Effects of Dietary Choline and *N,N*-Dimethylaminoethanol on Lung Phospholipid and Surfactant of Newborn Rats

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Summary

Pups delivered by rats fed during pregnancy a choline-deficient (CD) diet containing 1% *N,N*-dimethylaminoethanol (DME) die within 36 hr of birth. The concentrations of sphingomyelins, phosphatidyl cholines, and disaturated phosphatidyl cholines in the lungs of these pups are lower than those in the lungs of pups delivered by dams fed a choline-supplemented diet (CS). The amount of surfactant isolated from the lung of the pups was also reduced. These changes were accompanied by alterations in the activity of enzymes (choline kinase, EC 2.7.1.32; choline phosphotransferase, EC 2.7.8.2) involved in the synthesis of lung lecithins. These results strongly suggest that pups delivered by dams fed a

CD diet containing 1% DME die of respiratory distress syndrome due to altered metabolism of lung surfactant.

Speculation

The results presented in this paper show that synthesis of fetal lung phospholipids is influenced by the presence or absence of choline or its precursors in the diet of pregnant rats. This influence extends to the synthesis of lung surfactant. These observations indicate that a new experimental model of respiratory distress syndrome in the newborn may have been found, and raise the question of whether synthesis of lung surfactant in human fetuses may be also vulnerable to dietary conditions.

INTRODUCTION

In a previous communication (18) we described the effects of feeding a CD diet containing 1% N-methylaminoethanol on tissue phosphatides of adult rats. Large amounts of phosphatidyl N-methylaminoethanol (PMME) and of phosphatidyl N, N-dimethylaminoethanol (PDME) were found to accumulate in the lung and liver. A significant decrease in the liver content of lecithins also occurred. Furthermore, liver lecithins contained low proportions of palmitic acid and were instead enriched in stearic and oleic acids. These results led us to consider whether it would be possible to affect the content of surfactant in the lungs of newborns by feeding a CD diet containing either N, methylaminoethanol or N, N-dimethylaminoethanol (DME) to pregnant rats.

It is well established that the major active component of lung surfactant is a disaturated lecithin, dipalmitoyllecithin (1, 7). Moreover, it is known (8, 16) that a reduced content of lung surfactant is the major factor in the pathogenesis of respiratory distress syndrome in human infants. These results of the experiments described in this paper show that most of the newborn rats of dams fed a CD diet supplemented with DME die within 36 hours. In these newborns, the content of lung surfactant is severely reduced. This reduction is accompanied by changes in the activity of enzymes involved in the synthesis of lung lecithins. The results suggest that death of the newborns is probably due to the onset of a respiratory distress syndrome.

MATERIALS AND METHODS

Materials.

[methyl-³H] choline chloride (1.9 Ci/mol); Phospho-[methyl-¹⁴C] choline (16.7 m Ci/mol); CDP-[methyl-¹⁴C] choline (0.3 Ci/mol) and Aquasol were purchased from New England Nuclear, Boston, MA. CTP was obtained from Calbiochem, LaJolla, CA. DME was supplied by K and K Laboratories, Cleveland, OH. The base was adjusted to pH 7.0 with 9N hydrochloric acid before addition to the diets. All other chemicals were purchased from Sigma Chemicals, St. Louis, MO.

General Procedures.

Female Sprague-Dawley rats, weighing 250-350 g and at the 3rd day of pregnancy, were obtained from Zivic-Miller, Allison Park, PA. The animals were housed in individual plastic cages in an air conditioned room. On the 6th day of pregnancy, the animals were divided into groups and were fed for the remainder of pregnancy, and for two weeks thereafter, one of the following diets: a) choline deficient diet (CD); b) CD + 1% DME (CD-DME); and c) CD + 0.8% choline chloride (CS). Food and water were allowed ad libitum, and food consumption and body weight were recorded daily and twice a week, respectively. The semisynthetic, semipurified CD diet was prepared as indicated previously (18). Choline, MME and DME were added to the diet at the expense of sucrose. After delivery, some of the dams and their litters were left together and were observed for a week. Litters were separated from other dams and weighed, decapitated, their lungs removed and weighed. Lungs from pups of the same litter were pooled for analysis. The whole operation was completed within 1 hour of delivery.

