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A Novel Use of Digoxin Immune Fab Fragment in Identification and Isolation of an Endogenous Digitalis-like Factor Found in Preeclampsia

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A dissertation submitted to the faculty of
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in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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ABSTRACT

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The mechanisms mediating the hypertension of preeclampsia (PE) are unclear. Endogenous digitalis-like factors (EDLFs) are specific sodium pump (SP) inhibitors implicated in essential and experimental hypertension, but they have not been fully explored in the setting of PE. This study uses a digoxin antibody Fab fragment to address the question of whether such factors are present and increased in PE, to investigate a possible treatment of PE, and to isolate and characterize all EDLFs present in PE. Sera and placenta from women with PE did show a significant increase in SP inhibition in comparison to women with normal pregnancy and Digibind® was found to bind EDLFs and essentially block or reverse SP inhibition. Sera were collected in a Phase II, double-blind, placebo controlled clinical study in which women with severe preeclampsia were dosed with Digibind®, as a therapeutic, and the SP activity measured. Sera and placenta from women with PE was also investigated for their inhibitory effects on the SP. Known candidates for EDLFs were investigated for their SP inhibitory effects, as well as how digitalis antibody immune Fab fragments, Digibind® and DigiFab™, bound them and affected the SP activity. Digibind® is also a sufficient affinity material used to isolate and purify PE EDLFs. Additionally, the placentas of preeclamptic women have high levels of similar EDLFs. These studies provide evidence for the existence of EDLFs that circulate in women with PE, and Digibind® is an effective and novel tool to bind, isolate and purify EDLFs in PE.

Keywords: preeclampsia, Digibind, ouabain, Na^+/K^+-ATPase, placenta

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ABREVIATIONS

preeclampsia – PE

hemolysis, elevated liver enzymes, and low platelet count – HELLP

intrauterine growth restriction – IUGR

soluble fms-like tyrosine kinase 1 – sflt1

placental growth factor 1 – P1G1

sodium pump – SP

endogenous digitalis like factor – EDLF

ouabain – OBN, OUA

Digibind® - Digibind, Db

DigiFab™ – DigiFab

red blood cell – RBC

high pressure liquid chromatography – HPLC

proscillaridin A – ProA

rubidium – Rb

necrotizing enterocolitis – NEC

intraventrical hemorrhaging - IVH

graphite furnace atomic absorption – GFAA

Digibind Efficacy Evaluation of Preeclampsia – DEEP

radioactive immunoassay – RIA

polyethylene glycol – PEG

CHAPTER 1 – INTRODUCTION

PREECLAMPSIA

Preeclampsia (PE) is the leading cause of maternal death worldwide. Over 40,000 deaths a year worldwide can be attributed to PE. PE occurs in ~5% of all pregnancies. This percentage is closer to 15% to 20 % of pregnancies in small developing countries due to limited maternal care (Wallis, 2008) (Report, 2000). PE is also responsible for 15% of all premature births or approximately 70,000 early births anually (Report, 2000). PE is very difficult to diagnose early, so with very little warning of the onset of disease, severe complications can develop.

Eclampsia is the final and most severe phase of preeclampsia and can occur when PE is left untreated. Women with eclampsia have seizures and often die during or after pregnancy. Essentially, if PE can be controlled, then symptoms and cases of eclampsia can be prevented.

Symptoms of PE

The symptoms of PE are extensive. Women with PE report severe headaches, swelling of hands and feet, blurred vision, dizziness, nausea, and other flu like symptoms. Unfortunately, the flu is commonly diagnosed, and PE goes undetected. PE occurs in the second half of the pregnancy arising as early as 20 weeks gestation. It can arise quickly, sometimes in hours. Clinical symptoms of PE may include renal failure, edema, increased blood pressure, and those symptoms associated with HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome. The edema, raised blood pressure and protein in the urine are the first detectable physiological changes to occur to signal possible PE. Hypertension and proteinuria are considered diagnostic of PE.

PE not only manifests itself as a maternal disorder, but can also cause fetal complications, such as restricted growth or sudden fetal death. Prematurity and intrauterine growth restriction (IUGR) are the most common fetal complications, but there are others that seem more severe and harder to predict. For example, necrotizing enterocolitis and intraventricular hemorrhage are two severe, fetal complications that arise commonly with early PE and may be somewhat independent of the consequences of premature birth.

Risk Factors of PE

A detailed systematic review of the risk factors involved with PE was recently performed by Duckitt *et al.* (Duckitt, 2005). Reported risks of age (both 40 and older and younger than 17), nulliparity, previous PE, family history of PE, multiple pregnancy, pre-existing medical conditions (including insulin dependent diabetes, chronic hypertension, renal disease, autoimmune disease, antiphospholipid syndrome, extended time between pregnancies, increased body mass index, preexisting high blood pressure and proteinuria) all were investigated and reviewed both by past published data and current controlled cohort studies. The results of this extensive study showed that women over 40 years old have twice the risk of developing PE, but there was no consistent data that showed women under 17 had higher risk. Nulliparity, multiple pregnancy and family history of PE triples the risk of PE; previous PE actually increases the risk by seven fold; and insulin dependent diabetes nearly quadruples the risk of developing PE.

Only 29 out of 155 investigated cases of pre-existing hypertension actually developed severe PE, but those women who did had significantly higher rates of perinatal morbidity, low birth weight infants, and delivery before 32 weeks (Duckitt, 2005). Only one study investigated women with a history of renal disease or a history of urinary tract infections and scarred kidneys, and found that 2 out of 30 developed PE (Martinell, 1990). Existence of chronic autoimmune

disease and existence of antiphospholipid syndrome did increase the risk of developing PE with pregnancy. Although, interesting enough, women who were diagnosed with PE did not necessarily have increased risk of later development of diabetes, autoimmune diseases, chronic hypertension or renal disease (Duckitt, 2005). So, existence of these conditions can provide a red flag or warning to obstetricians, but does not necessarily determine whether PE will develop in individual cases of pregnancy and more importantly fails to provide a means of determining the cause of PE.

Causes of PE

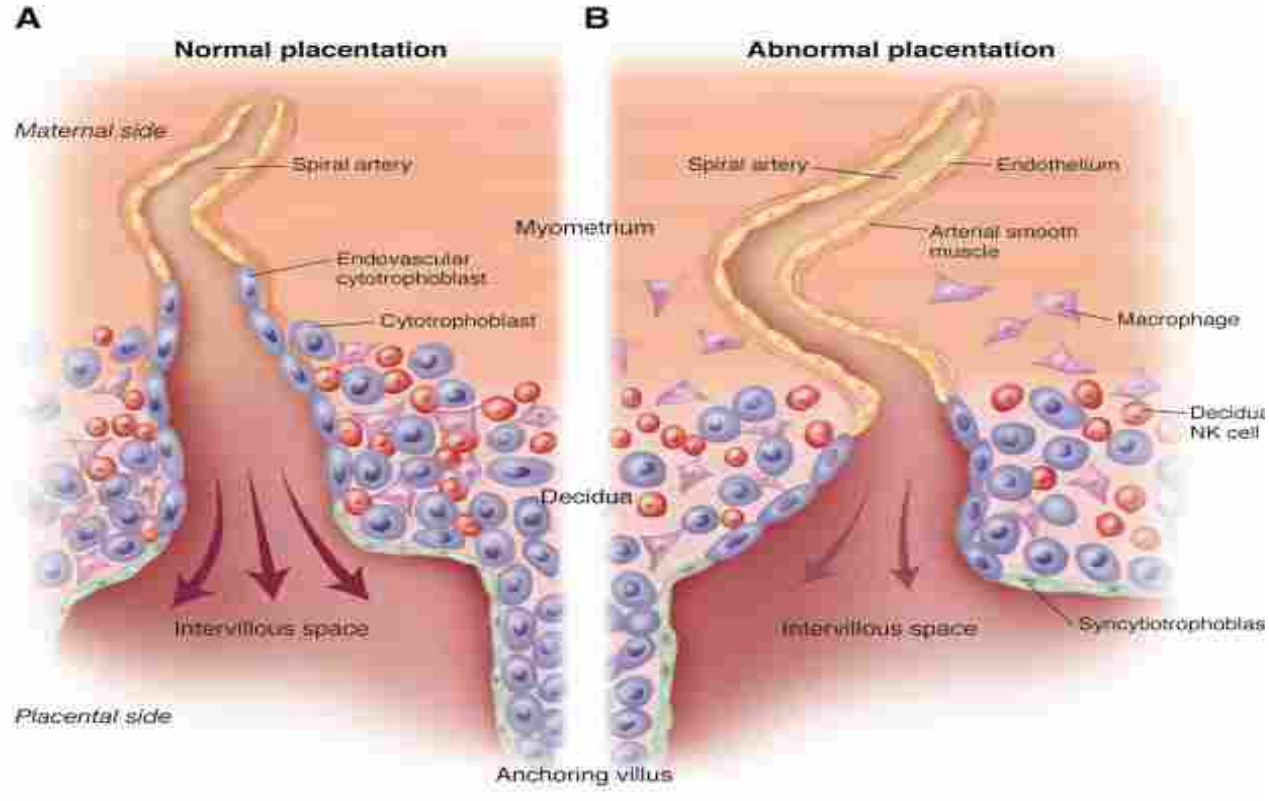
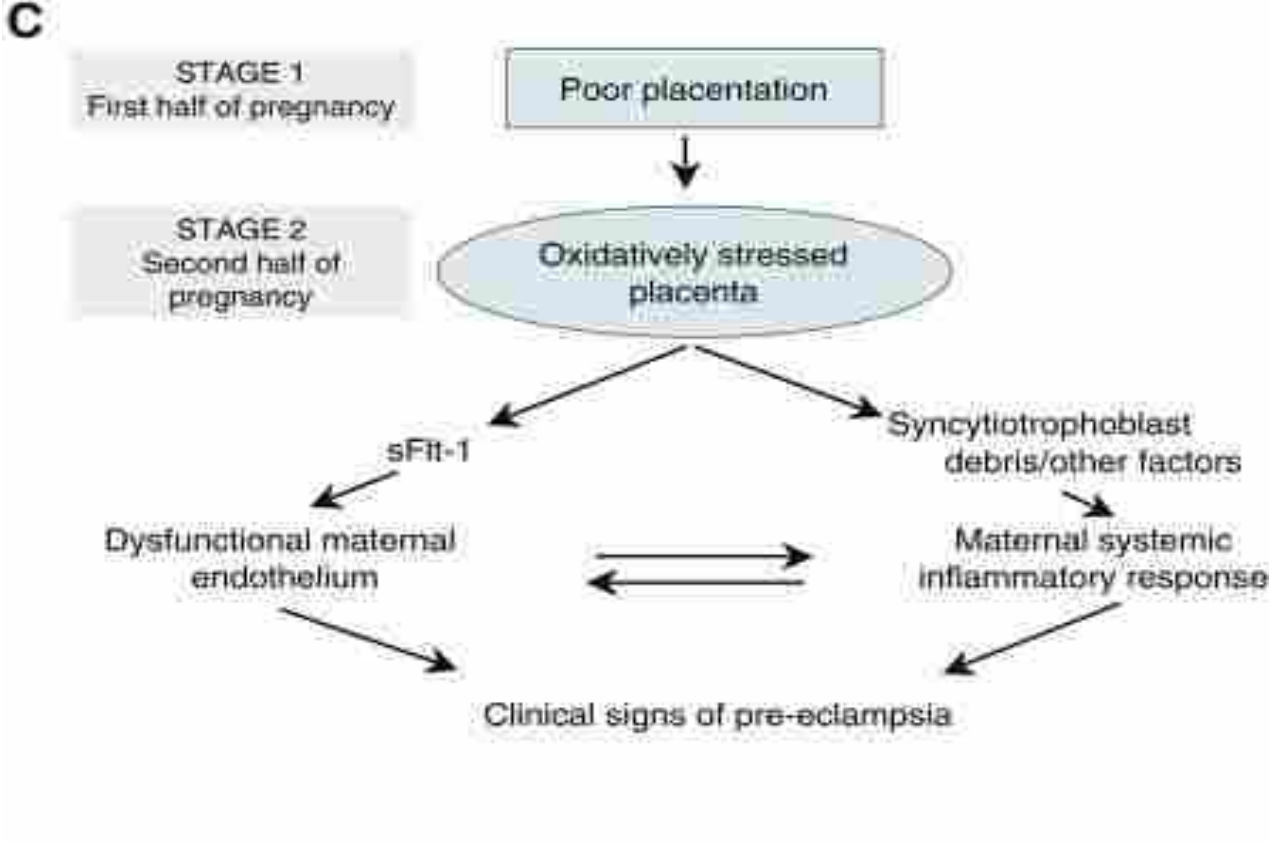


Figure 1-1. Remodeling of spiral arteries.
(A) shows normal placentation, sufficient trophoblast invasion and widening of blood vessels where (B) shows abnormal placentation, insufficient trophoblast invasion and decreased blood flow caused by the unsoftening of the arterial smooth muscle. Factors and macrophages are released in an immune response. This can lead to the situation detailed in (C) (next page, below) where insufficient blood flow leads to an oxidatively stressed placenta and release of factors leading to the critical stages of PE (Redman, 2005).



(Redman, 2005)

Many investigators believe that PE originates in the placenta (Redman, 2005). During the second trimester, the uterine spiral arteries are supposed to transform themselves from thick-walled, coiled, muscular vessels into more flaccid, uncoiled tubes to accommodate 10 times the blood flow needed for the developing fetus. Trophoblast cells enter the arterial walls and replace smooth muscle to allow the artery to dilate. In PE, this arterial remodeling or placentation is not completely successful. Redman and Sargent theorize that either there is a production failure of endothelial adhesion molecules from the trophoblast or there is a failure of immune response from the trophoblasts that prevent proper placentation (Redman, 2005). This is illustrated in *Fig. 1-1* above in parts A and B.

With improper placentation and reduced blood flow to the intervillous space of the placenta oxidative stress is introduced. This oxidative stress causes the release of factors, cytokines, and/or immunological responses that seem to cause or trigger changes eventually leading to PE. Whether these signals are sent maternally or neonatally is not known, but most

probably involve a combination of both. These signals or factors could be an important aspect in determining the cause of PE.

Although PE has been studied extensively, there are many models of the causes of PE; the cause has yet to be identified due to its multisystem character. It may even have multiple causes. Other possible causes continue to be researched in an effort to lower the prevalence of PE. Normal function of endothelial cells includes their lining all blood vessels to provide vessel wall integrity, preventing intravascular coagulation, maintain appropriate smooth muscle regulation and mediating immune and inflammatory responses. Accordingly endothelial cells are being researched extensively as a source for the cause of PE.

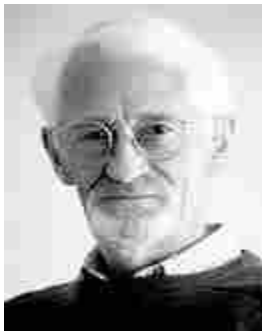
Another model of the cause of PE considers that placental vascular insufficiencies, in addition to, abnormal endothelial cell function, are the mechanism by which PE is brought about (Levine, 2004). Cytokines within these cells control cell development as well as secrete circulating factors that figure in fetal development. Levine et al. previously indicated that soluble fms-like tyrosine kinase 1 (sFlt-1), an anti-angiogenic factor, is increased in women with PE and placental growth factor 1 (PlG1), a pro-angiogenic and growth factor, is reduced. These changes were found not only when PE was clinically apparent, but several weeks earlier. Levine *et al*. hypothesized that the raised levels of sFlt-1 lead to reduced endothelial growth factors, including PIGF and VEGF, which in turn leads to placental vascular insufficiency.

Another suggested model of PE considers the hypertension associated with PE. Animal models of PE have revealed that the hypertension of PE may be due to increased circulating levels of Na^+/K^+-ATPase or sodium pump (SP) inhibitors (Hamlyn JM, 1996). Significantly, the inhibition of the SP leads to vascular smooth muscle contraction, vasoconstriction and potentially hypertension by membrane depolarization and increased Ca^{2+} entry into the cell.

These endogenous factors appear to be structurally similar to known cardioglycosides from plant sources, such as digitalis. *Digitalis purpurea*, the common foxglove plant, provides a pharmaceutical agent that is used in congestive heart failure patients to stimulate increased contraction of the heart muscle by inhibiting the SP found in cardiac muscle. Other factors similar to digitalis in structure and function have been found endogenously (D'Urso, 2004) (Hamlyn, 1996). These endogenous digitalis-like factors (EDLFs) have been isolated from animal tissues, such as bovine hypothalamus and adrenals (Laredo, 1994) (Murrell, 2005). Human EDLFs structural and chemical formulae have yet to be conclusively identified, although the work presented here will show a measured increase of EDLF activity in women with PE.

Na^+/K^+ATPASE

In 1997, Jens C. Skou was a Nobel Prize winner for his discovery of the Na^+/K^+ATPase. Since its discovery, consistent subsequent articles have been published each year regarding this ion transporting enzyme (Therien AG, 2000). The basic function of the Na^+/K^+-ATPase or SP is to maintain the high Na^+ and K^+ gradients across the plasma membrane of animal cells. The SP is the major determinant of low cytoplasmic Na^+. It has an important role in regulating cell volume, cytoplasmic pH, and Ca^{2+} levels driving a variety of secondary transport processes such as Na^+-dependent glucose and amino acid transport (Therien AG, 2000). The SP is, in turn, the target of many regulatory mechanisms.



Jens C. Skou
(http://nobelprize.org)

The SP helps maintain resting cell membrane potential and helps regulate cell volume as well as acts as a signal transducer for the MAPK pathway. This resting potential is dissipated

when K^+ channels take too long to close or with increased cell Na^+ and lead to Ca^{2+} entry into the cell, which can be a consequence of SP blockade resulting from depolarization. In cardiovascular tissue, this can cause increased contraction and increased cardiac output. Conversely, if the cell volume and osmotic pressure increase, the cell begins to swell, the SP is activated to pump ions with attendant water to the cell exterior. Cell volume expansion and increased cell Na^+ are symptoms of PE.

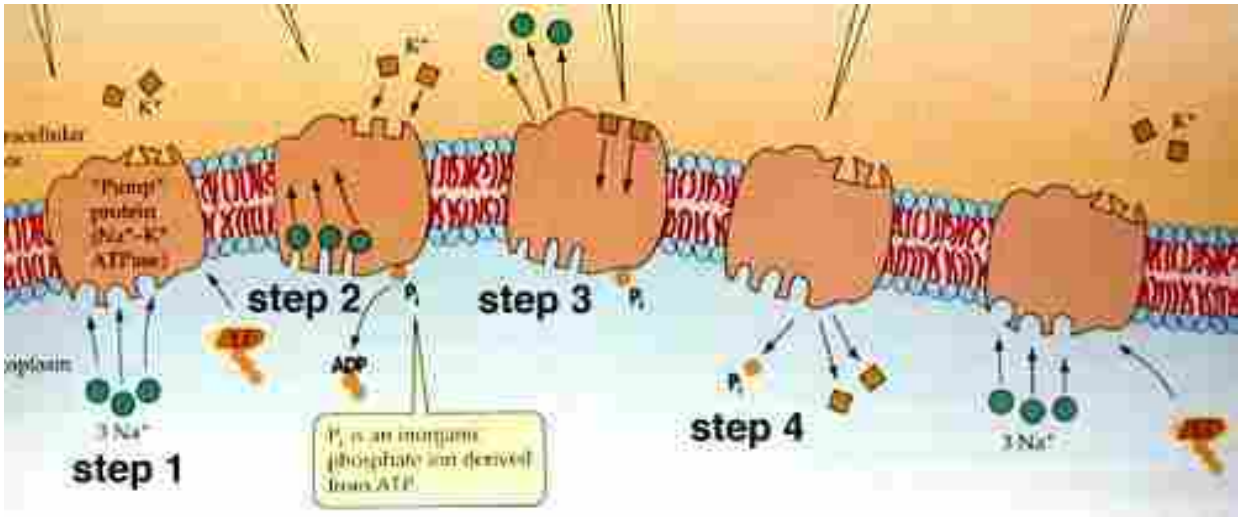


Figure 1-2. SP ionic transport activity.
Step 1 – SP binds three Na+ and ATP. Step 2 – Na+ exit cell through SP, ADP is release and two K+ are bound by SP. Step 3 – Two K+ are taken up by the SP. Step 4 – Two K+ and an inorganic phosphate ion are released from SP.
(http://academic.brooklyn.cuny.edu/biology/bio4fv/page/atp_ann.htm)

This membrane depolarization and Na^+ retention occurs at intensified levels when the SP is inhibited. This inhibition is known to occur with cardioglycosides, or with endogenous digitalis-like factors (EDLFs) circulating within the blood. Whether the EDLF found in PE is ouabain, or one of the other proposed SP inhibitors whose structures are known, or a new structural factor similar to one of these EDLFs still remains to be seen, but there is sufficient evidence, which will be shown within this work to indicate the presence of such SP inhibitors in

the circulation of pregnant women and evidence that these EDLFs may play an important factor in PE. Ouabain, a candidate EDLF is shown below in *Fig. 1-3* inhibiting the SP.

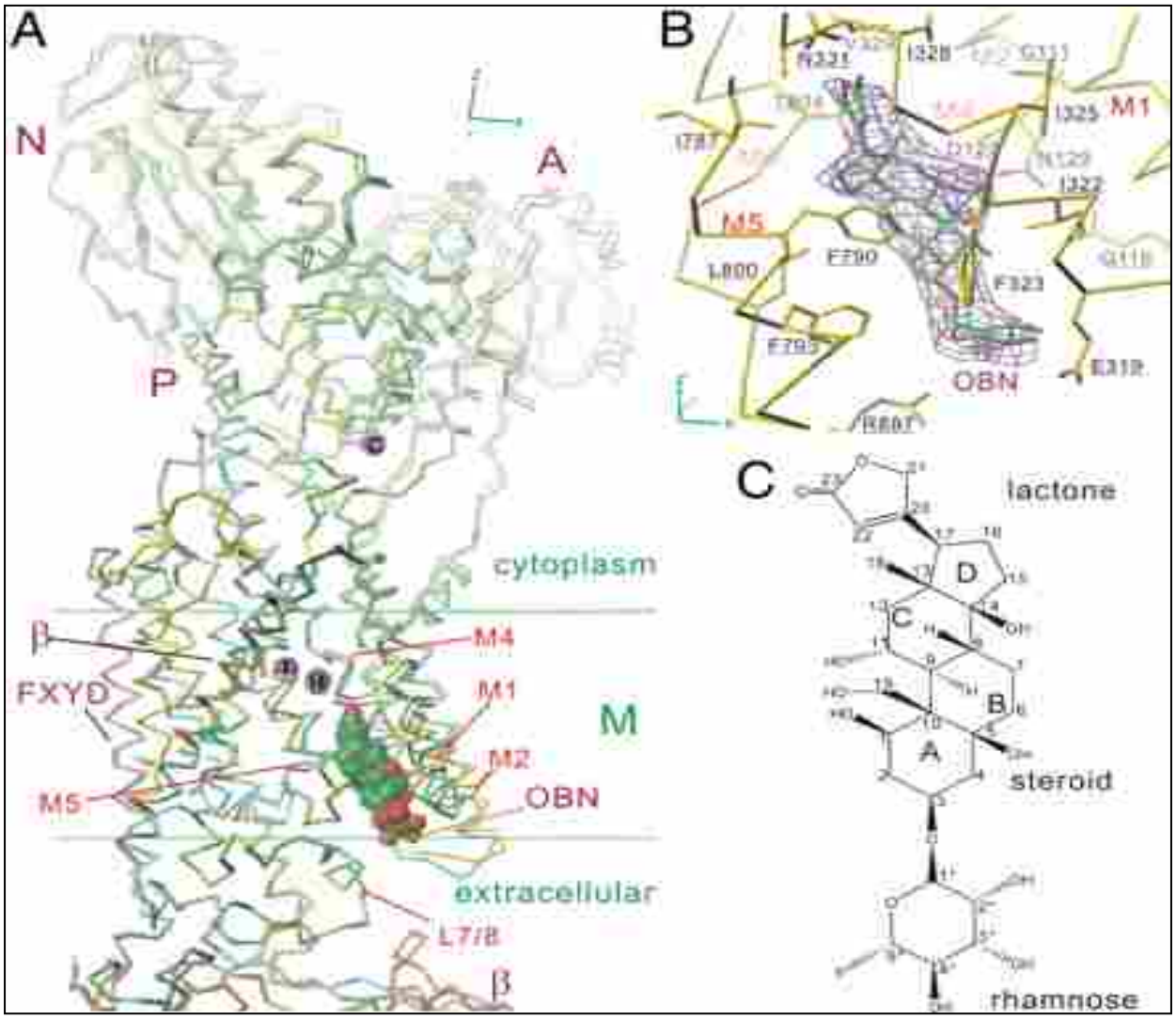


Figure 1-3. Crystal Structure of Na^+/K^+-ATPase Bound to Ouabain.
(***A***) Superimposition of Cα traces of Na^+,K^+-ATPase from shark rectal gland in ouabain-bound (yellow) and ouabain-unbound (cyan) forms, viewed along the membrane plane. Na^+,K^+-ATPase is fixed in a state analogous to $E2{\cdot}2K^+{\cdot}Pi$, with MgF_4^{2-} as a stable phosphate analog. Ouabain (OBN; green and red) and K^+ ions (I, II, and c; purple) are shown in space fill. The β-subunit, FXYD protein, 3 cytoplasmic domains (A, N, and P), and several transmembrane helices are marked. Green horizontal lines indicate the approximate position of lipid bilayer (M). (***B***) An $\boldsymbol{F_{obs} - F_{calc}}$ omit-annealed map of ouabain at 3σ, superimposed on the final atomic model. The underscores indicate that the residues have been identified as affecting ouabain binding by mutagenesis. (***C***) A diagram of ouabain (Ogawa, 2009).

