Clofibrate Feeding to Sprague-Dawley Rats Increases Endogenous Biosynthesis of Oxalate and Causes Hyperoxaluria

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The effects of clofibrate feeding (5 g/kg diet) on oxalate metabolism were investigated in male and female rats. Following clofibrate feeding, 24-hour urinary excretion of oxalate increased until 4 days and then reached a plateau. Whereas the contribution of dietary oxalate (1.4 g/kg diet, as potassium salt) to urinary oxalate was less than 5% in both control and clofibrate-treated male rats, the contribution of dietary glycolate (1.0 g/kg diet, as sodium salt) to urinary oxalate was six times higher in clofibrate-treated male rats compared with controls, indicating that the clofibrate-induced hyperoxaluria is due to increased endogenous biosynthesis of oxalate. This was supported by the increased lactate dehydrogenase (LDH) activity observed in liver supernatants of clofibrate-treated rats compared with controls, and the increased rate of conversion of glycolate and glyoxylate to oxalate by clofibrate-treated male rat liver supernatants. Female rats had lower excretion of urinary oxalate and lower levels of liver glycolic acid oxidase (GAO) as compared with males. Clofibrate-treated female rat liver supernatants had higher LDH levels and produced more oxalate from glyoxylate. Thus, it can be concluded that the increase in LDH activity may be the cause of the increased endogenous biosynthesis of oxalate leading to increased urinary excretion of oxalate in male and female rats treated with clofibrate.

LIVER PEROXISOMES occupy a key position in oxalate production in mammals. They contain enzymes for synthesis and removal of glyoxylate,[1] the immediate precursor of oxalate. We previously emphasized that although the generation of glyoxylate from glycolate occurs in peroxisomes, the conversion of glyoxylate to oxalate occurs in the cytosol via the glyoxylate dismutase activity of lactate dehydrogenase (LDH), and that thiols and sulfite may be the intracellular modulators of LDH activity.[2-4] An alteration in glyoxylate metabolism can thus be expected under conditions of altered peroxisomal metabolism, in turn influencing the rate of oxalate biosynthesis and the associated risk of calcium oxalate urolithiasis. In rodents, an alteration in peroxisomal metabolism can be caused by feeding (1) hypolipidemic drugs such as clofibrate, tiadenol, and niadenate, (2) a high-fat diet especially rich in very long-chain fatty acids, or (3) phthalate ester plasticizers. It has been reported that hepatocytes from male rats treated with clofibrate produce more oxalate from glycolate and glyoxylate.[5] Thus, it was of interest to investigate the effect of clofibrate on the enzymes of oxalate biosynthesis and urinary oxalate excretion in male and female rats. Based on the hyperoxaluric effects seen in the present study, the clinical use of fibrate drugs for hypolipidemia should be evaluated as a possible additional risk factor in calcium oxalate urolithiasis.

MATERIALS AND METHODS

Male and female Sprague-Dawley rats weighing approximately 300 g were kept under controlled conditions of temperature and humidity with a 12-hour light/dark cycle, and unless otherwise indicated, they were fed normal chow (no. 1324; Altromin, Lage, Germany) without and with clofibrate (5 g/kg diet; dose is commonly used in rats and differs from that recommended in humans) for a period of 6 days. The 24-hour urine specimens were collected in metabolism cages with free access to tap water and diet. Oxalate in urine was estimated by ion-exchange chromatography using AG4 and AS4 as guard and separator columns, respectively.[6] To ascertain the contribution of dietary oxalate and glycolate to clofibrate-induced oxaluria, male rats (300 g body weight) were kept in metabolism cages and fed (35 g/d) a semipurified diet (no. C-1000; Altromin) devoid of oxalate and glycolate without and with clofibrate (5 g/kg diet). From day 5 (selected based on the chow diet experiment wherein clofibrate-induced hyperoxaluria reached a plateau, Fig 1), diets were supplemented with potassium oxalate (1.4 g/kg diet, an amount of oxalate equal to that present in chow diet) for 3 days, followed by further supplementation with sodium glycolate (1.0 g/kg diet) for the next 3 days. During these days, daily food intake was recorded for all the animals (range, 16 to 24 g) and 24-hour urine samples were analyzed for oxalate.

