Subchronic Urinary Bladder Effects of Muraglitazar in Male Rats

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Muraglitazar, a PPARα/γ dual agonist, was dosed orally to rats once daily for 13 weeks to evaluate urinary and urothelial changes of potential relevance to urinary bladder tumorigenesis. Groups of 17 young or aged rats per sex were fed a normal or 1% NH₄Cl-supplemented diet and were dosed with 0, 1, or 50 mg/kg muraglitazar. Lithogenic ions and sediment were profiled from freshly voided urine samples collected 24 h after dosing, and drug exposures were measured. Urinary citrate, oxalate, and epidermal growth factor (EGF) were assayed from 18-h urine collections. Urothelium was assessed by light microscopy, scanning electron microscopy, and BrdU and TUNEL immunohistochemistry. When fed a normal diet, urine pH was higher in males (above 6.5). Urine volume/body weight was greater in females. Urine soluble/total calcium and magnesium and phosphorus/creatinine ratios were lower in male rats fed a normal diet. Urine citrate levels were decreased and oxalate was increased in young male rats treated with 50 mg/kg muraglitazar compared to age/sex/diet-matched controls. No changes in urine sediment were detected 24 h after dosing. In young male rats treated with 50 mg/kg on normal diet, multifocal urothelial necrosis and proliferation were observed, whereas urothelial apoptosis and urine EGF levels were unchanged compared to age/sex/diet-matched controls. Urothelial necrosis and proliferation were not correlated to systemic or urinary drug exposures and were prevented by dietary acidification. These data suggest that muraglitazar-associated changes in urine composition predispose to urothelial cytotoxicity and proliferation in the urinary bladder of young male rats and that urine sediment must be profiled at multiple daily timepoints to fully qualify drug-induced changes in urine composition.

Key Words: PPAR agonist; urinary bladder; tumorigenesis; crystalluria; citrate.

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There are several factors reportedly impacting the sensitivity of rats, particularly male rats, to urolithiasis and resultant urinary bladder tumorigenesis. First, the urine of rats is hyperosmolar and contains considerably higher amounts of calcium, magnesium, and phosphates than are present in mouse urine (Cohen, 1995b). In addition, the earlier onset and greater severity of spontaneous nephropathy in male compared to female rats may be predisposing to urolithiasis. Moreover, male rat urine contains high levels of α2u-globulin, a low–molecular weight protein that may act as a nucleus for crystal formation (Cohen, 2005; Hard, 1995). Expression of α2u-globulin has been shown to be highest in young male rats, suggesting that they might be most susceptible to certain agents causing crysalluria (Roy et al., 1983).

Other key factors that influence the rate of urinary crystal formation are the concentrations of urinary citrate and oxalate as well as urinary pH (Milliner et al., 2001; Schwille et al., 1992). Citrate acts as the primary chelator of urinary calcium, inhibiting urolithiasis, while oxalate promotes the formation of calcium oxalate crystals in urine. It has been demonstrated that the critical threshold pH in rats is 6.5, above which calcium- and magnesium-containing crystal formation readily occurs (Cohen, 1999, 2005). Mildly decreasing urinary pH below 6.5 by dietary acidification inhibits both the formation of urinary solids and urothelial proliferation caused by the administration of sodium saccharin in male rats (Cohen et al., 1995).

Urinary epidermal growth factor (EGF) has also been recognized as a potential stimulus for urothelial proliferation and has been identified at relatively high concentrations in the urine (Cohen, 1995b). In addition, the earlier onset and greater severity of spontaneous nephropathy in male compared to female rats may be predisposing to urolithiasis. Moreover, male rat urine contains high levels of α2u-globulin, a low–molecular weight protein that may act as a nucleus for crystal formation (Cohen, 2005; Hard, 1995). Expression of α2u-globulin has been shown to be highest in young male rats, suggesting that they might be most susceptible to certain agents causing crysalluria (Roy et al., 1983).

