Involvement of urinary proteins in the rat strain difference in sensitivity to ethylene glycol-induced renal toxicity

Yan Li, Marie C. McLaren, and Kenneth E. McMartin

Department of Pharmacology, Toxicology, and Neuroscience, Louisiana State University Health Sciences Center, Shreveport, Louisiana

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Li Y, McLaren MC, McMartin KE. Involvement of urinary proteins in the rat strain difference in sensitivity to ethylene glycol-induced renal toxicity. Am J Physiol Renal Physiol 299: F605–F615, 2010. First published June 9, 2010; doi:10.1152/ajprenal.00419.2009.—Ethylene glycol (EG) exposure is a common model for kidney stones, because animals accumulate calcium oxalate monohydrate (COM) in kidneys. Wistar rats are more sensitive to EG than Fischer 344 (F344) rats, with greater COM deposition in kidneys. The mechanisms by which COM accumulates differently among strains are poorly understood. Urinary proteins inhibit COM adhesion to renal cells, which could alter COM deposition in kidneys. We hypothesize that COM accumulates more in Wistar rat kidneys because of lower levels of inhibitory proteins in urine. Wistar and F344 rats were treated with 0.75% EG in drinking water for 8 wk. Twenty-four-hour urine was collected every 2 wk for analysis of urinary proteins. Similar studies were conducted for 2 wk using 2% hydroxyproline (HP) as an alternative oxalate source. Total urinary protein was higher in F344 than Wistar rats at all times. Tamm-Horsfall protein was not different between strains. Osteopontin (OPN) levels in Wistar urine and kidney tissue were higher and were further increased by EG treatment. This increase in OPN occurred before renal COM accumulation. Untreated F344 rats showed greater CD45 and ED-1 staining in kidneys than untreated Wistars; in contrast, EG treatment increased CD45 and ED-1 staining in Wistars more than in F344 rats, indicating macrophage infiltration. This increase occurred in parallel with the increase in OPN and before renal COM accumulation. Like EG, HP induced markedly greater oxalate concentrations in the plasma and urine of Wistar rats compared with F344 rats. These results suggest that OPN upregulation and macrophage infiltration do not completely protect against COM accumulation and may be a response to crystal retention. Because the two oxalate precursors, EG and HP, produced similar elevations of oxalate, the strain difference in COM accumulation may result more so from metabolic differences between strains than from differences in urinary proteins or inflammatory responses.

Calcium oxalate crystals; Tamm-Horsfall protein; osteopontin; macrophage infiltration; inflammation

Calcium Oxalate Monohydrate (COM) is the most prevalent crystalline constituent in kidney stones. Chronic low-dose administration of ethylene glycol (EG) to rats is often used to model stone formation (13, 26). Oxalate metabolism in humans and rats is considered to be similar (25). Chronic hyperoxaluria can induce oxalate crystal aggregate formation in both humans and rats (13, 26), although formation of stones per se in rats is rare and usually requires marked hyperoxaluria. The rat is the most commonly used animal among all animal models for the study of kidney stone disease (28). However, the variant sensitivity to COM accumulation among different rat strains is not widely known. Cruzan et al. (5) reported a strain-related difference in sensitivity to chronic COM accumulation between two rat strains, Wistar and Fischer 344 (F344) rats. Wistar rats treated for 16 wk at EG doses ≥500 mg·kg⁻¹·day⁻¹ show markedly more COM crystal deposition and renal damage than do F344 rats, and the degree of renal tissue damage is very closely related to the amount of COM crystals in kidney tissue in both strains. The mechanism for this strain-related difference is still under investigation. For example, the greater degree of COM-induced renal damage in Wistar rats is not due to a difference in the sensitivities to COM toxicity in the proximal tubule cells between the two rat strains (15). We showed that one factor in the strain-related difference in the deposition of COM crystals is the different supersaturation state of the calcium oxalate in urine, allowing for greater COM crystal formation in the Wistar rat (34).

