Glucose Utilization Rates and Insulin Sensitivity In Vivo in Tissues of Virgin and Pregnant Rats

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SUMMARY

In vivo studies have shown that insulin resistance in late pregnancy results from a decreased sensitivity of liver and peripheral tissues. In the present study, measurements of the rates of glucose utilization by skeletal muscles (soleus, extensor digitorum longus, epitrochlearis, and diaphragm), white adipose tissue, and brain of virgin and 19-day pregnant rats were performed in the basal condition and during a euglycemic, hyperinsulinemic (400 μU/ml) clamp to quantify the partition of glucose utilization and to identify the tissues other than liver responsible for insulin resistance. Fetal and placental glucose utilization rates were also measured in pregnant rats. The fetal glucose utilization rate (22 mg/min/kg) was very high and was not stimulated by physiologic maternal hyperinsulinemia. By contrast, the placental glucose utilization rate (29 mg/min/kg) was increased by 30% during hyperinsulinemia. The glucose utilization rate of the conceptus represented 23% of the maternal glucose utilization rate in the basal state. Glucose utilization rates in the basal condition were not statistically altered by pregnancy in brain, skeletal muscles, and white adipose tissue. During hyperinsulinemia (400 μU/ml), glucose utilization rates in extensor digitorum longus, epitrochlearis, and white adipose tissue were 30–70% lower in pregnant than in virgin rats. Insulin sensitivity of glucose metabolism in all the tissues tested other than brain was 50% lower in pregnant than in virgin rats. We conclude that skeletal muscles and, to a smaller extent, adipose tissue are involved in the insulin resistance of late pregnancy. DIABETES 1986; 35:172–77.

In vivo studies using the glucose tolerance test,[1,2] the insulin tolerance test,[2,3] or the euglycemic, hyperinsulinemic clamp technique[4] have shown that a state of insulin resistance develops in late pregnancy in the rat. Insulin resistance results from a decreased sensitivity to insulin of liver and peripheral tissues with preservation of normal maximal responses to insulin.[2,4] However, the euglycemic, hyperinsulinemic clamp technique does not allow identification of the peripheral tissues responsible for the decreased sensitivity of glucose utilization to insulin in vivo. The aim of the present study was to determine the rate of glucose utilization by different maternal tissues, the placenta, and the fetus under basal conditions and during euglycemic clamps performed at physiologic, hyperinsulinemic levels to identify the tissues responsible for insulin resistance during late pregnancy.

MATERIALS AND METHODS

Animals. Female rats of the Wistar strain bred in our laboratory were used. They were housed at 24°C with light from 0700 to 1900 h. They had free access to water and chow pellets (65% carbohydrate, 11% fat, and 24% protein) until 0800 h on the day of the experiment. Pregnant rats (317 ± 22 g, N = 12) were studied on day 19 of pregnancy. Pregnant rats with less than eight fetuses were not included in this study. Age-matched virgin rats (266 ± 6 g, N = 12) were used as controls.

Rats were anesthetized with pentobarbital (30 mg/kg body wt, i.p.). One carotid artery was catheterized and a tracheotomy was performed. Body temperature was maintained at 38°C.

Fetal blood was sampled via axillary vessels after laparotomy of the mother as described previously.[5] Two fetuses were sampled in each mother.

Euglycemic, hyperinsulinemic clamp studies. These studies were performed as described previously.[4] In brief, basal blood glucose was determined using a Yellow Springs Instruments 23A glucose analyzer (Yellow Springs, Ohio). Pork insulin (Novo Industry, Copenhagen, Denmark) was then infused at a constant rate of 0.4 U/kg/h in a saphenous vein to reach a plasma insulin concentration of 400 μU/ml. Blood (30 μl) was sampled from the carotid artery every 5 min and

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TABLE 1
In vitro determination of the lumped constant of different tissues in virgin and pregnant rats