In Vitro Experiments

Liver supernatants from 10% wt/vol homogenates were prepared (from livers of male and female rats on chow diet without and with clofibrate for 6 days) in 0.01 mol/L potassium phosphate buffer, pH 7.4, and centrifuged at 20,000 × *g* for 30 minutes. The supernatants were used for enzyme and oxalate biosynthesis assays.

Glycolic acid oxidase (GAO) was assayed by estimating glyoxylate[7] produced from glycolate at pH 7.4, with 1 U GAO being defined as 1 nmol glyoxylate formed per minute at 37°C.[8]

Lactate dehydrogenase (LDH) was assayed by monitoring the rate of decrease in absorbance at 340 nm due to oxidation of NADH by glyoxylate at pH 7.4. One unit of LDH was defined as a change in absorbance at 340 nm of 0.01/min at 25°C.[2]

Oxalate produced from glycolate and glyoxylate by in vitro systems was estimated by ion-exchange chromatography[6] using AG9 and AS9 as guard and separator columns, respectively.

Protein was estimated with Coomassie blue plus protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as standard.

The significance of differences was calculated using Student's *t* test.

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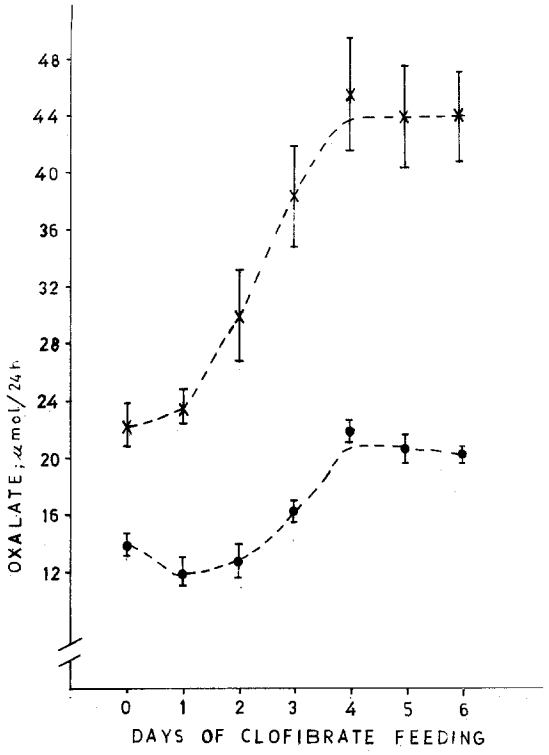

Fig 1. 24-Hour urinary oxalate excretion in male and female rats following clofibrate feeding. Values are the mean ± SEM of ≥ 6 animals. Clofibrate, 5 g/kg diet. (X) Male rats; (O) female rats.

RESULTS

24-Hour Urinary Excretion of Oxalate

Figure 1 shows 24-hour urinary excretion of oxalate following clofibrate feeding (5 g/kg diet) to male and female rats. It was observed that the basal excretion of oxalate in female rats is 60% of the level in males, and that following clofibrate feeding the urinary excretion of oxalate increased until day 4 and then reached a plateau. The increase in oxalate excretion due to clofibrate feeding was 100% in the case of males, whereas in females it caused a 57% increase, and the values were equal to those of male rats without clofibrate (Fig 1).

To ascertain the contribution of dietary glycolate and oxalate in clofibrate-induced hyperoxaluria, male rats were fed a semipurified diet devoid of glycolate and oxalate, without and with clofibrate (5 g/kg diet). The basal excretion of oxalate in male rats fed the semipurified diet is 7.98 ± 1.39 μmol/d (Table 1), which is 30% of the value observed in male rats fed a chow diet (22.8 ± 1.40 μmol/d; Fig 1). This may be attributed to a lack of oxalate and glycolate in the semipurified diet. Following 5 days of clofibrate feeding, a 130% increase in the 24-hour urinary excretion of oxalate was also observed in rats fed the semipurified diet (basal clofibrate *v* basal control, Table 1). Whereas with the addition of oxalate to the diet an increase of 3.19 μmol oxalate in urine (I and II, Table 1) was observed in controls, an increase of 7.59 μmol oxalate (I and II, Table 1) was observed in clofibrate-treated rats, which amounts to 2.0% and 4.3%, respectively, of the ingested oxalate (calculated from food intake and urinary excretion values). Whereas following the further addition of glycolate to the diet a 3.52-μmol increase in oxalate excretion was observed in controls (II and III, Table 1), an increase of 20.75 μmol was observed in clofibrate-fed rats (with urinary oxalate excretion values of 46.84 μmol/d, which is equal to that observed on days 4 to 6 in clofibrate-fed male rats on normal chow; Fig 1), indicating a sixfold increase in the rate of endogenous biosynthesis of oxalate from glycolate following clofibrate treatment.

