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Research Article

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Chronic Hyperglycemia with Secondary Hyperinsulinemia Inhibits the Maturational Response of Fetal Lamb Lungs to Cortisol

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ABSTRACT I tested the hypothesis that chronic hyperglycemia with secondary hyperinsulinemia inhibits the stimulation of fetal lung maturation by cortisol. Glucose was infused (16 ± 2 mg/kg per min, mean$\pm$SE) from 112 through 130 d gestation into five chronically catheterised twin fetal lambs from which tracheal fluid could be collected. In addition, cortisol was infused (420 μg/h) from 128 through 130 d gestation into both the five glucose-treated twins and the five twin controls. Serum glucose (48 ± 2 mg/dl) and insulin levels (45 ± 3 μU/ml) were significantly higher in the glucose-treated fetuses than serum glucose (23 ± 2 mg/dl, $P < 0.001$) and insulin (15 ± 3 μU/ml, $P < 0.001$) in the controls. Serum cortisol levels were <2 μg/dl before 128 d gestation and rose to >6 μg/dl, $P < 0.001$ during cortisol infusion in both the glucose-treated and control fetuses. Cortisol treatment of control fetuses was associated with a 4.8-fold increase in surface active material (SAM) flux into tracheal fluid, and a 7.7-fold increase in total phospholipid content, a 9.5-fold increase in mixed lecithin content, a 10.5-fold increase in disaturated phosphatidylcholine content, and a 5.6-fold increase in phosphatidylglycerol content of the tracheal fluid (all $P < 0.001$). In the glucose-treated fetuses there were no significant changes in the tracheal fluid SAM flux and phospholipid content following cortisol administration. In lung wash from the control fetuses treated with cortisol there was 8.9-fold more SAM, and on thin-layer chromatography there was 5.6-fold more total phospholipids, 3.9-fold more mixed lecithin, 6.2-fold more disaturated phosphatidylcholine, and 2.5-fold more phosphatidylglycerol when compared with lung wash from the glucose-treated fetuses treated with cortisol

(all $P < 0.001$). Lung volumes at maximal inflation pressure during air pressure-volume studies were 1.8-fold greater in the cortisol-treated control fetuses than in the glucose-treated fetuses, $P < 0.025$. Chronic hyperglycemia with secondary hyperinsulinemia inhibits the maturational response of fetal lamb lungs to cortisol. A similar mechanism may operate in utero to increase the incidence of respiratory distress syndrome in infants of diabetic mothers with poor maternal glucose homeostasis. Moreover, on the basis of these data, prenatal treatment of infants of diabetic mothers with corticosteroids might not be expected to enhance fetal lung maturation.

INTRODUCTION

The incidence of respiratory distress syndrome (RDS)[1] is reported by Robert et al. (1) to be increased 5.6-fold in infants of diabetic mothers (IDM), even when suitable corrections are made for gestational age. However, RDS may be less prevalent when maternal diabetes mellitus is mild and well controlled (2, 3). The IDM experiences hyperinsulinemia both in utero and in the postnatal period (4), particularly when glucose homeostasis is poor (4, 5).

Stubbs and Stubbs (6) have suggested that fetal hyperinsulinemia may be the common link between maternal diabetes mellitus and RDS. This hypothesis is supported by several in vitro experiments. Gross et al. (7) reported that insulin delays the morphologic maturation of fetal rat lung in organ culture, causing a decrease in the number of lamellar bodies in type II pneumocytes. In addition, Neufeld et al. (8) have also

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[1] *Abbreviations used in this paper:* IDM, infants of diabetic mothers; RDS, respiratory distress syndrome; SAM, surface active material.

reported that insulin inhibits surfactant production in fetal rabbit lung slices. However, in contrast, Epstein et al. (9) found increased rates of choline incorporation into phosphatidylcholine in lung slices obtained from premature fetuses of glucose-intolerant monkeys.

In the fetal lamb, infusion of insulin was reported by Warburton et al. (10) to reduce the flux of surface active material (SAM) into tracheal fluid, in contrast to the sharp rise toward term in SAM flux into tracheal fluid of control fetuses. However, the insulin-treated fetal lambs in this study became hypoglycemic relative to controls. In a subsequent study, Warburton (11) reported that chronic hyperglycemia with secondary hyperinsulinemia also reduces SAM flux into tracheal fluid of fetal lambs. In the human fetus, maternal diabetes mellitus leads to fetal hyperglycemia and hyperinsulinemia.