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In addition, EGF has been shown to inhibit PPARγ-mediated terminal differentiation in normal urothelial cells (Varley et al., 2004) by impeding translocation of PPARγ into the nucleus and preventing transcriptional changes in target genes (Camp and Tafuri, 1997).

Based on the demonstration of a dose-related increased incidence of urinary bladder tumors in male rats following lifetime treatment with 5–50 mg/kg/day muraglitazar (Simutis et al., 2006), the current study was designed to test the hypothesis that the male rat specificity of the tumorigenic response was a consequence of urothelial cytotoxicity secondary to age- and gender-specific increases in urinary solids.

**MATERIALS AND METHODS**

**Chemicals and Reagents**

Muraglitazar was synthesized by Bristol-Myers Squibb Pharmaceutical Development and analyzed for identity and purity by the Bristol-Myers Squibb Department of Analytical Research and Development (New Brunswick, NJ). Purity of the batch of bulk muraglitazar used in the present study was 99.96%. Muraglitazar dosing formulations (low and high concentrations) were analyzed for stability over 22 days of study storage conditions (refrigerated). Dose formulations (all concentrations) prepared for use at the beginning (week 1 of treatment), middle (week 7 of treatment), and end of the study (week 12 of treatment) were analyzed for muraglitazar content and determined to be acceptable (within 10% of target). Polyethylene glycol 400 (PEG 400), citric acid, sodium azide, and sodium hydroxide (5 N) were purchased from VWR International (West Chester, PA). BrdU was purchased from Sigma-Aldrich (St Louis, MO). Anti-BrdU primary antibody was purchased from Chemicon International (Temecula, CA). Horse anti-mouse secondary was purchased from Vector Laboratories (Burlingame, CA). TUNEL labeling kit (TACS 2 TdT In Situ Apoptosis Detection kit) was purchased from Trevigen Incorporated (Gaithersburg, MD).

**Animal Selection and Husbandry**

Twelve groups of 17 male and female Harlan Hsd:Sprague Dawley SD rats per group were selected for this study. Six groups comprised young animals (approximately 10–11 weeks old at the start of dosing) and six groups comprised aged animals (approximately 10–13 months old at the start of dosing) (Table 1). All animals were individually housed in stainless steel...
Table 2
Muraglitazar-Related Changes in Total and Soluble Calcium/Creatinine Ratios in Fresh-Void Urine in Female Rats 24 h after Dose during Weeks 4 and 12

<table>
<thead>
<tr>
<th>Age</th>
<th>Dose (mg/kg)</th>
<th>Total calcium/creatinine</th>
<th>Soluble calcium/creatinine</th>
<th>Total calcium/creatinine</th>
<th>Soluble calcium/creatinine</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Week 4</td>
<td></td>
<td>Week 4</td>
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<td>Normal diet</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>0</td>
<td>0.588 ± 0.267</td>
<td>0.535 ± 0.192</td>
<td>0.897 ± 0.433</td>
<td>0.812 ± 0.404</td>
</tr>
<tr>
<td>Young</td>
<td>1</td>
<td>0.457 ± 0.251</td>
<td>0.413 ± 0.228</td>
<td>0.563 ± 0.261</td>
<td>0.517 ± 0.206</td>
</tr>
<tr>
<td>Young</td>
<td>50</td>
<td>0.733 ± 0.369</td>
<td>0.577 ± 0.206</td>
<td>0.557 ± 0.300</td>
<td>0.448 ± 0.165*</td>
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<tr>
<td>Aged</td>
<td>0</td>
<td>0.944 ± 0.265</td>
<td>0.853 ± 0.242</td>
<td>0.867 ± 0.406</td>
<td>0.819 ± 0.409</td>
</tr>
<tr>
<td>Aged</td>
<td>1</td>
<td>0.614 ± 0.111*</td>
<td>0.498 ± 0.085**</td>
<td>0.775 ± 0.194</td>
<td>0.687 ± 0.151</td>
</tr>
<tr>
<td>Aged</td>
<td>50</td>
<td>0.659 ± 0.346*</td>
<td>0.483 ± 0.148**</td>
<td>0.679 ± 0.532</td>
<td>0.525 ± 0.223</td>
</tr>
<tr>
<td>Acidified diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>0</td>
<td>1.149 ± 0.437</td>
<td>1.111 ± 0.440</td>
<td>1.142 ± 0.482</td>
<td>1.146 ± 0.510</td>
</tr>
<tr>
<td>Young</td>
<td>1</td>
<td>1.140 ± 0.600</td>
<td>0.918 ± 0.487</td>
<td>0.830 ± 0.404</td>
<td>0.746 ± 0.381</td>
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<tr>
<td>Young</td>
<td>50</td>
<td>1.051 ± 0.485</td>
<td>0.994 ± 0.464</td>
<td>0.925 ± 0.417</td>
<td>0.898 ± 0.425</td>
</tr>
<tr>
<td>Aged</td>
<td>0</td>
<td>1.487 ± 0.351</td>
<td>1.387 ± 0.337</td>
<td>1.204 ± 0.504</td>
<td>1.176 ± 0.485</td>
</tr>
<tr>
<td>Aged</td>
<td>1</td>
<td>1.168 ± 0.202</td>
<td>1.103 ± 0.214</td>
<td>1.002 ± 0.322</td>
<td>0.984 ± 0.311</td>
</tr>
<tr>
<td>Aged</td>
<td>50</td>
<td>0.957 ± 0.402**</td>
<td>0.915 ± 0.369*</td>
<td>1.074 ± 0.428</td>
<td>1.060 ± 0.442</td>
</tr>
</tbody>
</table>

