Intrafetal glucose infusion alters glucocorticoid signaling and reduces surfactant protein mRNA expression in the lung of the late-gestation sheep fetus

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1Early Origins of Adult Health Research Group, School of Pharmacy and Medical Sciences, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia, Australia; and 2Molecular and Evolutionary Physiology of the Lung Laboratory, School of Pharmacy and Medical Sciences, Sansom Institute for Health Research, University of South Australia, Adelaide, South Australia, Australia

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McGillick EV, Morrison JL, McMillen IC, Orgeig S. Intrafetal glucose infusion alters glucocorticoid signaling and reduces surfactant protein mRNA expression in the lung of the late-gestation sheep fetus. Am J Physiol Regul Integr Comp Physiol 307: R538–R545, 2014. First published July 2, 2014; doi:10.1152/ajpregu.00053.2014.—Increased circulating fetal glucose and insulin concentrations are potential inhibitors of fetal lung maturation and may contribute to the pathogenesis of respiratory distress syndrome (RDS) in infants of diabetic mothers. In this study, we examined the effect of intrafetal glucose infusion on mRNA expression of glucose transporters, insulin-like growth factor signaling, glucocorticoid regulatory genes, and surfactant proteins in the lung of the late-gestation sheep fetus. The numerical density of the cells responsible for producing surfactant was determined using immunohistochemistry. Glucose infusion for 10 days did not affect mRNA expression of glucose transporters or IGFs but did decrease IGF-1R expression. There was reduced mRNA expression of the glucocorticoid-converting enzyme HSD11B-1 and the glucocorticoid receptor, potentially reducing glucocorticoid responsiveness in the fetal lung. Furthermore, surfactant protein (SFTP) mRNA expression was reduced in the lung following glucose infusion, while the number of SFTP-B-positive cells remained unchanged. These findings suggest the presence of a glucocorticoid-mediated mechanism regulating delayed maturation of the surfactant system in the sheep fetus following glucose infusion and provide evidence for the link between abnormal glycemic control during pregnancy and the increased risk of RDS in infants of uncontrolled diabetic mothers. glucose; diabetes; obesity; surfactant

IN THE FACE OF A WORLD-WIDE obesity epidemic, there has been an increase in the proportion of women entering pregnancy either overweight or obese (11, 29). With approximately 45% of women being overweight or obese at delivery (1), there has been an increase in obstetric complications in this population, including gestational diabetes and preterm birth (9), which are both associated with an increased risk of neonatal respiratory failure (59). Maternal metabolic regulation throughout pregnancy, particularly glucose homeostasis, is an important factor affecting fetal growth and development (3). Pregnancy is characterized by a state of insulin resistance in late gestation, which is essential to provide substrates to the developing fetus (13). While there is a natural decrease in insulin sensitivity through-out gestation, obese women are less insulin-sensitive than lean and overweight women prior to conception and remain this way throughout pregnancy (7, 8). As a result, they are at an increased risk of developing gestational diabetes. Worldwide, 3–10% of pregnancies are complicated by abnormal glycemic control, caused mostly by gestational diabetes (39). Uncontrolled diabetes in pregnancy results in exposure of the developing fetus to increased plasma glucose concentrations as a result of increased placental transfer, as well as increased secretion of insulin through fetal pancreatic activity in response to the increased fetal plasma glucose concentrations. Furthermore, exposure to maternal diabetes can alter fetal glucose transport due to changes in the expression of the solute carrier family 2 (facilitated glucose transporter, SLC2A), which is expressed in the lung (49). In addition, insulin plays an important role in lung development (28), and its effects are mediated by IGF signaling, including IGF-1, IGF-2, and IGF-1 receptor (IGF-1R). These growth factors have been associated with altered lung development (17) and respiratory complications, such as respiratory distress syndrome (RDS) following birth in neonates (10).

