Inhibitory Effect of Maternal Alcohol Ingestion on Rat Pup Hepatic 25-Hydroxyvitamin D Production

M. MILNE AND D. T. BARAN

Departments of Orthopedics and Medicine, Univeristy of Massachusetts Medical Center,
Worcester, Massachusetts

ABSTRACT. The mechanisms underlying the teratogenic effects of alcohol are unknown, and may reflect metabolic differences between adult and fetal tissues. The liver is the major site of alcohol metabolism and the sole site of 25 hydroxyvitamin D synthesis. We have compared 25-hydroxyvitamin D production in adult nonpregnant, maternal, and pup livers obtained from vitamin D-deficient animals and examined the effects of alcohol ingestion on hepatic vitamin D metabolism. 25 Hydroxyvitamin D production was comparable in adult nonpregnant and maternal livers, but was decreased in pup livers compared to their maternal controls (1.4 ± 0.2 versus 0.6 ± 0.1 pmol/g protein/h, $p < 0.001$). Alcohol ingestion for 18 days had no effect on hepatic synthesis of 25 hydroxyvitamin D in the adult livers, but inhibited production by the pup livers (0.6 ± 0.1 versus 0.3 ± 0.1 pmol/g protein/h, $p < 0.02$). To assess the physiologic significance of these observations, the effect of alcohol on 25-hydroxyvitamin D levels in vitamin D-replete mothers and fetuses was determined. Alcohol had no effect on circulating 25 hydroxyvitamin D levels in the mothers but lowered fetal 25 hydroxyvitamin D content (2.3 ± 0.3 versus 1.2 ± 0.3 ng/g fetus, $p < 0.02$) without altering fetal weight. The data indicate that differences exist in pup and adult hepatic metabolism of vitamin D and that alcohol has inhibitory effects on pup liver function not expressed in adult tissues. (*Pediatr Res* 19: 102–104, 1985)

Abbreviations

25 hydroxyvitamin D, 25 OH D
1,25 dihydroxyvitamin D, 125 (OH)$_2$D

The liver is the sole site of production of 25 OH D (1–3). Although chronic alcohol ingestion in nonpregnant man has been shown to result in decreased 25 OH D blood levels (4, 5), hepatic vitamin D 25-hydroxylation appears to be normal in alcoholic liver disease (5, 6). This suggests that the decreased blood levels are due to poor dietary intake and lack of sunlight (5, 6). Also, alcohol ingestion in nonpregnant vitamin D-depleted rats does not inhibit the conversion of vitamin D to 25 OH D following a pharmacologic dose of vitamin D (7). However, no studies have examined the effect of alcohol on the production of 25 OH D by livers obtained from rats ingesting alcohol while pregnant, or exposed to alcohol *in utero*.

Since alcohol ingestion during pregnancy has been reported to induce malformations specific to the fetus in man and rats (8), and since the liver is a major site of alcohol metabolism, we have compared the effects of ethanol on 25 OH D production by the livers of vitamin D-depleted adult nonpregnant, adult maternal rats and pups and on the 25 OH D content of alcohol-exposed vitamin D-replete fetuses. The data indicate that the ability of pup livers to produce 25 OH D is significantly diminished compared to their respective maternal livers, and that alcohol impairs 25 OH D production by pup livers, but not by the livers of adult rats. This impairment results in decreased fetal 25 OH D content.

METHODS

Three-week-old female weanling rats were fed a vitamin D-deficient diet (Teklad, Madison, WI) containing 0.2% calcium and 0.4% phosphate for 8 wk. This results in vitamin D deficiency characterized by 25 OH D levels less than 1 ng/ml (9). The rachitic rats were mated with vitamin D-replete male rats in the absence of uv light. Five pregnant rats (day 6) were given 20% ethanol in their drinking water supplemented with maltose, 37 g/dl, to insure ingestion until 2 days after delivery and given the Teklad food *ad libitum*. Five control pregnant rats were pair-fed with the Teklad food and maltose was isocalorically substituted for ethanol in the drinking water. Four nonpregnant female rats were administered alcohol and three control nonpregnant rats received maltose for the same 18-day period as did the pregnant rats.

