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Interaction of Digibind with endogenous cardiotonic steroids from preeclamptic placentae

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Abstract

Background—Preeclampsia (PE) is a major cause of maternal and fetal mortality, and its pathogenesis is not fully understood. Endogenous digitalis-like cardiotonic steroids (CTS) have been implicated in the pathophysiology of PE; this is illustrated by clinical observations that Digibind, a therapeutic digoxin antibody fragment which binds CTS, lowers blood pressure and reverses Na/K-ATPase inhibition in patients with PE. Recently we reported that plasma levels of marinobufagenin (MBG), a bufadienolide vasoconstrictor CTS, are increased four-fold in patients with severe PE.

Methods—In the present study, we compared levels of MBG in normal and PE placentae, as well as the interactions of Digibind and antibodies against MBG and ouabain with material purified from PE placentae using high-performance liquid chromatography (HPLC).

Results—Levels of endogenous MBG, but not that of endogenous ouabain, exhibited a four-fold elevation in PE placentae vs. normal placentae (13.6 ± 2.5 and 48.6 ± 7.0 nmoles/g tissue; $P < 0.01$). The elution time of endogenous placental MBG-like immunoreactive material from reverse-phase HPLC column was identical to that of authentic MBG. A competitive immunoassay based on Digibind exhibited reactivity to HPLC fractions having retention times similar to that seen with MBG and other bufadienolides, but no to ouabain-like immunoreactive material.

Conclusions—Our results suggest that elevated levels of endogenous bufadienolide CTS represent a potential target for immunoneutralization in patients with PE.

INTRODUCTION

Preeclampsia is a major cause for maternal and fetal mortality and morbidity, but its pathogenesis is still not well understood, and effective treatment other than delivery has not been developed [1]. Many factors have been implicated in pathogenesis of preeclampsia including endogenous digitalis-like cardiotonic steroids (CTS) [2]. CTS bind to the receptor site on the α -subunit of the Na/K-ATPase and induce natriuresis, vasoconstriction, and EGFR-dependent cellular signaling which involves the induction of oxidative stress [2]. In 1984, Gusdon et al. and Graves et al. demonstrated increased levels of CTS in pregnancy and

hypothesized that CTS were involved in the pathogenesis of pregnancy-induced hypertension and preeclampsia [3,4].

A convincing argument in favor of the role of CTS in preeclampsia comes from studies in which intravenously administered Digibind (the Fab₂ fragment of affinity purified ovine anti-digoxin antibodies) lowered the blood pressure in patients with preeclampsia. In 1988, Goodlin reported a successful use of Digibind in a preeclamptic patient [5]. Adair, et al presented another case of successful use of Digibind in preeclampsia [6], and subsequently, demonstrated that Digibind lowered the blood pressure in patients with post partum preeclampsia in a placebo controlled double-blinded study [7]. Most recently, a double-blind, placebo controlled study demonstrated that administration of Digibind was associated with an improvement of renal function and by reduction of plasma Na/K-ATPase inhibitory activity in severe preeclampsia [8]. Notably, Digibind did not exert adverse effects in any of these studies [5-8].

Endogenous mammalian CTS belong to either cardenolide (endogenous ouabain, EO) or bufadienolide (telocinobufagin and marinobufagenin - MBG) families; these families of CTS appear to differ with respect to their structure, targets, and physiological effects [9-11]. Previously, we demonstrated that plasma levels of EO and MBG increase by 2 and 4 times, respectively in patients with severe preeclampsia [12]. Later we reported that plasma levels of MBG, but not EO, become elevated in patients with moderate preeclampsia, and that ex vivo anti-MBG, but not anti-ouabain antibody, reversed the preeclampsia-induced inhibition of the Na/K-ATPase in erythrocytes [13]. Subsequently, we observed that MBG-immunoreactive material purified from preeclamptic placentae co-elutes with authentic MBG from reverse-phase chromatographic columns [14].

