Intranasal insulin affects adenyl cyclase system in rat tissues in neonatal diabetes

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Abstract: The changes in hormone-regulated adenyl cyclase (AC) signaling system implicated in control of the nervous, cardiovascular and reproductive systems may contribute to complications of diabetes mellitus (DM). We investigated the functional state of AC system in the brain, myocardium, ovary and uterus of rats with neonatal DM and examined the influence of intranasally administered insulin on the sensitivity of this system to biogenic amines and polypeptide hormones. The regulatory effects of somatostatin and 5-HT, R-agonist 5-nonyloxytryptamine acting via G protein-coupled receptors were significantly decreased in DM and partially restored in insulin-treated rats. The effects of hormones, activators of AC, are changed in tissue- and receptor-specific manner, and intranasal insulin restored the effects rather close to the level in control. In insulin-treated non-diabetic rats, AC stimulating effects of isoproterenol and relaxin in the myocardium and of human chorionic gonadotropin in the ovaries were decreased, while the effects of hormones, inhibitors of AC, were increased. These data indicate that with intranasal insulin, G protein-mediated signaling pathways continue to gain strength. The obtained data on the influence of hormones on AC system in the brain, myocardium, ovary and uterus allow looking anew into the mechanisms of therapeutic effects of intranasal insulin.

Keywords: Adenylyl cyclase signaling system • Brain • Diabetes mellitus • Gonadotropin • Insulin • Myocardium • Ovary • Somatostatin • Uterus

Abbreviations:

AC - adenylyl cyclase;
AC system - adenylyl cyclase signaling system;
DM - diabetes mellitus;
EMD-386088 - 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole;
GppNHp - βγ-imidoguanosine-5′-triphosphate;
5-HT receptor - 5-hydroxytryptamine receptor;
hCG - human chorionic gonadotropin;
PACAP-38 - pituitary adenylyl cyclase-activating peptide-38.

1. Introduction

Hypertension, coronary heart diseases, atherosclerosis, nephropathy, retinopathy, neuropathy, cognitive deficit and disorders of the reproductive system are the most common complications of diabetes mellitus (DM) [1-3]. Chronic hyperglycemia is quite often an important contributing factor in the development of these complications. Many hormone- and growth factor-regulated signaling pathways, such as the adenylyl cyclase (AC) signaling system, phospholipase C/protein kinase C signaling system and the cascade of mitogen-activated protein kinases are implicated in the regulation of the cardiovascular, nervous and reproductive systems and aberration of their functional activity in DM may contribute to complications of this disease [4-8]. It has been shown that in experimental types 1 and 2 DM the functional activity of hormone-sensitive AC system in the skeletal muscles, myocardium, testis, ovaries, uterus and brain of diabetic rats and the sensitivity of this system to hormones regulating AC activity are changed in tissue- and hormone-specific manner [6-12]. The most significant changes of hormonal sensitivity of AC system were found in the myocardium, testis and...
ovaries while in the brain the changes were not so pronounced, if affected at all. In most tissues of diabetic animals the effects of hormones acting on AC via G proteins of the inhibitory type (Gi) were changed to a greater degree compared with those of hormones acting on the enzyme via G proteins of the stimulating type (Gs), which is likely to be due to a decrease of protein expression and a reduction of their functional activity. Generally, in DM changes in the expression of some Gi- and Gs-coupled receptors, β-adrenergic receptors in particular, regulating the AC activity are also observed. However, the function of AC, catalytic component of AC system, and downstream signal proteins remain virtually unchanged.

In our view, the main disturbances in the AC system due to DM occur on the initial stages of signal transduction and are associated with changes in the expression of G proteins and hormonal receptors and violation of their functional interaction. There are reasons to regard the disturbances in hormone-regulated AC system, on the one hand, as a contribution in the development of dysfunctions of the cardiovascular, reproductive and other systems in DM leading to the complications of the disease and, on the other hand, as a compensatory response to physiological and biochemical changes occurring in a diabetic state [8,11]. It is known that the therapy of diabetic patients and experimental animals with insulin and other sugar-lowering drugs leads to normalization of the glucose level and restores the biochemical processes and physiological functions altered in DM. At the same time, the influence of the therapy on the functioning of hormonal signaling systems remains poorly studied and no information is available on the functional state of AC system in this case.

Insulin and its analogs with prolonged action find wide application in the treatment of insulin-sensitive DM, referred to as type 1 DM, as well as of insulin-independent DM, type 2 DM, especially at a later stage of this disease [13]. About 27% of diabetic patients continuously apply insulin for the management of their hyperglycemia [14]. In the recent years the method of insulin delivery by intranasal routes has been applied on a large scale. Intranasal insulin improves learning and memory, prevents cognitive decline, cerebral atrophy, and focal cerebral ischemia, and reduces food intake and body weight [15,16]. However, the molecular mechanisms and targets of intranasal insulin action have not been well defined. The involvement of hormonal signaling systems, AC system in particular, and their role in the intranasal insulin-mediated control of glucose homeostasis, energy metabolism, memory, and feeding behavior are not clear yet.