Enzymes of Oxalate Biosynthesis

Table 2 shows the activities of GAO and LDH in male and female rat livers following clofibrate feeding. The activity of GAO in female livers was significantly ($P < .001$) lower than in the livers of male rats, and whereas clofibrate feeding reduced GAO activity in male rat liver, the activity in female rat liver remained unaffected by clofibrate feeding for 6 days (Table 2). Liver LDH activities were similar in male and female rats, and clofibrate feeding significantly ($P < .001$) increased LDH activities in both male and female rat livers (Table 2).

In Vitro Biosynthesis of Oxalate From Glycolate and Glyoxylate

Table 3 shows the rate of oxalate biosynthesis from glycolate and glyoxylate by male and female rat liver supernatants. Female rat liver supernatants produced significantly ($P < .01$) less oxalate from both glycolate and glyoxylate compared with male rat liver supernatants (Table 3). Clofibrate feeding increased the rate of conversion of glycolate to oxalate only in male rats, while the rate of conversion of glyoxylate to oxalate was increased in both male and female rat liver supernatants (Table 3).

Body and Liver Weights

The 24-hour fasting body weight of male rats without and with clofibrate feeding at death was 330 ± 8.01 and 311 ± 4.28 g, respectively, and that of female rats was 272 ± 2.0 and 264 ± 1.83, respectively. Liver weights without and with clofibrate were 9.80 ± 0.27 and 15.48 ± 0.36 g, respectively, for males, whereas the values for females were 6.74 ± 0.18 and 7.80 ± 0.12 g, respectively. Thus, clofibrate feeding significantly ($P < .001$) increased the liver weight in both male and female rats; however, the effects are more prominent in male rats. This

Table 1. 24-Hour Urinary Excretion of Oxalate in Male Rats Fed a Semipurified Diet Devoid of Glycolate and Oxalate, Without and With Clofibrate: Effect of Addition of Oxalate and Glycolate to the Diet

	Urine Oxalate (μmol/24 h)	
	Control	Clofibrate-Treated
I. Basal	7.98 ± 1.39	18.5 ± 2.40
II. +Oxalate	11.17 ± 1.72	26.09 ± 2.39
III. +Oxalate + glycolate	14.69 ± 2.64	46.84 ± 7.04

NOTE. Values are the mean ± SD; basal (control) is for 6 animals on 2 consecutive days, and basal (clofibrate-treated) is for 3 animals on day 5 with the clofibrate diet. II and III, values for 3 consecutive 24-hour urine collections. n = 3 per group. Dosage: clofibrate 5 g/kg diet, oxalate as potassium salt 1.4 g/kg diet, and glycolate as sodium salt 1.0 g/kg diet.

Table 2. Enzymes of Oxalate Biosynthesis in Liver Supernatants: Effect of Feeding Clofibrate (5 g/kg diet) to Male and Female Rats

Parameter	Male		Female	
	Control (n = 12)	Clofibrate (n = 7)	Control (n = 6)	Clofibrate (n = 6)
GAO				
U/mg protein	10.73 ± 0.40	8.81 ± 0.53 (82)*	3.95 ± 0.28§	3.53 ± 0.34 (89)
U/g liver	429 ± 16	344 ± 21 (80)†	144 ± 10.8§	120 ± 11.4 (84)
U/liver × 10^3	44 ± 0.7	53 ± 3.3 (121)*	9.33 ± 0.60§	9.4 ± 0.93 (100)
LDH				
U/mg protein × 10^2	8.30 ± 0.89	10.99 ± 0.30 (132)†	8.32 ± 0.21	10.81 ± 0.32 (130)‡
U/g liver × 10^3	32.3 ± 1.6	43.0 ± 1.6 (133)‡	30.1 ± 0.73	37.3 ± 0.97 (124)‡
U/liver × 10^5	3.4 ± 0.3	6.6 ± 0.2 (198)‡	2.03 ± 0.06	2.92 ± 0.09 (144)‡

NOTE. Values are the mean ± SEM (% of controls). One unit of GAO is defined as 1 nmol glyoxylate formed/min at 37°C; 1 U LDH is defined as a change in A340 of 0.01/min at 25°C.

