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ABSTRACT

Some developmental dual-acting PPARα/γ agonists, such as ragaglitazar, have shown carcinogenic effects in the rodent urinary bladder urothelium after months-years of dosing. We examined early (precancerous) changes in the bladder urothelium of rats orally dosed with ragaglitazar, using a newly developed flow cytometric method. Following 3 weeks of oral ragaglitazar dosing, increases in physical size occurred in a generalized fashion in rat bladder urothelial cells, determined by flow cytometry. Protein/DNA measurements confirmed increased protein content of urothelial cells in the bladder, and hypertrophy was observed in the kidney pelvis urothelium by histopathology. In animals exhibiting urothelial hypertrophy, no cell cycle changes were detected in parallel samples of bladder urothelium. Interestingly, urothelial cells from normal rats were found to constitute a unique type of noncycling population, with high G2/M fractions. In summary, our findings showed that in the urothelium of ragaglitazar-treated animals, hypertrophy (increased size and protein content per cell) was an early change, that affected the whole bladder urothelial cell population. The urothelial hypertrophy was primary, i.e., occurred in the absence of similarly pronounced changes in cell cycle distributions. To our knowledge, this is the first report of a direct hypertrophic effect of a PPAR agonist. Urothelial hypertrophy might be a relevant early biological endpoint in mechanistic studies regarding the bladder-carcinogenic effect of PPAR agonists.

Keywords. Dual-acting PPARα/γ agonist; urothelial; carcinogenesis; hypertrophy; flow cytometry; cell cycle.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, belonging to the nuclear hormone receptor superfamily (Dreyer et al., 1992; Kersten et al., 2000; Abumrad, 2004; Nahle, 2004). Generally, PPARs act as intracellular sensors for fatty acids, allowing cells to adjust lipid metabolism (at the level of gene expression) after lipid availability (Abumrad, 2004; Kersten et al., 2000; Michalik et al., 2004). Three PPAR isoforms have been identified, PPARα, PPARβ/δ, and PPARγ (Dreyer et al., 1992). The PPARα and PPARγ isoforms have tissue-specific expression profiles, with highest expression levels in brown adipose tissue, liver, heart, kidney, and skeletal muscle (PPARα), or adipose tissue, colon, leukocytes, and retina (PPARγ). In contrast, PPARβ/δ exhibits a more ubiquitous expression (Kersten et al., 2000; Abumrad, 2004; Michalik et al., 2004; Nahle, 2004).

The importance of PPARs in regulating lipid metabolism and storage have made these nuclear receptors obvious targets for antidiabetic and anti-atherosclerotic drug development. Currently, small molecule agonists targeting either PPARα or PPARγ are on the market (so-called selective PPAR agonists) (Kersten et al., 2000). Selective PPARα agonists (fibrates) have blood lipid-lowering effect, and selective PPARγ agonists (thiazolidinediones) have insulin-sensitizing and blood glucose-lowering effect (Kersten et al., 2000). Regarding development of new PPAR agonists, dual-acting drugs that have agonist activity for both PPARα and PPARγ have been shown to have advantages over selective PPAR agonists for type II diabetes treatment, in humans as well as in animal models (Lohray et al., 2001; Brand et al., 2003; Ebdrup et al., 2003; Larsen et al., 2003; Ye et al., 2003; Saad et al., 2004). However, a high percentage of dual-acting PPARα/γ agonists, as well as some specific PPARγ agonists, have exhibited carcinogenic effect in rodents, especially affecting the urothelium (the epithelial lining of the urinary bladder, ureters, and kidney pelvis) (El-Hage, 2004). In contrast, specific PPARα agonists have not been associated with bladder cancers in rodents.

We believe that examination of very early (precancerous) changes in the urinary bladder urothelium in vivo (in rats) is highly relevant to elucidating the mechanisms by which PPAR agonists cause urothelial cancers in the longer term. Hypertrophy is potentially such an early change, because urothelial hypertrophy has been described as a precancerous change for some model bladder carcinogens in the rat (Lawson et al., 1970; Tiltman and Friedell, 1972). Unfortunately, because of the elastic nature of the urinary bladder wall, hypertrophic changes are difficult to score by histopathological methods. Therefore, in the present study, we developed a new in situ trypsinization method of rat urinary bladders, which allows simultaneous quantitation of hypertrophic (size) as well as hyperplastic (cell division)

Abbreviations: DNA: deoxyribonucleic acid; EDTA: ethylenediaminetetraacetic acid; FITC: fluorescein isothiocyanate; FSC: forward light scatter; PBSΔFCS: phosphate-buffered saline with 1% heat-inactivated fetal calf serum and 0.05% sodium azide; PI: propidium iodide; PPAR: peroxisome proliferator-activated receptor; PPARα: peroxisome proliferator-activated receptor, alpha isoform; PPARγ: peroxisome proliferator-activated receptor, gamma isoform; RNase A: ribonuclease A.

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changes in urothelial cells by flow cytometry. Using the new method, we examined the early changes that occur within 3 weeks in the urinary bladder urothelium of rats orally dosed with a dual-acting PPARα/γ agonist that is known to cause urothelial cancers in rodents after months-years of dosing. Data of this type is currently not available, and will provide valuable biological endpoints for studies of intracellular cell signalling changes during urothelial cancer progression.

Materials and Methods

Oral Treatment of Rats with Ragaglitazar

Male Sprague–Dawley rats were obtained from Charles River Wiga GMbH (Sandhoferweg 7, D-8741 Sutzfeld, Germany). Animals were acclimatized for 8 days prior to the start of the studies, at which point they were 6–7 weeks old, and weighed approximately 150 g. The rats were housed in type IV cages (1800 cm^2 floor area, 31 cm height), with 3–4 animals per cage, in a standard animal unit, using 12-hour-light/dark cycles. Water and feed were adlibitum throughout the animal experiment. All animal experiments were done according to national and company guidelines.