Infants of diabetic mothers were at a six-fold increased risk of developing RDS compared with infants of nondiabetic mothers (47). RDS is associated with both structural and biochemical immaturity of the neonatal lung; of particular importance is the delay in the maturation of the surfactant system (2). The presence of pulmonary surfactant, a complex mixture of lipids and proteins, at the air-liquid interface of the lung is required to prevent alveolar collapse throughout the breathing cycle and aids in the transition to air breathing at birth (12). The surfactant complex is synthesized, stored, and secreted from type II alveolar epithelial cells (AECs), which line the alveoli (34). Maturation of the surfactant system occurs in late gestation, in parallel with the prepartum cortisol surge and plays a vital role in preparing the fetus for the transition to extrauterine life (55). The lipid component of surfactant is primarily responsible for reducing surface tension; however, surfactant proteins (SFTP)-B and SFTP-C aid in their adsorption to the air-liquid interface and in the dynamic regulation of the functional surfactant film (43). SFTP-A and SFTP-D play important roles in innate immunity within the lung (24).

Maternal diabetes has been associated with a reduction in SFTP-A protein (51) and lipid profiles (35) in amniotic fluid, suggesting a delay in fetal lung maturation. In vitro and in vivo studies using rodents have shown that increased glucose and/or insulin concentrations result in a reduction of both the protein...
and lipid components of pulmonary surfactant (19, 20, 22, 23, 45). The molecular mechanism regulating this delayed maturation of the surfactant system has not been investigated. It was proposed that insulin may act indirectly by antagonizing the stimulatory effects of cortisol (14), the endogenous glucocorticoid (GC), in the lung, which normally plays a major role in stimulating lung and surfactant maturation. The bioavailability of cortisol in the lung may be affected, for example, through alterations in the regulatory enzyme isomorphs, hydroxysteroid (11-β) dehydrogenase (HSD11B)-1, which catalyzes the conversion of inactive cortisone to cortisol, or HSD11B-2, which catalyzes the conversion of bioactive cortisol to cortisone (56).

Alternatively, GC signaling may be affected in the levels of the GC receptor (nuclear receptor subfamily 3, group C, member 1; NR3C1) and the mineralocorticoid receptor (nuclear receptor subfamily 3, group C, member 2; NR3C2), the intracellular mediators of GC activity (37).

With the exception of one study examining factors, including glycogen regulation, surfactant lipid content (65), β-cell receptor binding (62), and surface-active material flux in tracheal fluid (60, 63) in the lung of the fetal sheep following exposure to hyperglycemia and hyperinsulinemia (analogous to the conditions experienced by a fetus in an uncontrolled diabetic pregnancy), no studies have evaluated the effect on regulation of SFTP expression in a large animal model with a developmental lung pattern similar to that of humans. Furthermore, no molecular mechanism has been identified to explain the historical link between uncontrolled diabetes in pregnancy and RDS in infants of diabetic mothers. Here, we investigate the impact of intrafetal glucose infusion on pathways that may regulate maturation of the surfactant system in the lung of the late-gestation sheep fetus.

METHODS

All procedures were approved by the University of Adelaide Animal Ethics Committee.

Animals and Surgery

Twenty-one pregnant Merino ewes were housed in individual pens in animal holding rooms, with a 12:12-h light-dark cycle, and fed once daily with water ad libitum. At 118–120 days of gestation (term 150 ± 3 days), general anesthesia was induced in the ewe with an intravenous injection of sodium thiopentone (1.25 g; Pentothal, Rhone Merieux, Pinkenba, QLD, Australia) and maintained with 2.5–4% halothane inhalation anesthetic (Fluothane; Imperial Chemical Industries (ICI) Ltd., Melbourne, VIC, Australia) in oxygen. Vascular catheters were implanted in the ewe’s jugular vein, a carotid artery, and jugular vein of the fetus, and in the amniotic cavity, as previously described (38). Ewes received an intramuscular injection of antibiotics (500 mg; pentamixin, 2 ml of 125 mg/ml dihydrostreptomycin in sterile saline (Sigma, St. Louis, MO) for 3 days following surgery. Antibiotics (500 mg; sodium ampicillin; Commonwealth Serum Laboratories, Melbourne, VIC, Australia) were administered intra-amniotically to all fetal sheep daily for 4 days postoperatively. Ewes were allowed at least 4 days to recover from surgery prior to the experimental protocol.