Glucocorticoids are capable of accelerating fetal lung maturation in numerous species including sheep and man (12, 13). However, since glucocorticoids can exacerbate maternal diabetes mellitus, they have not been widely used in diabetic pregnancies. Based on experiments with mixed fetal lung cell cultures, Smith et al. (14) concluded that insulin inhibition of surfactant phospholipid formation may be mediated by inhibition of the stimulatory effects of cortisol, rather than by direct inhibition of surfactant phospholipid formation by insulin per se.

Since the mechanism of inhibition of fetal lung maturation in the IDM is not fully understood, I sought to test the hypothesis that chronic hyperglycemia with secondary hyperinsulinemia inhibits the stimulation of fetal lung maturation by cortisol. The maturational response of the lungs of glucose-treated and control fetal lambs to exogenous cortisol were compared.

METHODS

Gestational age of sheep pregnant with twin fetal lambs was determined from the time of mating (Nebeker Farms, Santa Monica, CA). In addition, fetal age was estimated from ossification centers in utero, and by extrapolation of fetal weight and crown-rump measurements at delivery; dating by these methods agreed within 3 d (15, 16).

Between 108 and 110 d of pregnancy, ewes were operated upon under 0.5% xylocaine epidural anesthesia, as described by Platzker et al. (12). Polyvinyl chloride catheters were placed in a fetal carotid artery and jugular vein. A stiff polyethylene catheter was inserted into the fetal trachea. This catheter led to a 600-ml latex bag, which was left in the amniotic sac. A separate catheter leading from the latex bag, together with the fetal artery and vein catheters, was brought out through the ewe's flank. A polyvinyl chloride catheter was also placed in a maternal vein. After the operation, the ewes received 1.2 million U procaine penicillin and 1 g kanamycin (Bristol Laboratories, Syracuse, NY) intramuscularly for 5 d. The animals were allowed to recover for up to 3 d

following the operation. The fetuses received 200,000 U penicillin G and 10 mg kanamycin intravenously every day.

Hyperglycemia was induced by continuous intravenous infusion of 20% dextrose in water (American Hospital Supply Corp., Irvine, CA) using a constant infusion pump (IVAC Corp., San Diego, CA). Glucose infusions were given to five twin fetuses from 112 d gestation onwards at a rate of 16±2 mg/kg per min, mean±SE. The five untreated twins served as controls.

Between 128 and 130 d gestation, cortisol (hydrocortisone sodium succinate, Upjohn Co., Kalamazoo, MI) was infused at the rate of 420 μg/h into glucose-treated and control fetuses.

Arterial blood samples (3.5 ml; ~2% of fetal blood volume) were obtained every 48–72 h up to 127 d gestation, and daily from 128 through 130 d gestation.

Tracheal fluid was collected daily and stored at $-40°C$ for subsequent analysis. The SAM in each tracheal fluid sample and in the lung wash was measured on a surface balance (17). The SAM flux, in micrograms per kilogram per hour, was calculated by multiplying the tracheal fluid SAM concentration in micrograms per milliliter by tracheal fluid flow in milliliters per kilogram per hour. Fetal weight was extrapolated from the fetal weight data of Barcroft (16), using the actual fetal weight measured at necropsy.

Arterial blood gases were measured at 37°C using a Corning 175 blood gas analyzer (Corning Medical and Scientific, Medfield, MA). Arterial blood oxygen saturation was measured using a microöximeter (American Optical Co., Buffalo, NY).

Serum was separated from the arterial blood and stored at $-40°C$ for subsequent analysis. Serum glucose concentration in milligrams per deciliter was measured by the glucose oxidase method using a YSI-23A glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). Serum insulin concentration in microunits per milliliter was measured using a competitive binding radioimmunosassay (Beckton, Dickinson & Co., Orangeburg, NY). Serum cortisol levels in micrograms per deciliter were measured using a competitive binding radioimmunoassay (Clinical Assays Inc., Cambridge, MA). Hematocrit of fetal blood was determined using a microcentrifuge (International Equipment Co., Needham Heights, MA).