Chemical Analyses.

DNA and RNA were extracted from lung homogenates as outlined by Munro and Fleck (21). DNA was estimated by the method of Giles and Meyers (15), while RNA was quantitated by measurement of absorbance at 260 nm (21). Proteins were estimated by the method of Lowry, et al. (20).

Isolation of Surfactant.

Surfactant was isolated from homogenates of pooled lungs as described previously (17). Surfactant pellets were suspended in saline (0.9% NaCl, w/v), and aliquots of the suspensions were taken for analysis.

Lipid Analyses.

Whole lung homogenates (10% w/v) were prepared in saline using a Potter-Elvehjem type homogenizer. Total lipids were extracted (12) from aliquots of the homogenates and of the isolated surfactant preparations. Total and individual phospholipids, as well as disaturated lecithins, were separated and quantitated as described previously (17, 18).

Enzyme Assays.

Lungs were minced and homogenized with a Potter-Elvehjem type homogenizer in 9 volumes of 0.25 M sucrose containing 5 mM Tris-HCl, pH 7.4. The homogenates were filtered through 2 layers of gauze pads and were centrifuged at 1000 r.p.m. for 10 minutes (International Refrigerated Centrifuge, rotor 269) to sediment nuclei and cell debris. The supernatants were centrifuged at 13,000 r.p.m. for 30 minutes (Servall, rotor SS34) to sediment mitochondria. The activity of choline phosphate cytidyltransferase (CTP: choline phosphate cytidyltransferase, EC 2.7.7.15) was determined (2) in the supernatant. Aliquots of the same supernatants were then spun at 40,000 r.p.m. for 60 minutes (Spinco, Model L, ultracentrifuge, rotor 40) to sediment microsomes. The supernatant was used for assay (26) of choline kinase (ATP: choline phosphotransferase, EC 2.7.1.32). The microsomal pellet was washed twice with homogenizing medium. Choline phosphotransferase (CDP: choline: 1, 2-diacylglycerol choline phosphotransferase, EC 2.7.8.2) activity was estimated (25) in the microsomes after resuspending the pellet in 0.25M sucrose containing 5 mM Tris HCl, pH 7.4.

In the choline kinase assay, radioactive phosphoryl choline was separated (23) from choline on Ag-1 formate X4 columns (0.9 x 2.5 cm). Reaction rates were linear for 30 minutes, and were proportional to the amount of enzyme up to 1.5 mg of proteins per incubation. In the choline phosphate cytidyltransferase assay, radioactive phosphoryl choline was separated (22) from CDP-choline by elution from charcoal columns made from Pasteur pipettes. Reaction rates were linear up to 20 min., and were proportional to the amount of enzyme up to 1 mg of proteins per incubation. In the choline phosphotransferase assays, reaction rates were linear up to 20 min. and were proportional to the amount of enzyme up to 1 mg of proteins per incubation.

Other Analytical Procedures.

Radioactivity measurements were made with an Intertechnique 30 SL liquid scintillation spectrometer. Water (1 ml) and 10 ml of Aquasol were added to all radioactive samples.

Differences between means were checked with Student's test and regarded to be significant if $P \leq 0.05$.

RESULTS

Pregnancy progressed to term equally well in rats fed the various diets. Litters of similar sizes (10-14 pups) were delivered, with pups having only small differences in body weights (Table 1). All the pups delivered by dams fed the CD and CS diets survived for at least 15 days. In contrast, only 18 out of 253 survived for more than 36 hours in the group of pups delivered by mothers fed the CD-DME diet.