Arterial Blood Gas Measurements

Fetal arterial blood was collected throughout the infusion period, and at each time point, whole blood arterial partial pressure of oxygen (Pao2), arterial partial pressure of carbon dioxide (Paco2), pH, oxygen saturation (SaO2), and Hb content were measured using an ABL 520 analyzer (Radiometer, Copenhagen, Denmark) with the temperature corrected to 39°C.

Intrafetal Infusion Regime

Glucose-infused fetuses received an intravenous infusion of 50% dextrose in saline from 130–140 days of gestation (n = 9). Infusion began at an initial rate of 1.9 ml/h for 24 h and was then increased in a stepwise manner by 1.9 ml/h per day for the next 3 days. The final infusion rate of 7.5 ml/h obtained on the 4th day of the infusion was maintained until post mortem. Saline-infused fetuses received saline intravenously from 130 to 140 ± 1 day of gestation (n = 12). The timing of this infusion regime coincides with the time during which the surfactant system is undergoing maturation. The glucose infusion protocol resulted in a significant increase in both fetal plasma glucose and insulin concentrations (38), with mean values throughout the 10-day infusion period for plasma glucose (saline-infused, 1.10 ± 0.09 mmol/l; glucose infused, 2.37 ± 0.19 mmol/l; P < 0.05) and insulin (saline-infused, 4.93 ± 1.04 mmol/l; glucose infused, 10.26 ± 1.50 mmol/l; P < 0.05).

Post Mortem Procedures

At 140 ± 1 day gestation, ewes were humanely killed with an overdose of sodium pentobarbital administered via the jugular vein (Virbac, Peakhurst, NSW, Australia), and fetal sheep were delivered by hysterotomy. The lungs were removed, weighed, snap frozen in liquid nitrogen, and stored at −80°C for molecular analysis. A section of lung tissue was fixed in 4% paraformaldehyde for immunohistochemical analysis. Neuroendocrine function data from these fetuses have been published previously (38).

Quantification of mRNA Transcripts Within the Fetal Lung

Total RNA extraction. Total RNA was extracted from fetal lung samples (~50 mg) using Invitrogen TRIzol reagent solution and QIAGEN miRNeasy purification columns as per the manufacturer’s guidelines (Invitrogen, Carlsbad, CA) (18, 34). Total RNA integrity from all extracted tissue samples was assessed by running samples on an agarose gel stained with ethidium bromide. Total RNA was quantified by spectrophotometric measurements at 260 and 280 nm and checked for protein and DNA contamination. cDNA was synthesized using Superscript III First Strand Synthesis System (Invitrogen) using 2 μg of total RNA in a final volume of 20 μl, as per the manufacturer’s guidelines. Controls containing either no RNA transcript or no Superscript III were used to test for reagent contamination and genomic DNA contamination, respectively.

