Relationship Between Abnormal Cholesterol Synthesis and Retarded Learning in Rats

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We examined the relationship between brain sterol composition and associative learning (classical conditioning of the eyeblink response) in newly weaned rats fed BM 15.766 (BM) for 4 months. This compound inhibits 7-dehydrocholesterol-Δ^7-reductase, which catalyzes the conversion of 7-dehydrocholesterol to cholesterol, the last step in the synthetic pathway. As countertreatment, half of the BM-treated rats were fed 2% cholesterol during the last 2 months. With BM, cholesterol concentrations declined 91% in plasma, but with cholesterol feeding, the levels increased 50% compared with baseline values. 7-Dehydrocholesterol, which was not detected at baseline, increased to 55% of plasma sterols with BM but decreased to 5% of total plasma sterols when cholesterol was added. With BM, brain cholesterol levels decreased 60% and did not increase after cholesterol was added. However, 7-dehydrocholesterol, which comprised 39% of brain sterols with BM, decreased to 31% ($P < .05$) when cholesterol was fed. Hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase activity in the liver increased 2.2-fold with BM and declined 95% after adding cholesterol, but did not change in the brain. BM treatment for 4 months prevented learning of the conditioned eyeblink response as compared with controls. In contrast, BM-treated rats supplemented with cholesterol acquired the conditioned eyeblink response. Chronic inhibition of 7-dehydrocholesterol-Δ^7-reductase reduced cholesterol and increased 7-dehydrocholesterol levels in plasma and brain, and was associated with impaired learning. Cholesterol feeding corrected plasma and hepatic sterol levels and reduced brain 7-dehydrocholesterol concentrations to reestablish normal learning.

SMITH-LEMLI-OPITZ SYNDROME is the third most common recessively inherited birth defect in North American Caucasians, with an estimated frequency of 1 in 20,000 births and a carrier (heterozygote) prevalence of 1% to 2%.[1] The initial clinical description was published in 1964 by Smith, Lemli, and Opitz,[2] and the major features include distinctive facial dysmorphism, limb abnormalities, and malformations of the brain with incomplete myelination and mental retardation.

We recently described a severe defect[3] in late cholesterol biosynthesis involving incomplete conversion of the precursor, 7-dehydrocholesterol, to cholesterol (Fig 1). This step is catalyzed by the microsomal enzyme 7-dehydrocholesterol-Δ^7-reductase, which was markedly inhibited (11% of control) in the liver of four Smith-Lemli-Opitz homozygotes.[4] Because of the inherited defect in 7-dehydrocholesterol-Δ^7-reductase, Smith-Lemli-Opitz homozygotes cannot produce cholesterol normally and show a marked deficiency in plasma and tissues associated with the accumulation of the precursor, 7-dehydrocholesterol. However, it is not clear whether the multitude of neurologic and physical abnormalities present in homozygotes with Smith-Lemli-Opitz syndrome result from the lack of cholesterol, the increase in the precursor 7-dehydrocholesterol, or the combination of these biochemical defects.

In this study, we correlated brain and plasma sterol composition with sensory reactivity and new motor learning in newly weaned rats by measuring the classical conditioned eyeblink response. This reflex (eyeblink response) provides a convenient platform on which to observe the acquisition of causal relationships.[5,6] An advantage of this paradigm is that performance deficits can be measured; thus, an assessment of learning and memory can be made independently of performance factors. Furthermore, there is an extensive literature detailing the neural pathway involved in the learning of the eyeblink response.[7] Virtually the entire essential circuitry is located in the brainstem, while an intact cerebellum is also required to coordinate the muscular contractions involved in the eyeblink.[8] Another advantage of this paradigm is that corresponding learning can be quantified in both humans and other mammals.