The lungs of pups delivered by dams fed the CD and CD-DME diets weighed significantly less than those of pups delivered by dams fed the CS diet (Table 2). However, there were no significant differences in the concentrations of DNA, RNA and proteins between the various groups of pups. In pups delivered by the CD and CD-DME groups of dams, the concentrations of sphingomyelins and phosphatidyl choline were lower than those in pups delivered by dams fed the CS diet (Table 3). In addition, in the CD-DME group, there was a reduction in the amount of disaturated phosphatidyl choline, while 14% of the total phospholipids were present as PDME. No measurable quantity of PDME was found in the lungs of pups delivered by CD and CS group of dams. There were no significant differences in the concentrations of other phospholipids between the various groups of pups.

The amounts of protein and phospholipid constituents of surfactant isolated from the lungs of pups delivered by the CD group of dams were consistently lower than those in pups delivered by dams fed the CS diet (Table 4). A similar decrease, but somewhat more pronounced, was present in pups delivered by dams of the CD-DME group.

The results of the enzyme assays are shown in Table 5. In pups delivered by the CD group of dams, the specific activity of choline kinase was 60% and 100% higher than in pups delivered by dams fed the CS or CD-DME diet, respectively. The specific activity of cholinephosphate cytidyltransferase was the same in the CS and CD-DME groups of pups, and only 50% as high in the CD groups of pups. In the latter group of pups, the specific activity of choline phosphotransferase was about 50% and 70% of that in the CS and CD-DME groups of pups, respectively.

DISCUSSION

The contents of lecithins in whole lungs (Table 3) and in purified surfactant (Table 4) of newborn rats of dams fed through pregnancy a CD diet were found to be significantly lower than those in newborn rats of dams fed similarly a CS diet. Further decreases in these contents were caused by inclusion of DME in the CD diet. Thus, our results show that synthesis of fetal lung lecithins, and of lung surfactant lecithins, depends on the presence of choline in the diet of pregnant rats. The further decreases in total lecithins both in whole lung and purified lung surfactant, observed after inclusion of DME in the CD diet, are most likely attributable to competition by DME (3, 4, 6, 10) for the same enzyme system which catalyzes the conversion of preformed choline to lecithins, with accumulation of abnormal quantities of PDME (Tables 3 and 4). Of major interest were the findings that decreases occurred also in the contents of disaturated lecithins of both whole lungs and purified surfactant (Tables 3 and 4). Disaturated lecithins, especially dipalmitoyllecithin, are known to be the principal active component of lung surfactant (1, 7). However, dipalmitoyllecithin is synthesized primarily by the deacylation-reacylation and deacylation-transacylation pathways (24). Thus our results suggest that activity of these pathways may also be altered.

Farrell (11) has reported that choline phosphotransferase is a rate limiting enzyme in the synthesis of lung lecithins via the CDP-choline pathway, and that the activity of this enzyme, as well as that of choline kinase, increases during the late stages of lung maturation. On the other hand, Chida, et al. (9) and Weinhold (26) have presented evidence indicating that choline phosphate cytidyltransferase may be the rate limiting enzyme, and showed that the activity of this enzyme also increases gradually with lung maturation. In newborn rats of dams fed the CD diet, the specific activity of both choline phosphate cytidyltransferase and of choline phosphotransferase were only 50% those in newborns of dams fed the CS diet (Table 5). It appears, thus, that the activity of these enzymes in lungs of newborn rats is also influenced by the availability of choline in the diet of pregnant rats. Moreover, the decreased enzymatic activities constitute further evidence that synthesis of lung lecithins is curtailed by the choline deficient regimen. Inclusion of DME in the CD diet resulted in higher specific activities of both these enzymes. These findings can be accounted for by the fact that conversion of DME to PDME involves these enzymes as well (3). On the other hand, the specific activity of choline kinase was highest in the lung of the CD group of pups (Table 5). The reason for this finding is not readily apparent. It is worthy of note, however, that choline kinase has been suggested to be not a rate limiting enzyme, at least, in fetal lung (11, 26). In the CD-DME group of pups, the specific activity of choline kinase was lower not only than that in the CD group of pups but also that in the CS group. A plausible explanation for this finding may be the fact that this enzyme was assayed in the 100,000 g supernatants of the lung homogenates. Thus, presence of free DME in this supernatant would compete with the radioactive choline used as substrate in the assay, with formation of unlabeled phosphoryl-DME in place of labeled phosphorylcholine.

