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

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**AUTHOR CONTRIBUTIONS**

EM, ICM, SO and JLM were responsible for the conception and design of the experiments.

EM, ICM, SO and JLM were each involved in data acquisition.

EM, ICM, SO and JLM were involved in analysis and interpretation of the data.

EM, ICM, SO and JLM drafted the article and all authors contributed to the final version.

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**RUNNING TITLE:** Glucose infusion delays surfactant maturation

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ABSTRACT

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 signalling, 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 10d did not affect mRNA expression of glucose transporters or insulin like growth factors (IGF), 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.

KEYWORDS: glucose, diabetes, obesity, surfactant,

INTRODUCTION

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 45% of women being 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 characterised by a state of insulin resistance in late gestation, which is essential to provide substrates to the developing fetus (13). Whilst there is a natural decrease in insulin sensitivity throughout 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 signalling, including insulin-like growth factor (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 6-fold increased risk of developing RDS when compared to infants of non-diabetic 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 synthesised, 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 -C aid in their adsorption to the air-liquid interface and in the dynamic regulation of the functional surfactant film (43). SFTP-A and -D play important roles in innate immunity within the lung (24).

Maternal diabetes has also 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 antagonising 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 isoforms, hydroxysteroid (11-beta) dehydrogenase (HSD11B)-1, which catalyses the conversion of inactive cortisone to cortisol, or HSD11B-2, which catalyses the conversion of bioactive cortisol to cortisone (56). Alternatively, GC signalling may be affected by alterations 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 model 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.

GLOSSARY

AEC, alveolar epithelial cell; GC, glucocorticoid; GRE, glucocorticoid response element; Hb, Haemoglobin; HSD11B, hydroxysteroid (11-beta) dehydrogenase; IGF, insulin like growth factor; NR2C1, glucocorticoid receptor; PaCO₂, arterial partial pressure of carbon dioxide; PaO₂, arterial partial pressure of carbon dioxide; qRT-PCR, quantitative reverse transcription polymerase chain reaction; RDS, respiratory distress syndrome; SaO₂, oxygen saturation; SFTP, surfactant protein; SLC2A, solute carrier family 2 (facilitated glucose transporter).

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 12:12h light/dark cycle, and fed once daily with water ad libitum. At 118-120d
gestation, general anaesthesia was induced in the ewe with an intravenous injection of sodium thiopentone (1.25g, Pentothal, Rhone Merieux, Pinkenba, Qld, Australia) and maintained with 2.5-4% halothane inhalation anaesthetic (Fluothane, ICI, 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 (3.5ml of Norocillin (150mg/ml procaine penicillin and 112.5mg/ml benzathine penicillin; Norbrook Laboratories Ltd., Gisborne, Australia) and 2ml of 125mg/ml Dihydrostreptomycin in sterile saline (Sigma, St Louis, MO, USA) for 3d following surgery. Antibiotics (500mg; sodium ampicillin, Commonwealth Serum Laboratories) were administered intraamniotically to all fetal sheep daily for 4d post-operatively. Ewes were allowed at least 4d 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 (PaO$_2$), arterial partial pressure of carbon dioxide (PaCO$_2$), pH, oxygen saturation (SaO$_2$), and Hb content were measured using an ABL 520 analyser (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 130-140d gestation (n=9). Infusion began at an initial rate of 1.9ml/h for 24h and was then increased in a stepwise manner by 1.9ml/h per day for the next three days. The final infusion rate of 7.5ml/h obtained on the fourth day of the infusion was maintained until post mortem. Saline-infused fetuses received saline intravenously from 130-140±1d 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 10d infusion period for plasma glucose (saline-infused, 1.10±0.09mmol/l; glucose infused, 2.20±0.18mmol/l; \( P < 0.05 \)) and insulin (saline-infused, 4.93±1.03mmol/l; glucose infused, 9.77±1.38mmol/l; \( P < 0.05 \)).

**Post Mortem Procedures**

At 140±1d gestation, ewes were humanely killed with an overdose of sodium pentobarbitone administered via the jugular vein (Virbac Pty Ltd, Peakhurst, NSW, Australia) and fetal sheep were delivered by hysterectomy. The lungs were removed, weighed and 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. Fetal weight and neuroendocrine function data have been published previously (38).

