PROXIMITY TO DELIVERY ALTERS INSULIN SENSITIVITY AND GLUCOSE METABOLISM IN PREGNANT MICE

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ABSTRACT

In late pregnancy, maternal insulin resistance occurs to support fetal growth but little is known about insulin-glucose dynamics close to delivery. This study measured insulin sensitivity in mice in late pregnancy, day (D) 16, and near term, D19, (term 20.5D). Non-pregnant (NP) and pregnant mice were assessed for metabolite and hormone concentrations, body composition by dual energy X-ray absorptiometry, tissue insulin signalling protein abundance by Western blotting, glucose tolerance and utilisation, and insulin sensitivity using acute insulin administration and hyperinsulinaemic-euglycaemic clamps with ^{3}H-glucose infusion. Whole body insulin resistance occurred in D16 pregnant dams in association with basal hyperinsulinaemia, insulin-resistant endogenous glucose production and downregulation of several proteins in hepatic and skeletal muscle insulin signalling pathways relative to NP and D19 values. Insulin resistance was less pronounced at D19 with restoration of NP insulin concentrations, improved hepatic insulin sensitivity and increased abundance of hepatic insulin signalling proteins. At D16, insulin resistance at whole body, tissue and molecular levels will favour fetal glucose acquisition while improved D19 hepatic insulin sensitivity will conserve glucose for maternal use in anticipation of lactation. Tissue sensitivity to insulin, therefore, alters differentially with proximity to delivery in pregnant mice with implications for human and other species.

INTRODUCTION

During pregnancy, maternal metabolism adapts to support offspring growth. In particular, there are changes in insulin sensitivity, which affects the availability and fate of nutrients in both mother and conceptus (1,2). The specific adaptations depend on the stage of pregnancy as metabolic demands increase with expansion of the gravid uterus (3,4). In humans and rats, early pregnancy is a period of lipid accumulation and unchanged or increased insulin sensitivity, whereas, later pregnancy is characterised by lipid mobilisation and insulin resistance, common features of overt Type 2 diabetes (1,2,5,6). Indeed, whole body resistance to the hypoglycaemic action of insulin has been reported during late pregnancy in a wide range of species including rabbits, dogs, sheep, horses as well as rats and humans (7-13). It is often accompanied by reduced maternal glucose utilisation, particularly in skeletal muscle, although there is less consensus about the actions of insulin on hepatic glucogenesis during pregnancy (10,12,14,15). Most studies of insulin sensitivity during late pregnancy have been carried out between 60-85% of gestation with few measurements closer to term when fetal nutrient demands are maximal yet maternal nutrient requirements may also be changing in preparation for the imminent onset of labour and lactation.

Total conceptus mass varies not only with increasing gestational age but also between species both in total and as a percentage of maternal mass (16). Fetal growth rate is high during late mouse pregnancy and results in the gravid uterus accounting for 30% of maternal mass at term (16). This is a higher percentage than found in monotocous species like humans and sheep (5-9%) or the values of 12-25% seen in other polytocous animals like dogs, pigs and rats (16). Despite this, little is known about insulin sensitivity in pregnant mice, even though they are used widely in genetic and developmental studies in which variations in maternal insulin sensitivity may affect the ensuing offspring phenotype (17,18). The molecular basis of insulin resistance during late pregnancy is also still poorly understood in many species. Tissue insulin receptor (IR) abundance appears to be unaffected by pregnancy, although there is evidence for defects in the early stages of insulin signal transduction downstream of the IR in skeletal muscle of both rats in late pregnancy and pregnant women insulin resistant due to obesity or gestational diabetes (19-23). Hence, the aims of this study were to measure glucose-insulin dynamics, whole body insulin sensitivity and tissue insulin signalling proteins in non-pregnant (NP) mice and in pregnant dams in late gestation and close to term. C57B1/6

mice was used in the study because this strain has been used extensively to investigate the genetic and environmental regulation of feto-placental development (17,24).

MATERIALS AND METHODS

Animals

C57Bl/6 females (n=123) were group housed at 21ºC under 12hr dark:12hr light conditions with free access to water and food (RM3, Special Diet Services). Aged 8-12 weeks, females were time mated with C57Bl/6 males with the presence of a copulatory plug defined as day (D)1 of a D20.5 pregnancy. Pregnant (n=77) and remaining non-pregnant (NP, n=46) mice were weighed every five days while food intake was measured every three days. Mice were allocated to one of the following procedures: 1) tissue and blood collection (n=30), (2) dual energy X-ray absorptiometry (DEXA, n=29) scanning, (3) glucose tolerance tests (GTT, n=28) or insulin tolerance tests (ITT, n=20) or (4) a hyperinsulinaemic-euglycaemic clamp (HEC) together with D-^{3}H-glucose and 2-deoxyglucose (2DG) administration (n=18). All experiments were carried under the UK Animals (Scientific Procedures) Act 1986 after ethical review by the University of Cambridge.

Experimental procedures

Tissue and blood collection

Between 08.00-10.00 h in fed conditions, pregnant dams at D16 and D19 and age-matched NP females (n=10 mice per group) were weighed and anaesthetised (10 µl/g of fentanyl-fluanisone: midazolam in sterile water, 1:1:2, Jansen Animal Health, ip). A cardiac blood sample was taken before euthanasia by cervical dislocation. In NP mice, liver, heart, kidneys, skeletal muscle (*biceps femoris)* and retroperitoneal fat were dissected, weighed and snap frozen in liquid nitrogen. In pregnant dams, the gravid uterus, hysterectomised carcass and individual fetuses and placentas were weighed before maternal tissue collection. Blood glucose concentrations were measured on a hand held glucometer (One Touch Ultra, LifeScan, UK). After centrifugation, the plasma was stored at -20˚C to measure metabolite and hormone concentrations.

DEXA scanning

Whole body fat and lean mass content were determined by DEXA scanning (Lunar PIXImus densitometer; GE lunar Corp., Madison WI) in intact NP females (n=10) and hysterectomised pregnant mice killed by cervical dislocation between 08.00-10.00 h (D16 n=7, D19 n=10). Values were expressed as a proportion of total body weight in NP mice and of hysterectomised weight in pregnant dams.