*$P < .05$, †$P < .01$, ‡$P < .001$: *v* respective controls.

§$P < .001$ *v* males.

may be due to the low responsiveness of female rats to peroxisomal proliferation by clofibrate.[9]

DISCUSSION

Oxalic acid, an end product in mammalian metabolism, shows pathogenicity by forming insoluble salts with calcium, and a small increase in urinary excretion of oxalate (synonymous mild hyperoxaluria) is now considered a major risk factor for calcium oxalate urolithiasis.[10] The oxalate pool in the body arises either from endogenous biosynthesis (80% to 90% of the total) or from intestinal absorption of preformed oxalate present in the diet (10% to 20% of the total).[10,11] The latter does not seem to have a significant contribution to the clofibrate-induced hyperoxaluria observed in the present study (Fig 1), since less than 5.0% of the oxalate consumed in the diet appeared in urine samples of both controls and clofibrate-fed rats (Table 1). The increment in urinary oxalate excretion caused by oxalate supplementation is 22.3%, 16.2% of the total oxalate excreted in controls and clofibrate-treated groups where both glycolate and oxalate were added. These values are near the upper limits of the normal range reported for the contribution of dietary oxalate to urinary oxalate.[10,11] The higher percentage of ingested oxalate in urine (4.3%) in the case of clofibrate-treated rats as compared with 2.0% in controls, although not having significance in the present situation, may be a cause for concern during intake of a diet rich in oxalate, and thus intestinal absorption of oxalate under the influence of clofibrate needs investigation. Similarly, the effects of clofibrate, if any, on the renal clearance of oxalate need investigations. It has been reported that glycolate fed in the diet to rats is completely (100%) absorbed and that 3% of the glycolate consumed appears in urine as oxalate within 24 hours.[12] We have observed that in control rats, 1.0% to 2.67% of the glycolate consumed appears in urine as oxalate, whereas in the case of clofibrate-treated rats, 9.0% to 12.7% of the glycolate consumed appears in urine as oxalate. These results support our hypothesis that the rate of endogenous conversion of glycolate to oxalate is increased in clofibrate-treated rats, resulting in a sixfold increase in urinary oxalate excretion (Table 1).

The endogenous synthesis of oxalate from all its precursors

Table 3. Oxalate Biosynthesis From Glycolate and Glyoxylate by Liver Supernatants: Effect of Clofibrate Feeding to Male and Female Rats

Parameter	Male		Female	
	Control	Clofibrate	Control	Clofibrate
A: Glycolate $\xrightarrow{GAO}$ glyoxylate $\xrightarrow{LDH}$ oxalate				
Oxalate				
nmol/h/mg protein	196 ± 13.6	248 ± 15.2 (122)*	105 ± 9.6‖	96.5 ± 8.4 (92)
µmol/h/g liver	7.92 ± 0.54	9.69 ± 0.64 (122)*	3.78 ± 0.37‖	3.27 ± 0.28 (87)
µmol/h/liver	78.8 ± 6.8	148.7 ± 9.4 (189)‡	25.2 ± 2.1‖	25.7 ± 2.31 (102)
B: Glyoxylate $\xrightarrow{LDH}$ oxalate				
Oxalate				
nmol/h/mg protein × 10^2	13.50 ± 0.31	17.82 ± 0.50 (132)‡	11.98 ± 0.33§	15.75 ± 0.23 (131)‡
µmol/h/g liver	55.5 ± 1.53	69.73 ± 2.33 (126)†	43.8 ± 1.44‖	53.3 ± 0.72 (121)‡
µmol/h/liver × 10^2	5.44 ± 0.21	10.57 ± 0.43 (194)‡	2.94 ± 0.05‖	4.21 ± 0.09 (143)‡