Animals were randomly allocated to 2 groups, a treatment (ragaglitazar) group, and a negative control (vehicle) group. Animals in the treatment group received daily doses of ragaglitazar (arginine salt), by gastric gavage. Animals in the negative control (vehicle) group received daily doses of the fluid vehicle used for ragaglitazar formulation (ultrapure water with 0.6% w/w carboxymethylcellulose sodium and 4% w/w glycerol), by gastric gavage. The gastric gavage volume was the same in ragaglitazar and vehicle groups (5 ml/kg). Two independent animal experiments with this design were performed: In experiment 1, animals were dosed orally for 3 weeks (21 days), using 50 mg/kg ragaglitazar (7 rats) or vehicle (7 rats). In experiment 2, animals were dosed orally for 2 weeks, using 5 mg/kg ragaglitazar (10 rats) or vehicle (10 rats). The ragaglitazar doses were in the carcinogenic range, based on previous 2-year carcinogenicity studies.

Isolation of Bladder Urothelial Cells from Rats by Trypsinization In Situ of the Luminal Bladder Surface

Animals were anaesthetised using a cocktail of fentanyl citrate, fluanisone, and midazolam (79 ug/ml, 2.5 mg/ml, and 1.25 mg/ml, respectively), given by subcutaneous injection at 2 ml/kg. Treatment groups were randomized prior to euthanasia, to avoid the necropsy order influencing the results.

The abdomen was opened by a midline incision, and the urinary bladder was dissected free, but left in situ in the anaestetised rat. A venflon catheter (0.8 × 25 mm) was inserted by puncture through the surgically exposed outer surface of the bladder. The puncture was made caudally in the bladder, close to the neck of the bladder, after which the tip of the catheter was advanced cranially in the bladder lumen, towards the apex of the bladder, and the catheter was finally secured in place with a 3.0 silk suture around the bladder neck. Using a 1-ml tuberculin syringe (this syringe type has no hub dead volume), the bladder lumen was flushed twice, in quick succession, with 0.4 ml freshly prepared trypsin buffer (Hanks-based dissociation buffer, GIBCO Invitrogen, supplemented with 10 mM EDTA and 0.22 wt% trypsin). Then, 0.4 ml trypsin buffer was introduced into the bladder lumen, and left in place for 2 minutes, after which the buffer was withdrawn. This 2-minute trypsinization of the inner bladder surface was repeated up to 9 times, or until the bladder wall became translucent, or until blood started to appear in the trypsinization buffer, whichever occurred first (i.e., a maximum total of 10 × 2 min trypsinizations were done of the inner bladder surface). The volume of trypsin buffer introduced into the bladder lumen was adjusted to avoid overdistension of the bladder, but was 0.4 ml in most cases. The initial 2 bladder flushes, as well as the 10 2-minute-trypsinizations, were pooled in a single conical bottom centrifuge tube kept on crushed ice (0–4°C), containing 5 ml ice-cold PBSΔFCS (phosphate-buffered saline with 1% heat-inactivated fetal calf serum and 0.05% sodium azide). Rats were euthanized by exsanguination while still in full anaesthesia, immediately after the bladder trypsinization was performed.

In all cases, the in situ trypsinization procedure was performed in the morning, to avoid diurnal variation. Because the in situ trypsinization procedure was performed in live rats in deep surgical anaesthesia, and because the metal ion chelating agents in the bladder wash buffer would be expected to stabilize the cell samples, autolysis phenomena were avoided. The collected urothelial cell suspensions were kept strictly on wet ice, and analysed on the flow cytometer within 5 hours.

Flow Cytometric Analysis of Urothelial Cell Size and Cell Cycle Phase Distribution

The cells obtained by trypsinization in situ of the inner urinary bladder surface were spun down (300 g, 10 min at 4°C), and resuspended in ice cold PBSΔFCS. For analysis of cell size, half of the suspension of fresh (nonfixed) cells from an individual rat was added propidium iodide (1 ug/ml final concentration), and run on a FACSCalibur flow cytometer, using Cell Quest Pro software (Becton Dickinson, Brondby, Denmark). Analysis was done with Cell Quest software (Becton Dickinson), using propidium fluorescence to exclude dead cells, and forward light scatter (FSC) to evaluate cell size. Then, 10-um and 15-μm diameter size-reference beads (Molecular Probes, Leiden, The Netherlands) were used to adjust FSC detection settings on the flow cytometer between samples.

For analysis of cell cycle phase distribution, the other half of the cell suspension from an individual rat was fixed by adding a 10-fold excess volume of 70% ethanol, while vortexing. After 10 minutes at room temperature, the cells were spun down, washed once in PBSΔFCS, and resuspended in 50 ug/ml propidium iodide and 2 mg/ml RNAse A in citrate buffer. After a 10-minute incubation on ice in the dark, cell suspensions were analysed on a FACSCalibur flow cytometer, using FL3A/W dotplots to exclude cell doublets, and FL3H as a measure of DNA content, as recommended by the instrument manufacturer (Becton Dickinson). FL3H histogram modelling to estimate the percentage of cells in G0/G1, S, and G2/M was done with ModFit LT software (v2.0, Verity Software House, Topsham, ME, USA).

Isolation and Cell Cycle Analysis of Blood Leukocytes

At necropsy, blood was collected from anaestetised rats from the retroorbital plexus into sodium EDTA tubes. Blood leukocytes were isolated by lysing red blood cells with

ammonium chloride. The blood leukocytes were fixed in 70% ethanol, and subjected to flow cytometric cell cycle analysis as described above for urothelial cells.

Measurement of Protein/DNA Ratios

Lysates of urothelial cells were made by injecting a guanidine lysing solution into the bladder of anaesthetised rats, essentially as described above for isolation of suspensions of whole, live urothelial cells. The lysing solution was 4 M guanidine isothiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM citrate (pH 5.5). The lysing solution (0.5 ml) was left in the bladder lumen for 2 minutes, after which it was withdrawn with the syringe, and stored at −20°C.

Protein concentration in the urothelial lysates was determined using Bradford reagent, according to the manofacturers instructions (BioRad, Herlev, Denmark). DNA concentration in the urothelial lysates was determined using PicoGreen reagent, according to the manufacturers instruction (Molecular Probes).

