Abnormalities of glycosphingolipid, sulfatide, and ceramide in the polycystic (*cpk/cpk*) mouse

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Abstract Polycystic kidney disease is a disorder marked by aberrant renal tubular epithelial cell proliferation and transport abnormalities. Sphingolipids are ubiquitous membrane components implicated in several cellular functions including cell membrane sorting, signaling, growth, ion transport, and adhesion. To investigate a potential pathogenic role for sphingolipids in cystic kidney disease, we studied the sphingolipid content and associated enzymatic activities of the kidneys from *cpk/cpk* mice and their phenotypically normal litter mates. The neutral glycolipids, including glucosylceramide and lactosylceramide, displayed a striking increase in 3-week-old *cpk/cpk* mice as did the acidic lipid, ganglioside GM3. However, a correspondingly significant decrease in sulfoglycolipid and ceramide concentration was observed in the *cpk/cpk* kidneys. Glucosylceramide synthase activity was higher in the kidneys of the *cpk/cpk* mice than in those of the controls. Kinetic analysis of the glucosylceramide synthase revealed the presence of an endogenous activator in the cystic kidney. A marked decrease in sulfotransferase activity was observed in both whole kidney homogenates and in microsomal preparations that was consistent with the decrement in sulfolipid content. The increase in GM3, glucosyl- and lactosylceramide may therefore be the result of impaired sulfolipid synthesis at the 3-week time point. While sulfolipid and glucosylceramide concentrations are not different at 1 and 2 weeks of age, ceramide concentrations in cystic kidneys are significantly reduced compared to kidneys from phenotypically normal mice. These results suggest that sphingolipids may play a potential role in the proliferative and transport abnormalities associated with cystic renal disease and the development of azotemia.—**Deshmukh, G. D., N. S. Radin, V. H. Gattone II, and J. A. Shayman.** Abnormalities of glycosphingolipid, sulfatide, and ceramide in the polycystic (*cpk/cpk*) mouse. *J. Lipid Res.* 1994. **35:** 1611–1618.

Supplementary key words polycystic kidney • sulfotransferase

Polycystic kidney disease (PKD) is marked by several abnormalities, including tubular epithelial cell hyperplasia (1), abnormal differentiation (2), and altered tubular cell transport (3). The *cpk* strain of mice has been used as a model of autosomal recessive polycystic kidney disease where the phenotypic expression of cystic renal disease occurs in the homozygous mice (4).

Sphingolipids, in particular glycosphingolipids (GSLs), have been implicated as important mediators in a variety of cellular processes (5). These include growth (6), differentiation (7), interaction with extracellular matrix (8), and ion transport (9). Recent work has demonstrated a role for GSLs in growth and signaling events in a renal epithelial cell line, the Madin-Darby canine kidney cell (10, 11). MDCK cells have been used as an in vitro model of cyst formation (12).

The autosomal recessive C57BL/6J-*cpk* mouse has been used as a genetic murine model of human autosomal recessive polycystic kidney disease. Affected animals, the offspring of obligatory heterozygotes, appear normal at birth but develop renal failure after 3 to 4 weeks due to massive enlargement of the kidneys and collecting duct cyst formation. Although early changes in proximal tubules are observed at birth, the renal enlargement is due primarily to cystic changes in the collecting tubules (4, 13). The biochemical basis for polycystic kidney disease is presently undefined. Functionally, two features typify cyst formation, viz. increased epithelial cell proliferation and aberrant transtubular fluid transport.

A priori, GSLs might be considered to be important regulators of the growth response in cystic kidneys for three major reasons. First, both neutral and acidic glycolipids have been demonstrated to be important regulators of cell growth. In MDCK cells, for example, increasing the glucosylceramide content through inhibition of β-glucosidase promotes cell proliferation; decreasing glucosylceramide content through inhibition of glucosylceramide synthase blocks cell proliferation (10). In other systems, lactosylceramide (14) and ganglioside GM3 (15) have been reported to exert profound effects on cell growth. Several mechanisms have been proposed to explain these effects, including regulation of growth factor-stimulated tyrosine

Abbreviations: PKD, polycystic kidney disease; GSL, glycosphingolipid; MDCK, Madin-Darby canine kidney; EGF, epidermal growth factor; TLC, thin-layer chromatography.

