPARα and PPARγ agonists rosiglitazone and fenofibrate

(Figure 2B, compare lanes 2L, 3L and 4L). Intriguingly, combination treatment with rosiglitazone and fenofibrate induced a stable S6 phosphorylation, akin to that observed with ragaglitazar (Figure 2B, compare lanes 2L and 5L). Unfortunately, the study design did not allow examination of the statistical significance of these differences.

A representative example of ragaglitazar-induced c-Jun phosphorylation is shown in Figure 3. Oral ragaglitazar dosing caused a two-fold increase in c-Jun phosphorylation in the bladder urothelium (Figure 3). Similar findings were done with an antibody against phospho-serine 63, i.e. c-Jun became phosphorylated on serine 63 as well as 73 in response to ragaglitazar treatment (Figure 3 and data not shown). The ragaglitazar-induced changes were especially prominent through treatment days 7–15 (Figure 3). Thus, the c-Jun phosphorylation was a later change than S6 phosphorylation and Egr-1 production in ragaglitazar-dosed animals. Unfortunately, material was not available to examine c-Jun phosphorylation in rosiglitazone, fenofibrate and combination-treated animals.

To estimate the grade of the ragaglitazar-induced phosphorylation changes, we compared rat urothelial samples with commercially available NIH 3T3 cell lysates. Importantly, all the c-Jun and S6 antibodies were fully cross-reactive between rat and mouse (Cell Signaling, technical information). In PDGF-treated NIH 3T3 cells, a 2.5-fold increase in S6 phopshorylation was observed (Figure 2A), while in ultraviolet light-exposed NIH 3T3 cells, a 75-fold increase in c-Jun phosphorylation was seen (Figure 3). Thus, the two-fold increase in S6 phosphorylation caused by ragaglitazar in the rat urothelium could be classed as a relatively strong change (Figure 2), while the two-fold increase in c-Jun phosphorylation caused by ragaglitazar in the rat urothelium could be classed either as a mild change or, alternatively, a strong change affecting few urothelial cells (Figure 3). Note, however, that ragaglitazar also appeared to cause an increase in total levels of S6 and c-Jun protein, a phenomenon especially pronounced for the c-Jun protein (Figure 3). Thus, because phosphorylation was normalized to total protein levels (see the Materials and methods), the relative phosphorylation ratios reported here (Figures 2 and 3, bar diagrams) were likely slightly underestimated.

Discussion

The classical PPAR-associated tumours are the liver neoplasms seen in rodents after PPARα agonist treatment (Ashby et al. 1994). In hepatocytes, activated PPARα induces expression of genes involved in fatty acid peroxidation and peroxisome biogenesis, and associated generation of reactive oxygen species (Pauley et al. 2002). These biological effects of PPARα activation have been linked mechanistically to cancer development in rodents (Reddy & Rao 1989, Chu et al. 1995, Meyer et al. 2003, Rusyn et al. 2004). While the hepatocarcinogenic effect of PPARα agonist in rodents has been known for a long time, the link between PPARγ agonists and rodent carcinogenicity is more recent (Nahle 2004, Theocharis et al. 2004). Thus, it was recently reported that in rodents specific PPARγ agonists as well as dual-acting PPARα/γ agonists induce a variety of cancers such as hemangiosarcomas, liposarcomas, fibrosarcomas, liver tumours and urinary bladder transitional cell carcinomas. Urinary bladder carcinomas appeared to be especially prevalent for dual-acting PPARα/γ agonists (El-Hage 2004), perhaps reflecting the unique coexpression in the

urothelium of both PPARα and PPARγ isoforms (Guan et al. 1997). Clearly, an understanding of the mechanisms by which dual-acting PPARα/γ agonists induce bladder cancers in rodents, as well as development of early biomarkers for the carcinogenic effect, would aid in the development of this clinically superior class of oral antidiabetics.

Currently no data exists regarding the mechanisms by which PPAR agonists induce bladder cancers in rodents. In fact, paradoxically, some PPARγ agonists have antiproliferative effects in bladder cancer cell lines (Yoshimura et al. 2003a,b). Antiproliferative effects of PPARγ agonists have also been observed in liposarcoma and breast cancer cell lines, but clinical trials of PPARγ agonist failed to demonstrate benefits in those clinical settings (Burstein et al. 2003, Debrock et al. 2003, Nahle 2004). These examples highlight a fact that is becoming increasingly recognized, namely that PPAR agonists can have diametrically opposite effects *in vivo* (in intact animals) and *in vitro* (in cell cultures) (Burstein et al. 2003, Debrock et al. 2003, Michalik et al. 2004, Nahle 2004). As a further complication, the effects of PPAR agonists *in vitro* (in cell culture) appear to be highly dependent on dosage regimen and cell cycle stage (Ledwith et al. 1996, Clay et al. 2001, Chen et al. 2003).