BM 15.766 (BM) is a synthetic piperazine derivative that inhibits 7-dehydrocholesterol-Δ^7-reductase to block the conversion of 7-dehydrocholesterol to cholesterol.[9] When BM was fed to rats, plasma and tissue cholesterol levels declined and 7-dehydrocholesterol increased markedly to reproduce the biochemical abnormalities seen in Smith-Lemli-Opitz syndrome.[10] Recently, we tested different treatment strategies in this model and showed that feeding cholesterol might improve the biochemical abnormalities in this syndrome.[11] In this study, we correlated the sensory reactivity and associative learning as measured by the classically conditioned eyeblink response with the sterol composition of brain and plasma in rats treated with BM or BM plus cholesterol. Our results show that abnormal brain cholesterol metabolism impaired the learning of the conditioned eyeblink response and was normalized when cholesterol was fed.

MATERIALS AND METHODS

Chemicals

BM 15.766, 4-(2-[1-(4-chlorocinnamyl)piperazin-4-yl]ethyl)-benzoic acid, was a gift from Boehringer (Mannheim, Germany). 7-Dehydrocholesterol (5,7-cholestdien-3β-ol) was purchased from Aldrich Chemical (Milwaukee, WI), and cholesterol (5-cholesten-3β-ol)

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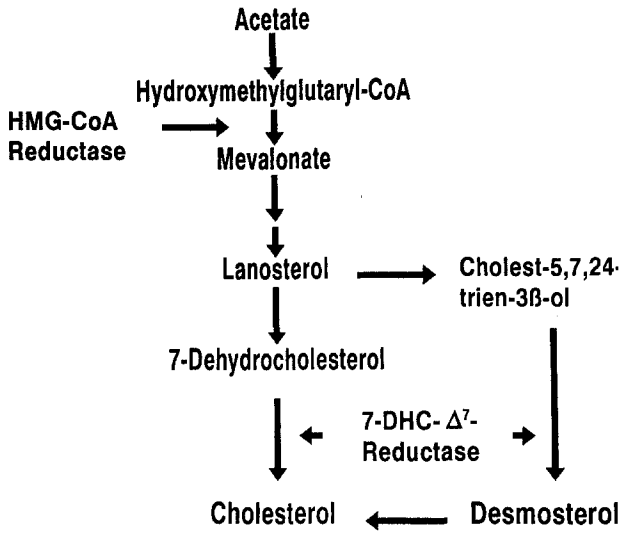

Fig 1. Diagram of cholesterol synthetic pathways showing the location of the key rate-controlling step catalyzed by HMG-CoA reductase and the conversion of 7-dehydrocholesterol to cholesterol catalyzed by 7-dehydrocholesterol(DHC)-Δ^7-reductase, which is defective in homozygotes with the Smith-Lemli-Opitz syndrome.

and 5α-cholestane were obtained from Sigma Chemical (St Louis, MO).

Animal Experiments

Eighteen newly weaned (50 to 70 g) Sprague-Dawley male rats (Charles River, Wilmington, MA) were divided into three groups: group 1 (n = 6) served as a control; group 2 (n = 6) was fed BM (30 mg/kg/d) by gavage for 4 months, with the BM suspended in water (10 mg/mL) by sonication; and group 3 (n = 6) was fed BM for 4 months plus rat chow containing 2% cholesterol for the last 2 months. During the last week of the 4-month treatment, the rats were tested for learning the conditioned eyeblink response. At completion of the experiment, the animals were killed and the blood, brain, and liver were obtained for measurements of sterol levels and hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase activity. The experimental protocol was approved by the committee on animal studies at the Veterans Affairs Medical Center (East Orange, NJ).

Conditioned Eyeblink Response Test

Microelectrodes were implanted subcutaneously to emerge through the right eyelid.[12] Of the four implanted electrodes, two delivered the unconditioned stimulus, a 100-ms, 0.7-mA periorbital shock, and another two transmitted eyelid electromyographic data that were filtered and amplified (10k) with a differential AC amplifier. The conditioned stimulus was a 600-ms, 90-dB white-noise burst.

Behavioral testing began with a 1-hour acclimation period, and sensory reactivity consisted of 10 exposures to the stimulus to be conditioned, prior to training. Thereafter, associative learning measured the acquisition of the classically conditioned eyeblink response. A delayed-type paradigm was used, ie, the 600-ms conditioned stimulus coterminated with the 100-ms unconditioned stimulus. Training consisted of 30 blocks of 10 trials. Each trial block consisted of a conditioned stimulus alone followed by four paired trials, an unconditioned stimulus alone, and four more paired trials. The intertrial interval was randomly determined as a minimum of 20 ± 10 seconds.

