



Urine acidification has no effect on peroxisome proliferator-activated receptor (PPAR) signaling or epidermal growth factor (EGF) expression in rat urinary bladder urothelium

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Received 1 February 2007; revised 12 June 2007; accepted 16 June 2007

Available online 3 July 2007

Abstract

We previously reported prevention of urolithiasis and associated rat urinary bladder tumors by urine acidification (via diet acidification) in male rats treated with the dual peroxisome proliferator-activated receptor (PPAR) α/γ agonist muraglitazar. Because urine acidification could potentially alter PPAR signaling and/or cellular proliferation in urothelium, we evaluated urothelial cell PPAR α , PPAR δ , PPAR γ , and epidermal growth factor receptor (EGFR) expression, PPAR signaling, and urothelial cell proliferation in rats fed either a normal or an acidified diet for 5, 18, or 33 days. A subset of rats in the 18-day study also received 63 mg/kg of the PPAR γ agonist pioglitazone daily for the final 3 days to directly assess the effects of diet acidification on responsiveness to PPAR γ agonism. Urothelial cell PPAR α and γ expression and signaling were evaluated in the 18- and 33-day studies by immunohistochemical assessment of PPAR protein (33-day study only) and quantitative real-time polymerase chain reaction (qRT-PCR) measurement of PPAR-regulated gene expression. In the 5-day study, EGFR expression and phosphorylation status were evaluated by immunohistochemical staining and *egfr* and *akt2* mRNA levels were assessed by qRT-PCR. Diet acidification did not alter PPAR α , δ , or γ mRNA or protein expression, PPAR α - or γ -regulated gene expression, total or phosphorylated EGFR protein, *egfr* or *akt2* gene expression, or proliferation in urothelium. Moreover, diet acidification had no effect on pioglitazone-induced changes in urothelial PPAR γ -regulated gene expression. These results support the contention that urine acidification does not prevent PPAR γ agonist-induced bladder tumors by altering PPAR α , γ , or EGFR expression or PPAR signaling in rat bladder urothelium.

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Keywords: PPAR; PPAR γ agonist; Urothelial; Urinary bladder; Carcinogenesis; Gene expression

Abbreviations: acaa1, acetyl-coenzyme A acyltransferase 1; acox1, acyl-coenzyme A oxidase 1 palmitoyl; adrp, adipose differentiation-related protein; apoA1, apolipoprotein A1; apoC3, apolipoprotein C3; B2mg, Beta-2 microglobulin; CD36a, CD36 antigen; fasn, fatty acid synthase; glyk, glycerol kinase; hsd11b1, hydroxysteroid 11-beta dehydrogenase 1; insrec, insulin receptor; lpl, lipoprotein lipase; muc-1, mucin 1; pdk4, pyruvate dehydrogenase kinase, isoenzyme 4; pex11a, peroxisomal biogenesis factor 11A; slc2a4, solute carrier family 2, member 4.

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of a nuclear hormone receptor superfamily of ligand-dependent transcription factors. Three PPAR isoforms (α , δ/β , and γ) have been identified with differing tissue distributions (Braissant et al., 1996) and cellular functions (Guan and Breyer, 2001; Cohen, 2005). Agonists against all 3 PPAR isoforms are being investigated as pharmaceutical agents for the treatment of dyslipidemia (PPAR α and δ) or insulin resistance (PPAR γ) (Gervois et al., 2004; Yki-Järvinen, 2004).

Insulin resistance is the hallmark of type 2 diabetes mellitus, a disease that is expected to afflict 29 million, or 7%, of the US population by 2050 (Boyle et al., 2001). Thiazolidinedione (TZD) PPAR γ agonists are effective agents for treating type 2 diabetes due to their ability to increase insulin sensitivity and lower plasma glucose level (Yki-Järvinen, 2004). There are currently two TZD PPAR γ agonists approved for treatment of type 2 diabetes, rosiglitazone and pioglitazone. Current efforts are focused on developing newer PPAR γ agonists along with dual PPAR α/γ agonists that promise to treat not only the hyperglycemia, but also the dyslipidemia often seen in type 2 diabetics (Gervois et al., 2004) while minimizing risk.

A number of TZD and non-TZD PPAR γ and dual PPAR α/γ agonists, including the marketed compound pioglitazone, have been shown to act as non-genotoxic carcinogens in rats, where they induce transitional cell tumors of the urinary bladder and/or renal pelvis (El-Hage, 2005). These findings suggest a relationship between PPAR γ agonism in the urothelium and tumor development; however, the current experimental evidence does not support this contention. For example, the dual PPAR α/γ agonist ragaglitazar induces urothelial cell hypertrophy in male rats treated with a dose known to induce bladder tumors (Oleksiewicz et al., 2005). However, based on *in vitro* studies showing that PPAR γ agonists suppress growth of non-neoplastic and neoplastic human urothelial cells (Nakashiro et al., 2001; Yoshimura et al., 2003a) and induce expression of urothelial differentiation markers (Varley et al., 2004a), it would be expected that PPAR γ agonists would directly inhibit, rather than induce, urothelial proliferation and/or tumorigenesis, suggesting that alternative mechanisms may be underlying the induction of urothelial cell tumor development.

PPAR γ agonist-induced alterations in urine composition, specifically increased endogenous urinary solids (uroolithiasis), are hypothesized to account for the urinary bladder tumors associated with PPAR γ agonist administration (Cohen, 2005). Similarly, urothelial injury secondary to uroolithiasis is the proposed mechanism underlying bladder tumors induced in rats by a number of non-genotoxic agents such as saccharin and melamine (Cohen et al., 1997). Increased urinary precipitate, crystals, and/or calculi cause urothelial necrosis, erosions, and ulcerations, resulting in increased cell proliferation and ultimately carcinoma formation via an epigenetic mechanism. Several factors can affect the burden of endogenous urinary solids, including urine pH. In the case of saccharin, feeding a urine-acidifying diet inhibits formation of both urinary solids and urothelial carcinomas (Cohen et al., 1997). Similar to PPAR γ agonists, sodium saccharin induces bladder tumors in rats, but not mice, with a generally higher incidence in males compared to females.

In support of the uroolithiasis hypothesis and its relationship to urinary tumor development in rats, our laboratories have previously shown that urine acidification prevents formation of rat urinary bladder tumors associated with the dual PPAR α/γ agonist, muraglitazar (Dominick et al., 2006). Oral administration of muraglitazar for up to 2 years caused a dose-dependent increase in urinary bladder tumors in male Harlan Sprague–Dawley rats. Cytotoxicity and proliferation of the ventral

bladder urothelium were observed as early as 1 month followed by development of urinary carcinomas by 9 months in muraglitazar-treated rats fed a normal (non-acidified) rodent diet. Microscopic examination of the urine and the urinary bladder revealed a drug-related increase in urinary solids and crystals after 1 month of treatment. Feeding a diet supplemented with 1% ammonium chloride (NH₄Cl) (acidified diet) resulted in urine acidification (pH \leq 6.5) that, in muraglitazar-treated rats, prevented the drug-related increase in urinary solids. Importantly, no drug-related urothelial cytotoxicity, proliferation, or carcinomas occurred in muraglitazar-treated rats fed the acidified diet. These studies support the hypothesis that PPAR γ agonist-induced alterations in urine composition predispose to urolithiasis and associated urothelial injury, proliferation, and tumor formation.