Supersaturation differences would lead to differences in COM crystal formation, but accumulation of COM in kidney tissues also depends on its binding and uptake by tubular cells. Thus, strain differences in COM accumulation could be regulated by factors that modify COM adherence. In vitro studies indicated that COM crystal adhesion to renal tubular cells can be affected by treatment with the total protein fraction in urine (32), suggesting that variations in urinary proteins might contribute to the strain difference in the accumulation of COM crystals in the kidney. Tamm-Horsfall protein (THP) (36), osteopontin (OPN) (49, 53), and urinary prothrombin fragment 1 (7) are proteins that appear to inhibit COM adhesion and/or aggregation. They have characteristic structures in common, which are long stretches of polyanion peptide chains that can bond with the surface calcium atoms of COM crystals, thus decreasing crystal growth, aggregation of small crystals into larger ones, and crystal adherence to tubular epithelium. THP is an abundant protein in human urine and can be found in the matrix in most kidney stones. This 80-kDa glycoprotein is synthesized mainly in the thick ascending limb of Henle’s loop (2). The role of THP in kidney stone formation remains controversial. Some studies show that the expression and urinary excretion of THP are unchanged in stone-forming patients (46), while other studies yield conflicting results that THP expression levels are either decreased or increased (12), or show a physico-chemically altered THP (23). In one study by Mo et al. (36), formation of spontaneous COM crystals is observed in kidneys of THP knockout mice, which indicates that a THP defect can promote COM accumulation in kidney tissue.

OPN is a glycoprotein that is expressed in different tissues, such as bone, liver, kidney, lung, bladder, pancreas, as well as macrophages (40). In the kidney, OPN is detected in the thick and thin ascending limbs of the loop of Henle, distal tubules,
and macula densa. OPN is a strong inhibitor of the nucleation, growth, and aggregation of COM crystals in vitro. The OPN levels normally present in human urine should be sufficient to inhibit COM crystallization in vitro. Studies in OPN also direct calcium oxalate crystallization to calcium oxalate dihydrate crystals (42), which significantly decreases the adhesion of renal tubular cells compared with COM crystals. COM crystals are observed to accumulate in the kidney in OPN knockout mice (47). In contrast to a potential protective effect against COM accumulation, OPN may be an important mediator of interstitial injury, suggesting a role in kidney stone disease (31). An increased expression of OPN in renal tubule cells is linked to an accumulation of macrophages in the damaged tissue (17, 51). OPN is also related to a reduction in inducible nitric oxide synthase levels and in macrophage attraction, which indicates a possible inflammatory response (21, 38).

We hypothesize that the accumulation of COM crystals can be regulated by the differing expression of urinary proteins between the rat strains and that COM crystals accumulate more in Wistar rats because its urinary proteome lacks an inhibitor of crystal formation or adherence to cells. To investigate our hypothesis, we treated age-matched male Wistar and F344 rats with EG for as long as 8 wk and observed the changes in total protein as well as in THP and OPN levels in urine in both rat strains. Because of the potential role that OPN might have in an inflammatory response, we also investigated strain-related differences in the macrophage infiltration in the kidney of these EG-treated rats. A goal of the study was to provide an insight into the rodent model that can best mimic the stone disease in humans and to provide more information about rat strain differences in assisting human health assessments of EG toxicity.

MATERIALS AND METHODS

Animal protocols. Male Wistar and F344 rats (Harlan, Indianapolis, IN) at 11 wk of age were housed in independent holding cages. Each strain was randomly divided into two groups. Group I control rats were given regular drinking water. Group II rats were administered 0.75% (vol/vol) EG (Sigma, St. Louis, MO) as their water source to induce a chronic mild hyperoxaluria and calcium oxalate deposition in kidneys (10, 13). Rats were treated for 2, 4, 6, and 8 wk, with 4 or 5 per treatment group. The animals were maintained under standard conditions of humidity, temperature (25 ± 2°C), and light (12:12-h light-dark) and were allowed free access to food (normal rat chow). Water and fluid consumption were recorded daily. Rats were weighed before and during the treatment to assess growth. Before and after 2-, 4-, 6-, and 8-wk treatment, the animals (4-5 per group) were anesthetized with pentobarbital sodium and the kidneys were rapidly removed. Half was fixed in neutral buffered 10% formalin for histological processing and the remaining was homogenized in ice-cold Tris·HCl buffer (0.1 M, pH 7.4) and was frozen at −80°C until further use. The protein concentration in urine was identified by a Non-Interfering Protein Assay Kit (Cayman Chemical, Ann Arbor, MI) for use as THP standards. The OPN level in urine was measured using a mouse OPN ELISA assay kit from R&D Systems (Minneapolis, MN).