INHIBITORS

The following is detailed information on each of the four known cardiac glycosides used in these studies, as well as those inhibitors that research may have demonstrated could be the EDLF of PE. They all act on the plasma membrane SP in a similar manner which can be understood by the similarity in the structure of these inhibitors. This information is not only important to the comparative study of Digibind and DigiFab highlighted in Chapter 4, but will be used as a basis for further identification of the EDLFs associated with PE in subsequent chapters.

Proscillaridin A

Proscillaridin A is isolated from plants from the genus *Scilla*. It is currently used to treat heart disease, it shows high cytotoxicity in tumor cells, and it is used as a very potent immunosuppressor. Proscillaridin A has a molecular weight of 530. 65 g/mol and its molecular formula is $C_{30}H_{42}O_8$.

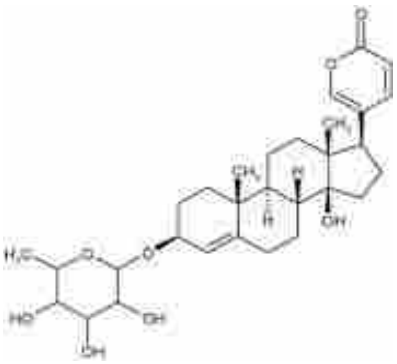


Figure 1-4.
Proscillaridin A

Bufalin

The source for bufalin is a milky substance found in the glands of the toads, *Bufo marinus* or *viridis*, which is secreted when the animal is injured, provoked or threatened. Bufalin has been investigated as an inhibitor against the multi-drug resistant human CCRF-CEM leukemia cells (Effereth, 2002) as well as inhibiting the proliferation of melanoma and prostate cancer cells (Yeh, 2002; Jing, 1994). Despite its extreme toxicity, bufalin has been used as a therapeutic for the treatment of heart disease for more than 200 years (Rhorer, 1982). The structure of

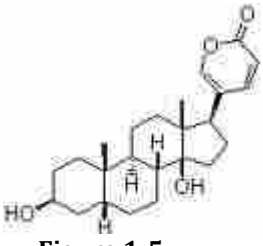


Figure 1-5.
Bufalin

bufalin is shown to the left in *Fig. 1-5* and is free of the sugar group and the A-ring double bond that appears on proscilliridin A. The molecular weight of bufalin is 386.54 g/mol and its molecular formula is $C_{24}H_{34}O_4$.

Ouabain

Ouabain is found in the bark of the African plant *Acokanthera ouabaio* and the ripe seeds of the African plant *Strophanthus gratus*. The water solubility of ouabain lends to its frequent use in research, compared to digoxin, which is more lipophilic. Research has also revealed that ouabain in low concentrations can actually have a stimulatory effect on the Na^+/K^+-ATPase, which is a characteristic that mechanistically is not fully understood (Gao, 2002).

Ouabain has been potentially identified in bovine hypothalamus (Schneider, 1998) (Laredo, 1994), human adrenals (Murrell, 2005), to be secreted by rat heart (D'Urso, 2004) and in the serum of rats exhibiting PE-like symptoms (Vu, 2005). Endogenous ouabain and ouabain-like inhibitors seem to be common in the human circulatory system, but the site and mechanism of production still remains to be identified. Ouabain contains a rhamnose sugar. Rhamnose does not appear to be made in mammals nor to be incorporated into known biomolecules in the human. This has led to concerns about ouabain's actual source.

Ouabain is used extensively in research. Ouabain's before mentioned water solubility makes it very useful as an *in vitro* SP inhibitor. Conversely, it is now rarely used *in vivo* or as a pharmaceutical, perhaps because of its shorter biological half-life. Historically, it was used by Somali tribesmen, as well as other ethnic groups, to poison the arrows of hunters.

Shown previously, in *Fig. 1-3* (*pg.21),* the SP is shown in its crystallized form with ouabain (OBN) bound (yellow) or absent ouabain (cyan). The chemical structure of ouabain *(C)*

is also shown in this figure. The molecular formula is $C_{29}H_{44}O_{12}$ and the molecular mass is 584.65 g/mol.

Digoxin

Digoxin is the principle active factor in digitalis and sometimes may be called digitalis. Digoxin is purified from the foxglove plant, *Digitalis lanata* or *purpurpea*. This cardiac glycoside has been used medicinally for more than two centuries.

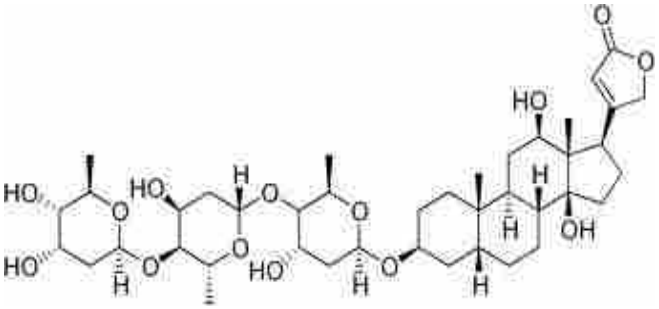


Figure 1-6. Digoxin

As mentioned before, it is used to treat progressive cardiac failure patients to increase myocardial contractibility. Digoxin has a molecular mass of 780.94 g/mol and a chemical formula of $C_{41}H_{64}O_{14}$. It is the largest of the cardiac glycosides highlighted here because of the large carbohydrate group attached to the 3' side.

Strophanthidin

Strophanthus is a genus that represents 35-40 species of flowering plants that are native to Africa. Several African tribes used these plants for arrow poison. Strophanthidin is also a cardenolide or cardiac glycoside with similar structure to digoxin and ouabain. It has a molecular mass of 404.50 g/mol and a molecular formula of $C_{23}H_{32}O_6$. Strophanthidin has never been proposed as an EDLF, but is included in our research as a SP inhibitor that was used to establish HPLC methods discussed in Chapter 5.

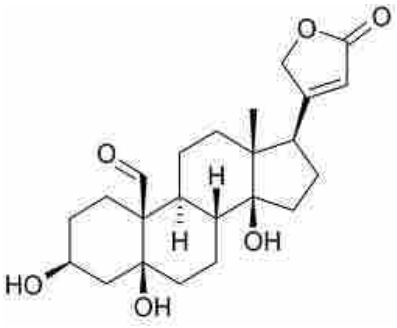


Figure 1-7. Stophanthidin

Serum from women with preeclampsia

Each of these cardioglycosides, as seen in the *Figs. 1-4 through 1-7* share a steroidal backbone, a 5- or 6-member unsaturated lactone ring and a variety of 3' -OH modifiers, e.g. rhamnose. The binding of these factors to the SP is primarily through the lactone ring. The first four cardiotonic inhibitors discussed here are tested for their binding efficiencies to Digibind, introduced next, and DigiFab, a newer, but similar compound.

TREATMENT OF PE

Currently, the only effective treatment for PE is delivery of the fetus. However, in many women, managed care with bed rest, and magnesium sulfate, to help prevent seizures, and antihypertensive drugs, can temporize the patient for days to weeks allowing the fetus to mature reducing fetal complications. While most PE occurs after 36 weeks gestation, it frequently results in premature delivery.

Although there are no FDA-approved therapeutics of PE, there are investigations of treatments as well as clinical cases of alternative treatments for women with severe PE. Of particular significance are the cases where an anti-digoxin antibody Fab fragment was used to treat women with severe PE on a compassionate use basis (Adair, 2009; Adair, 1996; Goodlin, 1988), as well as in a clinical trial that will be discussed extensively in Chapter 3 (Adair, 2010). Furthermore, In Chapter 4 there is an introduction to two such anti-digoxin immune Fab fragments studied extensively by this lab.

Digibind®

Digibind® (Digibind) represents an antibody Fab fragment from a polycolonal antiserum raised in sheep against digoxin, the digitalis derived pharmaceutical mentioned above. This

digoxin immune Fab (hereafter referred to by its commercial name, Digibind, GlaxoSmithKline, Research Triangle Park, NC) was originally developed to combat the potentially lethal effects of overdosing digoxin. Digibind has the ability to bind and inactivate digoxin interfering with its SP inhibition. The Fc portion of the antibody has been removed to reduce immunogenic effects in the patient (Adair 1996).

Digibind has been shown to bind digoxin specifically (Goodlin 1988). However, structurally related SP inhibitors, such as ouabain, bufalin and strophanthidin, as well as EDLFs found in PE, are also, though more weakly, bound to Digibind. Also, Digibind has been used to reverse the inhibitory effects of EDLFs on the SP. For example, when Digibind is added to assays that measure the ion transport activity of the SP and the effects of inhibitors thereon, inhibition is reduced (Hopoate-Sitake, 2011). The efficacy of Digibind in binding the EDLF of PE is of paramount importance to this study and so two complimentary studies are included in the next two chapters to demonstrate this interaction *in vitro* and *in vivo*.

DigiFab™

DigiFab™ (hereafter designated as DigiFab) produced by BTG, Inc. (London, UK), is also used here in comparative studies with Digibind. DigiFab, produced very much in the same way as Digibind, as a polyclonal antibody against digoxin, is a newer product and Chapter 4 will highlight how binding efficiency of the two immune Fab fragments with the above mentioned inhibitors, as well serum from women with PE, shows that the two digoxin immune Fab are very similar.

THESIS

Although PE is a multisystem disorder, EDLF may be one of the controllable circulating factors increased in PE that can be managed with the use of anti-digoxin immune Fab. Here we show that EDLF is increased in the serum and placenta of women with PE. We hypothesize that the use of Digibind or a similar anti-digoxin immune Fab may be effective in controlling the levels of EDLF *in vitro* as well as *in vivo*. In fact, the use of the digoxin-immune Fab is investigated as a possible therapeutic in the treatment of women who develop PE. Further, this work investigates another anti-digoxin immune Fab for its comparable effectiveness in binding known SP inhibitors as well as the EDLF found in PE. Finally, a unique protocol is created that uses Digibind as an affinity material to isolate and purify the EDLF found in women with PE.

CHAPTER 2: EDLF IS INCREASED IN WOMEN WITH PREECLAMPSIA

INTRODUCTION

Serum and placenta from pregnant women without or with varying degrees of PE were collected at the University of Tennessee, School of Medicine and sent to Brigham Young University for investigation of their inhibitory activity on the SP. This study was carried out using a Rb^+ uptake assay that utilizes the high unique absorbance spectrum (generated by free atoms produced at high temperatures) of Rb^+ which is taken up by the cell SP instead of the K^+ normally taken up in the cell. This spectrum is measured by graphite furnace atomic absorption (GFAA) spectrometry. The decrease in Rb^+ levels indicates a decrease in SP activity and an increase in inhibitory activity. It was our hypothesis that serum from women with PE would have an increased level of inhibition on the SP and therefore higher levels of EDLF.

It was also hypothesized that the use of the digoxin immune Fab, Digibind, in the Rb^+ uptake assay would bind EDLFs and reduce the inhibitory effect of the serum on SP activity. Studies of experimental animal hypertension, as discussed in *Chapter 1*, as well as previous use of digoxin antibody Fab treatment in women with PE further support this hypothesis (Goodlin, 1988). However, there still remains little direct evidence for this binding and neutralization of EDLFs by digoxin immune Fabs, such as Digibind, in PE. There is also very limited information about a source for such EDLFs in the context of PE.

Again, this study seeks to demonstrate that EDLFs exist at increased levels in the serum of women with PE and act as SP inhibitors and that EDLFs will be evident in placental tissues of women with PE and therefore suggests a possible source for the EDLFs. It also hypothesizes that Digibind can bind and inactivate these EDLFs found in PE sera or PE placenta.

METHODS

Patients and Patient Derived Specimens

All studies were reviewed and approved by Institutional Review Boards at both participating institutions, the University of Tennessee, School of Medicine, Chattanooga and Brigham Young University, Provo. All women who participated provided informed written consent prior to enrollment. Sera and placenta were collected at the time of delivery from women with third trimester uncomplicated pregnancies (controls) and from women whose pregnancies were complicated with PE. Specimens were frozen immediately in liquid nitrogen, handled on dry ice and stored at -80ºC until assay. Sera were thawed and assayed without further processing.

For those placentas obtained at delivery, a full thickness (2 x 2 x 2 cm) cut was removed, snap frozen in liquid nitrogen and stored at -80ºC until further processing and assay.

Placenta Homogenization

Frozen placenta (~1 g) was shaved into flakes using a surgical blade. The frozen flakes were then placed in a stainless steel ball mill cylinder along with 10 to 15 steel balls (Mikro-dismembrator, Sortorius Stedim, Bohemia, NY) and the entire cylinder, including placenta tissue contents, was submerged in liquid N_2 for 10 min. After thorough freezing of the cylinder, the cylinder was immediately fitted into the Mikro-dismembrator ball mill and set to shake at the maximum setting for 10 minutes. The process of submersion and shaking was repeated at least twice or until the contents became a well homogenized mixture. The tissue homogenate was removed from the cylinder by adding 1 ml of deionized H_2O sequentially 5 times. The total of 5 ml reconstituted homogenate solution was placed in a 50 ml conical tube.

To remove large and abundant proteins, the homogenate was treated further with methanol (MeOH). Two volumes (10 ml) of HPLC grade MeOH were added gradually in 500 µl increments to the homogenate over 5 min while intermittently vortexing the conical tube. After adding all 10 ml of MeOH, the conical tube containing the MeOH-treated homogenate was centrifuged for 10 min at 2000*x*g at 4ºC. The supernatant was collected and the precipitate discarded. The supernatant was then dried down *in vacuo* to completely remove the MeOH and then resuspended to original volume (5ml) using deionized H_2O. This protein-depleted placental homogenate was then stored at -80ºC until further processing and assay.

Cell Preparation

The primary assay used in these studies directly determined the ability of the cell Na^+/K^+-ATPase or sodium pump (SP) to import Rb^+ (in the place of K^+) into the cell. At the outset of these studies, the SP of two different cell types was assayed. The original intention was to find a contractile tissue cell related to pregnancy, and so a line of human myometrial cells was acquired. Human red blood cells (RBCs) were also assayed due to ease of availability and quantity of cells needed.

Myometrial cell preparation

Human myometrial cells were cultured in 20 ml of growth medium (100 ml of fetal bovine serum, 1 ml of 1000X Gentamicin, 1 L Modified Earl's Medium) in 150 mm culture dishes. Cells were passaged at 95 to 100% confluency by rinsing cells with 15 ml PBS with 10% EDTA and then incubating at 37ºC in another aliquot of 15 ml PBS with 10% EDTA containing 10 µl trypsin for 30 min. The cells were placed in a 15 ml conical tube and centrifuged for 10 min at 2000*x*g. The PBS containing 10% EDTA was removed, and then the cells were replated

in three 150 mm culture dishes containing 20 ml of the medium. All cell culture supplies were obtained from GIBCO, Carlsbad, CA.

When enough plates (1 plate for every inhibitor sample to be run in the Rb^+ uptake assay) had been cultured to 95 to 100% confluency, cells were lifted from the dish with 15 ml PBS and cell scrapers. Harvesting cells involved the same rinse step with 15 ml PBS with EDTA, followed by incubation in another 15 ml PBS with EDTA containing no trypsin for 30 min. Trypsin damages cell membrane proteins, including the SP located there, so it was not used to harvest the cells. Cells were again centrifuged and the supernatant removed. Packed cells were resuspended in 5.00 ml RbCl buffer containing in mmol/L: NaCL 135, RbCl 6.73, NaH_2PO_4 8.10, Na_2HPO_4 1.27, and $MgCl_2$ 1.0 at pH 7.45 and used immediately in the Rb^+ uptake assay described below.

Immediately after the harvesting of myometrial cells, the cells were prepared for use in the Rb^+ uptake assay by washing myometrial cells with K^+-free RbCl buffer 3 times, the cells were resuspended in 5.0 ml of RbCl buffer and combined with other washed cells (usually a total of ten tubes each containing cells from one 150 mm culture plate each) to create a single uniform pool (a Bradford protein assay was done in order to verify uniform cell content) of myometrial cells to be used as part of a single experiment.

Red Blood Cell Preparation

Red blood cells were freshly obtained from healthy men or non-pregnant women (not involved in the study group) by approved standard phlebotomy methods in a clinical setting. Blood was collected into tubes containing EDTA (to prevent clotting). The blood was allowed to sit for 1 hour at room temperature and then centrifuged at 3300*x*g, 4ºC for 10 minutes. The plasma and buffy coat were removed and the packed red cells (5 ml) were washed by

resuspension in 5.00 ml of the same RbCl buffer used in the treatment of myometrial cells. The mixture was vortexed well for 1 minute and then placed in the centrifuge once again to be re-spun at the same speed and time. This washing process was then repeated twice and finally cells were prepared as a 10% hematocrit suspension of RBC with the RbCl buffer (5 ml of packed RBCs: 45 ml of RbCl buffer) for use in the Rb^+ uptake bioassay. These cells were then used in the same way (i.e. washing three times with RbCl buffer) as the myometrial cells described above to assess inhibitors and the effect of the digoxin immune Fabs.

Rb^+ Uptake Assay

SP inhibition (or EDLF activity) was assayed using a Rb^+ uptake bioassay that was adapted from a previously published procedure (Zhen, 2005). This bioassay was performed immediately following cell preparation to optimize ouabain-sensitive SP activity. Essentially, less rubidium is taken up into the cytosol after the inhibitor is added to RBCs or myometrial cells in RbCl buffer. Digibind can also be added in addition to the inhibitor in each individual Eppendorf tube to test the SP inhibitor binding and inactivating capabilities of Digibind.

As soon as possible after cell harvesting or preparation, prepared cells were incubated with sera or placental homogenate and the specimens' effects on SP mediated Rb^+ uptake into cells were measured. The degree of SP inhibition was determined for different sera and placental homogenates using the absorbance data from the GFAA. Each assay contained sera from a healthy non-pregnant individual (baseline, no inhibition control), sera from women with normal pregnancy (normal control), ouabain at a final concentration of $1x10^{-3}M$ (positive, complete inhibition control) and sera or placental homogenate from subjects of interest without or with the diagnosis of PE cases. Each sample was run in triplicate.

100 µl of each serum (or placental homogenate) test solution was added to 900 µl of cells previously washed three times with RbCl buffer and then resuspended to produce a 10% hematocrit solution (5 ml cells: 45 ml buffer). Each Eppendorf tube containing cells and specimen was vortexed well for 60 seconds and then incubated in a shaker incubator (220 rpm, 37ºC) for 45 minutes. Following the incubation period each sample was spun in an Eppendorf Centrifuge 5415D (Hamburg, Germany) for 10 minutes at 220*x*g to pellet the cells and the supernatant was pipetted off. Each set of cells treated with serum sample was washed with 1 ml of RbCl washing buffer (containing in mmol/l: choline chloride 149, $MgCl_2$ 1.0, MOPS 5.88, Tris 2.12 at a pH of 7.40), and recentrifuged to thoroughly remove extracellular rubidium and allow only Rb^+ actually inside the cytosol of the cell to be measured. This washing was repeated two additional times. The myometrial cells or RBCs were then lysed with 1 ml ice cold deionized H_2O for further analysis. The cell ghosts were removed by centrifugation and the lysates collected and stored at -80ºC until analysis with GFAA.

GFAA analysis

Rb^+ content in the cell cytosol was measured by graphite furnace atomic absorption (GFAA, Graphite Furnace Atomic Absorption Instrument Model 4100Z, Perkin Elmer, Waltham, MA). Prior to the GFAA step, 10.0 µl of the cell lysate was diluted to 500.0 µl with deionized H_2O of which the auto sampler injected 10.0 µl three different times for assay in a standard GFAA method. This program in abbreviation is as follows: the furnace compartment where the sample is injected into a graphite tube which is rapidly heated to very high temperature (1800 K) to completely combust organic cell material which is carefully blown off by argon gas and to vaporize salts, but only the elemental Rb was measured by the spectrometer lamp which is

specific to Rb. Rubidium ion has a unique set of light absorbance spectral lines which allow it to be selectively measured.

In order to compensate for cell pool-to-pool variability in the Rb^+ uptake assay, the data were reported as percent inhibition. For each unknown assayed, three different determinations were carried out, each read 3 times by the GFAA instrument for a total of nine data points each. The GFAA instrument reported the average of three separate readings in absorbance units. The absorbance produced by pregnancy sera (higher inhibition lower Rb^+ reading), both normal and preeclamptic, was divided by absorbance of negative control, non-pregnant sera (lower inhibition and a higher Rb^+ reading), after subtracting the positive control (10^{-3}M ouabain) from any reading and the results expressed as a percentage. The positive control, 10^{-3}M ouabain, as mentioned above, is also considered for acceptability of the assay; if ouabain showed more than 50% inhibition of the negative control then the assay was considered useable. Actual Rb concentrations were also calculated for each absorbance reported by reference to a daily calculated calibration curve generated using aqueous Rb concentration standards.

Comparison of Myometrial Cells with RBCs for the Rb Uptake Assay

In these studies, both myometrial cells and RBCs were used and compared for measuring SP activity and SP inhibition. To insure similar assay results, Rb^+ uptake assays were done simultaneously on both human myometrial cells and human red blood cells (RBCs) using the same sera from individual women with PE and also with uncomplicated pregnancies and the results compared. All samples were assayed in triplicate. At least 6 assays of each myometrial cells and RBCs were run to determine the comparability of the two cell types.

Dose Dependency of PE Serum vs. Normal Pregnancy Serum

A concentration-dependence experiment was carried out to help determine if the reduction in SP activity was due to a true specific inhibitor and not a result of other interferences. Interferences often cause non specific reductions in SP activity by their mere presence and not in a dose-dependent manner. This study was carried out using a series of assays where differing amounts of PE sera were added to the prepared cells. Specifically, PE serum was added in increments of 20µl, 40µl, 60µl, or 100 µl to 900 µl of the Rb^+ buffer treated cells. The volume of each individual Eppendorf tube was brought to 1000 µl or 1.0 ml with deionized H_2O. Three to four experiments were done for each increment of PE sera. The same increments (3 or 4 experiments each) of sera from uncomplicated or normal pregnancies were also added to the prepared cells, and the effects of the sera on SP Rb^+ uptake were measured using GFAA. A total of 12 experiments were done for the dose dependency of PE sera and 12 total experiments were done with the sera from uncomplicated pregnancies showing dose dependency. All samples were assayed in triplicate.

Comparison of the Effects of PE and Normal Pregnancy Sera on SP Inhibition

The SP inhibition caused by sera of women with PE and sera of women with uncomplicated pregnancies were compared. Cells (900 µl of the 10% hematocrit cell solution) were incubated with 100 µl of each of the sera. Each specimen was tested separately with the same controls as mentioned before, so for each experiment there were twelve Eppendorfs because each sample is done in triplicate. A total of 9 experiments each were done for sera of women with PE and sera from women with uncomplicated pregnancies.

Placental Homogenate Effects on SP Inhibition

Protein-depleted placental homogenates from women with PE and women with normal pregnancies were added in 100 µl increments to 900 µl of 10% cell solution for measurement of SP inhibition effects. Cells were incubated for 45 min with the placental homogenate and processed in the same way as described above. The cellular Rb^+ uptake assay was once again measured by GFAA. Each specimen was assayed separately and done in triplicate. Sixteen experiments were done for both placental homogenates from women with PE and 16 experiments from placental homogenates from women with uncomplicated pregnancies.

