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ENDOGENOUS SODIUM PUMP INHIBITORS, DIABETES MELLITUS AND PREECLAMPSIA

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Abstract

Endogenous inhibitors of the Na/K-ATPase (NKA) and diabetes mellitus (DM) are both risk factors for preeclampsia and NaCl sensitive hypertension. Our goal was to test the hypothesis that NaCl supplementation, induces preeclampsia-like symptoms in pregnant rats with DM via stimulation of marinobufagenin (MBG), a natriuretic and vasoconstrictor inhibitor of the NKA. Type 2 DM in female Sprague-Dawley rats was induced by administration of 65 mg/kg streptozotocin at day 4 post partum. In intact rats, pregnancy was associated with a 2-fold increase in MBG levels and a mild impairment in glucose tolerance. Pregnant rats with DM exhibited fetal macrosomia, greater impairment of glucose tolerance, and higher levels of MBG as compared to that in normal pregnant rats. As compared to intact pregnant rats, NaCl supplementation of diabetic pregnant rats (drinking 1.8% NaCl during days 12-19 of pregnancy) was associated with an increase in systolic blood pressure, decreased fetal and placental weight, five-fold elevation of MBG excretion, and 42% inhibition of NKA in erythrocytes. In nonpregnant rats, in vivo pretreatment with anti-MBG antibody produced an exaggerated response of plasma levels of glucose and insulin in oral glucose tolerance test. These results suggest that MBG is a common factor in the pathogenesis of DM and preeclampsia, and that regulation of glucose tolerance may be one of the physiological functions of endogenous cardiotonic steroids.

Keywords

Diabetes mellitus; Glucose tolerance; Pregnancy; Preeclampsia; Hypertension; Salt-sensitivity; Na/K-ATPase; Marinobufagenin

1. INTRODUCTION

Endogenous digitalis-like Na/K-ATPase (NKA) inhibitors, i.e., cardiotonic steroids (CTS), are implicated in the pathogenesis of NaCl-sensitive hypertension, diabetes mellitus (DM), and preeclampsia (PE) [1-3]. Clinical data indicate that impairment of glucose tolerance and DM both represent potential risk factors of PE [4,5]. In diabetics, perturbed NKA function is linked to impaired tissue insulin resistance, renal sodium retention, and development of hypertension,

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generation of reactive oxygen species, and cardiovascular remodeling [3,6,7]. Recently, we demonstrated that in experimental rats and patients with type 1 and type 2 DM, inhibition of NKA is accompanied by elevated levels of marinobufagin (MBG), an endogenous bufadienolide CTS, a vasoconstrictor and a natriuretic [8,9].

Although PE is a major cause for maternal and fetal mortality and morbidity worldwide, its pathogenesis remains poorly understood. One of the factors contributing to the pathogenesis of PE are CTS [3,10,11]. Pregnancy is associated with renal sodium retention [12,13], a major stimulus for MBG production [14]. In normal pregnancy, moderately elevated levels of MBG do not alter vascular tone [10,13]. In patients with PE and in pregnant rats with PE-like symptoms, however, levels of MBG become substantially elevated and accompany inhibition of the NKA [11,13,15]. Accordingly, in rats with PE-like symptoms, in vivo administration of anti-MBG antibody reduced blood pressure and restored activity of the sodium pump in vascular sarcolemma [13]. Likewise, in patients with PE, anti-MBG antibody ex vivo restore activity of erythrocyte NKA [15].

Since MBG represents a common link in the pathogenesis of PE and DM, we hypothesized that, in experimental pregnant rats with DM, NaCl supplementation would facilitate excessive MBG production resulting in the development of PE-like symptoms.

2. METHODS

2.1. Experimental protocol

The study protocol was approved by the Research Council of Sechenov Institute of Evolutionary Physiology and Biochemistry, St. Petersburg, Russia in accordance with the rules and regulations of the National Institutes of Health, Bethesda, MD, USA. Type 2 (non-insulin-dependent) DM was produced by a single subcutaneous injection of 65 mg/kg streptozotocin (STZ) to 48 neonatal rats as described previously [16]. Control rats received a single intraperitoneal injection of a vehicle, citrate-buffered saline (pH 5.5)(n=24). Rats with type 2 DM exhibited moderate elevation of plasma glucose levels and substantial increases in the plasma levels of insulin (Results). Ten weeks following administration of STZ or vehicle, animals were divided into the following groups (n = 12 per groups): nondiabetic virgin rats, diabetic virgin rats, nondiabetic pregnant rats, nondiabetic pregnant rats supplemented with NaCl, diabetic pregnant rats, and diabetic pregnant rat supplemented with NaCl.