Kidney tissue processing. At the end of 2-, 4-, 6-, and 8-wk treatment, the animals (4-5 per group) were anesthetized with pentobarbital sodium and the kidneys were rapidly removed. Half was fixed in neutral buffered 10% formalin for histological processing and the remaining was homogenized in ice-cold Tris·HCl buffer (0.1 M, pH 7.4) and was frozen at −20°C for future analysis.

Histopathological studies. A slice of kidney tissue was fixed in 10% neutral buffered formalin. Tissues were washed and dehydrated in descending grades of isopropyl and finally cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 4-μm thickness and stained with hematoxylin and eosin. The sections were analyzed for COM crystal deposition and other histopathological changes with a Nikon Eclipse TE300 inverted polarizing microscope with Photometrics CoolSNAPfx monochrome 12-bit CCD camera (Nikon).

Immunohistochemical staining for OPN, CD45, and ED-1. The infiltration of leukocytes in the tissue is considered as an indication of inflammation. The expression of CD45 (leukocyte common antigen) and ED-1 (specific macrophage and monocyte antigen) in rat kidney were determined by immunohistochemistry. To further study the expression of OPN in the kidney and the relationship to the inflammatory response in the kidney tissues, OPN was also detected in a similar process. Briefly, after the deparaffinized and rehydrated sections were incubated with primary antibodies anti-mouse CD45 (BD Biosciences; diluted 1:100), anti-mouse monoclonal ED-1 (AbD SeroTec; diluted 1:200), or anti-mouse monoclonal recombiant OPN (Santa
Cruz Biotechnology; diluted 1:100), in 0.5% gelatin overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:1,000 for 1 h. Antigen-antibody reaction sites were detected with a DAB basic kit (Ventana Medical Systems). Sections were counterstained with hematoxylin and examined under a Nikon Eclipse TE300 inverted polarizing microscope (Nikon). To quantify the ED-1 staining, five sampled areas (magnification ×400, 0.038-mm² area) with positive staining were recorded from each animal. Fields to be scored were chosen at random and the observer (YL) was blinded as to the animal status. The number of positively stained cells was counted and expressed as average number per group.

Statistical analysis. Data were represented as means ± SE. Differences between groups were assessed by one- or two-way ANOVA with Student’s Newman-Keuls test to compare differences among treatment groups. Analysis of the ED-1-positive staining was performed using the nonparametric Kruskal-Wallis test followed by the Mann-Whitney test for group differences. The level of significance at P < 0.05 was considered significant.

RESULTS

COM crystal accumulation in kidney tissue. Visual characterization of retained crystals in kidney slices was examined under a light microscope, followed by microscopic visualization under dark field illumination with polarized light. Consistent with previous studies on the crystal accumulation in kidneys in the two rat strains (5, 34), crystal accumulation was observed only in Wistar rats after 4-, 6-, and 8-wk EG treatment, but not in F344 rats in any treatment group. The crystals were distinctively birefringent on dark field microscopy with polarized light illumination (Fig. 1), indicating that the crystals accumulated in kidney tissue were COM crystals.

Calcium oxalate crystals in stone-forming populations are commonly observed in the medullary and papillary interstitium in human kidney as well as in rat kidney (30). However, in the present study, the crystals mainly accumulated in the renal tubule lumen, mostly in the cortex and outer medulla, with fewer crystals in the inner medulla and papilla (Fig. 1, black arrows in B and C). In some tubules, crystals partially or completely blocked the tubule lumen. Some crystals were found at the basement membrane around the damaged renal tubule (Fig. 1, white arrows in B and C), whereas very few were in the interstitium. Progressive tissue damage was also observed in the adjacent renal tubular cells, including cell vacuolization, necrosis, and damage of normal tubule lumen structure.

