Initial Stages of the Insulin Signaling System in the Brain of Rats with Experimental Diabetes Mellitus

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We studied reactivity of insulin signal pathway elements, insulin receptor and insulin receptor substrate protein-2 (IRS2 protein), in rat brain in response to insulin insufficiency and insulin resistance during the development of experimental type 1 or type 2 diabetes mellitus. In type 1 diabetes mellitus characterized by acute insulin insufficiency, specific binding of insulin in rat brain increased 2-fold in comparison with the control and IRS2-gene expression in rat hypothalamus and cortex 2-4 fold surpassed the normal values. In type 2 diabetes mellitus (110 and 190 days of development), changes in the test parameters in rat brain were less pronounced. These findings attest to involvement of the brain insulin signal pathway into the response to systemic insulin deficiency in type 1 diabetes mellitus.

Key Words: *insulin; insulin receptor; IRS protein; brain; diabetes mellitus*

According to current views, insulin in CNS acts as a neurohormone that regulates a wide spectrum of physiological and biological parameters and processes, including glucose level, energy balance, reproductive function control, and memory processes [1,5,11]. Insulin signal pathway in the brain is similar to that in peripheral tissues [11]. Insulin receptor consists of two supermembrane hormone-binding α-subunits and two transmembrane β-subunits with tyrosine kinase activity. Insulin receptors were found in all brain regions, with highest concentration in the olfactory bulbs and hypothalamus arcuate nuclei [9]. Coupling of activated receptors with intracellular signaling pathways involves endogenous insulin receptor substrate proteins (IRS1-4 proteins) [11,14]. Substantial accumulation of IRS2 protein was observed in the brain, particularly in hypothalamus arcuate nuclei. Interaction of IRS protein with a variety of intracellular effector and adaptive proteins determines insulin regulation of mitogen-activated protein kinases, phospholipid pathway coupled with phosphatidyl inositol-3-kinase enzyme, and signal pathways dependent on protein tyrosine phosphatase activity [14].

Recent studies showed that dysfunction of the insulin signaling system in CNS leads to numerous metabolic disturbances and neurodegenerative diseases. Specific knockout of insulin receptor gene in mouse neurons results in the development of insulin resistance, diabetes mellitus (DM), and obesity [2]. IRS-2 protein gene homozygotic mice are phenotypically similar to knockout animals and have almost 3-fold lower brain weight in comparison with controls [12]. Despite substantial progress in understanding of the role of insulin in CNS functioning, molecular mechanisms of hormone action under normal and pathological conditions, particularly in DM of various etiology, remain poorly studied [7].

Here we studied the response of initial stages of insulin signal pathway in rat brain, insulin receptor and IRS2 protein, to systemic insulin deficiency and insulin resistance in the models of experimental type 1 and type 2 DM (DM1 and DM2).

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MATERIAL AND METHODS

Experiments were carried out on male Wistar rats. Experimental DM1 was induced by single streptozotocin administration (65 mg/kg, intraperitoneally) to 3-month old rats. Control animals received physiological saline (0.9% NaCl pH 4.5). DM1 development in rats was associated with pronounced hyperglycemia (22.1±1.0 mmol/liter), persistent glucosuria (28-56 mmol/liter) and polyuria. Neonatal DM2 was induced by streptozotocin administration (80 mg/kg) to 5-day old rat pups [6]. In 2.5-3 months, DM2 sings appeared detected using glucose tolerance test. Only rats with glycemia 1.5-2-fold surpassing the normal after glucose load test (2 mg/kg) were included in the study. Marked glucosuria (14-28 mmol/liter) was also noted in rats with DM2.

To obtain heterogeneous cell membrane fraction from rat brains, the cortex was homogenized in 1mM $NaHCO_3$ buffer pH 7.5. Homogenate was centrifuged at 8000*g* for 20 min (4°C). Supernatant was diluted in buffer and centrifuged at 80,000*g* for 30 min (4°C). Precipitate was resuspended and used in subsequent experiments. Protein concentration was measured by the method of Bradford; γ-globulin was used for construction of the calibration curve.

Specific binding of ^{125}I-insulin by rat brain cell membranes was assayed by radioligand method with plotting the curves of competitive displacement of receptor-bind labeled insulin by unlabeled hormone. The brain cell membrane fraction was incubated with ^{125}I-insulin in the presence of 0.1-1000 ng/ml of unlabeled hormone. Non-specific binding was determined by adding 10 μg/ml unlabeled insulin. Data of radioligand analysis was transformed into Scatchard coordinates to calculate K_D and R_O that characterize affinity and number of binding sites, respectively.

