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Development of Phosphatidyl Glycerol Biosynthesis in the Lungs of Fetuses of Diabetic Rats

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Summary. The lungs of fetuses of streptozotocin-diabetic rats were examined for their ability to incorporate U-^{14}C-glucose into phosphatidyl choline, phosphatidyl glycerol, phosphatidyl inositol and lysophosphatidyl choline. In the lungs of control rats an increased biosynthesis of phosphatidyl glycerol in late pregnancy suggested a close association between the production of this phospholipid and the terminal maturation of the fetal lung. In the offspring of diabetic rats the incorporation of ^{14}C-glucose into phosphatidyl choline, lysophosphatidyl choline and phosphatidyl glycerol was markedly decreased compared with the control rats on gestational day 20, whereas no difference was seen at day 22. Insulin treatment of the pregnant rats restored the biosynthesis of phosphatidyl choline and lysophosphatidyl choline towards normal on gestational day 20, while the ratio of phosphatidyl glycerol to phosphatidyl inositol incorporation of ^{14}C-glucose was decreased, suggesting that the biosynthesis of phosphatidyl glycerol is more sensitive than that of phosphatidyl choline and lysophosphatidyl choline to the metabolic disturbances inherent in maternal diabetes. The delayed fetal pulmonary maturation occurred without fetal hyperinsulinism which suggests that this latter feature may not be of crucial significance in the aetiology of the respiratory distress syndrome.

Key words: Phosphatidyl glycerol, diabetic pregnancy, fetal lung, rat.

Diabetes mellitus in the pregnant mother is associated with a delayed maturation of the fetal lung, leading to a high incidence of respiratory distress syndrome in the newborn [1–3]. In diabetic pregnancy, adequate production of the major surfactant phospholipid, disaturated phosphatidyl choline (lecithin) does not preclude the development of respiratory distress syndrome [4]. It has recently been claimed that the presence of the acidic phospholipid phosphatidyl glycerol (PG) in the amniotic fluid prevents respiratory distress syndrome in the newborn of the diabetic mother [4–6]. Delayed onset of the production of PG in the fetal lung may be one of the factors responsible for the decreased pulmonary surface activity predisposing to respiratory distress syndrome.

To elucidate this hypothesis further we have investigated the impact of maternal diabetes on PG production in relation to the biosynthesis of other surfactant phospholipids. For this purpose, we chose an experimental rat model which permits a study of well characterized degrees of glucose intolerance under strictly controlled conditions [7]. Using this model, we demonstrated previously that severe diabetes in the pregnant rat caused morphological immaturity and a decreased biosynthesis of phosphatidyl choline (PC) and lysophosphatidyl choline (LPC) in the fetal lung, as observed on gestational day 20 [8]. The aim of the present investigation was to examine the development of PG biosynthesis in late gestation in the fetuses of normal and diabetic rats; the incorporation of U-^{14}C-glucose into PC, LPC, phosphatidyl inositol (PI) and PG in fetal lung was determined after separation of the phospholipids by two-dimensional thin-layer chromatography.

Materials and Methods

Course and Termination of Pregnancy

Manifest diabetes was induced in 3-month-old virgin Sprague-Dawley rats (Anticimex, Sollentuna, Sweden) by a single IV injection of streptozotocin (kindly donated by Dr. W. E. Dulin, Upjohn, Kalamazoo, Michigan, USA) in a dose of 45 mg/kg body weight. Animals with a non-fasting serum glucose concentration of >22 mmol/l one week after the injection were classified as manifest diabetic.

Treatment with bovine insulin ultralente was commenced in half of the diabetic animals. The daily insulin dose (2–8 IU) was adjusted according to the serum glucose concentration, which was determined twice a week. Non-diabetic female rats of the same age and weight served as controls. All animals had access to commercially pelleted food (Ewos, Södertälje, Sweden) and water ad libitum. The animals were mated 2–5 weeks after the streptozotocin injection. The day on