Whereas urine acidification may have protected rats from muraglitazar-induced bladder tumors by inhibiting the formation of urinary solids, it may well have produced other protective effects. Specifically, diet acidification may have altered urothelial responsiveness to PPAR agonism and/or mitogenic signals such as epidermal growth factor (EGF). EGF receptor (EGFR)-mediated signaling plays an important role in the promotion of rat urinary bladder tumors (Hattori et al., 1998; el-Marjou et al., 2001). In addition, there is considerable evidence for crosstalk between EGFR and PPAR γ . Therefore, the objective of the present work was to evaluate PPAR α , PPAR δ , PPAR γ , and EGF receptor (EGFR) expression, PPAR signaling, and urothelial cell proliferation in the urothelium of rats fed a normal or urine-acidifying diet. To determine whether the urothelial cell response to PPAR γ agonism was altered by urine acidification, the effect of a urine-acidifying diet on PPAR γ agonist-induced gene expression in urothelium was also examined.

Materials and methods

Animals selection and husbandry

Random-bred, barrier-raised, male Hsd:Sprague–Dawley SD[®] rats were obtained from Harlan Laboratories, Frederick, MD, Indianapolis, IN, or Oregon, WI, and acclimated for 1 to 2 weeks prior to the initiation of the studies. The rats were approximately 7 to 10 weeks of age at study initiation and were individually housed in suspended stainless-steel wire-bottom cages in environmentally controlled rooms maintained on a 12-h light–dark cycle and at targeted humidity and temperature ranges of 30–70% and 64–79 °F, respectively. Food [HSD/ Teklad Certified Global Diet #8728C (normal diet) or the normal diet supplemented with 1% ammonium chloride (NH₄Cl) (acidified diet)] and water were provided *ad libitum*. The studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee (IACUC) guidelines. All studies were approved by the Institutional Animal Care and Use Committee prior to initiation and performed in American Association for Laboratory Animal Care (AALAC)-accredited facilities according to governmental guidelines (NRC, 1996).

Experimental design

Thirty-three-day diet acidification study. Rats were randomly assigned to two groups of 30/sex each based on body weights. One group was fed the normal diet and the other was fed the acidified diet for 1 month. The 1-month duration was chosen for this study because previously our studies had shown that sustained urothelial cytotoxicity and proliferation, with a predilection for the

ventral bladder, occurred after treatment with muraglitazar for 1 month. All rats were anesthetized by intraperitoneal administration of sodium pentobarbital. The urinary bladders from the first 15/sex in each group were collected for immunohistochemical evaluation while the bladders from the remaining rats were collected for gene expression evaluation. Following bladder collection, all rats were euthanized by exsanguination while under anesthesia.

Eighteen-day diet acidification study ± 3 days of pioglitazone. Male rats were randomly assigned to 4 groups of 6 each based on body weight. Two groups were fed the normal diet and 2 groups were fed the acidified diet. After 15 days, rats fed each diet were administered either 63 mg/kg/day pioglitazone in 0.5% Methocel or 0.5% Methocel alone by oral gavage for 3 days at a dose volume of 6 mL/kg. Urine pH was measured on freshly voided urine collected on several days prior to dosing and 3 h after dosing on the day of necropsy. After 3 days of dosing, which was considered sufficient for induction of PPAR γ response genes (data not shown), all rats were anesthetized by intraperitoneal administration of sodium pentobarbital. The urinary bladders were collected for gene expression evaluation. Following bladder collection, all rats were euthanized by exsanguination while under anesthesia.

Five-day diet acidification study. Male rats were randomly assigned to 2 groups of 15 rats each based on body weights. One group was fed the normal diet and the other group was fed the acidified diet. Urine pH was measured on urine collected overnight once prior to dosing and at the end of the dosing period. After 5 days, all rats were anesthetized by intraperitoneal administration of sodium pentobarbital. The urinary bladders were collected for gene expression evaluation. Following bladder collection, all rats were euthanized by exsanguination while under anesthesia.

Tissue/sample collection

Tissue harvest was conducted identically for all rats with the following exception. For cell proliferation assessment, 5 non-fasted rats/diet group were injected intraperitoneally with 100 mg/kg bromodeoxyuridine (BrdU) approximately 1 h prior to necropsy. At necropsy, urinary bladders were collected from all rats after achieving a deep plane (Stage IV) of anesthesia via an intraperitoneal injection of sodium pentobarbital. Urinary bladders were emptied of urine by gentle digital expression and subsequently inflated with fixative. Cold 4% paraformaldehyde was used for inflation and fixation of bladders for PPAR α , δ , and γ immunostaining, and 10% neutral-buffered formalin fixation was used for bladders designated for BrdU and EGFR immunostaining. A ligature was placed around the neck of the urinary bladder, and the prostate gland was dissected from the urethra. The urinary bladder was excised in its entirety at the neck and immediately immersed in fixative for 24 to 36 h (4% paraformaldehyde) or 72 h (10% neutral-buffered formalin). After fixation, urinary bladders were transected longitudinally and incised into 2- to 4-mm lengthwise strips, processed, and embedded in paraffin. Two (2) paraffin blocks/urinary bladder, corresponding to the dorsal and ventral aspects, were prepared for each rat. A section of proximal duodenum was also harvested for each rat designated for BrdU analysis and was processed and embedded in paraffin for use as a positive control for the incorporation of BrdU.

Urinary bladder urothelial cell lysate preparation

To obtain urothelial cells, the urinary bladder was dissected away from the prostate and extraneous tissue. After exposing the bladder as outlined above, it was everted onto an applicator stick and dissected from the animal. The bladder was secured to the applicator stick with the use of a ligature, rinsed in RNase-free ice-cold phosphate-buffered saline, and dropped into an RNase-free microfuge tube containing 350 μ L of lysis buffer comprised of Buffer RLT (Qiagen Inc., Valencia, CA) and 2-mercaptoethanol (Sigma-Aldrich). The bladder was vortexed for 1 min at half maximum speed to remove the urothelial cells. To confirm removal of urothelium, bladders were removed from the lysis buffer, placed in formalin, processed, embedded, and stained by hematoxylin and eosin for microscopic examination. The lysis buffer, now containing urinary bladder urothelial cells, was vortexed for an additional 30 s and then forced through a 25-gauge needle approximately 20 times using a 1-mL syringe to

ensure complete lysis of the cells. The microfuge tube was capped and placed on dry ice followed by storage at -80°C .