Digoxin Antibody Fab Effects on Cell SP Inhibition

As an added measure of confirmation that the SP inhibition came from a specific EDLF, Digibind was added to the Rb^+ uptake bioassay. In these experiments 800 µl of cell mixture was used and 100 µl of sera or placental homogenate from women with PE and also women with normal pregnancy. In addition, 100 µl of Digibind at $1x10^{-5}$M concentration was also added to the cell mixture and incubated for 45 min. Each specimen was assayed in triplicate with Digibind and also in triplicate with the same aliquot of deionized H_2O. In each case, Fab fragment was added to sera from women with PE versus women with uncomplicated pregnancies and Fab fragment was added to placental homogenates from women with PE versus women with uncomplicated pregnancies. There were 15 experiments done.

Statistics

Group data are expressed as the mean ±SE. Comparison of two groups was carried out by Students' t-test if the data were normally distributed or by Kruskal-Wallis analysis if they

were not. Analysis of the paired data was undertaken using Pearson's Product Moment Correlation Analysis. A p-value less than 0.05 was considered significant.

RESULTS

Human RBCs vs. Human Myometrial Cells

The potential involvement of EDLF in hypertension led us to assay EDLF using a contractile tissue associated with pregnancy as the biological assay. This led to our employing human myometrial cells in performing the Rb^+ uptake assay. The large number of slowly dividing cells required to assay unknowns in triplicate, as well as the number of studies needed, made it clear that the use of only myometrial cells would impose time and availability limitations on the studies. This led to our evaluating an additional cell source for measurement of SP ion transport, specifically human red blood cells (RBCs). These cells were obtained from healthy non-pregnant donors. The RBCs were readily available and provided a continuous fresh source for our SP inhibition studies

A series of experiments was done in which both Rb^+ uptake assays, employing both cell types were done. In order for a direct comparison to be done, the same unknowns containing EDLF were employed in both cell type assays. It was found that both cell types effectively measured SP activity and SP inhibition. As shown in *Fig. 2-1*, myometrial cells demonstrated somewhat greater inhibition (n=6, 44.4±9.7% inhibition of SP activity) than the RBC based assay (n=6, 26.4±4.9% inhibition of SP activity) but this difference was not significantly different (p=0.15), hence there was no compelling reason not to use human RBCs.

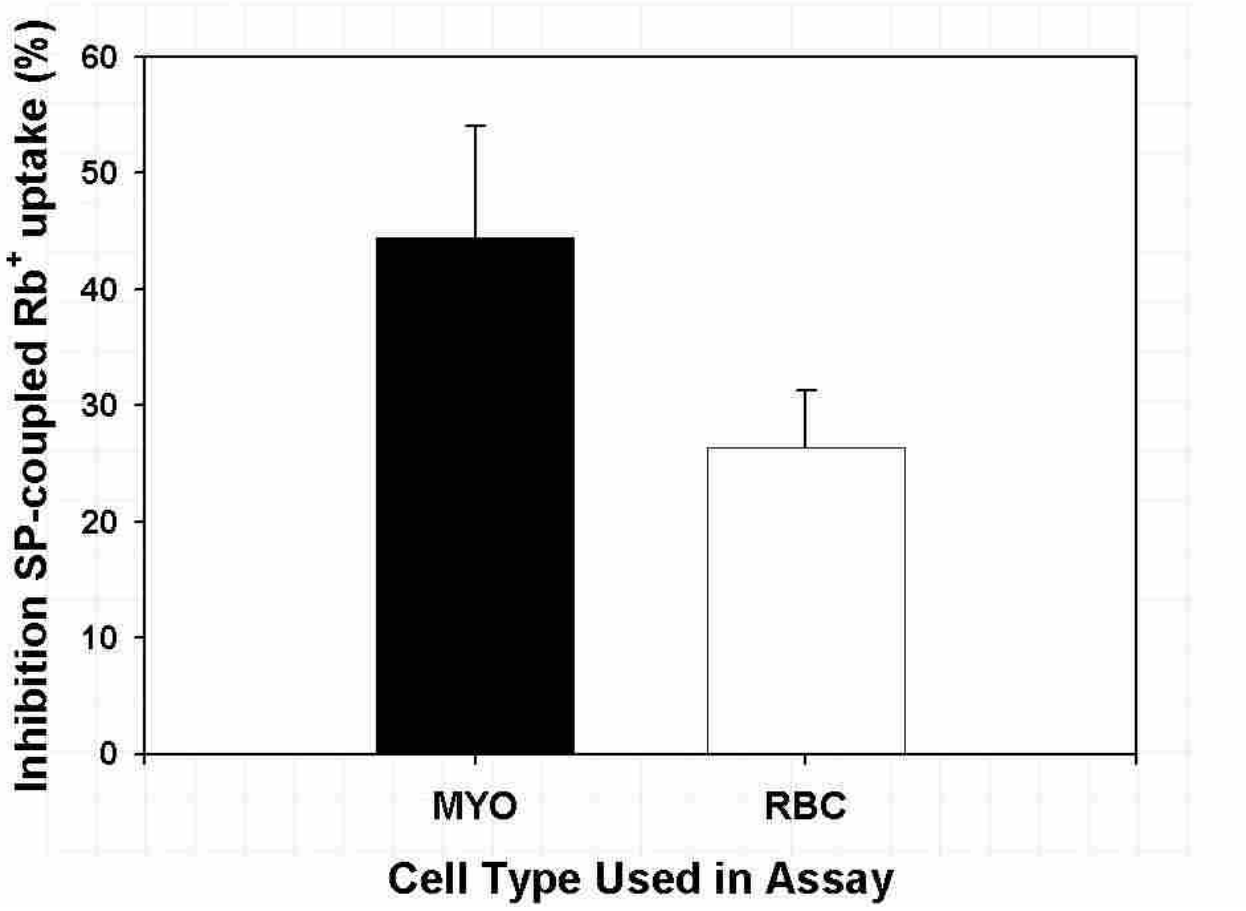


Figure 2-1. Myometrial Cells vs. Red Blood Cells.
Comparison of the Rb+ Uptake Assays Carried Out with Human Myometrial Cells (MYO) or with Human Red Blood Cells (RBC). Paired specimens were assayed against either cell type (n=6). Figure shows % SP activity measured by GFAA. The myometrial cells appeared to be more sensitive. RBCs showed significant activity.

Although myometrial cells may have been more sensitive, the difficulty of culturing large numbers of cells and the extended time needed to culture myometrial cells led us to use human RBCs as an acceptable alternative. Overall, myometrial cells were used in less than 10% of all assays, but represented a much more substantial fraction of early studies such as the initial comparison of control serum versus PE serum.

SP Inhibition Effects of PE Serum vs. Control Serum

SP activity as measured by Rb^+ uptake was significantly reduced when cells were incubated with sera from women with PE compared with serum from women with uncomplicated pregnancies (Control, n=9, 17.3±2.5% SP inhibition vs PE sera, n=9, 45.3±7.2% SP inhibition, p=0.0022). See *Fig. 2-2*.

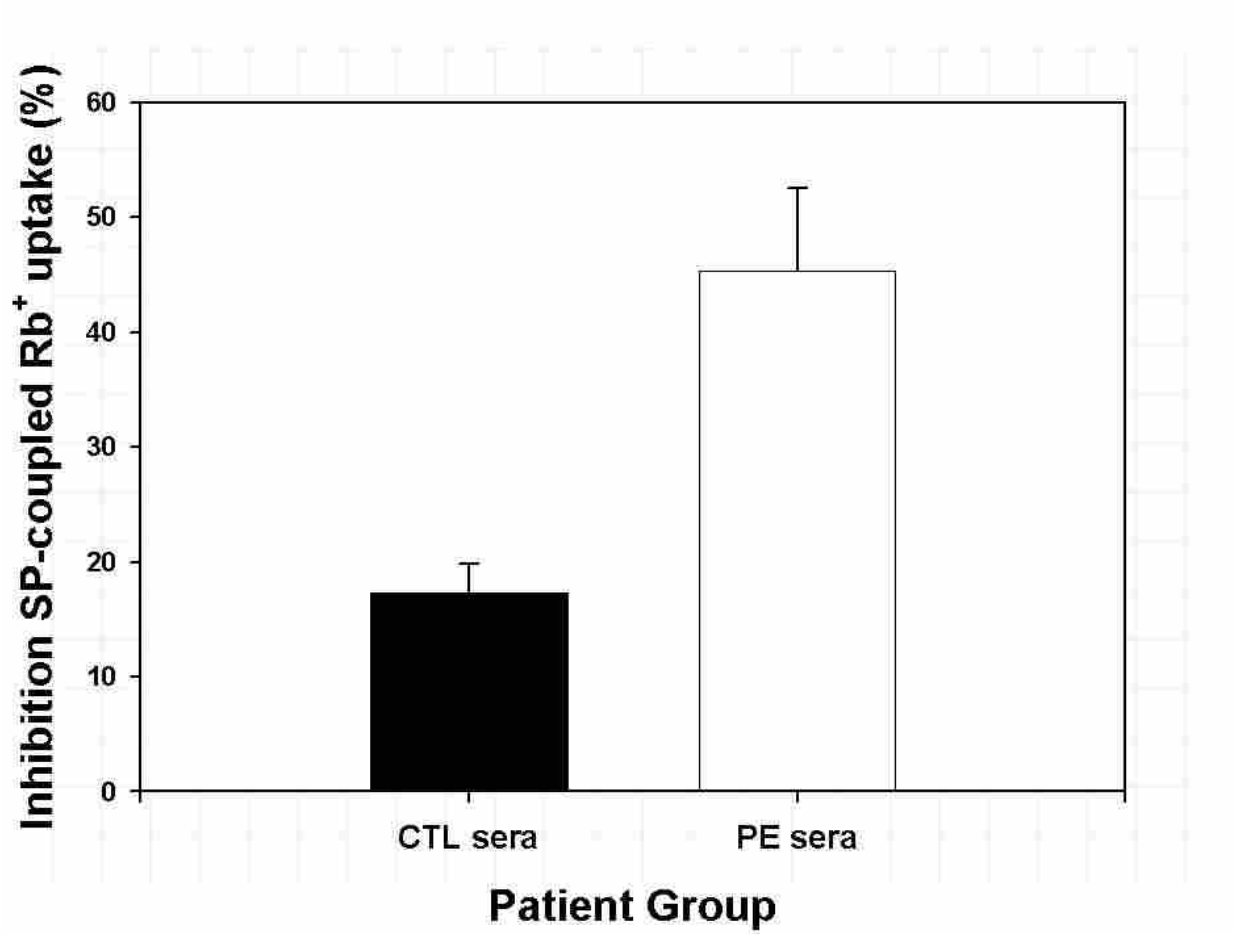


Figure 2-2. Effect of Serum on Cellular SP Activity.
The plots demonstrated the ability of sera from pregnant women with uncomplicated pregnancies (CTL sera, n=9) to inhibit the cell sodium pump as measured by diminished Rb+ ion uptake as compared with the inhibition produced by sera from women with preeclampsia (PE sera, n=9). Serum from pregnant women with PE produced significantly greater inhibition than serum from pregnant with uncomplicated pregnancies, p=0.002.

Dose Dependency of PE sera on SP inhibition

The effect of increasing serum concentration in the Rb^+ uptake assay was assessed to determine if there was a dose effect. Increasing amounts of serum from PE pregnancies caused a linear increase in SP inhibition (*Fig. 2-3*, n = 12, R = 0.948, p = 0.0003). When increasing amounts of sera from women with uncomplicated pregnancies were added to the cell Rb^+ uptake assay, unexpectedly, we actually saw an apparent reduction in SP inhibition (*Fig*. 2-4, n=12, R=-0.419, p=0.017).

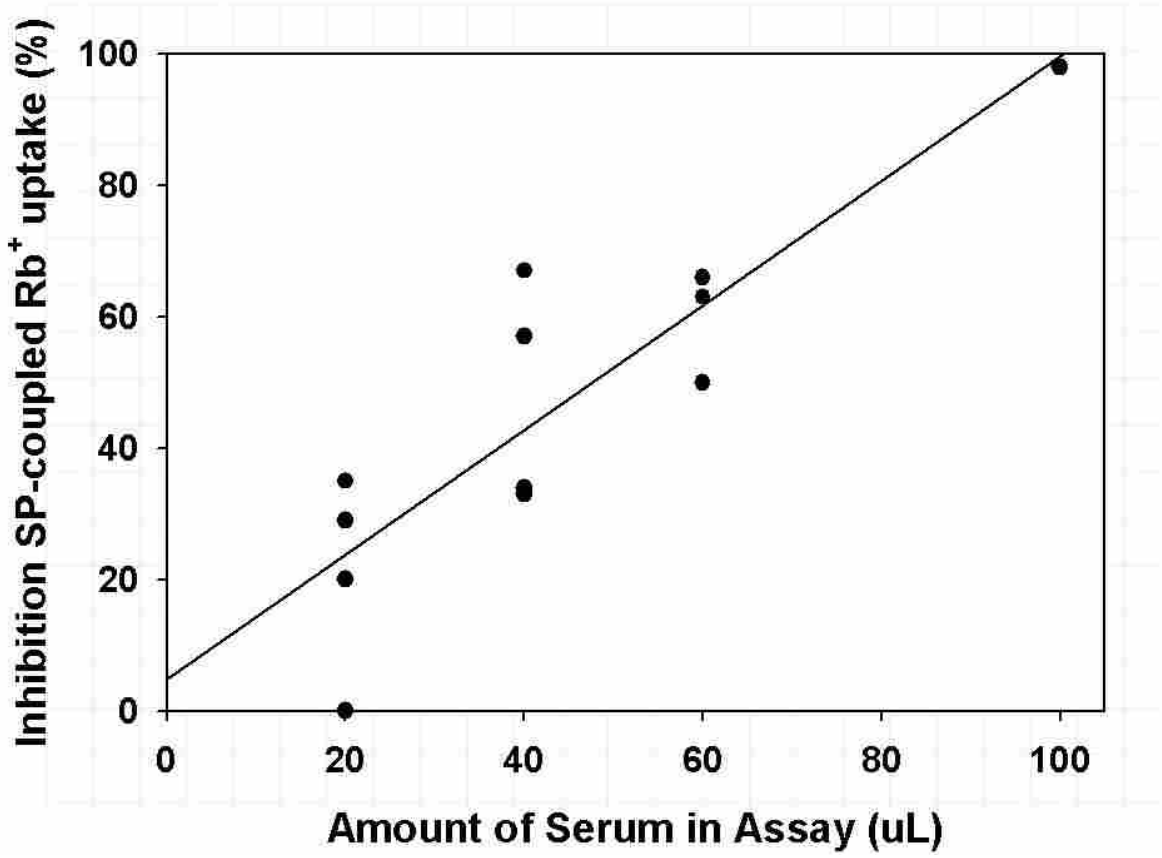


Figure 2-3. PE serum Dose Effect on SP Inhibition.
Varying amounts of serum from pregnant women with preeclamptic pregnancies (n=12) were added to the reaction mixture and the ability of serum to inhibit cell SP activity was determined. SP activity showed decrease in activity (and increases in apparent inhibition) with increasing PE serum (R=0.948, P=0.0003).

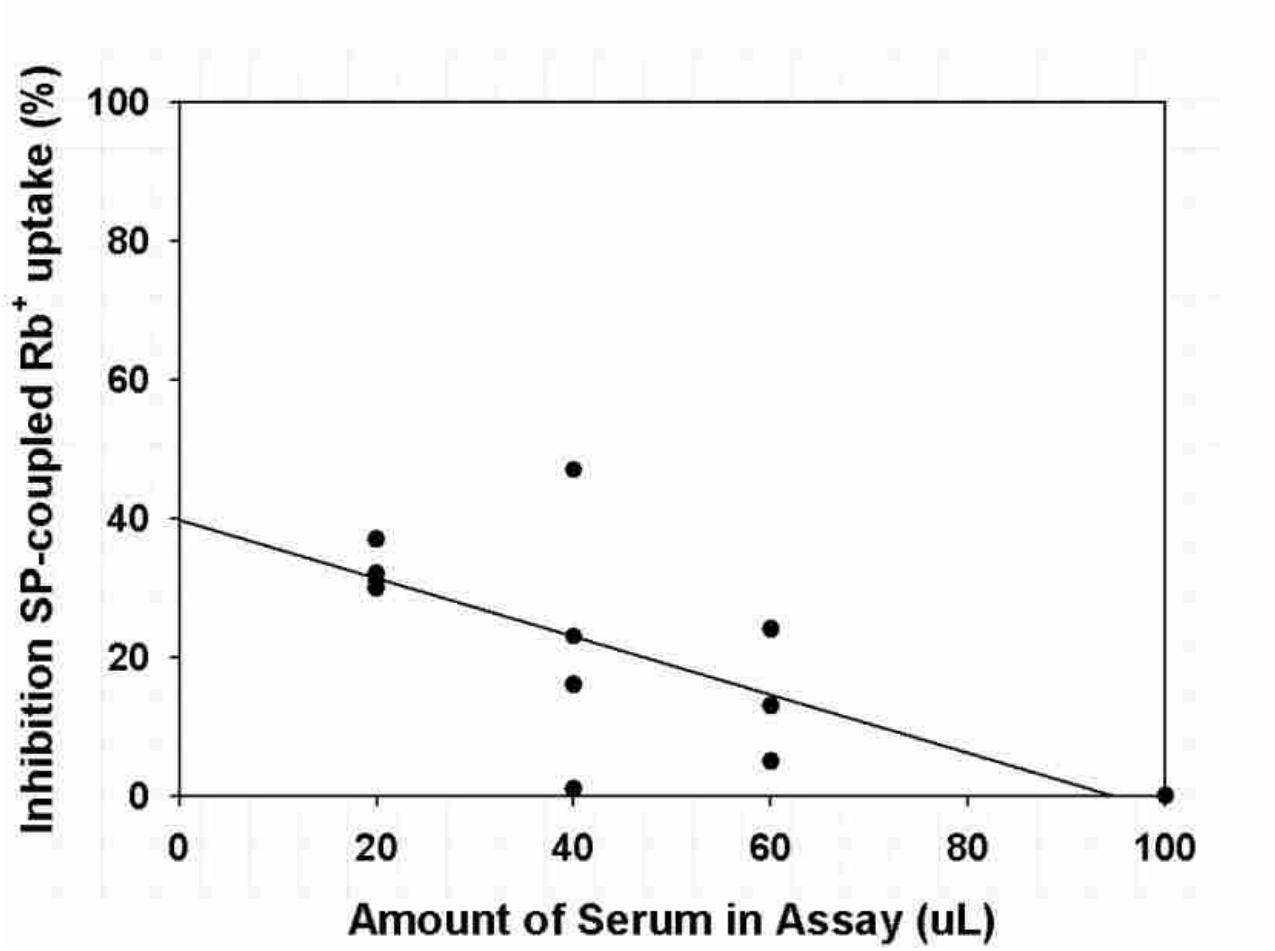


Figure 2-4. Dose Effect of Normal Pregnancy Sera on Cellular SP Inhibition.
Varying amounts of serum from pregnant women with uncomplicated pregnancies (n=12) were added to the reaction mixture and the ability of serum to inhibit cell SP activity was determined. SP activity showed modest increases in activity (and decreases in apparent inhibition) with increasing normal serum. (R=-0.419, p=0.017).

Digibind's Effect on Rb^+ Uptake in the Presence of Serum from Women with Uncomplicated Pregnancies and Sera from Women with PE

As a means to verify the SP inhibition seen with the addition of PE sera is definitely from an EDLF, Digibind was added to another set of specimens. Digibind is known to bind and inactivate EDLFs in studies of hypertension and in many animal models (Krep, 1995) (Krep, 1995). Digibind addition to sera from women with uncomplicated pregnancies brought about

very little change in SP inhibition (serum from uncomplicated pregnancies: n=5, 12.4±5.5% SP inhibition vs. serum from uncomplicated pregnancies + Digibind, n=5, 16.1±7.2% inhibition, p=0.39). In contrast, there was significant reversal (35% absolute change) of inhibition when Digibind was added to sera from women with PE (serum from women with PE, n =15: 47.6±9.4% SP inhibition vs same serum from women with PE + Digibind, n = 15, 12.6±4.7% SP inhibition, p=0.00099). See *Fig. 2-5 and 2-6.*

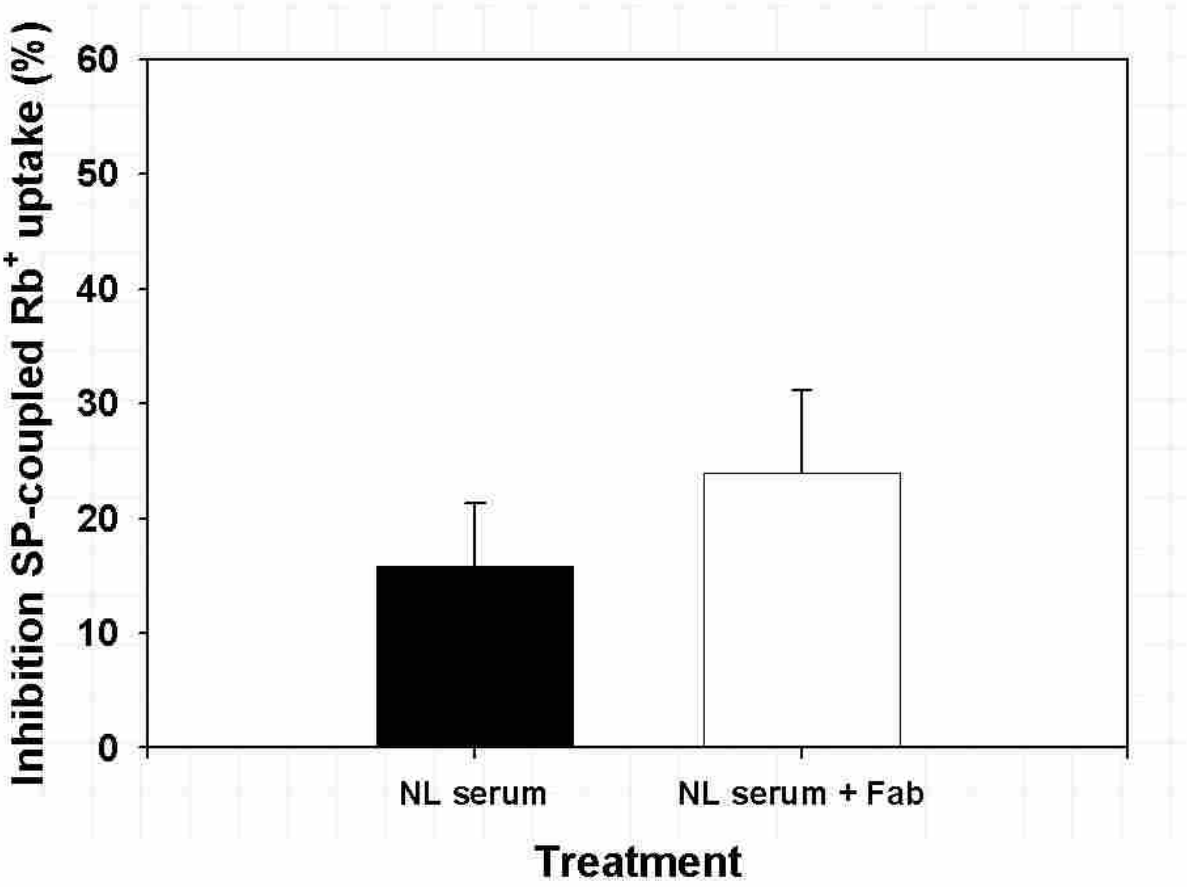


Figure 2-5. Effect on SP Inhibition with the Addition of Digibind to Serum from Women with Uncomplicated Pregnancies.

Serum from pregnant women having complicated pregnancies (n=15) was assayed for its ability to inhibit cellular SP activity directly (NL serum) or after treatment with 2x10-6M Digibind (NL serum+Fab). There was a non-significant change in SP inhibitory activity with Digibind treatment (p=0.39).

