Defects in insulin signal transduction in liver and muscle of pregnant rats

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Summary Pregnancy is known to induce insulin resistance, but the exact molecular mechanism involved is unknown. In the present study, we have examined the levels and phosphorylation state of the insulin receptor and of insulin receptor substrate 1 (IRS-1), as well as the association between IRS-1 and phosphatidylinositol 3-kinase (PI 3-kinase) in the liver and muscle of pregnant rats (day 20 of gestation) by immunoprecipitation and immunoblotting with anti-insulin receptor, anti-IRS-1, anti-PI 3-kinase and antiphosphotyrosine antibodies. There were no changes in the insulin receptor concentration in the liver and muscle of pregnant rats. However, insulin stimulation of receptor autophosphorylation, as determined by immunoblotting with antiphosphotyrosine antibody, was reduced by $30 \pm 6\%$ ($p < 0.02$) in muscle and $36 \pm 5\%$ ($p < 0.01$) in liver at day 20 of gestation. IRS-1 protein levels decreased by $45 \pm 6\%$

($p < 0.002$) in liver and by $56 \pm 9\%$ ($p < 0.002$) in muscle of pregnant rats. In samples previously immunoprecipitated with anti-IRS-1 antibody and blotted with antiphosphotyrosine antibody, the insulin-stimulated IRS-1 phosphorylation levels in the muscle and liver of pregnant rats decreased by $70 \pm 9\%$ ($p < 0.01$) and $75 \pm 8\%$ ($p < 0.01$), respectively. The insulin-stimulated IRS-1 association with PI 3-kinase decreased by $81 \pm 6\%$ in muscle ($p < 0.01$) and $79 \pm 11\%$ ($p < 0.01$) in the liver during pregnancy. These data suggest that changes in the early steps of insulin signal transduction may have a role in the insulin resistance observed in pregnancy. [Diabetologia (1997) 40: 179–186]

Keywords Insulin resistance, pregnancy, insulin action, insulin receptor, insulin receptor substrate, PI 3-kinase.

Insulin resistance during pregnancy has been reported both in women and in experimental animals [1–7], but the mechanism of this resistance remains unclear. *In vivo* studies have shown that pregnant rats become progressively resistant to insulin after day 16 of gestation [5]. In these animals, the insulin resistance of skeletal muscle has been clearly

demonstrated *in vitro*, while the insulin resistance *in vivo* in peripheral tissues and liver has been substantiated in studies using the euglycaemic-hyperinsulinaemic clamp technique [6, 7].

Insulin initiates its metabolic and growth-promoting effects by binding to the α subunit of its tetrameric receptor, thereby activating the kinase in the β subunit [8]. This interaction catalyses the intramolecular autophosphorylation of specific tyrosine residues of the β subunit which further enhances the tyrosine kinase activity of the receptor toward other protein substrates [8]. In most cells, this primary event leads to the subsequent tyrosyl phosphorylation of a cytoplasmic protein with an apparent molecular weight of 160–185 kDa, called insulin receptor substrate 1 (IRS-1) [9–11]. Considerable evidence indicates that insulin receptor tyrosine

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Abbreviations: IRS-1, Insulin receptor substrate 1; PI 3-kinase, phosphatidylinositol 3-kinase; SDS-PAGE, sodium dodecyl-sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; BSA, bovine serum albumin.

kinase and the associated IRS-1 phosphorylation are essential for many of the biological effects of insulin [12–16]. IRS-1 is also phosphorylated in response to insulin-like growth factor-1, interleukin-4 and angiotensin II [8–11, 17, 18] and, following its phosphorylation, it can associate with proteins containing Src homology 2 (SH2) domains through specific tyrosyl phosphorylation sites [19, 20]. In cells in culture and in animal tissues, phosphorylated IRS-1 associates with the lipid metabolizing enzyme phosphatidylinositol 3-kinase (PI 3-kinase) thereby activating the enzyme [21–23]. The insulin receptor, IRS-1 and PI 3-kinase therefore represent three of the earliest steps in insulin action, and each of these can be demonstrated and regulated in two of the main target tissues for the metabolic actions of insulin *in vivo*, namely liver and muscle [24–26].