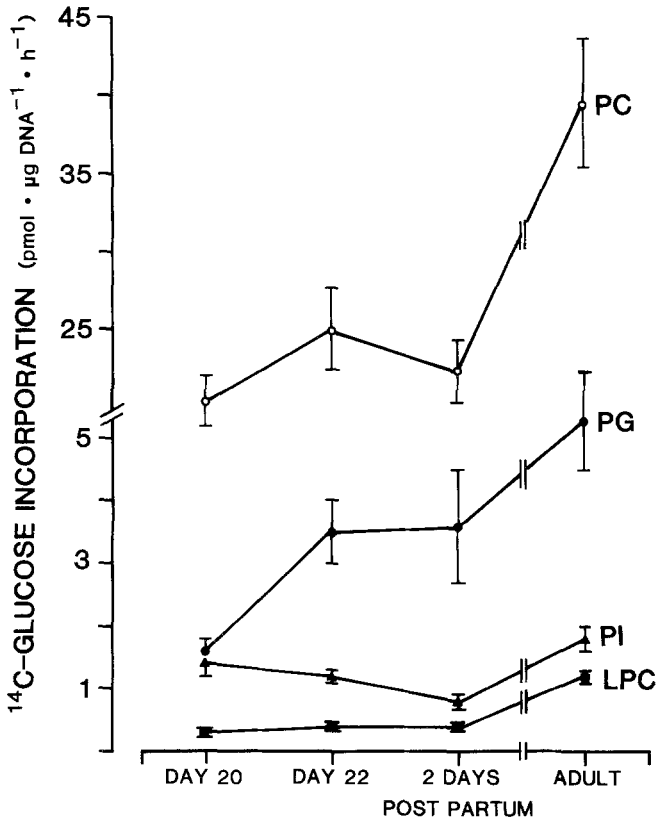

Fig. 1. Development of the biosynthesis of lung phospholipids in normal rat offspring in the perinatal period. Values from adult rats are included for comparison. Each point represents the mean ±SEM of four to seven determinations, corresponding to number of litters or adult animals, and shows the incorporation of ^{14}C-glucose into phosphatidyl choline O——O phosphatidyl glycerol ●——●, phosphatidyl inositol ▲——▲ and lysophosphatidyl choline ■——■

which a sperm-containing vaginal smear was found was denoted gestational day 0. The normal rats give birth spontaneously in the late afternoon of gestational day 22, and the diabetic and insulin-treated diabetic rats 1–5 days later [7]. On gestational days 20 and 22, the pregnant rats were killed by a blow on the neck. The fetuses were quickly dissected from the uterus and decapitated. The right lungs of three to six fetuses from each litter were excised and placed in ice-cold Hanks' medium [9]. In addition, lungs from spontaneously born normal offspring with a postnatal age of 2 days and lungs from adult, non-pregnant female rats were included for comparative purposes.

Incubation Protocol

The pooled lungs were cut into pieces (approximately 1 mm^3) in ice-cold Hank's medium. Duplicate samples of 10–15 pieces from each batch were transferred into 100 µl of a Gey-Gey buffer [10], pH 7.4, equilibrated with O_2:CO_2 (95%:5%) and containing D-U-^{14}C-glucose (5.5 mmol/l, spec. act. 18 mCi/mmol). After incubation in a shaking water bath for 90 min under an atmosphere of O_2:CO_2 (95%:5%), the reaction was terminated by the addition of D-glucose (200 µl, 28 mmol/l). The lung pieces were then washed twice in a salt solution [11] and disrupted ultrasonically for 20–30 s (Ultrasonic Disintegrator, MSE, London, UK) in 150 µl of the washing solution.

Extraction of the Phospholipids

After removal of duplicate samples (10 µl) for DNA estimation [12, 13], the lung homogenate was extracted for 1 h in 2 ml of $CHCl_3$:MeOH (2:1) containing 30 µl of carrier egg yolk lipids (Vitrum, Stockholm, Sweden). The lipid extract was washed ×4 to remove water-soluble radioactivity as described by Folch et al. [11] and the lipid-containing lowe phase (1 ml) was evaporated to dryness under N_2 and redissolved in 50 µl of $CHCl_3$:MeOH (2:1) containing 20 µl of a mixture of chromatographically pure phospholipids (2.5 mg/ml of each phospholipid). These lipids were: dipalmitoyl-PC, PI, PG, phosphatidyl ethanolamine (Sigma Chemicals, St. Louis, MO, USA), phosphatidyl serine, LPC and sphingomyelin (Koch-Light Laboratories, Colnbrook, Bucks, UK).

