Intrauterine Hyperglycemia Increases Insulin Binding Sites but Not Glucose Transporter Expression in Discrete Brain Areas in Term Rat Fetuses

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ABSTRACT

Diabetic pregnancy results in several metabolic and hormonal disorders, both in the embryo and the fetus of different species, including humans. Insulin is a potent modulator of brain development and is suggested to promote the differentiation and maturation of hypothalamic or related extrahypothalamic structures, which are directly involved in neural inputs to the pancreas. Because these structures are known to be specifically responsive both to insulin and glucose, we examined the effects of 48-h hyperglycemic clamps in unrestrained pregnant rats on insulin binding and glucose transporter expression in hypothalamic and extrahypothalamic-related areas of their fetal offspring. The main result was an increase in insulin binding in the ventromedial hypothalamic nucleus (VMH), the arcuate nucleus (AN), and the lateral hypothalamus (LH), and in the nucleus of the tractus solitarius (NTS) for extrahypothalamic areas (+30% in the VMH, +37% in the AN, +25.8% in the LH, and +37.3% in the NTS). The deleterious effect of brain hyperinsulinism during the late gestational stage does not seem to act through glucose transporter (GLUT) expression, inasmuch as no relation-

ship between GLUT level and hyperinsulinism in brain areas could be observed. The specific increase in insulin binding in areas involved in the nervous control of metabolism could be a factor in the increased glucose intolerance and impairment of insulin secretion that was previously observed in the adult rats from hyperglycemic mothers. (*Pediatr Res* 56: 263–267, 2004)

Abbreviations

GLUT, glucose transporter
VMH, ventromedial hypothalamic nucleus
AN, arcuate nucleus
LH, lateral hypothalamus
NTS, nucleus of the tractus solitarius
ANS, autonomic nervous system
PVN, paraventricular nucleus
qPCR, quantitative competitive reverse transcription polymerase chain reaction
TH, tyrosine hydroxylase
HI-HG, hyperinsulinemic-hyperglycemic

Diabetic pregnancy can lead to glucose intolerance and pancreatic dysfunction in offspring when they reach adult age (1–3). This situation seems to be mainly related to intrauterine hyperglycemia, inasmuch as mild hyperglycemia induced by glucose infusion during late gestation in normal rats is sufficient to induce persistent impairment of glucose regulation and insulin secretion in the adult offspring born of hyperglycemic mothers (4, 5). Interestingly, in this model, insulin deficiency was due to a perturbation of the control of insulin secretion by

the ANS and not to an intrinsic pancreatic β -cell defect (5). The activity of the ANS is under the influence of hypothalamic nuclei (PVN, VMH, and LH), themselves connected to preganglionic nuclei in the brainstem (6, 7). These areas present a neuronal population able to respond electrophysiologically to insulin and glucose (8, 9). Insulin, insulin receptors, and glucose transporters are present in the brain (8, 10). The defect in insulin secretion observed in the offspring of hyperglycemic mothers could be linked to impaired glucose and/or insulin sensing in the brain. We hypothesized that fetal hyperglycemia and/or hyperinsulinemia could hamper the functional development of glucose and/or insulin-sensitive brain areas. In fact, previous studies in models of perinatal hyperinsulinism (where hypothalamic insulin binding was increased) have demon-

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strated that hypothalamic nuclei were structurally altered in adult life (11, 12). We first addressed the question of whether hyperglycemia and/or hyperinsulinemia during late pregnancy alter insulin binding in fetal brain areas involved in the autonomic control of insulin secretion and glucose homeostasis.

Insulin controls glucose transport in insulin-sensitive tissues through GLUT4 (10). Some studies have reported that, in the brain, insulin stimulates glucose transport *in vitro* in glial cells (13). *In vivo*, hyperglycemia and hyperinsulinemia lead to increased glucose utilization in the hypothalamus (14). Altogether, these data suggest that insulin might be involved in glucose transport in some specific brain areas. Among the neural GLUT identified, GLUT1 (endothelial, glial cells) and GLUT3 (neuronal cells) are the main isoforms (10). We have recently demonstrated that GLUT2 and GLUT4 were also, at lower levels, expressed in adult hypothalamic and spinal cord areas, respectively, in astrocytic and neuronal cells (15, 16). Modifying the GLUT2 expression in the brain led to an abnormal control of energy metabolism and deranged insulin secretion (17). Moreover, in physiopathological model such as Zucker rats, GLUT4 expression was altered in hypothalamic nuclei (18).