Immunofluorescent Staining

Cell suspensions obtained by trypsinization in situ of the inner bladder surface were spun onto coated glass slides in a cytospin centrifuge (Shandon, Thermo Electron Corp., Witchford, UK). The glass slides were air-dried thoroughly, and immersed in acetone for 5 minutes at room temperature. The acetone-fixed cytospins were immunofluorescently stained using an anti-rat cytokeratin 17 monoclonal antibody (murine IgG2b, clone CK-E3, Sigma, Vallensbaek Strand, Denmark), which is known to label all differentiation stages of rat urothelial (epithelial) cells, but not other common cell types in the urinary bladder such as smooth muscle. FITC-conjugated goat anti-mouse immunoglobulin was used as secondary antibody (DakoCytomation, Glostrup, Denmark). Then, 1 ug/ml propidium iodide was used as nuclear counterstain.

Histology

For light microscopy, bladders and mid-sagittally cut kidneys were fixed in 4% neutral-buffered formaldehyde, and embedded in paraffin wax. H&E staining of 5-μm tissue sections was performed according to routine histochemical methods.

RESULTS

Trypsinization In Situ Yields Pure Preparations of Rat Urothelial Cells

To examine hypertrophic changes in the rat urinary bladder urothelium by flow cytometry, we developed a new in situ trypsinization method. The efficacy of the in situ trypsinization method was evaluated by histological examination of bladders that had been subjected to the procedure. In bladders from normal (nontreated) rats subjected to the in situ trypsinization procedure, areas with intact urothelium (Figure 1, A and B) were interspersed with denuded areas (Figure 1, C through I). In the denuded areas, all urothelial cell layers were typically removed (Figure 1, C through I). Thus, infusion of the trypsin/chelator solution into the bladder lumen appeared to break the integrity of the urothelial layer at focal positions, and at these entry sites, all layers of the urothelium were loosened (Figure 1E, arrowhead). In the denuded areas, underlying blood vessels were exposed (Figure 1, E through G, arrows), sometimes leading to bleeding into the bladder lumen (Figure 1, H and I, erythrocytes in lumen indicated by double arrows). In the bladder connective tissue, edema and hyperaemia were observed (Figure 1, A through I), in keeping with the macroscopically apparent edema and semitransparecy of stripped bladders (not shown). In short, as hoped, the in situ trypsinization procedure selectively and completely removed all urothelial layers, leaving deeper bladder structures intact. While areas with unaffected urothelium were also observed (Figure 1, A and B), urothelial cell yield was sufficient for FACS analysis in all cases.

Next, to confirm the purity of the cell suspensions produced by in situ trypsinization of the inner bladder surface, cytospins were stained with an antibody against cytokeratin 17 (an urothelial cell marker). Virtually all cells were cytokeratin 17-positive, showing the expected strong cytoplasmic staining pattern (Figure 2). Thus, as expected from the histopathological evaluation, the cell suspensions produced by by in situ trypsinization of the inner bladder surface consisted of pure urothelial cells (Figure 2). The urothelial cell suspensions obtained by our new in situ trypsinization procedure were >80% viable, judged by propidium iodide exclusion (not shown).

Finally, it should be mentioned that in up to one-third of the cases, blood was seen in the trypsin/chelator solution withdrawn from the bladder, typically after the first 4–5 washes, in keeping with the exposure and erosion of blood vessels in the bladder by the trypsin/chelator solution (Figure 1, E through I). While erythrocytes were easily distinguished from the larger urothelial cells by light scatter in flow cytometric analysis (not shown), contamination with leucocytes might interefere with urothelial cell analysis. This was solved by stopping the procedure as soon as blood appeared in the trypsin/chelator solution withdrawn from the bladder, and excluding bladder washes containing blood from the total pool of collected bladder wash fluid.

Ragaglitazar Induces Acute Increases in the Size and Protein Content of Rat Urothelial Cells

Using the in situ trypsinization method and flow cytometry, we found that oral ragaglitazar treatment significantly increased the physical size of the urothelial cells lining the rat urinary bladder (Table 1). Cell infiltration was not seen in the urothelium following ragaglitazar treatment, therefore, the flow cytometric data in Table 1 was not confounded by the presence of inflammatory cell infiltrates in the bladder urothelium (not shown).

To confirm that the increased forward light scatter was due to cellular hypertrophy (increased cellular protein content), urothelial lysates were made from 10 vehicle-treated rats and 10 ragaglitazar-treated rats by injecting a guanidine isothiocyanate buffer into the bladder lumen in situ. As shown in Table 2, the protein/DNA ratio was significantly higher for the ragaglitazar-treated rats than for vehicle-treated rats, confirming that the increased size of bladder urothelial cells observed by flow cytometry (Table 1 and Figure 3) was due to increased protein content on a per cell basis. Finally, to provide morphological support for the protein/DNA