The fetuses were removed from the uterus on day 130 of gestation immediately following euthanasia of the mother and fetus with sodium pentobarbital (Western Medical Supply, Arcadia, CA). The lungs were rapidly removed from the fetal thorax and chilled in ice-cold 0.9% saline solution. The left mainstem bronchus was cannulated and pressure-volume curves were recorded using a method similar to that of Avery et al. (18). The lungs were inflated to a pressure of at least 35 cm water. Each pressure was maintained for 2 min. The left lung was then lavaged four times with saline to produce a total wash volume of ~300 ml. Lung wash was stored at $-40°C$ for subsequent analysis. The right lung was cut into pieces and stored at $-40°C$ for subsequent analysis.

Lipids were extracted from the tracheal fluid, the lung wash, and the whole lung by the method of Bligh and Dyer (19). A 200,000-dpm aliquot of disaturated [^{14}C]phosphatidylcholine (50 μCi/μmol) (New England Nuclear, Boston, MA) was added during the lipid extraction step as a recovery standard. The extracted lipids were fractionated on thin-layer silica gel-G-coated plates (Analtech Inc., Newark, DE) activated for 2 h at 80°C, using as a developing solvent a mixture of chloroform, methanol, and 7 N ammonium hydroxide (70:30:3.5) (Mallinckrodt Inc,

Paris, KY). Regions on the thin-layer plate corresponding to authentic samples of phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and sphingomyelin (Applied Sciences Div., Milton Roy Co., Laboratory Group, State College, PA) were visualised with iodine vapor and collected into scintillation vials. Disaturated phosphatidylcholine was isolated and its content measured from an aliquot of total phosphatidylcholine by the method of Mason et al. (20). The recovery rates for lipid extraction and isolation of disaturated phosphatidylcholine were 90.4±2.1%, mean±SE, range 83 to 98%. Phospholipid content was expressed as micrograms of phospholipid phosphorus.

Radioactivity was measured using an aquasol (New England Nuclear) based scintillation system in a Beckman LS 8100 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Counting efficiency was determined by the channels-ratio method. Phospholipid content was measured using the phosphorus assay method of Bartlett (21).

Comparisons of serum glucose and insulin levels; arterial blood pH, PCO_2, PO_2, hematocrit, and oxygen saturation; tracheal fluid SAM flux and tracheal fluid and lung phospholipid content; and pressure-volume curve data, were made between glucose-treated and control animals using the Student's unpaired t test (22) (n for comparison = 10 times the number of days on which observations were collected). Comparisons of serum glucose, insulin, and cortisol tracheal fluid SAM flux and phospholipid content, before and after cortisol infusion, were made using the paired Student's t test (22).

RESULTS

Glucose infusions were given to five twin fetuses at a rate of 16±2 mg/kg per min, mean±SE, from 112 through 130 d gestation. Cortisol infusions were also given both to the five glucose-treated fetuses and to the five control fetuses at a rate of 420 μg/h from 128 through 130 d gestation. Term in the sheep is 150 d. Serial data on fetal serum glucose, insulin, and cortisol levels, arterial blood pH, PO_2, PCO_2, oxygen saturation, and hematocrit from both glucose-treated and control fetuses are shown in Fig. 1.

Serum glucose levels in the five glucose-treated fetuses, 48±2 mg/dl, were elevated twofold from 112 d gestation onwards in comparison with serum glucose levels in the five untreated twin control fetuses, 23±2 mg/dl, $P < 0.001$. The serum glucose levels in the mothers were 58±5 mg/dl and did not change as a result of glucose infusion to the fetuses.

Serum insulin levels in the glucose-treated fetuses, 45±3 μU/ml were elevated threefold from 112 d gestation onwards in comparisons with serum insulin levels in the control fetuses, 15±3 μU/ml, $P < 0.001$. The maternal serum insulin levels of 37±4 μU/ml did not change as a result of glucose infusion to the fetuses.

Arterial blood gases in the glucose-treated fetuses were pH 7.41±0.02, PO_2 23.1±0.8, PCO_2 39.2±1.1. Oxygen saturation in the glucose-treated fetuses was 73.6±0.8%. Arterial blood gases, oxygen saturation, and hematocrit in the glucose-treated fetuses were not significantly different from the same values in the control fetuses. Hematocrit did not fall significantly during the experiment.