In the second part of the study, we focused on the fact that a possible consequence of an alteration in insulin binding to specific brain areas could result in the modifications of expression of isoforms GLUT1, GLUT2, GLUT3, and GLUT4 in these areas during late development in the rat fetus.

The study was performed on rat fetuses at term from mothers made mildly hyperglycemic during the last 2 d of pregnancy by continuous glucose infusion.

METHODS

Animals. All rats were treated in accordance with European Community guidelines, and the experimentation was approved by our local institution. Three-month-old pregnant female Wistar rats weighing 250 g were used. They were allowed free access to water and standard laboratory chow pellets (UAR 113, Usine d'Alimentation Rationnelle, Villemoisson, France). At d 19 of pregnancy, a catheter was implanted under intraperitoneal pentobarbital anesthesia (125 mg/kg) in the right atrium *via* the jugular vein. The technique (19) for long-term infusion in unrestrained rats was used for glucose infusion (hypertonic 30% sterile glucose, Chaix & Du Marais, Paris, France). The infusion period started on d 2 after surgery and lasted 2 d. The initial infusion rate was 30 μ L/min. Control rats were infused with 0.9% NaCl. Blood glucose of infused rats was measured twice daily in samples drawn from caudal vessels. Glycemia was immediately measured using a glucose analyzer (LifeScan, Milpitas, CA, U.S.A.). These controls allowed us to adjust glucose flow rate throughout the infusion to maintain mild hyperglycemia. After centrifugation, the remaining plasma was stored at -20°C until insulin assay. Fetal blood samples originating from axillary vessels were used for measurements of both glycemia and insulinemia. Plasma immunoreactive insulin was measured by a RIA kit (DiaSorin, Saluggia, Italy).

At 21.5 d of pregnancy (normal term, 22 d), the infusions were disrupted and the fetuses were immediately removed after cesarean section. They were successively exteriorized from the uterus, leaving placenta and umbilical cord *in situ*; blood samples were taken from the axillary vessels, and the brains were rapidly removed and placed into freezing isopentane (-40°C) for 40 s. Fetal brains were stored until cryosection (20).

Brain region dissection. Fetal brains were removed and frozen by dry ice. Coronal sections (300 μm) were obtained using a cryostat and placed upon chilled microscope slides. Whole hypothalamus and rostral spinal cord were microdissected and used for either Northern blot, qcRT-PCR, or Western blot.

Quantification of brain insulin binding sites. The regional binding sites were localized and quantified by means of autoradiography (Biocom densitometer, Biocom, les Ulis, France) as already described (20). Autoradiograms were obtained by apposition of the sections on tritiated ultrafilm (LKB-ultrafilm, Amersham Biosciences AB, Uppsala, Sweden) over 7 d. Six to 10 readings were made for each discrete structure in each animal.

Northern blot. Frozen hypothalamic and extrahypothalamic tissues were used to extract total RNA using guanidine thiocyanate (21). Brain mRNA was further purified using an mRNA extraction kit (Amersham Biosciences AB) and the concentrations measured by absorbance at 260 nm. A Northern blot was performed, as described (22), using 20 μg of purified mRNA. The blots were hybridized with ^{32}P -labeled probe using a Megaprime labeling system kit (Amersham Biosciences AB). The probes for GLUT1, GLUT3, and GLUT4 corresponded to the 541–1623, 597–1411, and 334–979 cDNA sequences, respectively, and were constructed using PCR. Blots were exposed at -80°C with intensifying screens. Autoradiograms were scanned with an SI densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and signals were quantified using ImageQuant software (Molecular Dynamics).

