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DigiFab interacts with endogenous cardiotonic steroids and reverses preeclampsia-induced Na/K-ATPase inhibition

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Abstract

Elevated levels of endogenous Na/K-ATPase (NKA) inhibitors, cardiotonic steroids (CTS) including marinobufagenin (MBG), contribute to pathogenesis of preeclampsia (PE) and represent a target for immunoneutralization by Digibind (Ovine Digoxin Immune Antibody, Glaxo-Smith Kline). Because Digibind is no longer commercially available we studied whether DigiFab (BTG International Ltd, UK) can substitute Digibind for immunoneutralization of CTS in patients with PE. We compared DigiFab, Digibind and anti-MBG monoclonal antibody (mAb) with respect to their ability to interact with CTS in PE plasma and to restore NKA activity in erythrocytes from patients with PE. Using immunoassays based on DigiFab, Digibind, and anti-MBG mAb we studied the elution profile of CTS following HPLC-fractionation of PE plasma.

Seven patients with mild PE (28±2 years; gestational age, 39±0.5 weeks; blood pressure 156±5/94±2 mmHg) and six normotensive pregnant subjects (28±1 years; gestational age, 39±0.4 weeks; blood pressure 111±2/73±2 mmHg) were enrolled. PE was associated with a substantial inhibition of erythrocyte NKA (1.47±0.17 vs. 2.65±0.16 μmol Pi/mL/hr in control group, P<0.001). *Ex vivo*, at concentration 10 μg/mL, which is consistent with the clinical dosing of Digibind administered previously in PE, DigiFab and Digibind as well as anti-MBG mAb (0.5 μg/mL) restored erythrocyte NKA activity. Following HPLC fractionation of pooled PE and control plasma, PE-associated increase in CTS material was detected by Digibind (176 vs. 75 pmoles), DigiFab (221 vs. 70 pmoles) and anti-MBG mAb (1056 vs. 421 pmoles). Therefore, because DigiFab interacts with CTS from PE plasma and reverses PE-induced NKA inhibition, it can substitute Digibind for immunoneutralization of CTS in patients with PE.

Introduction

Preeclampsia (PE) represents one of the most serious complications of pregnancy, leading to maternal and fetal morbidity and mortality. The mechanisms of its pathogenesis are not well understood, and there is no effective cure or prophylaxis of this obstetric malady (1). One of the factors implicated in pathogenesis of PE are endogenous digitalis-like Na/K-ATPase inhibitors, i.e., cardiotonic steroids (CTS) (2–4). Specifically, elevated levels of endogenous

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CTS, including a bufadienolide marinobufagenin (MBG), contribute to the pathogenesis of PE via induction of vasoconstriction (5–7) and fibrosis (8), as well as by impairment of proliferation and invasion of the cytotrophoblast (9,10).

MBG induces natriuresis via inhibition of α-1 Na/K-ATPase in renal epithelium and reduction of sodium reabsorption (11). Exaggerated MBG production, however, causes inhibition of smooth muscle Na/K-ATPase, leading to vasoconstriction (7). Pregnancy is associated with water and sodium retention (12) and with moderately elevated plasma MBG concentrations (5,6,13). While in normal pregnancy moderately elevated MBG levels do not cause vasoconstriction, in patients with PE plasma levels of MBG exhibit dramatic elevations (5–7) and represent a potential target for therapy. Immunoneutralization of CTS is considered to be a novel approach in the treatment of PE (14). During the last 20 years, several successful cases of the treatment of PE by Digibind were reported (15–18). The antihypertensive activity of Digibind is based on the binding of CTS and reversal of the vasoconstriction. Thus, administration of Digibind to patients with PE is associated with restoration of activity of erythrocyte Na/K-ATPase, a target enzyme for CTS (19). Accordingly, *ex vivo* anti-MBG mAb and Digibind also restore PE-induced inhibition of erythrocyte Na/K-ATPase (6,7). Furthermore, in NaCl-supplemented pregnant rats exhibiting symptoms of PE, antihypertensive effect of Digibind and anti-MBG mAb is associated with restoration of the Na/K-ATPase in the vasculature (13). In 2010 Digibind production was terminated by its manufacturer and DigiFab™ became the only available anti-digoxin antibody, approved by FDA for administration to human patients in need of reversal of digoxin toxicity. The research aim of our study was to investigate whether DigiFab can substitute Digibind for immunoneutralization of CTS in patients with PE. For this, we compared DigiFab and Digibind with respect to their ability to *ex vivo* reverse PE-induced inhibition of erythrocyte Na/K-ATPase. Next, using competitive immunoassays based on DigiFab and Digibind, we studied profile of elution of CTS following fractionation of normal pregnant and PE plasma on reverse-phase high-performance liquid chromarography (HPLC) column. In our experiments we also compared effects of DigiFab and Digibind with that of 3E9 anti-MBG monoclonal antibody (mAb) which recently was shown to reverse PE-induced Na/K-ATPase inhibition (7) and to potently reverse deleterious effects of CTS in chronic renal failure (20).