To determine the occurrence of an eyeblink response, a comparison value was established for each trial. This number represented the maximum value of the rectified prestimulus baseline (spontaneous eyeblink) and was added to four times the standard deviation of that baseline. For tests of sensory reactivity, an eyeblink was scored if rectified electromyographic activity exceeded this value. During the tests of associative learning, a conditioned response was scored in conditioned stimulus–alone trials when rectified electromyographic activity in a 500-ms period beginning 100 ms after onset of the conditioned stimulus exceeded the comparison value (Fig 2). In trials involving unconditioned stimulus delivery, a conditioned response was scored if electromyographic activity exceeded the comparison value 100 ms after onset of the conditioned stimulus but before onset of the unconditioned stimulus. Eyeblink performance was computed as a percentage of conditioned responses produced in trials in which the conditioned stimulus was delivered over each session.

Chemical Analysis

Sterol assays. Neutral sterols were extracted with hexane from 1 mL plasma or 0.5 g brain tissue after saponification in 1N ethanolic NaOH at 70°C for 1 hour. 5α-Cholestane was added to the samples as an internal standard. Trimethylsilyl ether derivatives were prepared and quantified by capillary gas-liquid chromatography on a Hewlett-Packard model 5890A (Hewlett-Packard, Palo Alto, CA) equipped with a 25-m high-polarity capillary column (CP-Wax 57CB) as described previously.[13]

Hepatic and brain microsomal total HMG-CoA reductase activity. Microsomes from liver and brain were prepared by differential ultracentrifugation, and the protein content was determined according to the method of Lowry et al.[14] HMG-CoA reductase activity was measured by the production of mevalonic acid (picomoles per milligram protein per minute) according to the method of Nguyen et al.[15]

Statistics

ANOVA models were used to calculate statistical significance for the biochemical and behavioral data. Specific comparisons were made by Dunnett's test for multiple comparisons to the control value, Bonferroni's test for comparisons when more than two groups were tested, and Student's *t* test for comparisons between two groups. Significance levels were set at *P* less than .05. BMDP Statistical Software (BMDP

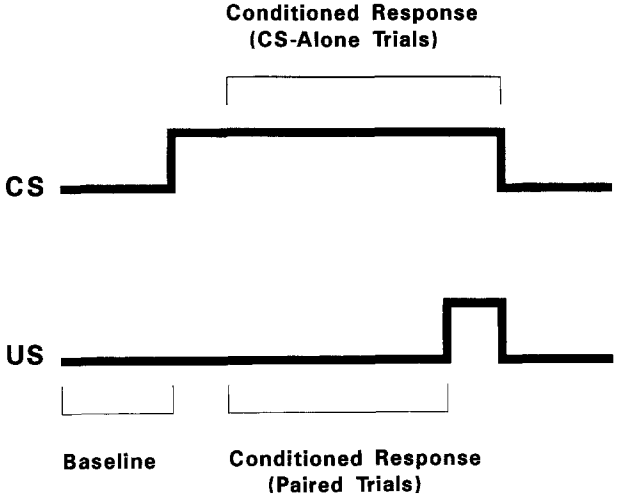

Fig 2. Delay-type classical conditioning paradigm. The baseline period was 200 ms. In trials where only the conditioned stimulus (CS) was delivered, a conditioned response was scored if significant electromyographic activity occurred from 100 ms after onset of the CS to offset of the CS. For trials where the unconditioned stimulus (US) was delivered, a conditioned response was scored if significant electromyographic activity occurred from 100 ms after onset of the CS to onset of the US.