Tissues	Virgin rats (N)	Pregnant rats (N)
Soleus	0.97 ± 0.07 (16)	0.94 ± 0.06 (6) NS
Extensor digitorum longus	1.05 ± 0.05 (16)	1.01 ± 0.13 (6) NS
Epitrochlearis	0.86 ± 0.06 (8)	0.74 ± 0.02 (2) NS
Diaphragm	0.90 ± 0.1 (10)	0.85 ± 0.1 (6) NS
White adipose tissue	0.67 ± 0.03 (8)	0.63 ± 0.07 (6) NS
Placenta		0.77 ± 0.07 (6)

Data are means ± SEM.
NS: no significant difference between virgin and pregnant rats.
(N) is the number of observations.

the rates of exogenous glucose infusion were adjusted to maintain euglycemia. Within 40 min, a plateau for the exogenous glucose infusion rate was reached. The rates of insulin clearance (ml/min) were calculated by dividing the insulin perfusion rate (μU/min) by the plasma insulin level (μU/ml).

Measurement of the rate of glucose utilization by individual tissues. Measurement was performed under the conditions of constant arterial blood glucose levels by measuring the accumulation of 2-deoxy-[1-^{3}H]glucose-6-phosphate in the tissue after a bolus injection of 2-deoxy-[1-^{3}H]glucose as described previously.[6] In brief, 30 μCi of 2-deoxy-[1-^{3}H]glucose (20 Ci/mmol, CEA, Saclay, France) was injected in 200 μl of 0.9% NaCl through the saphenous vein. Arterial blood was sampled between 1 and 60 min in the basal state and between 1 and 30 min in the euglycemic, hyperinsulinemic clamp studies. Blood samples were deproteinized with $Ba(OH)_2$-$ZnSO_4$ and immediately centrifuged. An aliquot (100 μl) of the supernatant was used for the determination of glucose concentration by a glucose-oxidase method (Boehringer, Meylan, France) and another aliquot (100 μl) was used for the determination of 2-deoxy-[1-^{3}H]glucose using a liquid scintillation counter (Betamatic II, Kontron, France). Plasma insulin was determined by radioimmunoassay[4] on aliquots of the last blood samples.

After the last blood sampling, the rats were killed by cervical dislocation. Soleus, extensor digitorum longus, epitrochlearis and diaphragm muscles, pieces of periovarian white adipose tissue, and cerebral hemispheres were sampled by three operators in <5 min. The tissues were placed in 0.5 ml of 1 M NaOH. In pregnant rats, three placentas and three fetuses were placed, respectively, in 1 ml and 10 ml of 1 M NaOH. After digestion (1 h at 60°C) and neutralization with 1 M HCl, the content of 2-deoxy-[1-^{3}H]glucose-6-phosphate in each tissue was measured. Two aliquots of 200 μl were added to $HClO_4$ (4% wt/vol) and into 1 ml $Ba(OH)_2$-$ZnSO_4$ and centrifuged. The $HClO_4$ supernatants contained 2-deoxy-[1-^{3}H]glucose plus 2-deoxy-[1-^{3}H]glucose-6-phosphate. The $Ba(OH)_2$-$ZnSO_4$ supernatants contained only 2-deoxy-[1-^{3}H]glucose. The radioactive 2-deoxy-[1-^{3}H]glucose-6-phosphate contained in each tissue was determined by the difference between the radioactivity contained in $HClO_4$ and $Ba(OH)_2$-$ZnSO_4$ supernatants. The rate of glucose utilization by each tissue was calculated by dividing the disintegrations per minute (dpm) of 2-deoxy-[1-^{3}H]glucose-6-phosphate in the tissue by the calculated integral of the ratio of arterial blood 2-deoxy-[1-^{3}H]glucose to glucose concentration and by the discrimination factor for 2-deoxyglucose in each tissue (lumped constant). The results are expressed in milligrams per minute per kilogram (mg/min/kg). The rates of glucose clearance were calculated by dividing the rate of glucose utilization by the blood glucose concentration and are expressed in milliliters per minute per kilogram (ml/min/kg).