The role of the phosphorylation of IRS-1, the endogenous substrate for the insulin receptor, and its association with PI 3-kinase in the insulin resistance of pregnancy are not known. In the present study, we have investigated the phosphorylation state of the insulin receptor and IRS-1, as well as the association of the latter with PI 3-kinase in the liver and muscle of pregnant rats stimulated with insulin.

Materials and methods

Materials. Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were from Bio-Rad (Richmond, Calif., USA). Tris, phenylmethylsulfonylfluoride (PMSF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol and bovine serum albumin (BSA, fraction V) were from Sigma Chemical Co. (St. Louis, Mo., USA). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), [¹²⁵I]protein A was from Amersham (Aylesbury, UK) and nitrocellulose paper (BA85, 0.2 mm) was from Schleicher & Schuell (Keene, N.H., USA). Sodium amobarbital (Amytal) and human recombinant insulin (Humulin R) were from Eli Lilly Co. (Indianapolis, Ind., USA). Monoclonal antiphosphotyrosine antibody and anti-PI 3-kinase antiserum was from UBI (Lake Placid, N.Y., USA). Anti-insulin receptor antibody and anti-IRS-1 antibodies were raised in rabbits using a synthetic peptide derived from the amino acid sequence to the C-terminus of these proteins and were kindly provided by Drs. C. R. Kahn and M. F. White (Joslin Diabetes Center, Boston, Mass., USA).

Method

Animals and tissue extracts. Eight-week-old female Wistar rats were mated. One group of 24 pregnant rats (day 20 of gestation) and age-matched non-pregnant rats were allowed access to standard rodent chow and water *ad libitum*. The animals were studied after 7–8 h of fasting. The studies were performed in parallel using control (virgin) and pregnant rats in each pair. All experiments involving animals were approved by the University of Campinas Ethical Committee.

The rats were anaesthetized with sodium amobarbital (15 mg/kg body weight, i. p.) and used as soon as anaesthesia

was assured by loss of the pedal and corneal reflexes. The abdominal cavity was opened, the portal vein exposed, and 0.5 ml of normal saline (0.9% NaCl), with or without 10⁻⁵ mol/l insulin, was injected. Thirty seconds later, the livers were removed, minced coarsely and homogenized immediately in approximately 10 volumes of solubilization buffer A in a water bath maintained at 100 °C using a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, N. Y., USA) operated at maximum speed (setting 10) for 30 s. The solubilization buffer A was composed of 1% SDS, 100 mmol/l Tris (pH 7.4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, and 10 mmol/l sodium vanadate. The homogenate was then boiled for a further 10 min and then cooled in an ice bath for 40 min.

Approximately 90 s after insulin injection, hindlimb muscles were excised and homogenized with a Polytron in 6 volumes of homogenization buffer A at 100 °C. The extracts were centrifuged at 55 000 rev/min at 4 °C in a Beckman 70.1 Ti rotor (Palo Alto, Calif., USA) for 60 min to remove insoluble material, and the supernatant was used as the sample. In some experiments, the tissues were extracted at 4 °C with homogenization buffer B (same as buffer A except that 1% Triton X-100 replaced 1% SDS and 2 mmol/l PMSF and 0.1 mg of aprotinin/ml were added), and after centrifugation the supernatant was used for immunoprecipitation with anti-IRS-1 antibody and Protein A-Sepharose 6 MB or protein A/G plus (Santa Cruz Technology, Santa Cruz, Calif., USA).