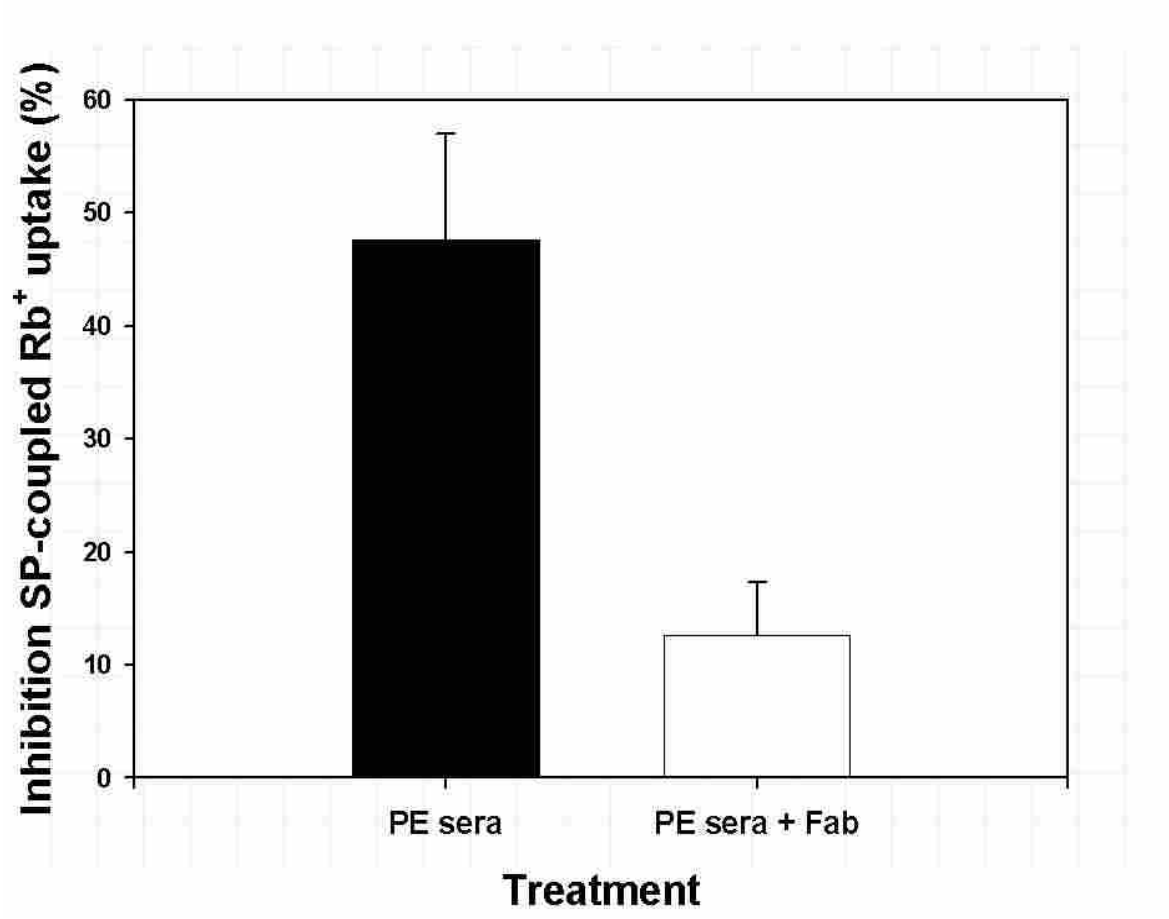


Figure 2-6. Effect on SP Inhibition with the Addition of Digibind to Serum from Women with Preeclamptic Pregnancies.
Serum from pregnant women having pregnancies complicated by PE (n=15) was assayed for its ability to inhibit cellular SP activity directly (PE sera) or after treatment with 2x10-6M Digibind (PE sera+Fab). There was a significant change in SP inhibitory activity with digibind treatment (p=0.00099).

Placenta Homogenate Effects on Rb^+ Uptake

Protein-depleted placental homogenates, from both women with a PE pregnancy and women with uncomplicated pregnancies, were also assayed for EDLF activity using the Rb^+ uptake assay. The RBC functional assay was used, but instead of serum being added, placental homogenates were added. The results showed that not only did placental homogenates from women with PE pregnancy demonstrate inhibition of the SP, but it also showed a significantly

greater degree of SP inhibition compared with the inhibition produced by placental homogenates from women with uncomplicated pregnancies (PE placental homogenate: n=16, 57.0±6.3% SP inhibition vs. uncomplicated pregnancy placental homogenates: n=16, 4.3±1.9% SP inhibition, $p<0.01$). See *Fig. 2-7*.

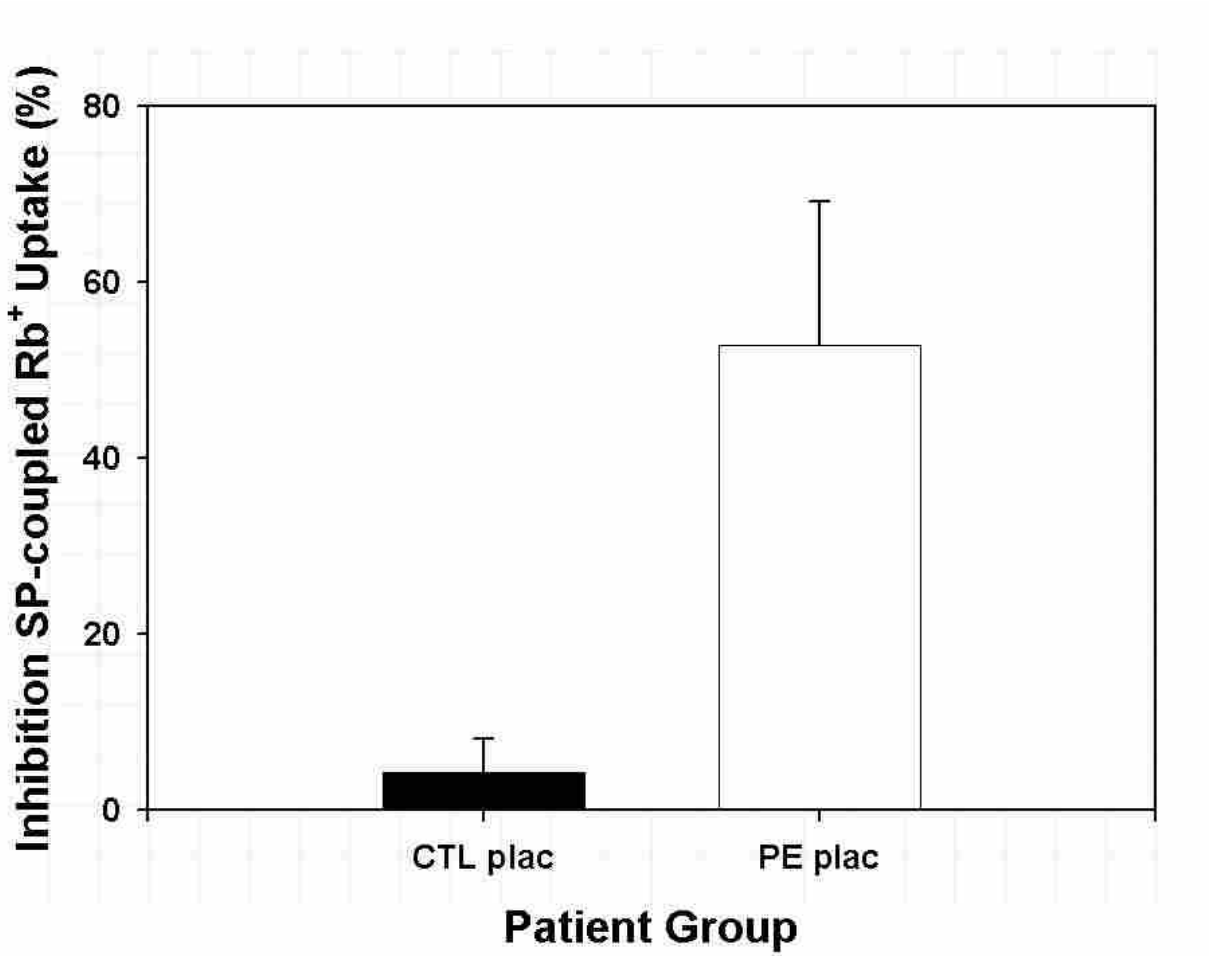


Figure 2-7. Effect of Placental Homogenate on SP Activity.
Placental homogenates from uncomplicated pregnancies (CTL, n=4) were compared with placental homogenates from women with PE (PE, n=4). Placental homogenates from pregnancies complicated by PE showed greater SP inhibition than placental homogenates from uncomplicated pregnancies (p=0.01).

Effect of Digibind on the SP Inhibition Caused by Placental Homogenates of PE Pregnancy

In an effort to further confirm that the SP inhibition caused by PE placental homogenates was due to an EDLF, similar RBC Rb uptake assays were done using PE placental homogenates without and with 2 x 10^{-6}M Digibind to determine the effects of Digibind on EDLF inhibition compared to control. Assay of the placental homogenates from uncomplicated pregnant women controls showed little effect of Digibind on SP inhibition (normal placental homogenates: n = 4, 4.3±1.9% SP inhibition vs. same normal placental homogenate + Digibind: n=4, 0.0% SP inhibition, p=0.23). However, the presence of Digibind brought about a 42.7% absolute reduction in inhibition when incubated with placental homogenates from women with PE (PE placental homogenates: n=16, 57.0±6.3% SP inhibition vs. same PE placental homogenates + Digibind: n=16, 14.3±4.3% SP inhibition, p=7.9 x 10^{-7}).

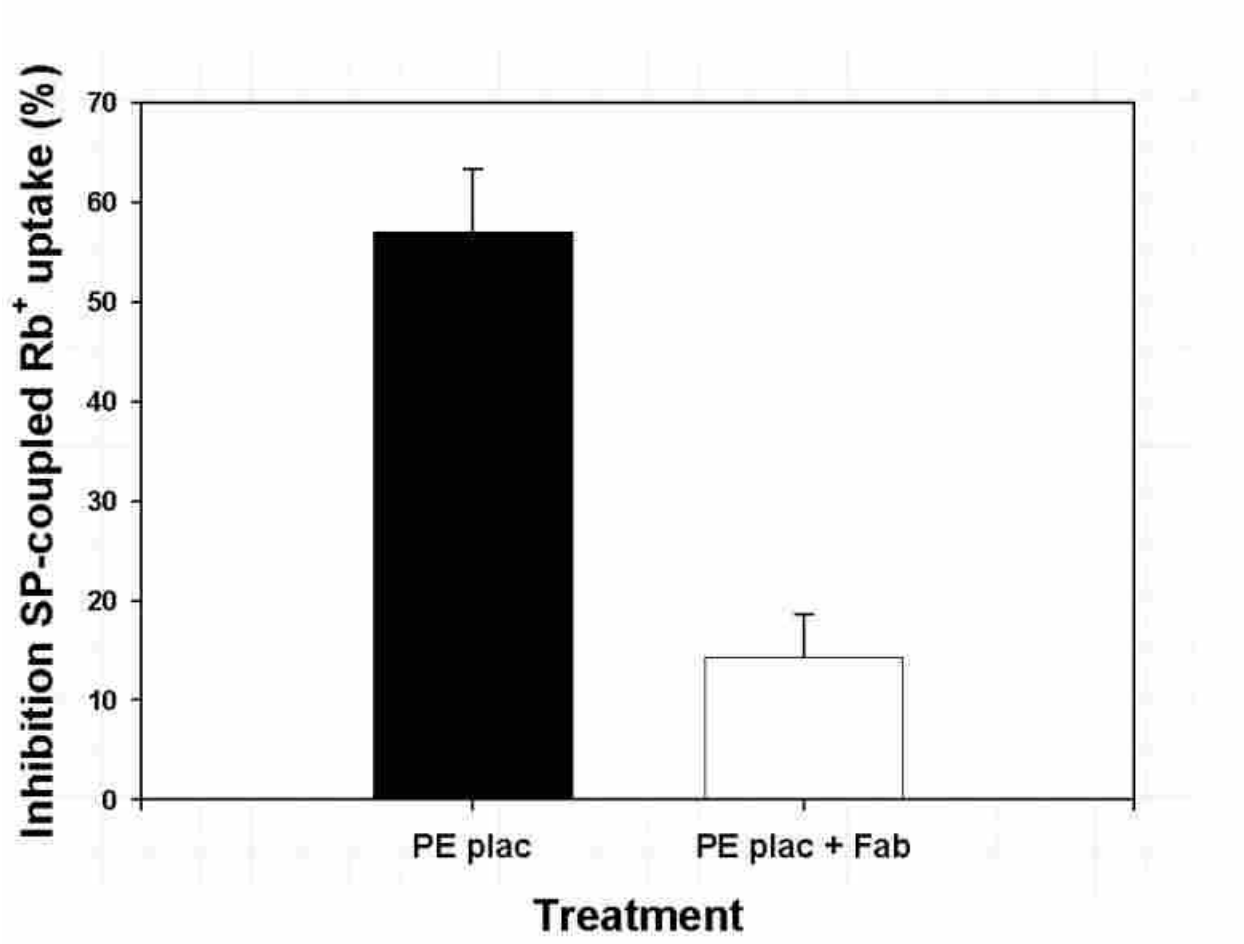


Figure 2-8. Effect of Digibind on the SP Inhibition Caused by Placental Homogenates from Preeclamptic Pregnancies.

Placental homogenates from women whose pregnancies were complicated by PE (n=16) were tested for their ability to inhibit the cellular SP in the absence and presence of 2.0×10^{-5}M Digibind. Digibind significantly attenuated SP inhibition ($p=7.9 \times 10^{-7}$).

DISCUSSION

Our first hypothesis was that SP inhibitory activity would be increased in women with PE. Our data showed that all SP inhibition in women with PE, whether from sera or placenta, was significantly higher (>20% and 50%, respectively) than the SP inhibition seen in specimens from women with uncomplicated pregnancies. It also showed that this inhibition is specific to

PE. Although EDLFs have been found in pregnant monkeys, and in some studies of human pregnancy, EDLFs specific to PE have not been identified (Kelly, 1986). Here we show strong evidence that there exists an EDLF within the serum and placenta of women with PE.

The binding of Digibind and inactivation of the SP inhibitory capabilities of PE's EDLF also give significant evidence as to the specific nature of the EDLF. Here we see that the addition of Digibind to the serum and placenta of women with PE markedly reduced or reversed the SP inhibition, whereas addition of Digibind to sera and placenta from women with normal pregnancies showed no change. This again demonstrates the specific nature of Digibind for EDLFs, in this case the EDLFs of PE.

Convincingly, not only did the sera of women with PE show increased inhibition, but also an even greater EDLF SP inhibition (>50%) was found in the placentas of women with PE. Previous research on the importance of placental growth and signaling factors in women with PE led to our hypothesis that the placenta may be a source for the EDLF in PE (Redman, 2005). The significantly increased concentration of SP inhibitory activity in the placenta suggests and supports this hypothesis. It should also be noted that symptoms of PE do disappear with the removal of the placenta.

Collectively these studies provide strong evidence of EDLF circulating in women with PE and the ability of Digibind to bind them *in vitro.* These studies and others led to a clinical study where Digibind was administered to women with severe preeclampsia as a therapeutic to reduce the effects of PE.

CHAPTER 3 - DIGIBIND EFFICACY EVALUATION OF PREECLAMPSIA (DEEP) STUDY

ABSTRACT

The *in vivo* therapeutic efficacy of Digibind, a digoxin immune Fab, was tested in a clinical trial. This Phase II, double-blinded, placebo controlled clinical study was a collaboration of over 15 different hospitals, universities and institutions (Adair, 2010). Fifty-one severely preeclamptic patients were enrolled and randomized; twenty-four women received Digibind and 27 received the placebo. Doctors, patients, and research personnel were unaware of which was given. As a part of the study, this lab was responsible for investigating the serum of each of these 51 subjects and measuring SP activity. Other investigations were also conducted at different institutions and the details of these studies will not be included here, but the result will be included in the Discussion as it lends to the investigations of Digibind and EDLFs.

Serum sodium pump inhibition was significantly decreased with Digibind treatmen compared to placebo at 24 hours after treatment (by 19%, p=0.03). This marks a significant reduction in EDLFs. Renal function, as measured by creatinine clearance, was also significantly better in the Digibind treatment group as compared with the placebo group. There is also evidence of fetal benefits.

INTRODUCTION

The previous chapter showed evidence that EDLF is raised in the circulation of women with PE and that the addition of Digibind *in vitro* reduces the inhibitory effects of EDLF on the SP. This study looked at the *in vivo* effects of Digibind as it is administered to women with severe PE upon admittance into the study.

Previous cases of Digibind being used as a therapeutic agent in treating women with eclampsia and preeclampsia exist. In these women, Digibind was used on a compassionate use basis (Adair, 2009; Adair, 1996; Goodlin, 1988). The particular study reported here is the first time in which Digibind was administered to preeclamptic women as part of a registered double-blind, placebo controlled drug trial. In this study, drug or placebo was given every 6 hours for 48 hours. The results from this *in vivo* study showed a positive Digibind effect with renal function being benefited. Thus, the evidence is compelling that Digibind is binding something, inactivating it, and preventing it from inhibiting the SP. Since Digibind is known to bind digoxin or EDLFs (and none of the subjects had ever had digoxin), it seems evident that if EDLFs mediate features of PE *in vivo*, then Digibind would bind EDLFs making them unavailable to cause these abnormalities.

Here, the effects of each subjects's plasma on SP inhibition and SP ion transport activity as part of a functional bioassay were measured at each time point. It was our hypothesis that improvement of SP activity in the assay, from SP activity at the first time point (t = 0) prior to any intervention, indicated that the digoxin immune Fab fragment had been administered, instead of the placebo, and that the Fab fragment bound EDLFs found in PE serum and eliminated their effects, ie. the binding of these EDLFs prohibited inhibition of the SP.

MATERIALS AND METHODS

For the purpose of this work, only those methods, materials and results relating to the red blood cell (RBC) assay of SP activity and inhibition by the serum of these women with severe PE are included here. A more complete description of all aspects of the clinical trial can be found in the original report by Adair et al. (Adair, 2010).

Specimen Handling

As mentioned above, all pregnant women admitted into the study had severe preeclampsia. IRB approval for each site was also obtained. Informed signed consent was obtained prior to enrollment. Criteria for admittance into the study included, a pregnancy at 23 weeks, 5 days to 34 weeks gestation, fulfillment of American College of Obstetricians and Gynecologists criteria for PE and severe PE, and probable delivery of fetus within 72 hours as judged by the primary physician (Adair, 2010). Digibind or placebo was given intravenously every 6 hours for eight total doses or as many as could be administered prior to delivery. Delivery was determined by clinical condition as judged by the primary physician and was not influenced by study participation. Blood was drawn from all women in the study upon admittance (and prior to drug or placebo), and 12, 24, and 48 hours (t = 0, 12, 24, 48hr) if possible. Blood was drawn into Li-heparin tubes, centrifuged, and plasma removed and aliquotted into plastic vials were snap frozen in liquid nitrogen, shipped on dry ice, and stored at -80º C until assay.

Red Blood Cell (RBC) Preparation

Red blood cells were processed in same way in preparation for the same Rb^+ uptake assay as described in Chapter 2. A description of the procedures used are included here for completeness and convenience.

Red blood cells were freshly obtained from healthy men or non-pregnant women (not involved in the study group) by approved standard phlebotomy methods in a clinical setting. Blood was collected into tubes containing EDTA (to prevent clotting). The blood was allowed to sit for 1 hour at room temperature and then centrifuged at 3300*x*g, 4ºC for 10 minutes. The serum and buffy coat were removed and the packed red cells (5 ml) were washed by

resuspension in 5.00 ml of the same RbCl buffer used in the treatment of myometrial cells. The mixture was vortexed well for 1 minute and then placed in the centrifuge once again to be re-spun at the same speed and time. This washing process was then repeated twice and finally cells were prepared as a 10% hematocrit suspension of RBC with RbCl buffer (5 ml of packed RBCs: 45 ml of RbCl buffer) for use in the Rb^+ uptake bioassay.

The cells were then used fresh to assess SP inhibitors and the effect of the digoxin immune Fab Digibind.

Rb^+ Uptake Assay

This method was described in Chapter 2 and was used extensively in the experiments dealing with the serum samples from these women involved in the DEEP study to further investigate the extent of SP inhibition, or when Digibind is administered, the reversal of inhibition. The details of the method are again described here.

This bioassay was performed immediately following cell preparation to optimize ouabain-sensitive SP activity. Essentially, less rubidium is taken up into the cytosol after the serum from the women with severe PE and included in the study is added to the RbCl buffer treated RBCs. Digibind was administered to 24 of the women included in the study and the plasma from these woman theoretically can also effect the SP activity or SP inhibition detected by the GFAA. As soon as possible after cell preparation detailed above, prepared cells were incubated with plasma from women involved in the study and the specimens' effects on SP mediated Rb^+ uptake into cells were measured. The degree of SP inhibition was determined using the absorbance data from the GFAA.

100 µl of each serum test solution was added to 900 µl of cells previously washed three times in RbCl buffer and then resuspended to produce a 10% hematocrit solution (5 ml cells: 45

ml buffer). Each Eppendorf tube containing cells and specimens was vortexed well for 60 seconds and then incubated in a shaker incubator (220 rpm, 37ºC) for 45 minutes. Following the incubation period each sample was spun in an Eppendorf Centrifuge 5415D (Hamburg, Germany) for 10 minutes at 220*x*g to pellet the cells and the supernatant was pipetted off. Each cell solution treated with serum sample was washed with 1 ml of RbCl washing buffer (containing in mmol/l: choline chloride 149, $MgCl_2$ 1.0, MOPS 5.88, Tris 2.12 at a pH of 7.40), and recentrifuged to thoroughly remove extracellular rubidium and allow only Rb^+ actually inside the cytosol of the cell to be measured. This washing was repeated two additional times. The RBCs were then lysed with 1 ml ice cold deionized H_2O until further analysis. The cell ghosts were removed by centrifugation and the lysates collected and stored at -80ºC until analysis with GFAA.

Rb^+ Uptake Serum Specimen Processing

All serum specimens for a single subject were assayed as part of a single run. The samples making up a run consisted of the following test solutions: 100 µl ouabain $2x10^{-5}$M (as an assay performance control); 100 µl positive SP inhibition control (ouabain $1x10^{-3}$M); 100 µl negative control represented by serum from a healthy, non-pregnant individual, or 100 µl each of the four different serum specimens representing the four time points previously mentioned (6 data points) plus 900 µl 10% hematocrit RBC suspension (total volume = 1000 µl. Individual specimens were received by this lab as masked samples with no additional information concerning the patient or treatment. These serum samples were run in random order and in triplicate.

GFAA Analysis

A summary of the GFAA analysis previously detailed in Chapter 2 follows as well as procedures unique to this study.

After a specimen or control was incubated with fresh RBCs for 45 min, cells were washed, lysed and the lysate stored in a -80ºC freezer. Each Eppendorf was thawed and a 10 µl aliquot was taken from each 1000 µl sample of treated RBCs and diluted with 490 µl of deionized H_20. This dilution is necessary to be in range on the graphite furnace atomic absorption spectrometer (GFAA). Rb^+ content in the cell cytosol was measured by graphite furnace atomic absorption (GFAA, Graphite Furnace Atomic Absorption Instrument Model 4100Z, Perkin Elmer, Waltham, MA). The GFAA spectrometer took a 10 µl sample of this mixture and ran it through a standard set program which measures the atomic absorption (AU) of Rb^+ in that 10 µl sample. The GFAA takes three replicates of each sample and averages the AU of each sample. The averages are applied to a standard curve generated using Rb^+ standard and the relative concentration of Rb^+ is reported.

This program in abbreviation heats the furnace compartment where the sample is injected into a graphite tube which is rapidly heated to very high temperature (1800 K) to completely combust organic cell material which is carefully blown off by argon gas and to vaporizing salts, but only the elemental Rb was measured by the spectrometer lamp which is specific to Rb. Rubidium ion has a unique set of light absorbance spectral lines which allow it to be uniquely measured.

In order to compensate for cell pool-to-pool variability in the Rb^+ uptake assay, the data were reported as percent inhibition. The absorbance produced by DEEP subjects sera (higher SP inhibition results in a lower Rb^+ reading), for 4 different time points, was divided by absorbance

of negative control, non-pregnant sera (lower inhibition and a higher Rb^+ reading), after subtracting the positive control (10^{-3}M ouabain) from any reading and expressing it as a percentage. The positive control, 10^{-3}M ouabain, as mentioned above, is also considered for acceptability of the assay; if ouabain at $2x10^{-5}$ M showed more than 50% inhibition of the negative control then the assay was considered useable. Actual Rb concentrations were also calculated for each absorbance reported by reference to a daily calculated calibration curve generated using aqueous Rb concentration standards. The daily variations in the absorbance units, due to variable GFAA sensitivity, led to using percent inhibition data as a means of normalizing data.