Expression of IRS2 protein gene was determined by the level of corresponding mRNA using RT-PCR. RNA isolation and purification were carried out using TRI-reagent (Sigma). Trace quantities of DNA were eliminated from RNA sample by DNAse I treatment (Fermentas). Reverse transcription was performed using RevertAid kit (Fermentas) in accordance with manufacturer specification using random primers. The following primers were used for PCR (for IRC2 protein mRNA): F: 5' GAG GAC TGA GGA AGA GGA C3', R: 5' GGT TAC TGC TGG AAC TCT TG3; product size 234 b.p. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a constitutive comparator gene: F: 5' CAA GGT CAT CCA TGA CAA CTT TG 3'; R: 5' GTC CAC CAC CCT GTT GCT GTA G 3'; product size 496 b.p. The reaction was carried out in a volume of 30 μl under the following conditions: (1) initial denaturation at 95°C for 5 min; (2) three-stage amplification consisting of 40 cycles, each included denaturation at 95°C for 20 sec, primer annealing (for IRS2 protein mRNA 52.9°C, for GAPDH mRNA 58°C) and elongation at 72°C for 30 sec; (3) final elongation for 3 min. Reaction mixture included PCR buffer (Fermentas) with $(NH_4)_2SO_4$, 0.2 mM each deoxyribonucleotide triphosphate, 2 mM $MgCl_2$, 1.5 μl cDNA, 15 pM primers, and 1 U Taq DNA polymerase (Fermentas). IRC2 protein gene expression was expressed as the ratio of PCR-product fluorescence to fluorescence of the amplificate encoding GAPDH. The obtained PCR product was separated from the gel using extraction kit (Fermentas) and sequenced (ATG Service Gene). The obtained sequences were verified to be the parts of IRS2 protein mRNA using BLAST software (www.ncbi.nlm.nih.gov).

The data were processed statistically using Student's *t* test for small samples and presented as mean± standard error of the mean from several independent experiments. The differences between the experimental and control samples were significant at $p<0.05$.

RESULTS

On day 30 of DM1 development, specific binding of ^{125}I-insulin in rat brain 2-fold surpassed that in control animals (Table 1). It was determined by changes in quantitative characteristics of both high- and low-affinity insulin receptors, which was seen from increased R_O values with constant K_i and K_D values for insulin receptors. On days 110 and 190 of DM2 development, specific binding of ^{125}I-insulin did not significantly dif-

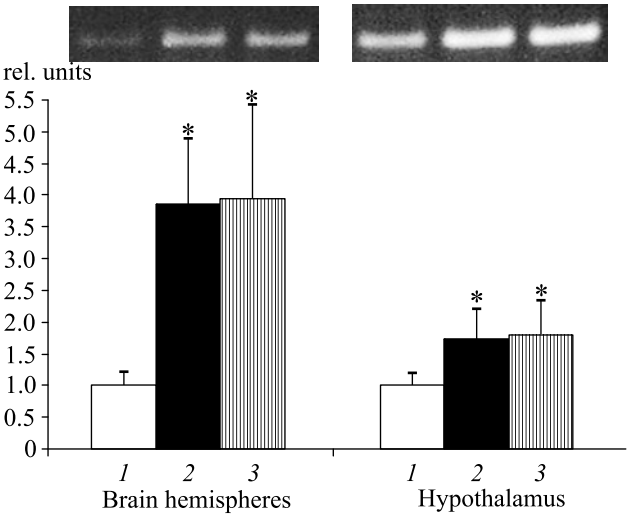

Fig. 1. Electrophoregrams (in 2% agarose gel) of IRS2 protein mRNA isolated from the cortex and hypothalamus of control rats (*1*) and rats with 15-day (*2*) and 30-day (*3*) DM1. Here and in Fig. 2: ordinate: IRS2 protein gene expression normalized to GAPDH gene and compared to respective values for control samples. *$p<0.05$ in comparison with the control.

