The Heterogeneity of the Developing Brain Insulin Receptor¹

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ABSTRACT. Comparison of the adult brain insulin receptor (IR) to other tissue IR demonstrates that the former migrates ~10 kD faster on sodium dodecyl sulfate-polyacrylamide gel electrophoresis due to deficient sialic acid content of the asparagine N-linked carbohydrate moieties. We studied these receptors in the fetal rat (18-day) brain (~125 kD) and liver (~135 kD), and demonstrated that similar differences are present during fetal life. These differences are not modified by hyperglycemia associated with both mild hyperinsulinemia and normoinsulinemia/hypoinsulinemia. We further studied the specific brain cell types: neurons, glial cells, and purified microvessel preparation, and demonstrated a heterogeneity in the N-linked glycosylation of the IR within an organ (brain). The neuronal (~125 kD) and microvascular (~125 kD, ~135 kD) IR are deficient in sialic acid, thus conferring neuraminidase-insensitivity to the whole brain, whereas the glial cell IR, similar to the liver IR, exhibits neuraminidase sensitivity and migrates intermediate (~128 kD) to the liver and brain IR. The functional significance of this receptor heterogeneity between various tissues and cells within the same organ (brain) remains to be determined. (*Pediatr Res* 24:683-692, 1988)

Abbreviations

IR, insulin receptor
kD, kilodaltons
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The IR, a heterotetrameric glycopeptide composed of two α - and two β -subunits linked by disulfide bonds, mediates the biologic effects of insulin (1). In the brain of both the adult (2) and developing animal (3-5), insulin and insulin receptors have been demonstrated. These brain IR are unique in the sense that they do not down-regulate in response to high insulin concentrations (5, 6), a phenomenon that has been observed in various fetal neural (5, 7) and nonneural tissues as well (8-10). There is evidence to support the fact that insulin is synthesized within the brain (11, 12) as well as the fact that circulating insulin traverses the blood-brain barrier and gains access to the brain (13), suggesting a dual origin for insulin within the central nervous system. Biologic effects of insulin, akin to those in other tissues such as

metabolic (14) and growth-promoting effects (15), have been demonstrated within the brain, specifically in glial cells; whereas the unique effect of neuromodulation has been observed only in neuronal cells (16, 17).

Structural studies of the adult brain IR in comparison with the IR of adipocytes and liver have revealed a heterogeneity. Specifically, the α -subunit which binds to the ligand has been demonstrated to be ~10 kD lighter in the brain than in other tissues (7, 18-20). Recent evidence supports the fact that a decrease in the sialic acid content of the brain IR N-linked carbohydrate moieties is responsible for this heterogeneity (21, 22). We studied this heterogeneity in the fetal rat to determine whether these structural IR differences are present developmentally as well. Additionally, we investigated the effect of hyperglycemia along with either mild hyperinsulinemia or normo/hypoinsulinemia on the N-linked glycosylation of the fetal and adult brain and liver IR (α -subunit) to determine whether there was any association between the IR structure and its ability either to down- or up-regulate in response to varying insulin concentrations, as previously observed (5-10).

We further studied the structure of the α -subunit of the IR in specific brain cell types such as the neurons, glial cells, and purified microvessels to investigate whether a heterogeneity in the structure of the IR determined the previously reported differential biologic effect of the hormone in the various cell types (14-17, 23).

MATERIALS AND METHODS

Animals. Two different species were used. The rat was used for the *in vivo* experiments in order to produce fetal hyperglycemia with small volumes of maternal glucose infusates (24, 25). The New Zealand White rabbit was preferred over the rat for isolation of brain cells as the yield was significantly better than in the rat, and the rabbit brain cell types have been extensively characterized previously by us (11).

Characterization of IR α -Subunit. Sprague-Dawley adult non-pregnant and fetal rats (18-day; term, ~21 days) were anesthetized with pentobarbital and their livers and brains excised. Tissues from an individual adult animal and pooled fetuses of a litter were homogenized and crude membranes prepared by differential centrifugation, as described previously (19). The protein content was determined by the Biorad assay (26) and these membranes used in the experiments designed to compare the fetal IR to the adult.

Diabetic model. Maternal diabetes was induced in 12-day pregnant rats by intraperitoneal injections of streptozotocin (60 mg/kg). Controls received saline injections alone. On day 20 of gestation, maternal and pooled fetal brain and liver membranes were prepared (19). These membranes were used in experiments designed to study the effect of hyperglycemia along with normoinsulinemia/hypoinsulinemia on the fetal and adult rat brain and liver IR.