Glucose tolerance test and insulin tolerance test

Conscious NP and pregnant mice received either a GTT (NP n=11, D16, n=9, D19 n-8) or ITT (n=6-8 mice per group) after fasting from 08.00 h for 6 h or 3.5 h, respectively. Blood samples (≤ 5µl) were taken from the tail vein immediately before intraperitoneal administration of either glucose (10% w/v, 1 g/kg body weight) or insulin (0.25 U/kg, human insulin, Actrapid, Novo Nordisk) and, thereafter, at 15 to 30 min intervals for 120 min to measure blood glucose concentrations as above. The mice were then killed by cervical dislocation.

Hyperinsulinaemic-euglycaemic clamp

The HEC was performed as described previously (25). Briefly, NP and pregnant mice fasted for 2.5 h (NP n=7, D16, n=5, D19 n=6) were anaesthetised with a mixture of ventranquil: dormicum: fentanyl (1:2:10 in 3 units of water, 10 µl/g body weight, ip, Janssen-Cilag, Tilburg, Netherlands) and maintained at 37ºC using a servo-controlled thermopad (Harvard Instruments, UK). After catheterising a tail vein, D-^{3}H-glucose was infused continuously (0.006MBq/min in PBS, 50µl/hr, iv, 370-740GBq/mmol Perkin Elmer, UK). After steady state was achieved at 60 min (basal state, ≈ 3.5 h fasted), two blood samples (≤ 50µl each) were taken 10 min apart from the tail. Insulin was then injected as a bolus (3.3 mU, iv, Actrapid, human insulin, Novo Nordisk) followed by infusion (0.09 mU/min) together with the D-^{3}H-glucose. Blood glucose levels was monitored every 5 min for the first 20 min after insulin administration and then at 10 min intervals until the end of the protocol (≤ 5 µl per sample). When a decrease in blood glucose concentration was detected 5-10 min after beginning the insulin infusion, a variable rate glucose infusion (12.5% w/v PBS, Sigma, iv) was begun and adjusted every 5-10 min thereafter to maintain blood glucose concentrations at mean basal levels. At 50 min after insulin infusion, 2-deoxy-glucose (^{14}C-2DG, specific

activity: 9.25-13.0GBq/mmol, Perkin Elmer, UK) was injected intravenously. By 70 min of insulin administration, blood glucose levels were clamped at basal concentrations and a further three blood samples (≤ 50µl each) were collected from the tail at 10 min intervals. The mice were then killed by cervical dislocation and samples of *biceps femoris* and retroperitoneal fat were collected from all animals together with the fetus and placenta adjacent to the cervix in each horn (n=2 fetuses and placentas per litter) for analysis of tissue ^{14}C-2DG content. The rates of glucose utilization and production in basal and hyperglycaemic states together with whole body and hepatic sensitivity to insulin were calculated as described previously (25,26).

Biochemical analyses

Hormone and metabolite concentrations

Plasma D-^{3}H-glucose concentrations were measured by scintillation counting (Hidex 300SL, LabLogic Ltd, Sheffield, UK), after samples were deproteinised with 20% trichloric acid and dried to eliminate tritiated water. Plasma leptin and insulin concentrations in the fed state were measured simultaneously using a 2-plex specific immunoassay (Meso Scale Discovery). The inter-assay coefficients of variation (CV) were 10.8% and 9.7%, respectively. Plasma insulin concentrations during the HEC were measured by ELISA (Crystal Chem Inc., 90090), which detected both murine and human insulin. The inter-assay CV was ≤ 10%. Plasma IGF1 levels were also measured by ELISA (ImmunoDiagnostic Systems) with an inter-assay CV of 4.6%. Enzymatic assay kits were used to determine the plasma concentrations of triglycerides (TG), total cholesterol (Siemens Healthcare, CV 5.5% and 4.9% respectively) and free fatty acids (Roche, CV 4.5%).

Tissue biochemical composition

Hepatic glycogen content was measured enzymatically, using amyloglucosidase as reported previously (27). The total fat content of the liver and pooled samples of skeletal muscle were measured using the modified Folch method (28). To determine tissue phosphorylated 2DG (p2DG) content, tissues were homogenised in 0.5% perochloric acid and the homogenates neutralised to separate p2DG from 2DG by precipitation, as described previously (29).

Protein expression in insulin signalling and lipid metabolism pathways

Proteins were extracted (~100 mg, NP n=10, D16 n=5, D19 n=5-6) from the liver and skeletal muscle and quantified using Western blotting as described previously [30]. Successful transfer and equal protein loading was confirmed by Ponceau-S staining of membranes before incubation with antibody (Table 1). Protein abundance was determined by measuring pixel intensity of the protein bands using ImageJ analysis software (U.S. National Institutes of Health, Bethesda, MD, USA).

Statistics

All statistical analyses and calculations were performed on the GraphPad Prism 4.0. For most of the data, differences between NP, D16 and D19 animals were analysed by one way analysis of variance (ANOVA) with Bonferroni *post hoc* test. When the ANOVA indicated an effect of pregnancy, differences between D16 and D19 of pregnancy were assessed separately by unpaired *t-test.* Changes within the same group were assessed by paired *t-test* or by *t-test* the mean change differing from zero. For GTT and ITT protocols, the changes in glucose concentration were analysed by two-way ANOVA with time as a repeated measure. The area above the curve (AAC) in the GTT and area under the curve (AUC) in the ITT for the changes in glucose concentrations were calculated using the trapezoid rule. Fetal and placental data were averaged for each litter before calculation of mean values at each gestational age.