Determination of the lumped constant of individual tissues. Since the lumped constant is the ratio of the fractional extraction rate of 2-deoxyglucose to glucose, it can be measured either in vivo when the vessels supplying and draining a tissue can be easily sampled,[7] or in vitro when arteriovenous sampling is not possible.[6]

For the brain, the lumped constant (0.51) determined by Sokoloff et al.[7] for the anesthetized rat was used. The lumped constants of muscles, adipose tissue, and placenta were determined in vitro as follows: soleus, extensor digitorum longus, epitrochlearis, pieces of adipose tissue, placenta, and hemi-diaphragms were incubated in 4 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 1.5% bovine serum albumin (Sigma, St. Louis, Missouri), 5 mmol/L glucose, 0.5 μCi/ml [5-^{3}H]glucose (New England Nuclear, Boston, Massachusetts), 0.5 μCi/ml 2-deoxy-[1-^{14}C]glucose (CEA). At time 0 (t_0) an aliquot of the incubation medium was sampled. Each flask was gassed with O_2/CO_2 (19/1) for 5 min and then sealed with a rubber stopper. After an incubation of 1 h at 37°C under constant shaking, another aliquot of the incubation medium was sampled (t_{60}). The samples were evaporated to dryness to eliminate the tritiated water, the only radioactive metabolite formed from [5-^{3}H]glucose and 2-deoxy-[1-^{14}C]glucose and released in the medium, and then counted in a liquid scintillation spectrometer with stored

TABLE 2
Blood glucose and plasma insulin in virgin and pregnant rats and their fetuses under basal conditions and during hyperinsulinemic euglycemic clamp (the glucose infusion rates to maintain euglycemia and the insulin clearance rates during the clamp are also indicated)

Animals	Body wt (g)	Plasma insulin (μU/ml)		Blood glucose (mg/ml)		Glucose infusion rate (mg/min)	Insulin clearance rate (ml/min)
		Basal	Clamp	Basal	Clamp		
Virgin	226 ± 6	108 ± 16	350 ± 50	1.13 ± 0.02	1.06 ± 0.03	5.0 ± 0.4	4.7 ± 0.5
Pregnant	317 ± 22†	102 ± 7 NS	450 ± 30 NS	0.81 ± 0.01‡	0.77 ± 0.02‡	4.0 ± 0.2*	5.0 ± 0.3 NS
Fetus	2.22 ± 0.06	103 ± 13	116 ± 17	0.35 ± 0.02	0.41 ± 0.03		

Data are means ± SEM of six experiments.
P-values are shown by *P < 0.05, †P < 0.01, and ‡P < 0.001.
NS: no significant difference between virgin and pregnant rats.

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correction curves for quenching and spillover of ^{14}C in the ^{3}H channel to determine the content in [5-^{3}H] glucose and 2-deoxy-[1-^{14}C]glucose.

The lumped constants of the placenta and the diaphragm were calculated as follows:

$$LC = \frac{\text{2-deoxy-[1-}^{14}\text{C]glucose }(t_0) - \text{2-deoxy-[1-}^{14}\text{C]glucose }(t_{60})}{\text{2-deoxy-[1-}^{14}\text{C]glucose }(t_0)} = \frac{\text{[5-}^{3}\text{H]glucose }(t_0) - \text{[5-}^{3}\text{H]glucose }(t_{60})}{\text{[5-}^{3}\text{H]glucose }(t_0)}$$

$$LC = \frac{\text{fractional extraction of 2-deoxy-[1-}^{14}\text{C]glucose}}{\text{fractional extraction of [5-}^{3}\text{H]glucose}}$$