Table 1. Sterol Concentrations in the Plasma, Brain, and Liver of Rats

Group	Plasma (mg/dL)			Brain (mg/g)			Liver (mg/g)		
	Ch	7DHC	%7DHC	Ch	7DHC	%7DHC	Ch	7DHC	%7DHC
Control (n = 6)	51 ± 7	ND	0	13.1 ± 1.2	ND	0	2.2 ± 0.8	ND	0
BM (n = 6)	10 ± 3*	12 ± 2	55 ± 8	5.2 ± 0.4*	3.3 ± 0.8	39 ± 5	0.5 ± 0.1*	0.9 ± 0.2	60 ± 7
BM + Ch (n = 6)	77 ± 12†	4 ± 2†	5 ± 2†	4.9 ± 0.9*	2.3 ± 0.5‡	31 ± 3‡	3.5 ± 1.5†	0.2 ± 0.1†	6 ± 3†

NOTE. Data are the mean ± SD. For comparisons of 7DHC and %7DHC, unpaired Student's *t* test was applied; Bonferroni's test was used for comparisons of Ch.

Abbreviations: ND, not detectable; BM, fed BM for 4 months; BM + Ch, fed BM for 4 months and 2% cholesterol during the last 2 months; Ch, cholesterol; 7DHC, 7-dehydrocholesterol; %7DHC, percentage of 7-dehydrocholesterol of total sterols.

*$P < .001$ *v* control.

†$P < .001$ *v* BM.

‡$P < .05$ *v* BM.

Statistical Software, Los Angeles, CA) was used for statistical evaluations.

RESULTS

Plasma sterol concentrations are listed in Table 1. After 4 months of feeding BM, plasma cholesterol declined 91% and the precursor 7-dehydrocholesterol, whose concentration in control plasma was almost nil, increased substantially to 12 ± 2 mg/dL and now accounted for 55% ± 8% of total sterols. Feeding 2% cholesterol (50 mg/d) in the diet for the last 2 months with BM increased plasma concentrations 7.7-fold and reduced 7-dehydrocholesterol levels 67% as compared with BM alone, so that the precursor now represented only 5% of plasma sterols.

Brain sterol concentrations are also reported in Table 1. Four months of BM treatment in newly weaned rats caused a 60% reduction in brain cholesterol levels and a marked increase in 7-dehydrocholesterol concentrations, so that the precursor accounted for 39% ± 5% of brain sterols as compared with the trace amount barely detected in control brains. Countertreatment with cholesterol for the last 2 months not only failed to increase brain cholesterol, but the concentrations actually decreased 62% as compared with the control. However, with cholesterol feeding, 7-dehydrocholesterol levels in the brain were 30% lower and the proportion of 7-dehydrocholesterol relative to total brain sterols decreased 20% ($P < .05$) as compared with BM treatment alone.

After BM treatment, hepatic cholesterol concentrations decreased 77%. When 2% cholesterol was added, liver cholesterol concentrations increased and were now 59% higher than in the controls ($P < .001$). Hepatic 7-dehydrocholesterol, which was not detected in controls, increased to 0.9 mg/g with BM treatment. After cholesterol was added to BM, hepatic 7-dehydrocholesterol concentrations decreased 78% and the percentage of 7-dehydrocholesterol in total hepatic sterols decreased 90% ($P < .001$; Table 1).

With BM treatment, HMG-CoA reductase activity increased 2.2-fold in the liver. Adding cholesterol with BM expanded the plasma and liver cholesterol pools (Table 1) and markedly inhibited the elevated HMG-CoA reductase activity 95% ($P < .001$), so the enzyme activity was even lower than the control level. Baseline HMG-CoA reductase activity in the brain was 58% lower ($P < .01$) than in the liver and did not increase when BM was administered despite the reduction in brain cholesterol. Cholesterol feeding with BM did not reduce HMG-CoA reductase activity in the brain (Table 2).

The spontaneous blink rates of BM- and BM + cholesterol-treated rats did not differ reliably from those of control rats (Table 3). Sensory reactivity was tested by exposing the rats to 10 white-noise bursts (the same auditory stimulus used in the training phase of testing). The proportion of eyeblinks elicited by the white-noise burst did not differ reliably among groups ($F(2,8) = 1.45$, $P = .28$). The latency time to respond, measured from the beginning of the white-noise burst to the onset of an eyeblink response, also did not reliably differ among groups ($F(2,8) = 3.05$, $P = .10$), although there was a tendency for a slower response in BM-treated rats. These data indicate that the sensory-motor apparatus for performing eyeblinks in BM-treated rats was intact.