The phospholipid to protein ratio of isolated lung surfactant is used as a criterion for its purity. As the data in Table 4 show, this ratio in our preparations was about 6.6 which is comparable to, or better than, those reported for lung surfactant isolated by other methods (13, 14, 19). Although the surface activity of the isolated surfactant was not measured, ultrastructurally, our surfactant preparations were found to contain both the intracellular (pool within the lamellar inclusion bodies) as well as the extracellular (pool present as tubular myelin figures [14]) surfactant. Quantitation of lung surfactant can also be made on the basis of its contents of phospholipids and proteins, which are the major constituents (19). The amount of surfactant (based on either phospholipid or protein content) present in the lungs of the CD group of pups was only about 70% that in the CS group, and was even lower (63%) in the CD-DME group.

Respiratory distress syndrome is a major cause of neonatal mortality in humans (5). The onset of this disease is known to be due to an insufficiency of lung surfactant at birth (8, 16). The results of the present studies show that in pups of rats fed through pregnancy a CD diet containing DME there is, at birth, a decreased amount of surfactant. Over 90% of the pups die within 36 hours of birth (Table 1). Were these two findings related, it is evident that a new experimental model of respiratory distress syndrome in the newborn would have been found.

CONCLUSIONS

Newborns of dams fed a CD diet containing 1% DME, die within 36 hours after birth. Their lungs show lowered contents of phosphatidyl choline, in particular, of disaturated species, and of surfactant. The activities of two enzymes (choline kinase and choline phosphotransferase) are also altered. The newborns may be dying due to onset of a respiratory distress syndrome.

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Table 1 Food Intake and Body Weight Gain of Pregnant Rats; Body Weight At Birth and Rate of Survival of Pups.

Pregnant Rats				Pups		
Diet*	Number	Average Daily Diet Intake (gm)**	Average Daily Gain in Body Weight (gm)**	Number Delivered	Body Weight at Birth (gm)***	Percent Surviving 36 Hours of birth
CS†	12	23.4 (18-26)	7.7 (7-9)	144	6.7 ± 0.1	100
CD	12	23.0 (22-25)	6.6 (5-9)	114	6.1 ± 0.2	100
CD-DME	23	22.1 (20-24)	6.2 (5-7)	253	6.1 ± 0.2	8

* Diet fed to the pregnant rats.

** Average (range).

*** Mean ± S.E.M.

† CS, Choline Supplemented; CD, Choline Deficient; CD-DME, CD + 1.0% N, N-dimethylaminoethanol.

Table 2 DNA, RNA and Protein Content in the Lungs
of Newborn Pups*

Diet**	Group	Lung			
		Wet Weight	DNA	RNA	Proteins
		g	mg/g wet weight		
CS***	a	0.138 ± 0.002 (4)	18.62 ± 1.0 (3)	5.64 ± 0.49 (3)	102.38 ± 8.79 (3)
CD	b	0.117 ± 0.005 # (4)	16.40 ± 1.46 (4)	4.91 ± 0.20 (4)	94.18 ± 6.62 (4)
CD-DME	c	0.118 ± 0.006 # (4)	19.19 ± 2.74 (2)	6.23 ± 0.12 (2)	101.73 ± 12.98 (2)

* Each value represents Mean ± S.E.M.; the number of pools of lungs are given in parentheses.