Effect of EG treatment on urinary proteins. Total urinary protein and levels of THP and OPN in the urine were measured to investigate the role of these proteins in the strain-related difference in COM accumulation after EG treatment over the 8-wk time course. Unlike proteins in other biological fluids, the concentration of proteins in the urine varies widely, due to the highly variable urine volume, which depends mainly on water consumption and other physiological factors. The 24-h creatinine excretion is relatively constant, so creatinine concentration is commonly used to normalize the variation in urine volume (4, 45). Thus, in the present study, excretion of proteins was examined both as urinary protein concentration (mg/ml or

Fig. 1. Crystal accumulation in kidney tissue in Wistar rats after 4-wk treatment with 0.75% ethylene glycol (EG). A large amount of crystals accumulated, specifically within the renal tubular lumen of the cortex. A: photomicrograph of a kidney section from a treated Wistar rat under dark field illumination with polarized light. Retained crystals exhibit strong birefringence (magnification ×40). B and C: photomicrograph of the field. Tissue section lightly stained with hematoxylin and eosin [magnification ×100 (B); magnification ×200 (C)]. Black arrows indicate crystals in the renal tubule lumen, and white arrows indicate the crystals precipitated at the basement membrane.
μg/ml) and as total daily excretion, with normalization to daily creatinine excretion. The total urinary protein excretion in F344 rats was much higher than that in Wistar rats (Fig. 2), both in terms of protein concentration and daily excretion normalized to creatinine. This strain difference was seen before treatment and remained consistent throughout the 8-wk EG treatment. EG treatment per se did not change the total urinary protein excretion in either rat strain.

Urinary THP levels, both in terms of THP concentration and daily excretion normalized to creatinine, varied among weeks along the time course in both rat strains (Fig. 3). However, there were no significant differences between the control and treated rats nor between strains at any time point. At 8 wk, F344 rats appeared to show a decreased THP level compared with That in Wistar rats, but this was not related to EG treatment (Fig. 3).

In contrast, Wistar rats had markedly higher OPN levels in urine compared with F344 rats, before as well as during EG treatment (Fig. 4), considering both OPN concentrations and daily excretion normalized to creatinine. EG treatment did not increase the OPN levels in urine in F344 rats. EG treatment appeared to modestly increase OPN concentrations in the urine of Wistar rats, but this was significant only at 8 wk (Fig. 4). EG treatment of Wistar rats increased total OPN excretion relative to creatinine, with significant increases at both 4 and 8 wk. As such, EG treatment increased the OPN levels in urine at about the same time as the accumulation of crystals was observed in the kidney of Wistar rats.

**Effect of EG treatment on expression of OPN in kidney tissue.** In numerous in vitro studies, OPN has been shown to inhibit COM crystal attachment to renal tubule cells and aggregation into large crystals (47), which should alleviate the toxicity of COM to renal tubule cells. However, as is shown in Fig. 4, a significantly higher OPN concentration was observed in the urine of Wistar rats compared with F344 rats before the EG treatment. EG treatment appeared to increase the OPN level in Wistar rats, which occurred at similar times as COM crystal precipitation in the kidneys. To further investigate the relationship between OPN and COM deposition in the kidney tissue, the expression level of OPN in kidney was determined by immunohistochemical staining. Similar to previous studies (52), OPN was constitutively expressed in kidney tissue, mainly in the loop of Henle and distal tubule, especially at the cortex-medulla transition zone (Fig. 5). Little difference in OPN expression was noted between the control rats of the two strains. Figure 5, inset, shows that under normal conditions, the OPN expression was generally localized to the renal epithelial cells along the tubular lumen, but not in the interstitial area. However, after EG treatment, the expression of OPN in the kidney tissue was markedly upregulated in Wistar rats, in contrast to the slight increase in F344 rats (Fig. 5). Moreover, the positive staining was also observed along the proximal tubule in the cortex and on the collecting duct in the papilla.
Positive OPN staining was noted at a small level in the interstitium in EG-treated Wistar rats.