A 3 × 7 treatment (group × trial block) ANOVA indicated only a main effect of treatment group ($F(2,8) = 7.2$, $P = .01$; Fig 3). Rats treated with BM alone exhibited fewer eyeblink conditioned responses over all trial blocks compared with control rats and rats treated with BM plus countertreatment with cholesterol. Specific comparisons indicated that rats treated with BM plus exogenous cholesterol displayed fewer eyeblink conditioned responses over the first trial block compared with control rats. Normal acquisition was exhibited in rats given countertreatment (BM plus cholesterol) from the second trial block to the end of training. None of the BM-treated rats reached the minimum criterion of eight eyeblink conditioned responses in nine consecutive trials within the 300-trial period, whereas all control rats and BM-treated rats fed cholesterol reached this criterion.

Table 2. HMG-CoA Reductase Activity (pmol/mg protein/min) in the Rat

Group	Liver	Brain
Control (n = 6)	52 ± 15	22 ± 11†
BM (n = 6)	114 ± 9	21 ± 9
BM + Ch (n = 6)	6 ± 4*	21 ± 8

NOTE. Data are the mean ± SD. Abbreviations are defined in Table 1.

*$P < .001$ *v* BM (Bonferroni test).

†$P < .01$ *v* control value of the liver (Student's *t* test).

Table 3. Sensory Reactivity of Rats Before Training

Group	Spontaneous Blink Rate (%)	Proportion of Eyeblink Responses (%)	Latency Time to Respond (ms)
Control (n = 3)	18 ± 3	88 ± 7	203 ± 21
BM (n = 3)	11 ± 4	60 ± 10	314 ± 66
BM + Ch (n = 3)	17 ± 3	70 ± 15	296 ± 19

NOTE. Data are the mean ± SD. Abbreviations are defined in Table 1. Dunnetts' test was applied for multiple comparisons to the control.

DISCUSSION

The results of this investigation demonstrated a strikingly negative effect on learning when the last step of cholesterol synthesis was inhibited. Brain, liver, and plasma cholesterol concentrations decreased 60%, 77%, and 91%, respectively, and the precursor 7-dehydrocholesterol, which usually cannot be detected, increased to 39% of brain, 60% of liver, and 55% of plasma sterols. Countertreatment with exogenous dietary cholesterol replenished cholesterol concentrations in the plasma such that the expanded pool inhibited de novo cholesterol biosynthesis in the liver and reduced the formation of the precursor 7-dehydrocholesterol, which declined markedly in the plasma to account for only 5% of total sterols. Although brain cholesterol concentrations did not increase, 7-dehydrocholesterol levels were also reduced 30% and the rats were then able to acquire the conditioned eyeblink response, albeit at a slightly slower learning rate in the first 20 trials (Fig 3). According to these results, the accumulation of 7-dehydrocholesterol in the brain with substitution of the precursor for cholesterol impaired the learning of the classically conditioned eyeblink response. The learning was normalized by feeding cholesterol, which reduced 7-dehydrocholesterol levels in the brain below a critical value.

Exogenous dietary cholesterol was absorbed and taken up into plasma lipoproteins and incorporated into the liver. The expanded cholesterol pool inhibited hepatic HMG-CoA reductase[16-18] and probably also lathosterol 5-dehydrogenase,[1] the first and next-to-last enzymes in the cholesterol biosynthetic pathway (Fig 1). The formation of precursors, specifically 7-dehydrocholesterol, was diminished such that the concentrations in the plasma and brain decreased. The enlarged cholesterol pool replenished virtually all tissue compartments with cholesterol except for the brain. The blood-brain barrier remained impermeable to circulating plasma cholesterol, and the cholesterol used for brain development must be synthesized within the brain.[19-21] Despite the considerable intake of dietary cholesterol (50 mg/d for 2 months), brain cholesterol concentrations even decreased. This presents a serious problem to overcome in designing treatment strategies for homozygotes with Smith-Lemli-Opitz syndrome, where the inherited defect in 7-dehydrocholesterol-Δ^7-reductase is expressed in every tissue and results in low concentrations of cholesterol with elevated amounts of the precursor 7-dehydrocholesterol. Countertreatment with exogenous cholesterol corrected hepatic and plasma cholesterol levels, but brain cholesterol concentrations remained low. We are not sure how feeding cholesterol, which corrects the abnormal cholesterol biosynthesis and significantly reduces 7-dehydrocholesterol in the liver and other tissues, can also decrease the proportion of 7-dehydrocholesterol in brain sterols. However, this is the most significant biochemical change in the brain after cholesterol feeding, and we postulate that this reduction in the proportion of 7-dehydrocholesterol is related to the restoration of normal learning.