Pregnancy was induced in ten-week old diabetic and nondiabetic rats. Day 1 of pregnancy was established when spermatozoa were found in morning vaginal smears. Nonpregnant rats were selected randomly through the estrous cycle and paired with pregnant ones. All animals were housed under controlled light (6 AM–6 PM) and temperature (22°C) and were fed a normal diet ad libitum. Control pregnant rats had tap water for the duration of the experiment. NaCl-supplemented rats drank 1.8% NaCl solution for 7 days, during days 12–19 of gestation. Systolic blood pressure was measured by the indirect tail-cuff method in unanesthetized rats, and 24-hr urine was collected to assess renal MBG excretion. Rats at day 19 of pregnancy, and virgin rats at the corresponding time were anaesthetized by 65 mg/kg ketamine and sacrificed by exsanguination. The placentas were removed and placental and fetal weights determined. Blood was collected for Na,K-ATPase measurements in erythrocytes [14], and for plasma MBG measurements [13].

2.2. Oral glucose tolerance test

Twelve nondiabetic female rats were food restricted the evening before the experiment and between 10:00 and 11:00 AM, underwent oral glucose tolerance test (OGTT) with a 2 g/kg body weight 50% glucose feeding by gavage as described previously in detail [17]. Three hours

before OGTT rats were intraperitoneally administered vehicle (saline; n=4) or anti-MBG antibody (n=4), at a concentration which blocks 75% of circulating MBG, as described previously in detail. Four control rats received water by gavage and served as controls.

2.3. Na/K-ATPase

Activity of NKA in erythrocytes was determined, as reported previously in detail [14]. Erythrocytes were preincubated with Tween-20 (0.5%) in sucrose (250 mmol/L) and Tris buffer (20 mmol/L; pH 7.4, 37°C) for 30 minutes, and were incubated for 30 minutes in the medium (mmol/L): Na 100, K 10, MgCl₂ 3, EDTA 0.5, Tris 50, ATP 2 (pH 7.4, 37°C) in the final dilution 1:40. The reaction was stopped by the addition of trichloroacetic acid to final concentration 7%. Total ATPase activity was measured by the production of inorganic phosphate (P_i), and NKA activity was estimated as the difference between ATPase activity in the presence and in the absence of 5 mmol/L ouabain.

2.4. Immunoassays

The MBG immunoassay was performed as recently described [13]. The assay is based on competition between immobilized antigen (MBG-thyroglobulin) and MBG within the sample for a limited amount of binding sites on polyclonal rabbit anti-MBG antibody MBG-P raised against MBG-glycoside-BSA (1:30,000). Secondary europium-labeled goat anti-rabbit antibody was obtained from Perkin-Elmer, Boston, MA. The cross-reactivity of MBG antibody was (%): MBG - 100, ouabain - 0.1, digoxin - 1.0, digitoxin - 3.0, bufalin - 1.0, cinobufagin - 1.0, prednisone - < 0.1, spironolactone - < 0.1, proscillaridin - 1.0, progesterone < 0.1, mixture of bufodienolides from *Bufo marinus* venom except MBG- < 5%. Plasma insulin levels were determined via enzyme immunoassay (Cayman Biochemical; Ann Arbor, MI). Chemicals used were obtained from Sigma Chemicals (St. Louis, MO) unless otherwise specified.

2.5. Statistical analyses

Data are presented as means $\pm$ S.E.M. Statistical analyses utilized repeated measures or one-way ANOVA followed by multiple comparisons Newman-Keuls test (GraphPad Instat and GraphPad Prism, GraphPad Software Inc., San Diego, CA). P values < 0.01 were considered significant.