Our laboratory is involved in examining the mechanisms by which ragaglitazar, a dual-acting PPARα/γ agonist, induces bladder cancers in rats. Because we believe that the examples given above demonstrate that it is currently not possible to use cell culture systems to examine the carcinogenic effects of PPAR agonists, we focused on describing the changes that occur *in vivo*, in the urothelium of rats orally dosed with ragaglitazar. Ragaglitazar is known to induce urothelial transitional cell carcinomas in rats after months/years of oral dosing, based on in-house carcinogenicity studies. The present study found that in the rat bladder urothelium, ragaglitazar induced the expression of Egr-1, and the expression as well as phosphorylation of c-Jun and S6 protein, after a few weeks of oral treatment. These proteins were identified based on DNA microarray studies, as well as targeted Western blot-based screening of proteins known to be involved in carcinogenesis.

Two of the proteins found to be regulated by ragaglitazar in the rat urothelium were transcription factors of the immediate/early gene family: c-Jun and Egr-1 (early growth response factor 1). Egr-1 is known by several synonyms, such as Zif268 (Christy et al. 1988), NGFI-A (Milbrandt 1987), TIS8 (Lim et al. 1987) and Krox-24 (Lemaire et al. 1988). Egr-1 is involved in cellular responses to mitogens, growth factors and stress stimuli (Lim et al. 1998, Thiel & Cibelli 2002). Outcomes of Egr-1 induction are cell and context specific, but may include proliferation, differentiation and apoptosis (Liu et al. 1998, Thiel & Cibelli 2002). Furthermore, Egr-1 is important in determining functional neuronal plasticity and learning (Beckmann & Wilce 1997). c-Jun forms part of the AP-1 transcription complex, and is a recognized oncogene (Hartl et al. 2003). For example, the viral v-Jun is sufficient to transform avian fibroblasts, and c-Jun is upregulated in some human tumours (Tiniakos et al. 1994, Hartl et al. 2003). The transcriptional activity of c-Jun is positively regulated by phosphorylation at serines 63 and 73 (Binetruy et al. 1991, Smeal et al. 1991). Thus, our findings of increased total c-Jun levels, as well as of increased phosphorylation of c-Jun at serines 73 (Figure 3) and 63 (data not shown), strongly suggest that the activity of c-Jun is increased in the bladder urothelium of rats following oral ragaglitazar treatment. For Egr-1, the rapid and robust increases in protein levels also suggest that the activity of this protein might be increased in the urothelium of

ragaglitazar-treated rats. However, because activity of Egr-1 is affected by co-regulatory proteins such as NAB1/2 (Russo et al. 1995, Svaren et al. 1996, Sevetson et al. 2000), further experiments are needed to establish the functional status of Egr-1 in the urothelium of ragaglitazar-treated rats.

