patient-oriented research

Increased nonsterol isoprenoids, dolichol and ubiquinone, in the Smith-Lemli-Opitz syndrome: effects of dietary cholesterol

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Abstract Smith-Lemli-Opitz syndrome (SLOS) is an inherited autosomal recessive cholesterol deficiency disorder. Our studies have shown that in SLOS children, urinary mevalonate excretion is normal and reflects hepatic HMG-CoA reductase activity but not ultimate sterol synthesis. Hence, we hypothesized that in SLOS there may be increased diversion of mevalonate to nonsterol isoprenoid synthesis. To test our hypothesis, we measured urinary dolichol and ubiquinone, two nonsterol isoprenoids, in 16 children with SLOS and 15 controls, all fed a low-cholesterol diet. The urinary excretion of both dolichol ($P < 0.002$) and ubiquinone ($P < 0.02$) in SLOS children was 7-fold higher than in control children, whereas mevalonate excretion was comparable. In a subset of 12 SLOS children, a high-cholesterol diet decreased urinary mevalonate excretion by 61% ($P < 0.001$), dolichol by 70% ($P < 0.001$), and ubiquinone by 67% ($P < 0.03$). Our hypothesis that in SLOS children, normal urinary mevalonate excretion results from increased diversion of mevalonate into the production of nonsterol isoprenoids is supported. Dietary cholesterol supplementation reduced urinary mevalonate and nonsterol isoprenoid excretion but did not change the relative ratios of their excretion. Therefore, in SLOS, a secondary peripheral regulation of isoprenoid synthesis may be stimulated.— Pappu, A. S., W. E. Connor, L. S. Merkens, J. M. Jordan, J. A. Penfield, D. R. Illingworth, and R. D. Steiner. **Increased nonsterol isoprenoids, dolichol and ubiquinone, in the Smith-Lemli-Opitz syndrome: effects of dietary cholesterol.** ***J. Lipid Res.*** **2006.** 47: **2789–2798.**

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Children with Smith-Lemli-Opitz syndrome (SLOS) have a defect in the cholesterol biosynthetic pathway at the step of sterol-Δ^7-reductase (EC 1.3.1.21), which converts 7-dehydrocholesterol (7-DHC) to cholesterol, resulting in increased levels of 7-DHC and low levels of cholesterol in plasma and tissues (1–3). The clinical manifestations of this inherited autosomal recessive metabolic syndrome include severe growth deficiency and congenital malformations as well as endocrine and neurological dysfunction, including in most cases mental retardation (4). It seems unlikely that such varied, multifaceted, and seemingly unrelated clinical abnormalities can be accounted for solely by a reduction in cholesterol and/or an accumulation of cholesterol precursors and their metabolites. In this study, we suggest other biochemical disturbances.

HMG-CoA reductase is the key rate-limiting enzyme in the biosynthesis of cholesterol and its precursors. Intermediates of cholesterol biosynthesis serve as precursors for the synthesis of a number of biologically active molecules, which play a vital role in maintaining cellular integrity and function. Changes in the activity of HMG-CoA reductase normally reflect changes in the rates of whole body cholesterol synthesis and can be readily measured by determining the urinary concentrations of its product mevalonate, the precursor of both cholesterol and nonsterol isoprenoids (5, 6). Studies in our laboratory have shown that urinary mevalonate excretion in SLOS children maintained on a very low-cholesterol diet is comparable with that observed in control children given the same diet. Mevalonate excretion is subject to sustained reduction when dietary cholesterol is increased (7). Thus, children with SLOS, who have low cholesterol levels, exhibit normal baseline HMG-CoA reductase activity and normal feedback inhibition by dietary cholesterol.

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Abbreviations: 7-DHC, 7-dehydrocholesterol; 8-DHC, 8-dehydrocholesterol; GCRC, General Clinical Research Center; SLOS, Smith-Lemli-Opitz syndrome.

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We questioned whether the distal block in sterol synthesis in SLOS would affect nonsterol isoprenoid synthesis from mevalonate. We hypothesized that the combination of cholesterol deficiency in SLOS with normal urinary mevalonate excretion may indicate increased diversion of mevalonate to nonsterol isoprenoid synthesis and/or the mevalonate shunt pathway (**Fig. 1**). The mevalonate shunt links the cholesterol biosynthetic pathway with mitochondrial acetyl-CoA metabolism through the intermediate 3-methyl-glutaconic acid (8). This intermediate is excreted in the urine when mevalonate production is stimulated and excessive. The studies of Kelley and Kratz (9) have shown that increased flux through the mevalonate shunt does occur in SLOS subjects, as they excrete significant quantities of 3-methyl-glutaconic acid. We investigated whether in addition to the increase in the mevalonate shunt pathway in SLOS, there may be an increase in nonsterol isoprenoid synthesis. Increased levels of biologically important isoprenoids could contribute to the SLOS phenotype.