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kinase activity (16), phosphorylation of the epidermal growth factor (EGF) receptor (17, 18) through sphingolipid-activated kinases (19), the regulation of phospholipase C (11, 20), the regulation of protein kinase C activity through sphingosine or dimethylsphingosine (21), or through diradylglycerides formed secondary to sphingomyelin synthesis (22). More generally, some agents that induce a cystic phenotype, e.g., glucocorticoids, are associated with changes in GSL formation (23) and cell differentiation (24).

Second, GSLs have been implicated as important regulators of ion transport. The addition of specific lipids to transporting epithelia, for example, increases sodium transport as measured by short circuit current or dome formation (9, 25). Sulfolipids have been implicated in the regulation of Na-K ATPase activity (26). Na-K ATPase expression and localization appear to be abnormal in the cystic epithelia of the *cpk/cpk* mouse (27). Transporting epithelia are polarized with respect to their apical and membrane lipids. GSLs are highly enriched in the outer leaflet of apical membranes (28). These lipids are sorted intracellularly in concert with membrane proteins and have been postulated to regulate protein sorting (although evidence in support of this latter point is limited), and membrane protein sorting appears to be abnormally regulated in polycystic kidney disease (3). In addition, the tight junction provides a barrier to the diffusion of GSLs between apical and basolateral surfaces (29, 30).

Third, GSLs are important determinants of cell-cell and cell-matrix interactions (6, 31). The binding of extracellular matrix proteins such as fibronectin and laminin to integrins may be regulated in part by GSLs (8, 32). Cells derived from polycystic kidneys appear to have altered cell matrix interactions (33). In addition, sulfolipids have been identified as potential receptors for the heparin binding domain of thrombospondin (34). A recent paper has reported the increased expression of a differentiation antigen representing a complex glycosphingolipid in autosomal dominant polycystic kidney disease raising the possibility that other abnormalities in sphingolipid metabolism may be found in polycystic kidney (2).

In the present study sphingolipid content and metabolism were studied in the kidneys of *cpk/cpk* mice and compared to those of the phenotypically normal litter mates.

METHODS

Materials

UDP-D-[1-^{3}H]glucose (7.8 Ci/mmol) and phosphoadenosine [^{35}S]sulfate (PAP^{35}S, 2.5 Ci/mmol) were from DuPont Company (Boston, MA). Galactosylceramide from bovine brain was from Serdary (Port Huron, MI). Ganglioside standards were from Matreya, Inc. (Pleasant Gap, PA). Other reagents, including ganglioside GM3, were from Sigma Chemical (St. Louis, MO). GlcCer was prepared from the spleen of a Gaucher patient (35).

Animals

Kidneys were obtained from a colony of C57BL/6J-*cpk* mice maintained at the University of Kansas Medical Center. This colony was established from mice originally obtained from the Jackson Laboratory, Bar Harbor, ME. Kidneys were obtained from the offspring of heterozygous male and female breeders, killed at 1, 2, or 3 weeks of age. Cystic kidneys from the affected animals were compared to phenotypically normal kidneys of litter mates. Mice were anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneally) prior to killing following institutional guidelines consistent with National Institutes of Health policy. The kidneys were frozen in liquid nitrogen and stored at −70°C until processed. For some analyses, kidneys from three mice were dissected into cortex and medulla (inner stripe of outer medulla and inner medulla).