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. Thirteen participants who were admitted to the Institute of Neonatology and Pediatrics, Almazov Federal Heart, Blood and Endocrinology Center in St Petersburg, Russia, were consented and enrolled in the study. Diagnosis of mild PE in seven patients was based on the criteria of American Congress of Obstetrics and Gynecology (21). This definition includes systolic blood pressure of at least 140 mmHg, or diastolic blood pressure of at least 90 mmHg, at least on two occasions 6 hours apart and new onset proteinuria (urinary protein excretion more than 0.3 g/24 hr or a urinary protein concentration of more than 1g/L in at least two random urine specimens collected 6 hours or more 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, chronic cardiovascular, renal, hepatic, or endocrine disorders. We specifically enrolled six age-matched normotensive uncomplicated pregnancies to serve as the control group.

Ten ml of venous blood were collected in heparinized tubes. 4.0 ml blood was centrifuged at 1,500 g for 15 min and plasma samples were kept at −80°C. 250 μL aliquots of plasma from PE and control subjects were extracted on Sep-Pak C-18 reverse-phase cartridges (Waters,

Milford, MA), and eluted with 80% acetonitrile. Individual samples were used for measurement of CTS using competitive fluoroimmunoassays based on a murine anti-MBG 4G4mAb, rabbit polyclonal antiouabain antibody "Anti-OU-M-2005", and rabbit polyclonal anti-digoxin antibody (Sigma Chemicals, St. Louis, MD) as recently described in detail (7,22). 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 cross-reactivity of anti-digoxin polyclonal antibody is (%): digoxin – 100, ouabain < 0.01, MBG – 0.2, digitoxin – 10, bufalin – 0.01, cinobufagin < 0.01. The cross-reactivity of anti-OU-M-2005 ouabain antibody is (%): ouabain – 100, ouabagenin - 52, digoxin - 1.8, 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, MBG – 0.036.

Six mL blood was used for the measurement of erythrocyte Na/K-ATPase activity in the presence and in the absence of 3E9 anti-MBG mAb, Digibind and DigiFab as reported previously in detail (6,7). The amount of Digibind and DigiFab for the *ex vivo* incubation with blood was 10 μg/mL, which corresponds to the dose used clinically in PE (16–18). Anti-MBG 3E9 mAb was used in concentration, which reversed IC_{50} for the inhibition of rat α-1 Na/K-ATPase from renal medulla by MBG (7). Cross reactivity of 3E9 anti-MBG mAb is (%): MBG – 100; marinobufotoxin – 4; telocinobufagin – 7; cinobufotalin – 44; cinobufagin – 1.4; resibufagenin – 0.5; bufalin – 0.3; proscillaridin A – 3; ouabain – 0.02; ouabagenin - <0.001; digoxin – 1.8; digitoxin – 0.7; aldosterone - <0.01; progesterone - <0.01; prednisone - <0.001.

250 μL of plasma samples from each group were pooled, extracted on C-18 cartridges as above, dried, reconstituted in 10% acetonitrile and fractionated on Agilent 1100 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 1ml/min, in linear (10 – 85.5 %) gradient of acetonitrile against 0.1% trifluoroacetic acid (TFA) for 45 min. Thirty 1.5-min fractions were collected and analyzed for MBG-immunoreactivity using assays based on 4G4 anti-MBG mAb (above) and on 3E9 anti-MBG mAb, an antibody previously reported to reduce blood pressure in experimental hypertension and to *ex vivo* reverse PE-induced Na/K-ATPase inhibition (7), and digoxin-like immunoreactivity using assays based on Digibind and DigiFab. For the digoxin immunoassay, we labeled the primary antibodies, Digibind and DigiFab using a Europium-labeling kit (Perkin Elmer, Wellesley, Massachusetts), as reported previously in detail for Digibind (7,23). The assays are 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 or DigiFab. The sensitivity of Digibind and DigiFab immunoassays for digoxin is 0.01 nmol/l, data on cross-immunoreactivity of Digibind and DigiFab are presented in Table 1.