In this study, we selected two nonsterol isoprenoids for measurement, ubiquinone and dolichol. These compounds are synthesized and excreted in quantities allowing their accurate determination in urine and have vital cellular functions, which make them attractive candidates for playing a role in the clinical abnormalities in SLOS. Ubiquinone serves as a universal antioxidant and, being a key member of the electron transport chain, participates in generating ATP by oxidative phosphorylation (10, 11). Dolichol increases cell membrane fluidity and permeability, participates in *n*-glycosylation of proteins essential for their transport and function, and is important in the synthesis of phosphatidylinositol anchors for membrane proteins (12). We have shown that plasma mevalonate exhibits diurnal variations (13). The 24 h urinary excretion of mevalonate reflects integrated plasma concentrations and provides a more practical way of assessing the flux of intermediates through the cholesterol biosynthetic pathway than blood. We assumed that there might be similar diurnal variations in blood isoprenoids. Hence, we did not measure these compounds in blood. Furthermore, a majority of the SLOS subjects (9 of 16) were children younger than 5 years of age. We were measuring many other metabolites in blood and are limited in the amount of blood we can safely collect in these small children.

To test our hypothesis, we measured ubiquinone and dolichol excretion in the urine of SLOS children versus controls. We also determined the effectiveness of a high-cholesterol diet in decreasing isoprenoid excretion in SLOS children.

MATERIALS AND METHODS

Subjects

Studies were conducted in the General Clinical Research Center (GCRC) at Oregon Health & Science University. Informed consent was obtained from the parents of each participant, and the Oregon Health and Science University Institutional Review Board approved the protocol. **Table 1** shows the sex, age, and body weight of the 16 SLOS subjects enrolled in the study,

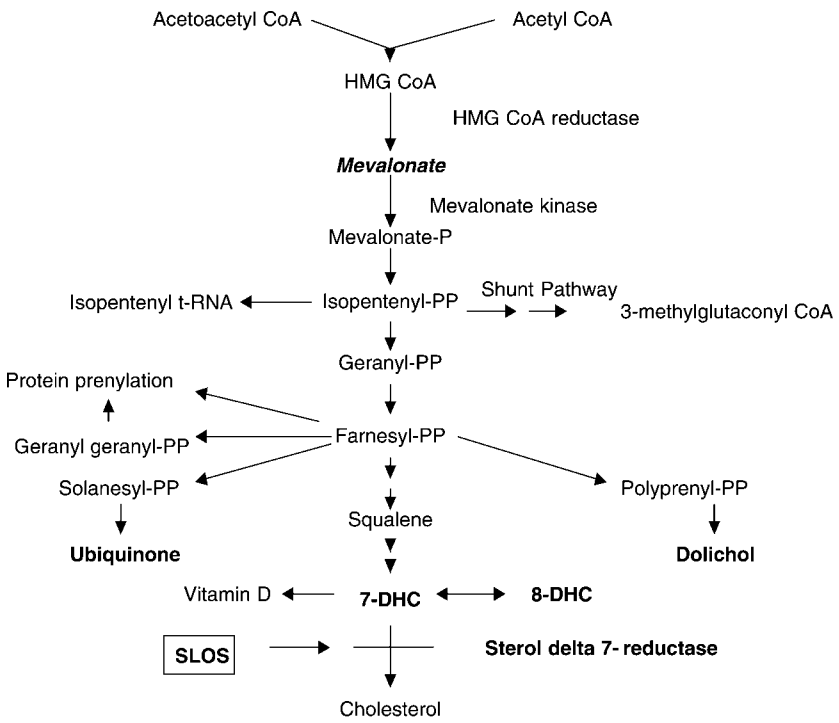

Fig. 1. Pathway of mevalonate metabolism for dolichol, ubiquinone, and sterol synthesis and the mevalonate shunt pathway with the intermediate 3-methyl-glutaconic acid. 7-DHC, 7-dehydrocholesterol; 8-DHC, 8-dehydrocholesterol; PP, pyrophosphate; SLOS, Smith-Lemli-Opitz syndrome.

TABLE 1. Description of experimental subjects

No.	SLOS Subjects							Control Subjects		
	Sex	Feeding	Age	Body Weight	Severity Score	Mutations		Sex	Age	Body Weight
			years	*kg*					*years*	*kg*
1	Male	Gastrostomy tube	0.5	5.4	11	A247V	IVS8-1G>C	Female	8.7	37.7
2	Male	Gastrostomy tube	2.3	8.5	72	W37X	Y143X	Male	12	24
3	Male	Oral	4.3	20.5	20	R352W	W151X	Female	8.4	39.1
4	Female	Oral	3.13	11.5	17	F284L	V326L	Female	2.4	10
5	Male	Oral	4.4	16.4	5	1A>g	R446Q	Female	3.2	15.3
6	Female	Oral	10.4	23.2	10	R450L	IVS8-1G>C	Male	7.9	21.7
7	Female	Oral	11.3	21.7	10	R450L	IVS8-1G>C	Female	2.8	12.3
8	Male	Oral	2	9.7	6	P51S	IVS8-1G>C	Female	17	58.6
9	Male	Gastrostomy tube	1.9	9.7	30	Y462H	IVS8-1G>C	Female	23	38
10	Male	Gastrostomy tube	13.6	29.2	17	F302L	L470Q	Female	21	42.4
11	Female	Gastrostomy tube	8.7	25.4	11	F302L	L470Q	Female	20	64
12	Male	Oral	12.3	61.8	6	C380Y	IVS8-1G>C	Male	34	74.6
13	Female	Oral	12	36.9	11	A247V	IVS8-1G>C	Male	5.5	22.3
14	Female	Oral	1	6.4	5	R242C	V466M	Male	5.1	18.2
15	Female	Oral	23.5	45.3	22	R352W	IVS8-1G>C	Male	8	23
16	Female	Oral	3	10.9	10	T154M	IVS8-1G>C			
Mean			7.1	22.4	16.4				11.9	33.4
SD			6.3	16.1	16.3				9.2	19.7