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Surgical model. To study the effect of hyperglycemia associated with mild hyperinsulinemia on the fetal and adult rat brain and liver IR, 13-day pregnant rats were given pentobarbital anesthesia and underwent cannulation of their external jugular vein. The procedure lasted 15 to 20 min, and the animals recovered soon after skin closure. The animals were moving freely in their cages and were allowed to recover from the stress of surgery for 24 h, after which some rats received 30% glucose water infusion at a 17 mg/kg/min rate; controls received a similar rate of sterile distilled water infusion until the rats were 18 days gestation. Both groups of animals were allowed free access to water and food. This previously described animal model results in fetal hyperglycemia and mild hyperinsulinemia (24, 25). Eighteen-day gestation maternal and pooled fetal brain and liver membranes were prepared as stated above.

Maternal and fetal plasma was collected for glucose and insulin concentration determinations (5) from both the diabetic and surgical models and their respective controls.

Rabbit Brain Cell Preparations. Brain microvessel-enriched tissue fraction was prepared and characterized as described before (27). The purity of the preparation was determined by the percentage recovery of the alkaline phosphatase enzyme activity when compared to the crude homogenate (27) and the contamination by cellular components (neurons and glia) determined by phase contrast microscopic examination of the final preparation.

Cell culture. Neuron-enriched and glial cell cultures were prepared from 10-day-old neonatal rabbits and characterized as previously described (11). The individual cell types were washed extensively with ice-cold Hepes buffer (50 mM Hepes, 2 mM phenylmethylsulfonyl fluoride, 10^3 U/liter aprotinin, 2 μ M pepstatin, 2 μ M leupeptin, 0.1 mg/ml bacitracin, pH 7.4) and scraped with a rubber policeman. The cell suspensions and the microvessel-enriched preparation were homogenized and centrifuged (20). The crude membrane pellet was used after protein estimation for characterization of the IR.

125 I-Insulin Binding Assay. Porcine insulin (Eli Lilly Co., Indianapolis, IN) was iodinated as previously described (28) to a specific activity of 100 to 150 μ Ci/ μ g and used as the ligand. $\sim 5 \times 10^6$ cpm of 125 I-insulin (used within a week of iodination with a trichloroacetic acid precipitability of $>95\%$) was incubated overnight at 4° C in the presence (nonspecific) and absence of 1×10^{-6} M native insulin along with 400 μ g of membrane protein, 35 μ g of aprotinin, and 1% bovine serum albumin in a final volume of 0.35 ml of Hepes buffer, pH 7.8. The bound fraction was separated from the free fraction by centrifugation. The 125 I-insulin that bound to the receptor was cross-linked with 0.1 mM disuccinimidyl suberate (29) as previously described.

Enzyme Studies. The cross-linked 125 I-insulin-bound receptor complex was incubated with specific enzymes such as neuraminidase (0.8 U), endoglucosidase H (0.23 IU), endoglucosidase F (10 μ l) or an equal volume of HEPES buffer (control) at 37° C (pH 6.0) for 2 h (30–32). The excess enzyme was removed by extensive washing, and the final pellet solubilized in Laemmli's buffer before subjecting the samples to SDS-PAGE and autoradiography (33).

RESULTS

Figure 1 demonstrates the adult rat liver α -subunit of the IR specifically displaced by native insulin to possess an Mr of ~ 135 kD. The neuraminidase enzyme digestion experiments demonstrating the cleavage of sialic acid moieties from the N-linked carbohydrates is shown in Figure 2A. Both the adult and fetal liver IR migrated ~ 10 kD higher than the respective brain IR (~ 125 kD). The fetal and adult liver IR mobility enhanced following the neuraminidase treatment, whereas the brain IR remained unchanged. After neuraminidase treatment, neither the adult nor fetal liver IR (~ 130 kD) co-migrated with the control adult and fetal brain IR (~ 125 kD), suggesting the absence (or deficiency) of other N-linked carbohydrate moieties

Fig. 1. Autoradiographs SDS-PAGE demonstrating the 125 I-insulin cross-linked receptor: adult liver insulin receptor (α -subunit) with an Mr of ~ 135 kD. A total of 1×10^{-6} M unlabeled insulin specifically displaced the labeled hormone from the receptor.

besides sialic acid. Endo H (cleaves the high mannose carbohydrate residues) enhanced the mobility of the adult and fetal liver and brain IR to the same extent (Fig. 2B). Again, after the enzyme treatment, the liver IR did not co-migrate with the control adult and fetal brain IR, suggesting other carbohydrate moieties, such as sialic acid, to be responsible for this discrepancy between the two organs. By contrast, Endo F (cleaves both the N-linked complex oligosaccharides and the high mannose carbohydrate residues) enhanced the mobility of the adult and fetal liver and brain IR, so that all the posttreated IR comigrated (Fig. 2C), implying that both the high mannose and complex oligosaccharide residues are similar in the two organs.