All chemicals were from Sigma-Aldrich (St. Louis, MO). Digibind™ was obtained from Glaxo-Smith Kline, Research Triangle Park, NC, and DigiFab™ from BTG International Ltd, London, UK. MBG (>98% purity by HPLC) was purified from the secretion from parotid glands of Bufo marinus toads (24), and anti-MBG 3E9 and 4G4 mAb were developed as reported recently in detail (7).

The results are presented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA) (intergroup analysis) or by repeated measures ANOVA (intragroup analysis) followed by Newman-Keuls test, and by two-tailed *t*-test when applicable (Graph

Pad Prism Software, San Diego, CA). A two-sided *P* value of less than 0.05 was considered to be statistically significant.

Results

Maternal demographics and data on levels of blood pressure are presented in Table 2. 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 increase in plasma MBG (Figure 1a) and a concomitant 50% inhibition of Na/K-ATPase activity in erythrocytes (Figure 1d). *Ex vivo* incubation of erythrocytes in the presence of Digibind, DigiFab and of 3E9 anti-MBG mAb restored activity of Na/K-ATPase (Figure 1d). As illustrated in Figure 1b, plasma levels of ouabain immunoreactivity in patients with PE did not differ significantly from that in control group, while levels of digoxin-like immunoreactivity exhibited significant elevation (Fig. 1c).

The retention times of cardenolide and bufadienolide CTS standards on Agilent Zorbax Eclipse XDB-C18 HPLC column were: ouabain – 8.5 minutes (fraction 6), digoxin – 18.0 minutes (fraction 13), telocinobufagin – 20.5 minutes (fraction 14), MBG – 22.6 minutes (fraction 16), and marinobufotoxin – 22.7 minutes (fraction 16), cinobufatalin – 22.9 minutes (fraction 16), bufalin – 24.5 minutes (fraction 17), and resibufagenin – 27.7 minutes (fraction 19). As presented in Figure 2 following HPLC fractionation of normal and PE plasma maximum ouabain-immunoreactivity co-eluted with ouabain standard. Levels of ouabain-like immunoreactivity in HPLC fractions from control plasma did not differ significantly from that in preeclamptic plasma (620 pmoles and 780 pmoles, respectively) (Figure 2c,d).

In addition to measuring MBG in C18-extracted plasma samples (Fig 1a), levels of MBG immunoreactivity were determined in HPLC fractions from control and PE plasma using immunoassays based on two anti-MBG mAbs, 4G4 which is used to measure plasma MBG levels, and 3E9 which potently reverses effects of endogenous MBG in vivo (7,20). Data are presented in Figure 2a,b (assay based on 4G4 anti-MBG mAb) and in Figure 3e,f (assay based on 3E9 anti-MBG mAb). As presented in Figure 2a, following HPLC fractionation of control plasma MBG immunoreactive material eluted in fractions 11–16. Immunoassay based on 4G4 mAb detected a two-fold increase in MBG in HPLC fractionated PE plasma (648 pmoles vs. 310 pmoles in HPLC fractions from normal pregnant plasma) with a maximum of MBG immunoreactivity co-eluting with MBG standard in fraction 16 (Figure 2c).