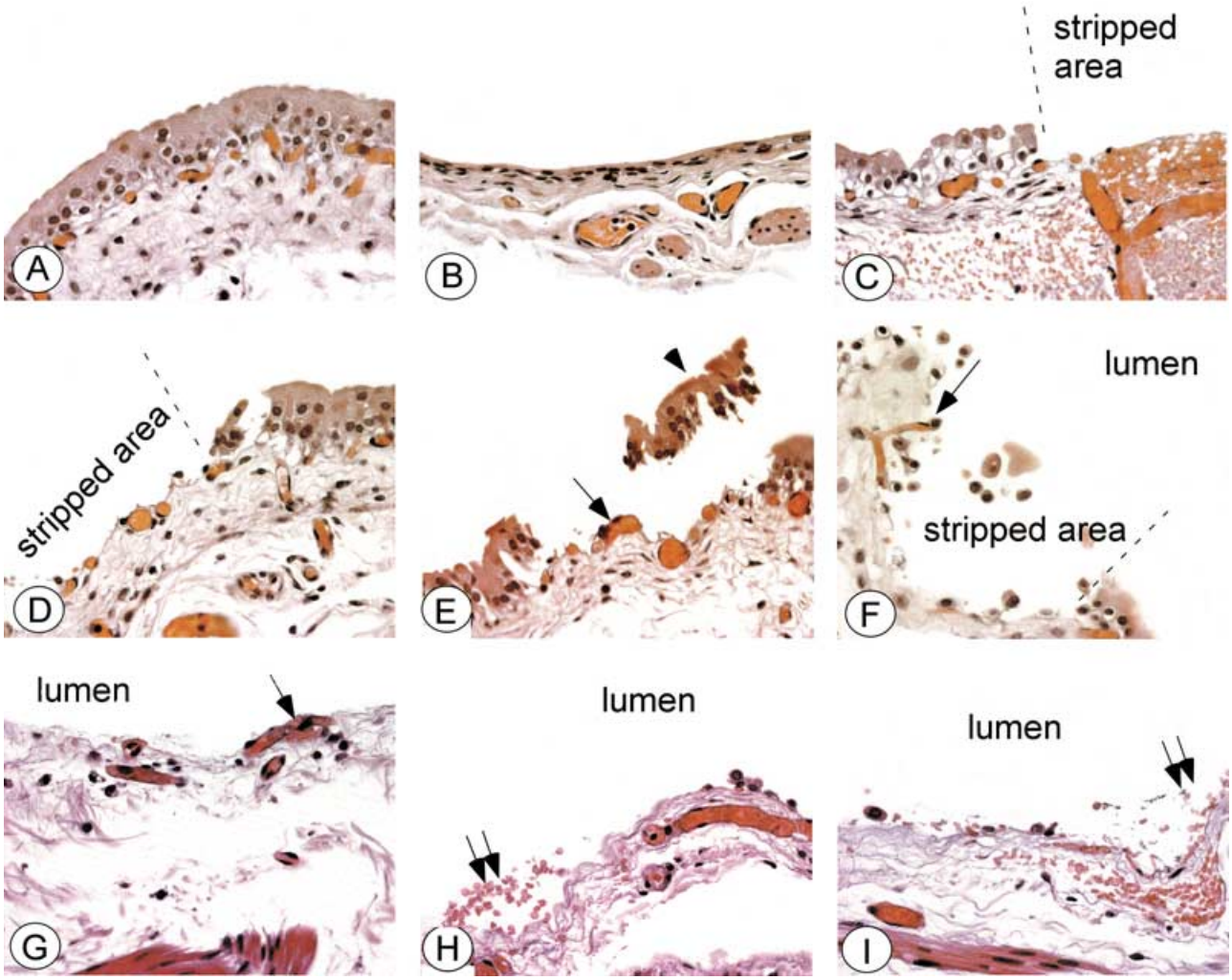


FIGURE 1.—In situ trypsinization removes all bladder urothelial cell layers. All photomicrographs are of in situ trypsinized bladder specimens, stained with H&E. All bladders were are from normal, nontreated rats. The in situ trypsinization procedure was performed on rats in full surgical anaesthesia. (A, B) Areas with intact urothelium, illustrating relaxed (A) and distended (B) situations. (C, D) transition between intact and stripped urothelium. Note that all urothelial layers have been removed in stripped area. (E) loosening of whole urothelial sheet (arrowhead), with exposure of underlying blood vessels (arrow). (F, G) areas completely stripped of urothelial layer, with exposure of underlying blood vessels (arrows). (H, I) bleeding into lumen in areas completely stripped of urothelial layer (erythrocytes in lumen indicated by double arrows).

measurements, samples of kidney pelvis urothelium were examined by light microscopy. As shown in Figure 3, robust hypertrophy was observed in the urothelium lining the kidney pelvis in ragaglitazar-treated rats. Together, the flow cytometric, protein/DNA ratio and histopathological results showed unequivocally that hypertrophy (increased physical size as well as increased protein content per urothelial cell) was an early change in the urothelium of rats orally treated with ragaglitazar.

Absence of Early Cell Cycle Disturbances in the Bladder Urothelium of Ragaglitazar-Treated Animals

It is known that cells increase in size during G1 progression (Zetterberg et al., 1984; Lane et al., 1993), and are reduced in size following cytokinesis. Thus, cell cycle and cell size data would be expected to be correlated. To our knowledge, urothelial cell cycle distribution reference data generated with modern flow cytometric equipment is not available for rats. Therefore, we first examined the normal cell cycle distribution of rat bladder urothelial cells, using paired blood leucocyte samples for comparison. In untreated rats, we observed that $<1\%$ of urothelial cells, and $<0.1\%$ of resting blood leukocytes, had S-phase DNA contents, i.e., both these cell types were, as expected, quiescent (Figure 4, not shown in detail). However, a striking difference was found in the proportion of G2/M cells between these 2 noncycling populations: while the mean G2/M fraction was 0.02% for leukocytes, it was 9.1%, i.e., more than $100\times$ higher, for urothelial cells (Figure 4). These findings suggested that urothelial cells represent a special noncycling cell population, that is characterized by a uniquely high proportion of G2/M cells. Then, we compared bladder urothelial cell cycle distributions of vehicle-treated and ragaglitazar-treated rats. No changes were observed in G0/G1 fractions (not shown), but the ragaglitazar-treated group had a slightly increased S-phase fraction (Table 1). The increased S-phase fraction in