Total RNA extraction and reverse transcription

Total RNA was extracted from the urothelial cell lysates using RNeasy Micro kits (Qiagen). The RNA concentration in each sample was calculated by measuring absorbance at 260 nm (A260); an RNA concentration ≥ 100 ng/ μ L was necessary for reverse transcription. The purity of each RNA sample was assessed by measuring absorbance at 260 and 280 nm and calculating the A260/A280 ratio, with a ratio between 1.9 and 2.1 indicative of high-purity samples. The integrity of each RNA sample was assessed using an Agilent 2100 Bioanalyzer to compare the 18S and 28S ribosomal RNA peaks.

Quantitative real-time polymerase chain reaction (qRT-PCR)

All qRT-PCR reactions were performed using 7900HT Sequence Detection Systems (Applied Biosystems, Foster City, CA). The specific genes evaluated for each analysis are shown in Table 1.

TaqMan reactions (18- and 33-day studies)

Pre-designed TaqMan assays containing gene-specific PCR primers and TaqMan probes were purchased from Applied Biosystems. Beta-2 microglobulin (B2mg) was used as an internal reference gene. Each reaction contained 2.5–20 ng template cDNA, 900 nM final primer concentration, and 250 nM final TaqMan probe concentration. Reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and the following thermocycling parameters:

- Stage 1: 50°C for 2 min (1 cycle)
- Stage 2: 95°C for 10 min (1 cycle)
- Stage 3: 95°C for 15 sec, then 60°C for 1 min (40 cycles)

The raw fluorescence data were collected and processed using the SDS v2.1 software (Applied Biosystems). The cycle threshold (Ct) values were determined for each target gene and normalized to B2mg. The data were

Table 1
Summary of duration, treatment and detection method utilized for the assessment of gene expression in urothelium from rats fed normal or acidified diet for 5–33 days with and without pioglitazone treatment

Treatment duration	Comparison	Detection method	Genes examined
18 days	Pioglitazone vs. vehicle, normal diet	TaqMan	PPAR α , PPAR γ , PPAR δ , <i>lpl</i> , <i>cd36a</i> , <i>gyk</i> , <i>slc2a4</i> , <i>fasn</i> , <i>hsd11b1</i> , <i>apoA1</i> , <i>apoC3</i> , <i>insrec</i> , <i>pdk4</i> , <i>acaal</i> , <i>acox1</i> , <i>adrp</i> , <i>muc-1</i> , <i>pex11a</i>
18 days	Vehicle, acidified vs. normal diet	TaqMan	<i>lpl</i> , <i>cd36a</i> , <i>acaal</i> , <i>acox1</i> , <i>adrp</i> , <i>pex11a</i>
18 days	Pioglitazone treatment, acidified vs. normal diet	TaqMan	<i>lpl</i> , <i>cd36a</i> , <i>acaal</i> , <i>acox1</i> , <i>adrp</i> , <i>pex11a</i>
33 days	Acidified vs. normal diet	TaqMan	<i>lpl</i> , <i>cd36a</i> , <i>acaal</i> , <i>acox1</i> , <i>adrp</i> , <i>pex11a</i>
5 days	Acidified vs. normal diet	Sybr green	<i>akt2</i> , <i>egf</i>

Applied Biosystems catalog numbers for commercially available TaqMan primer/probe sets are as follows: B2mg: Rn00560865_m1; PPAR α : Rn00566193_m1; PPAR γ : Rn00440945_m1; PPAR δ : Rn00565707_m1; *lpl*: Rn00561482_m1; *cd36a*: Rn00580728_m1; *gyk*: Rn00577740_m1; *slc2a4*: Rn00562597_m1; *fasn*: Rn00569117_m1; *hsd11b1*: Rn00567167_m1; *apoA1*: Rn00562483_q1; *apoC3*: Rn00560743_q1; *insrec*: Rn00567070_m1; *pdk4*: Rn00585577_m1; *acaal*: Rn00560616_m1; *acox1*: Rn00569216_m1; *adrp*: Rn01472318_m1; *muc-1*: Rn01462585_m1; *pex11a*: Rn00585152_m1.

exported into Microsoft Excel for analysis using the comparative Ct method developed by Applied Biosystems (2001) in which differences in gene expression (mRNA) levels in the acidified diet group compared to the normal diet group were reported as relative quantification (RQ) values. RQ values >1 indicate higher expression while values <1 indicate lower expression. Student's *t*-test was used to compare the Δ Ct (Ct of target gene - Ct of B2mg) values of the acidified diet group to the Δ Ct values of normal diet group. Statistically significant differences of $P < 0.05$ were considered biologically relevant if the RQ was ≥ 2.0 or ≤ 0.5 , which represents a 2-fold change in mRNA expression.

Sybr green reactions (5-day study)

Real-time PCR primer sets for *akt2* and *egfr* were designed using Primer Express software (Applied Biosystems). The primer sequences (5' to 3') for *akt2* and *egfr* and the housekeeping genes cyclophilin A and *gapdh* are as follows:

akt2 forward: 5'-GATGTGGGTACCGCGACATC-3'
akt2 reverse: 5'-CTCTTTGGACAAGCCAAAGTCA-3'
egfr forward: 5'-CCCACAGCAAGGCTTCTTCA-3'
egfr reverse: 5'-CACGGCAGCTCCATTCTA-3'
cyclophilin A forward: 5'-GGTCGGCTCTGCTTCGA-3'
cyclophilin A reverse: 5'-CATGAATCCTGGAATAATCTGTGAA-3'
gapdh forward: 5'-CTCAACTACATGGTCTACATGTTCCAGT-3'
gapdh reverse: 5'-GTAGACTCCACGACATACTCAGCAC-3'

Each reaction contained 50 ng template cDNA and 200 nM final primer concentration. Reactions were performed using Sybr Green PCR Master Mix (Applied Biosystems) and the default thermocycling profile:

Stage 1: 50 °C for 2 min (1 cycle)
Stage 2: 95 °C for 10 min (1 cycle)
Stage 3: 95 °C for 15 sec, then 60 °C for 1 min (40 cycles)
Stage 4 (dissociation curve): 95 °C 15 s followed by 60 °C to 95 °C at 2% ramp increase.

The raw fluorescence data were collected and processed using the SDS v2.1 software (Applied Biosystems). The Ct values were determined for each target gene and normalized to cyclophilin A and *gapdh*. The comparative quantification of bladder mucosal mRNA expression from rats fed an acidified versus normal diet was performed using the comparative Ct method developed by Applied Biosystems (2001).

Immunohistochemistry

PPAR α , δ , and γ . Prior to the comparative analyses of urinary bladder PPAR α and γ protein expression in rat urinary bladders in response to urine acidification, optimal methods, including reagent concentrations and staining conditions for each antibody, were developed. Development included assessment of the binding specificity, staining intensity, and cellular distribution as previously reported for each antibody in tissues chosen for their known expression of PPAR α , δ , and γ immunostaining *in vivo* (Braissant et al., 1996). These tissues included normal rat kidney, adipose tissue, and liver for PPAR α immunostaining and rat kidney and adipose tissue for PPAR γ staining. Parallel immunostaining was conducted in urinary bladder urothelium of mice as an additional positive control since PPAR γ has previously been demonstrated in the urothelium of mice (Jain et al., 1998).