Because Digibind was previously reported to have relatively low cross-reactivity with MBG and ouabain, [15] it is important to understand which CTS represent a potential target(s) for Digibind in preeclampsia. Since the placenta is a likely source of CTS [16,17], the goals of our study were to compare the levels of MBG and EO in preeclamptic and normal placentae, and to study the ability of Digibind to interact with MBG and ouabain-immunoreactive material purified from normal and preeclamptic placentae via reverse-phase high performance liquid chromatography (HPLC).

METHODS

The protocol for the human study was approved by the Research Council of St Petersburg School of Pediatric Medicine and by the Institutional Review Board of Medstar Research Institute, Washington, DC. Consecutive patients with preeclampsia (gestational age 37–39 weeks) admitted to Kolpino Obstetric Hospital and Snegirev Obstetric Hospital (St Petersburg, Russia) were enrolled in the study. Preeclampsia was diagnosed according to the criteria established by the American College of Obstetrics and Gynecology [18]. This definition includes at least two of the following criteria: 1) a diastolic blood pressure of at least 90 mmHg, a systolic blood pressure of at least 140 mmHg, an increase in the diastolic blood pressure of at least 15 mmHg, or an increase in systolic blood pressure of 30 mmHg on at least two occasions 6 h or more apart, 2) proteinuria defined by at least 300 mg protein in a 24-h urine collection or a protein concentration of 1 g or more per liter in two random urine specimens collected 6 h or more apart) and 3) edema defined as a generalized accumulation of fluid of greater than 1+ pitting edema after 12 h of bed rest or a weight gain of 5 pounds or more in 1 week in the setting of pregnancy after the 20th week of gestation. Gestationally age-matched subjects with uncomplicated pregnancies served as controls. None of the subjects studied had ever taken digitalis drugs or had a chronic disease known to be associated with increased levels of CTS (hypertension, renal and hepatic diseases, endocrine dysfunction).

Placentae were perfused with a solution containing (in mmol/l) NaCl 120; KCl 4; CaCl₂ 2.5; MgCl₂ 2.0; NaH₂PO₄ 1.1; NaHCO₃ 24; and glucose 5.6 until complete removal of blood was accomplished, and tissue was minced and homogenized. The homogenate was extracted with chloroform and dried under a vacuum. The dried extract was sonicated in water (1 : 5 w/v) and applied on a reverse-phase C-18 SepPak 'long body' cartridge, eluted with 80% acetonitrile, and dried in a SpeedVac centrifuge (Savant, Hicksville, New York, USA). The levels of MBG, EO and of digoxin-like immunoreactivity (using an assay based on Digibind) were determined in the prepurified extract as described below (Immunoassays). The C-18 purified extracts from normal and preeclamptic placentae equivalent to 0.4 mg tissue were fractionated on an Agilent 1100 series liquid chromatographic system. Specifically, this involved the use of an Agilent Zorbax Eclipse XDB-C18 (Agilent Technologies, Palo Alto, California, USA), 4.6_150mm, 5µm particle size, 80Å° column, a flow rate of 1 ml/min, and a linear (10–85.5%) gradient of acetonitrile against 0.1% trifluoroacetic (TFA) for 45 min. Thirty 1.5-min fractions were collected and analyzed for MBG-, ouabain- and digoxin-immunoreactivity.

MBG was measured using a fluoroimmunoassay (Dissociation Enhanced FluoroImmunoAssay - DELFIA) based on a murine anti-MBG 4G4 monoclonal antibody (mAb) recently described in detail [14]. This assay is based on competition between immobilized antigen (MBG-glycoside-thyroglobulin) and MBG, other cross-reactants, or endogenous CTS within the sample for a limited number of binding sites on an anti-MBG mAbs. Secondary (goat antimouse) antibody labeled with nonradioactive europium was obtained from Perkin-Elmer (Waltham, Massachusetts, USA). The cross-reactivity of 4G4 mAb is (%): 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.