Western blots. Total membranes were isolated from homogenized brain regions (*i.e.* hypothalamus and rostral spinal cord). A total membrane pellet was obtained by centrifugation at 10,000 g for 10 min at 4°C , then the supernatant was ultracentrifuged at 180,000 g for 35 min at 4°C . The membrane pellet was resuspended in a PBS buffer containing protease inhibitors, and protein content was determined (Bio-Rad S.A., Marnes-la-Coquette, France). Samples were stored at -80°C until assays. Fifty-microgram membrane samples were analyzed for GLUT content by classical Western blot analysis with the rabbit polyclonal GLUT1 (1/15,000) antibody (purchased from Biogenesis, Argene, France) and the goat polyclonal GLUT3 and GLUT4 antibodies (Santa Cruz Biotechnology, Tebu, France), 1/5000 and 1/4500, respectively. The immunoblot signals were detected using the Amersham ECL-detection kit system. Then, the nitrocellulose membranes were stripped of bound antibodies and incubated with a rabbit polyclonal antibody to $\beta 5$ unit of integrin protein (Chemicon, Temecula, CA, U.S.A.) (1/5000). Films of GLUT1, GLUT3, GLUT4, and $\beta 5$ -integrin were quantified as described for Northern blots.

Results were normalized by quantification of corresponding levels of β 5-integrin protein.

Statistical analysis. Values are presented as means $\pm$ SEM. All statistical analyses were done by unpaired *t* test with Prism software (GraphPad Software, San Diego, CA, U.S.A.). Statistical significance was accepted at $p < 0.05$.

RESULTS

Plasma glucose and insulin levels in mothers and fetuses.

As shown in Table 1, glucose infusion resulted in sustained hyperglycemia both in glucose-infused mothers and in fetuses when compared with their respective controls. For plasma insulin (Table 1), a 5-fold increase was observed in glucose-infused mothers and a nearly 7-fold increase was observed in their fetuses, compared with controls.

Insulin binding. Insulin receptor density was increased in several brain areas of fetuses from hyperglycemic mothers. The greatest differences with controls were observed in the VMH, the AN, and the LH for hypothalamic areas, and in the NTS for extrahypothalamic areas (Fig. 1).

mRNA levels of glucose transporters. GLUT2 mRNA were undetectable in all areas studied, whatever the glycemic and insulinemic status and the methodology used, *i.e.* classic Northern blot using mRNA preparation or the highly sensitive qcRT-PCR method (data not shown).

As shown in Figure 2, exposure of fetuses to hyperglycemia and hyperinsulinemia resulted in a marked increase in GLUT1 and GLUT3 mRNA concentrations, and a less pronounced increase in GLUT4 mRNA at the level of the rostral spinal cord (which includes the NTS). In contrast, hypothalamic GLUT3 and GLUT4 mRNA expression was not altered in hyperglycemic fetuses. Only a slight increase of GLUT1 transcripts could be observed (Fig. 2).

Protein quantification of glucose transporters. Total membranes prepared from hypothalamic and rostral spinal cord areas of three offspring for each status (HG-HI versus control) were analyzed by Western blot (Fig. 3A). The quantification of GLUT1, GLUT3, and GLUT4 levels in these samples, expressed as arbitrary standard units relative to the control offspring, is presented in Figure 3B. Hypothalamic as well as rostral spinal cord GLUT3 and GLUT4 levels were unchanged in HG-HI fetuses when compared with controls. The 55-kD GLUT1 (endothelial isoform) was also unaffected by the treatment, whatever the area studied. The 45-kD GLUT1 (glial and neuronal isoform) showed a significant increase, but only in the rostral spinal cord; there was no alteration in the hypothalamus.

Table 1. Plasma glucose and insulin levels in control (C) and hyperglycemic (HG) mothers and fetuses used for autoradiographic, Northern blot, and Western blot experiments

	Mothers (<i>n</i> = 6)		Fetuses (<i>n</i> = 45)	
	Control	Hyperglycemic	Control	Hyperglycemic
Glycemia (mM)	5.55 $\pm$ 0.04	15.0 $\pm$ 0.4*	4.44 $\pm$ 0.02	11.6 $\pm$ 0.2*
Insulinemia (pM)	308 $\pm$ 28	1729 $\pm$ 119 [†]	231 $\pm$ 10.5	1491 $\pm$ 63 [†]

Values are the mean $\pm$ SEM. All values are significantly higher (* $p < 0.05$, [†] $p < 0.0001$) in hyperglycemic compared with control animals.

