

Magnesium Sulfate Potentiates Effect of DigiFab on Marinobufagenin-Induced Na/K-ATPase Inhibition

Irina E. Zazerskaya,¹ Valentina V. Ishkaraeva,¹ Elena V. Frolova,² Nelly G. Solodovnikova,¹ Yulia N. Grigorova,^{1,3} C. David Adair,⁴ Olga V. Fedorova,³ and Alexei Y. Bagrov³

BACKGROUND

Immunoneutralization of elevated circulating levels of endogenous digitalis-like Na/K-ATPase inhibitors (i.e. cardiotonic steroids (CTS)) represents a novel approach in the treatment of preeclampsia (PE). Recently we demonstrated that DigiFab (Fab fragments of affinity-purified ovine digoxin antibody) restores PE-induced inhibition of Na/K-ATPase in erythrocytes *ex vivo*. Previously magnesium ions were shown to antagonize digitalis-induced toxicity, which is mediated by Na/K-ATPase inhibition. We hypothesized that magnesium sulfate would potentiate the effect of DigiFab in the reversal of CTS-induced Na/K-ATPase inhibition.

METHODS

To test this hypothesis, we studied the *ex vivo* effect of DigiFab on Na/K-ATPase activity in erythrocytes from patients with PE in the absence and in the presence of 3 mmol/L magnesium sulfate.

RESULTS

Compared with 11 normotensive pregnant subjects (29 ± 1 years; gestational age = 39.0 ± 0.2 weeks; blood pressure = 111 ± 2/73 ± 2 mm Hg),

the 12 patients with PE (30 ± 1 years; gestational age = 37.9 ± 0.3 weeks; blood pressure = 159 ± 5/99 ± 3 mm Hg) had plasma levels of marinobufagenin increased 3-fold (1.38 ± 0.40 vs. 0.38 ± 0.10 nmol/L; *P* < 0.01) and activity of Na/K-ATPase in erythrocytes was inhibited (1.16 ± 0.11 vs. 2.80 ± 0.20 μmol Pi/ml/h; *P* < 0.01). *Ex vivo*, DigiFab (1 μg/ml) restored erythrocyte Na/K-ATPase activity (1.72 ± 0.13 μmol Pi/ml/h; *P* < 0.01), and 3 mmol magnesium sulfate potentiated the effect of DigiFab (2.30 ± 0.20 μmol Pi/ml/h; *P* < 0.01).

CONCLUSIONS

Magnesium is capable of increasing the efficacy of immunoneutralization of marinobufagenin-induced Na/K-ATPase inhibition.

Keywords: blood pressure; cardiotonic steroids; digifab; digitalis-like factors; hypertension; immunotherapy; magnesium; marinobufagenin; Na/K-ATPase; preeclampsia.

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Although preeclampsia (PE) is a serious complication of pregnancy leading to maternal and fetal morbidity and mortality, mechanisms of its pathogenesis are not well understood, and there is no effective therapy for this syndrome.¹ One of the factors implicated in pathogenesis of PE is the presence of endogenous digitalis-like Na/K-ATPase inhibitors (i.e., cardiotonic steroids (CTSs)).^{2,3} CTSs, including a bufadienolide marinobufagenin (MBG), contribute to the pathogenesis of PE by induction of vasoconstriction,^{3,4} vascular fibrosis,⁵ and impairment of invasion of the cytotrophoblast.⁶

Immunoneutralization of CTSs is a novel approach in the treatment of PE, and several successful cases of the treatment of PE by Digibind have been reported.^{7,8} Digibind, the affinity-purified Fab fragments of sheep antidigoxin antibody, because of cross-reactivity with digitalis-like compounds,

is capable of reversing the effects of endogenous CTSs *in vivo*.^{4,7,8} The effect of Digibind in PE is based on the binding of CTSs and reversal of the vasoconstriction, which is associated with restoration of activity of erythrocyte Na/K-ATPase, a target enzyme for CTSs.^{7,13} DigiFab, which recently replaced Digibind on the market, is similar to Digibind in its ability to neutralize CTSs *ex vivo* and to restore PE-induced inhibition of erythrocyte Na/K-ATPase.¹⁴

Interactions between digitalis/CTSs and its receptor on the Na/K-ATPase are modulated by many factors, including magnesium (Mg) ions.^{10,11} Notably, *in vivo* Mg ions antagonize digitalis-induced toxicity, which is mediated by Na/K-ATPase inhibition.¹² Mg deficiency, on the contrary, sensitizes myocardium to the proarrhythmic action of digitalis.¹³ Because of the above evidence and because Mg sulfate (MgSO₄) exerts beneficial effects in PE,¹ we hypothesized that MgSO₄ would

Correspondence: Alexei Y. Bagrov (bagrova@mail.nih.gov).