Note. Data are presented as group mean ± SD. Statistically significant from age- and diet-matched control (*p < 0.05, **p < 0.01; Dunnett’s test).
FIG. 3. Soluble/total urinary calcium ratios in young rats. Urine soluble/total calcium excretion ratios (A, B) were assessed in freshly voided urines collected 24 h after dose during weeks 4 and 12 of treatment. There were no clear muraglitazar-related changes 24 h after dose. In young male rats, soluble/total calcium ratios (A) were considerably lower in animals on normal diet than in animals on acidified diet, a profile consistent with greater levels of urinary calcium solids in animals on normal diet. In female rats, there were no differences in soluble/total urinary calcium ratios between animals on normal and acidified diet (B). Data are presented as means ± SD (n = 3–12). Multiple means were compared within each sex per collection period using Tukey’s test. p < 0.05 was considered a statistically significant difference between mean values. Bars without common letters are significantly different from each other.

Toxicokinetics and Urinary Drug Concentrations

Urine samples were collected (chilled) in metabolism cages over a 24-h period from animals on the last six fasted animals per sex per group during weeks 4 and 12 of treatment for analysis of urinary muraglitazar levels. Collection tubes contained 800 mg of citric acid to prevent the potential hydrolysis of muraglitazar metabolites. Samples were stored frozen until processed and analyzed by LC/MS/MS using positive ion electrospray detection. \(^{13}\)C\(_6\)-muraglitazar was used as an internal standard (added to rat urine). Muraglitazar and the internal standard were extracted from a 0.05-ml aliquot of K\(_2\)EDTA rat urine using an automated liquid/liquid extraction method. Samples were reconstituted with mobile phase for analysis. The method was validated for 20–5000 ng/ml in rat urine, and quality control samples of 60, 2000, and 4000 ng/ml were assayed in triplicate in each run.

Steady state area under the concentration-time curve (AUC) exposures for muraglitazar were determined from blood samples collected at 1, 4, 8, and 24 h after dose from the first four animals per sex per group following 10 weeks of treatment. Blood samples were collected from the tail vein in 0.5-ml microtainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) containing K\(_2\)EDTA. Plasma was obtained by centrifugation. Plasma obtained was processed within 1 h and stored frozen prior to analysis by LC/MS/MS using positive ion electrospray detection. \(^{13}\)C\(_6\)-Muraglitazar was used as an internal standard (added to rat plasma). Muraglitazar and the internal standard were extracted from a 0.025-ml aliquot of K\(_2\)EDTA rat plasma using an automated liquid/liquid extraction method. Samples were reconstituted with mobile phase for analysis. The method was validated for 0.05–50 \(\mu\)g/ml in rat plasma, and quality control samples of 60, 2000, and 4000 ng/ml were assayed in triplicate in each run.