The lumped constant for the conceptus was measured in vivo as follows: a solution of 1 μCi/ml of [3-^{3}H]glucose and of 1 μCi/ml of 2-deoxy-[1-^{14}C]glucose in 0.9% NaCl was infused at a constant rate of 20 μl/min in a saphenous vein. Arterial blood (100 μl) was sampled at 60, 65, and 70 min to assess the constancy of the specific activity of the two tracers. After laparotomy, blood (100 μl) was sampled in the uterine vein at 65 min. The samples were deproteinized in $Ba(OH)_2$-$ZnSO_4$ and the supernatants were treated as described above to determine the content of 2-deoxy-[1-^{14}C]glucose and of [3-^{3}H]glucose in arterial and venous blood. The lumped constant for the conceptus (fetuses and placentas) was calculated by measuring the fractional extraction of the two tracers by the conceptus and was calculated by the following formula:

$$LC = \frac{\text{2-deoxy-[1-}^{14}\text{C]glucose}_{(\text{artery})} - \text{2-deoxy-[1-}^{14}\text{C]glucose}_{(\text{uterine vein})}}{\text{2-deoxy-[1-}^{14}\text{C]glucose}_{(\text{artery})}} \Big/ \frac{\text{[3-}^{3}\text{H]glucose}_{(\text{artery})} - \text{[3-}^{3}\text{H]glucose}_{(\text{uterine vein})}}{\text{[3-}^{3}\text{H]glucose}_{(\text{artery})}}$$

Statistics. Results are presented as means ± SEM of 5–6 determinations. Statistically significant differences were assessed using Student's *t*-test.

RESULTS

Determination of the lumped constant of skeletal muscles, diaphragm, adipose tissue, placenta, and conceptus. The lumped constants of four different muscles and adipose tissue determined in vitro were similar in virgin and pregnant rats (Table 1). The lumped constant of the placenta determined in vitro was 0.72 (Table 1); the lumped constant of the conceptus in vivo was 0.83 ± 0.02 (N = 5). As 85% of the conceptus mass was represented by the fetal mass, the lumped constant used to measure the rate of glucose utilization by the fetus was calculated to be 0.85.

Rates of glucose utilization in vivo by individual tissues of virgin and pregnant rats under basal conditions. Blood glucose concentrations in the basal state were lower in pregnant rats than in virgin rats (Table 2). Plasma insulin concentrations in the basal state were similar in pregnant and in virgin rats (Table 2). Blood glucose concentrations in the fetus were lower than in the mother, whereas fetal plasma insulin concentrations were similar (Table 2). By using the lumped constants determined in vitro (Table 1), the rates of glucose utilization by various tissues were determined in the basal state. The rates of basal glucose utilization by the brain, the diaphragm, the soleus muscle, the epitrochlearis muscle, and the periovarian white adipose tissue were not significantly altered during pregnancy (Figure 1 and Table 3). In contrast, the rate of basal glucose utilization in the extensor digitorum longus muscle was significantly decreased ($P < 0.01$) in pregnant compared with virgin rats: 3.0 ± 0.4 versus 5.0 ± 0.4 mg/min/kg (Figure 1 and Table 3). The rates of glucose utilization by the placenta (29 ± 1 mg/min/kg) and by the fetus (22 ± 1 mg/min/kg) were very high (Figure 2) and similar to the rates of glucose utilization by the maternal brain and diaphragm (Figure 1 and Table 3). In the basal state, the rates of glucose clearance of muscles and adipose tissue were similar in virgin and pregnant rats (Table 3).

Rates of glucose utilization in vivo by individual tissues of virgin and pregnant rats during the euglycemic, hyperinsulinemic clamp. Blood glucose concentrations were lower in pregnant than in virgin rats; thus, steady-state blood glucose concentrations during the hyperinsulinemic clamp were lower in pregnant than in virgin rats (Table 2). The glucose infusion rate necessary to maintain euglycemia in pregnant rats was lower ($P < 0.05$) than in virgin rats (Table 2). Steady-state plasma insulin concentrations reached during the clamp were not statistically different in virgin and in pregnant rats (Table 2), and insulin clearance rates were identical in virgin and pregnant rats (Table 2). Blood glucose and plasma insulin concentrations in the fetuses were not affected by the hyperinsulinemic clamp, and remained at basal values (Table 2).

