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Inhibition of Surfactant Production by Insulin in Fetal Rabbit Lung Slices

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Summary

Incorporation of labeled glucose and fatty acid residues into saturated phosphatidylcholine was significantly reduced in lung slices from 27.5 days of gestation fetal rabbits during 90 min incubation in the presence of 100 μU/ml insulin. When ^{14}C-glucose was used as substrate, incorporation into both phosphatidylcholine and saturated phosphatidylcholine was reduced by insulin. This occurred despite an increase in overall glucose utilization by the lung from 11.3 ± 3.9 to 16.3 ± 5.2 nmole/g tissue in the presence of insulin ($P < 0.05$). A decrease in incorporation of fatty acid residues into saturated phosphatidylcholine was also observed when ^{14}C-palmitate was used as substrate, from 102 ± 4 to 90 ± 5 nmole palmitate/g tissue ($P < 0.01$). In the presence of insulin, there were significant reductions of both substrates appearing in lysophosphatidylcholine, a precursor of saturated phosphatidylcholine. There was no significant change in incorporation of glucose residues into glycogen or lactate under these conditions.

Speculation

Hyperinsulinemia appears to be responsible for respiratory distress syndrome (RDS) in infants of diabetic mothers (IDM) and infants of gestational diabetic mothers (IGDM). Control of the maternal diabetic state to a degree which inhibits development of fetal hyperinsulinism reduces the incidence of RDS in IDM and IGDM by removing excess quantities of the factor, insulin, that is responsible for inhibition of surfactant production.

IDM are at greater risk for development of RDS than other infants even when other significant perinatal factors such as route of delivery, Apgar score, and antepartum hemorrhage, are taken into account (27). It appears, therefore, that influences inherent in the maternal diabetic state rather than factors involved at the time of delivery predispose the infant to development of RDS. It is now well established that deficiency of alveolar surfactant is important in the pathogenesis of RDS (1). This deficiency is usually characterized by diminished presence of the most abundant phospholipid constituent of pulmonary surface active material, dipalmitoyl lecithin (8), and is generally associated with diminution of the lecithin: sphingomyelin (L/S) ratio in amniotic fluid. If the L/S ratio is greater than 2, it is unlikely that either normal infants or IDM will develop RDS after an uncomplicated vaginal delivery (10); however, the risk is still considerably greater than normal for the IDM (6, 7). In IDM, it appears that deficiency of other components of surfactant such as phosphatidyl glycerol may be responsible for the development of RDS (15, 16).

Several lines of reasoning suggest that fetal hyperinsulinism may be a factor in the inhibition of formation of surfactant. First, insulin effects are often antagonized by adrenal glucocorticoids which are known to accelerate surfactant production (10) and which induce insulin resistance in several tissues (9). Second, it appears that lung glycogen may be involved in surfactant production because glycogen content of the lung diminishes in several mammalian species toward the end of gestation when formation of surfactant begins to occur (4, 20), and because glucose has been shown to be an important precursor of phosphatidylcholine synthesis in the lung (11, 14). It might be expected that insulin would inhibit lung glycogenolysis as it does in the liver and, thus, diminish availability of carbohydrates for synthesis of surfactant. In tissues such as liver and muscle, the glycolytic pathway is closely coupled to pathways leading to phospholipid synthesis (4). More direct evidence that insulin may be involved in inhibition of surfactant production was advanced by Smith *et al.* (29), who showed that insulin inhibits the corticosteroid-stimulated incorporation of choline into lecithin by type II pneumocytes grown in tissue culture.

The studies described here were carried out to determine if insulin, in concentrations found in the blood of IDM, inhibits incorporation of two precursors, glucose and palmitic acid, into surfactant and other metabolic products formed by rabbit fetal lung.

MATERIALS AND METHODS

Fetuses from 12 pregnant New Zealand Albino Rabbits, conception timed to within 3 hr, were studied. At 27.5 days of gestation, the fetuses were delivered by caesarean section as described previously (3). Serum from several fetuses was pooled for subsequent insulin analysis (30). Lungs were excised from the fetuses after decapitation and tissue slices of 1000 microns were prepared using a McIlwain slicer as described by O'Neil and Tierney (25).