Sphingolipid analyses

The kidneys were weighed and homogenized in 10 vol of chloroform-methanol 1:1 using a Polytron (Brinkmann Instruments). The homogenate was filtered through a sintered glass funnel. The pellet was extracted twice with 10 ml of chloroform-methanol 1:2. The remaining pellet was dried and weighed. The pooled extracts were evaporated to dryness under a stream of nitrogen and redissolved in chloroform-methanol-water 30:60:8. The lipid extract was applied to a 10 × 1 cm column of DEAE-Sephadex (acetate form) that was preequilibrated with chloroform-methanol-water 30:60:8. Neutral lipids were eluted with 30 ml of the same solvent, and the acidic lipids were eluted with 30 ml of chloroform-methanol-0.3 M NaOAc 30:60:8. The eluates were evaporated to complete dryness.

The neutral fraction was subjected to alkaline methanolysis by addition of 12 ml chloroform and 6 ml 0.21 N methanolic NaOH. The tubes were vortexed and left at room temperature for 60 min. Acetic acid (4.5 ml, 0.34 M) was added. The tubes were vortexed and then centrifuged at 1000 *g* for 5 min to effect phase separation. The lower layer was evaporated to dryness, suspended in 1 ml of chloroform-methanol 98:2, and subjected to silicic acid chromatography using 0.5 g Unisil (200-325 mesh). Ceramides were eluted with 35 ml chloroform-methanol 98:2 and cerebrosides were eluted with 35 ml of chloroform-methanol 80:20.

The acidic fraction was subjected to alkaline methanolysis by addition of 3 ml of methanolic NaOH (0.5 N) and heating for 60 min at 40°C. Freshly prepared methanolic acetic acid (0.5 N) was then added to neutralize the sample. Samples were evaporated under nitrogen and suspended in tap water by sonication. This fraction was dialyzed against tap water for 24 h at 4°C and dried in vacuo

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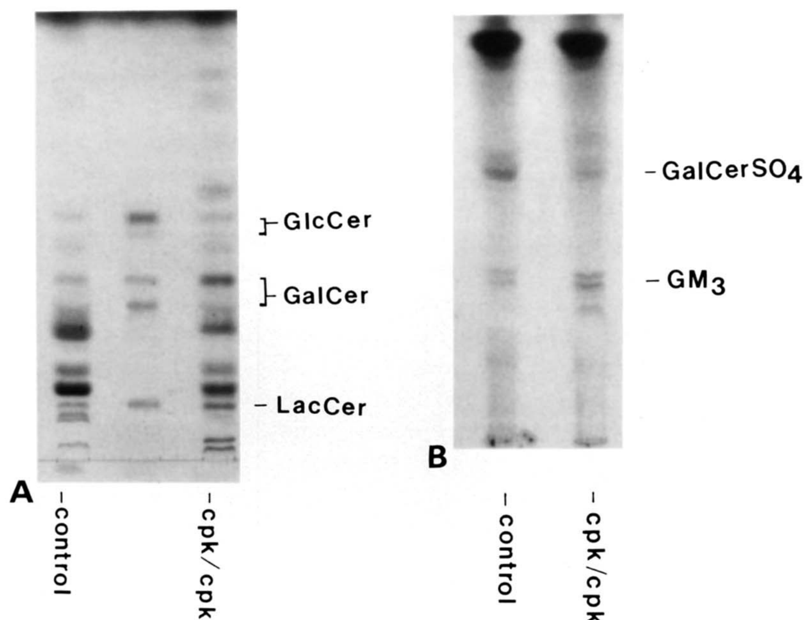

Fig. 1. HPTLC of neutral (A) and acidic (B) GSLs of kidneys from *cpk/cpk* and phenotypically normal mice. Lipids were extracted and isolated as described in Methods.

using a rotary evaporator. The lipids were redissolved in chloroform–methanol 1:2.

Samples were analyzed by high performance thin-layer chromatography (TLC). Only bands migrating with R_f values of known standards were quantified. Galactosylceramide, glucosylceramide, and lactosylceramide were separated on plates that were pretreated with 2.5% borax in methanol–water 1:1 and air-dried. The solvent system consisted of chloroform:methanol–water 65:25:4. The acidic GSLs, including galactosylceramide sulfate, were separated with chloroform–methanol–0.2% $CaCl_2 \cdot H_2O$ 60:40:9. Ceramide was developed with hexane–chloroform 1:1 followed by chloroform–methanol–acetic acid 91:2:3. Lipids were visualized by charring using a copper sulfate–phosphoric acid reagent. Lipid quantitation used a Bio-Rad 620 videodensitometer and 2-D Analyst software.