The adult rats were sacrificed under light ether anesthesia by exsanguination from the abdominal aorta, and the pups were sacrificed by decapitation. The livers were removed and the homogenate was prepared as we have previously described (10). The pup livers from an individual litter were pooled and two to three homogenate incubations performed on each of the five alcohol-treated and five control litters. Two pmoles of ^{3}H-vitamin-D$_3$ (Amersham Corp., Arlington Heights, IL) were added to each flask containing the liver homogenate in 25 μl of 99% ethanol. Incubations were carried out for a maximum of 2 h at 37°C and 120 oscillations/min with continuous oxygenation at which time the reaction was terminated by addition of 25 ml methanol plus 12.5 ml chloroform. The resulting single phase was allowed to extract overnight at 4°C. The extracts were filtered into separatory funnels to remove precipitated protein. Ten milliliters of water were added to each funnel to complete the separation of phases which occurred overnight at 4°C. ^{3}H-25 OH D production was determined by chromatography of the lipid fraction on Sephadex LH 20-100 columns as previously described (10) and identity confirmed by comigration with authentic cold 25 OH D (courtesy Upjohn Co., Kalamazoo, MI).

The protein content of the liver homogenates was determined

according to the procedure of Lowry et al. (11) with bovine serum albumin as standard. The amount of ^{3}H-25 OH D produced was calculated from results obtained from chromatography and expressed in pmol/g liver protein/h.

The effect of ethanol exposure on 25 OH D levels of vitamin D-replete animals was compared in adult pregnant rats and their fetuses. Ten 11-wk-old vitamin D-replete female rats were mated with vitamin D-replete male rats. Five pregnant rats (day 6) were given 20% ethanol in their drinking water and five pregnant control rats (day 6) were given the maltose solution. The two groups were pair-fed with Purina Rat Chow (St. Louis, MO). On day 22 of pregnancy, the rats were anesthetized with ether and the fetuses exposed by abdominal incision. Each fetus was removed, rinsed with 0.9% saline, and weighed. The mothers were exsanguinated and plasma 25 OH D levels were determined by the method of Haddad and Chyu (12). Twelve control and 12 alcohol-exposed fetuses were homogenized in 4 ml of 0.9% saline. ^{3}H-25 OH D tracer (300 cpm) was added to each homogenate and these were allowed to extract overnight in 12.5 ml CHCl$_3$ and 25 ml MeOH. The extracts were filtered into separatory funnels. Twevle and one-half milliliters CHCl$_3$ and 10 ml H$_2$O were added to complete the separation of phases overnight. The lipid phase was collected and dried. The residues were solubilized in 1 ml of 33% ether in n-hexane (V$^-$/V$^-$) and applied to 4 cm Pasteur pipet columns of silicic acid. The total 25 OH D level in the fetuses was then determined (12). All data are expressed as the mean ± SEM, and the probability of difference determined by Student's t test.

RESULTS

Alcohol constituted 52 ± 3% of the total caloric intake of the pregnant rats and 58 ± 3% of the total caloric intake of the nonpregnant rats. This level of alcohol consumption has previously been shown by us to result in blood levels of 98 mg/dl in pregnant rats (13) and 130 mg/dl in nonpregnant rats (14). Alcohol ingestion did not affect litter size. ^{3}H-25 OH D production was linear during the 2-h incubation in all tissues. Although the pup rat liver was able to synthesize ^{3}H-25 OH D, production by control pup liver homogenates was significantly less than maternal hepatic production (1.4 ± 0.2 versus 0.6 ± 0.1 pmol/g protein/h, $p < 0.001$) (Table 1). Alcohol consumption had no effect on ^{3}H-25 OH D production by liver homogenates of nonpregnant rats (1.3 ± 0.3 versus 1.0 ± 0.1 pmol/g protein/h) or rats ingesting alcohol while pregnant (1.4 ± 0.2 versus 1.2 ± 0.1 pmol/g protein/h). However, maternal alcohol ingestion significantly inhibited pup hepatic ^{3}H-25 OH D synthesis (0.6 ± 0.1 versus 0.3 ± 0.1 pmol/g protein/h, $p < 0.02$) (Table 1). Alcohol ingestion by vitamin D-replete rats did not affect maternal 25 OH D blood levels (12.3 ± 1.2 versus 11.6 ± 0.5 ng/ml) or fetal weight (2.63 ± 0.03 versus 2.65 ± 0.04 g/pup), but lowered fetal 25 OH D content (2.3 ± 0.3 versus 1.2 ± 0.3 ng/g fetus, $p < 0.02$) (Table 2).