Streptozotocin treatment resulted in maternal and fetal hyperglycemia along with hypoinsulinemia in the former and normoinsulinemia in the latter, whereas maternal infusions of glucose, while producing maternal and fetal hyperglycemia, resulted in mild hyperinsulinemia (Table 1). In both the models of hyperglycemia, adult and fetal liver and brain IR remained unchanged (~ 135 and ~ 125 kD) when compared to their respective controls. Further, the cleavage of sialic acid revealed the persistence of receptor heterogeneity between the liver and the brain in both the adult and fetus in response to hyperglycemia associated with either normo/hypo- or hyperinsulinemia.

Figure 3 demonstrates the IR in cultured neonatal rabbit brain cells, namely neurons and glial cells, whole brain membrane, and microvessel-enriched preparation in comparison with the neonatal rabbit liver IR. The rabbit whole brain (similar to the rat adult and fetal brain) migrated ~ 10 kD lighter than the liver

Fig. 2. Enzyme treatments: *A*, neuraminidase (0.8 U) treatment cleaved the sialic acid altering the molecular mass of the fetal and adult liver IR from ~135 kD to ~130 kD, but failed to alter the molecular mass of the fetal and adult brain IR (~125 kD); *B*, endoglucosidase H (0.023 IU) treatment altered the molecular mass of the fetal and adult brain IR from ~125 kD to ~120 kD and the fetal and adult liver IR from ~135 kD to ~130 kD; *C*, endoglucosidase F (10 μ l) treatment enhanced the molecular mass of the fetal and adult liver IR from ~135 kD to ~110 kD and the fetal and adult brain IR from ~125 kD to ~110 kD.

Table 1. Plasma glucose and insulin concentrations

Rats	Glucose infused (<i>n</i> = 5)	Controls (<i>n</i> = 4)	Streptozotocin treated (<i>n</i> = 4)	Controls (<i>n</i> = 5)
Mothers				
Plasma glucose (mg/dl)	368.8 $\pm$ 78.64	187 $\pm$ 6.67	317 $\pm$ 11†	109 $\pm$ 3
Plasma insulin (μ U/ml)	26 $\pm$ 0.41*	19 $\pm$ 2.65	4.7 $\pm$ 0.47*	10.5 $\pm$ 1.58
Fetuses				
Plasma glucose (mg/dl)	155 $\pm$ 25†	42.5 $\pm$ 2.5	214 $\pm$ 6†	60 $\pm$ 11.8
Plasma insulin (μ U/ml)	57.5 $\pm$ 2.5†	41.33 $\pm$ 1.33	40.6 $\pm$ 2.8	48.5 $\pm$ 4.3

* *t* test, *p* < 0.05.

† *t* test, *p* < 0.01.

IR (~135 kD). In comparison, the rabbit microvessel and neuronal IR were similar to that of the whole brain (~125 kD), whereas the glial IR was in between the brain and liver (~128 kD). Neuraminidase treatment revealed that the neonatal rabbit liver IR (~135 kD) was distinctly neuraminidase-sensitive, whereas the whole brain IR (~125 kD) was not, being similar to that of the rat. Additionally, rabbit neurons and microvessel IR which co-migrated with whole brain were neuraminidase-resistant. The glial cell IR, by contrast, migrated slightly higher than the whole brain and neurons (~128 kD) and was consistently neuraminidase-sensitive. The fetal rat brain microvessel IR, un-

like the rabbit microvessel IR, comigrated with the liver IR, but distinct from the liver, was neuraminidase-resistant. The IR in mixed brain cells (neurons and glial cells) and the particulate rabbit brain following the isolation of microvessels was observed to migrate at ~125 kD and to be neuraminidase-resistant. Phase contrast microscopic examination of the latter fraction, however, revealed the presence of some microvasculature contamination along with the presence of cellular elements. Further experiments with Endo H and Endo F, by contrast, resulted in similar increases in the mobility of the rabbit whole brain, microvessels, neurons, and glial cell IR, suggesting no major differences in the

Fig. 3. Brain cell IR (α -subunit): The IR (α -subunit) of the fetal rat brain microvessels (mic) (~ 135 kD), newborn rabbit liver (~ 135 kD), glial cell (~ 128 kD), neurons (~ 125 kD), rabbit brain microvessels (~ 125 kD) are compared. Although neuraminidase (0.8 U) treatment altered the mobility of the liver (~ 5 kD) and the glial cell IR (~ 3 kD) mildly, it failed to alter the molecular mass of the rabbit whole brain, microvascular, neuronal, and fetal rat brain microvascular IR.

high mannose and complex oligosaccharide moieties in the various cell types.