**Quantification of mRNA Transcripts within the Fetal Lung**

**Total RNA extraction:** Total RNA was extracted from fetal lung samples (~50mg) using Invitrogen Trizol Reagent Solution and Qiagen RNeasy purification columns as per the manufacturer’s guidelines (Invitrogen) (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 280nm and checked for protein and DNA contamination. cDNA was synthesised using Superscript III First Strand Synthesis System (Invitrogen) using 2µg of total RNA in a final volume of 20µl 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 qRT-PCR data output normalisation, three stable housekeeping genes (β-actin (ACTB; U39357), peptidylprolyl isomerase A (PPIA; AY251270) (42) and tyrosine 3-monooxygenase (YWHAZ; AY970970) (34)) were run in parallel with target genes as previously described (34, 52). Previously published or specifically designed (Table 1) primer sets were validated and optimised as previously described (41). The gene expression of the glucose transporters (SLC2A1 (U89029) and SLC2A4 (AB005283.1)), IGF signalling (IGF1 (DQ152962), IGF2 (M89789) and IGF1R (AY162434) (18)), GC regulatory genes (HSD11B-1, NM_001009395.1; HSD11B-2, NM_001009460.1; NR3CI, NM_001114186.1; NR3CI, AF349768.1 (34)), surfactant proteins (SFTP-A, AF211856; SFTP-B, AF07544; SFTP-C, AF076634 and SFTP-D, AJ133002 (34, 41)) were measured by qRT-PCR using Fast SYBR® Green Master Mix (Applied Biosystems) in a final volume of 6µl on a ViiA7 Fast Real-time PCR system (Applied Biosystems) as previously described (34). Each qRT-PCR well contained 3 µl Fast SYBR Green Master Mix (2X), 2µl of forward and reverse primer mixed with H₂O to obtain final primer concentrations (Table 1) and 1µl of diluted relevant cDNA. The abundance of each transcript relative to the abundance of stable housekeeping genes (27) was calculated using DataAssist 3.0 analysis software (Applied Biosystems) and expressed as mRNA mean normalised expression (MNE)±SEM (34, 52).
Quantification of type II AECs within the fetal lung

SP-B immunoreactivity to identify mature type II AECs: 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 7µm thickness were deparaffinised and rehydrated before endogenous peroxide solution activity was blocked, and followed with antigen retrieval. Slides were incubated overnight with the aforementioned SFTP-B antibody (1:1000) at 4°C. Negative control slides were performed in parallel with test slides. A Histostain-Plus broad spectrum kit (Zymed Laboratories Inc., California, USA) was utilised with horseradish peroxidase and 3,3-diaminobenzidine chromagen (Metal Enhanced DAB Substrate Kit, Pierce Biotechnology, Illinois, USA) for visualisation of SFTP-B positive cells. All sections were counterstained with Mayer’s Haematoxylin.

Quantitative assessment of type II AEC in the fetal lung: Sections were examined using Visiopharm NewCAST software (Visiopharm, Hoersholm, Denmark) as previously described (34). Analysis was carried out by a single trained individual who was blinded to treatment groups. Sixty counting frames (×400 magnification) of the alveolar epithelium were randomly selected per section. Point-counting using an unbiased counting frame with an area of 20,000µm² was used to estimate 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 SFTP-B positive cells per mm² of lung tissue was obtained using the following equation (5, 34):

\[
\frac{\sum Q^{(\text{SFTP-B Positive})}}{\sum P^{(\text{lung tissue})} \times \left[ \frac{\text{a (frame)}}{P} \right]} \times 10^6 ,
\]

where \( \sum Q^{(\text{SFTP-B Positive})} \) represents the total number of SFTP-B positive cells counted in all counting frames of one fetal lung tissue section; \( \sum P \) (lung) represents the total number of points falling on lung tissue (i.e. the reference space); \( P \) is the number of points which were used to count the points hitting the reference space (i.e. 4 corners per counting frame); and \( a \) was the area of the counting frame. Tissue sections were photographed using a digital camera DP72 (Olympus Australia Pty. Ltd), which was connected to a BX53 Research Microscope (Olympus Australia Pty. Ltd).

Statistical Analyses

\( \text{PaO}_2, \text{PaCO}_2, \text{pH}, \text{SaO}_2, \) and \( \text{Hb} \) 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 (SPSS) v20.0 (Chicago, USA). A Student’s unpaired \( t\)-test was used to compare all data between the saline- and glucose-infused fetuses. All data are presented as mean ± standard error of the mean (SEM). A probability level of 5% (\( P<0.05 \)) was considered significant.