The EO assay was based on a similar principle utilizing an ouabain–ovalbumin conjugate and ouabain antiserum (anti-OU-M-2005; 1 : 20 000) obtained from rabbits immunized with a ouabain-BSA conjugate [19]. The cross-reactivity of this ouabain antibody is (%) ouabain, 100; ouabagenin, 52, digoxin, 1.5; digitoxin, 0.47; progesterone, 0.002; prednisone, 0.001; proscillaridin, 0.03; bufalin, 0.10; aldosterone, 0.04; telocinobufagin, 0.02; resibufagin, 0.15; marinobufotoxin, 0.06; cinobufagin, 0.02; and MBG, 0.05.

For the digoxin immunoassay, we labeled the primary antibody (Digibind; Glaxo SmithKline, King of Prussia, PA) using a europium-labeling kit (Perkin Elmer, Wellesley, Massachusetts). The assay was based on a competition between the CTS in the sample and digoxin-BSA conjugate immobilized on the bottom of immunoprecipitation strips for a limited number of binding sites of the Digibind (0.125 µg/well). The sensitivity of Digibind immunoassay for digoxin is 0.01 nmol/l. The crossreactivity of Digibind in this assay is (%): digoxin – 100, ouabain – 0.4, ouabagenin – 0.1, MBG – 0.2, bufalin – 2.7, cinobufotalin – 4.3, cinobufagin – 0.02.

Calibration curves from immunoassays illustrating cross-reactivity of each antibody with MBG, ouabain, and digoxin are presented in Fig. 1 a-c.

MBG and telocinobufagin (>98% HPLC pure) were purified from toads (*Bufo marinus*) as reported previously in detail [10]. Bufalin, cinobufagin, cinobufotalin, and ouabain were purchased from Sigma Chemicals (St. Louis, MO). Proscillaridin A and resibufagenin were obtained from Axxora LLC (San Diego, CA), and marinobufotoxin was a generous gift by Dr. Vincent P. Butler, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY.

The results were analyzed statistically using two-tailed t-test (GraphPad Prism 3 software).

RESULTS

Placentae were obtained from 12 patients with preeclampsia and from 11 subjects with uncomplicated pregnancies. Two groups did not differ significantly with respect to age, gestational age, parity, and infant weight (Table 1). Levels of both systolic and diastolic blood pressure (Table 1) as well as concentrations of ouabain-, MBG- and digoxin-immunoreactivity (figure 1d) in placental chloroform extracts pre-purified on C18 cartridges are presented. Patients with preeclampsia exhibited elevated blood pressure (Fig. 1d). Levels of MBG and of digoxin-immunoreactivity in preeclamptic placentae significantly exceeded that in the placentae obtained from subjects with uncomplicated pregnancies, while levels of EO exhibited elevation of a borderline significance (Fig. 1d).

Fig. 2 demonstrates the elution of CTS standards and of endogenous MBG-, ouabain- and digoxin-immunoreactive material from Agilent Zorbax Eclipse XDB-C18 HPLC columns. Standards of ouabain, digoxin, and MBG eluted in 6, 14, and 16 minutes, respectively (Fig. 2. a-c). Endogenous MBG-, digoxin- and ouabain-immunoreactive material were detectable in the HPLC fractions from placenta obtained from subjects with uncomplicated pregnancies and eluted in 10-22, 10-24, and 3-6 minutes, respectively (Fig. 2d-f).