DISCUSSION

The adult brain IR (21) has been demonstrated to have a decreased sialic acid content altering its mobility on SDS-PAGE by ~ 10 kD when compared to either tumorous brain tissue, such as the rat (34), and to certain human neuroblastoma cells (35), human glioma tissue (36), or to other organs, such as the liver (19), and to adipocytes (18). We have demonstrated that this difference between the brain and liver IR exists in the near term fetus as well. Recently, Brennan demonstrated slight differences in the sialic acid content of the IR between the early (16-day) and late (20-day) fetal and adult rat brain IR. However, both the early and late fetal brain IR were lighter than the liver IR (37).

Similarly, studies in the chick embryo demonstrate that an IR deficient in sialic acid is present within the day 2 whole embryo and in the day 6 brain alone, suggesting that the "immature" receptor persists in the brain although differentiating in other tissues (22). In spite of this persistence of IR heterogeneity in the embryo, fetus, and adult, the fetal IR has been demonstrated to be distinct in other respects from the adult IR, for example, the IR in various fetal tissues is higher in number/mg (38–40), fails *in vivo* to down-regulate in response to high circulating insulin concentrations (8–10) and mediates an immature biologic response of insulin (41), specifically with regard to hepatic glucose to glycogen conversion (41). Similarly, the brain IR fails to down regulate in response to insulin both *in vivo* (5) and *in vitro* (6) and mediates a unique biologic effect of insulin within the central nervous system (16, 17). As suggested previously, if N-linked glycosylation were responsible for down-regulation of the IR, one would expect to see similar glycosylation patterns in the fetal and adult brain IR and the fetal liver IR alone (adult liver IR being different), because these receptors uniformly express an inability to down-regulate in response to insulin. That these patterns are different suggests that the N-linked glycosylation of the IR is not solely responsible for the differences observed between the fetal and adult liver IR, and generally between the liver and brain IR.

Hyperinsulinemia has been shown to down regulate (42), whereas hypoinsulinemia up-regulates the IR in adult tissues (43). However, in the fetus, both high circulating insulin concentrations secondary to maternal diabetes (human, rabbit) (8, 10) and the normo/hypoinsulinemia seen in the offspring of a streptozotocin-diabetic rat mother, did not down-regulate tissue IR (9). In this report, we examined the effect of both high and low insulin concentrations associated with hyperglycemia on the N-linked glycosylation of the adult and fetal brain and liver IR. Again, no differences were observed, suggesting that the N-linked glycosylation did not regulate the ability to down- or up-regulate the fetal and adult liver IR in response to varying insulin concentrations. One could argue that the brain IR N-linked glycosylation failed to change, possibly due to a lack of change in

brain insulin and/or insulin receptor number (5). However, the liver, which is exposed to circulating insulin concentrations, did not demonstrate a change in response to hormonal perturbations as well, negating this theory.

Studies using specific brain cell types have demonstrated that the brain type IR is present in crude brain membranes from the whole brain and neurons alone (20), whereas glial cells possessed a peripheral or liver type of receptor. Here, we have confirmed this observation by demonstrating neuronal IR to be neuraminidase resistant, and the glial cell IR to be neuraminidase sensitive. Mixed brain cell cultures consisting primarily of glial and neuronal cells also demonstrated a neuraminidase resistance. In contrast to previous reports (44, 45), we have demonstrated the rabbit brain microvascular IR to comigrate with the brain IR and express neuraminidase resistance. By contrast, the rat brain microvascular IR while co-migrating with the liver IR, similar to that observed in the human and bovine species (44, 45), was neuraminidase resistant. The migratory pattern of the microvascular IR may be species specific; however, in the two species we examined, the IR was uniformly deficient in sialic acid. Whether similar sialic acid deficiency is seen in the human and bovine species previously studied is unknown at the present time. Our results demonstrate that both the neurons and the microvascular IR are deficient in sialic acid, whereas the glial cell IR, like the liver, is neuraminidase sensitive, possessing varying amounts of sialic acid. Thus one can speculate that, while the sialic acid moiety is not associated with the receptor down-regulation phenomenon, it may be responsible for cell-cell interaction or specialized biologic functions specific to certain cell types alone.

In summary, similar to the adult, we have demonstrated the N-linked glycosylation of the fetal rat brain to be distinct from the liver IR (α -subunit). Further, mild hyperinsulinemia or normo/hypoinsulinemia along with hyperglycemia had no effect on the interorgan heterogeneity of the IR N-linked glycosylation. Examination of specific brain cell types revealed that the neuronal and microvascular IR are deficient in sialic acid, conferring the "brain type" of receptor to the whole brain tissue, whereas the glial cell IR, which is intermediate in size (between the liver and brain), resemble the peripheral type (liver) of receptor by not being deficient in sialic acid. Although the receptor N-linked glycosylation is not associated with the ability of the IR to down-regulate in response to insulin, this posttranslational modification may contribute to a specific biologic function that needs delineation.

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