Acquisition of the classically conditioned eyeblink response requires not only an intact sensory pathway (hearing and perception) but also a functioning cerebellum to coordinate impulses within a motor neuron pathway that innervates muscles of the eye so that an eyeblink can be performed. The integrity of the sensory-motor apparatus was verified through the test of sensory reactivity: eyeblink responses to loud noises were equivalent between BM-treated rats and controls. Thus, the inability of BM-treated rats to acquire the conditioned eyeblink response was likely unrelated to performance factors. Impaired learning as a result of inhibiting cholesterol biosynthe-

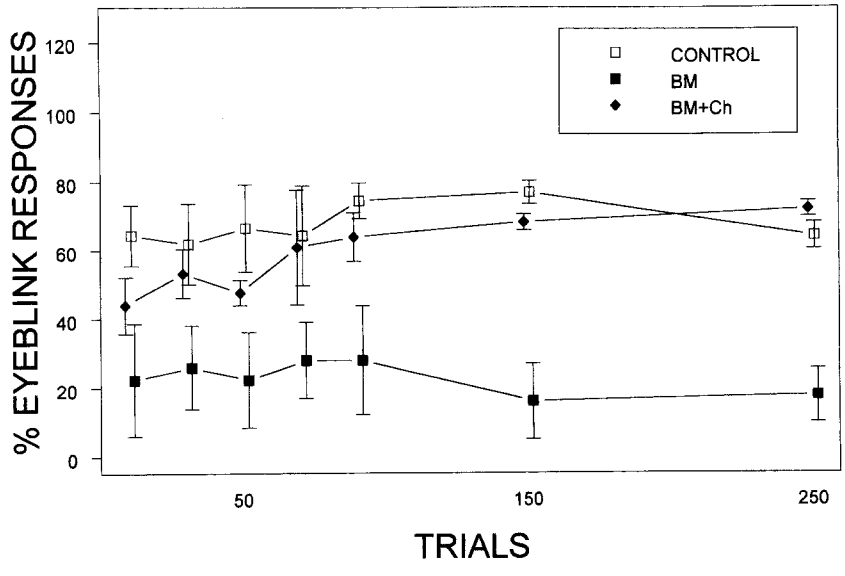

Fig 3. Acquisition of classically conditioned eyeblink response. Rats treated with BM emitted significantly fewer eyeblink responses over the 300-trial training period *v* control rats. Exogenous cholesterol (Ch) counteracted BM treatment; BM + Ch rats learned the eyeblink response similarly to control rats.

sis could be overcome by feeding cholesterol. The cholesterol-fed rats treated with BM were capable of learning the conditioned eyeblink response at a rate similar to that of the controls. These findings emphasize that a critical mass of 7-dehydrocholesterol in the brain is more important for interfering with brain function than low cholesterol levels alone. In BM-treated rats, brain cholesterol decreased and, unlike the plasma levels, did not increase when cholesterol was fed. However, cholesterol treatment significantly reduced the proportion of 7-dehydrocholesterol in brain sterols, which was associated with normalized learning of the eyeblink response.

At present, there are several trials testing the effect of cholesterol feeding in Smith-Lemli-Opitz syndrome homozygotes. The results are currently inconclusive, with some benefit being reported in a few homozygotes, while others are unable to document clinical improvement.[22] The results of this investigation emphasize the importance of increased 7-dehydrocholesterol in the pathogenesis of faulty brain function and the effect of dietary cholesterol, which indirectly reduces elevated brain 7-dehydrocholesterol concentrations without increasing brain cholesterol levels, that is associated with improved learning.

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