Figure 3 illustrates data on interaction of antibodies used to reverse PE-induced Na/K-ATPase inhibition, DigiFab, Digibind and 3E9 anti-MBG mAb, with CTS following fractionation of normal pregnant and PE plasma on reverse-phase HPLC column. As presented in Figure 3a,c,e, following fractionation of control plasma assays based on Digibind, DigiFab, and 3E9 anti-MBG mAb detected CTS in fractions 14–18. In HPLC-fractionated PE plasma material measured using Digibind and DigiFab eluted in fractions 17–22 with maximum in fraction 18 (Figure 3b,d). MBG-immunoreactive material from PE plasma measured by 3E9 mAb eluted in fractions 16–22 with distinctive a maximum eluting in fraction 16 (Figure 3b,d,f). The total amount of MBG-immunoreactive material determined by 3E9 anti-MBG mAb in HPLC fractions following fractionation of PE plasma was 2.5-fold greater than that in control samples (1056 pmoles vs 421 pmoles) (Figure 3e,f). The PE-associated increase in CTS material from was detected by Digibind (176 vs. 75 pmoles)(Figure 3c,d) and DigiFab (221 vs. 70 pmoles)(Figure 3a,b) and magnitude of PE-induced increase in the levels of digoxin-like immunoreactivity in HPLC fractions was similar to the magnitude of increase in the levels of MBG.

Discussion

The main observations of this study are that DigiFab, affinity-purified Fab fragments of anti-digoxin antibody, detects PE-induced increase in plasma levels of CTS and *ex vivo* reverse PE-induced inhibition of erythrocyte Na/K-ATPase similar to that of Digibind. These data suggest that DigiFab is capable to immunoneutralize CTS in PE and other conditions in which elevated levels of CTS exhibit deleterious effects.

Digitalis-like CTS act as endogenous ligands of the Na/K-ATPase and exhibit effects mediated by pumping and/or by signaling effects of this enzyme (4,11). According to the Concept of Natriuretic Hormone, CTS are produced with the adaptive aim to regulate sodium excretion via inhibition of the Na/K-ATPase in renal tubules (4). In patients with salt-sensitive hypertension exaggerated CTS response, however, produces inhibition of the sodium pump in the vasculature leading to vasoconstriction (4,11). In addition, an increasing body of evidence indicate that CTS bind to NKA and elicit cell signaling events leading to oxidative stress, hypertrophic growth and fibrosis, apoptosis, and cell differentiation (4). Previous studies have demonstrated that in mammals plasma levels of CTS, including MBG and endogenous ouabain, are moderately elevated throughout pregnancy indicating that these hormones are implicated in the physiology of gestation (5–7,13). In preeclampsia however, plasma and placental levels of CTS, and especially MBG, become substantially elevated and associate with heightened arterial pressure and inhibition of erythrocyte Na/K-ATPase (5–7,13). Moreover, in PE placental vascular bed exhibits heightened sensitivity to CTS-induced vasoconstriction (25). Recent evidence, however, indicates that the contribution of CTS to PE is not limited to only vasoconstriction. MBG in PE has been demonstrated to contribute to the impairment of vascular relaxation causing vascular fibrosis via inhibition of Fli-1, a nuclear transcription factor and a known negative regulator of collagen synthesis (8). In addition, MBG, acting via activation of Jnk, p38, and Src leads to increased apoptosis and IL-6 secretion, that has been reported to impair the proliferation, migration, and invasion of cytotrophoblast cell (9,10). Most recently elevated levels of MBG in pregnant NaCl-supplemented rats were shown to be associated with reduced number of nephrons in the offspring which is known to be a factor predisposing to the subsequent development of hypertension (26).

The above features make CTS attractive targets for therapy of PE, but interference with CTS at the level of cell signaling could interfere with other signaling pathways implicated in physiology of gestation (27,28). Therefore, in PE, immunoneutralization appears to be a promising approach to CTS antagonism. Because CTS share some of their antigenic properties with digitalis and digoxin, and antibodies raised to them have been used in humans to treat PE, the idea of immunoneutralization of CTS with Digibind has emerged (13). Goodlin was the first to report successful use of Digibind in a patient with PE (14). Following individual case reports (15,16). Digibind has been reported to reduce blood pressure in cohort of subjects with postpartum PE (17). Data from a recent phase II B randomized, double blinded, placebo controlled clinical trial of Digibind in women with severe antepartum PE suggest that CTS-induced abnormalities could be reversed by Digibind (18). The fact that in our previous (6,7) and present studies PE-induced inhibition of erythrocyte Na/KATPase was ex vivo reversed by immunoneutralization of CTS demonstrates that in PE there is a causative link between elevated levels of endogenous digitalis and sodium pump inhibition. In 2011 production of Digibind was terminated and currently DigiFab remains the only anti-digoxin antibody available for administration to human subjects. Our present data suggest that with respect to reversal to PE-induced Na/K-ATPase inhibition, DigiFab can substitute Digibind.