NOTE. Values are the mean ± SEM (% of controls). Assay system A (2.0 mL phosphate buffer, 0.01-mol/L, pH 7.4) contained 5.0 mmol/L glycolate, 0.14 mmol/L NAD, and 0.2 mL supernatant (0.65 to 0.84 mg protein). Assay system B (2.0 mL phosphate buffer, 0.01-mol/L, pH 7.4) contained 0.5 mmol/L glyoxylate, 0.14 mmol/L NAD, and 40 µL supernatant (0.13 to 0.168 mg protein). The reaction was stopped after 1 hour of incubation at 37°C by addition of 0.7 mL 7.0% wt/vol trichloroacetic acid (TCA). The tubes were then centrifuged to remove TCA-precipitated proteins; supernatants so obtained were used for estimation of oxalate by ion chromatography. Oxalate was not detectable in blanks without NAD. The lower detection limit is 2.75 nmol/mL.

*$P < .05$, †$P < .01$, ‡$P < .001$: *v* controls.

§$P < .01$, ‖$P < .001$: *v* control males.

except ascorbic acid occurs via the glycolate-glyoxylate-oxalate pathway[13] and involves three major enzymes: GAO,[14] GAD,[15] and LDH.[16] The relative importance of these enzymes in the intracellular production of oxalate is unresolved.[11,17] GAO is localized exclusively in liver peroxisomes and catalyzes the conversion of glycolate to glyoxylate. Although this enzyme has also been shown to catalyze the conversion of glyoxylate to oxalate, the kinetics of this enzyme do not favor this reaction. We have also reported that rat liver homogenates and 35% to 60% ammonium sulfate fractions capable of forming glyoxylate from glycolate are not able to form oxalate from glycolate and glyoxylate in the absence of added NAD or NADH[2,3] (Table 3). Similar results showing low oxalate production in the absence of NAD or NADH and an increase after their addition are available.[18,19] Thus, while the generation of glyoxylate from glycolate occurs in the peroxisomes and is mediated by GAO, its conversion to oxalate occurs in the cytosol and is mediated by LDH.

The role of sex hormones in the regulation of GAO activity in rats is well established. GAO activity in weanling male and female rats is similar,[8,20] and with age, it increases only in male rats.[21,22] Whereas estradiol administration to males decreased GAO activity,[8,23] testosterone administration to females increased GAO,[22] and castration in males reduced GAO levels while ovariectomy in females resulted in an increase of GAO.[24] The results from the present study showing low GAO activity in females (Table 2) corroborate the above results and are the cause of low oxalate production from glycolate in female rat liver supernatants (Table 3). Whereas in males GAO activity responded to clofibrate treatment, resulting in a decrease, the activity in females was unaltered by clofibrate treatment (Table 2). This can be explained on two bases: (1) GAO activity in females is already at the baseline (unchanged since weaning) and thus a further reduction may not be possible, and/or (2) the estradiol prevented the effects of clofibrate on GAO, similar to the preventive effects of estradiol observed on GAO under (1) a pyridoxine-deficient diet feeding,[23] or (2) glycolate feeding,[22] and on the prevention of nephrotoxic effects of glycolate and ethylene glycol feeding in female rats.[25] Although in male rats clofibrate feeding decreased GAO activity per gram liver, an increase in liver weight resulted in an increase in total GAO activity per liver, and this may contribute to some extent to the increased oxalate production observed on the diet without (Fig 1) and with 0.1% (Table 1) or 3.0% (data not shown) sodium glycolate. The decrease in GAO observed may be a consequence of product (glyoxylate) inhibition. The main reaction products of GAO are glyoxylate and H_2O_2, both of which can react, resulting in nonenzymatic decarboxylation of glyoxylate.[5,26] Under normal conditions, thus, only a fraction of the glyoxylate formed in the peroxisomes reaches the cytosol, where it is converted to oxalate by the action of LDH. Clofibrate feeding is associated with an increased catalase activity leading to a higher rate of removal of H_2O_2 and thereby preventing nonenzymatic decarboxylation of glyoxylate (conversely, inhibition of catalase by azide causes increased decarboxylation of glyoxylate and decreased oxalate production).[5,26] We previously observed that whereas short-term feeding (10 days) of glycolate (50 mg/rat/d) decreased GAO activity,[27] probably due to a sudden increase in glyoxylate levels caused by the glycolate load, long-term feeding (30 days) of glycolate at the same dose caused an increase in GAO activity.[21,22] Thus, it seems likely that a sudden increase in glyoxylate may cause an inhibition of GAO.