Enzyme assays

Ceramide:UDP-Glc glucosyltransferase (EC 2.4.1.80) was assayed with an improved method using NAD to protect the nucleotide sugar against pyrophosphatase (36).

Sulfotransferase activity was measured using a modification of the method of Farrell and McKhann (37) as described by Lingwood et al. (38). In brief, GSL substrate, 8 nmol galactosylceramide, was dissolved in 25 μl of chloroform–methanol 2:1 containing 1 mg Triton X-100. The solution was evaporated to dryness and resuspended in 0.1 ml of 100 mM imidazole buffer (pH 7.0) containing 20 mM $MgCl_2$, 4 mM dithiothreitol, 10 mM ATP, 52 mM $PAP^{35}S$, and 25 μl of kidney homogenate (~10 mg protein/ml). The mixture was incubated for 30 min at 37°C. The lipids were partitioned by the addition of 0.5 ml chloroform–methanol 2:1 and 0.5 ml of 0.88% KCl. The lipids in the lower phase were separated by TLC with chloroform–methanol–acetone–acetic acid–water 8:2:4:2:1. The radiolabeled products were identified by autofluorography, scraped, and quantified by liquid scintillation.

RESULTS

In 3-week-old mice the wet weights of the cystic kidneys (662 ± 84.6 mg) were significantly greater with those of the normal kidneys (71.6 ± 2.65 mg, $P < 0.005$). These changes were consistent with those previously reported in this model of cystic kidney disease (4). Analyses of neutral and acidic glycosphingolipids (**Figs. 1A and B**) revealed significant differences between the *cpk/cpk* and phenotypically normal kidneys. When neutral lipids were separated by TLC on a borate-impregnated plate, several bands were resolved. The analysis of sphingolipid content revealed significant increases in glucosylceramide, lactosylceramide, and ganglioside GM3 in the cystic kidneys for

TABLE 1. Sphingolipid content in normal versus cystic kidneys from 3-week-old mice

	Control (n = 6)	Cystic (n = 7)
Glucosylceramide	195 ± 8	337 ± 5[a]
Lactosylceramide	765 ± 121	1380 ± 74[a]
Ganglioside GM3	262 ± 12	323 ± 9[a]
Sulfolipid	2320 ± 598	909 ± 381[a]
Ceramide	1750 ± 184	334 ± 62[a]

The data are expressed as mg/g dry pellet weight ± SE. Protein concentrations in control versus cystic kidneys were 115 ± 3.0 and 21.5 ± 1.6 mg/g wet weight and 621 ± 23.7 versus 604 ± 31.5 mg/g dry pellet weight, respectively.
[a]Denotes $P < 0.05$ by unpaired t test.

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TABLE 2. Sphingolipid content in cortex and medulla of normal and cystic kidneys from 3-week-old mice

	Control	Cystic
Cortical glucosylceramide	105 ± 3.4	251 ± 19.4[a]
Medullary glucosylceramide	147 ± 6.0	182
Cortical GM3	206 ± 16.4	401 ± 29.9[a]
Medullary GM3	196 ± 35.5	360
Cortical sulfatide	1895 ± 131	919 ± 75[a]
Medullary sulfatide	4745 ± 607	1501

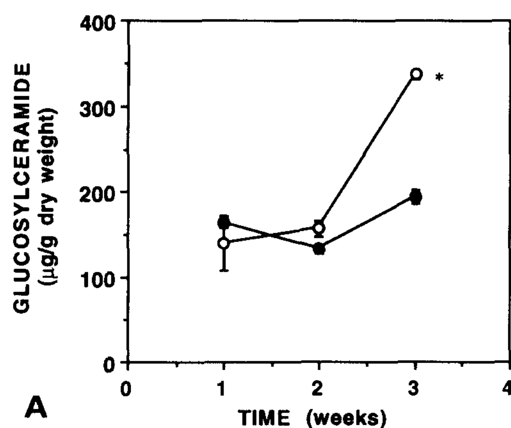
The data are expressed as mg/g dry pellet weight ± SE; n = 8 for control cortical samples; n = 3 for cystic cortical samples; and n = 3 (litters, pooled) for cystic medullary samples. The small tissue mass for cystic medullary samples required pooling samples from three kidneys.
[a]Denotes $P < 0.01$ cystic versus control by unpaired t test.