Paraffin blocks of dorsal and ventral urinary bladders from 15 male and 15 female rats fed a non-acidified or an acidified diet were sectioned at 5 μ m and immunoperoxidase staining was performed on tissue sections utilizing an avidin-biotin complex procedure. Deparaffinization and antigen retrieval at high temperature was achieved with Declere[®] (Cell Marque, Hot Springs, AR). Endogenous peroxidase activity was quenched by incubating tissue sections in 3% hydrogen peroxide for 10 min. Non-specific binding of reagents was blocked by incubation with sequential changes of avidin and biotin solutions (15 min each) and 10% normal goat serum for 20 min. Serial sections of dorsal and ventral urinary bladder were incubated with primary antibodies, either rabbit

anti-PPAR α [1:5000, PA1-822A, Affinity Bioreagents, Golden, CO], rabbit anti-PPAR δ [1:500, PA1-823, Affinity Bioreagents, Golden, CO], or rabbit anti-PPAR γ [1:5000, PA3-821, Affinity Bioreagents, Golden, CO] for 2 h at 24 °C. Additional negative control slides were produced by substitution of the primary antibody with a non-immune rabbit polyclonal antibody (negative control antibody) utilized at 1:500 on replicate sections of the same tissues. All primary antibodies were diluted in phosphate-buffered saline (PBS, pH 7.2) with 5% bovine serum albumin (BSA). All slides were incubated with biotinylated goat anti-rabbit secondary antibody for 30 min, followed by incubation with the Vectastain R.T.U. ABC Elite Peroxidase Kit (Vector Laboratories, Burlingame, CA) for an additional 30 min. Next, 3,3'-diaminobenzidine (DAB) was applied for 15 min as substrate for the peroxidase reaction. Slides were counterstained with light green, dehydrated, and coverslipped for light microscopic evaluation.

BrdU. Paraffin blocks of dorsal and ventral urinary bladder were sectioned at 5 μ m, and immunoperoxidase staining was performed on sections from 5 male rats/diet group. Deparaffinization was conducted and antigen retrieval achieved using high temperature (Decloaking Chamber Pro, Biocare Medical, Concord, CA) and Tris-buffered saline for 1 min. Non-specific binding of reagents was blocked by incubation with 20% rat serum/5% sheep serum/1% bovine serum albumin for 30 min followed by sequential changes of avidin and biotin solutions (10 min each). Serial sections of dorsal and ventral urinary bladder were incubated with biotinylated sheep anti-BrdU-antibody (1:200; Novus Biologicals, Littleton, CO) for 30 min. The positive control tissues (duodenum) were placed on the same slide as the bladder sections and stained in parallel. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide for 10 min. Antibody-antigen binding was detected by incubation with the Vectastain R.T.U. ABC Elite Peroxidase Kit (Vector Laboratories) for 30 min followed by incubation with DAB (Dako, Carpinteria, CA) for 15 min. Slides were counterstained with hematoxylin, Gills Formula (Vector Laboratories), dehydrated, cleared with xylene and permanently mounted under cover glass.

EGFR (total and phosphorylated). Paraffin blocks from 5 male rats/diet group were sectioned at 5 μ m and mounted on positively charged, coated slides for immunohistochemistry. Sections were deparaffinized and antigen retrieval was conducted using heat-induced epitope retrieval in a pressure cooker using a 1-mM EDTA solution at pH 8. For total EGFR staining, non-specific binding of reagents was blocked by incubation with 20% rat serum/5% donkey serum/1% bovine serum albumin for 30 min followed by sequential changes of avidin and biotin solutions (10 min each). For phosphorylated EGFR staining, non-specific binding of reagents was blocked by incubation with 20% rat serum/5% donkey serum/1% bovine serum albumin for 30 min. Serial sections were incubated with primary antibodies, either sheep anti-total EGFR [1:150, #ab10416, Abcam, Cambridge, MA] or rabbit anti-phosphorylated-EGFR (Tyr1173) 1:50, #sc1235, Santa Cruz Biotechnology, Santa Cruz, CA] for 90 min or 30 min, respectively, at room temperature. The anti-total EGFR primary antibody was followed by biotin-conjugated donkey anti-sheep secondary antibody (#713-065-147, Jackson ImmunoResearch, West Grove, PA). Antibody-antigen binding was detected by incubation with the Vectastain R.T.U. ABC Elite Peroxidase Kit (#PK-7100, Vector Laboratories), followed by incubation with DAB. The phosphorylated EGFR primary antibody was followed by application of the DakoCytomation EnVision Plus anti-Rabbit HRP Labeled Polymer System (#K4003, Dako) and visualized with the DAB Substrate Chromogen System (#K3466, Dako). Slides were counterstained with hematoxylin, then dehydrated through a graded series of alcohols to xylene. Coverslips were mounted using xylene-based mounting media. Slides were evaluated for phosphorylated and total (phosphorylated and unphosphorylated) EGFR. Human tonsil interfollicular lymphocytes were included as a positive control.

Analysis of results

Analysis of PPAR α , δ , or γ protein expression in rat urothelium was conducted using non-statistical methods. Staining intensity and the percentage of urothelial cells from the dorsal and ventral urinary bladder exhibiting positive staining (detected as brown colorimetric staining) were evaluated for each PPAR protein. Staining intensity was graded semi-quantitatively using the following

Table 2

Summary of PPAR α , δ , and γ staining intensity and percentage of positively staining urothelial cells in rats fed non-acidified or acidified diets

Groups and origin of urothelium	Males			Females		
	PPAR α intensity (% cells staining)	PPAR δ intensity (% cells staining)	PPAR γ intensity (% cells staining)	PPAR α intensity (% cells staining)	PPAR δ intensity (% cells staining)	PPAR γ intensity (% cells staining)
<i>Group 1—non-acidified diet</i>						
Urothelium from dorsal urinary bladder	1–3+ (>80%)	1–3+ (>80%)	1–2– (>80%)	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)
Urothelium from ventral urinary bladder	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)
<i>Group 2—acidified diet</i>						
Urothelium from dorsal urinary bladder	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)
Urothelium from ventral urinary bladder	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)	1–3+ (>80%)	1–3+ (>80%)	1–2+ (>80%)

– (negative), \pm (equivocal), 1+ (weak), 2+ (moderate), 3+ (strong), 4+ (intense).

scale: – (negative), \pm (equivocal), 1+ (weak), 2+ (moderate), 3+ (strong), 4+ (intense). Urothelium from the dorsal and ventral aspects of the bladder was assessed separately. In addition, the percentage of urothelial cells demonstrating targeted epitope expression was graded as follows: between 1% and 19% (<19%), between 20% and 39% (<39%), between 40% and 59% (<59%), between 60% and 79% (<79%), and greater than 80% (>80%).