Figure 1. Density of insulin receptors in the brain regions of rat fetuses. PCX, parietal cortex; FCX, frontal cortex; OB, olfactory bulbs; VMH, ventromedial hypothalamus; AN, arcuate nucleus; LH, lateral hypothalamus; AB, amygdaloid body; NTS, nucleus of the tractus solitarius; Ca3, field CA3 of Ammon's horn (hippocampus). Values are the mean $\pm$ SEM. Values are significantly higher (** $p < 0.01$, * $p < 0.05$) in hyperglycemic (*n* = 6) compared with control animals (*n* = 5).

Figure 2. Regional GLUT1, GLUT3, and GLUT4 mRNA levels in control and hyperglycemic-hyperinsulinemic brain fetuses. Five samples per region from 9 to 12 animals per group were analyzed. Results were normalized by quantitation of corresponding β -actin mRNA levels. Hypot, hypothalamus; SpCord, rostral spinal cord. Values are the mean $\pm$ SEM. Values are significantly higher (*** $p < 0.0005$, ** $p < 0.01$, * $p < 0.05$) in HG-HI compared with control fetuses.

DISCUSSION

Our working hypothesis was that hyperglycemia and hyperinsulinemia during intrauterine development could act independently or together to alter the neural control of glucose homeostasis and insulin secretion. We focused on fetal glucose

Figure 3. (A) Photographs of GLUT1, GLUT3, and GLUT4 Western blots. Only $\beta 5$ -integrin for the GLUT4 Western blot is presented. Similar images of $\beta 5$ -integrin have been obtained for the other GLUT Western blots. For details, see B and "Materials and Methods." (B) GLUT1, GLUT3, and GLUT4 proteins in hypothalamic and in rostral spinal cord brain membranes from control and HI-HG fetuses. Three samples per region from 8 to 12 animals per group were analyzed. A 50- μ g membrane fraction was analyzed for each GLUT content (for details, see "Materials and Methods"). Values are mean $\pm$ SEM. * $p < 0.05$ in hyperglycemic compared with control animals.

transporter expression and insulin binding in brain areas involved in this regulation.

The main finding of this study is that fetuses born of hyperglycemic mothers present a significant increase in insulin binding in some brain areas, *i.e.* hypothalamic and related extrahypothalamic nuclei. This suggests that the deterioration of glucose homeostasis and the impairment in insulin secretion in adult rats born of hyperglycemic mothers (5, 23) could at least in part originate from the development of brain areas involved in the control of glucose homeostasis in an abnormal intrauterine milieu. Therefore, high insulin and/or glycemic levels during fetal development may induce permanent disorders of some aspects of neuroendocrine systems. Previous studies in models of perinatal hyperinsulinism have focused on hypothalamic nuclei that are found to be structurally altered in adulthood (11, 12). A decrease in mean area of neuronal nuclei

and neuronal cytoplasm in VMH (anterior, central, and dorso-medial) and in AN were observed on d 21 of life in offspring from diabetic rats (24). VMH alterations were accompanied by an increased number of neurons expressing TH within the AN. It has been proposed that this increased TH neuron number might activate and overstimulate the differentiation of hypothalamic catecholaminergic systems due to hyperinsulinism. Moreover, neurons of this nucleus also express neuropeptides, such as NPY and galanin, involved in the control of food intake and metabolism, which are altered in the adult offspring of a diabetic mothers (25). Whether an increase in insulin receptors number could be linked to an abnormal development of monoaminergic neurons remains to be established. Although we describe here a significantly increased density of insulin receptors in the LH, these authors did not observe a similar disorganization in LH of hyperinsulinemic offspring (25). This could be explained by the more severe gestational diabetes status used in our study.