Statistical Analyses

Dunnett’s test was used to confirm/rule-out apparent dose-related trends. Tukey’s test was used to assess differences across multiple groups that did not appear to be dose related. Mean urinary citrate, oxalate, and EGF values and labeling indices from the TUNEL and BrdU assays were compared between dose groups and appropriate age- and diet-matched controls by Dunnett’s test (Dunnett, 1955, 1964). Mean systemic (plasma AUC) and urinary (concentration) exposure values, urinary soluble/total calcium ratios, urinary pH, urine volume/body weight ratios, and urinary magnesium and phosphorus excretion values were analyzed statistically using Tukey’s test for multiple comparisons (Mason et al., 1989). p Values < 0.05, resulting from group comparisons, were considered to be significantly different.

RESULTS

To evaluate potential effects of muraglitazar on urinary pH, freshly voided urines were collected at approximately 24 h after dosing from control and drug-treated animals during weeks 4 and 12 of treatment. No clear drug-related effects on urinary pH were noted. In general, male rats (Figs. 1A and 1B) had slightly higher urinary pH than female rats (Figs. 1C and 1D) when receiving normal diet (invariably above 6.5). The highest mean urinary pH values were in both young male and
female rats on normal diet during week 12 of the dosing period. As expected, acidification of the diet with 1% ammonium chloride reduced mean urine pH values to less than 6 in both male and female animals across all acidified diet groups. Muraglitazar treatment had no clear effect on urine volumes in male or female rats fed either a normal or acidified diet. However, mean urine volumes, normalized to animal body weights, were generally higher in control and muraglitazar-treated females than in males during weeks 5 (Figs. 2A and 2C) and 13 (Figs. 2B and 2D) of the treatment period. Although mild acidification of the diet had no effect on urinary volume in male rats, it appeared to slightly reduce urine volumes in females, especially during week 13 of the treatment period.

Urine soluble and total calcium levels were assayed from fresh-void urines collected approximately 24 h after dosing during weeks 4 and 12 of treatment as an indirect evaluation of the level of calcium-containing urinary sediment. Based on these endpoints, there was no apparent muraglitazar-related increase in the amount of calcium-containing urine solids 24 h after dosing. The urine total and soluble calcium/creatinine ratios for fresh-void urine were sporadically decreased in muraglitazar-treated female rats during weeks 4 and 12 of treatment (Table 2). This effect was generally restricted to animals receiving 50 mg/kg of muraglitazar but did not appear to be confined to any age or diet group except that aged male rats did not show this trend. There were no statistically significant differences in the urinary total or soluble calcium/creatinine ratios for fresh-void urine in males (data not shown).

Interestingly, soluble/total urine calcium ratios were significantly different between young male and female rats on normal diet, as well as between young male rats on normal diet and young male rats on acidified diet (Fig. 3). Young male rats on normal diet generally had ratios approximately half of those from male rats on acidified diet or females on either diet (Fig. 3A). In contrast, diet had no effect on the ratio of soluble/total calcium in female rats (Fig. 3B). A similar pattern was observed in the soluble/total urine calcium ratios in aged male and female animals (data not shown). This profile suggests a greater percentage of urinary calcium was present in solid form in control and muraglitazar-treated male rats on normal diet.