The serum cortisol level before 128 d gestation was <2 μg/dl in both the glucose-treated and control fetuses. During the continuous infusion of cortisol at a rate of 420 μg/h from 128 to 130 d gestation, the serum cortisol level rose significantly to >6 μg/dl, $P < 0.001$, in both the glucose-treated and control fetuses.

The rate of tracheal fluid production in the glucose-treated fetuses was 3.2±0.1 ml/kg per h. The rate of tracheal fluid production in the controls was 2.8±0.1 ml/kg per h (not significantly different). The tracheal fluid production rate did not change significantly with cortisol infusion. Two of the ewes went into early labor towards the end of the 48-h cortisol infusion.

SAM flux into the tracheal fluid of the fetal lambs is shown in Fig. 2. SAM flux became detectable in the tracheal fluid of the control fetuses at 124-d gestation, and was 17.2±2.1 μg/kg per h at 128 d gestation. Following treatment with cortisol, SAM flux rose 2.7-fold on day 129 to 45.8±3.2 μg/kg per h, $P < 0.001$, and 4.8-fold on day 130 to 83.4±3.3 μg/kg per h, $P < 0.001$. In contrast, SAM flux was detectable at much lower levels, <5 μg/kg per h, in only three of the glucose-treated fetuses before 128 d gestation. Following treatment with cortisol, SAM flux did not rise significantly on days 129, 2.6±1.4 μg/kg per h and 130, 2.6±1.5 μg/kg per h of gestation. The SAM flux in the glucose-treated fetuses was 17-fold lower on day 129, $P < 0.001$, and 27-fold lower on day 130, $P < 0.001$ of gestation than the corresponding levels of SAM flux in the control fetuses.

Lung wash obtained from the left lungs of cortisol-treated control fetuses contained 6,300±560 μg of SAM. This was 8.9-fold more than the 710±120 μg of SAM in the left lung wash of the glucose-treated fetuses, $P < 0.001$.

The phospholipids in the tracheal fluid and lung wash were separated into four major fractions (Table I). The fraction represented as sphingomyelin is a mixture of sphingomyelin, phosphatidylserine, and phosphatidylinosine, which were not completely separable by thin-layer chromatography.

Before 128 d gestation, the phospholipid content of the tracheal fluid was similar in the glucose-treated and control fetuses. There were significant increases in tracheal fluid phospholipid content following cortisol treatment in the control fetuses. In particular, there was a 7.7-fold increase in total phospholipid content, a 9.5-fold increase in mixed lecithin content, a 10.5-fold increase in disaturated phosphatidylcholine
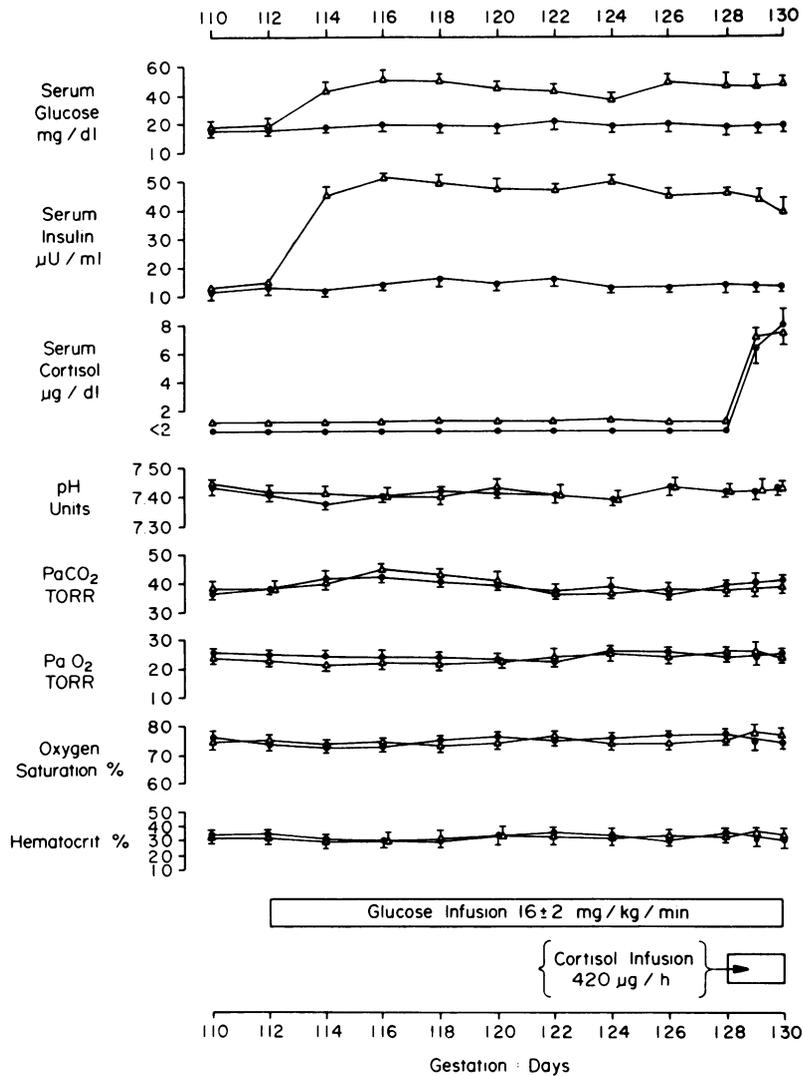
FIGURE 1 Serum glucose, insulin, and cortisol levels, arterial blood pH, PaO₂, PaCO₂, oxygen saturation, and hematocrit of five control (●) and five glucose-treated (Δ) twin chronically catheterised fetal lambs. The glucose infusion was given from 112 to 130 d gestation. The cortisol infusion was given to all 10 fetuses from 128 to 130 d gestation. Data represent mean±SE.