3. RESULTS

As presented in Figure 1a, within 12 weeks of STZ administration, fasting plasma levels of glucose were slightly but significantly elevated vs. those in control animals, while glucose tolerance following oral glucose change was impaired. Impairment of glucose tolerance in STZ-treated rats was associated with substantial elevation in fasting plasma insulin levels (Figure 1c) and moderate increase in renal excretion of MBG, as compared to that in virgin nondiabetic rats (10.6 $\pm$ 1.9 vs. 5.8 $\pm$ 0.6 pmoles per 24 hours; P<0.05).

At day 19 of gestation, glucose tolerance of nondiabetic rats did not differ from that in nonpregnant control animals (Figure 1a). Fasting plasma levels of insulin (Figure 1c) and renal excretion of MBG (10.5 $\pm$ 0.7 pmoles per 24 hours; P<0.05) in nondiabetic pregnant rats, however, significantly exceeded those in the virgin nondiabetic rats. NaCl supplementation of nondiabetic pregnant rats was associated with an increase in renal MBG excretion and inhibition of erythrocyte NKA (Figures 2 e,f), moderate increase in systolic blood pressure (Figure 2d), and reduction in fetal and placental weights (Figure 2 a,b), as compared to that in intact pregnant rat without NaCl supplementation. In nondiabetic rats, NaCl supplementation did not significantly affect fasting levels of plasma glucose and results of OGTT (data not presented).

As compared to that in pregnant nondiabetic animals, STZ-treated rats at day 19 of gestation exhibited a marked impairment of glucose tolerance in OGTT (Figure 1a), elevated levels of plasma insulin (Figure 1c), increased fetal and placental weight (Figure 2 a and b) and renal protein excretion (Figure 2c), and increased renal MBG excretion accompanied by inhibition of NKA in erythrocytes (Figures 2 e and f). Blood pressure in diabetic rats at day 19 of gestation did not differ from that in nondiabetic pregnant rats (Figure 2d).

As demonstrated in Figure 1b, NaCl supplementation of pregnant STZ treated rats was associated with an improvement of glucose tolerance following glucose oral challenge. Furthermore, NaCl supplementation in pregnant diabetic rats was associated with decreased fetal and placental weights (Figure 2 a and b), increased renal protein excretion (Figure 2c) and systolic blood pressure (Figure 2d), 42% (as in abstract) inhibition of erythrocyte NKA activity (Figure 2e), and a five-fold increase in renal MBG excretion (Figure 2f).

Figure 3 illustrates the results of experiment in which the impact of MBG immunoneutralization on plasma levels of glucose and insulin following oral glucose challenge was studied in nonpregnant nondiabetic rats. As compared to vehicle-treated rats, in vivo pre-treatment of rats with anti-MBG antibody at a concentration, which blocks 75% of circulating MBG, resulted in significantly elevated plasma levels of glucose and insulin within 30 minutes after oral glucose administration.

4. DISCUSSION

The main finding of the present study is that NaCl-supplemented pregnant rats with a mild type 2 DM develop some of the symptoms of PE, including elevation of arterial pressure, reduction in fetal and placental weight, and proteinuria. In these rats, development of PE-like symptoms is associated with markedly increased renal excretion of MBG and substantial inhibition of NKA in erythrocytes.

An increasing body of evidence indicates the importance of endogenous CTS in the pathogenesis of PE. DIGIBIND, an affinity-purified anti-digoxin antibody which recognizes CTS, has been successfully used in the treatment of PE [10]. Both experimental and clinical data indicated that MBG, rather than endogenous ouabain, becomes elevated and contributes to vasoconstriction in PE and represents a potential target for therapy of this disorder [13,15]. Our present observations provide further evidence for the involvement of MBG in the pathophysiology of PE. Besides acting as a direct vasoconstrictor, MBG is also capable of impairing trophoblast differentiation [18] and inducing oxidative stress [19]. Low concentrations of bufalin, a CTS that is structurally related to MBG, induces differentiation and apoptosis of human blood cells in vitro [20]. To what extent these mechanisms may be applicable to the PE model described in the present experiment remains to be studied.