Liver LDH activity is significantly increased in both male and female rats following clofibrate feeding, and the levels of LDH (units per liver) were 100% and 44% higher in males and females versus their respective controls (Table 2), corroborating the increase in oxaluria of 100% and 57% observed in males and females (Fig 1). Similarly, with in vitro systems, the increase in LDH activity (units per milligram protein; Table 2) can account for the increase in oxalate production observed from glycolate and glyoxylate in males and from glyoxylate in females (Table 3). Thus, it appears likely that the majority of the increased capability of the liver to produce oxalate following clofibrate treatment arises from increased LDH activity. Similarly, the rate of glyoxylate to oxalate conversion in females (micromoles per hour per liver) is 60% less than in males (Table 3), which corroborates the 60% decrease in oxalate excretion observed in females compared with males (Fig 1). Another evidence highlighting the importance of LDH in endogenous oxalate production comes from the observation that male rats fed a high-fat diet (20% wt/wt with oils rich in n-3, n-6, or n-9 fatty acids) for a short period had higher activity of liver LDH and produced more oxalate from glyoxylate in vitro as compared with controls fed normal chow (communication). The increase in LDH activity observed in the present study may be a consequence of activation caused by its substrate—glyoxylate.

Another enzyme responsible for removing glyoxylate from the cells is alanine-glyoxylate aminotransferase (AGT). This enzyme gains significance in primary hyperoxaluria type 1[28] and during pyridoxine deficiency, wherein a decrease in the activity of AGT increases glyoxylate levels, thereby causing hyperoxaluria. However, following clofibrate administration, it has been reported that the peroxisomal AGT is activated,[29] which in turn should remove glyoxylate from the cells. Thus, AGT activity appears to have no role in the increased oxalate production observed in the present study.

We previously proposed that hyperoxaluria observed in obese (*ob/ob*) mice[30] may be related to altered fatty acid or glyoxylate metabolism.[31] Based on the results of the present study and from high-fat diet feeding (communication), it appears likely that an increase in peroxisomal metabolism is associated with an increased glyoxylate pool in the cytosol, which may be responsible for the increase in LDH activity. Glyoxylate is an intermediate product formed during intracellular conversion of lipids to carbohydrates in higher plants, some unicellular organisms, and certain nematode parasites. The glyoxylate cycle, catalyzed by two unique enzymes, namely isocitrate lyase and malate synthase, allows the decarboxylation steps of the tricarboxylic acid cycle to be bypassed and the carbons derived from fatty acid oxidation to be preserved and converted to glucose.[32] Recently, many studies show the presence of glyoxylate cycle enzymes in various mammalian tissues, including human liver.[33] Thus, as a consequence of increased peroxisomal β-oxidation of fatty acids, it is likely that some acetate may be channeled into this pathway, thereby leading to an

increased glyoxylate pool in the body, a possibility that needs to be experimentally confirmed. Another factor having a role in glyoxylate to oxalate oxidation is the level of cytosolic catalase. We have also shown that catalase, by virtue of removing H_2O_2, prevents nonenzymatic decarboxylation of glyoxylate.[26] Reports showing higher decarboxylation of glyoxylate following catalase inhibition in hepatocytes from clofibrate-treated rats[5] and the selective induction of cytosolic catalase in rodents by peroxisome proliferators[34] are available. This situation of increased cytosolic catalase can also result in an increased glyoxylate pool in the cytosol, which in turn is converted to oxalate by LDH.

In conclusion, clofibrate-induced hyperoxaluria is associated with increased LDH activity in both male and female rats. Further study is required to establish a link between the altered peroxisomal/fatty acid metabolism and the increased glyoxylate/oxalate production via glyoxylate oxidation cycle enzymes and cytosolic catalase.

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