As presented in Fig. 2 g-i, in HPLC fractions from preeclamptic placentae levels of MBG and digoxin-like immunoreactivity became elevated as compared to that in HPLC fractions from normal placentae. MBG immunoreactivity eluted in 8-24 minutes with the maximum (35 %) eluting on minute 16, which coincided with the elution of the MBG standard (fig 2a). The total amount of MBG immunoreactivity in fractions from preeclamptic placentae exceeded that from the levels in normal placentae by 3.7 times (468 pmoles vs. 126 pmoles, respectively). The material which cross-reacted with Digibind eluted in 8-25 minutes (fig 2h) and overlapped with the elution of MBG- (10-24 minutes), but not ouabain-like (2-6 minutes) immunoreactive material. The total amount of digoxin-like immunoreactivity in HPLC fractions from preeclamptic placentae exceeded those from normal placentae by 3.5 times (484 pmoles vs. 137 pmoles), while the amount of ouabain-immunoreactivity in HPLC fractions from preeclamptic placentae was elevated by 25% only.

DISCUSSION

The main observation of our study is that in a competitive immunoassay, Digibind, a digoxin antibody fragment which lowers blood pressure in patients with preeclampsia, detects a four-fold elevation in the level of digoxin-immunoreactivity in preeclamptic vs. normal placentae. A 4-fold increase in the levels of MBG in preeclamptic placentae was proportional to that of digoxin-immunoreactivity, while levels of EO exhibited only a marginally significant increase. Our present data also demonstrate that in a competitive immunoassay Digibind cross-reacted with the material from preeclamptic placentae eluting from HPLC column at 8-25 minutes; these fractions correspond with the fractions where MBG-like (10-24 minutes), but not ouabain-immunoreactive (2-6 minutes) material eluted. These observations, together with our previous data [12,13,20] suggest that endogenous bufadienolides rather than EO represent a target of Digibind in preeclampsia. Pattern of preeclampsia-induced changes in placental levels of CTS in present study is consistent with previously reported pattern of changes in plasma CTS concentrations in preeclampsia, i.e., substantial increase in the levels of MBG and a lesser increase of EO [12-14]. Larger scale studies of associations of ante-partum plasma levels of CTS, other potential biomarkers of PE and clinical parameters with post-partum placental CTS levels are needed to better understand role of CTS in pathogenesis of preeclampsia.

Although the roles of CTS in pregnancy are not well understood, it seems very clear that they may potentially contribute to the pathogenesis of preeclampsia, particularly to the hypertension

which is a critical feature of the syndrome. Digibind has been shown to lower the blood pressure in patients with preeclampsia [5-8]. Observations of the antihypertensive activity of Digibind in preeclampsia agree with the results of studies demonstrating that Digibind produces vasorelaxation in isolated perfused preeclamptic placentae [16]. Moreover, it has also been observed that the Na/K-ATPase from preeclamptic placentae exhibited heightened sensitivity to CTS [21]. The mechanism underlying the antihypertensive effects of Digibind is based on the cross-reactivity with endogenous CTS; this is illustrated by several observations including the restoration of erythrocyte Na/K-ATPase activity by Digibind ex vivo [13,14] and the reduction in plasma Na/K-ATPase inhibitory activity following Digibind infusion [22]. Accordingly, in pregnant, NaCl-supplemented rats which exhibit symptoms of preeclampsia, the antihypertensive effects of Digibind as well as of anti-MBG mAb were associated with the restoration of vascular and erythrocyte Na/K-ATPase [14]. Interestingly, the pattern of CTS response to NaCl supplementation in pregnant rats is quite similar to that observed in the preeclamptic placentae in the present study. Specifically, this animal model is associated with an increase in the levels of MBG but not EO, indicating that endogenous bufadienolides represent a likely target for Digibind in preeclampsia. It should be noted that levels of MBG are elevated in normal pregnancy [12,14], and that MBG is implicated in regulation of tissue growth [2], apoptosis [23], and the epithelial-to-mesenchymal transition [24], i.e., processes which play an important role in fetal development. Although in previous reports no adverse effects were reported following short-term administration of Digibind to patients with preeclampsia [5-7], the safety of prolonged CTS immunoneutralization, as far as potential for fetal complications especially in early onset preeclampsia, has not been established. This issue merits further investigation.