The present work was undertaken with a view to study how intranasal delivery of insulin affects the hormone-sensitive AC system in the brain and peripheral tissues of female rats with neonatal model of type 2 DM similar by some characteristics to insulin-insensitive type 2 DM. We investigated the effects of 5-week treatment with intranasal insulin on the basal and hormone-regulated AC activity and G protein GppNHp-binding in the brain, myocardium and the tissues of reproductive system (uterus, ovaries) of diabetic animals, and compared them with those of diabetic rats without insulin therapy and with control animals. To estimate the tissue and hormone specificity of changes in DM in each case, we studied AC effects of hormones activating the enzyme via Gs protein-coupled receptors and hormones that, on the contrary, decrease AC activity and stimulate Gi proteins.

2. Experimental Procedures

2.1 Animals

For experiments, adult female Wistar rats housed in plastic sawdust-covered cages with a normal light–dark cycle and free access to food and water were obtained. The experiments were carried out under the guidelines of the National Institutes of Health regulations for the Care and Use of Animals for Scientific Purposes. All efforts were made to minimize animal suffering and reduce the number of animals used.

Neonatal insulin-independent DM was provoked by intraperitoneal administration of streptozotocin (STZ) dissolved in 0.9% NaCl solution, pH 4.5, at the dose of 80 mg/kg of body weight in newborn (5-day-old) rats [17]. Animals of control groups received acidified physiological solution. Using glucose tolerance test we showed that 5–6 month old rats with a neonatal model of DM have pronounced insulin resistance, typical of insulin-independent DM. It was shown that 2 hours after glucose loading (2 g/kg of body weight) in control rats, the concentration of sugar in blood reached a normal level, while in diabetic rats it did not. The glucose measurements in the whole blood from the tail vein were performed using test strips (One Touch Ultra, USA) and a glucometer (Life Scan Johnson & Johnson, Denmark). Diabetic rats had glucosuria. The monitoring of glucose in the urine was determined using test strips (Combi-Screen Analyticon, Germany). Alongside, we observed a moderate hypoinsulinemia in rats with neonatal DM, which is due to damage of β-cells in the neonatal period of rat development [18]. The insulin concentrations in the serum of diabetic and control rats were 0.6±0.3 and 1.1±0.2 μg/L, respectively. The insulin concentration...
in rat serum was determined using Rat Insulin ELISA (Mercodia AB, Sweden).

Four groups of animals were investigated: control animals \( (n=8) \), body weight at the end of experiment 283±17 g, the glucose level in whole blood 4.92±0.34 mM, Group 1), control animals with intranasally administered insulin \( (n=7) \), 271±14 g, 4.78±0.36 mM, Group 2), animals with 240-days neonatal model of type 2 DM \( (n=8) \), 313±15 g, 6.75±0.37 mM, Group 3), diabetic animals with intranasally administered insulin \( (n=7) \), 290±14 g, 6.21±0.31 mM, Group 4).

2.2 Intranasal delivery of insulin

Intranasal delivery of insulin to the rat brain was performed as described previously by Thorne and coworkers [19]. Crystalline insulin at concentration 24 IU/ml was dissolved in 0.9% NaCl solution, pH 4.5, and delivered intranasally to both diabetic and non-diabetic rats once a day. Each rat was placed in a supine position and then an average of 20 ml of insulin solution (0.48 IU) was administered by Eppendorf pipette as 5-ml drops in each nostril, in turn, every 1–2 min. Control animals were given the equal volume of saline, pH 4.5.

2.3 Chemicals and radiochemicals

The chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (San Diego, CA, USA). Streptozotocin, \( \beta,\gamma\)-imidoguanosine-5′-triphosphate (GppNHp), human chorionic gonadotropin (hCG), somatostatin-14, pituitary adenyl cyclase-activating peptide-38 (PACAP-38), serotonin, isoproterenol and bromocryptine were purchased from Sigma-Aldrich (St. Louis, MO, USA), 5-nonyloxytryptamine and 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H-indole (EMD-386088) were purchased from Tocris Cookson Ltd. (United Kingdom). Human relaxin-2 was kindly provided by Prof. J. Wade (Howard Florey Institute, University of Melbourne, Australia). \( [\alpha,\beta\gamma P] \)-ATP (4 Ci/mmol) was purchased from Isotope Company (St. Petersburg, Russia), \( \beta,\gamma\)-imidol[8-\( ^{3}H \)]-guanosine-5′-triphosphate (\( \beta,\gamma\)-GT) (5 Ci/mmol) was from Amersham (UK); the type HA 0.45 \( \mu \)m nitrocellulose filters were from Sigma-Aldrich Chemie GmbH (Germany).

2.4 Experimental design

The functional activity of AC system was investigated in the brain, myocardium and tissues of the reproductive system of diabetic and control rats (Figure 1). To study the efficiency of transduction of serotonin signal stimulating or inhibiting the AC activity in the brain, we used serotonin, a non-selective agonist of different types of 5-HT receptors coupled with AC in the stimulatory or inhibitory manner; 5-nonyloxytryptamine, a selective agonist 5-HT\(_{1A}\) receptor coupled with G\(_{i}\) proteins; and EMD-386088, a selective agonist of 5-HT\(_{6}\) receptor coupled with G\(_{s}\) proteins. To study the functional state of the dopaminergic AC system in the brain we investigated the inhibiting AC effect of bromocryptine, a selective agonist of D\(_{2}\)-dopamine receptors. Somatostatin realizing their effects via G\(_{i}\) protein-coupled somatostatin receptors was used to study the alterations in the somatostatin-regulated AC system in the brain, myocardium and reproductive tissues of diabetic rats. To study the efficiency of transduction of the stimulating signals via AC system in the myocardium we chose isoproterenol, a relatively selective agonist of \( \beta_{1}\)-adrenergic receptors, used in the treatment of bradycardia and heart block, and relaxin-2, peptide hormone of the insulin family acting via G\(_{s}\) protein-coupled relaxin receptor of type 1. In the tissues of the reproductive system we also studied the stimulating effects of PACAP-38 acting via G\(_{s}\) protein-coupled PAC1 receptor and hCG, a glycoprotein hormone produced during pregnancy, which binds to the cognate luteinizing hormone/hCG receptor coupled with AC via G\(_{s}\) protein. To study the guanine nucleotides binding ability of G proteins we used GppNHp, a synthetic non-hydrolysable analogue of GTP resistant to intrinsic GTPase activity of G protein \( \alpha \) subunits.