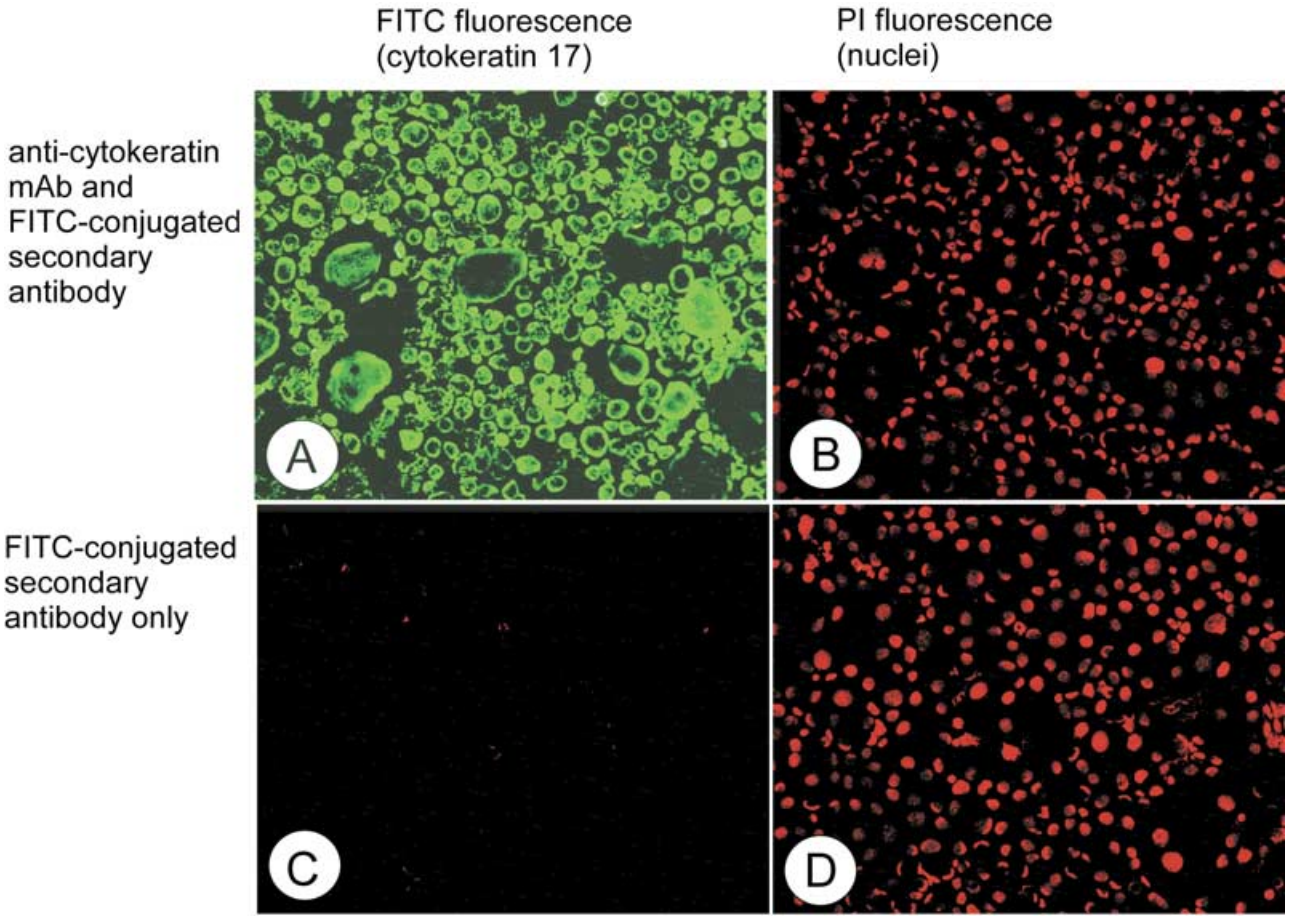


FIGURE 2.—Cell suspensions obtained by in situ trypsinization consist of cytokeratin 17-positive (urothelial) cells. Cytospins of cell suspensions obtained by in situ trypsinization of the inner surface of rat bladder were stained with an anti-cytokeratin 17 monoclonal antibody (A, B, same field of view) or secondary FITC-conjugated antibody only (C, D, same field of view). Nuclei were counterstained with propidium iodide. Stained slides were examined by fluorescence microscopy.

the ragaglitazar-treated group rested on a single animal with a high S-phase percentage (Table 1, rat 59).

DISCUSSION

Ragaglitazar is phenyl propanoic acid derivative, with agonist effect on PPARα as well as PPARγ (Lohray et al., 2001; Ebdrup et al., 2003). While ragaglitazar has proven clinical benefits over specific PPARγ agonists for treatment of type II diabetes (Lohray et al., 2001; Brand et al., 2003; Ebdrup et al., 2003; Larsen et al., 2003; Ye et al., 2003; Saad et al., 2004), it was withdrawn from development after the discovery that in rodents, long-term dosing with ragaglitazar induced transitional cell carcinomas in the urinary bladder. Recently, it has become obvious that a majority (5 of 6 drugs) of developmental dual-acting PPAR agonists, as well as some specific PPARγ agonists, had carcinogenic effects in the bladder of rodents (El-Hage, 2004). In short, carcinogenicity in rodents is a major obstacle to the development of this new, promising class of oral antidiabetics (Kersten et al., 2000; El-Hage, 2004). It might be supposed that an understanding of the mechanisms behind the bladder-carcinogenic effect of dual PPAR agonists could help in the design of new compunds without such serious side effects. Yet, the mechanisms behind PPAR agonist-induced cancers are unknown (El-Hage, 2004); in fact, not a single publication describing PPAR agonist induced bladder cancer has been published to our knowledge.

The urothelium is relatively unique in expressing both PPARα and PPARγ isoforms, albeit with PPARγ expression being highest (Guan et al., 1997). Since dual-acting PPARα/γ agonists appear to be more likely to give bladder cancer than selective PPARγ agonists (El-Hage, 2004), and because carcinogenic effect appears to relate to PPAR agonist potency (El-Hage, 2004), it is possible that the urothelial cancers caused by dual-acting PPARα/γ agonists arise because of regulation of hitherto unknown target genes by PPARα and PPARγ in urothelial cells. Interaction between PPARα and PPARγ regulated genes could be relevant for bladder cancer development, because specific PPARα agonists show no bladder carcinogenic effect in 2-year bioassays in rats and mice. Mechanistic DNA microarray studies performed in our laboratory indicate that gene expression changes do indeed occur within weeks in the urothelium of rats orally dosed with ragaglitazar (not shown). Yet, evaluating the relevance of such early gene expression data to bladder cancer progression is complex, especially as early gene expression changes cannot presently be linked to any biological endpoint, because early (precancerous) urothelial changes have not been

TABLE 1.—Effect of oral ragaglitazar dosing on size and cell cycle distribution of rat bladder urothelial cells.