RESULTS

Biometry

Pregnant dams were heavier than NP females due to the gravid uterus and increased weights of several maternal tissues, although, when weights were expressed as a percentage of total or hysterectomised body weight, only the liver and retroperitoneal fat pads were proportionately heavier during pregnancy (Table 2). However, DEXA scanning showed that no significant changes in body fat content during pregnancy (Table 2). As expected [31], the gravid uterus and individual fetuses weighed less while the placentas weighed more at D16 than D19 with

no difference in litter size between the two groups (Table 2). Hepatic fat and glycogen content were higher in total during pregnancy in line with the increased tissue weight but not when expressed per mg tissue (Table 2). Skeletal muscle fat content of pooled samples appeared to be greater in pregnant than NP animals when expressed per mg tissue (Table 2). Pregnant dams increased their food intake relative to NP females from D9 of pregnancy (data not shown).

Metabolites and hormones concentrations

Blood glucose concentrations were unaffected by pregnancy in the fed state but were significantly lower than NP values in both pregnant groups after 3.5 h of fasting (Table 3). At 6 h of fasting, blood glucose levels were similar to those seen at 3.5 h of fasting in NP and D19 groups but were significantly higher than at 3.5 h of fasting in D16 dams (Table 3). In fed animals, plasma FFA concentrations were significantly lower in both pregnant groups than in NP females with the lowest values in D16 dams (Table 3). Cholesterol concentrations were also significantly lower during pregnancy and declined significantly between D16 and D19 (Table 3). Insulin concentrations were significantly higher in D16 dams than NP females with intermediate values in D19 dams (Table 3). Plasma leptin concentrations were higher while plasma IGF1 levels were lower in pregnant than NP groups, with no significant differences with gestational age (Table 3).

Glucose tolerance

The increment in blood glucose concentrations, the time course of the concentration changes and the AUC did not differ with pregnancy or gestational age (Figure 1A). Blood glucose concentrations remained elevated for the entire 120 min after glucose injection in all 3 groups (Figure 1B).

Insulin sensitivity

Blood glucose concentrations were significantly lower than baseline 15 min after acute insulin injection in all 3 groups and remained depressed for up to 120 min (Figure 1B). However, the decrement in blood glucose concentrations was greater in NP than both pregnant groups from 15-90 min after insulin injection (Figure 1B). In D16 dams, blood glucose level returned to baseline by 60 min and were significantly greater than baseline 120 min after insulin injection (Figure 1B). The AAC also differed significantly with pregnancy

and gestational age, and indicated that pregnant dams were less sensitive to insulin than NP females, particularly at D16 (Figure 1B).

Whole body and hepatic insulin sensitivity were investigated in more detail by HEC coupled with ^{3}H-glucose infusion. In all 3 groups, blood glucose levels were clamped at basal, euglycaemic levels by 70-90 min after beginning insulin infusion (Figure 2A). At this time, plasma insulin concentrations were within the post-prandial range and 5-6 fold higher than the basal values in all 3 groups (Figure 2B). However, steady-state insulin concentrations during the clamp were significantly lower in the D19 than NP or D16 groups (Figure 2B). Whole body insulin sensitivity, measured as GIR, was lower in D16 dams than in the other two groups and similar in NP and D19 pregnant groups (Figure 2C). The findings were identical when GIR was adjusted for the differences in the increment in insulin concentration during the clamp period (data not shown). Whole body insulin sensitivity was also calculated as the difference between glucose utilisation in hyperinsulinaemic (R_d) and basal states (R_a). This difference varied widely between individuals, particularly in D16 dams, and was only a significant increment in NP females, indicative of insulin resistance in both D16 and D19 dams (Figure 2D). During hyperinsulinaemia, EGP continued at a significant rate in all 3 groups ($p<0.02$, greater than zero, all groups, *t-test*) and occurred at the highest rate in D16 dams (Figure 2D). Hepatic insulin sensitivity, calculated as a significant difference in glucose production between basal and hyperinsulinaemic states, was only detected in D19 dams and was greater in absolute value at D19 than D16 (Figure 2D).

Whole body and tissue glucose utilisation

Whole body glucose utilization was significantly greater in D19 dams than NP females in basal conditions (R_a) while, during hyperinsulinaemia (R_d), it was higher in D16 dams than NP females with intermediate values in D19 dams (Figure 2C). Tissue glucose utilization during hyperinsulinaemia, measured as p2DG content, in skeletal muscle and adipose tissue varied widely, particularly in NP females, but was not significantly different between pregnant and NP animals nor between D16 and D19 dams (Table 4). Fetal p2DG content was similar to that of maternal tissues at both ages (Table 4). Placental p2DG content was significantly higher than in the fetus or maternal tissues at D19, but not at D16 (Table 4).

Tissue insulin-IGF signalling and lipid metabolism

Hepatic insulin-IGF signalling pathway was largely downregulated in D16 dams compared with NP females (InsR, IGF1R, pAkt-T308, pMAPK, Figure 3A). In contrast, at D19, the insulin signalling pathway was largely upregulated, compared to NP and D16 pregnant groups (IGF1R, total Akt, pAkt-S473, total Gsk3, pGsk3, total S6K, total MAPK and pMAPK, Figure 3A). Pregnancy had less effect on insulin signalling pathways in skeletal muscle with increased abundance of p85α at D16 and of pAkt T308 and total MAPK at D19 compared to NP values and decreased total Akt and pS6K abundance at D19 compared to either NP or D16 values (Figure 3B).

Hepatic abundance of lipogenic SREBP was reduced at D16 but normalised by D19 while PPARα, PPARγ and LPL were reduced in both pregnant groups relative to NP values (Figure 3C). Hepatic abundance of PPARγ and LPL was significantly lower in D19 than D16 dams (Figure 3D). Hepatic FATP1 was lower in D19 dams than NP females (Figure 3C).In skeletal muscle, SREBP was reduced in D16 but not D19 dams while PPARγ and LPL abundance were lower in both pregnant groups relative to NP values (Figure 3D). Skeletal muscle expression of LPL was greater and FATP1 was less at D16 than D19 (Figure 3D).