Because several cardenolide and bufadienolide CTS exist in mammalian tissues, a question arises concerning the identity of targets of DigiFab and Digibind in PE. In the present study, Digibind and DigiFab, as well as anti-MBG mAb did not detect increases in the level of the material co-eluting with ouabain (HPLC fraction 6), which agrees with our previous data demonstrating that bufadienlide CTS, rather than endogenous ouabain, are implicated in pathogenesis of PE (7,8). In competitive immunoassays, both DigiFab and Digibind detected substantial increases in CTS levels in HPLC fractions 17–22. While 4G4 mAb detected increased levels of MBG mainly in HPLC fraction 16, 3E9 anti-MBG mAb was capable to detect PE-induced elevation of CTS in HPLC fractions 16–22, which overlaps with the material detected by assays based on DigiFab and Digibind. Thus, DigiFab and Digibind cross-reacted with MBG-immunoreactive material eluting in fractions 18–22 and corresponding to the elution time of several other bufadienolides, bufalin, resibufagenin, and cinobufagin. This pattern closely resembles that observed in our previous study investigating interactions of Digibind with CTS from preeclamptic placentae (23). In our previous study, following HPLC fractionation of chloroform extract of PE placentae, 3E9 mAb in addition to MBG per se, cross-reacted with more hydrophobic material having retention time greater than MBG. In the present study, PE-induced elevation in CTS levels in HPLC fractions eluting later than MBG was detected by 3E9 mAb, DigiFab and Digibind (23). Thus, our previous findings and our present data warrant search for yet unidentified hydrophobic bufadienolide CTS. The fact that 3E9 mAb cross-reacted with several HPLC fractions from PE plasma, may at least in part explain very high in vivo potency of this antibody. Thus, in rats with chronic renal failure, a single injection of 3E9 mAb induced a hypotensive effects lasting for 7 days, and reduced levels of oxidative stress and fibrosis in the myocardium (20).

In summary, our results demonstrate that DigiFab and Digibind detect identical CTS in PE plasma, exhibit comparable cross-immunoreactivity with CTS, and exert similar capacity to *ex vivo* reverse PE-induced Na/K-ATPase inhibition. Because DigiFab appears clinically similar to Digibind in its ability to immunoneutralize CTS, it holds promise for treatment of PE and other conditions in which CTS exhibit deleterious effects, including cerebral salt-wasting syndrome (29) and chronic kidney disease (20,30).