RESULTS

Impact of intrafetal glucose infusion on fetal blood gases and body and organ weight:

Mean \( \text{PaO}_2, \text{PaCO}_2, \) pH, \( \text{Hb} \) and \( \text{SaO}_2 \) were not different between saline- and glucose- infused fetuses on the day before post mortem (Table 2). Glucose infusion did not affect fetal weight,
crown-rump length, abdominal circumference, lung weight or relative lung weight to body weight ratio when compared to saline-infused controls (Table 3).

**Effect of intrafetal glucose infusion on expression of genes regulating glucose transport and insulin signalling in the fetal lung:** Lung mRNA expression of glucose transporters (*SLC2A1 and SLC2A4*), *IGF1*, *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).

**Effect of intrafetal glucose infusion on expression of genes regulating GC availability and signalling in the fetal lung:** Lung mRNA expression of *HSD11B-1* and *NR3C1* were reduced in glucose-infused fetuses (Figure 1A and C). There was no difference in mRNA expression of *HSD11B-2* or *NR3C2* (Figure 1 B and D) in the fetal lung.

**Effect of glucose infusion on surfactant protein mRNA expression and the numerical density of type II AECs in the fetal lung:** Glucose infusion resulted in a reduction of *SFTB*-A, -B, -C and -D mRNA expression in the fetal lung (Figure 2). The numerical density of *SFTP-B* positive cells in the alveolar epithelium of the fetal lung was not different between saline- and glucose-infused fetuses (Figure 3).

**DISCUSSION**

Intra-fetal glucose infusion increased plasma glucose and insulin concentrations (38), resulting in a reduction in the expression of genes regulating GC availability and signalling in the lung. These findings represent a potential mechanism regulating the observed reduction of *SFTP* mRNA expression in the lung of the hyperglycaemic 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).

Whilst 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 signalling 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 characterised in insulin responsive tissues. These factors are important as they have been implicated in the regulation of glucose signalling in the fetal lung by exposure to high or low concentrations of glucose in vitro (49).

It has been suggested that a limitation in utilisation 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 SCL2 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 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 signalling has also been shown to alter vascularisation and result in dramatic changes in fetal lung morphology in vitro (25). Murine models of IGF-1, -2 and -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 IFG-1 or –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 IGFI-R 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 characterised. 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 signalling (16) and inhibition of insulin secretion from the pancreatic β-cells (4, 30). Furthermore, the opposing effects have been characterised 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 -B mRNA expression (15), whilst this concentration 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, whilst the latter study demonstrated a negative impact of hyperglycemia and hyperinsulinemia, similar to that experienced in this study, on β-receptor binding in the lung, there was a greater effect in males than females (62). This represents a potential mechanism for male disadvantage in respiratory morbidity following exposure to uncontrolled diabetes during pregnancy. Although this presents as 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 observations in 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 HSDB11-1 mRNA expression in the fetal lung. This result suggests a reduction in the rate of cortisol activation, and thus glucose infusion may result in pre-receptor 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 \textit{NR3C1} in the lung of the glucose-infused fetus. The
\textit{NR3C1} is the intracellular mediator of GC function, which has been widely characterised
both by direct action on genes with a GC response element (GRE) 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 signalling provide
evidence for a molecular mechanism linking increased glucose and insulin concentrations
with delayed lung maturation \textit{in utero}.

Moreover, we have demonstrated that following exposure to increased glucose and
insulin concentrations, there is a concomitant reduction of \textit{SFTP} mRNA in the fetal sheep
lung in late gestation. While a rat model has previously been utilised to examine the effects of
induced diabetes on \textit{Sftp} mRNA expression in the fetal lung (22, 23), we have utilised a more
clinically relevant model to investigate the effect of increased glucose and insulin
concentrations \textit{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 when compared to humans (34). Whilst
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 \textit{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 if the changes in \textit{SFTP} 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. Whilst 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 signalling 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 & 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 signalling and are associated with downregulation of SFTP mRNA expression, which may complicate the fetuses’ 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. 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 lead to minimising the effects of excessive glucose and insulin concentrations on the molecular regulation of lung development and the risk of RDS in pregnancies complicated by diabetes.