Statistical Analysis

These results were calculated separately for each sample, compiled and sent to an independent auditor and statistical analysis team for further analysis to retain the blindedness and independence of statistical evaluation. A sample of one subject's results is provided in *Fig. 3-1*. These results were shown in relative [Rb ug/ml] as calculated by a daily standard curve. Our results were also reported as percent change in SP activity and percent change in SP inhibition relative to the time 0 value for each sample. This was done to normalize data due to daily vatiations in RBC activity and GFAA variability. *Table 3-1* shows the results of all data acquired from the DEEP specimens submitted. Data are presented as mean ± SD. Statistical analyses included Chi square and/or Fisher Exact analysis for categorical data. Continuous variables considered over multiple time points were analyzed by Analysis of Covariance. A p-value <0.05 was considered statistically significant.

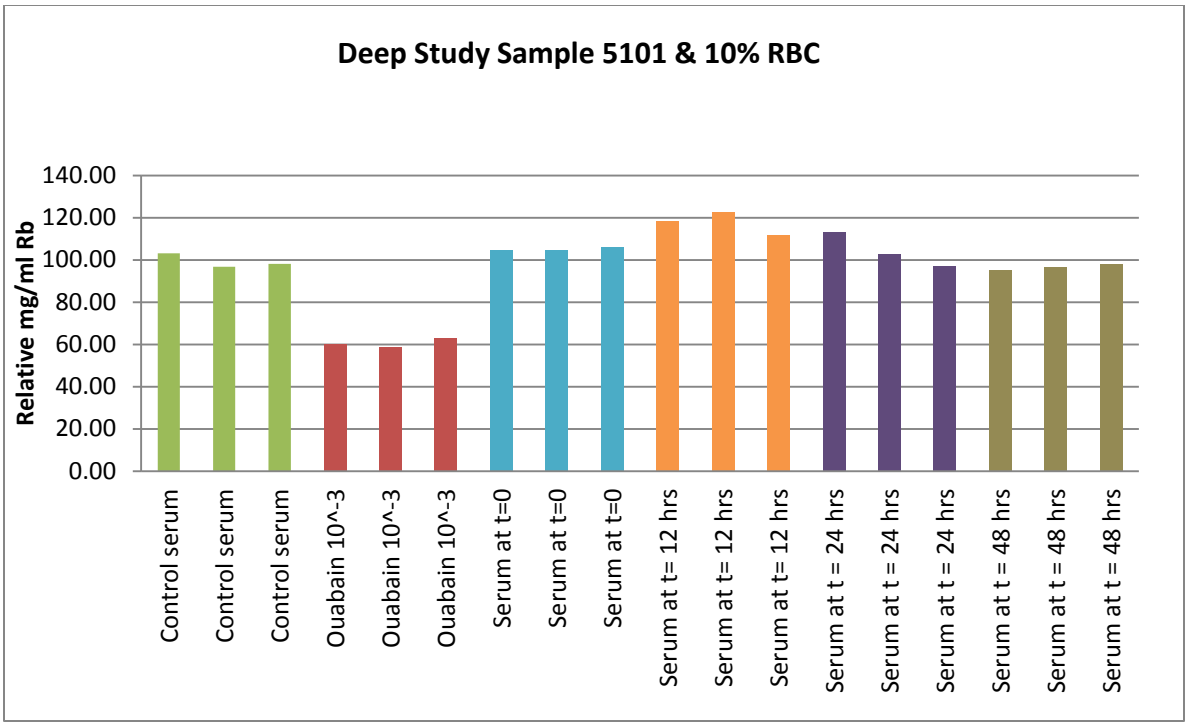


Figure 3-1. Sample #5101 as Example of Relative [Rb] as Measured by GFAA.
An example of one experiment in which SP dependent Rb uptake SP activity was measured. Data is reported directly as the absorbance (AU) by GFAA and referred to a standard curve to obtain relative concentration. Percent inhibition was attained from this data and sent to independent statistical analysis group. Control serum (green) is non-pregnant, healthy individuals, Ouabain (red) represents positive SP inhibition and the 4 different time points are represented by t=0 (blue), t=12 (orange), t=24 (purple) and t=48 (olive). All samples are done in triplicate as seen here. SP activity was not inhibited in this individual and may actually be slightly increased at t=12hr.

The calculated concentration (Rb µg/ml) data as well as percent SP inhibition data were sent to an independent data analysis core to further preserve the double blinded natures of this study. The statistical analysis team was lead by Nikhil Chauhan, Ph.D., of Protherics Corp of London, UK. A copy of the data (*Table 3-1*) sent to Dr. Chauhan by this lab is included on *Pg. 45 & 46* as a means of total disclosure, but this table is very detailed and further graphs and figures following in the Results section will provide summary details.

Creatinine clearance, maternal pulmonary edema, fetal necrotizing enterocolitis and intraventrical hemorrhaging were also considered in the DEEP study and their relationship to

EDLF was considered as part of a secondary analysis. Further details can be found in the paper cited earlier (Adair, 2010).

Baseline Secondary Analysis: EDLF vs. no EDLF

As a secondary analysis, women involved in the study were also stratified into three groups. Women whose plasma produced no inhibition of the SP were grouped together and labeled 'zero', women who plasma produced mild to moderate inhibition of the SP (less than or equal to 30% SP inhibition) were placed in a separate group and the last group included women whose plasma produced marked SP inhibition (more than 30%). Those PE women who had no SP inhibition were also called EDLF negative. Those women whose plasma did have SP inhibition were labeled as EDLF positive.

The data from for SP activity or inhibition, creatinine clearance maternal pulmonary edema, fetal necrotizing enterocolitis and intraventrical hemorrhage were all stratified according to these categories and the data was analyzed further. The Barnard Exact Test or Fisher Exact Analysis was used for categorical data while continuous variables were assessed by Analysis of Covariance.

Table 3-1: DEEP Study RBC % SP Inhibition Data

	% Inhibition				% Change from Initial (T=0)			
Study#	T=0	T=12	T=24	T=48	T=0	T=12	T-24	T=48
1101	0.00	0.00	0.00		0.00	3.43	5.76	
1102	41.35	38.62	32.00	42.39	0.00	1.20	4.10	-0.46
1103	53.34	60.97		0.00	0.00	-3.20		47.12
1104	25.01				0.00			
1201	23.78	17.85	0.00		0.00	1.38	9.15	
2101	42.20	0.00	0.00	0.00	0.00	29.78	19.95	22.93
3101	0.00				0.00			
3102	0.00	0.00	0.00	0.00	0.00	-0.46	-1.26	-4.05
3201	45.66	37.50	10.40		0.00	5.14	22.18	
3202	7.48	0.00			0.00	10.54		
3203	0.00	0.00	0.00		0.00	-4.96	5.23	
3204	42.79		33.84		0.00		5.73	
3205	0.00	0.00	0.00	0.00	0.00	3.04	1.35	-1.11
4101	96.43	77.20	158.71		0.00	4.45	-14.41	
4102	67.44				0.00			
4103	40.90	54.37			0.00	-7.03		
4104	39.40	31.89	24.19	22.89	0.00	5.67	11.48	12.46
4202	27.84	42.32	103.65	51.61	0.00	-11.70	-61.22	-19.19
4204	0.00	0.00	0.00	0.00	0.00	-10.11	-0.59	-8.77
4205	3.40	15.73	0.00	2.72	0.00	-3.83	3.36	0.21
4206	7.63	0.00	0.00		0.00	15.20	5.09	
5101	18.33	22.21	21.06	13.54	0.00	-2.08	-1.46	2.56
5102	55.49	52.63	22.14	54.52	0.00	0.72	8.39	0.24
5103	29.35	0.00	16.78	29.53	0.00	33.93	11.57	-0.16
5104	18.68	4.89	12.66	16.08	0.00	10.84	4.73	2.04
5105	31.44	19.64	22.26	30.68	0.00	7.83	6.09	0.50
5106	44.10	15.49	77.86		0.00	18.44	-21.75	
5107	0.00	0.00	0.00	0.00	0.00	-15.16	2.68	-2.11
5108	0.00	0.00	0.00	0.00	0.00	0.34	1.99	4.11
5201	0.00	0.00	0.00	0.00	0.00	-1.32	8.18	1.74
5202	48.26	34.90	21.95	43.72	0.00	9.34	18.40	3.17
5203	47.59	47.70	38.46	13.69	0.00	-0.03	2.55	9.48
5204	25.81		0.00		0.00		12.98	
5205	57.29	40.48	4.50		0.00	6.18	19.39	
5208		0.00	1.64	0.00	0.00	0.00	0.00	0.00
5209	84.39	93.14	27.09		0.00	-4.74	31.03	
5210	60.44		57.25	29.83	0.00		1.56	14.94
7101	22.51	0.00	4.34	31.88	0.00	14.09	6.87	-3.54
7201	16.93	0.00	0.00		0.00	24.13	13.03	
7202	11.79	14.71	5.64		0.00	-1.04	2.19	
8101	45.62	69.06	6.87	6.78	0.00	-14.10	23.30	23.35
8201	144.20				0.00			
8202	3.88	4.02		14.74	0.00	-0.04		-3.30
10101	1.33	6.41	19.85	31.33	0.00	-2.13	-7.77	-12.59
10201	59.83	14.76	0.00	0.00	0.00	28.45	41.60	48.47
7102	60.58	28.36	52.92	51.01	0.00	13.82	3.28	4.11
7203	9.72	18.66	18.93		0.00	-3.54	-3.64	

Table 3-1: DEEP Study RBC % SP Inhibition Data.

A detailed summary of the data obtained from Rb uptake assay of subjects in the DEEP study. The first column shows the number assigned to the patient involved in the study and the only data that this lab was privy to with regards to the woman involved in the study. The next four columns show the percent inhibition obtained from GFAA analysis of the Rb uptake assay. Blank cells of the table represent no serum sample available for that time point and so no data. The next four columns represent the percent change in SP inhibition that occurred after the Digibind or placebo was administered to the Deep subjects relative to t=0 value. Digibind was administered every 6 hours for a 48 hour period only but blood draws were at t=0, +12 hr, +24 hr, +48 hr.

RESULTS

The administration of Digibind significantly reduced the circulating EDLF levels and the attendant SP inhibition in those women who received it, as compared to the women who received the placebo. The SP activity levels in response to plasma from women who received the Digibind also increased significantly as compared to baseline when Digibind was administered, whereas the SP activity levels in response to plasma from the women who received placebo were not appreciably changed. Physiological evidence that Digibind had a positive effect on those women who received Digibind was significant for renal function also (data not presented here: See Adair 2010).

In vivo increases of assay SP activity for plasmas from treated women indicate the binding of Digibind to EDLFs that are present in the circulation of women suffering from PE. The binding of Digibind, an immune antibody Fab fragment, is very specific to substances with the same or similar structure to that of digoxin or digitalis. These results give evidence that there exists such a compound within the circulation of women with PE that when it is bound and inactivated provides better renal function of women receiving Digibind.

The following figures (*Fig. 3-2* through *Fig. 3.4*) illustrate the benefit of Digibind administered as part of the DEEP study. As mentioned previously, the clinical outcomes data considered here was not obtained in this laboratory but at other venues but is compared with EDLF data from our work. These results not only confirm that EDLF participates in some of the abnormalities of PE but also helps define possible actions of the EDLF found in PE. The data included here for SP activity and inhibition (*Figs. 3-2, 3-6a,* and *3-6b*) were all attained in this lab and the methods are included previously in the Methods section.

The first figure (*Fig. 3-2, pg. 63*) shows the difference in SP inhibition and activity comparing the plasma of women in the study who received Digibind and those that received placebo. Digibind treatment caused a significant reduction in SP inhibition from pretreatment baseline levels and likewise a significant increase in SP activity, but only in the Digibind-treated women. Placebo women demonstrated no significant changes. This indicates that the Digibind bound EDLF, removing it from the blood.

The next figure (*Fig. 3-3, pg. 64)* shows the change in creatinine clearance in response to Digibind or placebo. Creatinine clearance is a measure of renal function and a negative change from baseline indicates reduced or deteriorating renal function. Here Digibind preserved renal function as manifest by creatinine clearance over the course of treatment whereas PE subjects had significant reductions in creatinine clearance after receiving placebo.

The third figure in this section (*Fig. 3-4, pg. 65*) suggests fetal benefit. While the DEEP study focused primarily on maternal outcomes and benefit, secondary analyses have explored whether this anti-digoxin immune Fab might bring about fetal improvements. Intraventrical hemorrhaging (IVH) and necrotizing enterocolitis (NEC) are very serious complications that arise in newborn infants, especially those born prematurely. Such complications are seen in infants born to women with PE perhaps in excess of rates seen in preterm deliveries.

This figure (*Fig. 3-4*) counts the number of cases of IVH that occurred and compares the study women who received the Digibind with those that received the placebo. In this figure we can see that there is a significant reduction in cases of IVH when Digibind was administered to the mother compared with placebo (Digibind 0/18 IVH versus placebo 5/14 IVH, p=0.014, Barnard Exact) or in cases of severe IVH (Digibind 1/18 severe IVH verses placebo 3/14, p=0.028, Barnard Exact).

Also included in this figure (*Fig. 3-4*) is the number of infants who developed NEC. NEC cases appeared to be fewer in the infants whose mothers received the Digibind (Digibind 1/18 NEC versus placebo 3/14 NEC, p=0.11, Barnard Exact).

Figure 3-2. SP Inhibition in DEEP Study Women

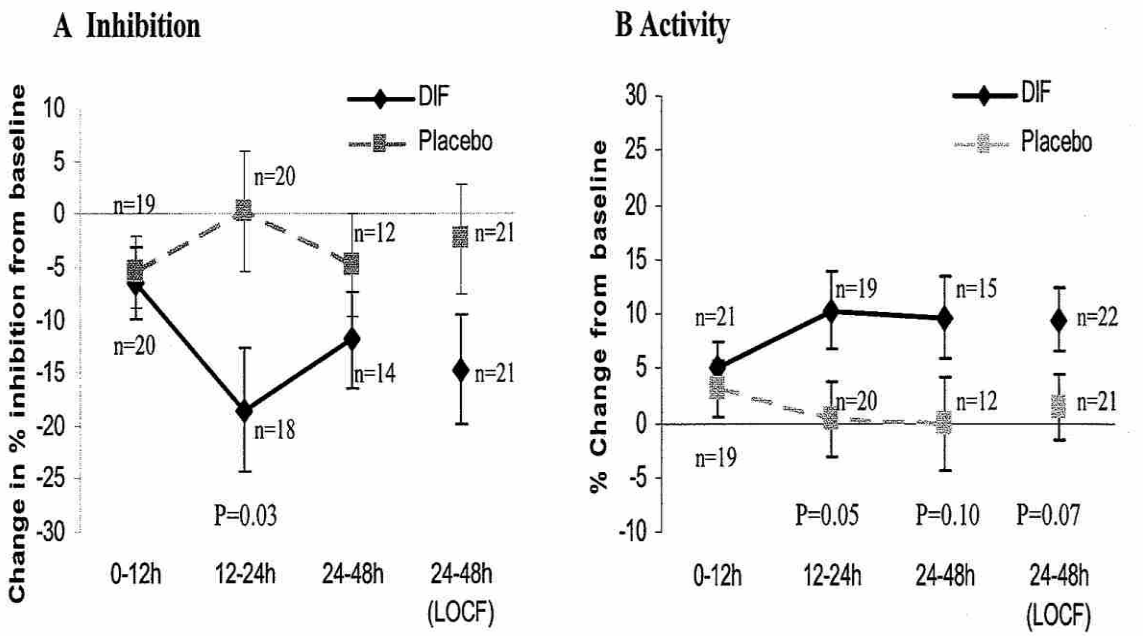


Figure 3-2. SP Inhibition in DEEP Study Women
(A) Sodium pump inhibition, a surrogate for endogenous digitalis-like factor activity, is presented (least squares mean and standard error bars) as change in percentage inhibition from baseline for the three sampling points during the treatment phase using available values. For the 48-hour sampling point, an LOCF analysis is also presented. (B) Change in SP activity (means and standard error bars). Inhibition is calculated as the reduction in activity relative to mean activity for normal controls (no inhibition) and complete inhibition as produced by ouabain. DIF, digoxin immune Fab (Digibind); LOCF, last observation carried forward (Adair 2010).

Figure 3-3. Change in Creatinine Clearance.

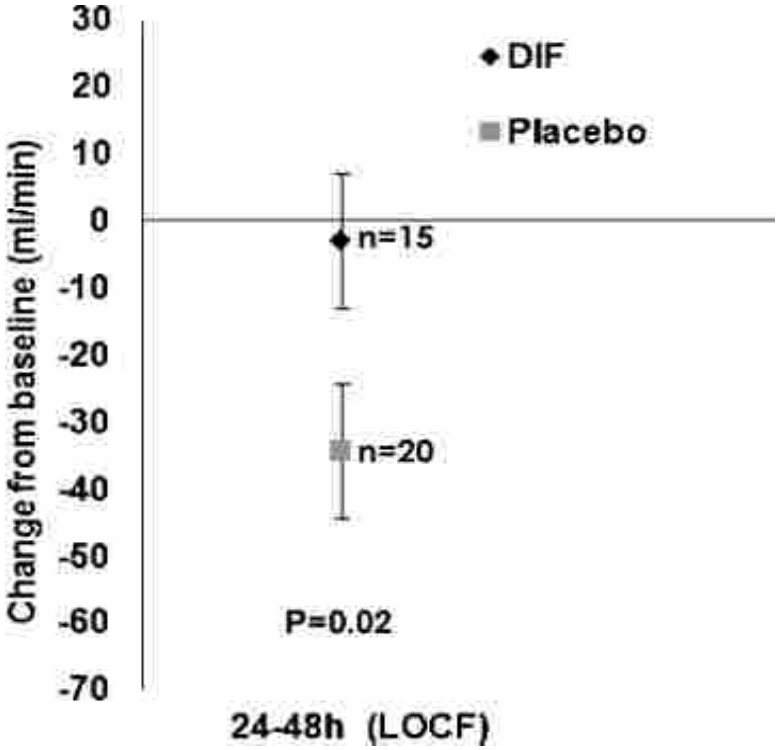


Figure 3-3. Change in creatinine clearance.
The change in creatinine clearance from baseline at the 24- to 48-hour time period (least squares mean and standard error bars) is presented using an LOCF analysis. DIF, digoxin immune Fab(Digibind); LOCF, last observation carried forward (Adair, 2010).

Figure 3-4. DEEP Study Infant Benefit

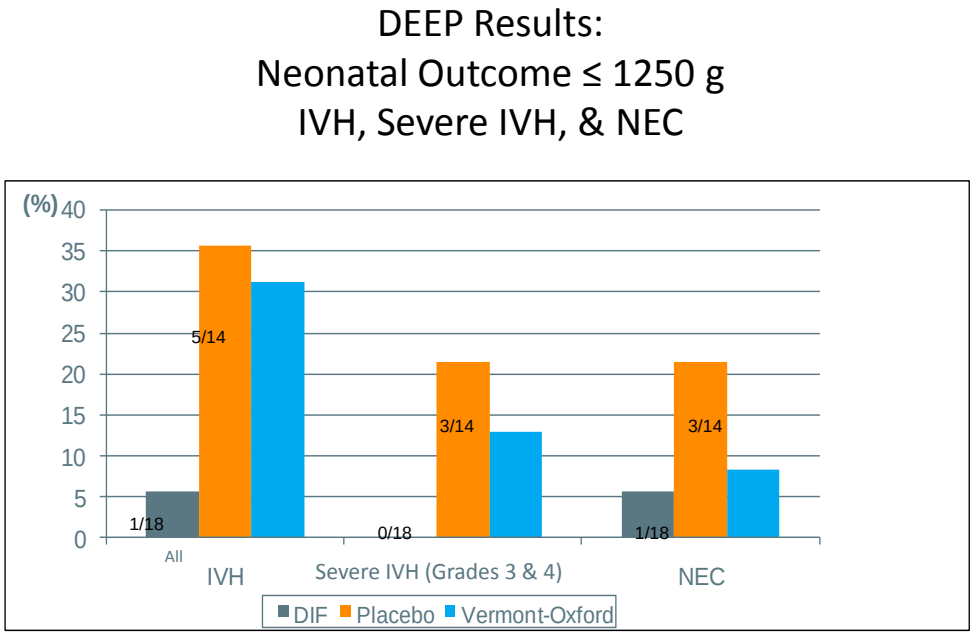


Figure 3-4. DEEP study Infant Benefit.
Cases of intraventrical hemorrhaging and necrotizing enterocolitis are fewer in those women that received Digibind (gray) compared to those women who received the placebo (orange). Vermont-Oxford standards (blue)(Adair, 2010).

EDLF Positive Women With PE

A more detailed examination of the results of the DEEP study was then made. Of interest and importance, not all women with severe PE had detectable plasma EDLF levels. It would be predicted that women's absent EDLF would not respond to Digibind treatment and hence it became useful to consider DEEP subjects based on their EDLF levels or more specifically to stratify responses based on the degree of SP inhibition produced by their plasma at the pretreatment time of admittance to the study (t = 0). This was considered a more appropriate way to determine whether EDLF contributed to their PE.

Among the 51 severe preeclamptic women studied, only 10 of these women (22%) were EDLF negative. PE, it should be remembered, is not a single disease but rather the result of several different disease processes. Importantly, there was the suggestion that PE women who showed increased levels of EDLF also showed severe disease. Not surprisingly, the EDLF positive women also showed increased Digibind response. Complications in those women who were EDLF negative (creatinine clearance, maternal pulmonary edema, fetal IVH, fetal NEC) were not altered by Digibind. This will be illustrated in the following figures.

As mentioned previously in the Methods, all these women met criteria for severe PE to be admitted to the study, so no SP inhibition, in this case does not mean absence of PE. In *Fig. 3-5a* and *3-5b* the PE women who received Digibind treatment responded better to treatment (DIF), i.e. even further increases in SP activity, as compared to all women enrolled and markedly higher increases than women who were EDLF negative (data not shown but no SP inhibition at t=0 and none whose plasma produced SP inhibition thereafter whether on Digibind or receiving placebo). The first figure *(Fig. 3-5a)* shows all women involved in the study that received Digibind, so EDLF negative women are included in these numbers, and *Fig. 3-5b* shows only the

women who originally were EDLF positive. The graph shows the SP activity when presented with plasma and demonstrates a recovery, i.e. removal of the EDLF, with Digibind treatment. The assay response to plasma is shown for the whole treatment period (t=0, 12, 24, 48, 48LOCF hours) and the change in response for those EDLF positive women is statistically significant in each of the blood draws that follow the initial admittance blood draw.

Figure 3-5a. Digibind Treatment of All DEEP Subjects

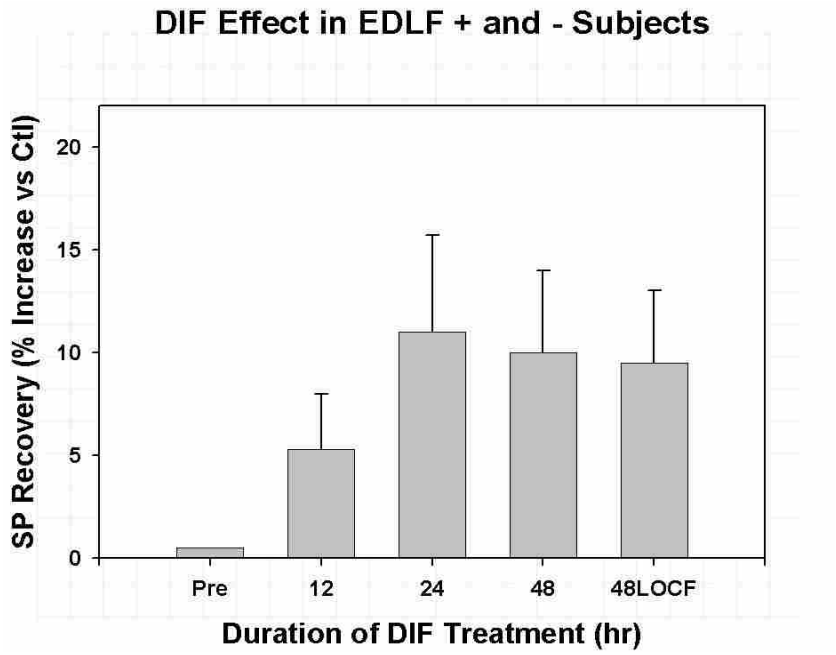


Figure 3-5b. Digibind Treatment of EDLF Positive Subjects

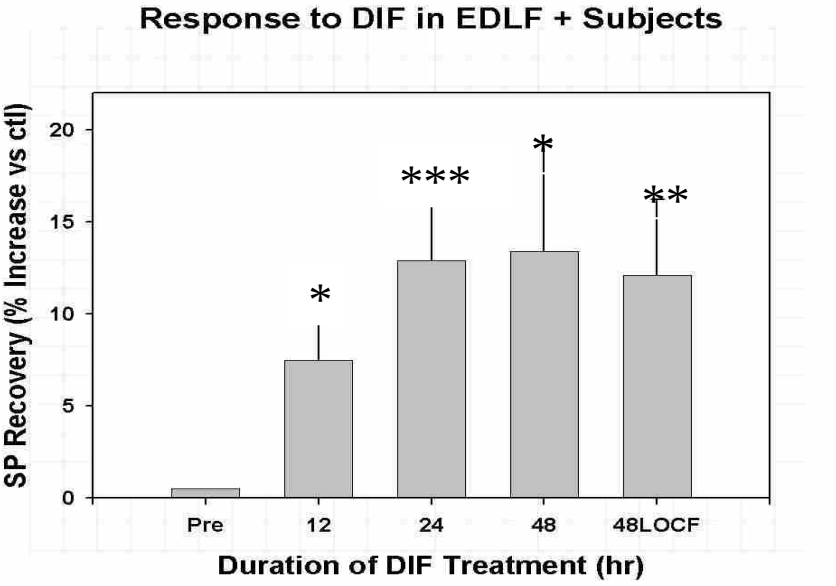


Figure 3-5a and Figure 3-5b:
Here the SP recovery (% increase in SP activity over control) at each time point is shown. In panel (a) plasma from EDLF negative women was included and in (b) plasma results from EDLF negative data were removed to show the improved SP activity (reduction in EDLF) in the EDLF positive women only. There is a marked increase in SP activity with plasma from those women who received the Digibind (DIF) (b) and were EDLF positive compared to all women who received the DIF (a). Time points (t=12, 24, 48, and 48LOCF) all show increase where (Pre) time point is obviously not changed because the DIF has not been administered yet. P-value =12 hr 0.04, 24 hr 0.0016, 48 hr 0.047; 48 hr LOCF 0.010.