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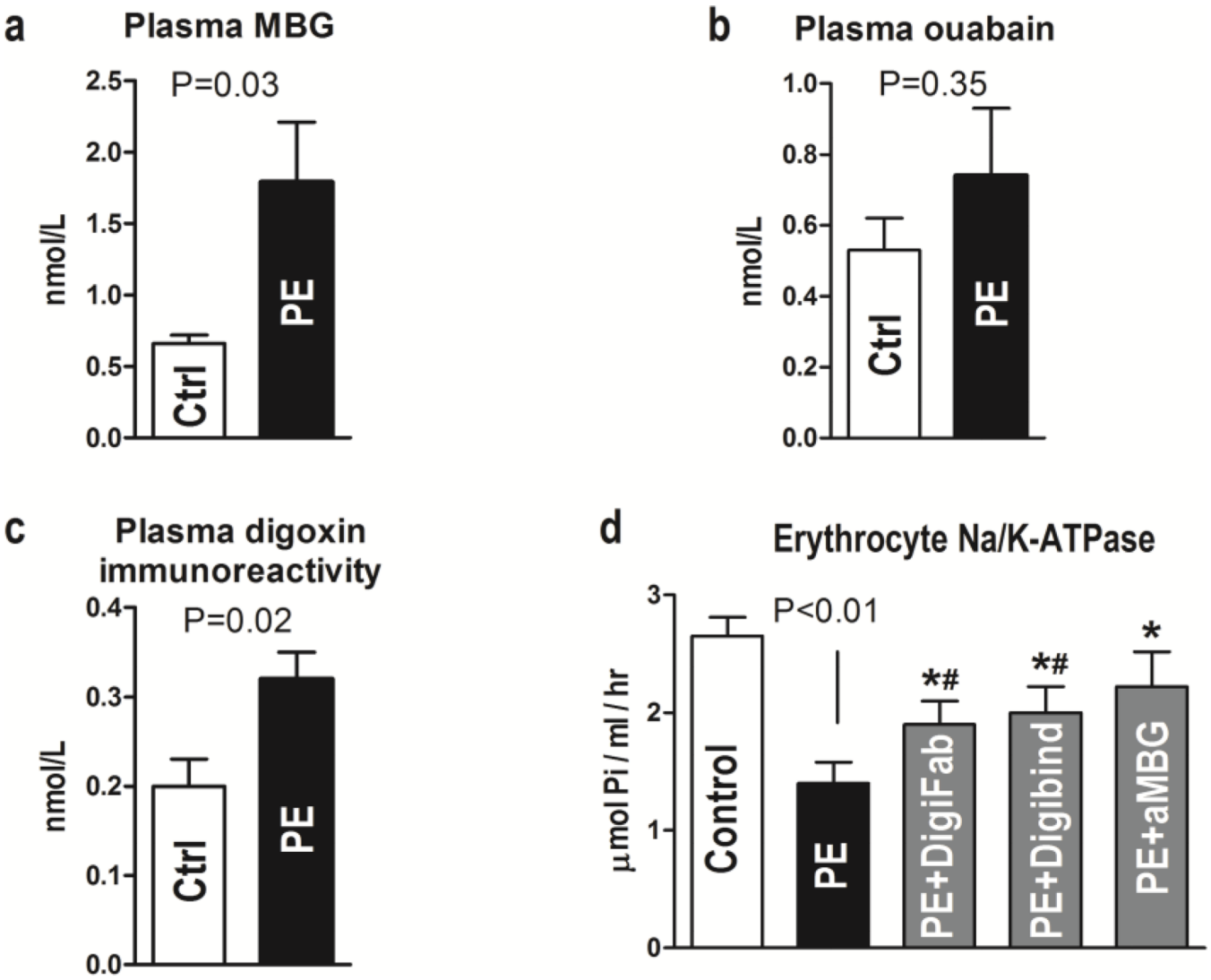

Figure 1.

Plasma levels of MBG (a), endogenous ouabain (b), and digoxin-like immunoreactivity (c) in normotensive pregnant subjects (Ctrl) and in patients with preeclampsia (PE). d - Erythrocyte Na/K-ATPase in normal pregnant subjects and in patients with preeclampsia, and effect of ex vivo treatment with DigiFab, Digibind and 3E9 anti-MBG mAb (aMBG) on erythrocyte Na/K-ATPase. * - P<0.01 vs. PE; # - P<0.01 vs. control by repeated measures ANOVA and Newman-Keuls test.