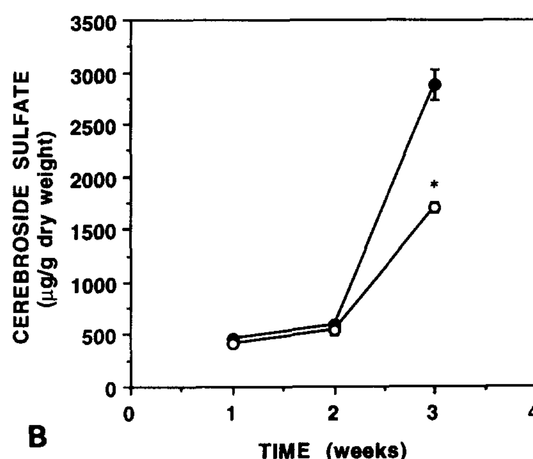
GSL species migrating with R_f values comparable to those of authentic standards (**Table 1**). A fast-moving band corresponding to GalCer was also increased in the *cpk/cpk* kidneys; however, a more slowly moving band perhaps representing a more polar species of GalCer was increased in the control kidneys. By contrast, ceramide and galactosylceramide sulfate levels were distinctly lower in the cystic kidneys (Fig. 1B and Table 1). A band was present in the neutral lipids from the *cpk/cpk* mice that was not seen in the control samples with an R_f value that was slightly greater than the GlcCer standard. In addition, a least one neutral compound of higher polarity was markedly increased in the *cpk/cpk* samples.

At 3 weeks of age, the changes in the cpk mouse kidney are reported to be due to cystic enlargement of the collecting ducts. In order to ascertain whether the observed differences of Table 1 could be accounted for by this enlargement, we analyzed the cortex and medulla separately (**Table 2**). Both sections exhibited markedly higher contents of GlcCer and ganglioside GM3 and lower concentrations of sulfatide.

Between 0.5 and 1.0 ml of cyst fluid was removed from the kidneys of three separate animals. Sphingolipids could not be detected in these samples. The protein concentration of the cyst fluid was 205 μg/ml, representing less than 2.5% of the total kidney protein. The differences in sphingolipid content between normal and cystic kidneys was therefore not simply the result of the presence or absence of cyst fluid. In addition, the contribution of the cyst fluid to total protein was insufficient to account for the differences in sphingolipid content.

The time-dependent expressions of glucosylceramide, cerebroside sulfate, and ceramide were measured in kidneys from 1-, 2-, and 3-week-old mice (**Figs. 2a-c**).





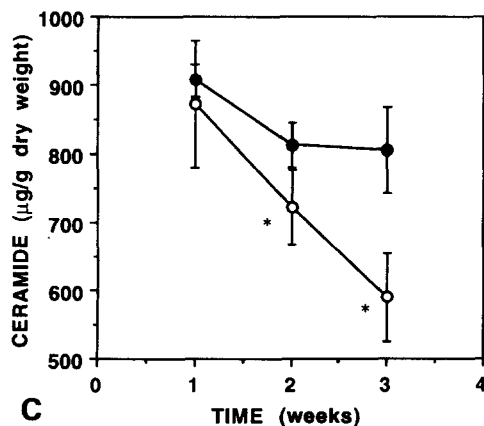


Fig. 2. Renal sphingolipid content as a function of age in normal (●) and cystic kidneys (○). A: glucosylceramide; B: cerebroside sulfate; C: ceramide. *Denotes $P < 0.05$ by unpaired t test.