Fig. 3-6 illustrates that EDLF-positive women do have a decreased change in baseline creatinine clearance despite their being administered Digibind. Digibind had no effect on those women whose plasma showed no inhibition of the SP (hence EDLF negative). Those women who were EDLF positive (increased SP inhibition at admittance [t=0]) showed greater response to the Digibind as compared with placebo. Digibind preserved function. This is especially evident in those women with higher EDLF levels who received placebo who showed marked deteriorationg in their creatinine clearance (notice the red bars). The lower the bar extends, the worse the renal function.

Figure 3-6. Creatinine Clearance Stratified by Degree of Inhibition

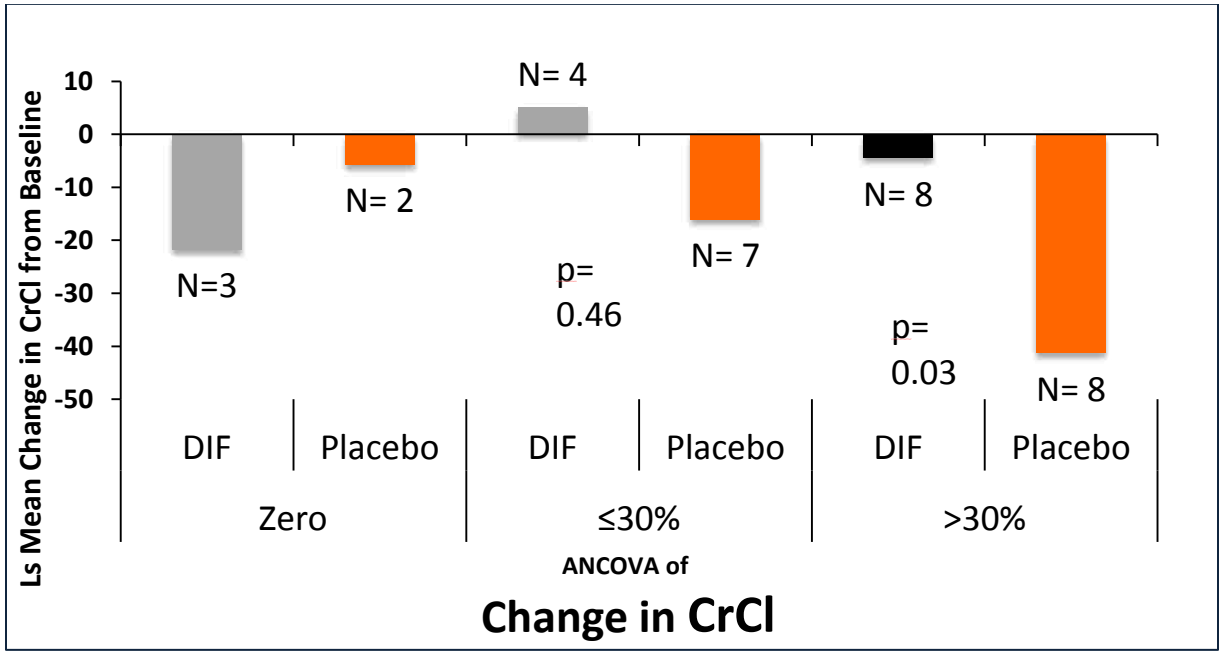


Figure 3-6. Creatinine Clearance Stratified by Degree of SP Inhibition.
The data has been stratified into 3 groups 1) those women with PE showing no EDLF activity or no inhibition [Zero], 2) those PE women showing modest to moderate SP inhibition levels or less than 30 percent [≤30%], and 3) PE women who show substantial PE inhibition [>30%]. Creatinine clearance was determined by standard clinical procedures by clinical staff and reported to the statistical core. Change was relative to pretreatment clearances. The data shows that those women who were EDLF positive, responded to the Digibind (DIF) and the change from baseline was much less in those women who were EDLF positive and received Digibind as compared to those who received the placebo.

Pulmonary edema is a very severe complication of PE. Previously, there has never been a link between EDLFs and pulmonary edema. Nevertheless, when secondary analyses were conducted, especially in the context of EDLF stratification, there was a potentially important finding. As illustrated in *Fig. 3-7*, only PE women who were EDLF positive developed pulmonary edema. This is most easily observed in considering the placebo results. This was statistically significant (p=0.0074, Barnard Exact). Additionally, among the EDLF positive women, those receiving Digibind had a lower incidence of pulmonary edema than those receiving placebo with near significance (SP inhibition ≤0%; Digibind; 1/7 cases versus placebo; 3/8 placebo cases, SP inhibitions > 30%; Digibind: 0/10 cases versus placebo: 2/10 cases, p=0.059, Barnard Exact).

Figure 3-7. Maternal Pulmonary Edema Stratified by Degree of Inhibition

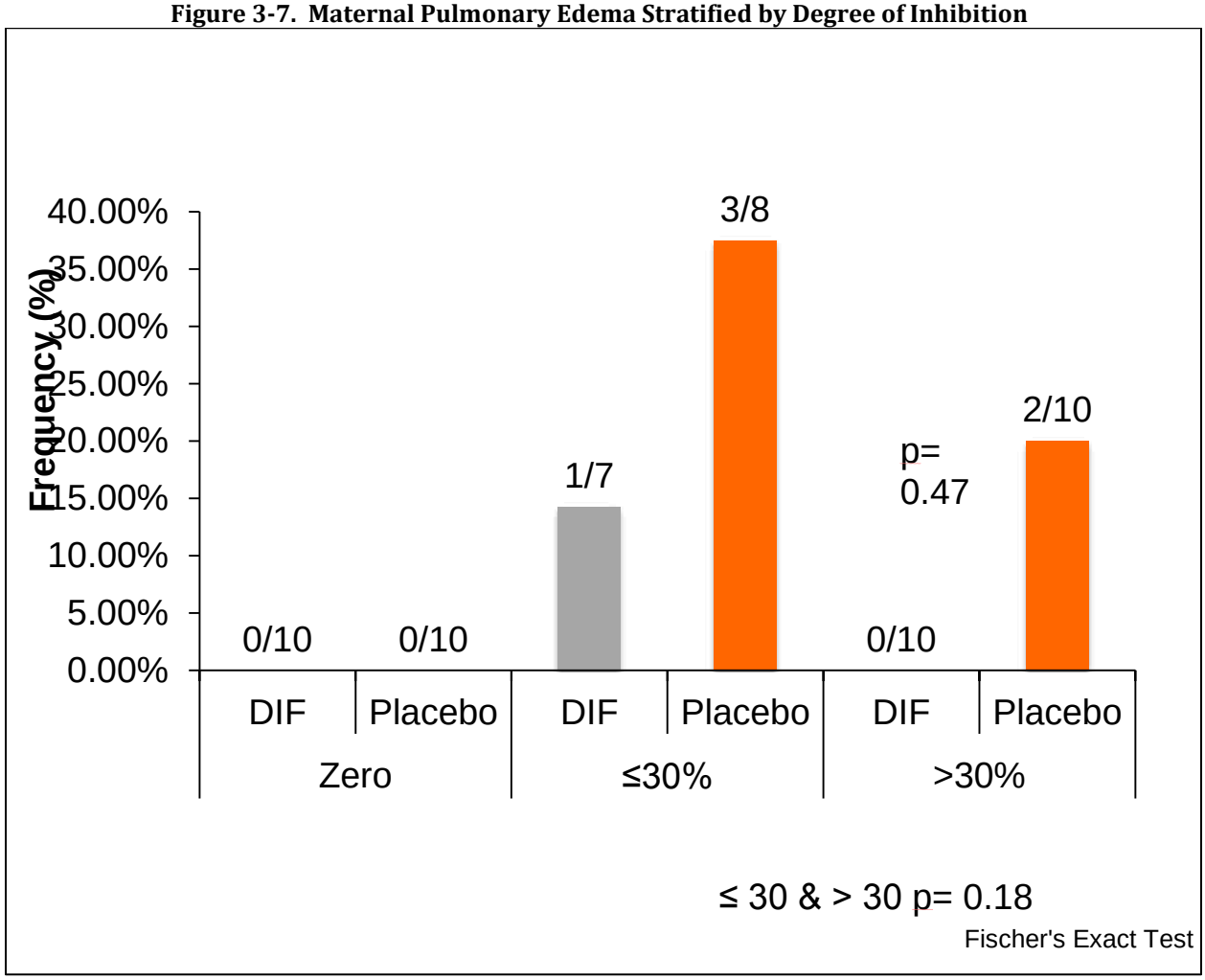


Figure 3-7. Maternal Pulmonary Edema.
Maternal pulmonary edema appears improved in EDLF posibive women when they received treatment as compared to those women who received placebo. Women who were EDLF negative (Zero) had no cases of pulmonary edema. Women with ≤30% SP inhibition had cases 1/7 DIF and 3/8 placebo and women with >30% SP inhibition showed 0/10 cases with Digibind and 2/10 with placebo.

As part of secondary analyses, life-threatening fetal complications were considered. The two most problematic neonatal complications are intraventrical hemorrhage (IVH) and necrotizing enterocolitis (NEC). When IVH complications were stratified by the extent of SP inhibition caused by maternal plasma, the evidence suggested that Digibind reduced the occurrence of IVH in EDLF positive mothers. Specifically, Digibind treatment resulted in no cases of IVH in 17 neonates compared with 4 cases in 18 neonates from the placebo group (p-0.021, Barnard Exact).

For NEC the numbers of cases were too few to carry out such stratification analyses.

Figure 3-8. IVH Stratified by EDLF Level

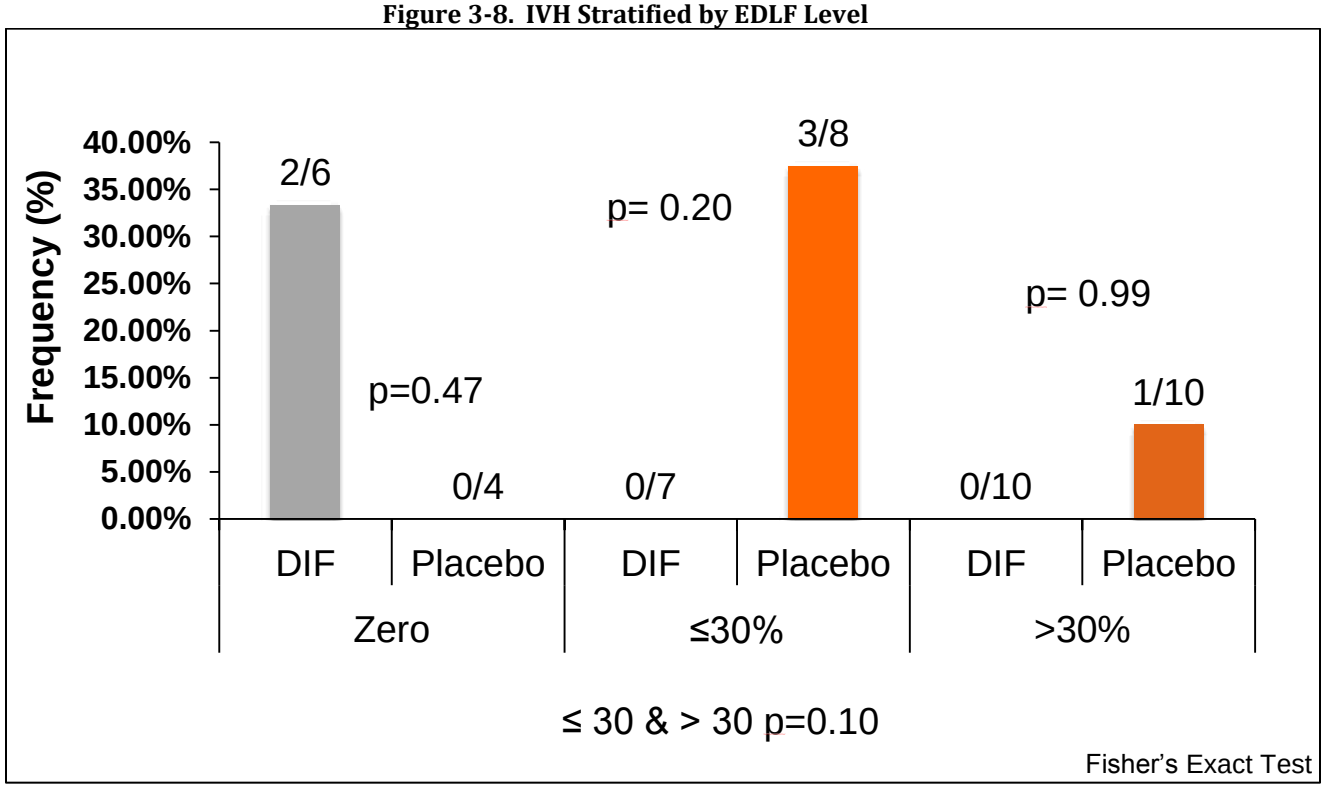


Figure 3-8. IVH Stratified by EDLF Level.
This Figure depicts the difference in IVH cases among those women who were EDLF negative and EDLF positive and the difference in response to Digibind (DIF) treatment. Of the women who were EDLF negative (Zero) the DIF had very little effect on the IVH (2/6 DIF vs. 0/4 placebo), but of the women who were EDLF positive or that had SP inhibition (≤30% and >30%) these infants who were born of the PE women showed improvement with the receipt of Digibind vs. placebo.

DISCUSSION

The DEEP study represented the first clinical trial where Digibind has been administered *in vivo* as a treatment for women with PE. It has been administered on a *compassionate use* basis in a few patients but never as a part of a double-blind, placebo-controlled, randomized trial. The benefits to the mother have been reported and can be summarized as a reduction in circulating EDLF (resulting in increased SP activity and reduced SP inhibition in the Rb^+ uptake assay in response to maternal plasma), significant preservation of creatinine clearance, and reduced maternal pulmonary edema experienced in the PE women who received Digibind instead of the placebo. This supports our hypothesis very well. A previously unrecognized complication of high EDLF levels was also suggested by these analyses to be pulmonary edema.

EDLF has been shown as part of many studies by this lab to be an *in vitro* SP inhibitor present and increased in the serum and plasma of women with PE and its effects reversed by Digibind (*Chapter 2)* but the DEEP study showed that Digibind not only binds EDLF *in vitro* but also *in vivo* and may be offered as a therapeutic for women with PE. The safety of the Digibind was included as part of the study (*see Adair 2010),* and no known side effects occurred. Nevertheless, it is recognized that the DEEP trial was small in number and a larger cohort will be needed to verify the data seen. That said, the data presented here support in vivo participation of EDLF in features of PE and the acceptable use of Digibind to treat women with PE.

The mechanism of Digibind complexation and removal of EDLF is also supported. This is especially clear in secondary data analyses, where it was found that a few women involved in the study did not show increased SP inhibitors in their circulation. The symptoms found in these EDLF negative women could not be due uniquely to an increase in circulating EDLF. However,

this does not exclude the possibility that EDLF can produce a comparable symptom or may contribute mechanistically to the symptom; nevertheless the use of Digibind in these women is unwarranted and as shown, does not provide benefit to those women who are EDLF negative. This EDLF negative nature of some women with PE suggest once again that PE has more than one cause and better diagnostic separation of different PE groups would be exceptionally valuable as causes and cause-specific therapies are considered. Clearly better PE classification needs to be studied more deeply, allowing division by mechanism, and mechanisms explored one by one. The definition of PE in use already very broadly defines PE as a multi-symptom, multi-system condition of pregnancy, and this has led to confusion about its cause.

This being acknowledge, those cases investigated here where EDLF was positive showed that the administration of Digibind did reduce symptoms of PE as compared to those women who received the placebo. Not only was EDLF reduced, but renal function measured by creatinine clearance and pulmonary edema were both improved in the treated EDLF positive PE women. Interestingly and unexpectedly, there also appeared to be fewer serious complications in neonates whose mothers received Digibind. This suggests a role (though not necessarily a cause) for EDLF in these serious neonatal problems at least in the setting of PE. Further studies are being planned to specifically address this possibility.

CHAPTER 4 – COMPARITIVE STUDIES OF DIGIBIND AND DIGIFAB

INTRODUCTION:

The previous chapters show the usefulness of Digibind in reversing SP inhibition caused by EDLFs that circulate within women who have PE. Digibind, a digoxin immune Fab, manufactured by SmithGlaxoKline, but they are discontinuing its production and BTG, Inc. (formerly Protherics, Inc., London, England) is now producing another digoxin immune Fab, DigiFab, to replace it. This chapter details a study done to compare the effectiveness of both digoxin immune Fabs, Digibind and DigiFab, in binding several known SP inhibitors, as well as the EDLF found in PE serum. Binding the known SP inhibitors, digoxin, bufalin, ouabain, and proscillaridin A, will disinhibit the SP and allow for an increase in SP activity or an increase in Rb ion uptake in our previously described bioassay. The binding of EDLFs by both of these Fab fragments is critical in further determining if circulating EDLFs exist in women with PE and more importantly in isolating from women with PE any EDLFs that do exist for further investigation. It is our hypothesis that both digoxin immune Fabs will bind all SP inhibitors, including EDLFs found in women with PE, and that concentrations of the two Fabs needed to reverse SP inhibition will be similar.

MATERIALS AND METHODS:

Most of the methods used here were already previously described in *Chapter 2* but are included here for completeness and convenience.

Patients and Patient Derived Specimens

All studies were reviewed and approved by Institutional Review Boards at both participation institutions, University of Tennessee, School of Medicine, Chattanooga and Brigham Young University, Provo. All women who participated provided informed written consent prior to enrollment. Sera were collected at the time of delivery from women with third trimester uncomplicated pregnancies (controls) and from women whose pregnancies were complicated with PE. Specimens were frozen immediately in liquid nitrogen, handled on dry ice and stored at -80ºC until assay. Sera were thawed and assayed without further processing.

Cell Preparation:

Both myometrial cells and red blood cells (RBCs) were prepared as described in previous chapters. These cells provide a means to measure the activity as well as the inhibitor EDLF effects on activity of the plasma membrane SP. The majority of the data obtained in this chapter was with RBCs. Cells are used as soon after isolation to maximize the activity of the SP.

Myometrial cell preparation

Human myometrial cells were cultured in 20 ml of growth medium (100 ml of fetal bovine serum, 1 ml of 1000X Gentamicin, 1 L MEM) in 150 mm culture dishes. Cells were passaged at 95 to 100% confluency by rinsing cells with 15 ml PBS with EDTA and then incubating at 37ºC in another aliquot of 15 ml PBS with EDTA containing 10 µl trypsin for 30 min. The cells were placed in a 15 ml conical tube and centrifuged for 10 min at 2000*xg*. The PBS containing EDTA was removed, and then the cells were replated in three 150 mm culture dishes containing 20 ml of the medium. All cell culture supplies were obtained from GIBCO, Carlsbad, CA.

When enough plates (1 plate for every inhibitor sample to be run in the Rb^+ uptake assay) had been cultured to 95 to 100% confluency, cells were removed from the dish with 15 ml PBS and cell scrapers. Harvesting cells involved the same rinse step with 15 ml PBS with EDTA, followed by incubation in another 15 ml PBS with EDTA containing no trypsin for 30 min. Trypsin damages cell membrane proteins, including the SP located there, so it was not used to harvest the cells. Cells were again centrifuged and the supernatant removed. Packed cells were resuspended in 5.00 ml RbCl buffer containing in mmol/L: NaCL 135, RbCl 6.73, NaH_2PO_4 8.10, Na_2HPO_4 1.27, and $MgCl_2$ 1.0 at pH 7.45 and used immediately in the Rb^+ uptake assay described below.

Immediately after the harvesting of myometrial cells, the cells were prepared for use in the Rb^+ uptake assay by washing myometrial cells with K^+-free RbCl buffer 3 times, the cells were resuspended in 5.0 ml of RbCl buffer and combined with other washed cells (usually a total of ten tubes each containing cells from one 150 mm culture plate each) to create a single uniform pool (a Bradford protein assay was done in order to verify uniform cell content) of myometrial cells to be used as part of a single experiment.

Red Blood Cell Preparation

Red blood cells were freshly obtained from healthy men or non-pregnant women (not involved in the study group) by approved standard phlebotomy methods in a clinical setting. Blood was collected into tubes containing EDTA (to prevent clotting). The blood was allowed to sit for 1 hour at room temperature and then centrifuged at 3300*x*g, 4ºC for 10 minutes. The plasma and buffy coat were removed and the packed red cells (5 ml) were washed by resuspension in 5.00 ml of the same RbCl buffer used in the treatment of myometrial cells. The mixture was vortexed well for 1 minute and then placed in the centrifuge once again to be re-

spun at the same speed and time. This washing process was then repeated twice and finally cells were prepared as a 10% hematocrit suspension of RBC with RbCl buffer (5 ml of packed RBCs: 45 ml of RbCl buffer) for use in the Rb^+ uptake bioassay. These cells were then used in the same way (i.e. washing three times with RbCl buffer) as the myometrial cells described above to assess inhibitors and the effect of the digoxin immune Fabs.

Rb^+ Uptake Assay

The Rb^+ uptake bioassay, which measures Rb^+, substituted for K^+, uptake was used to determine the comparability of two Fab fragments, Digibind and DigiFab. This bioassay can be done in the presence or absence of inhibitors, but will be used here in the presence of SP inhibitors, previously thought to be possible candidates for the EDLF in PE.

SP inhibition (or EDLF activity) was assayed using this Rb^+ uptake bioassay that was adapted from a previously published procedure (Zhen, 2005). This bioassay was performed immediately following cell preparation to optimize ATP dependent SP activity. Essentially, Rb^+ is taken up into the cytosol of RBCs or myometrial cells in RbCl buffer in the absence or presence of the inhibitor. After the effective dose of an inhibitor is determined, the Digibind or DigiFab (100 µl) is also added to individual Eppendorf tubes to test the binding and inactivating capabilities of the digoxin immune Fabs. Assay samples were then incubated in a shaker incubator (220 rpm, 37ºC) for 45 min. Following the incubation period each sample was spun in an Eppendorf Centrifuge 5415 (Hamburg, Germany) for 10 minutes at 2200*x*g to pellet the RBCs and the supernatant was pipetted off. Each RBC solution treated with sample was washed with 1 ml of RbCl washing buffer (containing in mmol/l: choline chloride 149, $MgCl_2$ 1.0, MOPS 5.88, Tris 2.12 at a pH of 7.40), and recentrifuged to thoroughly remove extracellular Rb^+ and allow only Rb^+ actually inside the cytosol of the cell to be measured. This washing was repeated two

additional times. The myometrial cells or RBCs were then lysed with 1 ml ice-cold deionized H_2O for further analysis.

As soon as possible after cell harvesting or preparation, prepared cells were incubated with known inhibitors or PE sera and the specimens' effects on SP mediated Rb^+ uptake into cells were measured. Four different known SP inhibitors was assayed in triplicate. The concentrations of inhibitors, Digibind, and DigiFab were varied.