Table 1. *Effect of alcohol on 25 OH D production by livers obtained from pregnant pup and nonpregnant vitamin D-deficient rats**

	Pregnant rats	Pups	Nonpregnant rats
	(p mol/g protein/hr)		
Control	1.4 ± 0.2	0.6 ± 0.1	1.3 ± 0.3
Alcohol	1.2 ± 0.1	0.3 ± 0.1	1.0 ± 0.1
Probability of difference	NS	$p < 0.02$	NS

* Hepatic 25 OH D production for each liver or litter was defined as the average of three homogenate incubations for the adult livers and two to three homogenate incubations on the pooled livers from each litter. Values represent the mean ± SEM of five control and five alcohol-exposed pregnant rat livers and the livers from each litter, and three control and four alcohol-exposed nonpregnant rat livers.

Table 2. *Effect of alcohol on fetal wt and 25 OH D content of vitamin D-replete fetuses**

	Fetal wt (g)	25 OH D (ng/g fetus)
Control	2.63 ± 0.03	2.3 ± 0.3
Alcohol	2.65 ± 0.04	1.2 ± 0.3
Probability of difference	NS	$p < 0.02$

* Values for fetal weight and fetal 25 OH D content represent the mean ± SEM of 12 control and 12 alcohol-treated fetuses obtained from five control and five alcohol-exposed litters.

DISCUSSION

Previous studies in our laboratory have demonstrated that 25 OH D and 1,25 (OH)$_2$D inhibit hepatic 25 OH D production in vitro (10). Recent observations in man indicate that treatment with 1,25 (OH)$_2$D impairs the conversion of administered vitamin D to 25 OH D (15) and lowers 25 OH D levels in postmenopausal women (16). Thus, it appears that the liver is capable of responding to increasing levels of 1,25 (OH)$_2$D by inhibiting production of the 25 OH D precursor and that mechanisms exist to regulate hepatic 25 OH D production.

Very little is known regarding the control of 25 OH D synthesis, which occurs in the microsomes (17) and mitochondria (18) of the hepatocyte. The microsomal 25-hydroxylation requires molecular oxygen and reduced pyridine nucleotide, appears to be stimulated by supernatnat protein, and behaves like a cytochrome P450-dependent monooxygenase (19). The mitochondrial 25-hydroxylase is catalyzed by a mixed function oxidase, depends on NADPH and ferrodoxin, and is inhibited by carbon monoxide, the latter suggesting that cytochrome P450 is involved (18). The hepatic microsomal 25-hydroxylation system is apparently regulated by the vitamin D status of the animal (20, 21) whereas the mitochondrial system does not appear to be affected by vitamin D status (18). Recent work has demonstrated that the reconstituted liver mitochondrial cytochrome P450 system active as a vitamin D 25-hydroxylase has characteristics different from the microsomal enzyme (22). This suggests that their control mechanisms might also differ.