Separation of Phospholipids

The lipid extract (30 µl) was spotted onto pre-coated plates of silica gel G (Merck, Darmstadt, FRG) and run 10–15 cm in a two-dimensional system [14] using $CHCl_3$:MeOH:H_2O:CH_3COOH (65:25:4:8) in the first direction and tetrahydrofuran: formaldehydedimethylacetal:MeOH:2N NH_4OH (40:28.5:7.8:4.2) in the second direction. After drying, the plates were sprayed with 2′,7′-dichlorofluorescein and the phospholipids visualized in ultraviolet light. The spots corresponding to PG, PC, PI and LPC were scraped off into vials containing MeOH (150 µl) [15] and Econofluor (2.5 ml, New England Nuclear, Boston, MA, USA) was added. Radioactivity was measured in a Packard Scintillation Counter, model 3255 (Packard Instruments, Downers Grove, IL, USA) with correction for quenching by the channels ratio method.

Statistics

Statistical calculations in the offspring and adult animals were based on the numbers of litters or individual adult animals, respectively. Probabilities *(p)* of chance differences between the different groups were estimated by Student's two-tailed t-test [16].

Results

Lung Phospholipid Biosynthesis in Normal Animals

The normal development of the biosynthesis of PC, PG, LPC and PI from gestational day 20 up to adulthood can be seen in Figure 1. The major increase in PC production occurred after the immediate neonatal period. The biosynthesis of LPC showed an analogous development, the major increase towards adult values being noted after the second postnatal day. The development of PG, however, exhibited a different pattern, with a rapid increase between days 20 and 22, a plateau during the neonatal period and a less pronounced increase up to adult values. Only the PI biosynthesis tended to decrease throughout the perinatal period, from 1.4 ± 0.2 pmol · µg DNA^{-1} · h^{-1} on day 20 to 0.8 ± 0.1 pmol · µg DNA^{-1} · h^{-1} on day 2 after birth ($0.05 < p < 0.10$).

Lung Phospholipid Biosynthesis in the Fetuses of Manifest Diabetic Mothers

The rates of biosynthesis of the four phospholipids on gestational days 20 and 22 are shown in Table 1. On day 20, the incorporation of ^{14}C-glucose into all of the phospholipids except PI was decreased. On day 22, however, the fetal lung phospholipid biosynthesis had accelerated markedly and was not significantly differ-

Table 1. Phospholipid biosynthesis in the rat fetal lung on gestational days 20 and 22

	D-U-^{14}C-glucose incorporation					
	Gestational day 20			Gestational day 22		
	Control rats	Manifest diabetic rats	Insulin treated rats	Control rats	Manifest diabetic rats	Insulin treated rats
Number of litters	8	10	11	9	10	7
Phosphatidyl choline	20.2 ±1.6	12.9 ±0.8[c]	18.7 ±1.2[f]	25.0 ±2.6	22.8 ±2.5	29.1 ±2.7
Lysophosphatidyl choline	0.34±0.05	0.23±0.02[b]	0.35±0.03[e]	0.45±0.03	0.39±0.02	0.64±0.07[b, d]
Phosphatidyl glycerol	1.6 ±0.2	0.5 ±0.1[c]	0.9 ±0.1[d]	3.5 ±0.5	3.1 ±0.5	3.9 ±0.5
Phosphatidyl inositol	1.4 ±0.2	1.1 ±0.1	1.6 ±0.1	1.2 ±0.1	1.2 ±0.1	1.5 ±0.2
PG/PI[a]	1.5 ±0.5	0.5 ±0.2[b]	0.6 ±0.1[b]	3.1 ±0.3	2.6 ±0.3	2.6 ±0.2

The results are expressed as pmol·μg DNA^{-1}·h^{-1} (mean±SEM)
[a] PG/PI denotes the ratio between the rates of incorporation into phosphatidyl glycerol and phosphatidyl inositol. Significances:
[b] $p<0.05$; [c] $p<0.001$ versus control fetuses of the same age; [d] $p<0.05$; [e] $p<0.01$; [f] $p<0.001$ versus fetuses of the same age from manifest diabetic rats

ent from that in the controls, although a tendency towards lowered average levels was observed. Consequently, the PG/PI ratio of ^{14}C-glucose incorporation in the fetuses of diabetic mothers increased significantly between gestational days 20 and 22 ($p<0.001$).

Lung Phospholipid Biosynthesis in the Fetuses of Insulin-Treated Diabetic Mothers

Insulin treatment of the mother markedly influenced the development of fetal lung phospholipid biosynthesis. On gestational day 20 the biosynthetic rates of all phospholipids (Table 1) in the fetuses of insulin-treated diabetic mothers were in the normal range. On that day, however, the PG/PI ratio of incorporation was significantly below that in the controls. Two days later, the lungs of fetuses from insulin-treated diabetic mothers showed a PG/PI ratio in the normal range, the production of PC was normal and that of LPC was slightly increased compared with the normal and manifest diabetic groups.