Treatment	Rat	†Urothelial cell size	‡Urothelial S-phase (%)	‡Urothelial G_2/M-phase (%)
Vehicle	15	485.05	Not done	
	17	434.74	1.29	4.76
	19	553.56	0.39	10.92
	16	473.39	0	11.41
	18	453.56	0.27	14.29
	20	616.30	0.87	4.21
	21	Not done	Not done	4.76
	Mean	502.77	0.56	9.12
	sd	68.84	0.51	4.43
Ragaglitazar (50 mg/kg/day for 3 weeks)	57	614.89	0.64	6.73
	59	544.35	6.38	6.43
	61	580.30	0.56	6.24
	58	571.01	0.63	10.51
	60	552.88	0.45	10,91
	62	752.75	1.45	4.55
	63	577.97	0.76	12.19
	Mean	599.16***	1.55	8.22
	sd	71.39	2.15	2.92

Urothelial cells were obtained by in situ trypsinization of bladders from animals in experiment 1. Urothelial cells from each individual animal were split on two tubes, and analysed for physical size and cell cycle distribution.

†Cell size, geometric means of the forward light scatter (FSC) of the live urothelial cell population. Determined with Cell Quest Pro software, with 700 to 7000 propidium-excluding urothelial cells analysed per animal. Size calibration particles were used to adjust flow cytometer FSC detection settings. The FSC unit is "channel values" (range 0–1023).

‡The cell cycle fractions were determined for parallel samples, by propidium iodide staining, doublet discrimination and cell-cycle-modelling with ModFit LT software.

***The urothelial cell sizes differed significantly between vehicle and ragaglitazar groups ($p = 0.03$, Student's t-test).

described at all in animals dosed with dual-acting PPARα/γ agonists.

It is known that cells traversing G0/G1 increase in size before entering S-phase (Zetterberg et al., 1984; Lane et al., 1993), and it is therefore to be expected, in a nondividing epithelium such as the urinary bladder urothelium (Jost, 1989; Jost et al., 1989), that hypertrophic changes might precede hyperplastic changes during cancer development. Furthermore, while hyperplasia is a more widely accepted type of precancerous change, increased translation (which is closely linked to cell hypertrophy) is being implicated as increasingly important for neoplastic transformation of cells (Fingar and Blenis 2004; Holland et al., 2004). Yet, methodologies for accurate evaluation of size changes in bladder urothelial cells are lacking. Therefore, we developed an in situ trypsinization procedure, which produced pure and viable suspensions of rat bladder urothelial cells, compatible with flow cytometric analysis of cell size as well as cell cycle. Other methods use overnight incubation in chelating solution, leading to stripping of the urothelium as sheets (Hutton et al., 1993; Southgate et al., 1994). In contrast, we believe our method is better suited to flow cytometric studies of cell size, because urothelial cells are isolated as single-cell suspensions, and can analysed within 1 hour, minimizing the risk of preparation artefacts. We validated the quality of the urothelial cell suspensions obtained by the in situ trypsinization method using histopathology and immunofluorescent staining (Figures 1 and 2). We found that the in situ trypsinization method sampled all urothelial layers in the urinary bladder, and yielded very pure and viable preparations of urothelial cells.

TABLE 2.—Effect of oral ragaglitazar dosing on protein/DNA ratios in rat bladder urothelial cells.

Treatment	Mean DNA concentration (μg/ml)	Mean protein concentration (μg/ml)	Mean protein/ DNA ratio	p-value
Vehicle (10 rats)	467.2	962.2	2.3	—
Ragaglitazar 5 mg/kg/day for 2 weeks (10 rats)	403.7	1871.0	4.8	0.048

In animal experiment 2, lysates of urothelial cells were made by injecting a guanidine isothiocyanate buffer into the urinary bladder lumen of anesthetised rats. DNA and protein concentrations in the urothelial cell lysates were determined by PicoGreen and Bradford assays, respectively. The shown values are means of 10 individual rats. p values were determined by Student's t-test.

Applying the in situ trypsinization technique and flow cytometry, we found that oral treatment with ragaglitazar induced a robust and significant increase in the size of urothelial cells (Table 1 and Figure 3). Ragaglitazar did not cause cell infiltration in the bladder urothelium (not shown). Therefore, the flow cytometric data were not confounded by the presence of contaminating cell types. Ragaglitazar also increased protein/DNA ratios in bladder urothelium (Table 2), i.e., the ragaglitazar-induced urothelial cell size increase was associated with increased protein content (true cellular hypertrophy), as opposed to for example disturbed water balance (intracellular edema). Finally, the presence of urothelial hypertrophy was confirmed by light microscopy in the kidney pelvis (Figure 3). Thus, it was unequivocally demonstrated that bladder urothelial hypertrophy is an early and robust change following oral ragaglitazar dosing of male rats. Our flow cytometric method allowed urothelial cell cycle analysis to be performed in parallel with urothelial cell size analysis on the same anaimals (see materials and methods). No significant differences in urothelial S-phase percentages were observed between vehicle-treated and ragaglitazar-treated animals (Table 1).