Quantitative real-time RT-PCR. Initially, the geNorm component of qbaseplus 2.0 software (Biogazelle, Zwijnaarde, Belgium) was used to determine the most stable reference genes from a panel of candidate genes (57) and the minimum number of reference genes required to calculate a stable normalization factor, as previously described (34, 52). For quantitative RT-PCR (qRT-PCR) data output normalization, three stable housekeeping genes—β-actin (ACTB; U39357), peptidylprolyl isomerase A (PPIA; AY251270) (42), and tyrosine 3-monooxygenase (YWHAZ; AF349768.1) (34)—were run in parallel with target genes, as previously described (34, 52). The gene expression of the glucose transporters [SLC2A1 (U89029.1) and SLC2A4 (AB005283)], IGF signaling [IGF1 (DQ152962), IGF2 (M89789), and IGF1R (AY162343)] (18), GC regulatory genes [HSD11B-1, NM_001009395.1; HSD11B-2, NM_001009460.1; NR3C1, NM_001141861.1; NR3C2, AF349768.1 (34)], surfactant proteins [SFTP-A, AF211856; SFTP-B, AF07544; SFTP-C, AF076634; and SFTP-D, AJ133002.1 (34, 41)] were measured by qRT-PCR using Fast SYBR Green master mix (Applied Biosystems, Foster City, CA) in a
Tissue sections were photographed using a digital camera DP72 were used to count the points hitting the reference space (i.e., four reverse primer mixed with H2O to obtain final primer concentrations and was blinded to treatment groups. Sixty counting frames (Visiopharm, Hoersholm, Denmark), as previously described tissue. Rockford, IL) for visualization of SFTP-B-positive cells. All sections formaldehyde-fixed, paraffin-processed lung tissue sections of 7-

Quantification of SFTP-B-Positive Cells in the Fetal Lung

Immunohistochemistry for identification of SFTP-B-positive cells. In a subset of animals (saline-infused, n = 5; glucose-infused, n = 6), immunohistochemistry was performed (34) using a monoclonal antibody to SFTP-B (produced by Dr. Y. Suzuki, Kyoto University, Japan and kindly donated by F. Possmayer, University of Western Ontario, Canada), staining of which is restricted to type II AECs in the alveolar epithelium and Clara cells in the bronchiolar epithelium (32). Paraformaldehyde-fixed, paraffin-processed lung tissue sections of 7-µm thickness were deparaffinized and rehydrated before endogenous peroxide solution activity was blocked, and followed with antigen retrieval. Slides were incubated overnight with the aforementioned SFTP-B antibody (1:1,000) at 4°C. Negative control slides were performed in parallel with test slides. A Histostain-Plus broad-spectrum kit (Zymed Laboratories, San Francisco, CA) was utilized with horseradish peroxidase and 3,3-diaminobenzidine chromagen (metal-enhanced diaminobenzidine substrate kit; Pierce Biotechnology, Rockford, IL) for visualization of SFTP-B-positive cells. All sections were counterstained with Mayer’s hematoxylin (Sigma-Aldrich, St. Louis, MO).

Quantitative assessment of SFTP-B-positive cells in fetal lung tissue. Sections were examined using Visiopharm NewCAST software (Visiopharm, Hoersholm, Denmark), as previously described (34). Analysis was carried out by a trained single individual, who was blinded to treatment groups. Sixty counting frames (×600 magnification) of the alveolar epithelium were randomly selected per section. Point-counting using an unbiased counting frame with an area of 20,000 µm² was used to determine the numerical density of SFTP-B-positive cells within the fetal lung. Using the four corners of the test frame, the reference space was estimated from the points falling on lung tissue. The numerical density of SFTP-B-positive cells expressed as mRNA mean normalized expression (MNE) ± SE (34, 52).

Table 1. Quantitative RT-PCR primer sequences for designed target genes

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5′ → 3′</th>
<th>Concentration, µM</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC2A1 Forward</td>
<td>ATCGTGGCCACATTTGCGCCTTG</td>
<td>0.45</td>
<td>U89029.1</td>
</tr>
<tr>
<td>SLC2A1 Reverse</td>
<td>CTGGAAGCAGCTGGCGCAAAAGA</td>
<td>0.45</td>
<td>AB805283</td>
</tr>
<tr>
<td>SLC2A4 Forward</td>
<td>TGGCACTTCTGGGAATTCTTG</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>SLC2A4 Reverse</td>
<td>AGGGCTCGCTATGGGAAGAAT</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

Accession numbers refer to the published cDNA sequences from which the primer sequences were designed.