content, and a 5.6-fold increase in phosphatidylglycerol content, all $P < 0.001$.

There were slight increases in the tracheal fluid phospholipid content among the glucose-treated fetuses following cortisol administration over days 129 and 130 of gestation, although none were large enough to reach statistical significance.

The total phospholipid content of whole lung was 398±24.3 μg/g wet wt in the control fetuses and was 315.6±22.1 μg/g wet wt in the glucose-treated fetuses (not significantly different). However, disaturated

phosphatidylcholine content of whole lung was 85.6±9.1 μg/g wet wt in the control fetuses and was 45.7±5.1 μg/g wet wt in the glucose-treated fetuses, a 1.9-fold difference, $P < 0.01$. The other subclasses of phospholipids of whole lung were not measured.

Pressure-volume curves from the left lungs of a pair of twins are shown in Fig. 3. The volume of the lungs at maximal pressure (V_{max}), 1.4 ± 0.2 ml/g wet wt in the cortisol-treated control fetuses was significantly greater than the V_{max}, 0.8±0.1 ml/g wet wt in the glucose-treated fetuses, $P < 0.025$. There was no sig-
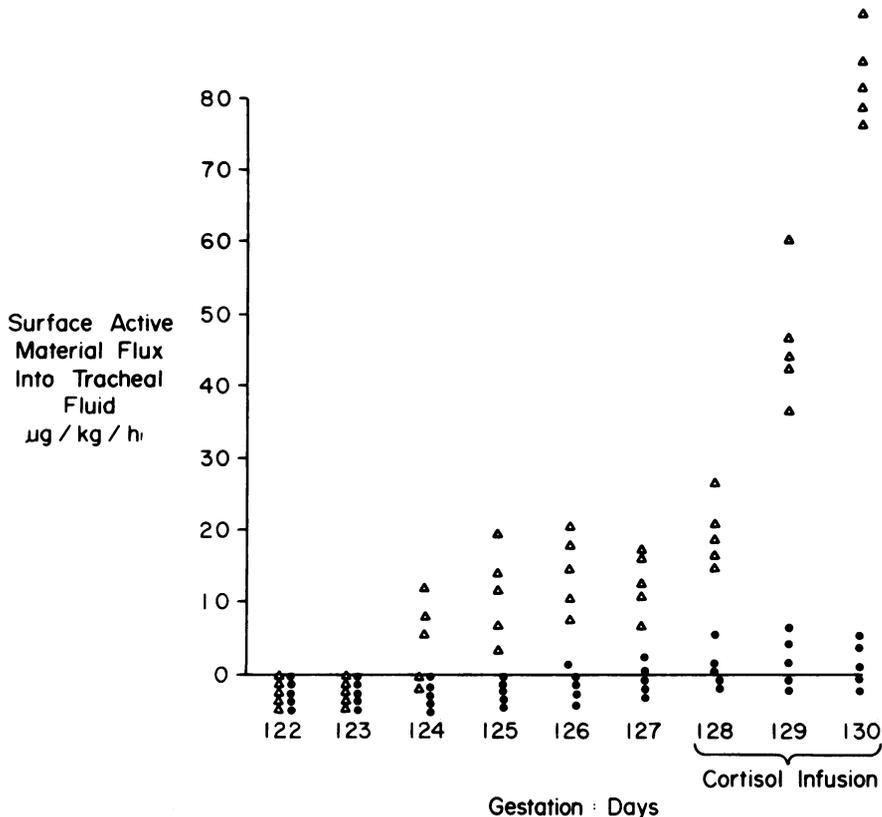
FIGURE 2 This figure shows SAM flux in micrograms per kilogram per hour of five control (Δ) and five glucose-treated (●) twin chronically catheterised fetal lambs both from 122 d gestation onwards and during cortisol infusion from 128 to 130 d gestation.