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Although glucosylceramide and cerebroside sulfate levels were significantly different at 3 weeks, no significant differences were observed at the earlier time points. Ceramide content, however, was significantly lower at 2 and 3 weeks of age in the kidneys from the cystic mice.

In an attempt to ascertain the basis for the differences in GSL content between the control and cystic kidneys, we measured glucosylceramide synthase and sulfotransferase activities in the cystic and phenotypically normal kidneys (**Table 3**). The sulfotransferase activity was significantly lower (40%) in the cystic kidneys.

In contrast, glucosylceramide synthase activity was significantly higher in the cystic kidney. When the synthase activity was measured as a function of protein concentration, it was found that the specific activity rose as higher tissue concentrations were used (**Fig. 3**). For example, the specific activity of the GlcCer synthase was 1472 pmol/h per mg protein when 338 μg of tissue protein was used in the assay and 2102 pmol/h per mg when 675 μg of tissue protein was used. This relationship is consistent with the presence of an endogenous activator of the synthase. In order to determine whether this may be the result of a stimulatory factor in the cystic fluid, the cerebroside synthase activity was measured in a 100,000 *g* pellet from homogenates of normal and cystic kidney. The enzyme activity was consistently lower in the control kidney homogenates than in the cystic homogenates. The presence of the putative activator in the cystic kidney was confirmed by sonication in water of the pellet resulting from the 100,000 *g* centrifugation for 1 h at 0°C. When the sonicate was repelleted at 100,000 *g*, the stimulatory activity was detected in the supernatant from cystic kidney and not the pellet (**Table 4**).

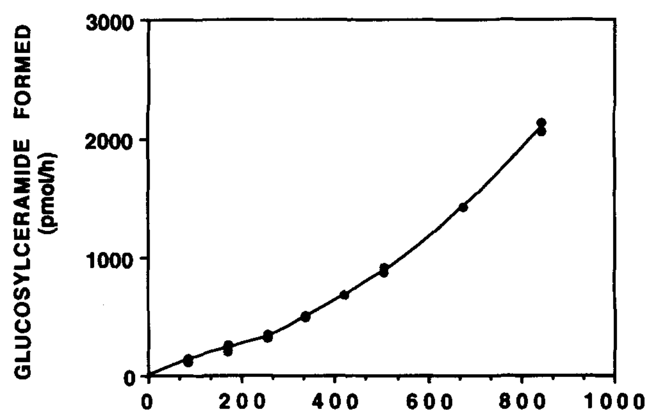

Fig. 3. Glucosylceramide synthase activity versus protein concentration. The activity of glucosylceramide synthase in a 100,000 *g* pellet from *cpk/cpk* kidney homogenate was assayed as a function of protein concentration. A nonlinear association was observed, with higher activities at higher protein concentrations. The pattern of enzyme activity is consistent with the presence of an activator of the cerebroside synthase.

DISCUSSION

The present study revealed abnormalities in the GSL content and synthase activities in the cystic kidney. First, the glucosphingolipids, glucosylceramide, lactosylceramide, and ganglioside GM3, are increased in the cystic compared to normal kidneys. Second, levels of sulfatide are markedly lower in the cystic compared to normal kidneys. These changes are opposite to what one would predict based on the known distribution of GSLs within the kidney. Both sulfatide content and sulfotransferase activities are elevated in medullary compared to cortical regions in normal kidney (39, 40) a finding confirmed in this study for the mouse kidney. Antisulfatide antibody staining identifies only collecting tubule epithelia (41). Thus, if the GSL content were simply a reflection of increased collecting tubule mass, then the sulfolipid and sulfotransferase activity should be higher, not lower, in the cystic kidneys. Changes in glucosylceramide synthase and sulfotransferase activities were noted to be in concert with these changes in glycolipid content. In only one other study has the expression of a GSL been evaluated in cystic kidney disease. Klingel et al. (2) observed that the expression of the differentiation antigen Exo-1 was expressed on up to 80% of cyst lining epithelial cells in autosomal dominant polycystic kidney disease but was not expressed

TABLE 3. Glycosphingolipid synthase activities in normal versus cystic kidneys

	Control	Cystic
Glucosylceramide synthase	1220 ± 45	1860 ± 136*
Sulfotransferase	182 ± 27.3	109 ± 33*

Activities are expressed as pmol/h/mg protein ± SE; n = 3.
*Denotes $P < 0.05$ by unpaired *t* test.