2.5 Plasma membrane preparation

The diabetic and non-diabetic animals were sacrificed 24 hours after the last intranasal administration of insulin or saline, and the brain, myocardium (ventricles), ovaries and uterus were rapidly removed and frozen before preparing plasma membrane fractions.

The preparation of cardiac membranes from the rat myocardium was performed according to Baker and Potter [20], with some modifications. The dissected hearts were placed in ice-cold 0.9% NaCl and atria, fat and valves were removed. The tissues were cut into small pieces, homogenized with a Polytron in 20 volumes of ice-cold 40 mM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl\(_{2}\), 320 mM sucrose and a cocktail of protease inhibitors 500 \( \mu \)M O-fenatrinol, 2 \( \mu \)M pepstatin and 1 mM phenylmethylsulphonyl fluoride (Buffer A) and centrifuged at 480xg for 10 min at 4°C. The pellet was discarded and the supernatant were centrifuged at 27500xg for 20 min at 4°C. The pellet was resuspended in the buffer A (without sucrose) and then centrifuged at 27500xg for 20 min.

The preparation of synaptosomal membranes from the rat brain was performed as described earlier [21]. The brain tissues were dissected on ice and homogenized with a Polytron in 30 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM
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MgCl₂, 2 mM EGTA, 10% (w/v) sucrose and a cocktail of protease inhibitors (Buffer B). The obtained material underwent centrifuge procedures, each performed at 4°C. The crude homogenate was centrifuged at 1000xg for 10 min; the resulting pellet was discarded and the supernatant was centrifuged at 9000xg for 20 min. The pellet was resuspended in the buffer B (without sucrose) and centrifuged at 35000xg for 10 min.

The isolation of plasma membrane fractions from ovaries and uterus was carried out as described previously [8]. The ovaries and uterus were placed in ice-cold Buffer A and homogenized with a Polytron. The homogenate was centrifuged at 1500xg for 10 min at 4°C. The supernatant was centrifuged at 20000xg for 30 min at 4°C. The resulting pellet was washed by resuspension in 10 volumes of Buffer A (without sucrose) and centrifuged again at 20000xg for 30 min.

The final pellet was resuspended in the 50 mM Tris-HCl buffer (pH 7.4) to produce the membrane fraction with a protein concentration range of 1–3 mg/ml and stored at -70°C. The protein concentration of each membrane preparation in all experiments was measured by the method of Lowry and colleagues using BSA as a standard.

2.6 Adenylyl cyclase assay

The adenylyl cyclase (AC) (EC 4.6.1.1) activity was measured using the method of Salomon and colleagues [22], with some modifications [12]. The reaction mixture (final volume 50 µl) contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM ATP, 1 µCi [α-³²P]-ATP, 0.1 mM cAMP, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 15–45 µg of the membrane protein. Incubation was carried out at 37°C for 10 min. The reaction was initiated by the addition of membrane protein and terminated by the addition of 100 µl of 0.5 M HCl, followed by immersing the tubes with mixture in a boiling water bath for 6 min. 100 µl of 1.5 M imidazole was added to each tube. In these conditions the AC activity was linear. [³²P]-cAMP formed as a result of

Figure 1. Hormone-sensitive adenylyl cyclase signaling system including the receptors of the serpentine type regulated by biogenic amines and peptide hormones. Through Gs proteins hormones stimulate functional activity of adenylyl cyclase (AC) catalyzing the formation of second messenger cAMP and trigger cAMP-dependent signaling cascades involved in the regulation of fundamental cellular processes, and through Gi proteins they also inhibit the enzyme activity and block cAMP-dependent pathways. Abbreviations: αsbg and αi bg, heterotrimeric Gs and Gi proteins; β2-AR, β2-adrenergic receptor; D2-DR, dopamine receptor of the type 2; LH/hCG-R, luteinizing hormone/ human chorionic gonadotropin receptor; 5-HT₂R and 5-HT₃R, 5-hydroxytryptamine receptors of the subtype 1B and the type 6, respectively; PAC1R, PACAP receptor of the type 1; RXFP1R, relaxin receptor of the type 1; SomR, somatostatin receptors of the types 1–5.
the enzyme reactions was separated using alumina for column chromatography. The samples were placed on neutral alumina columns and cAMP was eluted with 8 ml of 10 mM imidazole-HCl buffer (pH 7.4). The eluates were collected in scintillation vials and counted using a LS 6500 scintillation counter (Beckman Instruments Inc., USA). Each assay was carried out in triplicate at least three times, and the results were expressed as pmol cAMP/min per mg of membrane protein. The basal activity was measured in the absence of hormones and forskolin. To measure AC inhibition by hormones, the enzyme was activated by forskolin (10^{-5} M).