SLOS, Smith-Lemli-Opitz syndrome. The controls consisted of normal recruited volunteers. Some subjects with SLOS were fed by gastrostomy, whereas others took nourishment orally. All subjects were maintained on a very low-cholesterol diet (23.5 ± 19 mg/day).

together with the same values for 15 matched controls. The table also shows the anatomical severity score and *DHCR7* mutations of the SLOS subjects.

Study design

Sixteen children with SLOS and 15 healthy age- and sex-matched control subjects were initially fed a very low-cholesterol diet (23.5 ± 19 mg/day) for a period of 3–4 weeks to approximate a uniform steady state with respect to sterol homeostasis (Table 1). Of 16 children with SLOS (8 females, 8 males), 5 were fed by gastrostomy tube and the others took nourishment orally. Parents were given both oral and written dietary instructions by registered dietitians and were asked to maintain dietary intake records.

Subjects were admitted to the GCRC for 1 week periods at each dietary phase (low and high cholesterol) to obtain precise dietary control and urine collections. Some of the control subjects were outpatients. The parents provided 3 day dietary records.

All SLOS children receiving the very low-cholesterol diet exhibited low to low-normal plasma cholesterol levels and measurable levels of 7-DHC and its metabolites (8-dehydrocholesterol and cholestatriene 3β-ol) (**Table 2**). The mean plasma cholesterol level of the control subjects was 158 ± 23 mg/dl.

All dietary studies were carried out precisely as described in our earlier studies (7). The high-cholesterol diets consisted of the very low-cholesterol diet supplemented with hard-boiled egg yolk or crystalline cholesterol. A portion of the prepared food was analyzed as a measure of cholesterol intake. The cholesterol

TABLE 2. Plasma sterol composition in patients with SLOS fed a very low-cholesterol diet

No.	Total	Cholesterol	Plasma Sterols *mg/dl*					7-DHC/Cholesterol Ratio
			7-DHC	8-DHC	Cholestatriene 3β-ol	7-DHC and Its Metabolites	7-DHC and Its Metabolites	
							% total sterols	
1	116.3	88.3	13.2	12.9	2.1	28.2	24.2	0.15
2	32.6	8.0	14.3	9.2	1.1	24.6	75.5	1.79
3	56.5	31.6	12.1	10.3	2.4	24.8	43.9	0.38
4	123.2	121.1	0.6	1.5	0.0	2.1	1.7	0.00
5	143.1	142.0	0.2	0.5	0.1	0.8	0.6	0.00
6	105.0	95.0	4.7	5.3	0.4	10.4	9.9	0.05
7	95.0	84.3	5.0	5.3	0.5	10.8	11.4	0.06
8	100.5	94.8	4.8	2.6	0.3	7.7	7.7	0.05
9	73.6	56.0	9.4	7.1	1.2	17.7	24.0	0.17
10	63.5	39.8	12.6	9.3	1.8	23.7	37.3	0.32
11	71.8	49.5	11.9	8.8	1.6	22.3	31.1	0.24
12	132.5	104.5	13.6	11.8	2.6	28.0	21.1	0.13
13	90.7	78.1	5.2	5.8	1.6	12.6	13.9	0.07
14	103.0	84.7	9.3	8.3	0.7	18.3	17.8	0.11
15	65.9	44.9	10.8	9.5	0.7	21.0	31.9	0.24
16	106.7	101.6	2.3	2.5	0.3	5.1	4.8	0.02
Mean	92.5	76.5	8.1	6.9	1.1	16.1	22.3	0.24
SD	29.9	35.4	4.8	3.7	0.8	9.2	19.1	0.43

7-DHC, 7-dehydrocholesterol; 8-DHC, 8-dehydrocholesterol. Plasma sterol levels were determined by GC as described in Materials and Methods. 7-DHC and its metabolites include 7-DHC, 8-DHC, and cholestatriene 3β-ol. The mean plasma cholesterol level in control subjects was 158 ± 23 mg/dl.

intake of study subjects fed the very low-cholesterol diet was 26 ± 24 mg/day, and that for subjects fed the high-cholesterol diet was 495 ± 212 mg/day.

Collection of urine

Twenty-four hour urine was collected using preweighed cotton diapers from infants and children with SLOS with physical limitations, according to the procedure reported previously (7). From toilet-trained children, 24 h urine was collected. Urine from the diapers was extracted with a measured amount of distilled water. The total extract volume was calculated by adding urine weight and the volume of distilled water added for urine extraction. After noting the volume, aliquots of urine were taken for dolichol, ubiquinone, mevalonate, and creatinine measurements and stored at –20°C. The efficiency of extraction of mevalonate, dolichol, and ubiquinone from diaper was 99, 93, and 91%, respectively.