The insulin dose employed in this study was based on previous work in which the amount of insulin required to achieve a significant signal was determined [23, 24]. Thus, we know that a bolus injection of 10⁻⁵ mol/l insulin via the portal vein will lead to a transient rise in the peripheral concentration 5–10 times the postprandial levels at 90 s. The very high levels of insulin reached suggest that different levels of insulin cannot explain some of the variation observed.

Protein analysis by immunoblotting. The samples were treated with Laemmli sample buffer [27] containing 100 mmol/l DTT and heated in a boiling water bath for 4 min. For total extracts, similar sized aliquots (150 µg of protein) were subjected to SDS-PAGE (6% tris-acrylamide) in a Bio-Rad miniature lab gel apparatus (Mini-Protein, Bio-Rad). The electrotransfer of proteins from the gel to nitrocellulose was performed for 2 h at 120 V (constant) using a Bio-Rad miniature transfer apparatus (Mini-Protein) as described by Towbin et al. [28] but with 0.02% SDS added to the transfer buffer to enhance the elution of high molecular mass proteins. Non-specific protein binding to the nitrocellulose was reduced by preincubating the filter overnight at 4 °C in blocking buffer (3% BSA, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20). The prestained M_r standards were myosin (205 kDa), β galactosidase (116 kDa), (80 kDa), and ovalbumin (49.5 kDa).

The nitrocellulose blot was incubated for 4 h at 22 °C with antiphosphotyrosine antibody, anti-insulin receptor antibody, anti-IRS-1 antibody, anti-PI 3-kinase antibody or anti-SHPTP2 antibody (Santa Cruz Technology) diluted in blocking buffer; then washed for 60 min in blocking buffer without BSA. The blots were then incubated with 2 µCi of [¹²⁵I]protein A (30 µCi/µg) in 10 ml of blocking buffer for 1 h at 22 °C and washed again as described above for 2 h. [¹²⁵I]Protein A bound to the antibodies was detected by autoradiography using pre-flashed Kodak XAR film (Eastman Kodak, Rochester, N. Y., USA) with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, D. E., USA) at -70 °C for 12–48 h. Band intensities were quantitated by optical densitometry (model GS 300; Hoefer Scientific Instruments, San Francisco, Calif, USA) of the developed autoradiogram.

Other assays. Protein determination was performed by the Bradford dye-binding method [29] using the Bio-Rad reagent and BSA as the standard. The plasma glucose concentrations were measured by the oxidase method. Plasma insulin levels were measured by double-antibody radiimmunoassay (Diagnostic Products Corp., Los Angeles, Calif., USA). Some animals in both groups were also submitted to an insulin tolerance test (0.5 ml of 10^{-5} mol/l insulin, i.v.) and samples for plasma glucose determination were collected at 0 (basal), 4, 8, 12 and 16 min after insulin injection. The rate constant for plasma glucose disappearance (Kitt) was calculated using the formula $0.693/t_{1/2}$. The plasma glucose $t_{1/2}$ was calculated from the slope of the least square analysis of the plasma glucose concentrations during the linear decay phase.

Statistical analysis. Experiments were always performed by analysing samples from the pregnant rats in parallel with a control group. For comparisons, Student's unpaired *t*-test was used. The level of significance was set at $p < 0.05$.

Results

Animal characteristics. Fasting plasma glucose concentrations were lower in pregnant than in virgin rats (5.16 ± 0.27 vs 6.16 ± 0.22 mmol/l, $p < 0.02$) and fasting serum insulin concentrations were higher in pregnant rats than in virgin rats (2.78 ± 0.28 vs 1.94 ± 0.14 pmol/l, $p < 0.02$). Thus, the serum insulin concentration was inappropriately high for the low plasma glucose concentrations in pregnant rats [insulin-to-glucose ratio (pmol/mmol) 53.8 in pregnant rats vs 31.4 in virgin rats]. In order to demonstrate that the pregnant rats were insulin resistant, the animals were submitted to an insulin tolerance test. The glucose disappearance rate during this test was lower in pregnant than in virgin rats (pregnant 2.24 ± 0.84 vs virgin 4.84 ± 0.3 %/min), thus demonstrating a state of insulin resistance.