TABLE 4. Glucosylceramide synthase activator properties

	Control Activity	*cpk/cpk* Activity
100,000 *g* Pellet	100	100
Control supernatant	14 ± 2.5	
cpk/cpk Supernatant		5.7 ± 0.9
Pellet + control supernatant	101 ± 1.2	117 ± 3.2
Pellet + *cpk/cpk* supernatant	184 ± 3.4	164 ± 15.3

Evidence for the presence of an endogenous stimulator of glucosylceramide synthase activity in *cpk/cpk* kidneys. Kidneys were homogenized in 9 vol of water. The homogenate was centrifuged at 100,000 *g* for 1 h at 0°C. The pellet was resuspended in enough water to restore the original volume and used as the enzyme source. A portion of the enzyme was sonicated for 30 min at 37°C and centrifuged at 100,000 *g* for 1 h at 0°C. The supernatant was saved and assayed alone or in the presence of the first pellet. The second pellet had no stimulatory activity (data not shown). The data are expressed as percent of the specific activity of the 100,000 *g* pellet. The data represent the mean ± SE of three determinations.

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on normal renal epithelia. Exo-1 represents an uncharacterized neutral glycolipid that is very highly glycosylated (42).

Changes in sulfotransferase activity alone might be sufficient to account for the altered content of the glycolipids (**Fig. 4**). Two major sulfosphingolipids are present in kidney. These include galactosylceramide sulfate and lactosylceramide sulfate (43). In addition, glucosylceramide sulfate (44) and more complex sulfoglycosphingolipids have been found. Lower activity of the sulfotransferase, by slowing the utilization of GSLs, would be expected to cause the observed increases in the GSLs. Yet it is not clear why the slowing in sulfation would increase the activity of GlcCer synthase. The observed increases in the glucosphingolipids, GlcCer, lactosylceramide, and ganglioside GM3, are more readily explained by the increase in GlcCer synthase.

An unexpected and potentially significant finding in this study was the presence of an activator for the glucosylceramide synthase in the cystic kidneys. This activating factor was easily obtained from the sonicates of the 100,000 *g* pellet of the cystic kidneys. It stimulated the glucosylceramide synthase from both the phenotypically normal and cystic kidneys. The existence of activators for glycosidases has been known for many years (45). Deficiencies in these cohydrolases can result in variants of sphingolipidoses in which the hydrolase is normal. Inhibitors of glycosyltransferases have also been identified (46). However, activators of glycosyltransferases have not previously been described. To the best of our knowledge, these data provide the first evidence for the existence of an activator of a glycosyltransferase.

The evaluation of the time-dependent changes in sphingolipid content revealed that only ceramide levels were significantly lower in cystic kidneys prior to 3 weeks of age, a time when cystic changes were fully apparent. Thus, although changes in glucosylceramide, ganglioside GM3, or cerebroside sulfate levels may be useful markers of the severe cystic phenotype, they are unlikely to be causal factors in cyst development. Ceramide has recently been identified as a potentially important regulator of cellular growth and differentiation (47). In several hematopoetic cell lines, including the HL-60 cell line, ceramide levels increase in response to agonists such as TNF-α, γ-interferon, and vitamin D_3. Proposed mechanisms for these changes include the activation of the nuclear transcription factor NF-κB (48), stimulation of a mitogen-activated protein kinase (49), activation of a ceramide-dependent protein kinase (50), and activation of a ceramide-dependent, okadaic acid-sensitive protein phos-