As a natural part of examining the mechanisms behind ragaglitazar-induced urothelial carcinomas, we also wished to collect reference data on the cell cycle distribution of normal bladder urothelium from nontreated, healthy rats. The urothelium is known to be a noncycling epithelium (Jost, 1989; Jost et al., 1989). Despite being noncycling, the urothelium has a high metabolic activity, and reacts quickly to injury, with a robust wound healing response (Lewis, 2000). In contrast to for example the intestinal epithelium, all urothelial layers are thought to retain proliferative capacity (Kaneko et al., 1984). While flow cytometric cell cycle analysis is routinely performed for example as part of the diagnostic workup of human bladder cancers, no flow cytometric studies have to our knowledge been published about the cell cycle distribution of normal rat urothelial cells. We found that, as expected, normal bladder urothelial cells isolated from young, male Sprague Dawley rats exhibited very low S-phase percentages (<1%). However, urothelial cells were found to have an unexpectedly high percentage of cells residing in G2/M (Figure 4). In fact, the percentage of urothelial G2/M cells was more than 100× above that found in blood leukocytes isolated from the same rats (Figure 4). Others have suggested that a uniquely high proportion of G2/M cells might allow the urothelium to mount a very quick regenerative response following injury to the bladder surface (Kaneko et al., 1984). In this regard, it should be mentioned that we have so far not

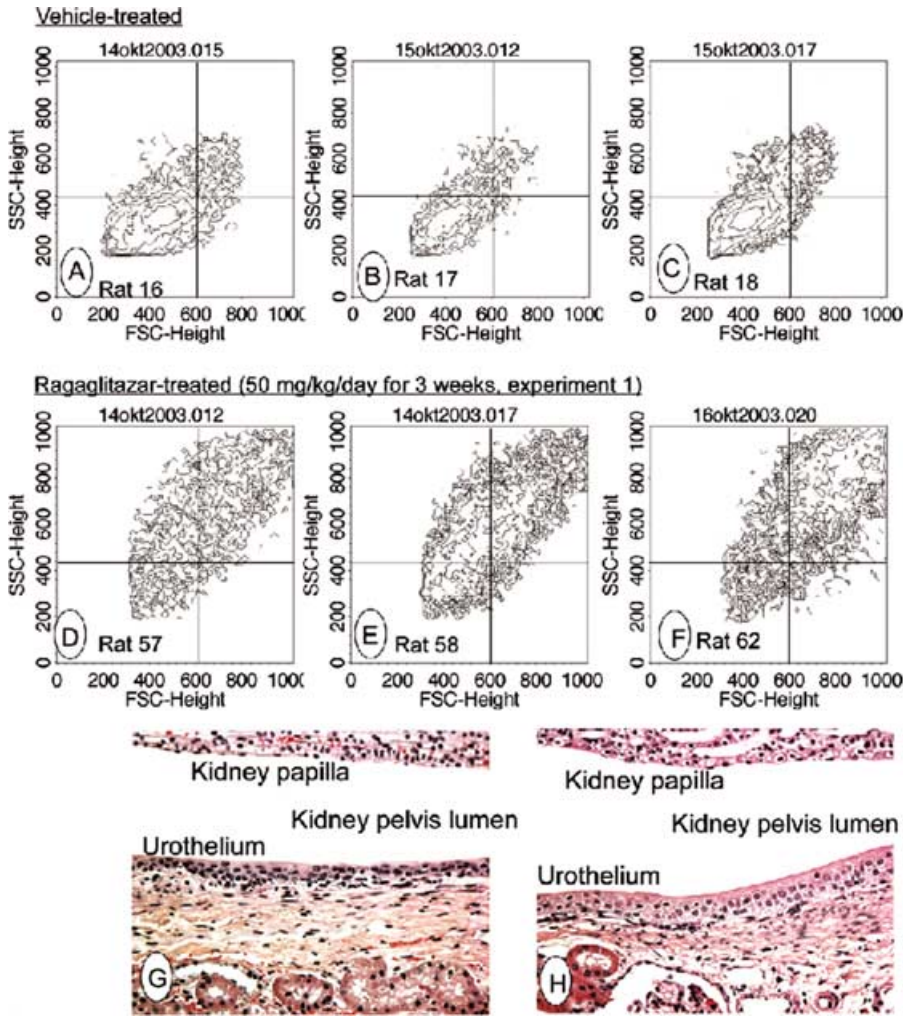
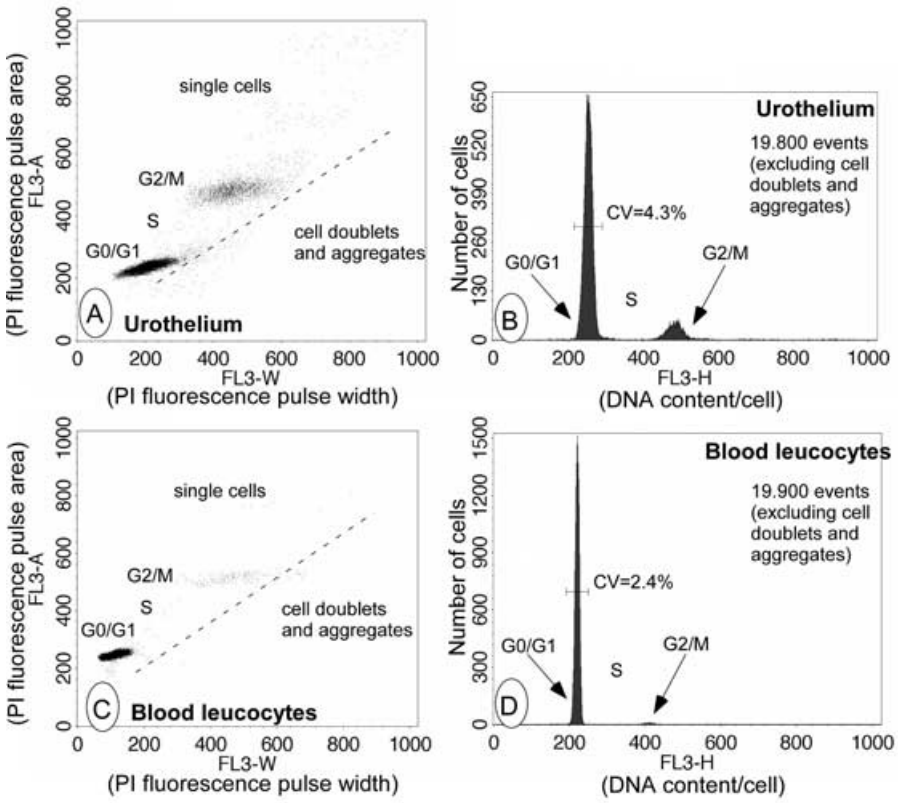