The effect of pregnancy on insulin receptor and IRS-1 phosphorylation in rat muscle. Figure 1A illustrates the protocol for assessing insulin receptor signalling in the intact rat. Control and pregnant rats were injected with saline or insulin and after 90 s hindlimb skeletal muscle was removed and homogenized as described in Methods. The extracts were then submitted to SDS-PAGE under reducing conditions and were blotted onto nitrocellulose membranes. These blots were subsequently analysed using anti-insulin receptor, anti-IRS-1, anti-PI 3-kinase and antiphosphotyrosine antibodies.

In pregnant rats, there were no changes in the insulin receptor levels in muscle as shown by immunoblotting with an antibody to the COOH terminus of the insulin receptor (Figs. 1B and 2A). In contrast, the levels of IRS-1 protein in muscle detected by immunoblotting were reduced by 56 ± 0.9 % ($p < 0.002$) in pregnant rats (Figs. 1C and 2A).

Following *in vivo* stimulation with insulin, a phosphotyrosine band of 95 kDa, previously identified

Fig. 1A-C. Insulin-stimulated tyrosine phosphorylation in the muscle of pregnant rats. Rats were anaesthetized and the abdominal wall incised to expose the viscera. 0.9 % NaCl (lanes 1 and 3, left to right) or 10^{-5} mol/l insulin (lanes 2 and 4) was administered into the portal vein as a bolus injection and 90 s later a muscle fragment was excised and homogenized in extraction buffer A at 100°C as described in Methods. After centrifugation, aliquots containing the same amount of protein were resolved by SDS-PAGE on 6 % gels, transferred to nitrocellulose, and detected with antiphosphotyrosine antibody (A), anti-insulin receptor antibody (B), or anti-IRS-1 antibody (C), and [^{125}I]protein A, and then subjected to autoradiography

as the β -subunit of the insulin receptor, appeared and became prominently phosphorylated. The level of phosphorylation of this band was reduced by 30 ± 6 % ($p < 0.02$) in pregnant rats (Figs. 1A and 2B). In the same antiphosphotyrosine blots of whole tissue extracts, in addition to the 95 kDa band seen after insulin injection, a broad band migrating between 165 and 185 kDa was also detectable. This band is known as pp185 and contains IRS-1 as one of its components [8–11]. The phosphorylation of pp185 was reduced by 48 ± 5 % ($p < 0.002$) in pregnant animals (Figs. 1A and 2B).

In samples from muscle previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine antibody, there was a clear decrease in insulin-stimulated IRS-1 phosphorylation to 30 ± 9 % of the control value ($p < 0.01$) in pregnant rats (Figs. 2C and 3A). In order to investigate the stoichiometry of IRS-1 phosphorylation (phosphate to protein ratio), we stripped these nitrocellulose

Fig 2A-D. Insulin receptor (IR) and IRS-1 protein levels (**A**) and tyrosine phosphorylation of IR and pp185 (**B**) in the muscle of virgin and pregnant rats. Scanning densitometry was performed on autoradiograms of eight experiments for insulin receptor and IRS-1 concentrations, and seven experiments for tyrosine phosphorylation levels of IR and pp185. IRS-1 tyrosine phosphorylation levels (**C**) and association with PI 3-kinase in muscle of virgin and pregnant rats (**D**). Scanning densitometry was performed on autoradiograms of four experiments for IRS-1 phosphorylation levels and four experiments for the association between IRS-1 and PI 3-kinase. Data are expressed as mean $\pm$ SEM and are normalized for protein content. * $p < 0.05$

membranes and incubated them with anti-IRS-1 antibody, after which the phosphorylation of IRS-1 relative to the total amount of IRS-1 was calculated. Following insulin stimulation this stoichiometry was reduced by approximately 35 % in the muscle of pregnant rats ($n = 4$).