Urinary magnesium and phosphorus were assayed in freshly voided urines collected during weeks 4 and 12 of treatment. Urinary magnesium and phosphorus levels were assessed in freshly voided urines collected during weeks 4 and 12 of the treatment period. There were no clear drug-related changes in magnesium/creatinine or phosphorous/creatinine ratios when urines were collected only at 24 h after dose. However, male rats on normal diet had lower levels of urinary magnesium than males on acidified diet (A). A similar pattern was not present in females (B). Male rats, and to a lesser extent female rats, on normal diet had lower urinary phosphorous/creatinine ratios compared to rats on acidified diet (C, D). Data are presented as means ± SD (n = 3–12). Multiple means were compared within each sex per collection period using Tukey’s test. p < 0.05 was considered a statistically significant difference between mean values. Bars without common letters are significantly different from each other.
in any age/sex/diet-matched animals (Figs. 4A and 4B). However, young male animals on the acidified diet had slightly higher (approximately 140% greater) urinary magnesium than young male rats on normal diet (Fig. 4A). In addition, young male rats on normal diet (Fig. 4A) had approximately 70% of the urine magnesium levels of young female rats on normal diet (Fig. 4B) during weeks 4 and 12 of treatment. No apparent diet-related differences in magnesium levels were present in the urine of females (Fig. 4B). A similar pattern was observed in urine magnesium levels from aged animals compared to that observed in young rats of the same sex (data not shown).

No clear muraglitazar-related effects on urinary phosphorus/creatinine ratios were noted in urine of age/sex/diet-matched groups. Interestingly, when compared to animals on normal diet, urinary phosphorus levels appeared to be increased to some degree in all of the age- and sex-matched groups receiving acidified diet (Figs. 4C and 4D) with clearly significant differences in the young males on acidified diet compared to young males on normal diet. In young male rats, the difference in phosphorus levels (Fig. 4C) was approximately three-fold during week 4 and 12 of treatment. No statistically significant differences were observed between any of the young female treatment and diet combinations. A similar pattern was seen in aged animals (data not shown).

Citrate and oxalate levels were assayed in urine samples collected during weeks 5 and 13 of treatment because of the role these anions play in the formation of urinary solids. Muraglitazar treatment decreased urinary citrate levels dose-dependently in young and aged male rats and aged female rats on acidified diet (Figs. 5A, 5B, and 5D), whereas citrate was decreased only at 50 mg/kg/day in the same groups on normal diet. In young and aged 50 mg/kg male rats receiving normal diet, the decreases were statistically significant relative to controls during weeks 5 and/or 13 of treatment. There was a trend (not statistically significant) for reduced urinary citrate in aged females on normal diet at weeks 5 and 13, but not in young female rats on normal diet (Fig. 5C). As expected, acidification of the urine further decreased urinary citrate due to increased proximal tubular uptake (Bushinsky et al., 2001), but, importantly, did not mask muraglitazar-related decreases in urinary citrate.

At weeks 5 and 13, there were generally increased mean urinary concentrations of oxalate in male and female rats dosed with 50 mg/kg/day muraglitazar when compared to age- and diet-matched controls (Figs. 6A–D). The highest levels of urinary oxalate were observed at week 5 in young male rats on normal diet receiving 50 mg/kg/day muraglitazar (Fig. 6A). In comparison, treated and control aged male rats on normal diet

![FIG. 5. Muraglitazar- and diet-related changes in urinary citrate: Muraglitazar treatment dose-dependently decreased urinary citrate concentrations in young male rats and aged male and female rats on acidified diet, whereas citrate was decreased only at 50 mg/kg/day in the same groups on normal diet (A, B, D). Muraglitazar did not affect urinary citrate levels in young female rats (C). Acidification of the urine by dietary acidification further decreased urinary citrate. Data are presented graphically as the mean ± SD (n = 9–12). *Indicates a significant difference from age-, sex-, and diet-matched controls as determined by Dunnett’s test (p < 0.05).]
had generally lower levels of urinary oxalate as compared to treated and control young male rats (Figs. 6A and 6B). Moreover, increases in urinary oxalate concentrations were generally somewhat higher in young male and female animals on normal diet than those on acidified diet (Figs. 6A and 6C). In aged animals, dietary and consequent urinary acidification appeared to have little or no effect on urinary oxalate levels.

Urinary EGF levels were determined from samples of urines collected during weeks 5 and 13 of the treatment period because of the known proliferative effects of urinary EGF on the urinary bladder urothelium (Momose et al., 1991). There were no clear muraglitazar-related effects on urinary EGF concentrations (Fig. 7). The few statistically significant differences in EGF levels (Figs. 7A and 7B) were considered unrelated to treatment due to the absence of any group consistency in the direction of the response.