A number of immediate early genes, including Egr-1 and c-Jun, have been described to be induced in rodent hepatocyte cell cultures (*in vitro*) by PPARα agonists, and this was related to the hepatocarcinogenic effect of PPARα agonists in rodents (Ledwith et al. 1993, 1996, Rokos & Ledwith 1997, Pauley et al. 2002). Also, PPARγ agonists have been described as being able to induce as well as suppress Egr-1 in various settings, and this was related to antiproliferative and anti-inflammatory effects of PPARγ agonist (Okada et al. 2002, Baek et al. 2003, 2004, Cheng et al. 2004). Those studies, together with the present data, suggest that modulation of immediate/early genes is a general feature of PPARα as PPARγ well as agonists, and that the immediate/early gene induction can generally occur in various tissue and cell types, *in vivo* as well as *in vitro*. We asked whether immediate/early gene induction is related to the side-effects of PPAR agonist drugs, i.e. whether immediate/early gene induction might have a biomarker value. For that purpose, our study contained PPAR agonists that are known bladder carcinogens in rodents, as well as PPAR agonists that are not carcinogenic in the rodent urinary bladder (Table I). The doses were equalized to reflect approximately 10–20× expected human exposure. Furthermore, the dose of the bladder carcinogenic compound (ragaglitazar) was in the carcinogenic range, based on previous 2-year carcinogenicity studies (data not shown). Intriguingly, it was found that the bladder carcinogen ragaglitazar induced higher levels of Egr-1 in the urothelium than rosiglitazone and fenofibrate, which are not bladder carcinogens, albeit that the latter agonist is a liver carcinogen in rodents (Table I and Figure 1). A more subtle difference was found for S6 protein phosphorylation: in this case, all compounds induced comparable levels of S6 phosphorylation (two-fold increases; Figure 2), but for the non-carcinogenic compounds the changes were transient (data not shown), while the carcinogenic compound induced permanent changes (Figure 2). The differences between the PPAR compounds in Egr-1 and S6 perturbation could be explained by postulating that acute changes in Egr-1 and S6 are causally related to later urothelial cancer development, with amplitude (Egr-1) as well as duration (S6) of the acute protein changes influencing later cancer development (Murphy et al. 2004). Alternatively, it is possible that the differences we observed between the PPAR agonists in Egr-1 and S6 perturbation were not related to carcinogenic potential, but to PPAR agonist dosage. Finally, it could be argued that the difference between ragaglitazar on one hand and rosiglitazone and fenofibrate on the other hand in Egr-1 and S6 perturbation (Figures 1 and 2) was not related to bladder-carcinogenic potential, but to compound selectivity for the PPARα and PPARγ isoforms (Table I). Thus, examination of a wider range of doses and a larger panel of PPAR agonists, especially dual-acting PPARα/γ agonists without rodent bladder carcinogenic effect, are required to validate Egr-1 and S6 as biomarkers. Importantly, our reference values from orally dosed rats (Figures 1 and 2) might aid in validating cell culture systems for future biomarker and mechanistic studies.

Since PPAR agonists are well known to have biological effects not mediated by the PPAR receptor (Ledwith et al. 1993), we were interested in determining whether the acute induction of Egr-1 levels and S6 phosphorylation caused by ragaglitazar

(Figures 1 and 2) was a PPAR–mediated effect. We observed that the specific agonists rosiglitazone (PPARγ) and fenofibrate (PPARα) differed to the dual–acting ragaglitazar with respect to the amplitude (Egr-1; Figure 1) or duration (S6; Figure 2) of induced protein changes. However, combination treatment of animals with rosiglitazone and fenofibrate produced Egr-1 and S6 protein changes that resembled those observed in ragaglitazar–treated animals (Figures 1 and 2). This suggested that the ragaglitazar–induced Egr-1 and S6 protein changes might be mediated by PPARα and PPARγ isoforms, which are known to be co-expressed in the urothelium. However, the further studies with, for example, specific PPAR antagonists are required to substantiate this hypothesis.

It is currently completely unknown whether the acute c-Jun, Egr-1 and S6 changes are causally involved in later urothelial cancer development. Yet, there is indirect evidence to support that the observed modulation of c-Jun and Egr-1 may be relevant for ragaglitazar-induced bladder cancers: expression of c-Jun correlates to urothelial cancer grade in humans (Tiniakos et al. 1994), and Egr-1 as well as c-Jun have been found to be induced in the urothelium of rats treated with arsenic, another bladder carcinogenic compound (Simeonova et al. 2000). Likewise, the S6 protein is part of the 40S ribosomal subunit, and phosphorylation of the S6 protein at serines 235 and 236, as observed in our study (Figure 2 and data not shown), is known to stimulate translation of so-called 5′TOP mRNAs, which mostly code for proteins required for translation. Thus, phosphorylation of the S6 protein stimulates ribosome biogenesis, and increases the cellular translational capacity, adaptations that are increasingly being recognized as important for cellular transformation (Thomas 2002, Holland 2004, Holland et al. 2004). By virtue of increasing cellular protein content, phosphorylation of the S6 protein is also known to increase cellular size (cause hypertrophy) (Shima et al. 1998, Montagne et al. 1999). Intriguingly, we have found that ragaglitazar does cause hypertrophy in rat bladder urothelial cells after few weeks of oral dosing (Oleksiewicz et al. 2005). Again, the significance for later bladder cancer development of the acute S6 protein phosphorylation and cellular hypertrophy induced by ragaglitazar is unknown, but increased translation as well as hypertrophy have been described as early precancerous changes in the rodent urothelium following dosing with model bladder carcinogens (Lawson et al. 1970, Pai et al. 1974).

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