2.7 GppNHp binding assay

[8-3H]-GppNHp binding to G proteins was determined using the method of McIntire and colleagues [23], with some modifications [12]. The reaction mixture (final volume 50 μl) contained 25 mM HEPES-Na buffer (pH 7.4), 1 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 1 μM GppNHp, 0.1% BSA, 0.5–1 μCi [8-3H]-GppNHp. The reaction was started by the addition of 50–100 μg of membrane protein and carried out at 30°C for 45 min. After incubating, the reaction mixture was rapidly diluted with 100 μl of the washing buffer (20 mM K⁺-Na⁺ phosphate buffer, pH 8.0) containing 0.1% Lubrol-PX and the samples were filtered under vacuum through 0.45 μm nitrocellulose filters (type HA). In each case, the filter was washed three times with 2 ml of washing buffer and dried. The filter-bound radioactivity was estimated in a toluene scintillator using a LKB 1209/1215 RackBeta scintillation counter. 10 mM GppNHp was added to the reaction mixture to estimate non-specific binding. The specific GppNHp binding was determined as the difference between a total and non-specific binding. Each assay was carried out in triplicate at least three times and the results were expressed as pmol [8-3H]-GppNHp per mg of membrane protein.

2.8 Statistical analysis

The data is presented as the mean ± SEM. The difference in the basal activity of AC or the basal level of GppNHp binding in the tissue membrane fractions of control and diabetic animals as well as the difference in the AC activity or GppNHp binding in the membrane fractions treated by hormones and non-hormonal AC regulators in each case was statistically assessed using one-way analysis of variance (ANOVA) and considered significant at P<0.05.

3. Results

The AC activity in the brain and the uterus in control and diabetic animals is changed insignificantly after intranasal administration of insulin (Table 1). In the myocardium of diabetic animals (Group 3) the basal activity of the enzyme is increased and intranasal administration of insulin returns it to control level. In the ovaries of diabetic rats the basal AC activity is markedly decreased compared with control and partially restored in insulin-treated animals. GppNHp, non-hydrolysable analog of GTP, at 10^{-5} M concentration stimulates AC activity in the brain, myocardium, ovaries and uterus by 181, 223, 144 and 204% over its basal level, respectively. The stimulating effects of GppNHp on AC activity in the ovaries and, to a lesser extent, in the uterus of diabetic rats are decreased, but in the ovaries of insulin-treated rats, unlike in the uterus, they are restored to control level (Figure 2). At the same time, GppNHp effects in the myocardium and brain of diabetic animals do not differ significantly from those in control (data not shown). AC stimulating effect of diterpene forskolin (10^{-5} M) interacting directly with the catalytic site of the enzyme practically does not differ in the tissues of all animals under study (data not shown). The basal level of GppNHp binding shows a 12–28% decrease in the myocardium, ovaries and uterus of diabetic rats while in the brain it does not differ from control (Table 2). Insulin administration does not influence significantly the basal level of GppNHp binding in control animals and partially restores it in rats with DM. These data give evidence that in the case of neonatal DM changes in the basal activity of

<table>
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<tr>
<th>The group of animals</th>
<th>Myocardium</th>
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<th>Ovaries</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Control rats (Group 1)</td>
<td>26.7 ± 1.3</td>
<td>23.6 ± 1.0</td>
<td>13.4 ± 1.1</td>
<td>19.7 ± 1.8</td>
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<td>Control rats + insulin (Group 2)</td>
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<td>18.4 ± 0.7</td>
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<td>Diabetic rats (Group 3)</td>
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<td>22.2 ± 0.5*</td>
<td>10.1 ± 1.3*</td>
<td>18.8 ± 1.5</td>
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<td>Diabetic rats + insulin (Group 4)</td>
<td>26.6 ± 1.3*</td>
<td>23.0 ± 1.1</td>
<td>12.3 ± 1.1*</td>
<td>19.0 ± 1.6</td>
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Table 1. The basal activity of adenylyl cyclase (AC) in the tissues of control and diabetic rats without treatment by insulin and with intranasal insulin (5 weeks, a daily dose of hormone is 0.48 IU per each animal).

Values are expressed as the mean ± SEM of three individual experiments. * P<0.05 vs. Group 1, # P<0.05 vs. Group 3.
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AC and in the basal level of GppNHp binding are tissue-specific and are best expressed in the myocardium and ovaries. The fact that forskolin-stimulated AC activity in the tissues of all groups of animals remains the same, while the basal and GppNHp-stimulated enzyme activity and the basal level of GppNHp binding change in DM, suggests that the disturbances in the AC system in neonatal DM originate mostly at the level of functional coupling between receptors and G proteins.