As mentioned, ouabain at a final concentration of $1x10^{-3}$M was used here and throughout all experiments as a positive inhibition control. Ouabain at this concentration caused 100% SP inhibition whereas a concentration of 2×10^{-6}M produced ~50-80% inhibition. The degree of inhibition is expressed as a percentage. This is calculated as the decrease in Rb^+ uptake of a given specimen as compared with Rb^+ uptake with no inhibition (normal human control serum). The value for complete inhibition (10^{-3}M ouabain control) must show more than 85 percent reduction in SP activity to be acceptable.

Known SP Inhibitors

Each inhibitor at each concentration was assayed in triplicate. Each individual assay consisted of a negative control to determine baseline (no inhibition): for these studies, serum from normal healthy men and non-pregnant women; a positive control: ouabain [$1x10^{-3}$M]; inhibitor or serum of interest without immune Fab (a deionized H_2O blank was substituted); or inhibitor or serum of interest with Digibind or DigiFab.

Inhibitors used were ouabain (at lower concentrations than ouabain used as a positive control), bufalin, proscillaridin A, and digoxin. They were obtained from Sigma, St. Louis, MO and were prepared by dilution to proper concentrations with deionized H_2O. Each inhibitor was prepared at varying concentrations (i.e. $1.00x10^{-5}$M, $1.00x10^{-6}$M, $1.00x10^{-7}$M, and $1.00x10^{-8}$M)

and assayed by Rb^+ uptake to identify a concentration that produced ~50% inhibition. This represents the most sensitive concentration region of SP inhibition. This concentration was then used for that inhibitor in later experiments. This was done by adding each inhibitor to the cell bioassay described above in increments of 100 µl per Eppendorf tube.

Serum from Preeclamptic Women

Serum from preeclamptic women, containing an endogenous SP inhibitor or EDLF, was also used in these studies and was used undiluted, but preparatory inhibition assays were done to ensure adequate inhibition levels.

Sera from women with established PE and from third trimester pregnant women with uncomplicated pregnancies (CTL) were collected as part of an IRB approved study at the University of Tennessee Medical School Chattanooga. Women provided signed informed consent prior to participation in the study. Blood samples were collected and processed immediately into serum, snap frozen in liquid nitrogen, and maintained frozen until assay. Specimens were transferred on dry ice in batches to this laboratory (Brigham Young University) for assay of their EDLF activity. Sera were stored at -80^{o}C until they were assayed for their ability to inhibit SP dependent Rb^+ uptake into fresh human RBCs collected from healthy volunteers as described above. It should be noted that most of the women with PE in this particular study did not have a severe form of the disease. This was not the case with the women described in Chapters 2 and 3.

For these comparability studies to be carried out, an initial determination of the EDLF activity had to be made. While most sera from women with PE had increased capacity to inhibit RBC SP activity as compared with uncomplicated pregnancy controls, it was necessary that the actual specimens used in the DigiFab and Digibind SP inhibition reversal studies produced

adequate SP inhibition, i.e. adequate EDLF activity, so that reversal of inhibition could be readily observed. Consequently, the ideal PE serum caused greater than 50% inhibition of SP activity in the initial screen.

The degree of SP inhibition was determined for different sera using the absorbance data from the GFAA. This assay was detailed previously.

Digoxin Immune Fab Addition

After the concentration at which each SP inhibitor produced ~50% inhibition was found, DigiFab and Digibind were prepared at varying concentrations to see if a reversal of at least 20% could be attained with these antibody immune Fab fragments. In general, initial concentrations of $1x10^{-5}$M to $1x10^{-7}$M inhibitor were evaluated and the one producing ~50% inhibition chosen for the Fab experiment. Each immune Fab concentration was varied separately so that the remaining percentage of SP inhibition was comparable for both Fab's. Digibind and DigiFab were dissolved in deionized H_2O and stored at 4ºC until used in the bioassay. Typically, a concentration of $1x10^{-6}$M Digibind and $1x10^{-6}$M DigiFab were used in conjunction with the chosen concentration of inhibitor. The DigiFab concentration was then adjusted to be equivalent to the effect of $1x10^{-6}$M Digibind in its ability to reverse or block inhibition. An aliquot of 100 µl immune Fab was used in each bioassay. Deionized water was added as a blank.

GFAA Analysis

Rb^+ content in the cell cytosol was measured by graphite furnace atomic absorption (GFAA, Graphite Furnace Atomic Absorption Instrument Model 4100Z, Perkin Elmer, Waltham, MA). Prior to the GFAA step, 10.0 µl of the cell lysate was diluted to 500.0 µl with deionized H_2O of which the auto sampler injected 10.0 µl three different times for assay in a

standard GFAA method. This program in brief heats the furnace compartment where the sample is injected to very high temperature (1800 K) to completely combust organic cell material which is carefully blown off by argon gas leaving salts and to ionize salts, but only the elemental rubidium was measured by the absorption lamp specific to Rb.

In order to compensate for cell pool-to-pool variability (i.e. caused by varying initial activities of the SP) in the Rb^+ uptake assay, the data were reported as percent inhibition. For each unknown assayed, three different determinations were carried out in separate Eppendorf tubes, each read 3 times by the GFAA instrument for a total of nine data points each. The GFAA instrument reported the average of three separate readings in absorbance units. The absorbance produced by inhibitor or PE sera (higher inhibition lower Rb^+ reading) was divided by absorbance of the negative control, (non-pregnant sera, set to equal no inhibition and a higher Rb^+ reading), minus the positive control (10^{-3}M ouabain, complete SP inhibition) and expressed as a percentage. The positive control, 10^{-3}M ouabain, as mentioned above, was also considered as a criterion of accuracy; if ouabain at 10^{-3} M showed more than 50% reduction of the SP activity then the assay was considered acceptable. Actual Rb concentrations were also calculated for each absorbance reported by reference to a daily calculated calibration curve generated using aqueous Rb concentration standards. The individual results of each single bioassay allowed us to determine the next step and the next concentration of inhibitor or anti-digoxin immune Fab to use systematically, working closer to the previously determined percentage inhibition and/or reversal of inhibition guidelines.

RESULTS

Digoxin

First, the concentration of digoxin that caused ~50% inhibition of the SP was determined. Concentrations of $1x10^{-5}$M, $1x10^{-6}$M, and $1x10^{-7}$M digoxin were tested in a fresh human RBC Rb^{+} uptake assay. The following *Table 4-1* and *Fig. 4-1* show the results of the assays using digoxin as the SP inhibitor.

Of the three concentrations tested $1x10^{-6}$M digoxin produced 47.6 % inhibition of the SP in RBCs and the concentration was closest to the predetermined 50% inhibition level.

TABLE 4-1: Dose Response of Digoxin

CONCENTRATION OF INHIBITOR	DIGOXIN N=3	
	GFAA AU	% INH
$1X10^{-5}$ M	0.263 ±0.040	73.7
$1X10^{-6}$ M	0.524±0.094	47.6
$1X10^{-7}$ M	0.743±0.177	25.7

Percent Inhibition was determined by GFAA reading for digoxin dose divided by the GFAA reading for the negative control (non pregnant healthy serum) and then taken as a percent. Ouabain at $1x10^{-3}$M was also included as a positive control in each assay, and assay was only considered functional when it showed more than 50% inhibition.

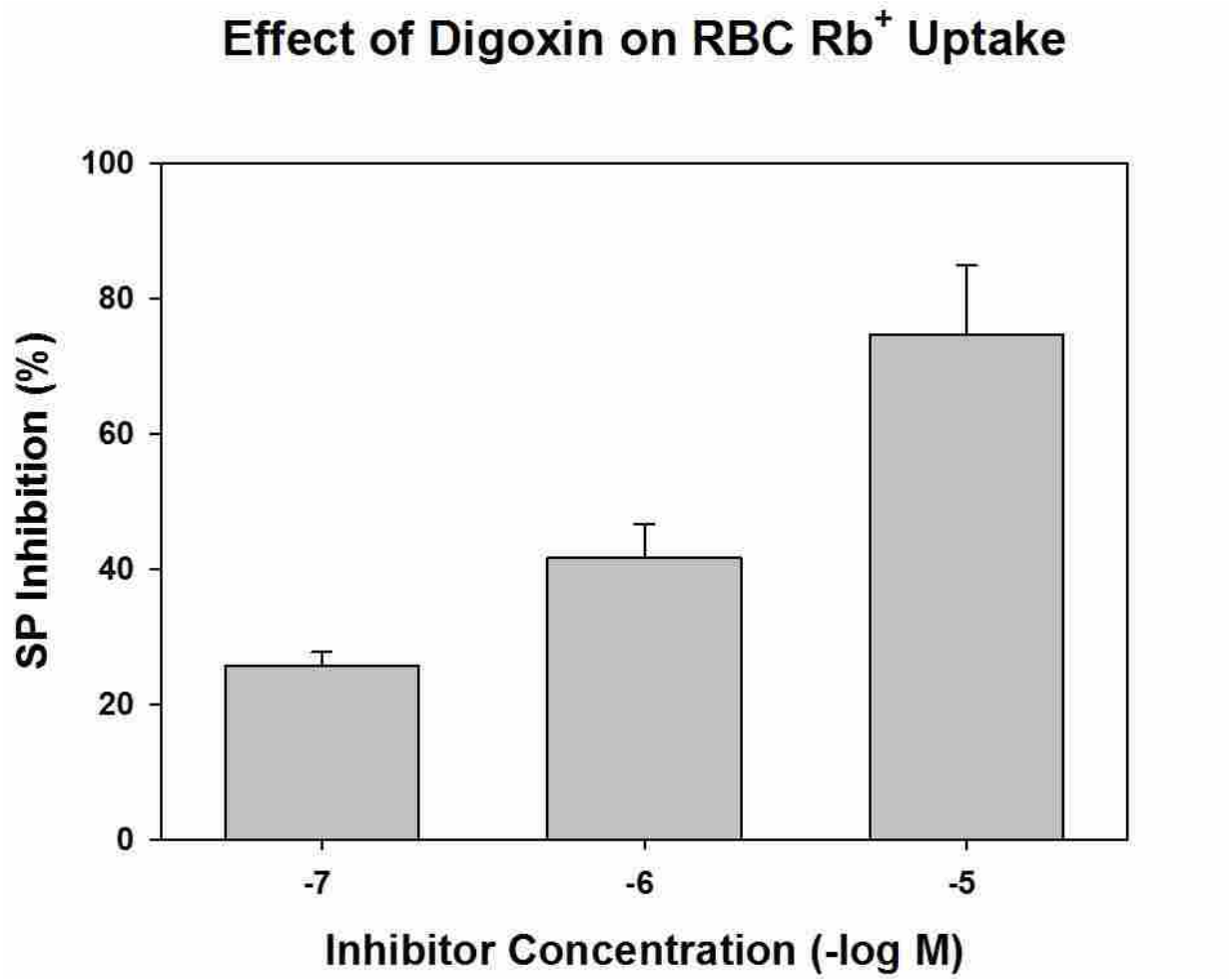


Figure 4-1. Effect of Digoxin on RBC Rb^+ Uptake

The effect of graded concentrations of digoxin (D) on SP mediated Rb ion transport into fresh human red blood cells (RBC). Digoxin at 1 x 10^{-7}M (-7 D), at 1 x 10^{-6}M (-6 D) or 1 x 10^{-5}M (-5 D) each showed a significant (ANOVA $p<0.01$, all post hoc pairwise comparisons $p<0.05$) and progressive increased inhibition.

After determining that the concentration of digoxin used for the investigation of the two Fabs was to be $1x10^{-6}$M, we then proceeded to investigate the concentrations of the two different Fabs needed for at least a ~20% reversal of inhibition. In the following table (*Table 4-2*) each row represents a set of experiments. The inhibitor is first tested alone and then the Fabs are added separately to the inhibitor, in this case digoxin, and the percent inhibition is again calculated to see if the Fabs reverse the original inhibition.

TABLE 4-2: Anti-digoxin Immune Fab Dose Determination on Digoxin

	N=	Inhibitor and Fab	[Inhibitor]	[DigiFab]	[Digibind]	% INH	GFAA AU	SD in AU
SET 1	3	DIGOXIN ALONE	$1X10^{-6}$ M			49.6	0.504	0.042
		DIGOXIN+DIGIFAB	$1X10^{-6}$ M	$1X10^{-6}$ M		7.20	0.928	0.094
		DIGOXIN+DIGIBIND	$1X10^{-6}$ M		$1X10^{-6}$ M	8.20	0.918	0.181
SET 2	2	DIGOXIN	$1X10^{-6}$ M			63.3	0.367	0.061
		DIGOXIN+DIGIFAB	$1X10^{-6}$ M	$1X10^{-6}$ M		0.00	1.033	0.050
		DIGOXIN+DIGIBIND	$1X10^{-6}$ M		$2X10^{-6}$ M	0.00	1.144	0.012
SET 3	2	DIGOXIN	$1X10^{-6}$ M			60.2	0.398	0.008
		DIGOXIN+DIGIFAB	$1X10^{-6}$ M	$1X10^{-6}$ M		8.60	0.912	0.048
		DIGOXIN+DIGIBIND	$1X10^{-6}$ M		$1.5X10^{-6}$ M	6.00	0.940	0.002

A summary of assays performed to determine when the concentration of DigiFab and the concentration of Digibind give similar percent inhibition and is as close to 20% inhibition as possible. This represents a reversal of inhibition caused by the digitalis like factor, digoxin.

The target concentration of the inhibitor was determined to be $1x10^{-6}$M, it causing ~50% SP inhibition, and afterwards the effects of the Fab's were assessed. Digibind and DigiFab were added in graded concentrations to determine the concentration of each immune Fab necessary to reverse the inhibition of digoxin. A series of assays that included digoxin at $1x10^{-6}$M treated with selected concentrations of DigiFab or Digibind were performed to determine the concentrations of each Fab needed for similar restored SP activity. Table 4-2 shows the summary of these assays.

The first set of experiments showed that both digoxin Fabs used could bind and inactivate the investigated inhibitor digoxin. At equal molar concentrations of the digoxin with either one of the Fabs, most SP inhibition was overcome and SP activity rescued. DigiFab seemed to have an equivalent effect to Digibind, although DigiFab seemed to very slightly, work better, against this inhibitor. Increasing Digibind concentration appeared to produce effects indistinguishable to DigiFab. This can be visualized in *Fig. 4-2*.

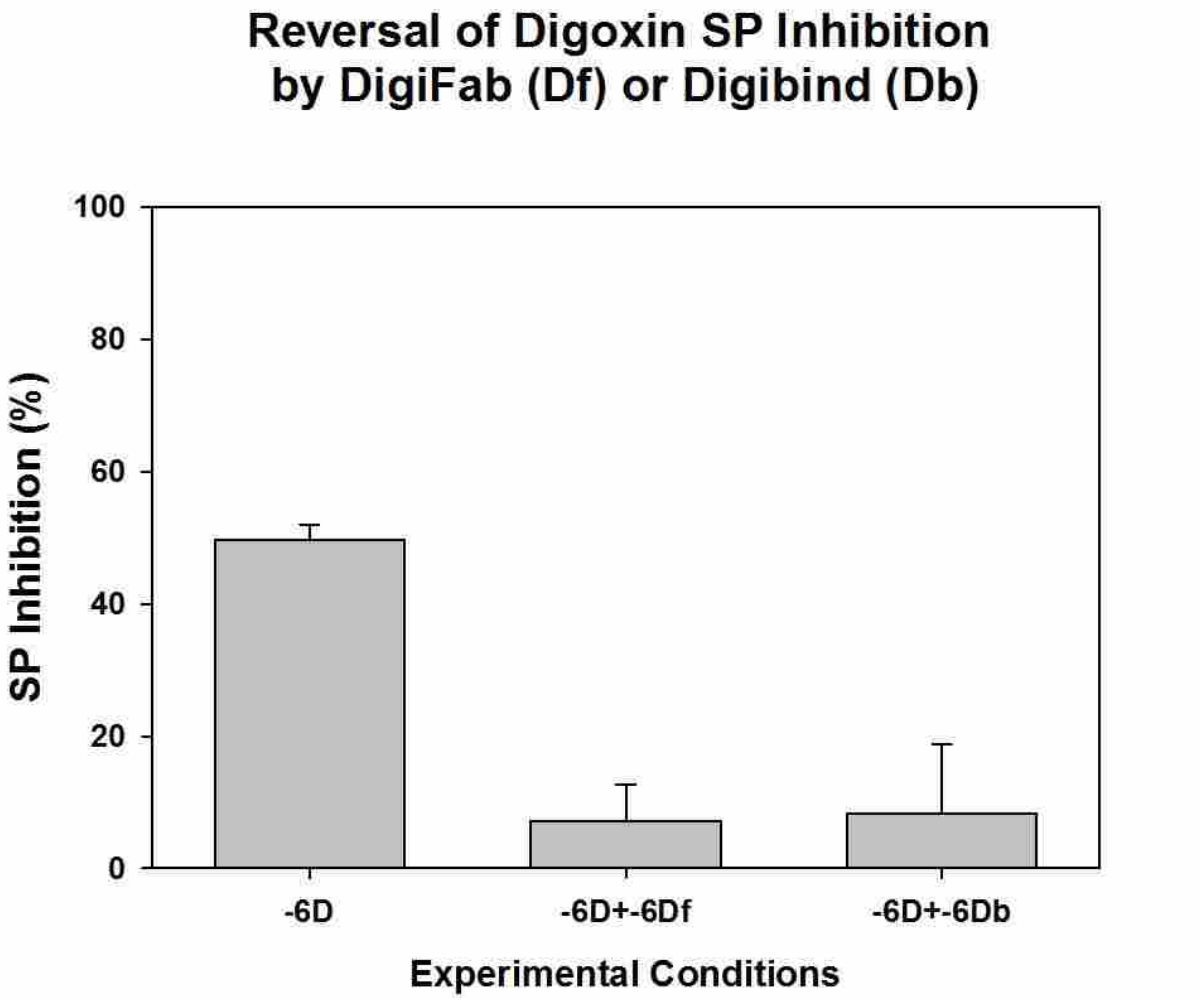


Figure 4-2. Reversal of Digoxin SP Inhibition by DigiFab (Df) or Digibind (Db). Effect of DigiFab (Df) or Digibind (Db) in Reversing the Digoxin Inhibition of SP Mediated Rb Ion Transport into Fresh Human RBCs. A concentration of 1 x 10^{-6}M digoxin (-6 D) was used to produce inhibition and this concentration was also assayed in the presence of 1 x 10^{-6}M DigiFab (-6Df) or 1 x 10^{-6}M Digibind (-6Db). The reversal was significant ($p = 0.01$) and comparable for DigiFab and Digibind ($p>0.05$).

Ouabain

The results of the 3 assays used with the inhibitor ouabain were performed in order to determine which concentration produced approximately 50% inhibition of the SP. The results showed that $1x10^{-6}$M ouabain produced more than 80% inhibition, whereas $1x10^{-7}$M ouabain produced only 33% inhibition, and $1x10^{-5}$M ouabain produced nearly 96% inhibition. See also Table 4-3 and *Fig. 4-3*. We determined to use $1x10^{-6}$M ouabain to test the two digitalis Fabs. This concentration did show somewhat more than 50% inhibition but it is important to have enough inhibition to show the ability of the immune Fabs to reverse the inhibition.

TABLE 4-3: Dose Response of Ouabain

CONCENTRATION OF INHIBITOR	OUABAIN N=3	
	GFAA AU	% INH
$1X10^{-5}$ M	0.044±0.025	95.6
$1X10^{-6}$ M	0.185±0.050	81.4
$1X10^{-7}$ M	0.679±0.085	33.1

Table 4-3 is a summary of the experiments done to determine dose response of SP in RBCs to ouabain. Concentrations of $1x10^{-6}$M showed closest inhibition to 50%.

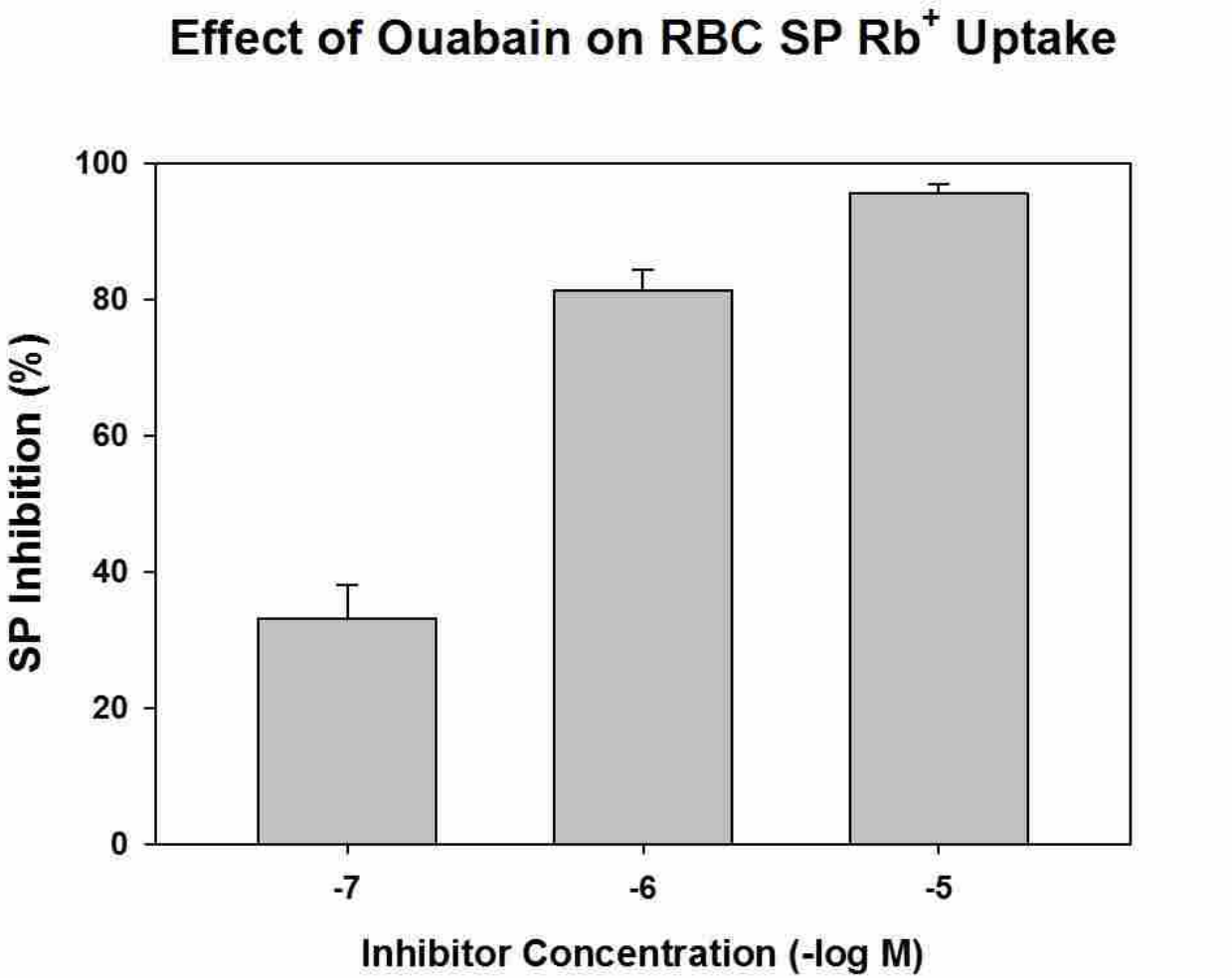


Figure 4-3. The Effect of Ouabain (O) on RBC SP Rb+.
A graded concentration of ouabain at 1 x 10^{-7}M (-7 O), at 1 x 10^{-6}M (-6 O) and 1 x 10-5M (-5 O) added to RBCs and the effect seen on rubidium transport on SP. Each experiment showed a significant inhibition (ANOVA $p<0.01$, all post hoc pairwise comparisons $p<0.05$) and progressive increased inhibition with increased ouabain.

The use of 1x10^{-6}M ouabain in the next series of experiments demonstrated that slightly more DigiFab was needed to equal the binding and inactivating capabilities of Digibind. Digibind at 1x10^{-6}M showed an SP activity increase of ~20% whereas DigiFab at the same concentration showed no improvement in SP activity. When we doubled the DigiFab concentration to 2x10^{-6}M the SP activity was reversed completely. Using a DigiFab concentration in the middle (1.5x10^{-6}M) of these two concentrations, we were able to more

closely match the binding and inactivating capabilities of Digibind. In summary, with ouabain $1x10^{-6}$M, the addition of DigiFab at $1.5x10^{-6}$M showed 73.3% inhibition and Digibind at $1x10^{-6}$M showed 71.6% inhibition, a 12% and 14% reduction in SP inhibition respectively.