Measurement of mevalonate

Aliquots of urine were thawed and centrifuged at 2,000 *g* for 30 min at 4°C to remove insoluble residues. Mevalonic acid in the supernatant was phosphorylated using [^{32}P]-γ-ATP (New England Nuclear) and purified pig liver mevalonate kinase. Mevalonate phosphate was extracted with ethanol and quantified using a modification of the isotope dilution chromatography method of Popjak et al. (14). The coefficient of variation within experiment and between experiments was <5%. The presence of minute amounts of stool in the urine did not interfere with mevalonate estimations. Urinary mevalonate excretion in a given individual may vary by 35% under stable metabolic conditions (15). Hence, we measured mevalonate in 24 h urine samples collected for 5 consecutive days of each dietary period. Mevalonate values are expressed as ng excreted/mg creatinine. Each urinary mevalonate value reported is the mean of mevalonate measurements from five independent determinations for each individual patient.

Measurement of dolichol

Urinary dolichol was extracted and measured by reverse-phase HPLC. Dolichol was extracted from urine using Waters Sep-Pak® Vac Rc (500 mg) C18 cartridges using a modified procedure (16). Fifteen to 40 ml of urine was applied to a Sep-Pak C18 column, which was previously washed with 8 ml of ethanol-methanol-2-propanol (90:5:5, v/v) and equilibrated with 8 ml of methanol and 8 ml of water. After washing the column with 8 ml of water and 8 ml of methanol, the dolichol was eluted with 8 ml of ethanol-methanol-2-propanol (90:5:5, v/v) and dried under N_2, and the residue was dissolved in 100 μl of 2-propanol-methanol (72:28, v/v). Analytical recovery was determined by spiking urine samples with a known amount of dolichol-23 (Larodan AB, Malmo, Sweden). The recovery from extraction from the column was ~85%.

Separation of dolichol was performed using the HPLC system from the Waters 717 plus autosampler with the Waters 2487 dual absorbance detector (17). The sample was injected onto a reverse-phase Phenomenex Luna 3u (C18) column (150 × 4.6 mm) and eluted with the mobile phase 2-propanol-methanol (72:28, v/v) at a flow rate of 0.8 ml/min, and the elute was monitored at a wavelength of 210 nm.

The intra-assay and interassay variations were <8%. Urinary dolichol values for a given individual may vary by 23% under stable metabolic conditions. Hence, we measured dolichol levels in 24 h urine samples collected for 3 consecutive days of each dietary period. Therefore, each dolichol value is the average of three independent determinations. Urinary dolichol is expressed as ng excreted/mg creatinine.

Measurement of ubiquinone

Ubiquinone from urine was extracted and measured by HPLC as described by Okamoto et al. (18). Five milliliters of urine with 10 ml of methanol was extracted with 20 ml of hexane. After vigorous shaking, the hexane layer was collected and washed twice with 5 ml of distilled water and dried under N_2. For dilute urine extracted from diapers, two to three 5 ml aliquots were processed and combined. The residue was dissolved in 100 μl of ethanol with 1 μg butylated hydroxytoluene/ml and subjected to HPLC. Ubiquinone was quantified using a Phenomenex Luna 3u (C18) column with a mobile phase of methanol-hexane (72:28, v/v) and detected at a wavelength of 275 nm.

The intra-assay and interassay variations were <5%. Urinary ubiquinone values for a given individual may vary by 18% under stable metabolic conditions. Hence, we measured ubiquinone levels in 24 h urine samples collected for 3 consecutive days of each dietary period. Therefore, each ubiquinone value is the average of three independent determinations. Urinary ubiquinone is expressed as ng excreted/mg creatinine.

Other biochemical analyses

For urinary creatinine, the GCRC Core Laboratory determined creatinine concentrations in the urine samples. For plasma sterols, venous blood samples were collected in EDTA-containing tubes from each individual when they were admitted to the GCRC. Plasma was separated by centrifugation of collected blood, and sterols were extracted from plasma, saponified with alcoholic KOH, and then converted to trisilyl derivatives, which were quantified by gas chromatography as described previously (19).

Statistical analysis

Statistical analysis was performed using Student's *t*-test, paired *t*-tests, and ANOVA using SPSS software package 10.00 (SPSS, Inc.). Results are expressed as means ± SD.

RESULTS

The urinary excretion of dolichol, ubiquinone, and mevalonate in children with SLOS and controls is summarized in **Figs. 2**, **3**. The individual values of all subjects are given in Fig. 2, and the mean of each group is given in Fig. 3. With a very low-cholesterol diet, urinary mevalonate excretion in the SLOS subjects was similar to that in control subjects (760 ± 970 and 600 ± 810 ng/mg creatinine, SLOS vs. control). These results are in agreement with our earlier reported studies indicating that HMG-CoA reductase activity in children with SLOS is unaltered despite a reduction in plasma and cellular cholesterol (7).