DISCUSSION

This is the first study to measure insulin sensitivity of glucose metabolism in pregnant mice and shows significant changes in insulin-glucose dynamics with both pregnancy and gestational age over the last 20% of gestation. In particular, there were changes in insulin concentration, glucose utilization and production, and in whole body and tissue insulin sensitivity between D16 and D19 of pregnancy. Protein abundance in the hepatic and skeletal muscle insulin signalling pathways also differed during pregnancy in line with the gestational changes in insulin sensitivity and glucose metabolism. Overall, insulin resistance and EGP capacity were more pronounced at D16 than D19, which indicates that mice adapt their metabolic strategy for supporting pregnancy as delivery approaches. There were also changes in body composition, tissue lipogenic signalling proteins and circulating concentrations of leptin and IGFI during pregnancy which indicate that, like other species (2,5), mice accumulate fat in specific deposits during early but not late pregnancy and are resistant to the

actions of leptin, as well as insulin, in late pregnancy, when their food intake increases despite hyperleptinaemia.

While glucose tolerance was unaffected by pregnancy, insulin resistance was evident in D16 pregnant mice. These dams were hyperinsulinaemic in both fed and fasted states, had a significantly smaller hypoglycaemic response to acute insulin administration and required 80-85% less exogenous glucose to maintain euglycaemia during the HEC than the other two groups of animals. This decrement in GIR from NP values was greater than seen in rats, dogs and women at an equivalent stage of pregnancy (7,9,12). In addition, insulin failed to inhibit EGP in D16 mouse dams. Similar findings have been made in pregnant rats but, in women, rabbits and dogs with a proportionately smaller gravid uterus, insulin continues to reduce EGP during late pregnancy, although not always as effectively as in the NP state (9,10,12,14,15). Furthermore, whole body glucose utilisation varied widely and did not increase significantly during hyperinsulinaemia in D16 pregnant mice, unlike the significant increment seen in NP females. Although there were no significant changes in tissue p2DG content, skeletal muscle expression of p85α, a known regulator of human muscle insulin resistance (32), was increased while hepatic expression of IR and several downstream insulin signalling proteins were decreased in D16 mouse dams, consistent with their reduced whole body and liver insulin sensitivity. Collectively, these findings indicate that insulin resistance occurs at whole body, tissue and molecular levels at D16 of mouse pregnancy.

By D19, insulin concentrations in fed and fasted states had returned to NP levels and the hypoglycaemic response to acute insulin administration was greater than at D16, although still less than in NP females. Insulin concentrations were also lower during the clamp in D19 dams which suggests increased insulin clearance consistent with findings in dogs during late pregnancy (12). A degree of hepatic insulin sensitivity was restored in D19 pregnant mice as insulin infusion now reduced EGP. Hepatic expression of several proteins in the insulin signalling pathway were also up-regulated at D19 relative to the other two groups. Improved hepatic insulin signalling at D19 is also suggested by normalised expression of the insulin-regulated transcription factor, SREBP (33). Furthermore, the GIR required to maintain euglycamia during hyperinsulinaemia was significantly greater at D19 than D16, indicative of improved whole body insulin sensitivity near term. However, tissue p2DG content showed no change between D16 and D19, and whole body glucose utilisation was unresponsive to insulin at D19, which suggests that a degree of insulin resistance still persists in pregnant

mice close to delivery. These apparent contradictions probably reflect the non-insulin dependent, transplacental flux of glucose, which would increase GIR and lead to overestimation of the actual maternal insulin sensitivity, particularly when fetal glucose demands are high near term (2,34). Insulin sensitivity, therefore, appears to change differentially in individual maternal tissues with proximity to delivery in pregnant mice.

In basal conditions, the rate of endogenous glucose production (R_a) in the NP females fasted for 3.5hr in the present study was higher than that seen previously in older male mice fasted overnight (25). Insulin also had little effect on the rate of EGP in NP females in the present study compared to its inhibitory actions in males published previously (25). Whether these differences are sex-linked or due to the differing ages and length of fasting remain unclear but insulin sensitivity is known to be greater in juvenile than adult animals and in adult women than men (35,36). In the present study, basal glucose production (R_a) was higher in pregnant than NP females as seen previously in dogs, sheep and women during late pregnancy (11,12,14). In mice, this is consistent with the greater total availability of hepatic glycogen associated with the increased relative size of the liver during pregnancy. Particularly at D16, EGP was activated readily and sustained for several hours. Blood glucose concentrations were higher after fasting for 6 h than 3.5 h and could be increased above pre-treatment levels 2 h after initiating an acute hypoglycaemic challenge at D16 but not D19. EGP were also significantly higher during hyperinsulinaemia in D16 dams than in the other two groups. Since hepatic glycogen content and activity of glucose-6-phosphatase are similar at D16 and D19 (37), the results indicate that, in addition to differences in hepatic insulin sensitivity, there may also be a greater gluconeogenic capacity and/or a more robust counter-regulatory response to hypoglycaemia at D16 than D19. Mice may, therefore, appear to rely more heavily on glucose production to meet the glucose demands of the gravid uterus at D16 than D19. Although the causes of the changes in insulin sensitivity and glucose production with proximity to delivery remain unknown, they closely parallel gestational changes in maternal concentrations of corticosterone, a known insulin antagonist (4,37), with maximal values at D16 and progressively declining concentrations thereafter towards term (38).

Fetal glucose utilisation tended to be less at D19 than D16, consistent with the changes in glucose metabolism seen in fetal sheep towards term (39). At D19, weight specific rates of glucose utilisation by the fetus (≈ 39 μmol/min/kg) and placenta (≈ 120 μmol/min/kg),

estimated from their p2DG contents, were within the range of values reported previously for other species at ≥ 90% of gestation (40-42). Estimation of total feto-placental glucose utilization by the whole litter indicates that this increases by 15-20% between D16 and D19 while total conceptus mass increases by 120%. Mouse pups must, therefore, be using nutrients other than glucose to sustain their growth rate during late gestation. Indeed, previous studies have shown that their growth is positively correlated to placental glucose transport at D16, but not at D19, and becomes progressively more dependent on placental amino acid transport towards term (43). A greater ATP requirement for active amino acid transport by the D19 placenta is consistent with its high rate of glucose utilization relative to other fetal and maternal tissues at this age. In addition, the lower maternal FFA and cholesterol concentrations during pregnancy indicate that lipids may also provide alternative substrates for feto-placental tissues during late gestation (44).