The study of the sensitivity of AC and Gs proteins to hormones, activators of AC system, in the case of neonatal DM and the influence of intranasal insulin treatment on this system gives the following results. In the myocardium of diabetic rats, the AC stimulating effect of β-adrenergic agonist isoproterenol is slightly increased, while the corresponding effect of peptide hormone relaxin is a little decreased (Figure 3A). Insulin treatment of diabetic rats decreases AC effect of isoproterenol, compared to its values in control groups, but does not influence AC effect of relaxin. The stimulation of GppNHp binding by isoproterenol in the myocardium of diabetic animals is similar to that in the control group and does not change in insulin-treated diabetic rats (Figure 4A). In the brain of diabetic animals, the stimulating effects of serotonin acting on different types of 5-HT receptors and EMD-386088, a selective agonist of 5-HT6 receptor, are slightly increased (Figure 3B) At the same time, the GppNHp binding stimulating effect of serotonin in the brain is drastically decreased and, in the insulin-treated rats, partially restored (Figure 4B). In the ovaries of diabetic animals the weakening of both AC and GppNHp binding stimulating effects of hCG and PACAP-38 is observed (Figure 3C and 4C). Intranasal insulin restores to some extent the stimulating effects of hCG in the ovary, but does not influence the corresponding effects of PACAP-38. In the uterus of diabetic rats AC and GppNHp binding stimulating effects of relaxin are slightly decreased, while the corresponding effects of PACAP-38 do not change (Figure 3D and 4D). The effects of these hormones in the uterus of diabetic rats with and without insulin treatment are the same. All this suggests that the alterations of sensitivity of the AC system to hormones stimulating the enzyme activity are tissue- and receptor-specific, and the molecular mechanisms underlying a change of sensitivity in each case depend on a particular hormone. In some cases, insulin treatment has a significant influence on AC and Gs protein stimulating effects of hormones, which change in DM, and leads to their complete or partial restoration.

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It is very important that the intranasal delivery of insulin in control rats induced changes in the sensitivity of the AC system to a number of hormones stimulating AC and Gs proteins. It was shown that AC stimulating effects of isoproterenol and relaxin in the myocardium and of hCG in the ovaries are decreased but the effect of serotonin in the brain is slightly increased (Figure 3). The stimulating effects of serotonin and EMD-386088 in the brain and PACAP-38 in the uterus on GppNHp

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<tr>
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<td>Diabetic rats (Group 3)</td>
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<td>4.4 ± 0.1</td>
<td>1.1 ± 0.1*</td>
<td>1.7 ± 0.2*</td>
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<td>Diabetic rats + insulin (Group 4)</td>
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<td>4.6 ± 0.1</td>
<td>1.3 ± 0.1*&amp;</td>
<td>1.9 ± 0.1</td>
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Table 2. The basal level of GppNHp binding in the tissues of control and diabetic rats with and without treatment by insulin.

Values are expressed as the mean ± SEM of three individual experiments. * P<0.05 vs. Group 1, & P<0.05 vs. Group 2.
Figure 3. The stimulating effects of hormones on adenylyl cyclase activity in the myocardium (A), brain (B), ovaries (C) and uterus (D) of diabetic and control rats. 1, 2, 3 and 4 – Groups 1, 2, 3 and 4, respectively. Values are the mean ± SEM of four individual experiments, each performed in triplicate. * P<0.05 vs. Group 1, & P<0.05 vs. Group 2, # P<0.05 vs. Group 3.

Figure 4. The stimulating effects of hormones on GppNHp binding in the myocardium (A), brain (B), ovaries (C) and uterus (D) of diabetic and control rats. 1, 2, 3 and 4 – Groups 1, 2, 3 and 4, respectively. Values are the mean ± SEM of three individual experiments, each performed in triplicate. * P<0.05 vs. Group 1, & P<0.05 vs. Group 2, # P<0.05 vs. Group 3.
binding were found to gain strength in insulin-treated control rats (Figure 4). These data show that intranasal insulin influences in tissue- and receptor-specific manner the response of the AC system to hormonal activators of AC and G proteins not only in rats with neonatal DM characterized by insulin resistance but also in control rats with functionally active insulin signaling system both in the brain and the peripheral tissues.

Then we studied the regulatory effects of hormones, inhibitors of AC activity, in the tissues of control and diabetic animals. AC inhibiting effect of hormones is estimated by their influence on the enzyme activity stimulated by forskolin ($10^{-5}$ M). The inhibitory effects of peptide hormone somatostatin on forskolin-stimulated AC activity and somatostatin-induced stimulation of GppNHp binding in the myocardium and uterus of diabetic rats are significantly decreased but in the ovaries and brain only a little (Figure 5 and 6). Intranasal insulin partially or completely restored the effects of somatostatin. It is shown that AC inhibiting effect of 5-nonyloxytryptamine, a selective agonist of 5-HT$_{1B}$ receptor, and its stimulating effect on GppNHp binding in the brain of diabetic rats are both significantly decreased, while the corresponding effects of bromocryptine, agonist of D$_2$-dopamine receptor, remain unchanged (Figure 5). The intranasal insulin leads to complete restoration of AC inhibiting effect of 5-nonyloxytryptamine and significantly increases its stimulating effect on GppNHp-binding in the brain of diabetic rats. It should be pointed out that the insulin treatment of control animals induces increase of both AC inhibiting and GppNHp stimulating effects of somatostatin in all tissues under study; the same refers to the effects of 5-nonyloxytryptamine and bromocryptine in the brain (Figure 5 and 6). Thus, in DM the regulatory effects of hormones, inhibitors of AC, with the exception of D$_2$-agonist bromocryptine, are decreased giving evidence for the existence of a common mechanism responsible for attenuation of G$_i$ protein-coupled AC signaling in the tissues of diabetic rats. The intranasal

**Figure 5.** The inhibitory effects of somatostatin, 5-nonyloxytryptamine and bromocryptine on forskolin-stimulated adenylyl cyclase activity (A) and their stimulating effects on the basal level of GppNHp binding (B) in the brain of diabetic and control rats. 1, 2, 3 and 4 – Groups 1, 2, 3 and 4, respectively. Adenylyl cyclase stimulating effect of forskolin ($10^{-5}$ M) is 263% and taken as 100%. Values are the mean ± SEM of three individual experiments, each performed in triplicate. * P<0.05 vs. Group 1, & P<0.05 vs. Group 2, # P<0.05 vs. Group 3.