FIGURE 3.—The hypertrophic effects of ragaglitazar affect the whole rat urothelial cell population. From the data shown in Table 1 (animal experiment 1), 3 vehicle-treated rats with average urothelial cell sizes were selected (panels A, B, and C). Also, 3 ragaglitazar-treated rats that responded with strong changes in urothelial cell size were selected (panels D, E, and F). The density plots show the forward light scatter (FSC)/side light scatter (SSC) distribution of live (propidium iodide-excluding) urothelial cells obtained by in situ trypsinization of urinary bladders. The density gradients in each plot show 50% log density differences. FSC is a measure of cell size, and SSC is a measure of cell granularity. Size calibration particles were used to adjust flow cytometer FSC detection settings between samples. From animal experiment 2, histopathological examination was performed of H&E stained sections encompassing the urothelial layer lining the lateral wall of the kidney pelvis. (G) vehicle-treated rat. (H) ragaglitazar-treated rat. Note the urothelial hypertrophy in the ragaglitazar-treated animal.

found significant changes in G2/M percentages after 3 weeks ragaglitazar treatment (Table I).

Urothelial cell size did not provide a perfect discrimination between vehicle- and ragaglitazar-treated groups; some vehicle-treated animals had larger urothelial cells that some ragaglitazar-treated animals (Table 1, for example compare rats 20 and 59). Nevertheless, as the ragaglitazar effect on cell size was significant at the treatment group level (Table 1), we examined the urothelial cell size increase in more detail for strongly responding animals (Figure 3). Interestingly, in strongly responding animals, ragaglitazar appeared to shift the whole population of bladder urothelial cells towards higher size distributions (Figure 3). These findings suggested that the trophic effects of ragaglitazar were diffuse and generalized in the bladder urothelium, with all layers and all cells being affected.

Taken together, our results showed that urothelial cell hypertrophy, affecting all bladder urothelial cells, was a primary response, occurring in the absence of pronounced cell cycle changes (Tables 1 and 2, and Figure 3). This interpretation was supported by BrdU labelling studies (not shown). It is well known that cell size correlates with cellular content of protein and ribosomal RNA (Cohen and Studzinski, 1967; Gaub et al., 1975; Baxter and Stanners, 1978). Furthermore, it has been reported that increased protein synthesis, rather than decreased catabolism, is the most likely explanation for increased cell size (Pai et al., 1974; Baxter and Stanners 1978). Thus, the hypertrophic effect of ragaglitazar in the rat was likely exerted at the level of increased protein synthesis (translation) and ribosome biogenesis. Phosphorylation of the S6 protein is known to regulate cell size, independently of the cell cycle (Shima et al., 1998; Montagne et al., 1999; Holland et al., 2004). Intriguingly, we have found that oral ragaglitazar treatment increases S6 protein phosphorylation in the rat bladder urothelium (F. L. Egerod, accepted for publication).



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Rat	G2/M urothelium (%)	G2/M blood leucocytes (%)
1	4.76	0.02
2	10.92	0.02
3	11.41	0.02
4	14.29	0.03
5	4.21	0.01
Mean:	9.12***	0.02

FIGURE 4.—Rat urothelial cells have an unusually large G2/M fraction. Urothelial cells isolated by in situ trypsinization of rat bladders, and leucocytes isolated by NH_4Cl lysis of whole blood from the same rat, were subjected to flow cytometric cell cycle analysis. A through D, typical results for urothelial cells (A and B) and leukocytes (C and D). The DNA histograms show that urothelial cells have a larger G2/M population than leucocytes (compare B and D). This finding was not an artefact of cell aggregation, because doublet discrimination was performed for all samples, as demonstrated in dot plots A and C. The dashed line in dot plots A and C indicates the division between the expected positions of single cells, and cell aggregates. CV, coefficient of variation, determined by Cell Quest software using the histogram markers shown in B and D. E, summary of cell cycle analysis for 5 rats. Urothelial cells and leukocyte samples were paired (isolated from the same rats). Doublet discrimination was used throughout, and G2/M percentages were obtained by DNA histogram modeling with ModFit LT software. The difference in G2/M percentages between urothelial cells and blood leukocytes was significant ($p = 0.002$, student's t-test).

It is currently completely unknown whether there is a cause-effect relationship between early hypertrophic changes and later tumor development. Yet, some arguments exist in favor of such a hypothesis: First, hypertrophy and increased protein synthesis have been described as early precancerous changes in the bladder of rodents exposed to model bladder carcinogens (Lawson et al., 1970; Tiltman and Friedell, 1972). Second, increased translation (which is closely linked to cellular hypertrophy) has been suggested to play a part during neoplastic transformation of cells (Fingar and Blenis, 2004; Holland et al., 2004). Urothelial hypertrophy also occurs with noncarcinogenic drugs, for example diuretics and carbonic anhydrase inhibitors (Molon-Noblot et al., 1992). Thus, urothelial hypertrophy is not a carcinogen-specific change. Nevertheless, in other cellular systems, it is well established that cell proliferation requires a hypertrophic stimulus followed by a mitogenic stimulus (Coulonval et al., 2000). Thus, in a multistage carcinogenesis model, a substance that chronically stimulates protein synthesis might be hypothetized to create a milieu favorable to the later emergence of neoplastically transformed cells. In short, our current hypothesis is that hypertrophy may be required, but cannot be sufficient, for the emergence of ragaglitazar-induced bladder tumors.

In summary, in a mechanistical study of the carcinogenic effect of ragaglitazar in the rat urothelium, we examined early changes in bladder urothelial cells using a newly developed flow cytometric method. We found that cellular hypertrophy was an early, generalized and primary change, that preceded cellular proliferation. To our knowledge, this is the first study

to show a hypertrophic effect of a PPAR agonists, with other studies describing the exactly opposite (anti-hypertrophic) effects (Palakurthi et al., 2001; Liang et al., 2003). More work is required to show whether the hypertrophic effect of ragaglitazar is mediated by PPARα and γ in the rat urothelium, or whether hypertrophy is a PPAR-independent event, perhaps even mediated by drug metabolites.

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