In summary, the marked insulin resistance of glucose utilisation and production at D16 will favour fetal glucose acquisition when fetuses are entering their maximal growth phase (31). When the fetuses have nearly reached term weight and can use other substrates at D19, insulin sensitivity of maternal tissues like the liver improves, which will conserve proportionally more glucose for maternal use in anticipation of the imminent demands of labour and lactation. This late change in insulin-glucose dynamics is likely to be particularly important in mice in which the gravid uterus accounts for such a large proportion of maternal weight at term but may also have a significant role in the maternal metabolic preparations for delivery in other species when the primary site of maternal-offspring nutrient allocation switches from the uterus to the mammary gland.

AUTHOR CONTRIBUTIONS

BM, ANS-P, ALF and SEO designed the study. BM, DSF-T, PV and ALF carried out the clamp studies. ANS-P, ORV, BM and ALF collected the tissues. BM, ANS-P and ALF carried out the glucose and insulin tolerance tests. BM carried out the Western blotting. All authors contributed to writing the paper.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

GUARANTOR STATEMENT

Professor Fowden is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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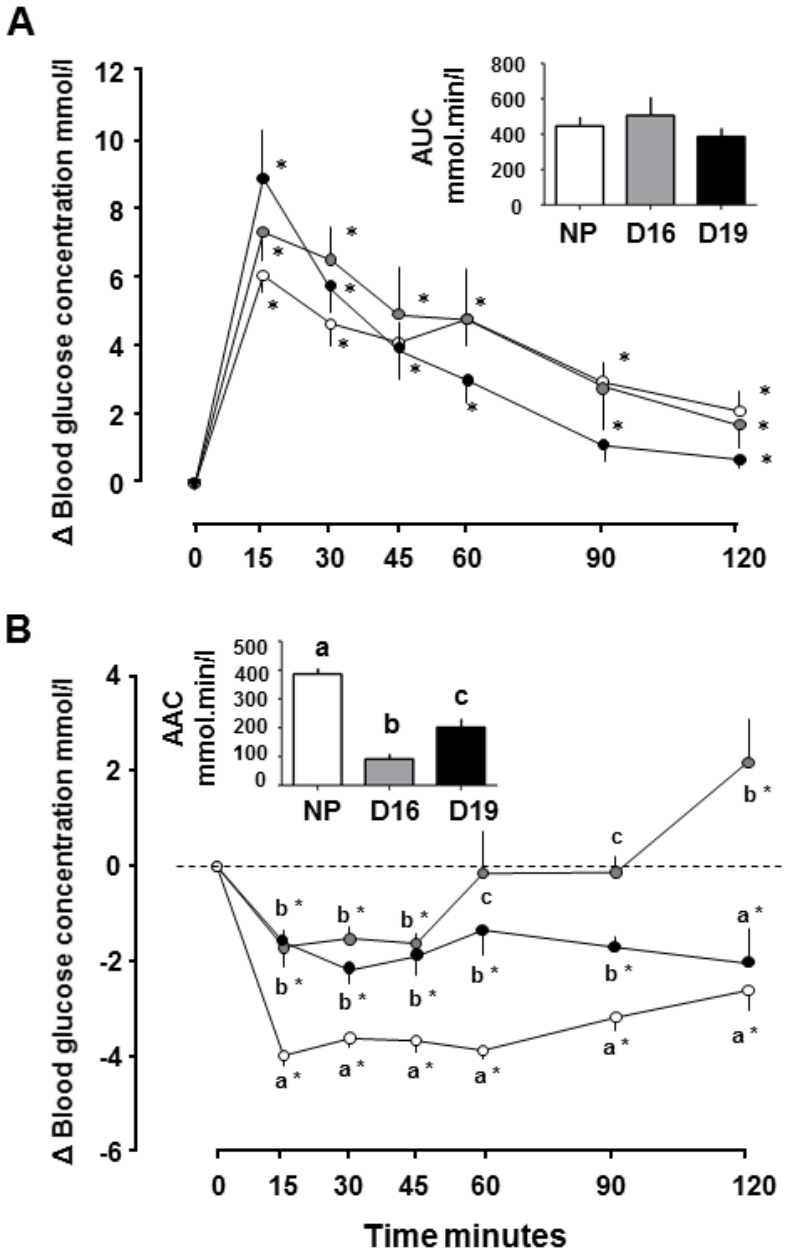
FIGURE LEGENDS

Figure 1. Mean ± SEM changes in blood glucose concentrations from basal pre-treatment values with time after intraperitoneal administration of (A) glucose (1 g/kg) and (B) insulin (0.25 U/kg) in non-pregnant (NP) females (n=8-11, open symbols) and pregnant dams at day (D)16 (n=6-9, grey symbols) and D19 (n=6-8, black symbols). Inserts show area (a) under the glucose curve (AUC) and (b) above the glucose curve (AAC). In (a) and (b), *significant increment above baseline values in each group ($p<0.05$, paired *t-test*). In (b) values at each sampling time with different letters indicate significant differences between groups ($p<0.05$, one way ANOVA).

Figure 2. Mean ± SEM concentrations of (A) blood glucose and (B) plasma insulin in the basal (stippled columns) and hyperinsulinaemic (Stripped columns) periods of the hyperinsulinaemic-euglycaemic clamp, rates of glucose infusion (GIR), appearance (R_a) and disappearance (R_d) measured directly (C) or derived indirectly as differences in rates (D) from tritiated glucose infusion and the HEC protocol (whole body and hepatic insulin sensitivity and endogenous glucose production, EGP) in non-pregnant (NP) females (n=7, white columns) and pregnant dams at D16 (n=5, grey columns) and D19 (n=6, black columns). In (B) *significant difference in concentration from the basal period ($p<0.01$, paired *t-test*) while, within each period of the clamp, values with different superscripts are significantly different from each other ($p<0.05$, one-way ANOVA). In (C) and (D) rates with different superscripts are significantly different from each other ($p<0.05$, one-way ANOVA). In (D) * significant difference between the values in the two states of the clamp ($P<0.02$, paired *t-test*).