The data in this report demonstrate that differences exist between the maternal and fetal hepatic metabolism of vitamin D. These differences may indicate either an alteration in the amount of mitochondrial or microsomal enzyme available, or a change in the affinity of enzyme for vitamin D. It is unlikely that these differences reflect varying levels of vitamin D metabolites in mothers and pups. 25 OH D levels in cord blood of vitamin D-replete rats correlate closely with maternal levels of 25 OH D (23). There is poor correlation between maternal and cord levels of 1,25 (OH)$_2$D in the vitamin D-replete rat (23), with cord levels being lower. However, since 1,25 (OH)$_2$D inhibits 25 OH D production in vitro (14) and in vivo (15, 16), lower pup levels of 1,25 (OH)$_2$D would not explain decreased pup hepatic synthesis of 25 OH D relative to maternal production. These differences between the ability of adult and pup hepatic tissues to metabolize vitamin D may underline the susceptibility of pup livers to alcohol-induced inhibition of 25 OH D synthesis. In preliminary studies we have demostrated that calcium inhibits synthesis of 25 OH D by liver homogenates in a dose-dependent fashion, with inhibition being demonstrated at a calcium concentration of 0.4 mM (24). Coupled with the observation that chronic ethanol ingestion increases calcium uptake by liver microsomes (25), it is tempting to speculate that the effects of alcohol on pup hepatic 25 OH D production are mediated by enhanced susceptibility to alterations in calcium fluxes. Alternatively, ethanol rapidly crosses the placenta and it has been shown to retard growth and differentiation in cultured rat embryos during organogenesis (26), suggesting that the observed effects of alcohol may be the result of a direct toxic effect on the pup hepatic microsomes and/or mitochondria.

This study indicates that differences exist in the pup and adult

hepatic 25-hydroxylation of vitamin D and that alcohol, while having no effect on 25 OH D production in adult livers, inhibits synthesis in pup livers. Alcohol-mediated decreases in pup hepatic 25 OH D production as well as decreases in 25 OH D transport from mother to fetus (13) may contribute to the diminished 25 OH D content of alcohol-exposed vitamin D-replete pups.

REFERENCES

1. Ponchon G, Kennan AL, DeLuca HF 1969 Activation of vitamin D by the liver. J Clin Invest 48:2032
2. Olson EB Jr, Knutson JC, Bhattacharyya MH, DeLuca HF 1976 The effect of hepatectomy on the synthesis of 25 hydroxyvitamin D_3. J Clin Invest 57:1213
3. Ponchon G, DeLuca HF 1979 The role of the liver in the metabolism of vitamin D. J Clin Invest 48:1273
4. Lund B. Sorenson OH, Hilden M, Lund B 1977 The hepatic conversion of vitamin D in alcoholics with varying degrees of liver affection. Acta Med Scand 202:221
5. Posner DB, Russell RM, Absood S, Connor TB, Davis C, Martin L, Williams JB, Norris AH, Merchant C 1978 Effective 25-hydroxylation of vitamin D_2 in alcoholic cirrhosis. Gastroenterology 74:866
6. Barragry JM, Long RG, France MW, Wills MR, Boucher BJ, Sherlock S 1979 Intestinal absorption of cholecalciferol in alcoholic liver disease and primary biliary cirrhosis. Gut 20:559
7. Gascon-Barre M 1982 Plasma 25 OH D response to a pharmacological dose of vitamin D or 25 OH D during chronic ethanol administration in the rat. Horm Metabol Res 14:332
8. Streissguth AP, Lundesman-Dwyer S, Martin JC, Smith DW 1980 Teratogenic effects of alcohol in humans and laboratory animals. Science 209:353
9. Baran DT 1983 Effect of chronic (8 week) phenobarbital treatment on the metabolism of ^{3}H-vitamin D by the perfused rachitic rat liver in vitro. Am J Physiol 245:E55
10. Baran DT, Milne M 1983 1,25$(OH)_2$D induced inhibition of ^{3}H-25 OH D production by the rachitic rat liver in vitro. Calcif Tissue Int 35:461
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the folin-phenol reagent. J Biol Chem 193:265
12. Haddad JG, Chyu KJ 1971 Competitive protein binding assay for 25 hydroxycholecalciferol. J Clin Endocrinol Metab 33:992
13. Baran DT 1982 Alcohol-induced inhibition of fetal ^{3}H-25 OH D and ^{14}C-α-aminoisobutyric acid accumulation in the pregnant rat. Endocrinology 111:1109
14. Baran DT, Bryant C, Robson D 1982 Alcohol induced alterations in calcium metabolism in the pregnant rat. Am J Clin Nutr 36:41
15. Bell NH, Shaw S, Turner RT 1984 Evidence that 1,25 dihydroxy-vitamin D inhibits the hepatic production of 25 hydroxyvitamin D in man. J Clin Invest 74:1540
16. Lore F, DiCairano G, Periti P, Caniggia A 1982 Effect of the administration of 1,25 dihydroxyvitamin D_3 on serum levels of 25 hydroxyvitamin D in postmenopausal osteoporosis. Calcif Tissue Int 34:539
17. Bhattacharyya MH, DeLuca HF 1974 Subcellular location of rat liver calciferol-25-hydroxylase. Arch Biochem Biophys 160:58
18. Bjorkhem I, Holmberg I 1978 Assay and properties of a mitochondrial 25 hydroxylase active on vitamin D. J Biol Chem 253:842
19. Madhok TC, DeLuca HF 1979 Characteristics of the rat liver microsomal enzyme system converting cholecalciferol into 25 hydroxycholecalciferol. Biochem J 184:491
20. Bhattacharyya MH, DeLuca HF 1973 The regulation of rat liver calciferol-25-hydroxylase. J Biol Chem 248:2969
21. Delvin EE, Arabian A, Glorieux FH 1978 Kinetics of liver microsomal cholecalciferol-25-hydroxylase in vitamin D deplete and replete rats. Biochem J 172:417
22. Bjorkhem I, Holmberg I, Oftebro H, Pedersen JI 1980 Properties of a reconstituted vitamin D-25-hydroxylase from rat liver mitochondria. J Biol Chem 255:5244
23. Halloran BP, Barthell EN, DeLuca HF 1979 Vitamin D metabolism during pregnancy and lactation in the rat. Proc Nat Acad Sci 76:5549
24. Baran DT, Milne M 1983 Inhibitory effect of calcium on ^{3}H-25 OH D production by the rachitic rat liver in vitro. Clin Res 31:693 (abstr)
25. Ponappa BC, Waring AJ, Hoek JB, Rottenberg H, Rubin E 1982 Chronic ethanol ingestion increases calcium uptake and resistance to molecular disordering by ethanol in liver microsomes. J Biol Chem 257:10141
26. Brown NA, Goulding EH, Fabri S 1979 Ethanol embryotoxocity: direct effects on mammalian embryos in vitro. Science 206:573