Table 2. Mean arterial blood gas and pH values on the day before post mortem in saline- and glucose-infused fetuses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline-Infused (n = 12)</th>
<th>Glucose-Infused (n = 9)</th>
</tr>
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<tbody>
<tr>
<td>PaO2, mmHg</td>
<td>21.6 ± 0.5</td>
<td>21.4 ± 1.0</td>
</tr>
<tr>
<td>PaCO2, mmHg</td>
<td>51.1 ± 1.2</td>
<td>55.5 ± 0.7</td>
</tr>
<tr>
<td>pH</td>
<td>7.383 ± 0.008</td>
<td>7.395 ± 0.007</td>
</tr>
<tr>
<td>SaO2, %</td>
<td>64.9 ± 1.7</td>
<td>62.0 ± 2.6</td>
</tr>
<tr>
<td>Hb, ml/dl</td>
<td>10.4 ± 0.3</td>
<td>11.2 ± 0.7</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Data were analyzed by a Student’s unpaired t-test. *P < 0.05 was considered statistically significant. PaO2, arterial partial pressure of oxygen; PaCO2, arterial partial pressure of carbon dioxide; SaO2, oxygen saturation; Hb, hemoglobin.

Table 3. Mean arterial blood gas and pH values on the day before post mortem in saline- and glucose-infused fetuses

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Data are expressed as means ± SE. Data were analyzed by a Student’s unpaired t-test. *P < 0.05 was considered statistically significant.

RESULTS

Impact of Intraperitoneal Glucose Infusion on Fetal Blood Gases and Body and Organ Weight

Mean PaO2, PaCO2, pH, SaO2, and Hb were calculated as the mean of the values collected on the day before post mortem. Fetal weight, crown-rump length, lung weight, and relative lung weight were recorded at post mortem. All statistical analyses were carried out using Statistical Package for Social Sciences v20.0 (Chicago, IL). A Student’s unpaired t-test was used to compare all data between the saline- and glucose-infused fetuses. All data are presented as means ± SE. A probability level of 5% (P < 0.05) was considered significant.

Effect of Intrafetal Glucose Infusion on Expression of Genes Regulating Glucose Transport and Insulin Signaling in the Fetal Lung

Lung mRNA expression of glucose transporters (SLC2A1 and SLC2A4), IGF1, and IGF2 were not different between the saline- and glucose-infused fetuses. However, IGF1R mRNA expression was reduced in the lung of the glucose-infused fetus (Table 4).

Table 3. Effect of intravenous saline and glucose infusion on fetal and lung growth

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline-Infused (n = 12)</th>
<th>Glucose-Infused (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at post mortem, days</td>
<td>140 ± 1</td>
<td>140 ± 0*</td>
</tr>
<tr>
<td>Fetal weight, kg</td>
<td>4.81 ± 0.16</td>
<td>5.12 ± 0.13</td>
</tr>
<tr>
<td>Crown-rump length, cm</td>
<td>58.3 ± 1.4</td>
<td>56.9 ± 1.3</td>
</tr>
<tr>
<td>Lung weight, g</td>
<td>157.97 ± 6.7</td>
<td>158.04 ± 8.9</td>
</tr>
<tr>
<td>Relative lung weight, g/kg</td>
<td>33.1 ± 1.4</td>
<td>30.8 ± 1.3</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Data were analyzed by a Student’s unpaired t-test. *P < 0.05 was considered statistically significant.
Table 4. Effect of intravenous infusion on the mRNA expression of genes regulating glucose uptake and insulin signaling in the fetal lung

<table>
<thead>
<tr>
<th>Gene</th>
<th>Saline-Infused (n = 12)</th>
<th>Glucose-Infused (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC2A1</td>
<td>0.0155 ± 0.0012</td>
<td>0.0138 ± 0.0008</td>
</tr>
<tr>
<td>SLC2A4</td>
<td>0.0015 ± 0.0003</td>
<td>0.0008 ± 0.0001</td>
</tr>
<tr>
<td>IGF1</td>
<td>0.0347 ± 0.0041</td>
<td>0.0393 ± 0.0042</td>
</tr>
<tr>
<td>IGF2</td>
<td>14.10 ± 0.61</td>
<td>11.91 ± 0.97</td>
</tr>
<tr>
<td>IGF1R</td>
<td>0.0837 ± 0.0029</td>
<td>0.0689 ± 0.0037*</td>
</tr>
</tbody>
</table>

Data are expressed as mean normalized expression (MNE) ± SE. Data were analyzed by a Student’s unpaired t-test between saline- and glucose-infused fetuses. *P < 0.05 was considered statistically significant.