For assessment of nuclear BrdU incorporation, dorsal and ventral urinary bladder and duodenum samples were evaluated on an automated image analysis system, Ariol SL-50 (Applied Imaging Corp.). The Kisight assay was utilized and reconfigured to conduct counts on the transitional epithelial cells of the mucosa for both positive and negative cells. Cells located in the submucosa were excluded. Ten (10) urothelial segments of equal size were placed along the length of each bladder section for analysis (a total of 40

segments/animal). As urinary bladder over-inflation markedly attenuated the bladder epithelium, rendering the enumeration of non-labeled cells inaccurate, a proliferation index was not calculated. Data are therefore expressed as the total number of positive cells from 40 evaluated regions of approximately equal area (0.1 \times 0.04 mm).

Results

Confirmation of urine acidification

In the 5- and 18-day studies, mean urine pH in rats fed the acidified diet ranged from 5.8 to 6.3 compared to a range of 6.8

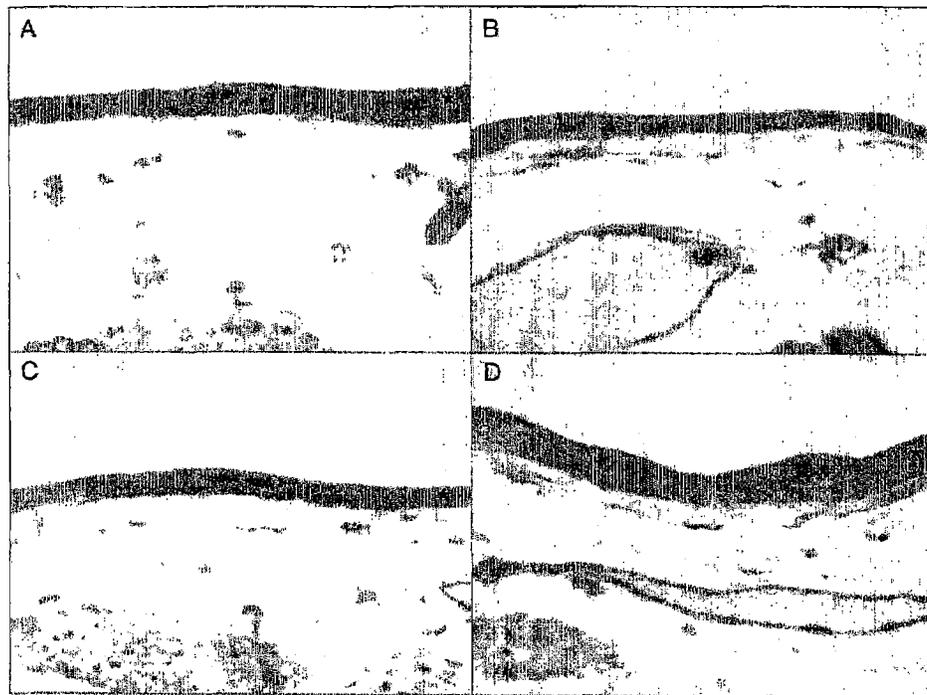


Fig. 1. Representative examples of PPAR α protein expression in dorsal and ventral urinary bladder of male rats fed a normal or acidified diet after 33 days. (A) Normal diet, dorsal urothelium; (B) acidified diet, dorsal urothelium; (C) normal diet, ventral urothelium; (D) acidified diet, ventral urothelium. In all panels, the urothelium is oriented toward the top and marked with an asterisk. Protein expression is detected by the localization of brown staining within cells. Green staining was used as a counterstain to identify cells. Immunohistochemistry, \times 40 magnification.

to 7.6 for rats fed the normal diet (data not shown). Because there was sufficient evidence from these shorter-term studies that diet acidification resulted in urine acidification, urine pH was not measured in the 1-month study.

Effects of urine acidification on PPAR α , δ and γ expression and signaling

PPAR α , δ , and γ protein expression

PPAR α , δ , and γ protein expression in the urothelium from male and female rats fed an acidified or non-acidified diet was evaluated by immunohistochemistry to determine if there were qualitative or semi-quantitative differences in the expression of these proteins based on diet acidification. The results are summarized in Table 2 and illustrated in Figs. 1 and 2. Table 2 outlines PPAR α , δ , and γ protein expression based on cellular location, staining intensity and percentage of positively staining urothelium in the dorsal and ventral bladders of male and female rats fed either a normal or an acidified diet. The results show that there were no differences in the location, staining intensity or percentage of cells expressing PPAR α , δ , or γ proteins in the urothelium of bladders of male and female rats regardless of diet. Weak to strong PPAR α staining was localized in the nuclei of >80% urothelial cells in a random pattern in the basal, intermediate, and superficial cell layers of the dorsal and ventral bladder mucosa (Fig. 1). This range of staining intensity was

detected in the bladder of each animal, regardless of gender, and did not demonstrate a predilection for mucosal cell layer or orientation. Punctate, weak PPAR α staining was observed within the cytoplasm of >80% cells in all dorsal and ventral urothelial cells in an evenly distributed pattern. This pattern did not change regardless of location within the urothelial layer or bladder mucosal orientation. Randomly distributed weak to strong PPAR δ immunostaining was also observed in the nuclei of >80% urothelial cells. This pattern was identical in all bladders from all animals, regardless of orientation, distribution with the urothelial mucosa, gender, or diet (not shown). Finally, PPAR γ was detected as weak to moderate immunostaining in the nuclei of >80% urothelial cells in the dorsal and ventral mucosae of urinary bladders of each male and female rat (Fig. 2). Urothelial cells expressing this range of staining intensity were distributed randomly throughout all mucosal cell layers of the urinary bladder and no differences were observed in the distribution, staining intensities, or percentage of urothelial cells expressing PPAR γ protein regardless of diet.

PPAR target gene expression

PPAR signaling was assessed by examining urothelial expression of genes reported to be transcriptionally activated by PPAR α or γ . Compared to rats fed a normal diet, there were no biologically relevant effects on the urinary bladder urothelial