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¹Institutes of Neonatology and Heart and Vessels, Almazov Federal Heart, Blood and Endocrinology Center, St. Petersburg, Russia;

²Sechenov Institute of Evolutionary Physiology and Biochemistry, St. Petersburg, Russia; ³National Institute on Aging, National Institutes of Health, Baltimore, MD; ⁴Department of Obstetrics and Gynecology, University of Tennessee, Chattanooga, Tennessee.

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potentiate the effect of DigiFab with respect to Na/K-ATPase restoration and reduction of blood pressure in PE. To test this hypothesis, in erythrocytes from patients with PE we studied the effect of DigiFab on Na/K-ATPase activity in the absence and in the presence of 3 mmol/L MgSO₄ *ex vivo*.

METHODS

The protocol for this study was approved by the ethical committee of Almazov Federal Heart, Blood and Endocrinology Center, and by the institutional review board of Medstar Research Institute, Washington, DC. Twelve participants who were admitted to the Institute of Neonatology and Pediatrics, Almazov Federal Heart, Blood and Endocrinology Center in St Petersburg, Russia, were enrolled in the study after giving informed consent. Diagnosis of mild PE in 7 patients was based on the criteria of the American Congress of Obstetrics and Gynecology.¹⁴ This definition includes systolic blood pressure of at least 140 mm Hg or diastolic blood pressure of at least 90 mm Hg on at least 2 occasions 6 hours apart and new onset proteinuria (urinary protein excretion >0.3 g/24 hours or a urinary protein concentration of >1 g/L in at least 2 random urine specimens collected ≥6 hours apart) in a pregnancy after the 20th week of gestation. Exclusion criteria included patients with a clinical need for digitalis drugs, antecedent history of essential hypertension, and chronic cardiovascular, renal, hepatic, or endocrine disorders. We specifically enrolled 11 age-matched normotensive women with uncomplicated pregnancies to serve as the control group.

Ten milliliters of venous blood were collected. Four milliliters of blood were centrifuged at 1,500 g for 15 minutes, and plasma samples were kept at -80 °C for measurement of MBG. Each plasma sample was extracted on C18 SepPak cartridges, dried, reconstituted in 10% acetonitrile, and fractionated on Agilent 1,100 series high performance liquid chromatography system using Agilent Zorbax Eclipse XDB-C18 (Agilent Technologies, Palo Alto, CA), 4.6×150 mm, 5 μm particle size, 80 Å column, flow rate 1 ml/minute, in linear (10%–85.5%) gradient of acetonitrile against 0.1% trifluoroacetic acid for 45 minutes. Levels of MBG were determined in chromatographic fractions coeluting with authentic MBG (16 minutes) using fluoroimmunoassay (Dissociation Enhanced FluoroImmunoAssay (DELFI)) based on a murine anti-MBG 4G4 monoclonal antibody recently described in detail.⁴ This assay is based on competition between immobilized antigen (MBG-glycoside-thyroglobulin) and MBG, other cross-reactants, or endogenous CTSs within the sample for a limited number of binding sites on an anti-MBG monoclonal antibodies. Secondary (goat antimouse) antibody labeled with nonradioactive Europium was obtained from Perkin-Elmer (Waltham, MA). The sensitivity of this MBG DELFIA is 0.05 nmol/L, and the cross-reactivity of 4G4 monoclonal antibody used in this assay with other steroids is as follows: MBG: 100%; marinobufotoxin: 43%; cinobufotalin: 40%; telocinobufagin: 14%; resibufagenin: 0.5%; bufalin: 0.08%; cinobufagin: 0.07%; digoxin: 0.03%; ouabain: 0.005%; digoxigenin: 0.004%; proscillaridin A, digitoxin, aldosterone, progesterone, prednisone, corticosterone, and thyroglobulin: <0.001%.