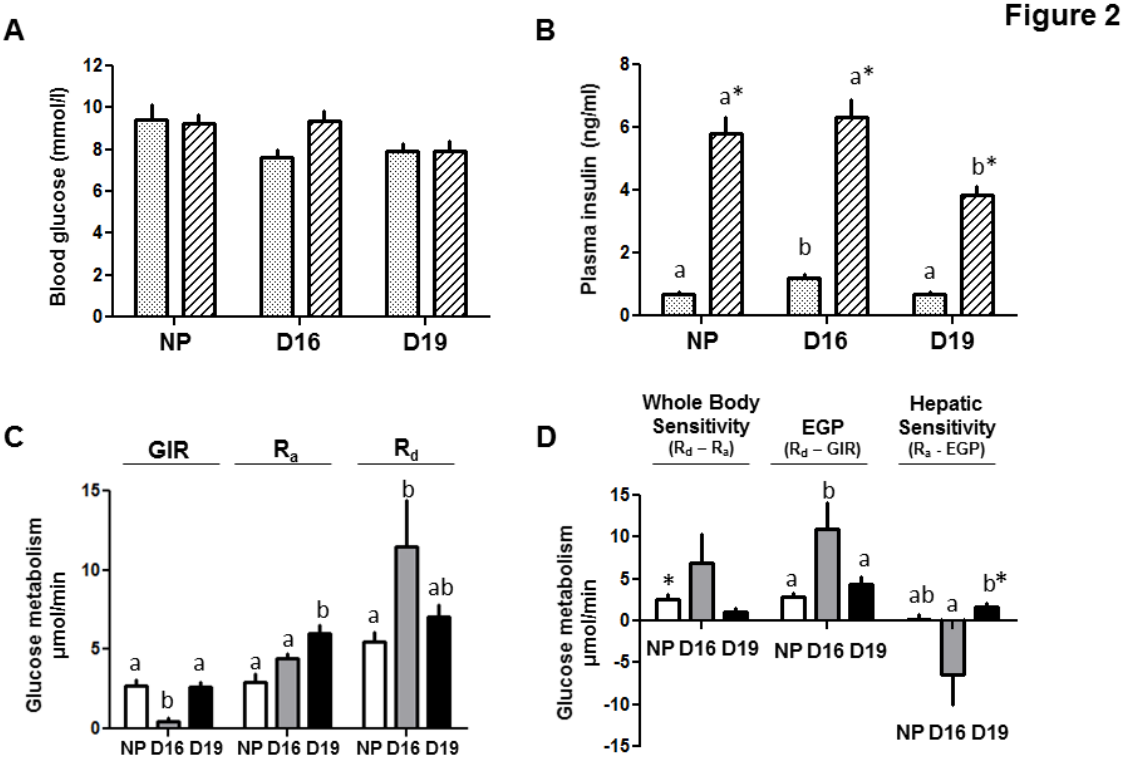
Figure 3. Mean ± SEM abundance of insulin signalling (a,b) and lipid metabolism (c,d) proteins in liver (A, C) and skeletal muscle (B, D) of non-pregnant (NP) females (n=10, white columns) and pregnant dams at D16 (n=5, grey columns) and D19 (n=5-6, black columns). Values with different superscripts are significantly different from each other ($p<0.05$, one way ANOVA). *significant difference between D16 and D19 dams ($p<0.05$, *t-test*).

Figure 1


A
Δ Blood glucose concentration mmol/l
12
10
8
6
4
2
0
0
15
30
45
60
90
120
AUC
mmol.min/l
800
600
400
200
0
NP
D16
D19
B
Δ Blood glucose concentration mmol/l
4
2
0
-2
-4
-6
AAC
mmol.min/l
500
400
300
200
100
0
a
b
c
NP
D16
D19
Time minutes

609

Figure 2


A
Blood glucose (mmol/l)
12
10
8
6
4
2
0
NP
D16
D19
B
Plasma insulin (ng/ml)
8
6
4
2
0
a
a*
b
a*
a
b*
NP
D16
D19
C
GIR
R_a
R_d
Glucose metabolism µmol/min
15
10
5
0
a
b
a
a
a
b
a
b
ab
NP D16 D19
NP D16 D19
NP D16 D19
D
Whole Body Sensitivity ($R_d - R_a$)
EGP (R_d – GIR)
Hepatic Sensitivity (R_a - EGP)
Glucose metabolism µmol/min
15
10
5
0
-5
-10
-15
*
a
b
a
ab
a
b*
NP D16 D19
NP D16 D19
NP D16 D19