ACKNOWLEDGEMENTS

We acknowledge the assistance of Esther Marrocco, Beverley Mühlhäusler, Anne Jurisevic and Laura O’Carroll in performing the surgical procedures, providing expert post-surgical care of the ewe and her fetus and performing the glucose infusion. We thank Darran Tosh for assistance with real-time PCR and Stacey Dunn and Kimberley Botting for assistance with immunohistochemistry and cell counting, respectively.
The animal component of the work was funded by a NHMRC Program Grant (ICM). The molecular component of the work and JLM were funded by a South Australian Cardiovascular Research Network Fellowship (CR10A4988).

DISCLOSURES

The authors have no conflict of interest.
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Table 1. qRT-PCR primer sequences for designed target genes.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ ➔ 3’</th>
<th>Primer Concentration (μM)</th>
<th>Accession No.</th>
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<tr>
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<td>U89029.1</td>
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<td>Reverse</td>
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<td>Reverse</td>
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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>
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<tr>
<th></th>
<th>Saline-infused (n=12)</th>
<th>Glucose-infused (n=9)</th>
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</thead>
<tbody>
<tr>
<td>( \text{PaO}_2 ) (mmHg)</td>
<td>21.6 ± 0.5</td>
<td>21.4 ± 1.0</td>
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<tr>
<td>( \text{PaCO}_2 ) (mmHg)</td>
<td>51.1 ± 1.2</td>
<td>53.5 ± 0.7</td>
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<td>pH</td>
<td>7.383 ± 0.008</td>
<td>7.393 ± 0.007</td>
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<td>( \text{SaO}_2 ) (%)</td>
<td>64.9 ± 1.7</td>
<td>62.0 ± 2.6</td>
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<td>Hb (ml/dl)</td>
<td>10.4 ± 0.3</td>
<td>11.2 ± 0.7</td>
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</tbody>
</table>

Data expressed as mean±SEM. Data were analysed by a Student’s unpaired \( t \)-test. \( P<0.05 \) was considered statistically significant. \( \text{PaO}_2 \), arterial partial pressure of oxygen; \( \text{PaCO}_2 \), arterial partial pressure of carbon dioxide; \( \text{SaO}_2 \), oxygen saturation; Hb, Haemoglobin.
Table 3. Effect of intravenous saline and glucose infusion on fetal and lung growth.

<table>
<thead>
<tr>
<th></th>
<th>Saline-infused (n=12)</th>
<th>Glucose-infused (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at post-mortem (d)</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 expressed as mean±SEM. Data were analysed 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 signalling in the fetal lung.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Saline-infused (MNE ±SEM)</th>
<th>Glucose-infused (MNE ±SEM)</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 expressed as mean normalised expression (MNE)±SEM. Data were analysed by a Student’s unpaired *t*-test between saline- (n=12) and glucose-infused (n=9) fetuses. *P<0.05 was considered statistically significant.
Legends for Figures

Figure 1. Intrafetal glucose infusion decreased GC activating enzyme and receptor mRNA expression in the fetal lung. Lung mRNA expression of 11β hydroxysteroid dehydrogenase isoform -1 (HSD11B-1, A) and glucocorticoid receptor (NR3C1, C) decreased in glucose-infused fetuses. There was no change in expression of HSD11B-2 (B) or mineralocorticoid receptor (NR3C2, D). Data expressed as mean±SEM. *P<0.05 was considered significant. Saline-infused fetuses, open bars (n=12); Glucose-infused fetuses, closed bars (n=9).

Figure 2. Intrafetal glucose infusion decreased SFTP mRNA expression in the fetal lung. Normalised mRNA expression 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 expressed as mean±SEM. *P<0.05 was considered significant. Saline-infused fetuses, open bars (n=12); Glucose-infused fetuses, closed bars (n=9).

Figure 3. Evaluation of SFTP-B positive alveolar epithelial cells (AEC) in the fetal lung. Micrographs demonstrating SFTP-B immunoreactivity of type II AEC in the saline-infused (A) and glucose-infused (B) fetal lung (200x magnification, scale bar = 50µm). Glucose infusion did not change the numerical density of SFTP-B positive type II AEC per mm² of lung tissue (C). Data expressed as mean±SEM. *P<0.05 was considered significant. Saline-infused fetuses, open bars (n=5); Glucose-infused fetuses, closed bars (n=6).
Figure 1