Six milliliters of blood were used for the measurement of erythrocyte Na/K-ATPase activity in the presence and in the absence of DigiFab, 3 mmol/L MgSO₄, and their combination as reported previously in detail.⁴ The amount of DigiFab for the *ex vivo* incubation with blood was 1 μg/ml, which corresponds to our recent observation of *ex vivo* activity of DigiFab⁹ and to the dose of Digibind used clinically in PE.⁸ In a separate experiment in erythrocytes obtained from normotensive pregnant subjects, we studied the effect of MgSO₄ on MBG-induced Na/K-ATPase inhibition. For that, aliquots of the whole blood (0.5 ml) were preincubated at room temperature for 30 minutes with MBG in the absence and in the presence of MgSO₄. Erythrocytes were washed 3 times in an isotonic medium (145 mmol/L sodium chloride in 20 mmol/L Tris buffer; pH = 7.6, 4 °C). Activity of Na/K-ATPase was determined, as reported previously in detail.⁴ 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: sodium chloride 100 mmol/L, potassium chloride 10 mmol/L, magnesium chloride 3 mmol/L, ethylenediaminetetraacetic acid 0.5 mmol/L, Tris 50 mmol/L, ATP 2 mmol/L (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 Na/K-ATPase activity was estimated by the difference between ATPase activity in the presence and in the absence of 5 mmol/L ouabain.

All chemicals were from Sigma-Aldrich (St. Louis, MO). DigiFab was obtained from BTG International (London, UK). MBG (>98% purity by high performance liquid chromatography (HPLC)) was purified from the secretion from parotid glands of *Bufo marinus* toads, and 4G4 monoclonal antibody were developed as reported recently in detail.⁷

The results are presented as mean ± SEM. Data were analyzed using 1-way analysis of variance (ANOVA) (intergroup analysis) or by repeated measures ANOVA (intragroup analysis) followed by Newman-Keuls test, and by 2-tailed *t* test when applicable (Graph Pad Prism Software, San Diego, CA). A 2-sided *P* < 0.05 was considered to be statistically significant.

RESULTS

Maternal demographics and data on levels of blood pressure are presented in Table 1. Data on levels of plasma MBG and erythrocyte Na/K-ATPase activity in patients with PE and in control subjects are summarized in Figure 1. In patients with PE, elevated blood pressure was accompanied by a 4-fold increase in plasma MBG concentration (Figure 1a) and a concomitant 61% inhibition of Na/K-ATPase activity in erythrocytes (Figure 1b). *Ex vivo* incubation of erythrocytes in the presence of 1 μg/ml DigiFab or 3 mmol/L MgSO₄ produced a comparable increase in Na/K-ATPase activity. Treatment of erythrocytes with a combination of DigiFab and MgSO₄ produced a more substantial increase in Na/K-ATPase activity, which exceeded the effect of the each individual treatment.

Table 1. Characteristics of study subjects and levels of blood pressure

Characteristic	Normal pregnancy (n = 11)	Preeclampsia (n = 12)
Maternal age, y	29 ± 1	30 ± 1
Gestational weeks at delivery	39 ± 0.2	37.9 ± 0.6
No. of primigravida (%)	5 (45%)	5 (42%)
Cesarean section, No.	4	7
Vaginal delivery, No.	7	5
Infant birth weight, g	3796 ± 121	3404 ± 127*
Systolic blood pressure, mm Hg	111 ± 2	159 ± 5**
Diastolic blood pressure, mm Hg	73 ± 2	99 ± 3**

Data are given as means ± SEMs unless otherwise noted.

* $P < 0.05$, ** $P < 0.01$ versus normal pregnant subjects, 2-tailed t test.