In control subjects, the urinary excretion of ubiquinone varied from 12.8 to 370 (mean, 87.5 ± 87.8) ng/mg creatinine. In our control subjects, ubiquinone excretion was slightly above the range reported by Okamoto et al. (18), 20–85 μg/g creatinine for adults. As the average age in our control subjects was 12.3 years, the higher levels seen in our studies compared with adult controls from the literature likely are a reflection of the measurement of ubiquinone in children versus adults. In SLOS subjects, the urinary ubiquinone excretion also varied widely from

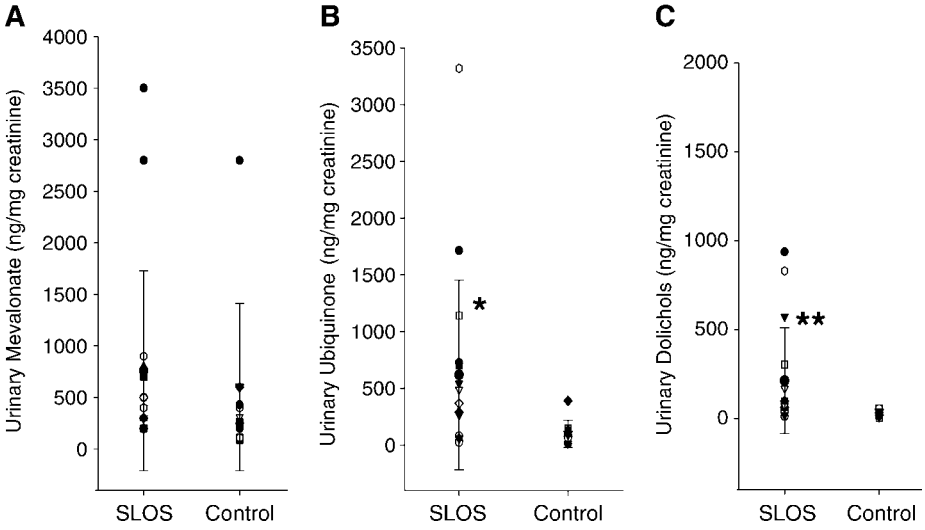

Fig. 2. Individual values for urinary excretion of mevalonate (A), ubiquinone (B), and dolichol (C) in SLOS children (n = 16) and control children (n = 15) maintained on a very low-cholesterol diet. Urinary mevalonate is expressed as ng/mg creatinine and is an average of five independent determinations for each individual. Urinary dolichol and ubiquinone are expressed as ng/mg creatinine and are averages of three independent measurements. Values shown are means ± SD. * Significantly different from the corresponding value of control subjects ($P < 0.02$); ** significantly different from the corresponding value of control subjects ($P < 0.002$).

24 to 3,320 (mean, 618 ± 857) ng/mg creatinine. However, the average ubiquinone excretion in SLOS subjects was significantly higher than that observed in control subjects ($P < 0.02$). One control subject, No. 7, had a very high urinary ubiquinone value of 370 ng/mg creatinine; however, this was significantly lower than that of her affected sibling, No. 14, with a value of 1,714 ng/mg creatinine. These subjects illustrate the wide variability that exists in urinary excretion of ubiquinone. Thus, ubiquinone excretion seems to vary widely in both SLOS and control children.

In control subjects, urinary dolichol concentrations varied between 5.6 and 62.2 (mean, 31.5 ± 17.6) ng/mg creatinine. The values observed in our control subjects are comparable to previously published values for dolichol excretion, 23–106 μg/g creatinine (17). Twenty-four hour dolichol excretion in SLOS subjects varied from 12 to 938 (mean, 213 ± 298) ng/mg creatinine.

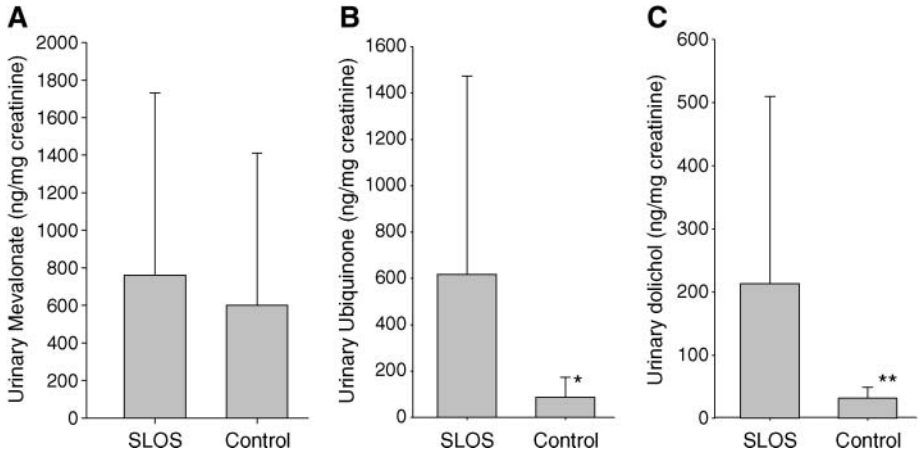

Fig. 3. Average values of urinary excretion of mevalonate (A), ubiquinone (B), and dolichol (C) in SLOS children (n = 16) and control children (n = 15) maintained on a very low-cholesterol diet. Urinary mevalonate is expressed as ng/mg creatinine and is an average of five independent determinations for each individual. Urinary dolichol and ubiquinone are expressed as ng/mg creatinine and are averages of three independent measurements. Values shown are means ± SD. * Significantly different from the corresponding value of control subjects ($P < 0.02$); ** significantly different from the corresponding value of control subjects ($P < 0.002$).