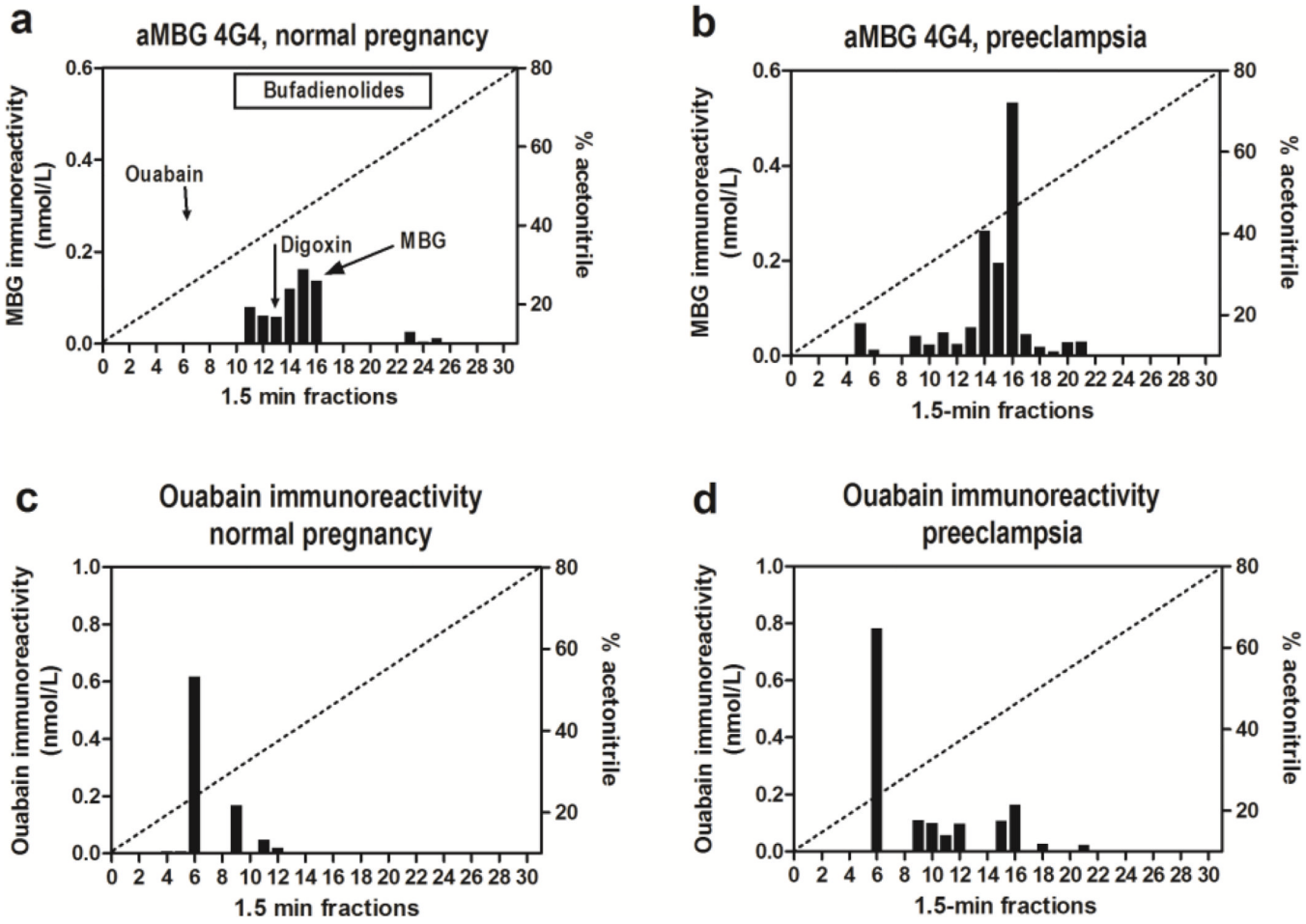

Figure 2.

Fractionation of endogenous CTS from normal and preeclamptic plasma on Zorbax Eclipse XDB-C18 HPLC columns. (a-b) – Pattern of elution of endogenous MBGimmunoreactive material determined by assay based on anti-MBG 4G4 mAb. Retention times of MBG, ouabain and digoxin standards are indicated by arrows. (c-d) - Pattern of elution of endogenous ouabain-immunoreactive material. Bar on the top of panel “a” indicates retention times of bufadienolide standards.