TABLE 1. Receptor Binding of ^{125}I-Insulin in the Brain of Control and Diabetic Rats

Group	Binding, %/0.5 mg	K_i, nM	High-affinity receptors		Low-affinity receptors	
			K_D, nM	R_O, fmol/0.5 mg	K_D, nM	R_O, fmol/0.5 mg
Control, (*n*=7)	6.1±1.0	25.6±4.4	1.58±0.12	99±21	41.0±4.5	1153±290
DM1 15 days (*n*=5)	11.2±2.7	19.1±6.0	1.22±0.24	138±24	33.9±7.0	1219±206
DM1 30 days (*n*=5)	11.6±1.1*	26.4±4.9	1.47±0.15	189±30*	42.9±2.2	2154±281*
DM2 110 days (*n*=6)	5.4±0.6	31.7±7.7	1.50±0.19	89±21	48.7±4.7	1138±224
DM2 190 days (*n*=5)	4.8±0.7	25.7±1.4	1.60±0.05	75±12	55.8±10.8	1188±343

Note. *p<0.05 in comparison with the control.

fer from the control. Published data concerning insulin receptor reaction in the CNS to changes in hormone concentration are ambiguous. According to some experiments, the number of insulin receptors [10] and the level of the expression of corresponding gene [13] remained unchanged in DM, which was associated with independence of insulin receptors in CNS to the serum hormone level fluctuations. On the other hand, noticeable reduction of specific insulin binding and reduction of expression of insulin receptor gene in rats were observed after streptozotocin injection into brain ventricles [8]. At the same time, tyrosine kinase activity of insulin receptor β-subunits is decreased against the background of insulin resistance [3]. These data indicate disturbances in the receptor element of the insulin signaling system and impaired hormonal signal transduction through it. Our findings indicate that the insulin signaling system in rat brain responds to insulin deficiency (DM1) by enhanced receptor binding of the hormone, whereas insulin resistance (DM2) little affects binding properties of insulin receptors.

The expression of IRS2 protein in brain tissues from rats with 15-day DM1 4-fold surpassed that in the control in both hemispheres and 1.8-fold in the hypothalamus (Fig. 1). These changes persisted on day 30 of DM1. These findings attest to functional importance of IRS2 protein for the realization of insulin effect in the brain of diabetic rats. Increased IRS2 protein expression in the brain of DM1 rat was found to result in stimulation of initially suppressed phosphatidyl inositol-3-kinase activity, which finally potentiates the glycemic response to insulin administration [4]. At the same time, we found no significant differences between the levels of IRS2 protein mRNA in the cortex and hypothalamus of DM2 rats and control rats (Fig. 2). Only a trend toward stimulation of IRS2 protein gene expression was observed with increasing DM2 duration from 110 days to 190 days.

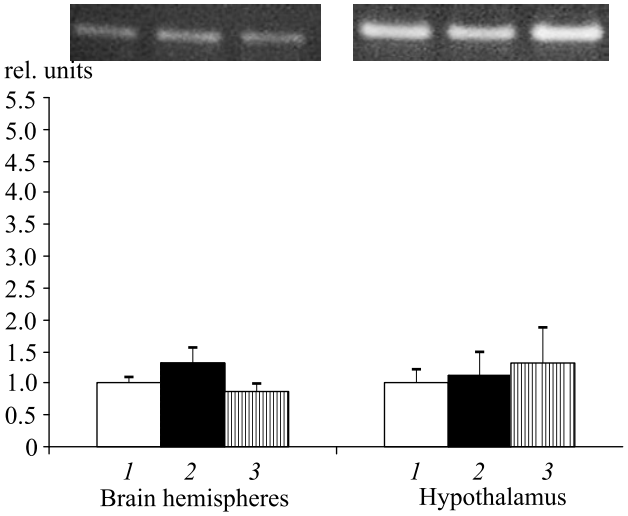

Fig. 2. Electrophoregrams (in 2% agarose gel) of IRS2 protein mRNA isolated from the cortex and hypothalamus of control rats (*1*) and rats with 110- (*2*) and 190-day (*3*) DM2.

Thus, experimental DM1 is associated with a 2-fold increase in the number of insulin receptors in rat brain and with 2-4-fold increase in the level of IRS2 proteins that are functionally coupled with them. It appears to be a compensatory reaction to acute hormone deficiency typical of DM1. At the same time, the profile of receptor binding and IRS2 protein expressionin the brain of rats with neonatal DM2 model remained practically unchanged, which attests to stability of the initial elements of insulin signaling system in DM2.

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