Discussion

Intrafetal glucose infusion increased plasma glucose and insulin concentrations (38), resulting in a reduction in the expression of genes regulating GC availability and signaling in the lung. These findings represent a potential mechanism regulating the observed reduction of SFTP mRNA expression in the lung of the hyperglycemic and hyperinsulinemic late-gestation sheep fetus. These findings provide evidence for a molecular mechanism regulating the delay in surfactant system maturation in infants of uncontrolled diabetic mothers and may explain the increased incidence of RDS (47, 59).

While increased plasma glucose and insulin concentrations are experienced by the fetus in a diabetic pregnancy, the specific effects at the cellular level can be determined by the extent of glucose uptake due to modulation of alterations in glucose signaling or indirect action on other pathways normally regulating fetal lung development. In fetal life, SLC2A1 is the primary regulator of cellular glucose uptake across the plasma membrane by carrier-mediated facilitated diffusion in a wide variety of tissues, including the lung (49). Furthermore, in response to the secondary hyperinsulinemia induced by fetal glucose infusion, it is necessary to consider the role of the insulin-dependent glucose transporter, SLC2A4, whose action on glucose uptake has been widely characterized in insulin-responsive tissues. These factors are important as they have been implicated in the regulation of glucose signaling in the fetal lung by exposure to high or low concentrations of glucose in vitro (49). It has been suggested that a limitation in utilization of the glucose as a substrate for surfactant synthesis or...
lung liquid clearance may correlate with the historical incidence of RDS in infants of poorly controlled diabetic mothers (49). Despite these findings on SLC2A expression following exposure to increased glucose and/or insulin concentrations, we observed no change in the mRNA expression of either SLC2A1 or SLC2A4 in the lung of the sheep fetus following glucose infusion in this study. These results suggest that it is unlikely that the observed changes in the fetal lung following glucose infusion are regulated directly by glucose availability; rather, changes occur by indirect regulation by the action of these factors at the tissue level.

In addition to regulation of fetal development by glucose, insulin and IGF-1 are key mediators of normal lung development (46, 53). Within the fetal lung, IGF1 and IGF2 both bind to the IGF-1 receptor (IGF1R) to regulate normal lung development and cellular proliferation during both fetal and postnatal life (17, 28). It has previously been demonstrated that changes in expression of these growth factors between lung

Fig. 2. Intrafetal glucose infusion decreased SFTP mRNA expression in the fetal lung. mRNA mean-normalized expression (MNE) of surfactant protein (SFTP)-A (A), SFTP-B (B), SFTP-C (C), and SFTP-D (D) decreased in the lung of glucose-infused fetuses. Data are expressed as means ± SE. *P < 0.05 was considered significant. Saline-infused fetuses are represented by open bars (n = 12), while glucose-infused fetuses are represented by closed bars (n = 9).