To examine drug-related urothelial changes, urinary bladders were assessed by light microscopy, SEM, and TUNEL and BrdU immunohistochemistry. Assessment of urinary bladders by light microscopy revealed minimal multifocal urothelial hyperplasia of the urinary bladder in two young males (of 12) receiving 50 mg/kg/day muraglitazar and two aged males (of 12) receiving 50 mg/kg/day muraglitazar and fed a normal diet. In comparison, evaluation of the urothelium by SEM revealed muraglitazar-related urothelial necrosis and hyperplasia in seven (of 12) young male rats given 50 mg/kg/day muraglitazar and fed a normal diet (Figs. 8B and 8C). The close proximity of these lesions suggested that urothelial cytotoxicity (necrosis) preceded the urothelial hyperplasia (i.e., regenerative in nature) (Fig. 8C). Additionally, superficial urothelial erosions and ulcers were evident by SEM in the urinary bladders from some of the young male rats given 50 mg/kg/day muraglitazar and fed a normal diet. In contrast, there were no muraglitazar-related SEM findings in the urinary bladders from young male rats given 50 mg/kg/day muraglitazar and fed an acidified diet (Fig. 8D) or in aged male rats given 50 mg/kg/day muraglitazar and fed either a normal or acidified diet (data not shown). Urinary bladders from 50 mg/kg young (Fig. 8D) and aged male rats on acidified diet were indistinguishable from urinary bladders of untreated young (Fig. 8A) and aged male rats, respectively. Additionally, bladders from young females receiving 50 mg/kg/day muraglitazar and fed a normal diet were unaffected by muraglitazar treatment (Fig. 8E). There were no muraglitazar-related lesions in the urinary bladders from young or aged, male or female rats receiving 1 mg/kg/day muraglitazar.

Urothelial proliferation was evaluated by nuclear BrdU labeling. After 13 weeks of treatment, statistically significant increases in the overall urothelial proliferative index (ventral and dorsal bladder indices combined) were observed in young
male animals receiving 50 mg/kg/day muraglitazar and fed a normal diet (Table 3). The overall response in the five (of 10) 50 mg/kg young male rats demonstrating an increased proliferative index ranged from approximately 6- to 34-fold the mean overall BrdU labeling index of controls, and in two of these animals, correlated with the light microscopic finding of minimal, multifocal urothelial hyperplasia. In all affected animals, the proliferative response tended to be multifocal rather than diffuse in nature. The proliferative response in the ventral urinary bladder mucosa was similar to (one of five animals) or greater than (three of five animals) the response in the dorsal urinary bladder mucosa. Importantly, urinary acidification prevented the increase in urothelial proliferation in young male animals dosed with 50 mg/kg/day muraglitazar, but had no apparent effect on background rates of urothelial proliferation (Table 3).

| DISCUSSION |

In the present study, an indirect mechanism of muraglitazar-induced urothelial cytotoxicity and regenerative hyperplasia involving the formation of urinary solids is supported by the predisposition of young male rats to develop these changes in the presence of systemic drug exposures comparable to those observed in aged male rats and young and aged female rats. Although muraglitazar-related increases in urinary solids were not detected in this study, the distribution of the cytotoxic and proliferative responses in young male rats and the primarily ventral location of the minimal proliferative response in aged male rats were consistent with an etiology of urinary solids. Moreover, the ability to prevent the development of these lesions via mild urinary acidification was consistent with urinary solids as the inciting agent. And finally, when freshly voided urine samples were collected at multiple daily time-points throughout the light and dark cycle in a time course study in rats, large increases in calcium- and magnesium-containing solids were detected in the urine of male Harlan rats given 50 mg/kg/day muraglitazar (Dominick et al., 2006).
It is well known that male rats are particularly susceptible to the development of urinary bladder tumors secondary to urinary solids because of their generally alkaline and highly osmolar urine and due to high urinary concentrations of calcium, phosphorus, and protein (Cohen, 1999). In the present study with muraglitazar, urine pH of rats fed a normal diet was generally higher in males than in females 24 h after dosing, suggesting that male Harlan rats may be more predisposed to urolithiasis than female Harlan rats. Interestingly, control and drug-treated female rats also produced more urine in relation to their body weights than males, a finding supported by the work of others (Schmidt et al., 2001). Therefore, increased urine volume per unit weight in females administered muraglitazar would be expected to account, in part, for the decreased susceptibility of females to urinary bladder tumors if the mode involved increased formation of urinary solids.