610

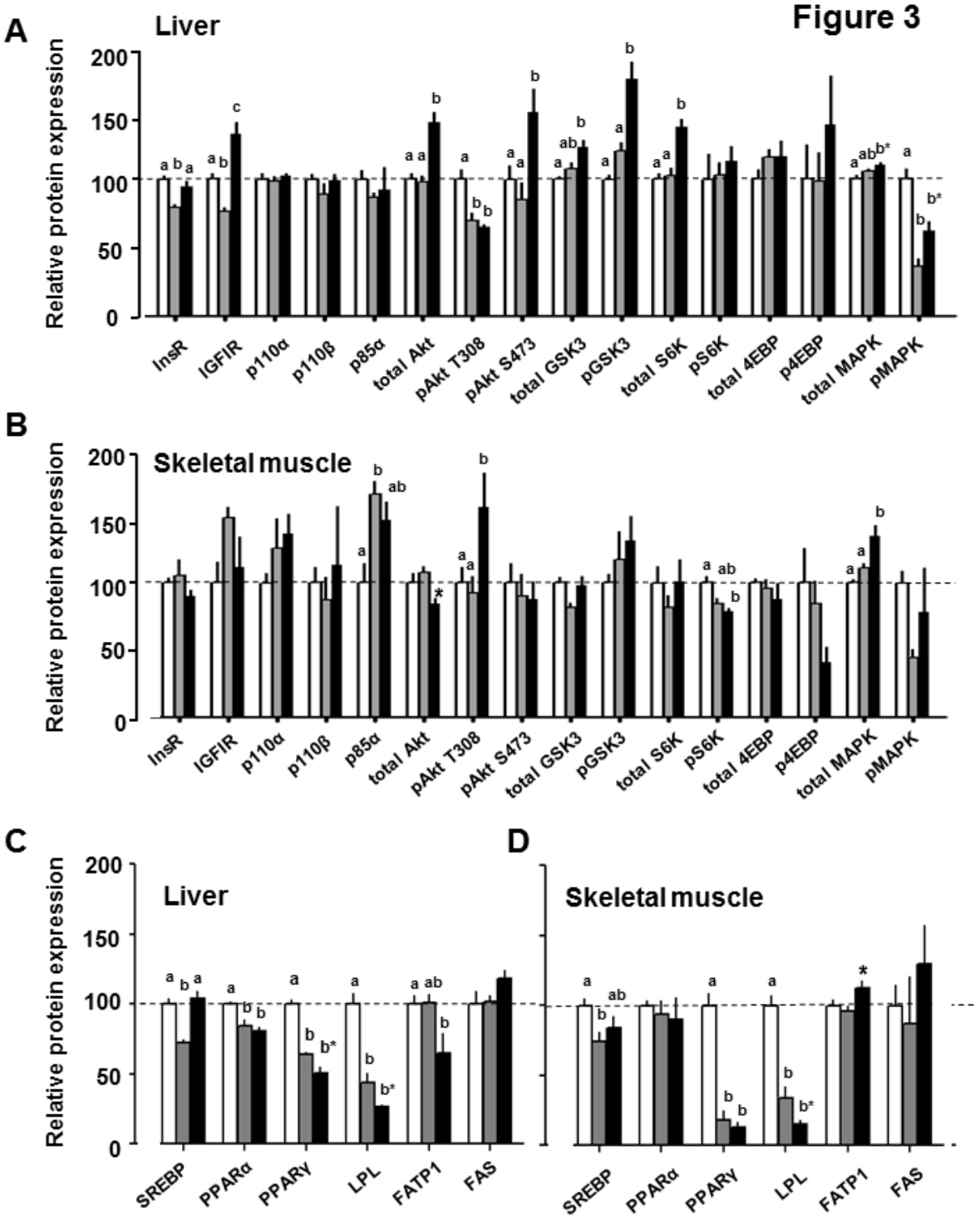


Figure 3
A
Liver
Relative protein expression
200
150
100
50
0
InsR
IGFIR
p110α
p110β
p85α
total Akt
pAkt T308
pAkt S473
total GSK3
pGSK3
total S6K
pS6K
total 4EBP
p4EBP
total MAPK
pMAPK
B
Skeletal muscle
Relative protein expression
C
Liver
Relative protein expression
SREBP
PPARα
PPARγ
LPL
FATP1
FAS
D
Skeletal muscle

Table 1. List of primary antibodies used in this study.

Primary antibody	Manufacturer	Dilution
Insulin receptor (InsR)	Santa Cruz, sc-711	1/400
Insulin like growth factor receptor type 1 IGF1R	Santa Cruz, sc-713	1/400
Catalytic subunits of phosphoinositide 3-kinase (p110α/β)	Cell Signalling, 4249, 3011	1/1000
Regulatory subunits of phosphoinositide 3-kinase (p85α)	Millipore, 06-195	1/5000 in 1% milk
Kinase Akt	Cell Signalling, 9272	1/1000
Phosphorylated (p)Akt Thr308	Cell Signalling, 9275	1/1000
pAkt Ser473	Cell Signalling, 9271	1/1000
Glycogen synthase kinase 3 (GSK3)	Cell Signalling, 9315	1/1000
pGSK3 Ser21/9	Cell Signalling, 9331	1/1000
Ribosomal S6 kinase (S6K)	Cell Signalling, 2708	1/1000
pS6K Thr 389	Cell Signalling, 9234	1/1000
Eukaryotic translocation initiation factor 4 binding protein (4EBP)	Cell Signalling, 9644	1/1000
p4EBP Ser65	Cell Signalling, 9451	1/1000
Mitogen activated protein kinase (MAPK)	Cell Signalling, 4695	1/1000
pMAPK Thr202/Tyr204	Cell Signalling, 4370	1/1000
Lipoprotein lipase (LPL)	Abcam, 21356	1/1000
Sterol regulatory element binding protein (SREBP)	Abcam, 3259	1/200
Fatty acid synthase (FAS)	Cell Signalling, 3180	1/1000
Peroxisome proliferator – activated receptor alpha (PPARα)	Abcam, 8934	1/1500
Peroxisome proliferator – activated receptor gamma (PPARγ)	Santa Cruz, sc-7273	1/200
Fatty acid transport protein 1 (FATP1)	Santa Cruz, sc-31955	1/400

Table 2. The effect of pregnancy and gestational age on the biometry and biochemical composition in non-pregnant females and pregnant dams at day (D)16 and D19 of pregnancy.