In addition to NTS, we found that the insulin binding sites were significantly increased. Moreover, this extrahypothalamic structure has been demonstrated to be directly linked to hypothalamic pathways in regulating glucose levels (26, 27). In a previous study, Devaskar *et al.* (28) did not find any modification in whole brain (cerebral) insulin binding in HG-HI fetuses, whereas the number of liver insulin receptors was largely increased. We note here the changes in insulin binding sites were restricted to hypothalamic and related extrahypothalamic nuclei involved in the control of energy metabolism. Therefore, the results of both studies reinforce the idea that the brain targets of fetal hyperglycemia and/or hyperinsulinemia are specifically located in nuclei including neuronal populations participating in the control of glucose homeostasis. The modifications in insulin binding in these specific areas may result in a more general alteration in neuronal function leading to further glucose intolerance in impaired insulin secretion.

Whether alterations in brain insulin binding were associated with changes in glucose transporters of these specific structures were next investigated. Although GLUT1 and GLUT3 mRNA were increased in the hypothalamus of the HI-HG offspring, only the 45-kD GLUT1 protein, the glial form, was significantly increased in the spinal cord. Previous studies on fetal brains have focused on severe hyperglycemia, associated with hypoinsulinemia, and quantified the protein levels of GLUT1 and GLUT3 (29). In this severe diabetic state, total fetal brain GLUT1 and GLUT3 protein were decreased, but this decrease was also present in the streptozotocin-nondiabetic group. This suggests a chemical effect of the drug independent from maternal diabetes. The authors concluded that hyperglycemia failed to alter fetal brain glucose transporter expression (29). Therefore, this suggests that hyperinsulinemia rather than hyperglycemia controls GLUT1 and GLUT3 expression in fetal brain. Other studies have examined the effects of hyperglycemia on adult brain GLUT1 level without consistent results. Indeed, it has been shown that GLUT1 expression was decreased, increased, or unchanged (30). The only modification that we observed was in the 45-kD GLUT1 glial isoform. A significant increase in this transporter was present only at the spinal cord level, where insulin binding was also increased.

Why hyperinsulinism should increase the 45-kD GLUT1 isoform only in this region without affecting the hypothalamic level is difficult to explain. To our knowledge, there are no data describing the effect of hyperinsulinism on glucose transport in the spinal cord nuclei which can explain disturbances in nervous control of metabolism. Except for the 45-kD GLUT1 in the spinal cord, the general absence of modification of glucose transporters in hypothalamus and spinal cord of HI-HG fetuses leads us to think that the hypothalamic alterations described above in hyperinsulinemic fetuses might not be due to glucose transport capacity, at least at the level of the neurons and the endothelial cells that composed the blood-brain barrier.

Apart from GLUT1 and GLUT3 isoforms, we have recently demonstrated that GLUT2 and GLUT4 were expressed in adult hypothalamic and spinal cord areas (15, 16). GLUT2 mRNA are undetectable at this age, whatever the technique used (data not shown).

Regarding GLUT4, its level is not affected except for increased mRNA in the spinal cords of HG-HI offspring which did not translate to the protein level. Such a discrepancy between mRNA and protein expression has already been reported in other tissues (31). GLUT4 mRNA and protein levels have already been described to be regulated in developing rat brain under hypoxia or throughout embryonic development (32, 33), thus supporting the view that insulin and/or glucose are probably not the main regulators of GLUT4 expression in the brain. The absence of modification of the insulin-dependant glucose transporter GLUT4 is concomitant with a large change in insulin binding, which is in line with the idea of an insulin-independent GLUT4 regulation in fetal brain. Recent studies have provided evidence that the cerebral insulin pathway could act independent of glucose transport to modify glucose homeostasis (33, 34).

In summary, our study provides evidence that insulin binding is increased in hypothalamic and extrahypothalamic-related nuclei in the offspring of hyperglycemic mothers. This increase was specific to areas involved in the nervous control of metabolism and could be a factor in glucose intolerance and impairment of insulin secretion exhibited by young-adult rats from HI-HG mothers. The deleterious effect of brain hyperinsulinism during the late gestation does not seem to act through glucose transport expression, inasmuch as no relationship between GLUT level and hyperinsulinism in brain areas has been observed. This does not exclude alterations in other targets of insulin action.

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