Previous studies [19–23] have suggested that there is a relatively stable, high affinity interaction between IRS-1 and the 85 kDa subunit of PI 3-kinase, such that both proteins are co-precipitated by antibodies

Fig 3A, B. Insulin-stimulated tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase in the muscle of virgin and pregnant rats. The proteins were isolated with extraction buffer B, as described in Methods, and kept on ice. After centrifugation, aliquots of the supernatant were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antiphosphotyrosine (**A**) and anti-PI 3-kinase (85 kDa subunit) (**B**) antibodies and [125 I]protein A, and then subjected to autoradiography

to each protein. In samples from muscle previously immunoprecipitated with anti-IRS-1 antibody and immunoblotted with antibodies directed against the 85 kDa subunit of PI 3-kinase, there was little or no detectable PI 3-kinase immunoreactivity in the basal state in either virgin or pregnant rats (Fig. 3B). After stimulation with insulin, a band with the expected molecular weight of the regulatory subunit of PI 3-kinase (85 kDa) was present in anti-IRS-1 antibody immunoprecipitates of muscle from both groups of animals. This observation is consistent with the existence of a stable complex between IRS-1 and PI 3-kinase. However, the amount of PI 3-kinase associated with IRS-1 was reduced by $81 \pm 6\%$ ($p < 0.01$) in pregnant rats (Figs. 2D and 3B), thus suggesting a reduced affinity between these two proteins. IRS-1 can also bind and activate other proteins including the phosphotyrosine phosphatase SHPTP2 [30–34]. We next investigated whether there is any change in IRS-1/SHPTP2 interaction during pregnancy, by performing immunoprecipitation with anti-IRS-1 antibody and immunoblotting with antibodies directed against SHPTP2. After stimulation with insulin, SHPTP2 is co-precipitated with IRS-1, but this association is reduced approximately 50 % ($n = 3$) in the muscle of pregnant rats (data not shown).

The effect of pregnancy on insulin receptor and IRS-1 phosphorylation in rat liver: Pregnancy had a similar effect on insulin receptor and IRS-1 phosphorylation in liver as in muscle (Fig. 4A). There was no change in the hepatic insulin receptor level as determined by immunoblotting (Fig. 4B). The immunoblotting of

Fig. 4A–C. Insulin-stimulated tyrosine phosphorylation in the liver of pregnant rats. Rats were anaesthetized and the abdominal wall incised to expose the viscera. Normal saline (lanes 1 and 3, left to right) or 10^{-5} mol/l insulin (lanes 2 and 4) was administered into the portal vein as a bolus injection and 30 s later a liver fragment was excised and homogenized in extraction buffer A at 100°C as described in Methods. After centrifugation, aliquots containing the same amount of protein were resolved by SDS-PAGE on 6% gels, transferred to nitrocellulose, and detected with antiphosphotyrosine antibody (**A**), anti-insulin receptor antibody (**B**), or anti-IRS-1 antibody (**C**), and $[^{125}\text{I}]$ protein A, and then subjected to autoradiography

liver extracts with antibodies to IRS-1 revealed that the level of IRS-1 decreased by $45 \pm 6\%$ ($p < 0.002$) in pregnant rats compared to virgin rats (Fig. 4C and 5A).

After insulin treatment, insulin receptor phosphorylation was reduced by $36 \pm 5\%$ ($p < 0.01$; Figs. 4A and 5B) while the tyrosine phosphorylation of pp185 decreased by $58 \pm 8\%$ ($p < 0.002$; Figs. 4A and 5B) in the pregnant rats compared to the controls.