Urinary dolichol excretion in SLOS subjects was significantly higher than that observed in control subjects ($P < 0.002$).

Our subjects exhibited wide variation in the excretion of nonsterol isoprenoids, especially ubiquinone, which may be attributable to the wide variation in age (20–23). In SLOS children, a significant negative correlation was found between age and ubiquinone excretion ($r = -0.75$, $P < 0.001$). However, the correlation between age and dolichol excretion was not significant ($r = -0.308$, $P < 0.25$). Yet, when the subjects from the two groups were combined, a significant negative correlation was found between the age and both urinary ubiquinone excretion ($r = -0.473$, $P < 0.017$) and urinary dolichol excretion ($r = -0.042$, $P < 0.035$). There were no correlations between urinary excretion of nonsterol isoprenoids and plasma 7-DHC or 7-DHC/cholesterol ratio. There was no correlation between anatomical severity score and urinary excretion of mevalonate, ubiquinone, and dolichol. With regard to the *DHCR7* mutation, our subjects are heterogeneous, making it difficult to appreciate any possible correlations between the mutation and nonsterol isoprenoid excretion.

The SLOS children fed by gastrostomy tube were smaller in size and body weight and had significantly lower plasma cholesterol levels and high 7-DHC plus metabolites and high 7-DHC/cholesterol ratios compared with orally fed SLOS children. However, there were no significant differences between the two groups of SLOS children with regard to urinary mevalonate and nonsterol isoprenoid excretion.

A subset of subjects with SLOS (n = 12) was fed a very low-cholesterol diet for 3–4 weeks and then a high-cholesterol diet for an average of 1.9 ± 1.2 years (**Table 3**). Our earlier studies have shown that one of the main desired effect in SLOS subjects, namely the reduction of plasma 7-DHC levels, occurred in most subjects only when maintained on a high-cholesterol diet for almost 24 months (7). Hence, we wanted to measure urinary isoprenoids in our subjects over a similar period of intervention. Furthermore, only one subject was local, and many others traveled from great distances to our site. We had to plan our studies according to the convenience of the families to accommodate their travel and availability, as they had to stay in the GCRC for 1 week periods.

The cholesterol content of the diet was increased from a very low value of 26 ± 23.8 mg/day to a high of 494.6 ± 212.4 mg/day. The plasma cholesterol levels in these subjects increased significantly by 34.5% on the high-cholesterol diet ($P < 0.001$). The ratio of 7-DHC to cholesterol, which has been suggested as a measure of severity of the disease, showed a significant reduction of 25% ($P < 0.009$). However, the total 7-DHC and its metabolites was not reduced. This is in contrast with our earlier observation that long-term treatment (2 years) with a high-cholesterol diet reduced 7-DHC and its metabolites in children with SLOS (7). In 12 subjects, 5 exhibited a reduction and 7 exhibited slight increases in plasma concentrations of 7-DHC and its metabolites. There was a direct correlation between the percentage reduction in 7-DHC and its metabolites and the duration of the high-cholesterol diet ($r = 0.61$, $P < 0.036$).

The individual urinary mevalonate (A), ubiquinone (B), and dolichol (C) concentrations in SLOS subjects fed the very low- and high-cholesterol diets are illustrated in **Fig. 4**. The individual values exhibit wide variations. The cholesterol added to the diet decreased urinary mevalonate excretion from 705 ± 890 to 272 ± 156 ng/mg creatinine. This reduction of 61% was highly significant ($P < 0.001$). There was a significant correlation between the percentage reduction of urinary mevalonate and the baseline urinary mevalonate concentrations in the low-cholesterol diet group ($r = 0.61$, $P < 0.03$). These results confirm our reported observations that the high-cholesterol diet in six SLOS subjects decreased the urinary excretion of mevalonate, the precursor of isoprenoid synthesis. There was a direct correlation between the percentage reduction in mevalonate concentrations and the percentage reductions in 7-DHC and its metabolites ($r = 0.67$, $P < 0.016$) and also with the percentage reduction in 7-DHC/cholesterol ratio ($r = 0.76$, $P < 0.004$).

Dietary cholesterol decreased urinary ubiquinone excretion in these patients by 67% ($P < 0.03$), from a baseline mean of 575 ± 935 to 191 ± 350 ng/mg creatinine, and urinary dolichol excretion decreased by 70% ($P < 0.001$), from a baseline mean of 262 ± 32 to 66 ± 115 ng/mg creatinine. There was a significant correlation between the percentage reduction in urinary dolichol and baseline dolichol levels in the very low-cholesterol diet group ($r = 0.64$, $P < 0.024$). However, there was no correlation

TABLE 3. Effect of dietary cholesterol on plasma sterols in patients with SLOS

Type of Diet	Value	Dietary Cholesterol	Body Weight	Plasma Sterols						
				Total	Cholesterol	7-DHC	8-DHC	Cholestatriene 3β-ola-	7-DHC and Its Metabolites	7-DHC/ Cholesterol Ratio
		mg/day	*kg*	*mg/dl*						
Very low-cholesterol diet	Mean	26.10	24.22	95.45	79.83	7.84	6.87	1.19	15.80	0.12
	SD	23.80	16.97	22.93	26.36	4.57	3.69	0.80	8.89	0.10
High-cholesterol diet	Mean	494.61	30.03	121.73	107.43	7.21	6.26	0.85	14.31	0.09
	SD	212.40	21.69	31.59	38.39	4.46	3.27	0.69	8.28	0.08
P		0.000		0.001	0.000	0.970	0.850	0.390	0.470	0.009

The children were fed the high-cholesterol diet for a period of 1.9 ± 1.25 years. 7-DHC and its metabolites include 7-DHC, 8-DHC, and cholestatriene 3β-ol. Plasma sterol values were from single determinations for each individual at each dietary phase. Each value is expressed as mean ± SD from 12 individual values. *P* values are significantly different from the corresponding values on a very low-cholesterol diet.