Discussion

In rat fetuses a considerable acceleration of lung maturation occurs between gestational day 20 and birth [17]. Therefore, the present investigation on the impact of maternal diabetes upon lung maturation appertained to this particular period of gestation.

In confirmation of the results obtained with tritiated choline [8], we observed a similar trend towards increased phosphatidyl choline biosynthesis between day 20 and day 22 and a further increase up to adult values using ^{14}C-glucose. PC was responsible for the greatest fractional incorporation of radioactivity from ^{14}C-glucose, reaching 85% of the total phospholipids. An increase in the phosphatidyl glycerol biosynthesis of >100% between days 20 and 22 is consistent with the idea of a strong link between accelerated PG production and the terminal maturation of the fetal lung [18–20].

In the offspring of manifest diabetic rats the 70% reduction of PG biosynthesis accompanied by a minor inhibition of phosphatidyl inositol biosynthesis on day 20 indicates that one of the major consequences of maternal diabetes is disturbed regulation of the enzymatic steps diverting cytidine diphosphoglyceride from PI to PG production in late pregnancy. The other major effect of maternal diabetes on the biochemical maturation of fetal lungs was marked impairment of the biosynthesis of PC and lysophosphatidyl choline in the fetuses of the manifest diabetic rats, irrespective of whether ^{3}H-choline or ^{14}C-glucose was used as the precursor. This observation, which has been found to be connected with signs of morphological immaturity of Type II pneumocytes [8] led to the speculation that the production of disaturated PC through the 'LPC pathway' [21] could be inhibited in the fetuses of manifest diabetic rats. The demonstration in lung lavage, of reduced disaturated PC in fetuses of alloxan-diabetic rabbits [22] and in the newborn of streptozotocin-diabetic rat mothers [23] supports this conclusion.

Correction of the maternal metabolic disturbance by insulin treatment was accompanied by a return to normal of surfactant biosynthesis in the fetus. On day 20 of gestation the sole exception was only partial correction of PG biosynthesis, as shown by the decreased ratio of incorporation of ^{14}C-glucose into PG and PI. Interestingly, this finding has a parallel in the development of human diabetic pregnancy, in which the maturation of PC biosynthesis precedes that of PG [4, 5], indicating that the delicate mechanism regulating the terminal rise in PG production is very sensitive to the metabolic disturbance caused by maternal diabetes, even when insulin treatment has resulted in near-normalization of the blood glucose.

In an attempt to explain the delayed lung maturation in diabetic pregnancy, the frequently observed fetal hyperinsulinism, which is responsible for several of the

features of the diabetic fetopathy syndrome has been invoked to play a role. In studies performed in vitro, insulin inhibits the corticosteroid – induced increase in PC biosynthesis in fetal lungs [24, 25]. Recently Gross et al. [26] reported an inhibition of the production of disaturated PC in fetal rabbit lungs in organ cultures after 24 h of exposure to insulin. On the other hand, insulin stimulates biosynthesis of glycogen, structural lipids and PG. Therefore, factors other than insulin must participate in the process leading to the inhibition of both PC and PG production in diabetic pregnancy. Indeed, further characterization of the fetal endocrine pancreas in the manifest diabetic group has shown retarded development of the *B* cells [7, 27–29]. Direct measurement of serum insulin in these fetuses [8] and in the offspring of alloxan-diabetic rabbits [22] has failed also to demonstrate hyperinsulinism. The fact that the retarded development of the rat fetal lung occurs without fetal hyperinsulinism [8, 30, 31] suggests that this latter feature is not of crucial significance to the aetiology of delayed pulmonary maturation in man [32].

Other possible explanations for the delayed lung maturation in diabetic pregnancy may involve a shortage of substrate for phospholipid biosynthesis, e.g. fatty acids [33]. Alternatively deficient corticosteroid action either in the production of hormone [34] or in the number of receptors [35] may lead to delayed induction of surfactant biosynthesis. Both lack of substrate and deficient corticosteroid action would lead to decreased production of both PG and disaturated PC, which could explain the mechanism of delayed lung maturation in diabetic pregnancy.

The present results underline the close association between enhanced biosynthesis of PG and late fetal lung development and lend support to the suggested measurement of PG in amniotic fluid as an index of pulmonary maturation. The study stresses the role of maternal diabetes in delayed biosynthesis of lung surfactant in the fetus, with the consequently increased risk of pulmonary immaturity in the case of pre-term delivery.

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