**Figure 6.** The inhibitory effects of somatostatin on forskolin-stimulated adenylyl cyclase activity (A) and its stimulating effects on the basal level of GppNHp binding (B) in the myocardium, ovaries and uterus of diabetic and control rats. 1, 2, 3 and 4 – Groups 1, 2, 3 and 4, respectively. Adenylyl cyclase stimulating effects of forskolin ($10^{-5}$M) in the myocardium, ovaries and uterus reach 439, 212 and 354%, respectively, and taken as 100%. Values are the mean ± SEM of three individual experiments, each performed in triplicate. * P<0.05 vs. Group 1, & P<0.05 vs. Group 2, # P<0.05 vs. Group 3.
insulin induces partial or complete restoration of the effects of these hormones in diabetic rats and gives a substantial, though to a different extent, contribution to the effects in the tissues of control animals. These findings suggest that intranasal insulin has influence on the functional activity and/or expression of G proteins responsible for the transduction of inhibiting signal from G\textsubscript{i}-coupled receptors.

4. Discussion

As demonstrated in our previous studies, chronic hyperglycemia characteristic of insulin-deficient streptozotocin type 1 DM and short-time hyperglycemia induced by acute glucose load lead to changes in the basal level of GTP binding of G proteins [6,11,24]. It has been shown that in the skeletal muscles, myocardium and reproductive tissues of rats with 10–30-days streptozotocin type 1 DM the basal level of GppNHp binding is below control and changes a little only in the brain [11,12]. In the present study we used rats with 240-days neonatal DM, similar to type 2 DM in humans, induced by STZ treatment of newborn rats [17]. Typically, STZ treatment leads to destruction of \(\beta\)-cells and, as a result, insulin production is almost completely blocked. However, at the early stages of rat development (the first week after birth) a partial restoration of insulin-producing function of \(\beta\)-cells is observed owing to their regeneration. As a result, STZ treatment of infant rats induces type 2 DM in 70% of the animals at the age of 2.5–3 months [18]. It is shown that GppNHp binding in the membrane preparations isolated from the myocardium, uterus and ovaries of rats with neonatal DM is decreased, as in the case of type 1 DM, but to a lesser degree. This is likely to be due to a weaker form of hyperglycemia, typical of neonatal DM, which is one of the key factors regulating the expression of genes encoding G protein \(\alpha\) subunits and, as a consequence, responsible for their level in cells [25].

In our view, a low basal level of GppNHp binding in the myocardium and tissues of the reproductive system of rats with neonatal DM should primarily be ascribed to a decreased expression of G\(_i\) proteins, rather than of the other types of G proteins. This is in good agreement with many reports about abnormalities in expression and functional activity of heterotrimeric G proteins, G\(_i\) proteins in particular, in the case of DM and a high level of plasma glucose [9,25-32]. A decrease of expression of G\(_i\) protein \(\alpha\) subunits is revealed in the tissues of diabetic patients and animals with different models of DM and insulin resistance-associated obesity. The expression of G\(_{\alpha_{i1}}\) and G\(_{\alpha_{i2}}\) is decreased by 40% in the liver of patients with type 2 DM [28]. The expression of G\(_{\alpha_{i1}}\) and G\(_{\alpha_{i2}}\) is also markedly decreased in the membranes isolated from the liver of rats with streptozotocin DM and diabetic db/db mice [9,26]. The level of all subtypes of G\(_{\alpha_{i1}}\) subunits is modestly decreased in the blood vessels from rats with streptozotocin-induced DM [30]. In the platelets from patients with type 2 DM the levels of G\(_{\alpha_{i1}}\) and G\(_{\alpha_{i3}}\) subunits are 49 and 75%, respectively, of those in platelets from control subjects, while G\(_{\alpha_{i1}}\) is not expressed at all [27]. A similar picture has been observed in the hepatocytes from streptozotocin-treated rats [33]. Studying G protein expression in various tissues of diabetic ob/ob mice, it has been shown that the alteration in expression of G\(_{\alpha_{i1}}\) subunits is tissue-specific. The expression of G\(_{\alpha_{i1}}\) in the myocardium, G\(_{\alpha_{i3}}\) in the liver and G\(_{\alpha_{i3}}\) in adipose tissue is decreased, while the expression of G\(_{\alpha_{i2}}\) subunits in the brain and the testes does not differ significantly in lean and ob/ob mice [29]. The levels of G\(_{\alpha_{i2}}\) and G\(_{\alpha_{i3}}\) subunits are markedly decreased both in aortic vascular smooth muscle cells from streptozotocin-diabetic rats and in A10 vascular smooth muscle cells line from rat embryonic thoracic aorta exposed to high concentrations of glucose [25].