In rats fed an acidified diet, the higher levels of urinary phosphorus in males compared to females (Figs. 4C and 4D) suggests that male rats likely have a higher innate burden of urinary solids containing phosphorus than females. For example, when the urine of male rats is acidified (preventing solid formation) via dietary acidification, there is a dramatic increase in urinary phosphorus levels. Despite the increase in urinary phosphorus in animals on acidified diet, urinary solid formation is prevented because the proportion of divalent and trivalent forms of phosphate ions available for complexing with calcium and magnesium are markedly reduced (Brown and Purich, 1992).

It is noteworthy that muraglitazar treatment reduced urinary citrate levels in this study since citrate is known to be the major chelating agent of calcium in urine and to be a powerful inhibitor of urinary crystal formation, growth, and aggregation (Brown et al., 1989; Hamm and Hering-Smith, 2002; Lieske and Coe, 1996; McLean et al., 1990; Pak, 1987; Trinchieri...
TABLE 3
Muraglitazar-Related Changes in BrdU Labeling Indices

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>Diet</th>
<th>Dose (mg/kg/day)</th>
<th>Overall BrdU labeling index (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td>Male</td>
<td>Young</td>
<td>Normal</td>
<td>0</td>
<td>0.13 ± 0.09</td>
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<tr>
<td>Male</td>
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Note. Statistically significant from age- and diet-matched control (*p < 0.05; Dunnett’s test).

FIG. 9. Systemic exposures to muraglitazar. Systemic muraglitazar exposures (Plasma AUC values) were generally similar across age, diet, and gender and increased approximately in proportion to dose. Rats were dosed with 1 (A, males; B, females) or 50 (C, males; D, females) mg/kg/day muraglitazar, and blood samples were collected following a daily dose during week 11 of the treatment period. Data are presented as means ± SD (n = 4). Multiple means were compared within each sex per dose using Tukey’s test. p < 0.05 was considered a statistically significant difference between mean values. Bars without common letters are significantly different from each other.
et al., 2006). Even a mild reduction in urinary citrate excretion is associated with an increased potential for urinary stone formation in humans predisposed to nephrolithiasis (Hamm and Hering-Smith, 2002).

As previously reported, urinary acidification decreased urinary citrate levels in both control and muraglitazar-treated rats (Gordon, 1962; Simpson, 1983), but is inconsequential to urinary solid formation as the lower urinary pH inhibits the formation of urinary solids. A mild decrease in urinary pH can result in a dramatic shift in the proportion of trivalent to divalent urine citrate, the preferred substrate for the sodium dicarboxylate cotransporter 1 (NaDC1) which is the predominant citrate transporter on the brush border membrane of the proximal tubules (Hering-Smith et al., 2000).

The mechanism for PPAR-mediated reductions in urinary citrate may be related to alterations in citrate metabolism. For example, adipocyte differentiation (driven by PPARγ) has been shown to induce the expression of aconitase (Mueller et al., 2002), an enzyme which converts citrate to isocitrate (possibly depleting citrate levels). Additionally, PPARγ agonists stimulate adipogenesis, which utilizes citrate as a substrate for fatty acid synthesis (Ball, 1966). Alterations in citrate levels caused by this mechanism would likely occur in adipose tissue rather than directly in the urinary bladder. In agreement, decreases in serum citrate in rats have also been observed following muraglitazar treatment (Dominick et al., 2006).