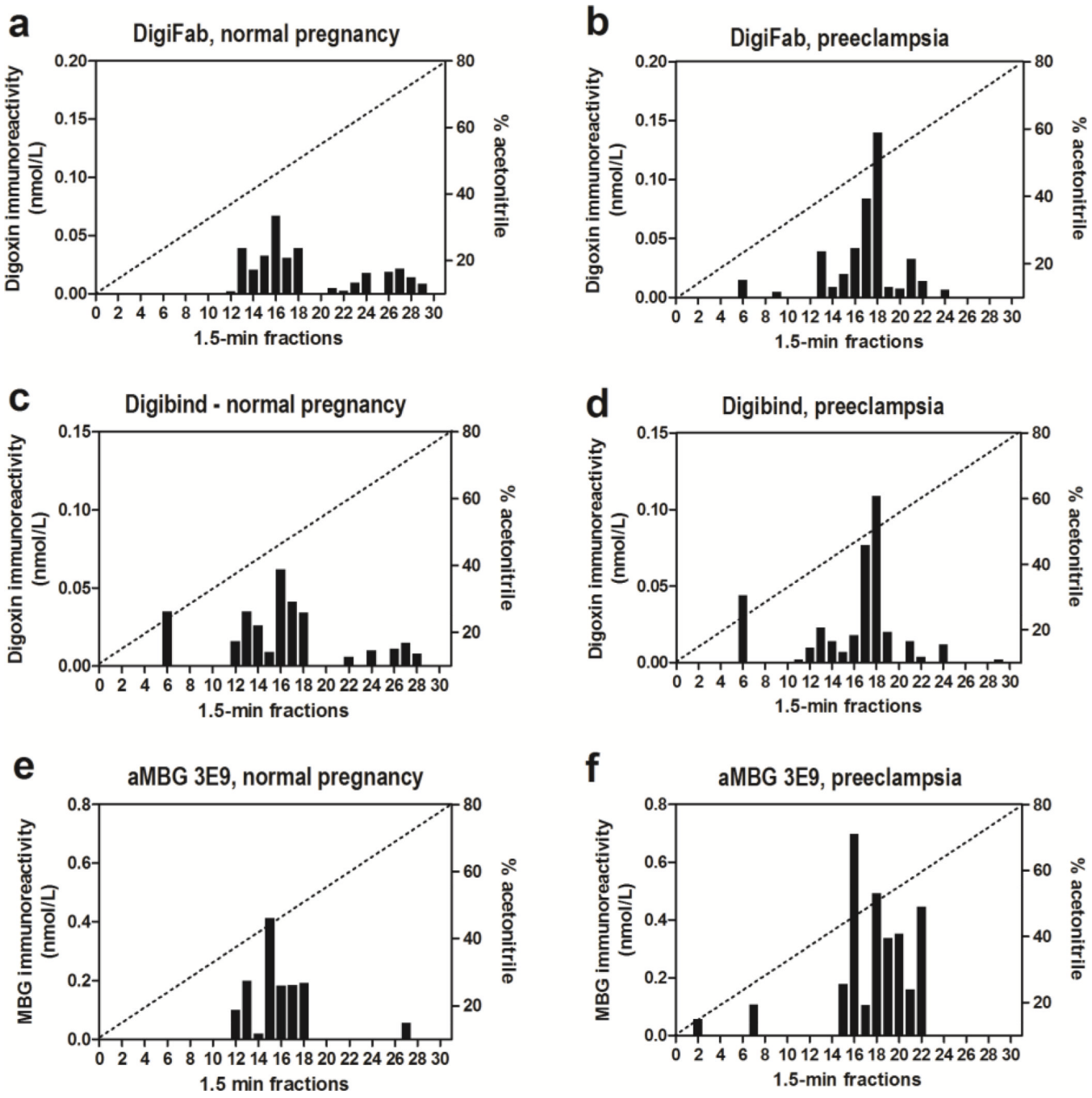

Figure 3.

Fractionation of endogenous CTS from normal and preeclamptic plasma on Zorbax Eclipse XDB-C18 HPLC columns. (a-d) Pattern of elution of digoxin-immunoreactivity measured by assays based on Digibind and DigiFab. (e-f) - Pattern of elution of endogenous MBG-immunoreactivity measured by immunoassay based on anti-MBG 3E9 mAb.

Table 1

Cross-immunoreactiviy of Digibind and DigiFab

Cross-reactant	% cross-reactivity	
	Digibind	**DigiFab**
Digoxin	100	100
Digitoxin	1.4	1.3
Digoxigenin	76	100
Ouabain	0.4	0.14
Ouabagenin	0.1	0.025
MBG	0.2	0.29
Marinobufotoxin	0.06	0.02
Telocinobufagin	1.0	0.34
Bufalin	2.7	0.9
Cinobufagin	0.02	0.03
Cinobufotalin	0.2	0.006
Resibufagenin	1	0.02
Proscillaridin-A	1.46	0.2
Prednisone	< 0.001	<0.001
Progesterone	< 0.001	< 0.001

Table 2

Characteristics of study subjects and levels of blood pressure

	Normal pregnancy (*n* = 6)	**Preeclampsia (*n* = 7)**
Maternal age (years)	28 ± 1	28 ± 2
Gestational weeks at delivery	39.0 ± 0.4	39 ± 0.5
No of primigravida (%)	6 (100%)	6 (86%)
Cesarean section (n)	3	2
Vaginal delivery (n)	3	5
Infant birth weight (grams)	3796 ± 121	3401 ± 201
Systolic blood pressure (mmHg)	111 ± 2	156 ± 5 *
Diastolic blood pressure (mmHg)	73 ± 2	94 ± 2 *

Means ± SEM. (*) – P < 0.001 vs. normal pregnant subjects, two-tailed t-test.