Fig. 4. Individual values of urinary excretion of mevalonate (A), ubiquinone (B), and dolichol (C) in SLOS children (n = 12) maintained on a very low-cholesterol diet and a high-cholesterol diet. The results are expressed as ng excreted/mg creatinine. * Significantly different from the corresponding value on a very low-cholesterol diet ($P < 0.001$); ** significantly different from the corresponding value on a very low-cholesterol diet ($P < 0.03$).

between the percentage reduction in urinary ubiquinone excretion and the baseline values.

DISCUSSION

SLOS is an autosomal recessive disorder caused by a defect in the enzyme sterol-Δ^7-reductase, which catalyzes the final step in cholesterol synthesis. This results in low concentrations of cholesterol and high concentrations of 7-DHC in plasma and tissues (24–26). The clinical manifestations are diverse and include congenital malformations, facial anomalies, mental retardation, and behavioral problems (27, 28). These clinical symptoms are believed to result from a deficiency of cholesterol, an increase in 7-DHC, or a combination of both. We hypothesized that nonsterol isoprenoid excess could also play a role.

Cholesterol is an integral part of cellular membranes, the precursor of steroid hormones, and serves as a cofactor and covalent adduct for normal autoprocessing of Sonic hedgehog (29, 30). The rate-limiting enzyme in cholesterol biosynthesis is HMG-CoA reductase, which is subject to multifactorial regulation both by sterols at the level of transcription and by nonsterols and sterols at the post-transcriptional level (31). Measuring the concentration of its product mevalonic acid in plasma and urine can assess HMG-CoA reductase activity in vivo (5, 6). The present studies confirm our earlier findings that urinary mevalonate levels in SLOS subjects do not differ from those in control subjects and represent normal HMG-CoA reductase activity in vivo. Thus, the SLOS mevalonate levels do not reflect the rate of hepatic or whole body cholesterol synthesis that is reduced (7).

Sterol balance studies in our laboratory have shown that in children with SLOS fed a very low-cholesterol diet, total sterol synthesis was reduced to 60% of that in controls fed a similar diet (19). At the same time, urinary mevalonate excretion in SLOS subjects does not differ from those of control subjects (7). Normal mevalonate excretion, suggestive of normal flux via HMG-CoA reductase through the cholesterol biosynthetic pathway despite reduction in total sterol synthesis, could be explained by increased diversion of mevalonate for the production of nonsterol isoprenoids and/or shunting into the mitochondrial mevalonate shunt pathway. The mevalonate shunt links the cholesterol biosynthetic pathway with mitochondrial acetyl-CoA metabolism through the intermediate 3-methyl-glutaconic acid (Fig. 1). Kelley and Kratz (9) found evidence for increased mevalonate shunt activity in SLOS children, as demonstrated by increased urinary and plasma 3-methyl-glutaconic acid, an intermediate in the pathway. They suggested that a comprehensive analysis of metabolites of isoprenoid and sterol biosynthesis would be necessary to test the hypothesis that in SLOS there is overproduction of nonsterol isoprenoids that may be responsible for some of the phenotypes. Increased levels of biologically important isoprenoids could contribute to the SLOS phenotype.

To determine whether the distal block in cholesterol synthesis in SLOS would indeed result in increased diversion of mevalonate to the synthesis of nonsterol isoprenoids, we measured ubiquinone and dolichol (Fig. 1). These two nonsterol isoprenoids are excreted in urine in measurable concentrations. Hence, we measured urinary ubiquinone and dolichol excretion in SLOS subjects as a simple noninvasive method to assess disturbances in nonsterol isoprenoid metabolism.

In humans, the main ubiquinone is ubiquinone 10, or CoQ10, with 10 isoprene units. Ubiquinone performs two major functions: as an electron carrier in mitochondrial oxidative phosphorylation, and as a universal lipid-soluble antioxidant (32). Ubiquinone concentration has been shown to vary with age and in diseases with oxidative stress (33–36). Dolichol participates in the glycosylation of proteins that facilitate their transport and function.

Dolichyl-phosphate is an active participant in the synthesis of glycoproteins as an intermediate in *N*-glycosylation and membrane phosphatidylinositol anchors (12). The distribution of dolichol in tissues and subcellular structures varies more widely than that of ubiquinone and cholesterol (37).