Because the blood glucose concentration was clamped at a lower level in pregnant rats than in virgin rats, and because glucose utilization is partly dependent on glycemia, the lower rate of glucose utilization observed in pregnant rats at high plasma insulin concentrations could result from relative hypoglycemia. Thus, we have also expressed our data as glucose clearance rates (Table 3).

The rates of glucose utilization by the brain of virgin or

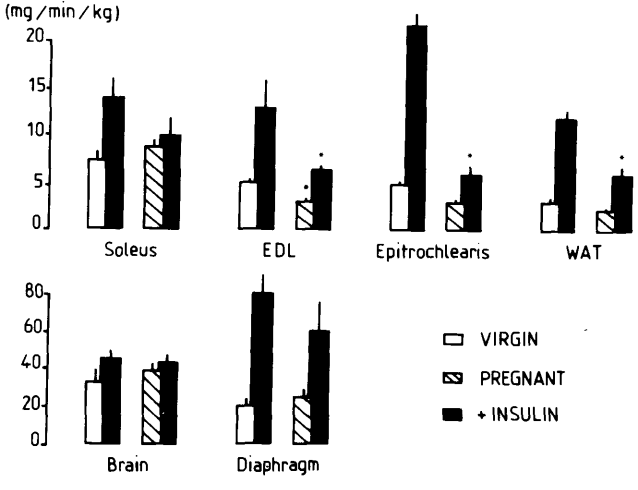

FIGURE 1. In vivo glucose utilization rates in soleus, extensor digitorum longus (EDL), and epitrochlearis muscles, and periovarian white adipose tissue (WAT), brain, and diaphragm in virgin and 19-day pregnant rats in the basal state and during euglycemic, hyperinsulinemic clamp. Results are presented as means ± SEM for six determinations. *Difference significant at $P < 0.05$ when compared with the respective virgin controls.

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TABLE 3
Glucose utilization and glucose clearance rates under basal conditions and during euglycemic, hyperinsulinemic clamp in virgin and 19-day pregnant rats

		Diaphragm	Soleus	Extensor digitorum longus	Epitrochlearis	White adipose tissue
Glucose utilization rates (mg/min/kg)						
Basal	Virgin	20 ± 4	7.4 ± 1	5 ± 0.4	5.3 ± 1	3.3 ± 0.7
	Pregnant	25 ± 3	8.8 ± 0.8	3 ± 0.4	3.0 ± 0.1	2.4 ± 0.1
Clamp	Virgin	80 ± 10	14 ± 2	13 ± 3	22 ± 2	12 ± 2.7
	Pregnant	60 ± 15	10 ± 2	6.5 ± 0.6*	7.7 ± 1†	6 ± 1*
Glucose clearance rates (ml/min/kg)						
Basal	Virgin	18 ± 4	7 ± 1	4.5 ± 0.3	4.7 ± 1	2.9 ± 0.6
	Pregnant	30 ± 3	11 ± 1	3.7 ± 0.4	3.7 ± 0.1	2.9 ± 0.1
Clamp	Virgin	71 ± 16	13 ± 2	12 ± 2	19 ± 2	11 ± 2
	Pregnant	74 ± 18	12 ± 1	8 ± 1	9.5 ± 2*	7.4 ± 1
Percent increase of glucose utilization or clearance rate over basal value during hyperinsulinemia						
	Virgin	300	89	160	315	263
	Pregnant	140	14	116	157	150

Data are means ± SEM of six experiments.
P-values are shown by *$P < 0.05$ and †$P < 0.001$ between virgin and pregnant tissues.
Percent increases over basal values are calculated from means.