Approximately 300 mg of lung slices was placed in center well flasks containing 4.5 ml Krebs-Ringer bicarbonate buffer pH 7.4, and D-(U-^{14}C) glucose (10 mM, 2.5 μCi/flask) or (L-^{14}C) palmitic acid. ^{14}C-L palmitic acid was saponified with 1 N KOH with heating to 80°C, and allowed to cool to room temperature, and then added to Krebs-Ringer bicarbonate buffer, pH 7.5 containing 5% bovine serum albumin and 35 mM unlabeled palmitic acid, to obtain a specific activity of 0.65 μCi/flask (37). To some flasks, insulin was added to a final concentration of 10 and 100 μU/ml. After equilibration for 45 sec with 95% O_2-5% CO_2, flasks were incubated at 37°C in a metabolic shaker at 120 oscillations/min for up to 90 min. A time of 90 min was chosen because incorporation is linear with time over this period. In one experiment, incorporation of ^{14}C-palmitate into lecithin was 75, 150, and 210 nmoles/g of tissue at 30, 60, and 90 min respectively. At 90 min of incubation, sufficient quantities of radioactivity were incorporated to permit adequate measurement in the tissue. Those flasks used for 0 time controls were kept on ice, and processed as described. After 90 min of incubation, the remaining flasks were placed on ice and in those in which oxygen consumption was to be measured, 0.4 ml hyamine was injected onto coiled strips of filter paper previously placed in center wells. The reaction was stopped with 1.5 ml 0.1 M HCl, and CO_2 was collected on the moistened filter paper over 1 hr at 25°C with shaking. The strips were then placed in vials containing 12 ml Aquasol and 6 ml ethanol, and ^{14}C radioactivity was determined in a liquid scintillation counter. Aliquots of the tissue and medium were analyzed for glucose and glycogen as described previously (14). To 1 ml medium was added 2 ml 0.6 M perchloric acid and lactate was determined on the supernatant after centrifugation for 5 min at

1500 *g*. Radioactivity in the lactate fraction was determined in the neutralized PCA extract as described by Davidson and Berliner (5). Tissue and medium in the remaining flasks were lyophilized and stored at −20°C for lipid extraction and analyses as described previously (13, 14). Saturated phosphatidylcholine was extracted and determined by the method of Shimojo *et al.* (28).

Sources of chemicals were: Bovine serum albumin, Fraction V, powder, Miles Research Labs; ^{14}C-U-labeled glucose and ^{14}C-L-palmitate, New England Nuclear; Glucagon free insulin, Sigma Chemical Co.; Lactate assay kit, Cal Biochem; Aquasol scintillation fluid, New England Nuclear. Radioactivity was measured in an Isocap 300 liquid scintillation counter. Statistical analyses were performed using the paired *t* test.

RESULTS

Effects of insulin on precursor incorporation into tissues were measured at insulin concentrations of 10 μU/ml and 100 μU/ml. No significant differences from controls were found in the presence of 10 μU/ml. The following data refer only to observations made in the presence of 100 μU/ml. The insulin concentration in the plasma of the newborn animals was 21.5 ± 9.9 μ/ml (mean + SD).

Table 1 lists analyses of lipid content of the tissues in the presence and absence of 100 μU/ml insulin. After incubation for 90 min, it was not possible to detect significant effects of insulin on total tissue content of any of the lipids. Analysis of the medium (data not shown) also did not show significant differences between control and insulin containing media.

Table 2 shows that glucose uptake calculated from glucose concentration in the medium at the end of 90 min incubation is significantly increased in the presence of insulin as compared to controls, 16.35 ± 5.23 vs. 11.39 ± 3.9 μmole/g tissue ($P < 0.05$). Incorporation of the glucose label into total lipids and phosphatidylcholine is not significantly different in the presence of insulin. When incorporation of glucose into saturated phosphatidylcholine is measured, however, significant inhibition occurs in the presence of insulin. Similarly, incorporation of the glucose label into lysophosphatidylcholine is significantly inhibited in the presence of insulin.