To define better the levels of IRS-1 phosphorylation, we performed Western blot analysis of tyrosyl-phosphorylated proteins in anti-IRS-1 immunoprecipitates before and after stimulation with insulin in both groups of animals. Figures 6A and 5C show that there was a marked reduction (to $25 \pm 8\%$ of the control values; $p < 0.01$) in insulin-stimulated IRS-1 phosphorylation in the liver of pregnant animals. When these nitrocellulose membranes were stripped and immunoblotted with anti-IRS-1, the stoichiometry of IRS-1 phosphorylation was seen to be reduced by approximately 50% in the liver of pregnant rats ($n = 4$). Association of the 85 kDa subunit of PI

Fig. 5A–D. Insulin receptor (IR) and IRS-1 protein levels (**A**) and tyrosine phosphorylation of IR and pp185 (**B**) in the liver of virgin and pregnant rats. Scanning densitometry was performed on autoradiograms of eight experiments for insulin receptor and IRS-1 concentrations, and also for tyrosine phosphorylation levels of IR and pp185. IRS-1 tyrosine phosphorylation levels (**C**) and association with PI 3-kinase in liver of control and pregnant rats (**D**). Scanning densitometry was performed on autoradiograms of four experiments for IRS-1 phosphorylation levels and four experiments for the association between IRS-1 and PI 3-kinase. The data are expressed as the mean $\pm$ SEM and are normalized for protein content. $*p < 0.05$

3-kinase with IRS-1 was examined by incubating the same blot with antibodies to this subunit (Fig. 6B). As expected, in both groups, a band of 85 kDa was present in the IRS-1 immunoprecipitates after exposure to insulin, although there was a $79 \pm 11\%$ ($p < 0.01$) decrease in the intensity of this band in pregnant rats (Fig. 5D). This suggests that in pregnant rats there is a reduced association between hepatic IRS-1 and PI 3-kinase after insulin stimulation. It is interesting that in liver the association of IRS-1/PI

Fig 6A, B. Insulin-stimulated tyrosine phosphorylation of IRS-1 and its association with PI 3-kinase in the liver of virgin and pregnant rats. The proteins were isolated with extraction buffer B as described in Methods, and kept on ice. After centrifugation, aliquots of the supernatant were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-phosphotyrosine (**A**) and anti-PI 3-kinase (85 kDa subunit) (**B**) antibodies and [125 I]protein A, and then subjected to autoradiography

3-kinase in the basal state is higher than in muscle both of virgin and pregnant rats. When we compared liver and muscle in both groups of animals, the IRS-1 and PI 3-kinase protein levels were higher in the former. The amount of insulin reaching the liver under basal conditions is higher than in muscle, and this may enhance the association of p85 with IRS-1 in this organ. The association of IRS-1 with SHPTP2 after insulin stimulation, determined by immunoprecipitation with anti-IRS-1 antibody and immunoblotting with SHPTP2 antibody, was reduced to approximately 45 % ($n=3$) in the liver of pregnant rats (data not shown).

Discussion

In the present study, we investigated the importance of insulin receptor and IRS-1 phosphorylation and of the association of PI 3-kinase with IRS-1 in the liver and muscle of pregnant rats. It is known that pregnancy induces marked insulin resistance [1–7] and that it is accompanied by a reduced glucose uptake in peripheral tissues and an increase in hepatic glucose output. Our results indicate that pregnancy has no significant effect on the levels of the insulin receptor in rat liver and muscle. Despite the normal number of receptors, there was a 36 % reduction in insulin receptor autophosphorylation in the liver and a 30 % reduction in muscle after stimulation with insulin. The phosphorylation of IRS-1 (reflecting the kinase activity of the insulin receptor towards its endogenous substrate) was reduced by 75 % in the liver and