**Increased inflammation in kidney in EG-treated Wistar rats.** Previous work suggested that the renal damage caused by COM crystal deposition in the kidney may induce a noninfectious inflammation, as indicated by the large amount of macrophage infiltration for removal of the crystals and for tissue repair (6, 29). To identify whether there is a different inflammatory response between the two rat strains, the expressions of a leukocyte cell surface biomarker CD45, which is a leukocyte common antigen, and of a more specific macrophage marker ED-1 were examined in rat kidney sections by immunohistochemistry. The CD45-positive cells were observed clustering, especially in the interstitium of the cortex-medulla transition zone in the control rats in both strains. It is interesting that a greater degree of positive staining of CD45 was observed in control F344 rats compared with control Wistar rats (data not shown). In EG-treated F344 rats, the positive labeling was slightly increased and limited local leukocyte infiltration was also observed in the cortex. However, in EG-treated Wistar rats, CD45 positively stained cells were ubiquitously clustered as infiltrates in the interstitium of the cortex, the papilla, as well as the medulla (data not shown).

Among all the leukocytes, macrophage infiltration is considered as an index for tissue damage, inflammatory response, and tissue repair. To gain a further insight into the migration of macrophages during COM accumulation in the kidney, ED-1 was used to specifically detect monocytes and macrophages (17, 33). The dark brown cells represented the positive labeling (Fig. 6), which were counted as an index of macrophage infiltration. The quantification of ED-1 staining is shown in Fig. 7. Similar to the results for CD45 staining, control F344 rats had more ED-1 positively stained cells in the kidney than control Wistar rats. No change was observed in ED-1-positive
cells in EG-treated F344 rats at 2-wk endpoint, and only a small increase appeared by 4 wk compared with the control. In Wistar rats, however, there was a small increase in the number of macrophages in the kidney tissue by EG treatment at 2 wk, but a marked increase by 4 wk. The nonparametric Kruskal-Wallis test showed a marginal statistical significance ($P = 0.0547$), but the Mann-Whitney test did not show any significant differences among the groups, due to the large variation in treated Wistar rats.

During the tissue processing of blank kidney sections, most large COM crystals were apparently removed. However, under polarized light microscopy, tiny crystal residues with strong birefringence were found attached to the damaged renal tubule, with a large amount of macrophage aggregation around the tubule (Fig. 8). This result suggests that the macrophage infiltration and aggregation in the kidney tissue may be induced either directly by the accumulated COM crystals or by the tissue damage caused by these crystals. However, there was no crystal deposition in the F344 rats, despite a higher inflammatory status, indicating that an inflammatory state was not a prerequisite for COM deposition.

**Strain difference in oxalate accumulation in hydroxyproline-treated rats.** A recent dose-response study (50) showed that ingestion of hydroxyproline (HP) at the 2% level in drinking water by Brown Norway rats is sufficient to produce markedly increased urinary oxalate excretion (compared with controls) within 2 wk and COM crystal deposition in the kidney by 5 wk. EG is metabolized to glycolaldehyde, glycolate, and glyoxy-late, which acts as the precursor to oxalate. HP is metabolized via 4-hydroxy-2-ketoglutarate to glyoxylate, thus acting as an alternative source of oxalate and bypassing the production of glycolate and the potential adverse effects therefrom (27). The results in Fig. 9 demonstrate that Wistar rats, treated with HP for 2 wk, showed marked increases in both urinary and plasma oxalate concentrations, whereas F344 rats showed no significant increases in oxalate levels in either urine or plasma. This is exactly the same pattern that was observed in these two strains when they were treated with EG (0.75%) in their drinking water for 2 wk (34).