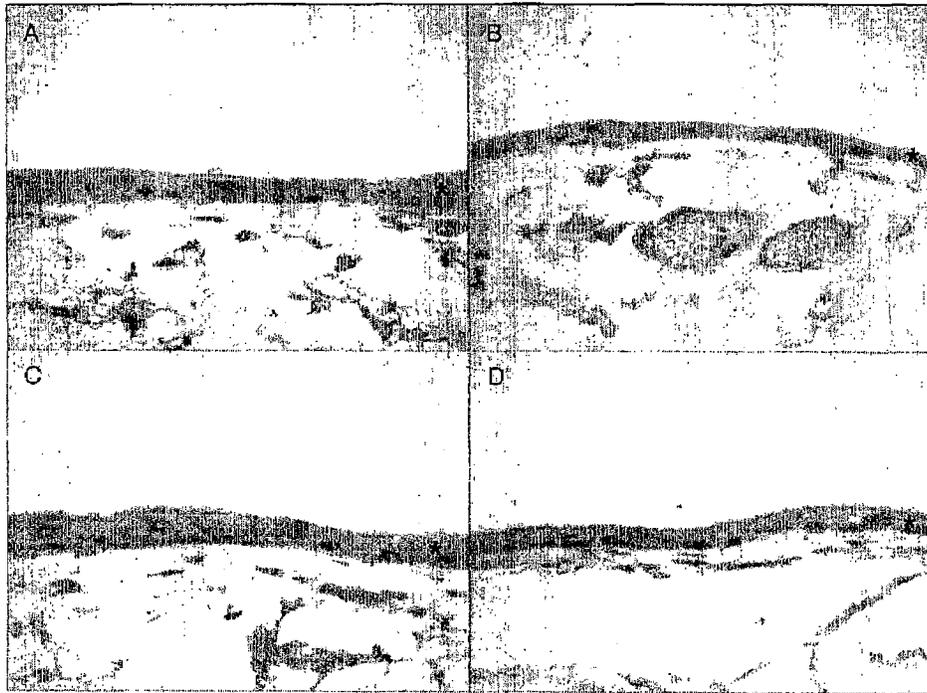


Fig. 2. Representative examples of PPAR γ expression in dorsal and ventral urinary bladder of male rats fed a normal or acidified diet after 33 days. (A) Normal diet, dorsal urothelium; (B) acidified diet, dorsal urothelium; (C) normal diet, ventral urothelium; (D) acidified diet, ventral urothelium. In all panels, the urothelium is oriented toward the top and marked with an asterisk. Protein expression is detected by the localization of brown staining within cells. Green staining was used as a counterstain to identify cells. Immunohistochemistry, $\times 40$ magnification.

Table 3
Effect of urine acidification on urothelial expression of PPAR-transactivated genes

Gene	Treatment duration (days)	Male		Female ^a	
		RQ	P-value	RQ	P-value
<i>lpl</i>	18	1.126	0.559	–	–
	33	0.878	0.214	0.995	0.972
<i>cd36a</i>	18	0.772	0.430	–	–
	33	0.909	0.639	1.384	0.402
<i>acaa1</i>	18	1.080	0.250	–	–
	33	0.992	0.931	1.017	0.735
<i>acox1</i>	18	1.129	0.398	–	–
	33	0.822	0.060	0.965	0.757
<i>adrp</i>	18	0.963	0.782	–	–
	33	0.616	0.0001	1.004	0.963
<i>pex11a</i>	18	1.077	0.704	–	–
	33	1.001	0.994	1.010	0.934

^a Only male rats were used in the 18-day study.

expression of PPAR α - or γ -transactivated genes in male and female rats fed an acidified diet for up to 1 month (Table 3).

egfr/akt2 expression

There were no biologically relevant effects of the acidified diet on *akt2* (0.9-fold control) or *egfr* (0.9-fold control) gene expression in urinary bladder urothelium of male rats.

Pioglitazone-induced PPAR signaling

In rats fed a normal diet, oral pioglitazone administration for 3 days was associated with a significant reduction in PPAR γ (RQ=0.40) gene expression and significant increases in *adrp* (RQ=4.15), *lpl* (RQ=1.97), and *pex11a* (RQ=2.56) gene expression in male rat urinary bladder urothelium compared to vehicle-treated controls (Table 4). Statistically significant changes in *gylk*, *acaa1*, and *acox1* expression did not meet the criteria for biological significance (RQ ≥ 2 or ≤ 0.5). No statistically significant expression changes were noted for PPAR α , PPAR δ , *cd36a*, *slc2a4*, *fasn*, *hsl11b1*, *pdk4*, *muc-1*, or *insrec*. RQ values were not calculated for *apoA1* and *apoC3* due to the low expression level of these genes in both drug-treated and control samples. A subsequent comparison of urothelial gene expression in rats administered pioglitazone and

Table 4

Comparison of PPAR α - and PPAR γ -transactivated gene expression in urinary bladder urothelium of male rats fed a normal diet and administered either pioglitazone or vehicle

Gene	RQ	P-value	Gene	RQ	P-value
PPAR α	0.969	0.875	<i>pdk4</i>	1.522	0.141
PPAR γ	0.400	<0.05	<i>acaa1</i>	1.367	<0.05
PPAR δ	1.183	0.435	<i>acox1</i>	1.785	<0.05
<i>lpl</i>	1.969	<0.05	<i>adrp</i>	4.150	<0.05
<i>cd36a</i>	1.837	0.178	<i>muc-1</i>	0.769	0.122
<i>gylk</i>	0.672	<0.05	<i>pex11a</i>	2.564	<0.05
<i>slc2a4</i>	1.485	0.297	<i>insrec</i>	1.129	0.332
<i>fasn</i>	1.255	0.353	<i>apoA1</i>	*	NA
<i>hsl11b1</i>	1.130	0.711	<i>apoC3</i>	*	NA

*RQ values not calculated due to low expression levels and variability in data from individual samples; NA = not applicable.

Table 5

Comparison of PPAR-transactivated gene expression in urinary bladder urothelium of male rats administered pioglitazone and fed either a normal or urine-acidifying diet

Gene	RQ	P-value
<i>lpl</i>	0.94	0.688
<i>cd36a</i>	0.92	0.841
<i>acaa1</i>	0.95	0.446
<i>acox1</i>	0.92	0.306
<i>adrp</i>	0.87	0.382
<i>pex11a</i>	0.97	0.783

fed either a normal or acidified diet revealed no biologically relevant expression differences in *lpl*, *cd36a*, *acaa1*, *acox1*, *adrp*, or *pex11a* expression (Table 5). Due to insufficient RNA remaining at the completion of the analyses described above, urothelial gene expression in rats fed an acidified diet and administered either vehicle or pioglitazone could not be performed.

Effects of urine acidification on urothelial cell proliferation

BrdU incorporation

The mean number of BrdU-labeled urothelial cells from 5 male rats/group with 40 urothelial segments of approximately equivalent size evaluated per animal did not significantly differ between rats fed a normal diet (10.2 cells) compared to those fed an acidified diet (8.2 cells) (Table 6). Labeling indices (labeled cells as a percentage of total cells) were not calculated due to extensive attenuation of the epithelium resulting from over-inflation.

Effects of urine acidification on total and phosphorylated EGFR expression

Immunohistochemical staining for both phosphorylated and total (phosphorylated and unphosphorylated) EGFR was comparable between rats fed a non-acidified versus an acidified diet (Table 7) for 5 days. Total EGFR staining in the urothelium of urinary bladders from rats fed either an acidified or non-acidified diet was specific to the membrane and varied similarly

Table 6

Summary of BrdU immunostaining in the urothelium of rats fed non-acidified or acidified diets

Diet groups	Animal number	BrdU-positive cells
Non-acidified diet	1101	16
	1102	14
	1103	15
	1104	2
	1105	4
	Mean \pm SD	10.2 \pm 6.6
Acidified diet	2101	10
	2102	8
	2103	12
	2104	1
	2105	10
	Mean \pm SD	8.2 \pm 6.6

Table 7
Summary of total and phosphorylated EGFR staining intensity in the urothelium of rats fed non-acidified or acidified diets

Diet groups	Animal number	Total EGFR	Phosphorylated EGFR
Non-acidified diet	1106	2+	2+
	1107	1+	2+
	1108	2+	2+
	1109	3+	2+
	1110	2+	2+
Acidified diet	2106	1+	2+
	2107	3+	2+
	2108	2+	2+
	2109	2+	2+
	2110	1+	2+

– (negative), ± (equivocal), 1+ (weak), 2+ (moderate), 3+ (strong).

in intensity from least to most intense. Phosphorylated EGFR staining of urothelium demonstrated equally intense membranous staining between the two diet groups (Fig. 3).