Fatty Acid Abnormalities in Cystic Fibrosis

PHILIP M. FARRELL, ELAINE H. MISCHLER, MICHAEL J. ENGLE, D. JEANNETTE BROWN,
AND SZE-MEI LAU

*Departments of Pediatrics and Nutritional Sciences, University of Wisconsin-Madison, Madison,
Wisconsin 53792*

ABSTRACT. Fatty acids were measured by gas chromatography in lipid extracts of plasma and tissues obtained from three categories of 46 patients with cystic fibrosis. Low levels of the major essential fatty acid linoleate were found in plasma total lipids of patients who had malabsorption but not in those without evidence of steatorrhea. Circulating arachidonic acid was only slightly decreased, and the unusual triene reflecting pathologically altered fatty acid metabolism (20:3ω9) was generally not detected, nor was the triene/tetraene ratio abnormal except for in two patients. There was no correlation between plasma linoleate and age, clinical severity score, or vitamin E status. Decreased linoleate did correlate with two indices of malabsorption, namely plasma carotene (r = 0.64) and fecal fat excretion (r = 0.76). Our data therefore indicate that the abnormality in linoleate is associated with (secondary to) malabsorption of dietary fat despite pancreatic enzyme replacement therapy and consumption of a regular diet. The frequency of this alteration was determined to be quite high in 40 patients with steatorrhea, 85% of whom showed values below the lower limit of normal for plasma linoleate. It was of interest to find markedly decreased levels of linoleate in adipose tissue, cardiac muscle, and lung and lesser reductions in liver and psoas muscle taken at autopsies. Tissue arachidonic acid percentage was normal, however, and 20:3ω9 was rarely present. Thus, the physiological significance of this common abnormality in