**DISCUSSION**

Strain-related differences in physiology and pharmacology can have a critical effect on the results of a study, so such
differences need to be evaluated before choosing animals for a study. In considering use of various rat strains to model hyperoxaluria, F344 rats show more resistance to the renal accumulation of COM after EG exposure compared with Wistar rats (5, 34), so that the Wistar rat may be the preferred animal model for the study of hyperoxaluria (28). The present results show that the Wistar rat is also much more sensitive to hyperoxaluria when HP is used as the oxalate source. The mechanism for the variant sensitivity to COM among different rat strains has not been widely studied. In our previous study (34), a markedly increased calcium oxalate supersaturation (CaOx SS) in EG-treated Wistar rats, with only a small increase in EG-treated F344 rats, contributed to the strain difference in COM crystal accumulation, i.e., COM crystals were retained in the kidney only in Wistar rats and not in F344 rats. However, we noticed that CaOx SS was moderately elevated in EG-treated F344 rats after 8 wk, yet no crystals were found in the F344 rat kidney. These results indicate that factors other than supersaturation alone are involved in this strain-related difference in COM formation and kidney damage. Because urinary macromolecules may regulate COM crystal growth and adhesion (36, 49, 53), a higher level of certain urinary proteins in EG-treated F344 rats might have contributed to the lack of COM accumulation in F344 rats despite the moderately elevated CaOx SS in our previous study.

The current studies of urine protein differences between the two rat strains were developed to evaluate the hypothesis that F344 rats have a greater amount of protein(s) in the urine that inhibit crystal formation or adherence to cells, thus decreasing their susceptibility to EG-induced COM crystal accumulation. Hard (16) reported that rats have very high concentrations of protein in urine compared with humans, which may explain why spontaneous calcium oxalate stones are rare in rats under normal conditions (28, 54). In vitro studies indicated that COM crystal adhesion to renal tubular cells can be inhibited or promoted by cotreatment with urinary proteins (32, 43), which might alter the accumulation of COM in the kidney tissue. Some urinary proteins can also affect the crystal nucleation and/or aggregation in the urine (37, 48). Thus, the accumulation of COM crystals in the kidney might be controlled by differences in the concentration of the urinary protein(s). In the present study, F344 rats had much higher protein concentrations in urine than Wistar rats through 8 wk (Fig. 2). Also, the difference was maintained even with the EG treatment. The higher total protein levels in F344 rats might contribute to an inhibition of COM crystal deposition in the kidney in situations where CaOx SS is only moderately elevated (as in EG-treated F344 rats) (34).

We subsequently measured the concentrations of certain specific proteins in the urine to determine whether they may play a role in the lesser accumulation of COM in the F344 rats. THP is an abundant protein in the urine of normal humans as well as animals, and has been shown to inhibit COM growth and adhesion in vitro (36), so it is a very likely candidate. Although some clinical studies support the hypothesis that a defect in the amount of THP in urine may be responsible for the individual susceptibility to COM deposition (1, 9), other studies failed to show quantitative differences in THP between healthy people and stone formers (18). Tiselius et al. (44) reported a reduced inhibitory effect of THP on CaOx aggre-

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**Fig. 8.** Relationship between macrophage infiltration and calcium oxalate monohydrate (COM) crystal accumulation in the kidney tissue in EG-treated Wistar rats (at the 4-wk endpoint). A: photomicrographs of the renal cortex showing macrophage (red arrows) aggregation around a damaged renal tubule with COM crystal residues attached. B: COM crystals (white arrows) showed strong birefringence under polarized light.

**Fig. 9.** Hydroxyproline (HP) administration increases urinary and plasma oxalate concentrations in Wister rats, but not F344 rats. Rats were treated with 2.0% HP or water for 2 wk. Data were represented as means ± SE (n = 6 per group). *Significant difference from water-treated control; #Significant difference from HP-treated F344 rat. One-way ANOVA followed by Tukey’s test (P < 0.05).
gation in male stone formers, indicating that a functional defect in THP in the urine might also contribute to stone formation. To better understand the role of this specific protein, knockout mouse models tested the role of THP in kidney stone formation, but produced somewhat controversial results. In the study by Mo et al. (36), spontaneous formation of COM crystals is observed in kidneys in 4 of 25 THP knockout mice without EG exposure, and after EG treatment, diffuse crystals are observed in 13 of 17 THP knockout mice, but none in wild-type mice. These results indicate that THP may be a critical inhibitory factor during COM crystal deposition and that a THP defect can promote COM accumulation in kidney tissue. However, Bachmann et al. (3) compared the renal function and urinary electrolytes between wild-type and THP−/− mice and found that the THP−/− mice have significantly decreased creatinine clearance and an upregulation in major distal transporters, which results in an increased ion load in the renal tubule. These results suggest that the different sensitivity to oxalate overload between wild-type and THP−/− mice may be caused more by the changes in the transporters than by a direct effect of THP deficiency. So, the role of THP in COM deposition in vivo is still unclear.