Discussion

We previously reported that rat urinary bladder tumors induced by muraglitazar, a dual PPAR α / γ agonist, can be prevented by feeding a urine-acidifying diet (Dominick et al.,

2006). The goal of the present work was to explore the potential for altered PPAR and EGFR expression, PPAR signaling, and/or urothelial cell proliferation as a consequence of urinary acidification. There were no observed effects of urine acidification for up to 1 month on urothelial PPAR α , PPAR γ , or EGFR mRNA or protein levels, EGFR phosphorylation status, expression of PPAR-regulated genes, cell proliferation, or responsiveness to pioglitazone, a PPAR γ agonist known to induce rat urinary bladder tumors. These findings indicate that prevention of muraglitazar-induced bladder tumors by urine acidification is not due to alterations in either urothelial responsiveness to PPAR agonism or mitogenic potential.

The use of single PPAR α and γ agonists to treat dyslipidemia and insulin resistance, respectively, is well documented (Willson et al., 2000). Given that both disorders are often seen in patients with type 2 diabetes, there has been considerable interest in developing dual PPAR α / γ agonists for clinical use (Gervois et al., 2004). However, the propensity of dual PPAR α / γ agonists to induce urinary bladder tumors in rats has proved to be a major hurdle in their development. The FDA's review of 2-year rodent carcinogenicity studies for 6 dual PPAR α / γ agonists has shown that 5 of 6 compounds, as well as the marketed PPAR γ agonist pioglitazone, induce transitional cell tumors of the urinary bladder and/or renal pelvis

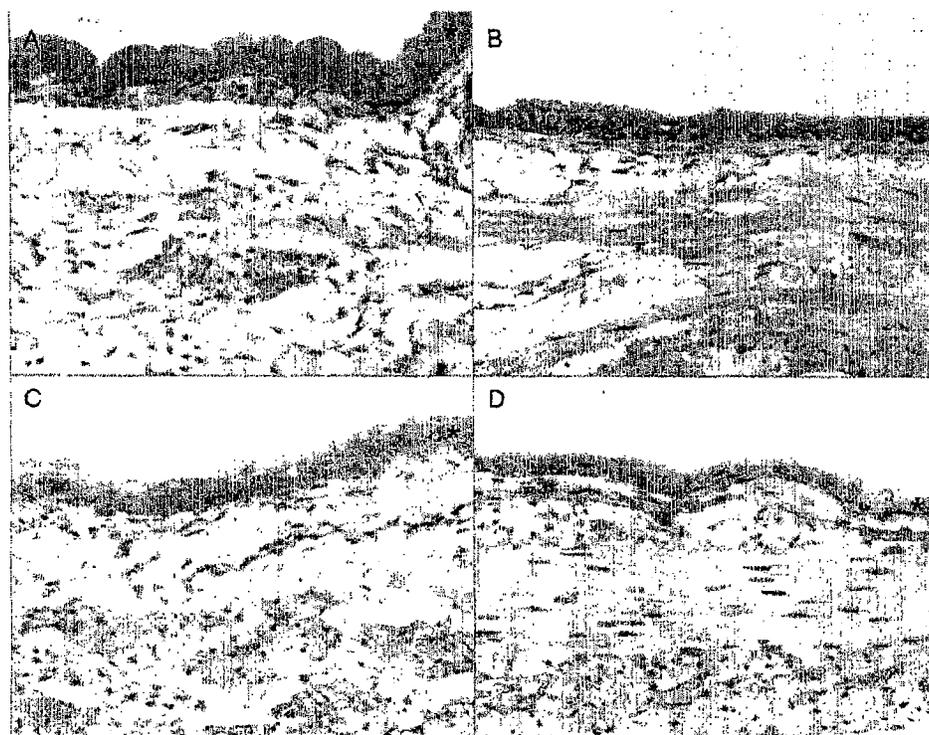


Fig. 3. Representative examples of total or phosphorylated EGFR protein expression in urinary bladder of male rats fed a normal or acidified diet. (A) normal diet, total EGFR protein; (B) acidified diet, total EGFR protein; (C) normal diet, phosphorylated EGFR protein; (D) acidified diet, phosphorylated EGFR protein. In all panels, the urothelium is oriented toward the top and marked with an asterisk. Protein expression is detected by the localization of brown staining within cells. Hematoxylin was used as a nuclear counterstain as a means to identify cells. Immunohistochemistry, $\times 40$ magnification.

in the rat (El-Hage, 2005). These tumors are confined to the ventral bladder and occur predominantly in males (Cohen, 2005). Interestingly, none of the compounds reviewed by the FDA induce bladder tumors in mice of either sex (El-Hage, 2005).

The mechanism underlying PPAR γ agonist induction of urinary bladder tumors has been the subject of considerable debate. A pharmacologic basis for these tumors is suggested by the observation that rat urinary bladder tumors are induced by both TZD and non-TZD agonists (El-Hage, 2005). In recent studies conducted with the dual agonist ragaglitazar, the authors propose that direct urothelial PPAR activation may be a factor in the observed tumorigenic response with that agent (Oleksiewicz et al., 2005; Egerod et al., 2005), although the cellular effects observed in these studies (urothelial hypertrophy, increased Egr-1 expression, and increased c-Jun phosphorylation) are not PPAR-specific. There are a number of compelling arguments against these tumors being induced by direct urothelial PPAR activation. PPAR α and γ are strongly expressed in mouse bladder (Jain et al., 1998), yet there is no report of mouse bladder tumor induction by dual PPAR α/γ agonists or pioglitazone. A direct pharmacologically mediated mechanism is also inconsistent with the clear gender bias in tumor formation since the present work shows identical urothelial PPAR α , δ , and γ expression in male and female rats. Furthermore, the predisposition of the urothelial proliferative response to the ventral aspect of the urinary bladder cannot be ascribed to regional differences in PPAR α or γ expression since the present study also demonstrates that both PPAR α and γ are identically expressed in the dorsal and the ventral bladder urothelium of rats. Lastly, the majority of published studies indicate that PPAR γ activation stimulates differentiation, inhibits growth, and/or induces apoptosis in normal and neoplastic urothelium (Nakashiro et al., 2001; Guan, 2002; Kawakami et al., 2002; Possati et al., 2002; Yoshimura et al., 2003a; Varley et al., 2004a; Kassouf et al., 2006). A minority of reports, including studies by Fauconnet et al. (2002), have suggested PPAR γ activation can potentiate urinary bladder carcinogenesis.