Fig. 3. Evaluation of SFTP-B-positive cells in the alveolar epithelium. Micrographs demonstrating SFTP-B immunoreactivity in the alveolar epithelium in the saline-infused (A) and glucose-infused (B) fetal lung (×200 magnification; scale bar = 50 μm). Glucose infusion did not change the numerical density of SFTP-B-positive cells per mm² of lung tissue (C). Data are expressed as means ± SE. P < 0.05 was considered significant. Saline-infused fetuses are represented by open bars (n = 5), while glucose-infused fetuses are represented by solid bars (n = 6).
tissue components are associated with respiratory complications, including RDS and BPD (10), in addition to lethal respiratory failure following birth (33). Disruption of normal IGF-1R signaling has also been shown to alter vascularization and result in dramatic changes in fetal lung morphology in vitro (25). Murine models of IGF-1, IGF-2, and IGF-1R-null mutations display effects on global growth, organ hypoplasia, and neonatal survival, demonstrating the key role of these factors in normal growth and postnatal survival (33). More specifically, IGF-1R knockout studies have demonstrated effects on distal lung branching morphogenesis in the mouse lung (17), and targeted deletion of IGF-1 or IGF-2 in mice leads to delayed lung maturation, as evidenced by morphological and structural changes to AEC proportions and lung tissue density (36, 44, 48). Despite no change in IGF1 or IGF2 mRNA expression following glucose infusion, there was a decrease in IGF1R mRNA expression in the lung of the glucose-infused fetuses, suggesting that there may be alterations to cellular proliferation and airway development in the lung, which may be associated with increased risk of complications at birth in infants of poorly controlled diabetic mothers (17, 33).

While the maturational effects of endogenous/exogenous GCs on the fetal lung are widely understood, the impact of glucose and insulin either individually or synergistically, and their interaction with GC on SFTP expression, is less well characterized. Insulin has been shown to inhibit the GC-stimulated incorporation of choline into the major surfactant phospholipid, phosphatidylcholine, by fetal rat type II AECs in culture (50). Furthermore, the presence of hyperglycemia and secondary hyperinsulinemia was found to inhibit the stimulatory effects of cortisol on surface-active material flux into tracheal fluid (61). An antagonistic relationship between GCs and insulin is well established in insulin-sensitive tissues and includes impairment of insulin-dependent glucose uptake, increased lipolysis and enhanced gluconeogenesis (26), altered insulin signaling (16), and inhibition of insulin secretion from the pancreatic β-cells (4, 30). Furthermore, the opposing effects have been characterized in clinical situations such as Type 2 diabetes, in which treatment with GCs decreased both insulin sensitivity (40) and NRC31 expression in skeletal muscle (58). Interestingly, high plasma concentrations of insulin alone decrease both SFTP-A and SFTP-B mRNA expression (15), while increased insulin in combination with cortisol increases SFTP-A expression in a dose-dependent manner in human fetal lung explants (14). It has been suggested that a link between hyperinsulinemia and RDS may involve a reduction in glycerol-3-phosphate and dihydroxyacetone phosphate production, which impairs surfactant phospholipid synthesis in the lung (54). A further potential mechanism regulating changes in the fetal lung following exposure to hyperglycemia and hyperinsulinemia involves β-receptor binding, which plays a vital role in surfactant release and reabsorption of fetal lung liquid (62, 64). Interestingly, although the latter study demonstrated a negative impact of hyperglycemia and hyperinsulinemia, similar to that experienced in this study, regarding β-receptor binding in the lung, there was a greater effect in males than females (62). This represents a potential mechanism for male infants to suffer disadvantage in respiratory morbidity following exposure to uncontrolled diabetes during pregnancy. Although this presents an interesting factor, a limitation of the current study is that there was not sufficient statistical power to determine differences due to fetal sex.

Despite the above-mentioned observations from previous studies, a molecular mechanism regulating the maturation of the protein component of the surfactant system under these conditions during fetal life has not been established. Here, we have demonstrated that following exposure to increased glucose and insulin concentrations in utero, there is a reduction in HSD11B–1 mRNA expression in the fetal lung. This result suggests a reduction in the rate of cortisol activation, and thus, glucose infusion may result in prereceptor regulation of GC action in the fetal lung in late gestation. In addition to regulation of GC availability, there is a reduction in the mRNA expression of NR3C1 in the lung of the glucose-infused fetus. The NR3C1 is the intracellular mediator of GC function, which has been widely characterized by both direct action on genes with a GC response element and indirect mechanisms via the action of transcription factors and cofactors to promote both normal lung and surfactant system maturation (37). These changes in GC availability and signaling provide evidence for a molecular mechanism linking increased glucose and insulin concentrations with delayed lung maturation in utero.