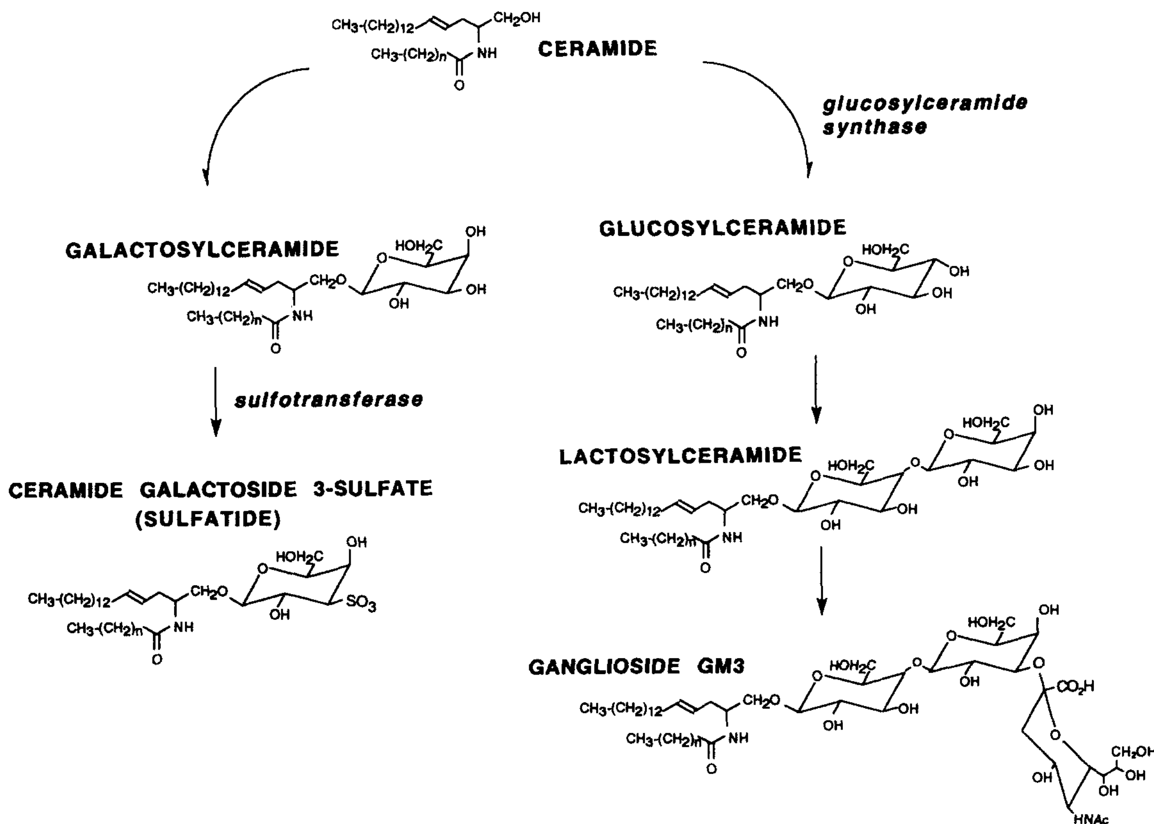

Fig. 4. Pathways for synthesis of simple neutral and acidic GSLs in kidney.

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phatase (51). Ceramide has also been identified as a potential mediator of programmed cell death (52). The growing list of potentially important biological activities regulated by ceramide, therefore, raises the interesting possibility that ceramide or ceramide metabolites may be causative factors in disorders of aberrant differentiation. In the present model, decreased levels of ceramide may result in a delayed differentiation response in the cystic epithelia. Discerning whether or not there is a causal association between ceramide metabolism and renal cyst development will require further investigation.

In summary, GSL abnormalities exist in the kidneys of cystic mice and may participate in the pathogenesis of polycystic kidney disease. Alterations in GSLs may help interrelate the various abnormalities associated with polycystic kidney disease including epithelial hyperplasia, altered states of differentiation, abnormal cell sorting of membrane-associated proteins, and altered cell matrix interactions.

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