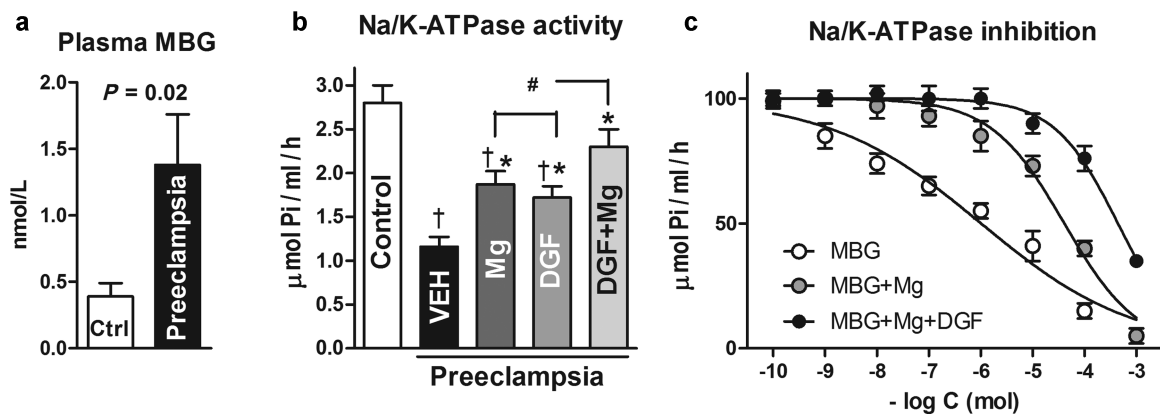


Figure 1. Plasma marinobufagenin, erythrocyte Na/K-ATPase activity, and Na/K-ATPase inhibition. (a) Plasma levels of marinobufagenin (MBG) in 11 subjects with noncomplicated pregnancy (control) and in 12 patients with preeclampsia (two-tailed t test). (b) Activity of sodium (Na)/potassium (K)-ATPase in erythrocytes from 11 subjects with noncomplicated pregnancy (control) and in 12 patients with preeclampsia in the presence of vehicle (VEH), DigiFab (DGF, 1 $\mu\text{g}/\text{ml}$), magnesium sulfate (Mg, 3 mmol/L), and their combination (DGF + Mg). Means \pm SEMs. By 1-way analysis of variance (ANOVA) and Newman-Keuls test: $^{\dagger}P < 0.01$ vs. control. By repeated measures ANOVA and Newman-Keuls test: $^{*}P < 0.01$ vs. VEH and $^{*}P < 0.05$ vs. DGF and Mg. (c) Inhibition of Na/K-ATPase in the erythrocytes from healthy pregnant subjects by MBG in the absence and in the presence of 3 mmol/L magnesium sulfate. Means \pm SEM. By repeated measures ANOVA and Newman-Keuls test: MBG vs. MBG + Mg and MBG + Mg + DGF: $P < 0.01$; MBG + Mg vs. MBG + Mg + DGF: $P < 0.05$.

As presented in Figure 1c, MBG inhibited activity of Na/K-ATPase in erythrocyte from control subjects in a concentration-dependent manner. MgSO_4 did not affect the activity of the Na/K-ATPase at baseline, but the addition of 3 mmol/L MgSO_4 to the incubation medium markedly reduced sensitivity of Na/K-ATPase to MBG. Thus, the median inhibitory concentration (IC_{50}) to the Na/K-ATPase inhibitory effect of MBG in the absence and in the presence of MgSO_4 was $1.1 \pm 0.2 \mu\text{mol}/\text{L}$ and $40 \pm 10 \mu\text{mol}/\text{L}$, respectively. Addition of 1.0 $\mu\text{mol}/\text{L}$ DigiFab to the incubation medium further desensitized Na/K-ATPase to MBG ($\text{IC}_{50} = 290 \pm 49 \mu\text{mol}/\text{L}$).

DISCUSSION

The main observation of this study is that MgSO_4 potentiates the effect of DigiFab with respect to reversal

of PE-induced inhibition of Na/K-ATPase by an endogenous CTS, MBG. In PE, elevated plasma levels of MBG exhibit prohypertensive effect by inhibition of vascular Na/K-ATPase.³ Erythrocyte Na/K-ATPase is sensitive to endogenous CTSs, and changes in its activity could serve as a marker characterizing magnitude to CTS-dependent effects.^{4,9} Accordingly, in this study heightened levels of MBG in patients with PE were associated with substantial inhibition of erythrocyte Na/K-ATPase, whereas *ex vivo* incubation of PE blood in the presence of the CTS-neutralizing antibody DigiFab restored Na/K-ATPase activity. In this study DigiFab restored Na/K-ATPase activity *ex vivo*, and an increase in levels of Mg^{2+} ions in incubation medium potentiated the effect of DigiFab. Accordingly, *in vitro*, MBG potentially inhibited Na/K-ATPase in erythrocytes from control subjects and Mg^{2+} desensitized Na/K-ATPase to MBG. These findings agree with previous clinical data demonstrating that MgSO_4 is capable of offsetting the

deleterious cardiac effects of digitalis overdose¹² and that Mg²⁺ ions are capable of changing Na/K-ATPase conformation.¹⁰ Our observations suggest that MgSO₄ is capable of increasing the efficacy of DigiFab-mediated immunoneutralization of MBG-induced Na/K-ATPase inhibition in pathological states in which levels of CTSs are elevated.

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DISCLOSURE

The authors declared no conflict of interest.

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