In the current study, there were no differences in THP levels in the urine between the two rat strains with or without EG treatment. It is interesting that the total protein level was much higher in the F344 rats, but there was no strain difference in THP levels, considering that THP has been reported to be the higher in the F344 rats, but there was no strain difference in treatment. It is interesting that the total protein level was much higher in the urine between the two rat strains with or without EG treatment. Nevertheless, OPN expression in the kidney tissue, located at the epithelial cells lining the renal tubule, was increased after EG treatment in Wistar rats, but not in F344 rats. The increase in OPN in the Wistar rats, accompanied in time by the appearance of COM deposition in the kidney, would suggest that OPN may not protect against COM accumulation in vivo. Alternatively, without OPN as in the case of EG-treated OPN knockout mice, the EG-treated rats might have been more prone to crystal deposition, such that the increase in OPN might still reflect a compensatory increase to minimize crystal retention. In the latter case, it is obvious in the Wistar rat that any defensive ability of OPN was easily overcome by the amount of COM crystal formation from EG exposure.

An interesting dichotomy in these results is that urinary OPN levels were higher in untreated Wistar rats compared with untreated F344 rats, while renal tissue expression of OPN was not markedly different between the two strains before EG treatment. Wistar rats are larger than F344 rats, so the greater excretion of OPN could result from the greater kidney size, i.e., more kidney cells releasing or shedding OPN into the urine, leading to a higher OPN excretion. Alternatively, it is possible that the OPN in the kidney tissue of the Wistar rat could be a more labile form, such that the release of OPN from the Wistar kidney tissue was higher, thus leading to a greater excretion. Nevertheless, OPN levels increased in both the kidney tissue and the urine of EG-treated Wistar rats, while not changing in either in F344 rats. As such, these results suggest that the higher level of OPN in the Wistar urine was not sufficient to protect against COM deposition in the kidney and might actually promote COM retention.

A possible limitation of the present study is that we treated both strains of rats with the same doses of EG (or HP), which then resulted in higher levels of oxalate and a greater CaOx SS in the urine of Wistar rats. As an alternative, it might have been possible to treat the F344 rats with a greater amount of EG than the 0.75% for the Wistar rat, such that the resulting urinary oxalate excretions would be relatively the same in the two strains. Under those circumstances, the CaOx SS would have been comparable between the two strains and then the role of urinary proteins, both in general and in terms of THP and OPN, and of the inflammatory response in regulating the COM crystal deposition and the renal histology might have been tested. However, in the present design, the total urinary protein, the urinary OPN levels, and the background inflammatory status were already different between the two strains (so even without any hyperoxaluria). Changing the EG treatment to
produce similar hyperoxaluria between strains would not have changed these inherent differences between strains in total urinary protein, urinary OPN excretion, or macrophage infiltration, although the response of the proteins to the EG challenge might have changed. Another issue is that the need to administer a larger dose of EG to F344 rats to produce similar oxalate levels would probably have produced additional off-target effects in the F344 rats, including metabolic acidosis, which would have greatly complicated the interpretation of results. Lastly, the present studies were designed to examine possible reasons for the strain difference in oxalate accumulation, which would seem to require us to reproduce the strain difference. The alternative approach would essentially eliminate the strain difference per se.