When the data are expressed as percentage of glucose taken up in the different lipid fractions, (Table 3) insulin significantly inhibits incorporation of the glucose label into total lipids, total phosphatidylcholine, saturated phosphatidylcholine, and lysophosphatidylcholine.

Table 4 lists the results of experiments in which incorporation of ^{14}C-L-palmitate into various lipid fractions was measured. Insulin did not change incorporation into lipids or total phosphatidylcholine to a significant degree. On the other hand, a significant inhibition of incorporation of palmitate into saturated phosphatidylcholine and lysophosphatidylcholine was observed.

Table 5 lists results of experiments in which incorporation of glucose into CO_2, glycogen and lactate was measured. Despite increased uptake of glucose by the lung slices in the presence of insulin, significant inhibition of glucose oxidation to carbon dioxide occurred. Insulin did not affect the conversion of glucose to glycogen or lactate.

DISCUSSION

This study was undertaken to test the hypothesis that hyperinsulinism inhibits the production of surfactant and could, therefore, account for the clinical observation of the higher incidence of RDS seen in IDM. The results indicate that insulin at a concentration comparable to that observed in IDM at birth (18) was able to inhibit incorporation of both palmitate and glucose residues into saturated phosphatidylcholine, the major surface active lipid found in neonatal lung at term. Insulin also reduced the incorporation of both substrates into lysophosphatidylcholine, a precursor through which the lung enriches its pool of saturated phosphatidylcholine. Utilization of lysophosphatidylcholine for saturated phosphatidylcholine synthesis has been reported to be very active in fetal lung particularly in the later stages of development, and alterations in its activity would be critical to maintenance of surfactant production (32, 34).

Incorporation of glucose residues into saturated phosphatidylcholine occurs at a much slower rate than incorporation of palmi-

Table 1. *Total lipid content of lung slices (mean ± SD) after 90 min incubation*

	Control $n = 12$	Insulin $n = 12$
Neutral Lipid (mg/g)	2.05 ± 0.29	1.95 ± 0.19
Sphingomyelin (μmole/g)	1.01 ± 0.11	0.98 ± 0.21
Phosphatidylcholine (μmole/g)	4.77 ± 0.52	4.51 ± 0.51
Saturated phosphatidylcholine (μmole/g)	1.42 ± 0.30	1.25 ± 0.30
Lysophosphatidylcholine (μmole/g)	0.16 ± 0.02	0.15 ± 0.02

Table 2. *Uptake and incorporation of ^{14}C-U labeled glucose residues into various products over 90 min incubation (mean ± SD)*

	N	Control	Insulin	*P*
Glucose uptake (μmole/g)	9	11.3 ± 3.9	16.3 ± 5.2	<0.05
Total lipids $\frac{(\text{nmole})}{(\text{g})}$	8	73.3 ± 10.8	67.9 ± 16.8	NS
Total phosphatidylcholine $\frac{(\text{nmole})}{(\text{g})}$	8	40.6 ± 12.9	32.8 ± 7.9	NS
Saturated phosphatidylcholine $\frac{(\text{nmole})}{(\text{g})}$	8	6.71 ± 1.15	5.43 ± 1.31	<0.05
Lysophosphatidylcholine $\frac{(\text{nmole})}{(\text{g})}$	6	1.63 ± 0.37	1.02 ± 0.48	<0.005

Table 3. *Uptake and incorporation of ^{14}C-U labeled glucose residues into various products over 90 min incubation expressed as percent of glucose uptake into product (mean ± SD)*

	N	Control	Insulin	*P*
Glucose uptake (μmole/g)	9	11.3 ± 3.9	16.3 ± 5.2	<0.05
Total lipids	8	1.69 ± 1.44	1.11 ± 0.89	<0.05
Phosphatidylcholine	8	0.93 ± 0.50	0.59 ± 0.42	<0.01
Saturated phosphatidylcholine	8	0.115 ± 0.059	0.071 ± 0.057	<0.02
Lysophosphatidylcholine	7	0.025 ± 0.02	0.013 ± 0.01	<0.02