In the present study, STZ-treated pregnant rats exhibited impaired glucose tolerance, increased placental weight and increased fetal weight, i.e., fetal macrosomia, a feature characteristic to human diabetic pregnancies [21]. As compared to that in nondiabetic pregnant rats, renal MBG excretion in pregnant diabetic rats was increased two-fold, and NKA activity was inhibited by 20%. A ten-day NaCl supplementation of pregnant diabetic rats, however, resulted in a significant reduction in fetal and placental weights, a five-fold increase in MBG excretion and a 42% inhibition of erythrocyte NKA. As compared to that in NaCl-supplemented nondiabetic rats, NaCl supplementation of diabetic animals was associated with a greater reduction of fetal and placental weights, higher arterial pressure, MBG levels and renal protein excretion, and lower NKA activity.

In the present experiment, a profound inhibition of inhibition of the NKA in NaCl-supplemented diabetic pregnant rats was unexpectedly found to be associated with a marked

improvement of glucose tolerance. We hypothesize that there could be a causative link between MBG and glucose tolerance, and that exaggerated MBG production in pregnant diabetic NaCl-supplemented rats could have contributed to the improvement of glucose tolerance, as compared to that in pregnant diabetic rats. Accordingly, in healthy nonpregnant rats following oral glucose challenge, the *in vivo* immunoneutralization of MBG produced elevations of plasma levels of glucose and insulin (Figure 3), i.e., blockade of circulating MBG results in the reduction of glucose tolerance. We, therefore, further hypothesize that, along with regulation of sodium metabolism, adaptation to impaired glucose tolerance may be one of the functions of CTS in pregnancy. This hypothesis is consistent with the results obtained by Melander et al., who demonstrated that NaCl loading of healthy human subjects, a condition which may stimulate MBG production [22], is associated with an improvement of insulin sensitivity [23].

In conclusion, our data demonstrate that pregnant rats with a combination of mild type 2 DM and NaCl supplementation exhibit some of the symptoms of PE, inhibition of the sodium pump, and high levels of MBG. The physiological roles of CTS in pregnancy remain poorly understood. It has been hypothesized that the primary role of CTS during pregnancy is control of water-electrolyte balance via induction of natriuresis [3]. Growing evidence for implication of CTS including MBG, in tissue growth and differentiation [20,24,25], raises the possibility that the fetus may be a primary target for MBG. Our present data suggest that regulation of carbohydrate metabolism and modulation of glucose tolerance may also be one of the roles of MBG in pregnancy.

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Figure 1.

(a) Plasma glucose following oral glucose challenge in nondiabetic (Virgin) and diabetic (Virgin STZ) virgin rats, and in nondiabetic (N pr) and diabetic (Pr STZ) pregnant rats. Means $\pm$ SEM from 12 observations. (b) Plasma glucose following oral glucose challenge in pregnant diabetic rats with (Pr STZ+NaCl) or without NaCl supplementation (Pr STZ). Repeated measures ANOVA followed by Newman-Keuls test: Virgin vs. Virgin STZ – $P < 0.05$; N Pr vs. Pr STZ – $P < 0.05$; Pr STZ vs. Pr STZ+NaCl – $P < 0.05$. (c) – Fasting plasma levels of insulin. One-way ANOVA followed by Newman-Keuls test: * - $P < 0.01$ vs. Virgin; # - $P < 0.01$ vs. N Pr and Virgin STZ.

Figure 2.

(a) – Fetal weight, (b) – placental weight, (c) renal protein excretion, (d) – systolic blood pressure, (e) – activity of Na/K-ATPase in erythrocytes, and (f) 24 hour renal excretion of MBG in nondiabetic pregnant rats and in pregnant rats with type 2 DM with (NaCl+) or without (NaCl-) NaCl-supplementation at day 19 of gestation. Means ± SEM from 12 observations. By one-way ANOVA followed by Newman-Keuls test: * - $P < 0.05$, ** - $P < 0.01$ vs. nondiabetic, NaCl(-); # - $P < 0.05$, ## - $P < 0.01$ vs. diabetic, NaCl(-).

Figure 3.

Plasma levels of glucose (a) and immunoreactive insulin (b) in control rats (Ctrl) and 30 minutes following glucose oral challenge (OGTT) in nonpregnant rats following in vivo administration of anti-MBG antibody - aM(+), or vehicle - aM(-). By one-way ANOVA followed by Newman-Keuls test: * - $P < 0.05$, ** - $P < 0.01$ vs. Bl; # - $P < 0.05$, ## - $P < 0.01$ vs. aM(-).