Macrophage infiltration is a common feature of tissue injury (20, 22). The renal damage caused by COM crystal deposition in the kidney may induce an inflammatory response, in which the injured renal tubule cells produce various cytokines including OPN to attract macrophages, neutrophils, and multinucleate cells, which help to remove the crystals and in tissue repair. Also, COM crystals may be transported into the interstitium, where they can be surrounded by macrophages for removal. On the other hand, OPN can also be produced by activated macrophages through certain signaling pathways (17, 41), such that OPN may act as an important mediator of interstitial injury. Thus, we investigated whether the inflammatory response might be different between the two rat strains or following the EG treatment regimen. Our studies showed that control F344 rats had a higher background level of macrophage infiltration than did Wistar rats (both CD45 and ED-1 staining). Also, EG-treated F344 rats had a mild increase in numbers of infiltrated macrophages (ED-1 staining) compared with control F344 rats, but this increase was much less than the increase seen in EG-treated Wistar rats at both 2 and 4 wk. The higher background level of macrophage infiltration in the F344 rats compared with that in Wistar rats might have played a role in crystal removal in the treated F344 rats (since no crystals were observed in F344 rats). However, the high degree of macrophage infiltration in the EG-treated Wistar rat was not sufficient to prevent COM accumulation in the kidney tissue. The macrophage infiltration in these Wistar rats thus appears to be a response to COM accumulation, or even a contributor to it, rather than an action to prevent COM accumulation.

OPN is also involved in biological processes such as tissue repair and inflammation through different pathways. OPN is known to correlate with and be upregulated by the macrophage infiltration in various animal models (17, 41). In our study, the increased OPN expression in EG-treated Wistar rats was associated with an increased macrophage infiltration in the kidney tissue as early as 2 wk of EG treatment. OPN expression was increased primarily along the renal tubular epithelium, including both the cortex and medulla. The increase in macrophages occurred primarily in the interstitium, as has been expected for an inflammatory response. The tubular cell upregulation of OPN with interstitial increase in macrophages has also been reported in other cases of OPN-induced inflammation (17). The likely explanation is that OPN is acting as a chemoattractant, such that its upregulation initiates signaling pathways that then attract macrophage infiltration into the interstitium. There was a further increase in both OPN level and macrophage infiltration by 4 wk, yet visible COM deposition in the kidney. These results suggest that before the visible crystal deposition in the kidney tissue, presence of micro-crystals may have induced an increasing macrophage infiltration and increasing OPN expression in the renal tubule cells, which then induced more crystal attachment and deposition.

Both EG and HP act as oxalate precursors and can produce hyperoxaluria when administered in sufficient doses. The two compounds are metabolized in the liver through different pathways to glyoxylate, which is the ultimate oxalate source. The present studies showed that both EG and HP produce a similar strain difference in that treatment markedly increased oxalate levels in Wistar rats, whereas no increase in oxalate was observed in treated F344 rats compared with control F344 rats. These results imply that there probably is an important metabolic component in the strain difference in hyperoxaluria. Possible explanations include a difference in a key metabolic enzyme involved in glyoxylate metabolism, in glyoxylate intercompartmental transport, or in oxalate transport into or out of kidney cells (thus resulting in different renal oxalate clearances).

In summary, several key findings were suggested in this study. Because the two oxalate precursors, EG and HP, produced similar elevations of oxalate, the strain difference in COM accumulation may result more from metabolic differences between strains than from differences in urinary proteins or inflammatory responses. Nevertheless, the higher total urinary protein levels in F344 rats suggest that urinary proteins may play a minor role in preventing COM deposition and that this prevention might be nonspecific but strain related. THP and OPN, two proteins that have been widely suggested to play a role in modulating COM accumulation in kidney tissue, did not show strain-related differences in expression, suggesting that the strain difference in COM deposition is not due to differences in these two proteins. In contrast, an increased OPN expression, which occurred in treated Wistar rats only, might be a response to COM accumulation, or even a promoter of, rather than an inhibitor of COM accumulation. Because EG treatment induces marked hyperoxaluria, these studies do not preclude a role for either THP or OPN in protecting against formation of kidney stones per se. Third, the inflammatory response that accompanies increased OPN expression in the kidney also precedes COM deposition, thus it appears to be a response to crystals and not a defense against COM accumulation.

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