pregnant rats were not significantly modified during the euglycemic, hyperinsulinemic clamp (Figure 1 and Table 3). In contrast, the rate of glucose utilization in muscles and adipose tissue of virgin and pregnant rats was significantly increased by hyperinsulinemia. However, the rate of glucose utilization at 400 μU/ml plasma insulin was significantly less in pregnant rats when compared with virgin rats in extensor digitorum longus muscle ($P < 0.05$), epitrochlearis muscle ($P < 0.001$), and white adipose tissue ($P < 0.01$), whereas it was not statistically different in diaphragm and soleus muscles. Furthermore, in all the tissues studied except the brain, the extent of the stimulation of glucose utilization or glucose clearance by hyperinsulinemia was much lower in muscles and adipose tissue of pregnant than of virgin rats (Table 3).

As expected, the rate of glucose utilization by the fetuses was not altered by maternal hyperinsulinemia (Figure 2). In contrast, the rate of glucose utilization by the placenta was increased by 30% in the presence of maternal hyperinsulinemia (Figure 2).

DISCUSSION

Radioactive 2-deoxyglucose has been used previously to measure glucose utilization in vivo by the brain[7] and various tissues.[6,8,9] The correction factor for the discrimination of 2-deoxyglucose in glucose metabolic pathways, necessary to measure the rate of glucose utilization by individual tissues, has been determined previously for the brain,[7] skeletal muscles, and adipose tissue.[6] The present study extends the use of this technique to the measurement of glucose utilization in vivo by the diaphragm, fetus, and placenta. Since glucose-6-phosphatase activity is very low in the liver and kidney of the fetus,[10] in the placenta,[11,12] as well as in the diaphragm,[13] skeletal muscles, and adipose tissue (see ref. 6) of the rat, the hydrolysis of 2-deoxyglucose-6-phosphate back to 2-deoxyglucose is considered to be negligible.

Rates of glucose utilization by the fetus and the placenta. In the basal state, the rate of glucose utilization by the fetus (22 mg/min/kg) is twofold higher than the rate of glucose utilization by the 19-day pregnant rat (9 mg/min/kg).[14] Since the rate of glucose utilization by the placenta is 29 mg/min/kg, it is possible to calculate glucose utilization by the rat conceptus. For a conceptus of 10 fetus and 10 placentas, the rate of glucose utilization is 0.7 mg/min, i.e., 23% of the maternal glucose utilization rate. This value is comparable with values found in other species: near the term, the rate of glucose utilization of the gravid uterus represents 30–36% of the maternal glucose utilization in the sheep,[15] the rabbit,[16] and the pig.[17]

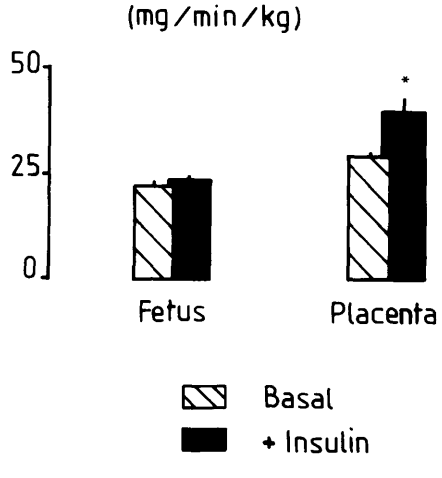

FIGURE 2. In vivo glucose utilization rates in fetus and placenta of 19-day pregnant rats in the basal condition and during euglycemic, hyperinsulinemic clamp. Results are presented as means ± SEM for six determinations. *Difference significant at $P < 0.05$.