Proceeding from what is mentioned above on a decrease of the basal level of GTP-binding in neonatal DM, we put forward a suggestion that weakening of AC inhibitory signaling pathways triggered by hormones that act via G\(_i\)-coupled receptors detected in the tissues of diabetic rats may well be associated with a decrease of the expression and functional activity of G proteins, especially so as this extends to various tissues and is typical of hormones of different nature. In our case, there was weakening of the inhibiting effect of somatostatin on forskolin-stimulated AC activity and of the stimulating effect on GppNHp binding in the tissues of diabetic animals. The most significant changes in somatostatin effects are found in the muscle tissues. In the brain of rats with DM AC inhibiting effect of 5-nonyloxytryptamine, a selective agonist of 5-HT\(_{1B}\) receptor, and its stimulating effect on GppNHp binding were decreased. As it is known, the regulatory action of somatostatin and 5-HT\(_{1B}\)-agonists on AC activity is realized via receptors of the serpentine type coupled with G\(_i\) proteins [34,35]. This is in good agreement with our data and that of other authors about weakening of AC response to hormones acting via G\(_i\)-coupled receptors in the tissues of rats with the experimental models of DM and with acute hyperglycemia [4,7,11,24,32]. At the same time, a decrease of expression of G\(_i\) proteins is not the only factor affecting the functional activity of G\(_i\)-coupled signaling cascades. In the case of bromocriptine that acts via G\(_i\)-coupled dopamine
receptor of type 2 the AC inhibitory effect in the brain of diabetic rats was shown to be slightly different from that of control animals. This might be due to enhanced expression of D₂-dopamine receptors or to the changes of their binding characteristics, which compensates for a decreased level of G protein expression and maintains the efficiency of bromocryptine-induced signaling at the level similar to that in control. This suggestion finds confirmation in the recent investigation showing that the Bₘₐₓ of a total dopamine receptor binding and the expression of D₁- and D₂-dopamine receptors in the cerebral cortex of rats with streptozotocin DM are increased [36,37]. Some authors have pointed out that in their investigations, the expression of Gαs and Gαq subunits in diabetic tissues are, as a rule, either unchanged or increased [9,27-31,33,38]. Using Western blot analysis, a 2.5-fold increase in the level of Gαq11 subunits has been shown in aortic membranes of rats with type 1 DM compared with control animals and in adipocyte membranes from the db/db mice [30,31]. No changes in Gαq subunit have been detected in the platelets and in the liver of diabetic patients with type 2 DM [27,28], in the blood vessels, brain, heart, skeletal muscle, kidney and adipose tissue of streptozotocin-treated rats [30,33,38], in the brain of ob/ob mice and in the liver of db/db mice [9,29], while the level of Gαq subunits in hepatocyte membranes from rats with type 1 DM is significantly increased [26]. Our data demonstrated that the basal AC activity in the myocardium of diabetic rats is increased and the AC stimulating effects of GppNHp and forskolin are unchanged. There were no significant changes of the basal, forskolin-and GppNHp-stimulated AC activity in the brain and uterus of diabetic rats. The stimulating effects of hormones, the activators of AC, on the enzyme activity and GppNHp binding of G proteins remained the same or in some cases were decreased a little. All this serves as indication that the function of Gs proteins in the case of neonatal type 2 DM is preserved, while the function of Gi proteins, as mentioned above, is impaired.

We observed a slight increase of AC stimulating effect of β-agonist isoproterenol in the myocardium of DM rats. It coincides with the data on preservation or strengthening of Gs-coupled pathway regulated by adrenergic agonists that mediate the AC stimulation in the tissues of animals with type 2 DM and DM-like states characterized by insulin resistance and obesity [7,39,40]. In the liver membranes obtained from 8-12-week ob/ob mice with hyperglycemia and hyperinsulinemia, there is a threefold increase in the number of β²-adrenergic receptor binding sites and a threefold increase in the response to catecholamines compared to the preparations from control mice [39]. In the myocardium of spontaneously diabetic Chinese hamsters with cardiomyopathy, there is a considerable increase of the density of β-adrenergceptors and the response of AC to isoproterenol, preceding the development of cardiac hypertrophy [40]. At the same time, the functioning of adrenergic signaling system in type 1 DM differs from that in type 2 DM. In animals with streptozotocin insulin-deficient DM some authors have revealed redistribution of the β-adrenergceptors [41-43] and weakening of their functional activity [44,45], whereas others have found no changes in their functional activity [46-48]. Despite a decreased density of β-adrenergceptors in the myocardial membranes from rats and swine with type 1 DM, there are no differences in the number of high-affinity β-adrenergceptors, neither in the ability of isoproterenol to stimulate AC activity in diabetic and non-diabetic animals [46,47]. In the myocardium of rats with 14-week type 1 DM, the mRNA level encoding β₁-adrenergceptors is decreased while the mRNA levels of β₂- and β₃-adrenergceptors are significantly increased [41]. Our results and the data of other authors suggest that insulin therapy gives a partial or complete recovery of functional activity of hormone-sensitive AC signaling system and its sensitivity to hormones [12,41,49-51]. The insulin treatment returns the level of mRNA encoding β₂- and β₃-adrenergceptors to control [41]. A chronic treatment with insulin brings back the abnormalities of Gs-coupled β₁-adrenergceptors signaling in the myocardium of streptozotocin-induced diabetic rats [49]. The treatment with insulin reverses the increased concentrations of norepinephrine, dopamine and serotonin in the brain of diabetic rats with type 1 DM to control levels [50]. The insulin therapy leads to restoration of regulatory effects of relaxin and adrenergic ligands on functional activity of AC system in the skeletal muscles of rats with short-time streptozotocin DM [12]. However, the data on the influence of insulin therapy on the functioning of hormonal signaling systems were generally obtained using the methods of intraperitoneal, subcutaneous and intravenous injections of insulin. The influence of intranasal insulin on the functional state of hormone-regulated signaling pathways in DM has been poorly studied. There is a significant difference in the mechanisms of action of centrally and peripherally administered insulin on organisms, therefore the study of action of intranasal insulin on the hormonal signaling systems in normal subjects and those with DM is an urgent task of molecular endocrinology and practical medicine [16,52]. Insulin in the brain functions as a neuropeptide and binds specifically to brain insulin receptors whose density in the olfactory bulbs, hypothalamus and hippocampus is the highest [53]. Thus, the brain can be
regarded as an insulin-sensitive and a glucose-sensitive organ. The localization of insulin receptors in the brain areas and their high density in astrocytes and neurons suggest that insulin affects a variety of physiological functions. Intranasal insulin increases vagally mediated secretion of peripheral insulin from pancreatic β-cells and contributes to a subsequent decrease of blood glucose, reduces food intake and body weight, increases fertility and reproduction, improves learning and memory and modifies attention [16,54]. These data speak in favor of the fact that intranasal insulin regulates many signaling cascades in the brain and, thus, controls the functioning of hormonal signaling systems in peripheral tissues. As an example, the activation of insulin receptors expressed in hypothalamic neurons producing gonadotropin-releasing hormone and mediating its synthesis by intranasal insulin induces the spermatogenesis and maturation of the ovarian follicles [55].