nificant difference between the volumes of the lungs at 10 cm H_2O pressure, which was 0.1 ml/g wet wt in both groups of fetuses.

DISCUSSION

Continuous intravenous infusion of glucose produced significant elevation of serum glucose and insulin levels in five chronically catheterised twin lamb fetuses.

The lungs of the fetal lamb produce fluid that flows from the trachea as early as 94 d gestation. Mean tracheal fluid production rates previously reported by Warburton (10, 11) and others (23) were similar to those reported in the current study.

Kikkawa et al. (24) found lamellar inclusion bodies were first visible on electron microscopy of type II pneumocytes in fetal lamb lungs at 113 d gestation. Lamellar inclusion bodies are the most probable storage form of pulmonary surfactant. Hence, chronic hyperglycemia with secondary hyperinsulinemia was present from the probable time of inception of sur-

factant phospholipid synthesis and packaging for secretion.

Warburton (10, 11) has reported that both primary hyperinsulinemia with concomitant hypoglycemia, and chronic hyperglycemia with secondary hyperinsulinemia reduce SAM flux into tracheal fluid of fetal lambs. Before 128 d gestation, SAM flux into tracheal fluid in the current experiment was similar to Warburton's previously reported data.

Glucocorticoids are known to stimulate fetal lung maturation in numerous species including sheep and man (12, 13). The control fetuses reported here underwent significant lung maturation in response to cortisol, as reflected by large and statistically significant increases in SAM flux into their tracheal fluid and in their tracheal fluid phospholipid content, which were much greater than those that occur between days 128 and 130 of gestation during normal maturation of fetal lamb lungs (10, 11, 23). By contrast, glucose-treated fetuses underwent no significant lung maturation. In addition, the stability of lung expansion and the sur-

TABLE I

Phospholipid Content of Tracheal Fluid SAM Collected Daily from Five Chronically Catheterised Glucose-treated and Five Twin Control Lambs from 112 to 130 d Gestation, and of Lung Wash Obtained from the Left Lungs of All 10 Fetuses at 130 d Gestation

Gestation	Total phospholipids	Mixed lecithin	Disaturated phosphatidylcholine	Sphingomyelin	Phosphatidyl-glycerol	Phosphatidyl-ethanlamine	
d							
<128	56.3±10.3	34.3±5.2	24.5±3.8	9.8±1.2	3.3±0.8	8.9±1.1	Control
	50.0±8.2	29.9±6.8	23.0±4.2	8.2±0.9	1.5±0.6	6.6±1.4	Glucose
			Cortisol infusion began				
129	110.6±9.3*	94.8±8.7*	84.5±8.3*	10.0±0.8	5.0±0.9	5.8±1.3	Control
	61.2±6.5‡	42.9±8.2§	26.4±3.4§	11.1±1.3	1.7±0.7	8.0±1.4	Glucose
130	432.8±21.3*	325.9±9.3*	256.0±4.3*	27.2±2.1*	8.4±1.2	30.4±1.6*	Control
	74.0±10.2§	59.1±8.4§	35.8±8.4§	8.6±1.8§	3.4±1.3	8.6±1.3§	Glucose
Lung wash	343.8±12.2	225.7±10.2	195.3±5.2	7.8±2.5	17.2±1.1	11.1±2.0	Control
	61.8±8.5§	57.7±7.8§	37.4±9.0§	14.3±2.1	7.0±0.8§	14.9±1.6	Glucose