Table 4. *Incorporation over 90 min incubation of ^{14}C-L palmitate residues into various products expressed as nmoles palmitate per g of tissue (mean ± SD)*

	N	Control	Insulin	*P*
Total lipids	3	300.0 ± 45.0	267.0 ± 64.9	NS
Total phosphatidylcholine	3	206.0 ± 6.6	195.3 ± 10.4	NS
Saturated phosphatidylcholine	3	102.0 ± 4.0	90.0 ± 5.0	<0.01
Lysophosphatidylcholine	3	3.2 ± 0.87	2.6 ± 0.57	<0.05

Table 5. *Incorporation of ^{14}C-U-glucose residues into nonlipid products and net glycogen accumulation over 90 min (mean ± SD)*[1]

	N	Control	Insulin	*P*
CO_2	9	1.08 ± 0.36	0.65 ± 0.18	<0.02
Glycogen	9	2.42 ± 0.81	1.76 ± 0.77	NS
Lactate	9	1.78 ± 1.09	1.72 ± 0.63	NS

[1] Values expressed as μmoles glucose incorporated per g tissue.

tate. The degree of inhibition of both processes by insulin is the same and it appears, therefore, that the major effect of insulin is not exerted through glucose incorporation, but through inhibition of esterification of glycerol with saturated fatty acids.

Insulin increased uptake of glucose by lung slices from the suspending medium. This is consistent with the effect that insulin has on other tissues, notably muscle and adipose tissue. However, unlike the effect seen on adipose tissue, glucose oxidation is significantly inhibited in fetal lung slices in the presence of insulin. One possible explanation for the apparent decrease in glucose oxidation in the presence of increased glucose consumption may be an increase in the relative activity of the hexose monophosphate shunt under the influence of insulin. If the activity of this pathway was increased, one would observe an increase in $^{14}CO_2$ appearance from the C-1 carbon; however an increase in cytoplasmic NADPH would allow for increased fatty acid biosynthesis diverting acetyl CoA from the Krebs cycle and resulting in relatively decreased oxidation of carbons C-2 through C-6 (23). Several factors make this explanation logical. First, the hexose monophosphate pathway is active in fetal lung (35). Second, insulin is known to increase activity of this pathway in many tissues (12, 36). Third, Leroux et al. (22) have observed effects of insulin on human granulocytes which are similar to those reported here in fetal lung.

The finding that insulin accelerated the uptake of glucose by the lung tissue suggests that the inhibitory effects which have been observed with insulin on other steps in this system are specific and probably not due to a toxic contaminant in the insulin preparation. The lack of a direct stimulatory effect on glycogen synthesis in this system is not surprising. While insulin has been shown to enhance glycogen synthesis activity under certain conditions, an effect of insulin alone on net glycogen production has not been demonstrated in short term experiments in other tissues.

Recent studies suggest that administration of glucocorticoids to the mother before premature delivery of the infant may promote lung maturation and prevent RDS. This mode of therapy, however, may not be useful to the IDM. First, glucocorticoids administered in pharmacologic doses to the mother may aggravate her diabetic state. Second, as shown by Smith *et al.* (29), whereas glucocorticoids will stimulate incorporation of choline into lecithin by fetal lung cells, the presence of insulin at concentrations similar to that used in this study abolishes this effect.

It has been shown that the hyperinsulinism observed in IDM is due to the hyperglycemia of the mother, which results in an increased fetal glucose load due to placental transfer of glucose (26). It is also possible that transplacental transmission of amino acids may stimulate fetal insulin secretion (24). It should be possible to prevent the hyperinsulinism of the fetus if the maternal diabetic state is well controlled during the last part of the pregnancy. It has been shown that careful regulation of the blood sugar in pregnant diabetics in the last trimester resulted in infants with birth weights comparable to normals and reduction of perinatal mortality of infants to as low as 2–3% (19, 34).

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