In contrast to direct urothelial PPAR γ activation, drug-induced urolithiasis is currently hypothesized to be the primary mechanism underlying PPAR γ agonist and dual PPAR α/γ agonist induction of rat urinary bladder tumors (Cohen, 2005). This mechanism is consistent with our observations to date and, more importantly, is considered to be rat-specific and not relevant to humans. Male rats are more sensitive than female rats to urinary solid formation and the associated bladder effects (Cohen, 1995), which addresses the gender bias in PPAR γ agonist-induced tumor formation. With respect to the lack of PPAR γ agonist-induced bladder tumors in mice, rats are more sensitive to urinary solid formation than mice (Cohen, 1995). Finally, urinary solids sedimentation account for the ventral bladder-specific distribution of PPAR γ -induced tumors. Our studies support this hypothesis as we have previously reported increases in urinary solids and associated induction of rat urinary bladder tumors by the dual PPAR α/γ agonist muraglitazar, which can be prevented by feeding a urine-acidifying diet (Dominick et al., 2006).

The effects of urine pH on urinary bladder tumor formation are well documented (Cohen et al., 1997). Whereas urine alkalization has a general tumor-promoting effect, urine acidification has generally not been shown to be tumor-protective. For example, Lina and van Garderen-Hoetmer (1999) demonstrated that urine acidification offers no protection against chemically initiated bladder tumors in Wistar rats. Furthermore, mild urine acidification with 1% NH₄Cl does not alter the incidence of spontaneous urinary bladder proliferative lesions in rats (Lina and Kuijpers, 2004). Interestingly, urine acidification has been cited as preventing the induction of urinary bladder tumors by certain non-genotoxic carcinogens (de Groot et al., 1988; Garland et al., 1989; Cohen et al., 2000); however, under these experimental conditions, tumor prevention is generally observed when the underlying carcinogenic mechanism involves agent-induced urine alterations counteracted by urine acidification. For example, monosodium glutamate induces rat bladder tumors through its ability to increase urine pH. Feeding a urine-acidifying diet prevents both urine alkalization and bladder tumorigenesis (de Groot et al., 1988). Sodium saccharin, as well as other sodium salts, induces urinary bladder tumors in rats through the induction of calcium-rich urinary solids and calculi formation (Cohen et al., 2000). Reminiscent of what was seen with muraglitazar, sodium saccharin-induced urinary solid formation and urinary bladder tumors were prevented by urine acidification (Garland et al., 1989). In our previous studies, no differences in cell kinetics or hyperplasia were observed in the urothelium of rats fed a urine-acidifying diet compared to rats fed a normal diet (Dominick et al., 2006; Van Vleet et al., 2007). In the current studies, our data further support that urothelial cell function as measured by PPAR $\alpha/\delta/\gamma$ expression, cell turnover, or microanatomic organization is not altered by mild urinary acidification. Therefore, these data collectively support the hypothesis that urine acidification protects against PPAR γ agonist-induced bladder tumors by preventing drug-induced urine composition changes rather than altering urothelial cell structure or function.

The role of PPAR γ in bladder cancer has been the subject of intense investigation both *in vivo* and *in vitro*. Urothelial cell expression of PPAR γ has previously been demonstrated in mouse, rabbit, and human (Jain et al., 1998; Guan et al., 1997; Nakashiro et al., 2001), although Yoshimura et al. (2003b) failed to detect expression of PPAR γ in human bladder samples, including the urothelium. Despite this discrepancy, PPAR γ expression has been demonstrated in human urinary bladder cancer samples as well as a number of non-neoplastic and neoplastic human urothelial cell lines (Guan et al., 1999; Nakashiro et al., 2001; Possati et al., 2002; Yoshimura et al., 2003b). Importantly, both natural and synthetic PPAR γ agonists suppress growth of these cell lines (Guan et al., 1999; Nakashiro et al., 2001; Yoshimura et al., 2003a) and induce expression of urothelial differentiation markers (Varley et al., 2004a), suggesting that PPAR γ agonism directly inhibits, rather than induces, urothelial cell proliferation and/or tumorigenesis. Furthermore, PPAR γ agonists have been shown to inhibit angiogenesis, a critical process in tumor growth and progression, both *in vivo* and *in vitro* (Margeli et al., 2003). Taken

together, these findings support an antitumorigenic, rather than a pro-tumorigenic, effect of PPAR γ agonists on the urinary bladder urothelium.

Despite the consistency of our current data with a non-pharmacologically mediated mechanism of bladder tumorigenicity by muraglitazar, the possibility remained that regulation of PPAR γ function through EGFR-mediated signaling may play a role in the development of urinary bladder tumors in our studies. EGFR is a receptor tyrosine kinase overexpressed in a number of human cancers, including bladder cancer (Sebastian et al., 2006). Overexpression of EGFR in bladder cancer directly correlates with poor clinical prognosis (Popov et al., 2004; Memon et al., 2006). Data supporting a role for EGF and its receptor as pro-tumorigenic in the urinary bladder has previously been shown in animal models including rats. For example, instillation of EGF in a rat model significantly enhances the tumorigenic effects of *N*-methyl-*N*-nitrosourea-initiated, heterotopically transplanted urinary bladder tumors (Hattori et al., 1998). Conversely, treatment with a specific EGFR inhibitor reduces both the incidence and multiplicity of *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)-induced rat urinary bladder tumors (Hattori et al., 2006). Although the mechanisms underlying tumor development by EGFR activation are currently unknown, crosstalk between EGFR and PPAR γ has previously been demonstrated. Studies evaluating cultured human urothelial cells suggest that PPAR γ agonist-mediated effects on uroplakin gene expression and cellular differentiation are enhanced by concurrent treatment with inhibitors of EGFR, mitogen-activated protein (MAP) kinase kinase (which activates ERK), or PI3K and inhibited by concurrent treatment with EGFR agonists (Varley et al., 2004b). In addition, there are reports of PPAR γ agonist activation of ERK in a PPAR-independent manner (Gardner et al., 2005). Because of the potential interaction between EGFR with PPAR γ , our laboratory evaluated whether urothelial cell EGFR protein or gene expression may be altered by dietary-induced urine acidification. Our analyses showed no differences in EGFR protein or gene expression in rat urothelium regardless of diet suggesting that the absence of effects of urinary acidification on urothelial PPAR γ responsiveness was not related to alterations in urothelial EGFR expression.

In conclusion, the present work indicates that neither PPAR $\alpha/\delta/\gamma$ nor EGFR expression or PPAR signaling pathways are altered in the urinary bladder urothelium of rats fed a urine-acidifying diet. These findings support our conclusion from previous work (Dominick et al., 2006) that urine acidification prevents muraglitazar-induced rat bladder tumors by preventing drug-induced urine composition changes.

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