Phospholipid content was expressed as micrograms phospholipid phosphorus per day (mean±SE). All 10 fetuses received cortisol infusions from day 128 through day 130 of gestation.
* P < 0.001 by Student's paired t test (before and after cortisol infusion).
‡ P < 0.05.
§ P < 0.001 by Student's unpaired t test (glucose-treated vs. controls).

face activity and phospholipid content of alveolar washings were significantly lower in the glucose-treated fetuses. These data indicate that the surfactant supply of fetal lambs was not augmented by exogenous
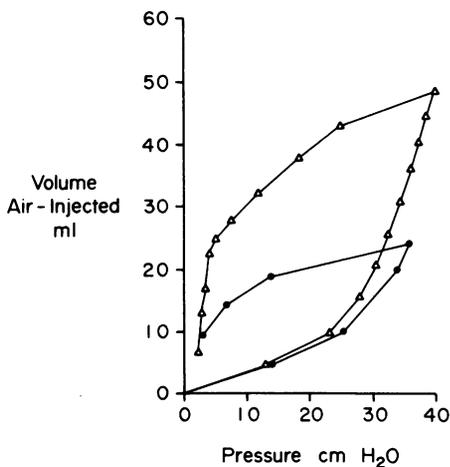

FIGURE 3 Pressure-volume curves measured on the left lungs of a pair of twin chronically catheterised fetal lambs. One of the lambs (●) was infused with glucose from 112 through 130 d gestation while the other (△) served as control. Both lambs received cortisol infusions from 128 through 130 d gestation.

cortisol in the presence of chronic hyperglycemia with secondary hyperinsulinemia.

The dose of cortisol infused to both glucose-treated and control fetuses (420 μg/h) was calculated to be approximately twice the cortisol production rate into an adrenal vein of term fetal lambs reported by Chester-Jones et al. (25). Significant elevations in serum cortisol levels occurred in both control and glucose-treated fetuses to levels approximating those seen in the near-term fetal lamb (23).

The relationship between fetal hyperinsulinemia and the inhibition of cortisol-induced fetal lung maturation is complex. Neufeld et al. (26) found increased numbers and affinity of insulin receptors in membranes obtained from lungs of fetuses from alloxan diabetic rabbits. This suggests that not only are the fetal lungs of the IDM exposed to increased circulating concentrations of glucose and insulin, but also that any intracellular insulin effects might be further increased. Gewolb et al. (27) recently noted delayed depletion of pulmonary glycogen in fetuses of streptozotocin diabetic rats, and suggested that this finding might be attributed to a concomitant decrease in the activity of the glycogenolytic enzyme phosphorylase A, which might in turn be mediated by insulin. Decreased glycogen availability as a substrate reservoir for surfactant phospholipid component synthesis might explain the increased incidence of pulmonary immaturity in IDM.

However, it should be noted that Gewolb et al. (27) and other workers (28), could find no evidence of fetal hyperinsulinemia in their animal models of diabetic pregnancy that depend on chemical induction of maternal diabetes. The lack of fetal hyperinsulinemia may explain a discrepancy between the report of Sosenko et al. (28), who induced lung maturation in fetuses of alloxan diabetic rabbits with cortisol, and the inhibition of cortisol-induced lung maturation reported here in the presence of both fetal hyperglycemia and hyperinsulinemia. However, this discrepancy might also be explained by a species difference. Boutwell and Goldman (29) reported that uptake of tritiated dexamethasone into fetal lung nuclei of streptozotocin diabetic rats was reduced. Thus, altered substrate availability, or inhibition of cortisol effects mediated in the nucleus might at least partially explain the inhibition of cortisol-induced fetal lung maturation by insulin described both here in a live animal model, and by Smith et al. (14) in tissue culture.

In conclusion, chronic hyperglycemia with secondary hyperinsulinemia inhibits the maturational response of fetal lamb lungs to cortisol. A similar mechanism may operate in utero to increase the incidence of RDS in the IDM with poor maternal glucose homeostasis. Moreover, on the basis of the data presented in this paper, prenatal treatment of IDM with glucocorticoids might not be expected to enhance fetal lung maturation.

ACKNOWLEDGMENTS

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