by 70 % in the muscle of pregnant rats. The reduction in IRS-1 protein levels may have a role in the decrease in insulin-stimulated IRS-1 phosphorylation. However, the stoichiometry of IRS-1 phosphorylation was also reduced in the liver and muscle of pregnant rats, demonstrating a reduction in IRS-1 phosphorylation greater than when taking into account the decrease in IRS-1 protein. In this context, changes in the insulin receptor kinase in the same direction may have at least a permissive role, since the IRS-1 protein levels are not the only determinant of IRS-1 phosphorylation levels [35, 36]. Whether these regulatory defects induced by chronic hyperinsulinaemia reflect alterations in receptor and IRS-1 serine phosphorylation or some other regulatory event is unknown. Interestingly, the amount of PI 3-kinase associated with IRS-1 was also reduced in the liver and muscle of these animals. The decrease in the three early steps of insulin action after hormone binding may have an important role in the insulin resistance observed during pregnancy. Insulin increases glucose uptake into cells, partly through the translocation of GLUT4 from intracellular compartments to the plasma membrane in muscle and adipose tissues [37]. Distinct experimental approaches have led to the conclusion that PI 3-kinase is necessary for insulin-stimulated GLUT4 translocation [38–44]. Evidence from other sources has also demonstrated a correlation between PI 3-kinase activity and glycogen metabolism [45, 46]. Thus, it is reasonable to speculate that the IRS-1/PI 3-kinase pathway may be linked to the activation of glucose transport in muscle and glycogen synthesis in liver and muscle, and that a reduction in this association in pregnancy may have a role in the insulin resistance in liver and muscle of pregnant rats.

In addition to PI 3-kinase other SH2 proteins, including Nck, GRB2 [8] and SHPTP2 (also known as Syp and phosphotyrosine phosphatase 1D) [30–34] were found to associate with IRS-1. The association of SHPTP2 with IRS-1 may activate the phosphatase, in a manner analogous to the activation of PI 3-kinase [30, 31]. Recent data suggest that SHPTP2 has a role in insulin-stimulated mitogenesis and also indicate that this phosphatase is necessary for insulin-stimulated expression of GLUT1 [32]. There is evidence that SHPTP2 can dephosphorylate IRS-1 [30, 31], although this has not been uniformly observed [33, 34]. Our results showed that the association of IRS-1/SHPTP2 after insulin stimulation was also reduced in the liver and muscle of pregnant rats, suggesting that alterations in this association do not account for the reduction in IRS-1 phosphorylation in these animals. Instead, the decrease in IRS-1/SHPTP2 interaction in pregnant rats may reflect a reduction in IRS-1 phosphorylation.

The mechanisms whereby pregnancy induces the above alterations are unknown but the hormonal

milieu may have a role. An increase in circulating hormones such as progesterone and placental lactogen, which act as antagonists of insulin, may contribute to insulin resistance during pregnancy. In this regard, maternal plasma corticosterone levels are increased on day 18 and remained high until parturition [47]. However, the influence of progesterone and placental lactogen on the early steps of insulin action have not yet been investigated. The effect of an excess of glucocorticoid on these steps is well established in the liver and muscle of animals [36, 48], and also in cell culture [49]. In animals treated with dexamethasone, there is a decrease in insulin receptor and IRS-1 phosphorylation in the liver, and a decrease in PI 3-kinase activity in both liver and muscle. Interestingly, there is also a decrease in the IRS-1 protein levels in muscle [36, 48]. In 3T3-F442A adipocytes, the administration of dexamethasone reduced the insulin receptor and IRS-1 tyrosine phosphorylation levels as well as PI 3-kinase activity [49]. Taken together, these findings suggest that high cortisol levels may have a role in the alterations seen in the early steps of insulin action in these rats. Hyperinsulinaemia per se can also induce similar alterations in early steps of insulin action, as demonstrated in previous studies of animal models of insulin resistance and in cell culture [35, 49, 50]. However, as the insulin levels observed in pregnant rats are only slightly higher than in virgin animals, this increase may not be responsible for all of the observed effects.

In summary, this study has shown that a reduced expression and/or phosphorylation of IRS-1 may lead to reduced association between IRS-1/PI 3-kinase in the liver and muscle of pregnant rats. These data suggest that changes in the early steps of insulin action may have a role in the insulin resistance observed in these animals.

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