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It is of interest to note that the rate of glucose utilization by the conceptus of small mammals such as the rat (present study), rabbit,[16] and guinea pig[18] is in the range of 23–42 mg/min/kg, whereas it is lower (7–15 mg/min/kg) for the conceptus of large mammals such as the pig,[17] sheep,[15] cow,[20] and mare.[20]

Maternal hyperinsulinemia does not affect the rate of glucose utilization by the fetus. This is not surprising, since fetal plasma insulin and fetal blood glucose concentrations do not change during maternal hyperinsulinemia (Table 2). Similar results have been observed in the fetal lamb.[19] In contrast, glucose utilization by the placenta is increased by 30% during physiologic hyperinsulinemia in the pregnant rat. This is not totally unexpected, since insulin receptors have been found in rat placenta.[21] However, recent studies in the sheep have shown that the rate of placental glucose utilization was not increased by maternal hyperinsulinemia.[19] Since insulin receptors are also present in the sheep placenta,[22] the reasons for this discrepancy are unclear but could be due to the higher plasma insulin levels reached in the present study (400 μU/ml) than in the study in sheep (280 μU/ml).

Rates of glucose utilization in peripheral tissues. As previously described,[6,8] the rate of glucose utilization by the brain is neither affected by physiologic hyperinsulinemia nor by pregnancy (Figure 1).

In late-pregnant rats, an insulin resistance has been found in peripheral tissues.[4] However, the tissues responsible for insulin resistance and the mechanism involved remain largely unknown. In vitro studies have given conflicting results concerning the two principal insulin-sensitive tissues: skeletal muscle and adipose tissue. It has been reported that the stimulation of glucose uptake by insulin in perfused hindlimb was 40% lower in late-pregnant rats than in virgin rats,[23] whereas we have not observed any insulin resistance in isolated strips of soleus muscle.[24] A decreased insulin sensitivity and responsiveness has been reported in isolated periovarian adipocytes of late-pregnant rats,[25,26] whereas no insulin resistance has been observed in pieces of periovarian adipose tissue [27,28] or in periovarian adipocytes from late-pregnant rats.[29]

In vivo, the basal rates of glucose utilization by skeletal muscles and adipose tissue are similar in virgin and pregnant rats (Figure 1) and close to those found previously in adult female rats.[6] The rate of glucose utilization by the diaphragm is high compared with other skeletal muscles. This is probably due to the fact that, among the muscles studied, diaphragm is the only one continuously working during the experiment. Indeed, muscle contraction has been shown to stimulate glucose utilization.[30]

Physiologic hyperinsulinemia induces an increase in the rate of glucose utilization in these tissues. Nevertheless, glucose utilization rates after hyperinsulinemia are 50–70% lower in the extensor digitorum longus and epitrochlearis muscles, as well as in periovarian adipose tissue of the pregnant rats compared with virgin rats. This cannot be totally explained by the 25% lower blood glucose concentration found in pregnant rats (Tables 2 and 3). In soleus and diaphragm muscles, insulin-stimulated glucose utilization is similar in virgin and pregnant animals. It must be stated that these two red muscles have to provide a higher work load during pregnancy, since (1) soleus muscle is a postural muscle that has to adapt to the increased body weight of pregnant rats and (2) pregnancy is associated with hyperventilation and thus the work load of the diaphragm increases. This higher work load might overcome the insulin resistance, since it has been shown that exercise and training increases the sensitivity of muscles to insulin.[30] When expressed as glucose clearance, to take into account the difference in blood glucose levels, the difference between virgin and pregnant tissues is minimized.

Nevertheless, the insulin sensitivity of the tissues expressed in percent increase over basal value of glucose utilization (or clearance) of all the tissues tested is reduced by about 50% during pregnancy even in diaphragm and soleus muscle. The present study thus demonstrates that in vivo skeletal muscles and adipose tissue are involved in the insulin resistance of pregnancy. As the mass of skeletal muscle represents 36–40% of the body weight and adipose tissue only 10–13%,[31–33] the absolute amount of glucose spared is quantitatively more important in muscles than in adipose tissue.

In summary, this study shows that (1) the rate of glucose utilization by the rat conceptus represents 23% of the maternal glucose utilization, and (2) insulin stimulation of glucose metabolism is reduced by 50% in skeletal muscles and adipose tissue of pregnant rats. However, the mechanisms involved in insulin resistance in these tissues remain to be determined.

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