We found significantly increased excretion of ubiquinone and dolichol in SLOS subjects compared with controls. Increased excretion could be attributable to decreased catabolism rather than increased synthesis. However, the observed reduced excretion of these isoprenoids in SLOS subjects with increasing cholesterol content of the diet indicates that the increased excretion in dolichol and ubiquinone at baseline is more likely the result of increased synthesis. In SLOS subjects, the significant increase in the urinary excretion of the nonsterol isoprenoids, ubiquinone and dolichol, was abolished by a high-cholesterol diet. This is in keeping with results from animal studies reported previously. In male C57BL/CJ mice, cholesterol feeding depressed hepatic dolichol levels but not ubiquinone levels (38). This lack of decrease in ubiquinone level is attributable to adaptation by these animals to more efficient use of ubiquinone from the diet (38). The ubiquinone content of our very low-cholesterol diet was 4 ± 2 mg/kg diet, and that of the high-cholesterol diet was 13.8 ± 6 mg/kg diet. The reduction in urinary ubiquinone with the high-cholesterol diet would likely have been even more pronounced if the ubiquinone content of the diet remained low. The wide variation in ubiquinone excretion in the subjects on both diets could potentially be explained by variation in the absorption of dietary ubiquinone. An increase in isoprenoid synthesis could account for some of the mevalonate excess generated by the reduction of sterol synthesis in SLOS subjects.

Variations in serum isoprenoid concentrations have been reported in neurological disorders, including Parkinson disease, multiple sclerosis, and metabolic syndrome with vascular dementia, and in alcohol and steroid abusers (39–42). It has been speculated that changes in the isoprenoid levels participate in the clinical manifestations of those disorders. Thus, a reduction in isoprenoid synthesis may be beneficial in these conditions with increased isoprenoid levels. Statin drugs, which may reduce the risk of dementia, Alzheimer disease, and ischemic stoke, may do so in part as a result of their ability to reduce isoprenoid synthesis by inhibiting HMG-CoA reductase (43).

What are the consequences of the increased accumulation of isoprenoids in cells and tissues? Although these isoprenoids are present in all subcellular structures, the highest concentrations are found in Golgi vesicles and lysosomes, which are maintained in a very narrow range (10). We looked for previously published data on the effects of isoprenoids in SLOS and could not find any studies of isoprenoids in patients with SLOS or even in an animal model. Keller, Arnold, and Fliesler (44), studying a SLOS rat model, have shown that dolichol content is much lower in rat membrane rafts in brain than in whole brain. However, dolichol levels were determined only in control animals and not in their SLOS rat model. Accumulation of isoprenoids in membranes could destabilize the membranes, as these compounds are fusogenic and can alter fluidity and membrane permeability and function (10). Tulenko et al. (45) have shown that disturbances in membrane sterol levels in SLOS may be responsible in part for the pathogenesis of SLOS. However, conversely, increased synthesis of these isoprenoids (especially ubiquinone) in SLOS patients may be initially protective to help relieve oxidative stress caused by the accumulation of 7-DHC, which is highly prone to oxidation and the formation of potentially toxic oxysterols (46–48).

The regulation of nonsterol isoprenoid synthesis is incompletely understood. Ericsson and colleagues (49–51) have shown that the first committed enzymes of dolichol and ubiquinone biosynthesis have a very low K_m and hence higher affinities for the branch-point intermediate farnesyl pyrophosphate leading to nonsterol synthesis than squalene synthase leading to cholesterol synthesis. Even if the concentration of farnesyl pyrophosphate decreases far below the K_m for squalene synthase, the concentration is sufficient for the saturation of other enzymes at this branch point. Hence, it was suggested that nonsterol isoprenoid synthesis is subjected to primary regulation at the HMG-CoA reductase level as well as secondary peripheral regulation at the branch point of farnesyl pyrophosphate.

Based on the results presented here, there is evidence of coordinated regulation of mevalonate and nonsterol isoprenoid synthesis in SLOS. The ratio of urinary mevalonate to dolichol to ubiquinone is 100:31:82, which is much higher in SLOS subjects than in controls (100:7.5:20), indicating that a higher proportion of the mevalonate is converted to isoprenoids in SLOS subjects. However, increasing dietary cholesterol reduced the excretion of both mevalonate and its nonsterol isoprenoid products but did not alter their ratios. Hence, the increased synthesis of nonsterol isoprenoids in SLOS may be a consequence of altered affinities of prenylating enzymes that use farnesyl pyrophosphate. It is unclear whether increased nonsterol synthesis is unique to SLOS or is a consequence in general of a distal block in the cholesterol synthetic pathway. In the latter case, disorders involving each step of post-squalene cholesterol biosynthesis should exhibit increased production and excretion of isoprenoids. Studies are planned to investigate that hypothesis.

In conclusion, our studies indicate that increased excretion of the nonsterol isoprenoids in SLOS is likely from increased synthesis, resulting from the distal block in cholesterol biosynthesis that diverts mevalonate to the nonsterol isoprenoid pathway. Increasing dietary cholesterol reduced nonsterol isoprenoid excretion likely as a result of the feedback inhibition of HMG-CoA reductase leading to decreased mevalonate availability. Increased nonsterol isoprenoid synthesis in SLOS could theoretically play a role in the pathophysiology of SLOS. Conversely, increased ubiquinone, in particular, could be protective in SLOS as an antioxidant. Further studies are needed to help assess whether increased isoprenoids in SLOS are protective or damaging. This could have therapeutic implications for this condition for which no proven therapy exists.

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