	Non-pregnant	Pregnant	
		D16	D19
Biometry			
Total body weight BW (g)	21.0 ± 0.4^{a} (20)	31.0 ± 1.1^{b} (13)	35.0 ± 1.2^{b} (16)
Hysterectomised weight HW (g)	-	24.0 ± 0.7^{b} (13)	23.0 ± 0.8^{b} (15)
Liver (mg)	1099 ± 28^{a} (10)	1903 ± 57^{b} (13)	1856 ± 76^{b} (16)
(%)†	5.5 ± 0.2^{a} (10)	7.9 ± 0.1^{b} (13)	8.1 ± 0.3^{b} (16)
Kidney (mg)	234.0 ± 6.5^{a} (10)	277 ± 12^{b} (11)	271 ± 11^{b} (15)
(%)†	1.20 ± 0.02 (10)	1.10 ± 0.02 (11)	1.20 ± 0.04 (15)
Heart (mg)	106.0 ± 6.5^{a} (10)	132 ± 5^{b} (13)	137 ± 7^{b} (16)
(%)†	0.50 ± 0.03 (10)	0.50 ± 0.01 (13)	0.60 ± 0.03 (16)
Retroperitoneal fat (mg)	30 ± 2^{a} (16)	73 ± 7^{b} (13)	63 ± 6^{b} (16)
(%)†	0.1 ± 2.0^{a} (16)	0.30 ± 0.03^{b} (13)	0.30 ± 0.03^{b} (16)
Gravid uterus (g)	-	7.3 ± 0.4 (13)	11.2 ± 0.7* (15)
Placenta average per litter (mg)	-	104.0 ± 1.8 (13)	88.0 ± 2.7* (16)
Fetus average per litter (mg)	-	427 ± 12 (13)	1204 ± 17* (16)
Litter size	-	7.8 ± 0.5 (13)	7.1 ± 0.4 (16)
Biochemical composition			
DEXA absolute fat mass (g)†	3.9 ± 0.2 (10)	4.4 ± 0.3 (7)	4.5 ± 0.2 (7)
DEXA fat mass (%)†	17.6 ± 0.9 (10)	17.8 ± 1.2 (7)	19.2 ± 0.5 (7)
DEXA absolute lean mass (g)†	18.2 ± 0.6^{a} (10)	20.3 ± 0.5^{b} (7)	18.7 ± 0.7^{ab} (10)
DEXA lean mass (%)†	82.4 ± 0.9 (10)	82.3 ± 1.2 (7)	80.9 ± 0.5 (10)
Hepatic glycogen (mg/g)	55 ± 3 (15)	49 ± 1 (6)	57 ± 6 (6)
Total hepatic glycogen (mg)	61 ± 4^{a} (15)	86 ± 3^{b} (6)	103 ± 11^{b} (6)
Hepatic fat content (%)	5.4 ± 0.3 (15)	5.1 ± 0.3 (6)	4.7 ± 0.3 (6)
Total hepatic fat content (mg)	59 ± 5^{a} (15)	91 ± 6^{b} (6)	86 ± 6^{b} (6)
Skeletal muscle fat content (%)§	4.0	15.4	20.4

Data are expressed as mean ± SEM with the number of dams/litters in parentheses. Values with different superscripts are significantly different from each other ($p<0.05$, one way ANOVA). *significant difference between D16 and D19 pregnant dams ($p<0.05$, *t-test*). † Organ weights and DEXA results were expressed as % of total body weight for NP females and of hysterectomised for pregnant dams. § skeletal muscle fat content was measured on samples pooled from 3-5 animals from each group.

Table 3. The effect of pregnancy and gestational age on blood glucose concentrations in the fed and fasted state, plasma concentrations of free fatty acids (FFA), triglycerides (TG), cholesterol, insulin, leptin and insulin-like growth factor (IGF)1 in non-pregnant females and dams at day (D)16 and D19 of pregnancy in the fed state.

		Non-pregnant	Pregnant	
			D16	D19
Glucose	Fed state (mmol/l)	9.3 ± 0.5 (10)	10.3 ± 0.3 (11)	9.4 ± 0.6 (16)
	Fasted 3.5h (mmol/l)	7.1 ± 0.4^{a} (8)	5.3 ± 0.3^{b} (6)	5.5 ± 0.3^{b} (6)
	Fasted 6h (mmol/l)	6.9 ± 0.3^{a} (11)	6.8 ± 0.2^{a}† (9)	5.2 ± 0.3^{b} (8)
FFA	Fed (μmol/l)	637 ± 49^{a} (10)	256 ± 42^{b} (9)	381 ± 41^{b}*(10)
TG	Fed (mmol/l)	1.0 ± 0.1 (10)	1.2 ± 0.2 (9)	1.0 ± 0.1 (10)
Cholesterol	Fed (mmol/l)	1.8 ± 0.1^{a} (10)	1.4 ± 0.1^{b} (9)	1.0 ± 0.1^{c} (10)
Insulin	Fed (μg/L)	0.19 ± 0.04^{a} (10)	1.3 ± 0.3^{b} (10)	0.6 ± 0.1^{ab} (10)
Leptin	Fed (pg/ml)	766 ± 71^{a} (10)	4058 ± 656^{b} (10)	3927 ± 709^{b} (10)
IGF1	Fed (pg/ml)	487 ± 68^{a} (10)	299 ± 23^{b} (12)	253 ± 20^{b} (12)

Data are expressed as mean±SEM with the number of animals shown in parentheses. Values with different superscripts are significantly different from each other ($p<0.05$, one way ANOVA). The asterisk indicates a significant difference between D16 and D19 of pregnancy ($p<0.05$, *t-test*). † indicates a significantly different values between animals fasted for 3.5h and 6h in the same group.

Table 4. Glucose utilisation by skeletal muscle and adipose tissue of the adult non-pregnant and pregnant mice and by the feto-placental tissues of the pregnant dams at day (D)16 and D19 of pregnancy.

p2DG content (nmol/mg)	Non-pregnant	Pregnant	
		D16	D19
Skeletal muscle	6.8 ± 2.4	4.2 ± 2.1	2.0 ± 0.6
White adipose tissue	3.2 ± 1.3	1.3 ± 0.3	1.3 ± 0.8
Placenta	-	4.8 ± 1.9	5.4 ± 1.2†
Fetus	-	3.5 ± 1.8	1.7 ± 0.3

Data are expressed as mean ± SEM for non-pregnant females (n=7) and pregnant dams/litters (D16 n=5, D19 n=6 with 2 conceptuses per litter). † Significantly different from the other tissues at this stage of pregnancy ($p<0.01$, one-way ANOVA).