** Diet fed to pregnant rats.

*** CS, choline supplemented; CD, choline deficient; CD-DME, choline deficient + 1% N, N-dimethyl-aminoethanol.

P ≤ 0.05 when compared with group a.

Table 3 Phospholipid Content of Lungs of Newborn Rats.*

	Diet**		
	CS***	CD	CD-DME
Total Phospholipids	24.2 ± 0.3	21.2 ± 0.9 #	22.8 ± 1.8
Sphingomyelins	1.9 ± 0.09	1.5 ± 0.04 #	1.4 ± 0.1 #
Phosphatidyl choline	16.0 ± 0.34	13.3 ± 0.51 #	11.9 ± 0.35 #
Phosphatidyl ethanolamines	3.1 ± 0.14	3.6 ± 0.14	3.4 ± 0.15
Phosphatidyl glycerols	1.4 ± 0.01	1.1 ± 0.12	1.1 ± 0.05
Phosphatidyl Serines plus Phosphatidyl Inositols	1.8 ± 0.25	1.6 ± 0.35	1.0 ± 0.05
Phosphatidyl dimethyl aminoethanols	N.D.	N.D.	3.2 ± 0.14
Disaturated Phosphatidyl choline	7.1 ± 0.2	6.0 ± 0.5	5.5 ± 0.3 #

* Each value represents the Mean ± S.E.M. of 4 pools of lungs.

** Diet fed to pregnant rats.

*** CS, choline supplemented; CD, choline deficient; CD-DME, choline deficient + 1% N, N-dimethylaminoethanol.

P ≤ 0.05 when compared with CS group.

N.D. Not detected.

Table 5 Choline Kinase, Cholinephosphate Cytidyltransferase and
Choline Phosphotransferase Activities in the Lungs of Newborn Rats.*

Diet**	Group	Choline Kinase	Cholinephosphate Cytidyl Transferase	Choline Phosphotransferase
			n moles/hr/mg Protein	
CS***	a	32.0 ± 0.7 (4)	385 ± 21 (4)	5.7 ± 0.6 (4)
CD	b	50.4 ± 3.9 # (3)	179 ± 18 # (3)	2.3 ± 0.1 # (3)
CD-DME	c	24.4 ± 0.6 # (4)	362 ± 13 (4)	3.4 ± 0.1 # (4)

* Each value represents the Mean ± S.E.M. of the number of pools of lungs shown in parenthesis.

** Diet fed to pregnant rats.

*** CS, choline supplemented; CD, choline deficient; CD-DME, choline deficient + 1% N, N-dimethylaminoethanol.

P ≤ 0.05, when compared with group a.

Table 4 Protein and Phospholipid of Surfactant Isolated from the Lung of Newborn Rats.*

	Diet**		
	CS***	CD	CD-DME
Total protein	900 ± 34	628 ± 21 #	568 ± 46 #
Total phospholipids	5995 ± 428	4200 ± 315 #	3843 ± 321 #
Sphingomyelins	253 ± 14	199 ± 19	132 ± 11 #
Phosphatidyl choline	4222 ± 366	2915 ± 172 #	2427 ± 202 #
Phosphatidyl ethanolamines	745 ± 32	559 ± 28 #	395 ± 33 #
Phosphatidyl glycerols	351 ± 30	298 ± 23	245 ± 21
Phosphatidyl serines plus Phosphatidyl inositols	422 ± 24	306 ± 18 #	235 ± 20 #
Phosphatidyl dimethyl- aminoethanols	N.D.	N.D.	400 ± 33
Disaturated phosphatidyl choline	2127 ± 243	1415 ± 91	1165 ± 98 #

* Each value represents the Mean ± S.E.M. of 3 pools of lungs.

** Diet fed to the pregnant rats.

*** CS, Choline supplemented; CD, Choline deficient; CD-DME, Choline deficient + 1% N, N-dimethylaminoethanol.

P ≤ 0.05 when compared with CS group.

N.D. Not detected.