We showed that intranasal insulin influences the sensitivity of AC signaling system to some hormones in the tissues of control rats and is effective in correcting the hormonal disturbances that occur in experimental neonatal DM, which, like type 2 DM of human, is characterized by insulin resistance. In our experiments, intranasal insulin partially or completely restored both G protein- and Gs protein-coupled AC systems in the tissues of diabetic animals. Insulin treatment decreased AC stimulating effect of β-adrenergic agonist isoproterenol elevated in DM and increased AC inhibitory effect of somatostatin decreased in DM. In the brain of insulin-treated diabetic rats, the regulatory effects of the agonists of 5-HT receptors and somatostatin acting via Gi protein-coupled receptors were also partially restored. The same picture was observed in the case of the effects of somatostatin in the tissues of reproductive system. It was also shown that insulin treatment leads to an increase of AC and GppNHP binding stimulating effects of hCG in the ovaries. These data give evidence that intranasal insulin has a highly positive influence on the functioning of hormonal signaling systems and the transduction of hormonal signals. The ability of intranasal insulin to change the functional activity of hormonal signaling systems and to restore it to the level in healthy animals may be responsible for the therapeutic effects of this hormone when administered intranasally. Among the best studied therapeutic effects of intranasal insulin in human DM are the regulation of food intake and body weight, and the prevention of cognitive decline, cerebral atrophy, and focal cerebral ischemia [15,56,57].

Along with the influence on AC system in rats with neonatal DM, insulin caused changes of the sensitivity of this system to some hormones in the tissues of control rats. The stimulating effects of isoproterenol and relaxin in the myocardium and the effects of hCG in the ovaries were decreased in insulin-treated control rats, while the regulatory effects of serotonin in the brain, on the contrary, were enhanced. The regulatory effects of hormones acting via Gi protein-mediated signaling pathways in the tissues of animals treated with intranasal insulin. These results can bring us to the understanding of the molecular mechanisms that govern the action of central insulin on the brain and peripheral tissues, including the regulation and modulation of hormonal signaling systems and the sensitivity of different tissues and organs to hormones. These findings are consistent with the reports describing the influence of intranasal insulin on functions of the CNS, such as the feeding behavior, learning and memory, and of the reproductive system – spermatogenesis and ovarian folliculogenesis in particular – and the influence of intranasal insulin-like growth factor-1 on the brain, myocardium and other peripheral tissues [15,16,19,55,58,59] (Figure 7).

In conclusion, experimental neonatal DM elicits changes in the functional activity of the AC system sensitive to biogenic amines and polypeptide hormones, especially in Gi protein-coupled AC signaling cascades, involved in the control of many physiological and biochemical processes in organisms. Intranasal administration of insulin restores, partially or completely, the functioning of the AC system, which confirms a high efficiency of therapeutic action of central insulin on CNS and peripheral tissues and organs. In addition, the influence of intranasal insulin on the AC system in control rats was also shown. Thus, we can suggest that insulin in the brain has a key role in the regulation and modulation of hormonal signaling systems in the nervous and peripheral systems, its action being mediated by a complex network of hypothalamic neuropeptides and neurotransmitters. The work must be carried on to clarify the mechanisms of hormonal action and to establish the role of brain insulin in regulation of functions of the cardiovascular and reproductive systems.

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Intranasal insulin affects adenyl cyclase system in rat tissues in neonatal diabetes

Figure 7. Intranasal insulin action on the central nervous, cardiovascular and reproductive systems.

References

[9] Palmer T.M., Houslay M.D., Determination of G-protein levels, ADP-ribosylation by cholera and pertussis toxins and the regulation of adenyl cyclase activity in liver plasma membranes from
lean and genetically diabetic (db/db) mice, Biochim. Biophys. Acta, 1991, 1097, 193-204
Intranasal insulin affects adenyl cyclase system in rat tissues in neonatal diabetes


[56] Hallschmid M., Benedict C., Born J., Fehm H.L., Kern W., Manipulating central nervous mechanisms

of food intake and body weight regulation by intranasal administration of neuropeptides in man, Physiol. Behav., 2004, 83, 55-64