In the present study, in a competitive immunoassay, Digibind exhibited substantial (3-4%) cross-reactivity with two bufadienolides, bufalin and cinobufagin. Although the pathways of biosynthesis of mammalian bufadienolides are still poorly understood, the placenta is considered a major source of CTS elaboration, and previously, bufadienolide molecules were purified from this tissue [17]. Assuming that pathways of biosynthesis of mammalian bufadienolides share some features with the pathways of "classic" steroidogenesis, the placentae may be expected to also contain several yet unidentified bufadienolides. Thus, in toads, in which bufadienolides originate from cholesterol, several bufadienolides with varying structures and biological activities are present [25]. Notably, Digibind has been successfully used to treat bufadienolide poisoning in humans [26,27].

Another possible explanation for the efficacy of Digibind in the face of its low affinity to prototypical CTS, may come from a recent study by Pullen et al, in which it has been demonstrated that the ability of Digibind to immunoneutralize CTS does not equate with its specificity defined in competitive immunoassays [28]. In fact, Digibind represents Fab fragments of polyclonal anti-digoxin antibodies, and, using specific radioligands, Pullen et al. [28] demonstrated that Digibind contains the small but possibly clinically relevant subpopulations of Fab fragments exhibiting high ouabain affinity. The presence of a similar subpopulation of anti-digoxin Fabs with high affinity to MBG or other bufadienolides might have explained the efficacy of Digibind in preeclampsia and in experimental hypertensive models, in which levels of bufadienolides are elevated.

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Figure 1.

Displacement of binding of anti-MBG 4G4 mAb (a), aOU-M anti-ouabain antibody (b), and Digibind (c) to conjugated antigens by cardiotonic steroids in a competitive fluoroimmunoassays. (d) levels of endogenous ouabain, marinobufagenin (MBG), and of digoxin-like immunoreactivity determined by an assay based on Digibind ("Digoxin") in placentae from subjects with uncomplicated pregnancies and in patients with preeclampsia. Ctrl – uncomplicated pregnancy; Preecl – Preeclampsia. Bars represent means $\pm$ S.E.M. Two-tailed t-test.

Figure 2.

Purification of endogenous cardiotonic steroids from preeclamptic placentae on Zorbax Eclipse XDB-C18 HPLC columns. (a-c) – Elution of standards of marinobufagenin (MBG), digoxin and ouabain. (d-f) Pattern of elution of endogenous MBG-immunoreactivity, digoxin-immunoreactivity measured by an assay based of Digibind, and ouabain-immunoreactivity following HPLC fractionation of extracts of placentae from subjects with uncomplicated pregnancy. (g-i) - Pattern of elution of endogenous MBG-immunoreactivity, digoxin-immunoreactivity measured by an assay based of Digibind, and ouabain-immunoreactivity following HPLC fractionation of extracts of placentae from subjects with preeclampsia. Bar on the top of panel “h” indicates retention times of bufadienolide standards (telocinobufagin - 14 min, MBG - 16 min, cinobufatalin - 16 min, bufalin - 17 min, resibufagenin - 19 min, cinobufagin - 19 min).

Table 1

Characteristics of study subjects

	Normal pregnancy (<i>n</i> = 11)	Preeclampsia (<i>n</i> = 12)
Maternal age (years)	26 ± 1	29 ± 2
Gestational weeks at delivery	38.5 ± 0.5	37.4 ± 0.6
No of primigravida (%)	6 (45%)	6 (50%)
Cesarean section (n)	0	3
Vaginal delivery (n)	12	9
Infant birth weight (grams)	3350 ± 68	2823 ± 466
Systolic blood pressure (mmHg)	108 ± 4	165 ± 9 [*]
Diastolic blood pressure (mmHg)	71 ± 3	104 ± 5 [*]

Means ± SEM.

^{*} P < 0.001 vs. normal pregnant subjects, two-tailed t-test.