Moreover, we have demonstrated that following exposure to increased glucose and insulin concentrations, there is a concomitant reduction of SFTP mRNA in the fetal sheep lung in late gestation. Although a rat model has previously been utilized to examine the effects of induced diabetes on Sftp mRNA expression in the fetal lung (22, 23), we have utilized a more clinically relevant model to investigate the effect of increased glucose and insulin concentrations in utero on lung development and pulmonary surfactant maturation. The fetal sheep is an ideal model of human lung development because of its similar phasic pattern of development and relative proportions of gestation compared with humans (34). While increased glucose concentrations, similar to the ones achieved in this study, have been associated with abnormalities in surfactant phospholipid content, lung stability, and glycogen regulation in the fetal sheep lung (65), here, we provide evidence for changes in the protein component of the surfactant system. A reduction in all four SFTPs is likely to impair both the surface tension regulating and the innate immune functions (24, 31) of the surfactant system, potentially impairing the smooth transition to postnatal life and increasing the risk of RDS following birth in infants of diabetic mothers.

To determine whether the changes in SFTP mRNA expression observed at the molecular level in this study were due to changes at the cellular level within the lung, we evaluated the number of SFTP-B-positive cells in the alveolar epithelium, which are able to produce the components of pulmonary surfactant. While it has previously been demonstrated that insulin inhibits type II AEC differentiation in fetal rat lung explants (21), the impact of high glucose and insulin at the cellular level has not been evaluated in vivo. In this study, we found no difference in the number of SFTP-B-positive cells in the alveolar epithelium between the saline- and glucose-infused fetuses. These results suggest that there were no structural changes in cell density within the fetal lung; however, it is likely that the overall reduction in SFTP mRNA expression observed in the glucose-infused fetuses in this study was due to a reduced functional capacity of the surfactant-producing cells.
that are present in the lung of the glucose-infused fetus during late gestation.

Following glucose infusion in the late-gestation sheep fetus, we have identified changes in GC signaling as a mechanism regulating the observed delay in maturation of the surfactant system following exposure to increased glucose and insulin concentrations. It is likely that this effect on SFTP mRNA expression is mediated at the molecular level through an alteration to the functional output of cells able to produce surfactant, as there was no change to the number of SFTP-B-positive cells present in the alveolar epithelium.

The mRNA changes observed in this study are seen in whole lung tissue; thus, it is important to consider that changes in expression of some genes cannot be compared as simply as the expression of surfactant protein markers that are primarily restricted to the respiratory epithelium and alveolar type II cells in vivo. Despite this, the changes observed in this study highlight the multifactorial impact that exposure to increased plasma glucose and insulin concentrations has to modulate lung development on a whole, which ultimately leads to changes at the surfactant protein mRNA expression level and correlates with the historically observed increase in RDS in infants of poorly controlled diabetic mothers.

**Perspectives and Significance**

It is undisputed that there are more women of reproductive age who are classified as overweight or obese (11, 29) and that factors associated with obese obstetric populations and diabetic pregnancies contribute to an increased risk of maternal and fetal complications, preterm birth, and an increased risk of RDS following birth (6, 9). By investigating the effects in vivo of increased glucose and insulin concentrations in the lung of the late-gestation sheep fetus, we have demonstrated that conditions analogous to those experienced by a fetus in an uncontrolled diabetic pregnancy result in changes to GC signaling and are associated with downregulation of SFTP mRNA expression, which may complicate the fetus’s transition to air breathing at birth. These findings provide evidence for a GC-mediated mechanism to support the link between abnormal glycemic control in utero and RDS observed in infants of mothers with uncontrolled diabetes (47). What is clear is that glucose regulation and homeostatic feedback mechanisms throughout pregnancy are important and that early intervention to maintain tight maternal glycemic control throughout gestation is a key factor that will minimize the detrimental effects of excessive glucose and insulin concentrations on the molecular regulation of lung development and the risk of RDS in pregnancies complicated by diabetes.

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