In the present study, young male rats (control and muraglitazar treated) had higher baseline levels of urinary oxalate than young female rats (Figs. 6A and 6C). Interestingly, PPARα agonists have been shown to inhibit the expression of alanine/glyoxalate aminotransferase, an enzyme critical in depleting glyoxalate (Genolet et al., 2005; Kersten et al., 2001), the major substrate converted to oxalate by lactate dehydrogenase (LDH) and glycolate oxidase (GaO) in the liver (Poore et al., 1997). It is also noteworthy that PPARα agonists have been shown to increase the activity of LDH and GaO, the major oxalate-producing enzymes in the liver (Sharma and Schwille, 1997).

The urothelial cytotoxic and proliferative responses were observed in male rats (predominantly young males) receiving 50 mg/kg muraglitazar and fed a normal diet. However, the magnitude of these responses did not correlate with the level of muraglitazar exposure in the urine. This is evident from the observation that 50 mg/kg–dosed aged male rats on normal diet had the highest concentrations of muraglitazar in the urine (Figs. 10A–D), whereas 50 mg/kg–dosed young male rats on normal diet had the highest levels of urothelial cytotoxicity and proliferation (Table 3). Additionally, systemic drug exposures were similar between young and aged male and female rats.
and there were no urothelial changes in the urinary bladder of 50 mg/kg–dosed animals on acidified diet in the presence of comparable systemic and urinary drug exposures. Because of age-dependent differences in urine composition, it is expected that there would be age-dependent differences in crystalluria and proliferative changes of the urothelium.

The assessment of urinary bladder mucosa by SEM strongly supported that the urothelial proliferative response was secondary to mucosal injury. That is, the urinary bladder mucosa of high-dose young male animals on normal diet contained areas of multifocal urothelial injury proximal to urothelial hyperplasia. A profile of urothelial necrosis and associated regenerative hyperplasia is consistent with an indirect, non-genotoxic mode of urinary bladder tumorigenesis (Cohen et al., 2001). Importantly, when urinary pH was lowered by dietary acidification, there was no evidence of urothelial necrosis or proliferation by any endpoint as previously reported in cases involving calcium-containing urinary solids and resultant microabrasion of the urothelium (Chappel, 1992).

Although data from this study support the urothelial cytotoxic and proliferative responses observed in male rats were due to alterations in urine composition, no clear increases in crystals were observed in freshly voided urines collected prior to dosing during weeks 5 and 13 of treatment in muraglitazar-treated rats. Considering the toxicokinetic profile of muraglitazar and the diurnal fluctuations in urine pH (Fisher et al., 1989), this single point analysis of urine composition was clearly inadequate for the detection of potential drug-related changes in urinary solids. At the 24-h postdose timepoint in which urines were sampled during the present study, plasma levels of muraglitazar were lowest and the animals were entering a phase of fasting and reduced activity, which results in decreased urinary pH and solids. Therefore, additional investigative studies were undertaken with multiple daily sampling periods during both the light and dark phase to optimize the potential for detecting drug-related changes in urinary solids. In those studies, the 50-mg/kg tumorigenic dose of muraglitazar resulted in a large increase in urinary calcium- and magnesium-containing solids (Dominick et al., 2006).

The bulk of evidence from the present study suggests that muraglitazar-induced urinary bladder cytotoxicity and proliferation occur via an indirect mode of action, likely involving increased urinary solids. The male rat specificity of muraglitazar-induced urinary bladder effects was considered to be the result of a combination of the predisposition of male rats to urinary solid formation and the drug-related prolithogenic changes in urine. Importantly, this mode of action has been shown to be a true threshold phenomenon, and in many agents, it is a rodent-specific phenomenon without significant relevance to human carcinogenic risk (Elcock and Morgan, 1993). Subsequent work by our laboratory fully characterizing the extent and types of urinary solid formation occurring in male rats treated with muraglitazar has provided more conclusive evidence of an indirect mode of urinary bladder tumor development in muraglitazar-treated male rats (Dominick et al., 2006).

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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