TABLE 4-4: Anti-digoxin Immune Fab Dose Determination on Ouabain

	N=	Inhibitor and Fab	[Inhibitor]	[DigiFab]	[Digibind]	% INH	GFAA AU	SD in AU
SET 1	3	OUABAIN	$1X10^{-6}$ M			83.6	0.164	0.109
		OUABAIN+DIGIFAB	$1X10^{-6}$ M	$1X10^{-6}$ M		89.1	0.109	0.12
		OUABAIN+DIGIBIND	$1X10^{-6}$ M		$1X10^{-6}$ M	67.7	0.393	0.118
SET 2	4	OUABAIN	$1X10^{-6}$ M			85.4	0.146	0.088
		OUABAIN+DIGIFAB	$1X10^{-6}$ M	$2X10^{-6}$ M		0.00	0.963	0.087
		OUABAIN+DIGIBIND	$1X10^{-6}$ M		$1X10^{-6}$ M	67.9	0.321	0.16
SET 3	2	OUABAIN	$1X10^{-6}$ M			84.3	0.157	0.0085
		OUABAIN+DIGIFAB	$1X10^{-6}$ M	$1.5X10^{-6}$ M		73.3	0.267	0.004
		OUABAIN+DIGIBIND	$1X10^{-6}$ M		$1X10^{-6}$ M	71.6	0.284	0.000001

Table 4-4 is a summary of experiments done to find equivalent disinhibiting performance of two digoxin immune Fab fragments against ouabain. It was found that 1.5 times the concentration of DigiFab was needed to equal that of Digibind.

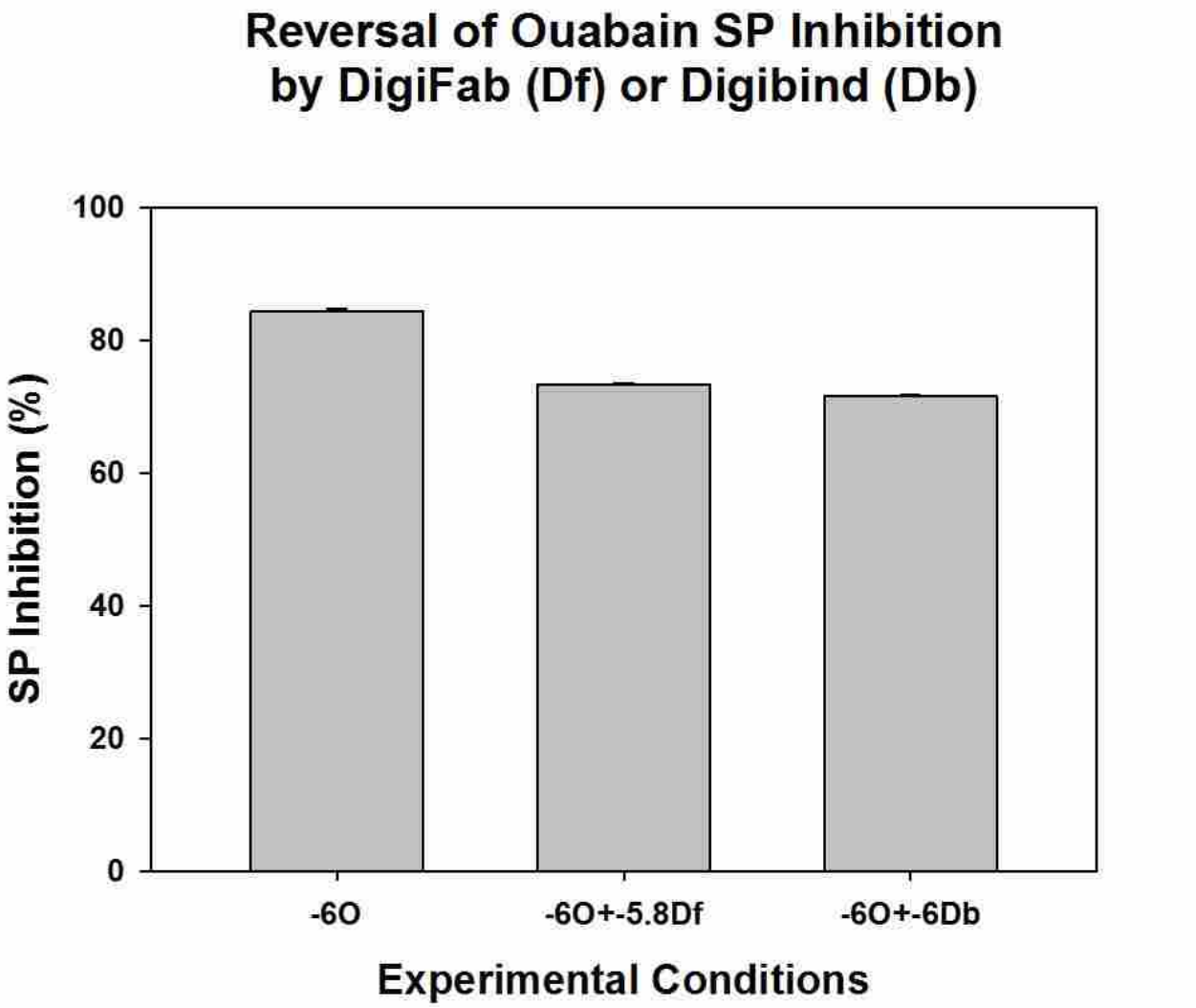


Figure 4-4. Reversal of Ouabain SP Inhibition by DigiFab and Digibind.
Effect of DigiFab (Df) or Digibind (Db) in reversing the inhibition of SP mediated Rb ion transport into fresh human red blood cells (RBC). A concentration of 1x10-6M ouabain (-6O) was used to produce inhibition and this combination was also assayed in the presence of 1.5x10-6M DigiFab (-5.8Df) or 1.0x10-6M Digibind (-6Db). The reversal was significant ($p<0.05$) and comparable for DigiFab and Digibind for these respective concentrations ($p>0.05$).

Proscillaridin A

TABLE 4-5: Dose Response of Proscillaridin A

CONCENTRATION OF INHIBITOR	PROSCILLARIDIN A N=3	
	GFAA AU	% INH
1X10^{-5} M	0.044±0.013	95.6
1X10^{-6} M	0.131±0.085	86.9
1X10^{-7} M	0.498±0.108	50.2

Table 4-5 is a summary of experiments to show dose dependency of Proscillaridin A against SP activity in RBCs.

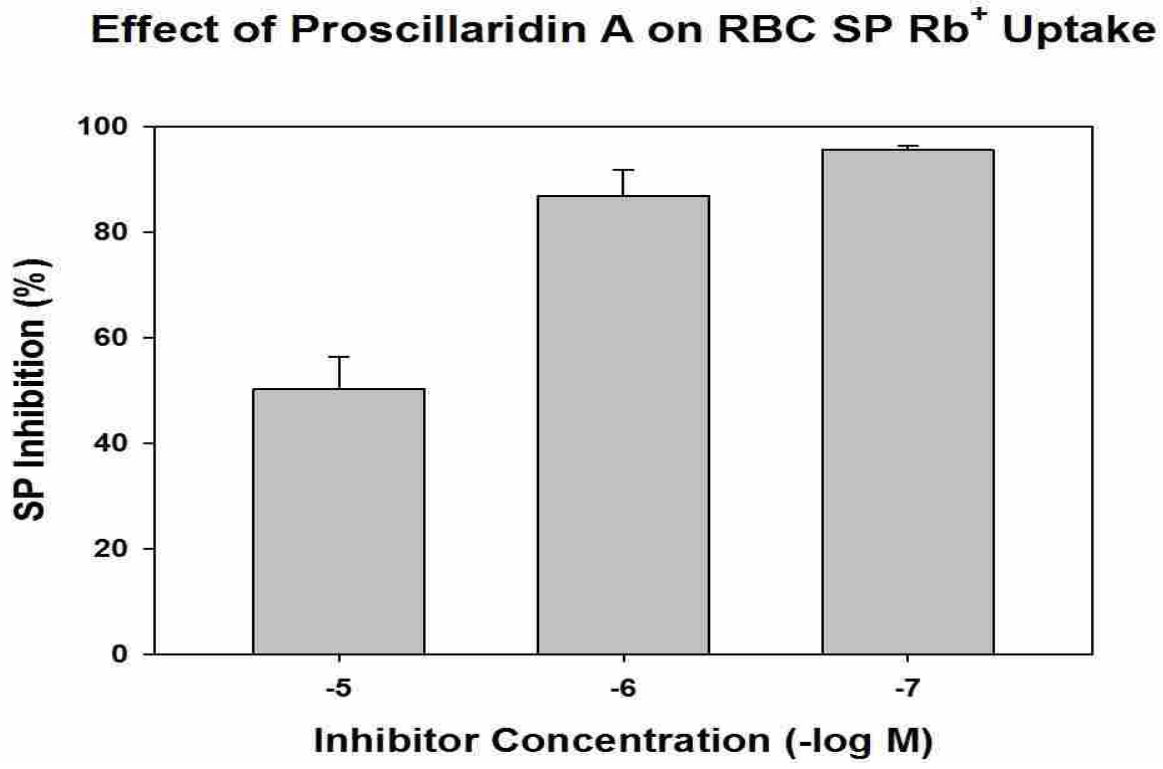


Figure 4-5. Effect of Proscillaridin A on RBC SP Rb+ Uptake.
The effect of graded concentrations of proscillaridin A (ProA) on SP mediated Rb ion transport into fresh human RBC. Proscillaridin A at 1 x 10-7M (-7 P), at 1 x 10-6M (-6 P) or 1 x 10-5M (-5 P) each showed a significant (ANOVA $p<0.01$, all post hoc pairwise comparisons $p<0.05$) and increased inhibition.

Proscillaridin A (ProA) has been proposed as a candidate for an endogenous SP inhibitor. In our studies, it appeared to have a higher binding affinity to the SP of RBCs than ouabain or digoxin and the required concentration was ~10 fold lower. Of the three concentrations tested ($1x10^{-5}$M, $1x10^{-6}$M, $1x10^{-7}$M), $1x10^{-7}$M produced inhibition closest to the 50% inhibition level sought (50.2%). See *Table 4-5* and *Fig. 4-5* above.

As done previously with ouabain and digoxin, we next investigated the ability of DigiFab and Digibind to bind to ProA and decrease inhibition of the SP in RBC(s). With the use of ProA at $1x10^{-7}$M we again found that ~1.5 times more DigiFab was needed to equal the SP inhibition reversing capabilities of Digibind. ProA at $1x10^{-7}$M showed ~75% SP inhibition alone, and when DigiFab was added at $1x10^{-7}$M the SP inhibition was reduced by ~12%. In contrast, when $1x10^{-7}$M Digibind was added, to the ProA, SP inhibition was reduced by ~30%.

Increasing DigiFab to $2x10^{-7}$M reduced the inhibition to 35.4% which again was slightly more than that of Digibind at the same concentration used previously ($1x10^{-7}$M). As with ouabain, adding 1.5 times the concentration of Digibind, DigiFab at $1.5x10^{-7}$M to the ProA RBC mixture, more closely matched the reduction in SP inhibition provided by $1x10^{-7}$M Digibind; 48.1% and 48.9% respectively. See Table 4-6 for a more detailed summary of these experiments. *Fig. 4-6* also shows a graph summarizing these experiments.

TABLE 4-6: Anti-digoxin Immune Fab Dose Determination on Proscillaridin A

	N=	Inhibitor and Fab	[Inhibitor]	[DigiFab]	[Digibind]	% INH	GFAA AU	SD in AU
SET 1	3	PROSCILLARIDIN A	$1X10^{-7}$ M			72.6	0.274	0.0684
		PRO+DIGIFAB	$1X10^{-7}$ M	$1X10^{-7}$ M		57.2	0.428	0.360
		PRO+DIGIBIND	$1X10^{-7}$ M		$1X10^{-7}$ M	39.5	0.605	0.280
SET 2	2	PROSCILLARIDIN A	$1X10^{-7}$ M			76.6	0.234	0.0410
		PRO+DIGIFAB	$1X10^{-7}$ M	$2X10^{-7}$ M		35.4	0.646	0.188
		PRO+DIGIBIND	$1X10^{-7}$ M		$1X10^{-7}$ M	43.7	0.563	0.207
SET 3	6	PROSCILLARIDIN A	$1X10^{-7}$ M			75.6	0.244	0.128
		PRO+DIGIFAB	$1X10^{-7}$ M	$1.5X10^{-7}$ M		48.1	0.516	0.275
		PRO+DIGIBIND	$1X10^{-7}$ M		$1X10^{-7}$ M	48.7	0.513	0.270

Table 4-6 is a summary of experiments done to find equivalent disinhibiting performance of two digoxin immune Fab fragments against proscillaridin A. It was found that 1.5 times the concentration of DigiFab was needed to equal that of Digibind.

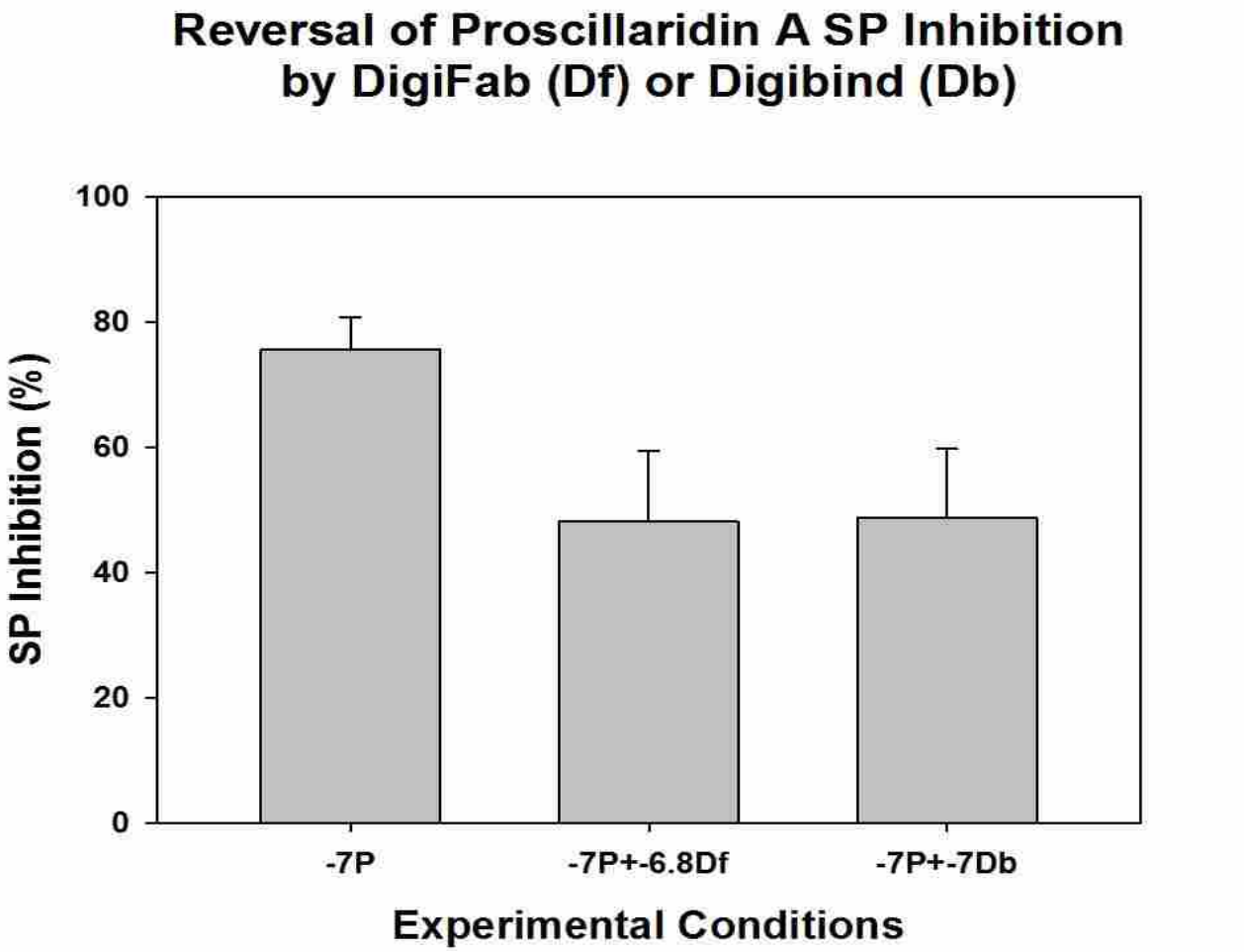


Figure 4-6. Reversal of Proscillaridin A SP Inhibition by DibiFab or Digibind.
Effect of DigiFab (Df) or Digibind (Db) to reverse the inhibition of SP mediated Rb ion transport into fresh human red blood cells (RBC). A concentration of 1 x 10-7M proscillaridin A (-7P) was used to produce inhibition and this combination was also assayed in the presence of 1.5 x 10-7M DigiFab (-6.8Df) or 1.0 x 10-7M Digibind (-7Db). The reversal was significant ($p<0.05$) and comparable for DigiFab and Digibind for these respective concentrations ($p>0.05$).

Bufalin

Bufalin and related compounds from similar sources have been proposed as EDLFs. Four experiments were done to determine the most sensitive concentration of bufalin or the concentration that most closely achieved ~50% inhibition of the SP in RBCs. Of the 4 concentrations tested ($1x10^{-5}$M, $1x10^{-6}$M, $1x10^{-7}$M, $1x10^{-8}$M), $1x10^{-6}$M seemed to provide the most sensitive concentration although the inhibition provided was higher than the normally desired 50% SP inhibition. Table 4-7 shows the data of the SP activity provided when bufalin was added in three different concentrations as an inhibitor. *Fig. 4-7* shows this information in bar graph format as was shown previously for the other known candidates of EDLF.

TABLE 4-7: Dose Response of Bufalin

CONCENTRATION OF INHIBITOR	BUFALIN N=4	
	GFAA AU	% INH
$1X10^{-5}$ M	0.020±0.017	98.0
$1X10^{-6}$ M	0.161±0.154	83.9
$1X10^{-7}$ M	0.898±0.128	11.2

Table 4-7 is a summary of the experiments done to determine dose response of SP in RBCs to bufalin. Concentrations of 1x10-6M showed closest inhibition to 50% even though it was much higher than 50% this was the concentration of bufalin chosen in further assays of bufalin to investigate the concentration of digoxin immune Fab fragments to reverse inhibition caused by bufalin.

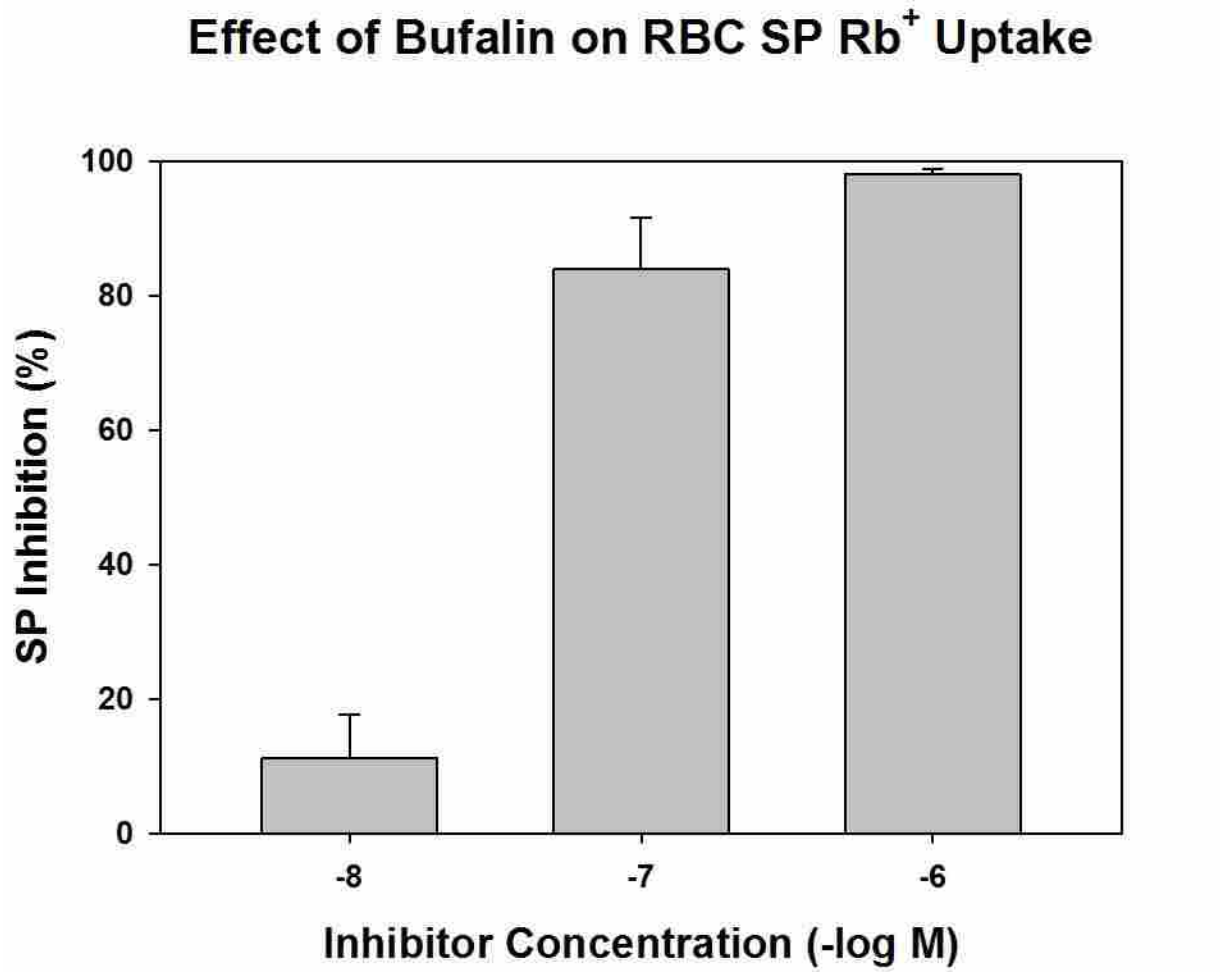


Figure 4-7. Effect of Bufalin on RBC SP Rb^+ Uptake.
The effect of graded concentrations of bufalin (B) on SP mediated Rb ion transport into fresh human red blood cells (RBC). Bufalin at 1 x 10-8M (-8 B), 1 x 10-7M (-7 B), at 1 x 10-6M (-6 B) each showed a significant (ANOVA $p<0.01$, all post hoc pairwise comparisons $p<0.05$, and increased inhibition.

A substantial inhibition of the SP was found when $1x10^{-7}$M bufalin was added to the RBCs showing an increased sensitivity of the RBCs SP to bufalin compared to digoxin. Previously we saw this increased sensitivity also with ProA. For the next step, this concentration of bufalin was added to the treated washed RBC mixture in addition to the two Fabs. The same pattern of experiments was carried out to determine at what concentration of the two Fabs are most similar in their efficiency of reversing the SP inhibition produced by bufalin.

TABLE 4-8: Anti-digoxin Immune Fab Dose Determination on Bufalin

	N=	Inhibitor and Fab	[Inhibitor]	[DigiFab]	[Digibind]	% INH	GFAA AU	SD in AU
SET 1	3	BUFALIN	$1X10^{-7}$ M			77.6	0.224	0.213
		BUFALIN+DIGIFAB	$1X10^{-7}$ M	$1X10^{-7}$ M		65.5	0.345	0.015
		BUFALIN+DIGIBIND	$1X10^{-7}$ M		$1X10^{-7}$ M	0.0	1.066	0.041
SET 2	4	BUFALIN	$1X10^{-7}$ M			62.0	0.380	0.192
		BUFALIN+DIGIFAB	$1X10^{-7}$ M	$1.5X10^{-7}$ M		0.0	1.203	0.432
		BUFALIN+DIGIBIND	$1X10^{-7}$ M		$1X10^{-6}$ M	0.0	1.012	0.295
SET 3	2	BUFALIN	$1X10^{-7}$ M			64.9	0.351	0.076
		BUFALIN+DIGIFAB	$1X10^{-7}$ M	$2X10^{-7}$ M		2.5	0.975	0.013
		BUFALIN+DIGIBIND	$1X10^{-7}$ M		$1X10^{-7}$ M	16.0	0.840	0.079

Table 4-8 is a summary showing data of the three sets of experiments used to determine at what concentration the two Fabs are most similar in reversing the SP inhibition caused by bufalin. Concentrations of Digibind at 1x10-7M and DigiFab at 1.5x10-7M were found to be most similar.

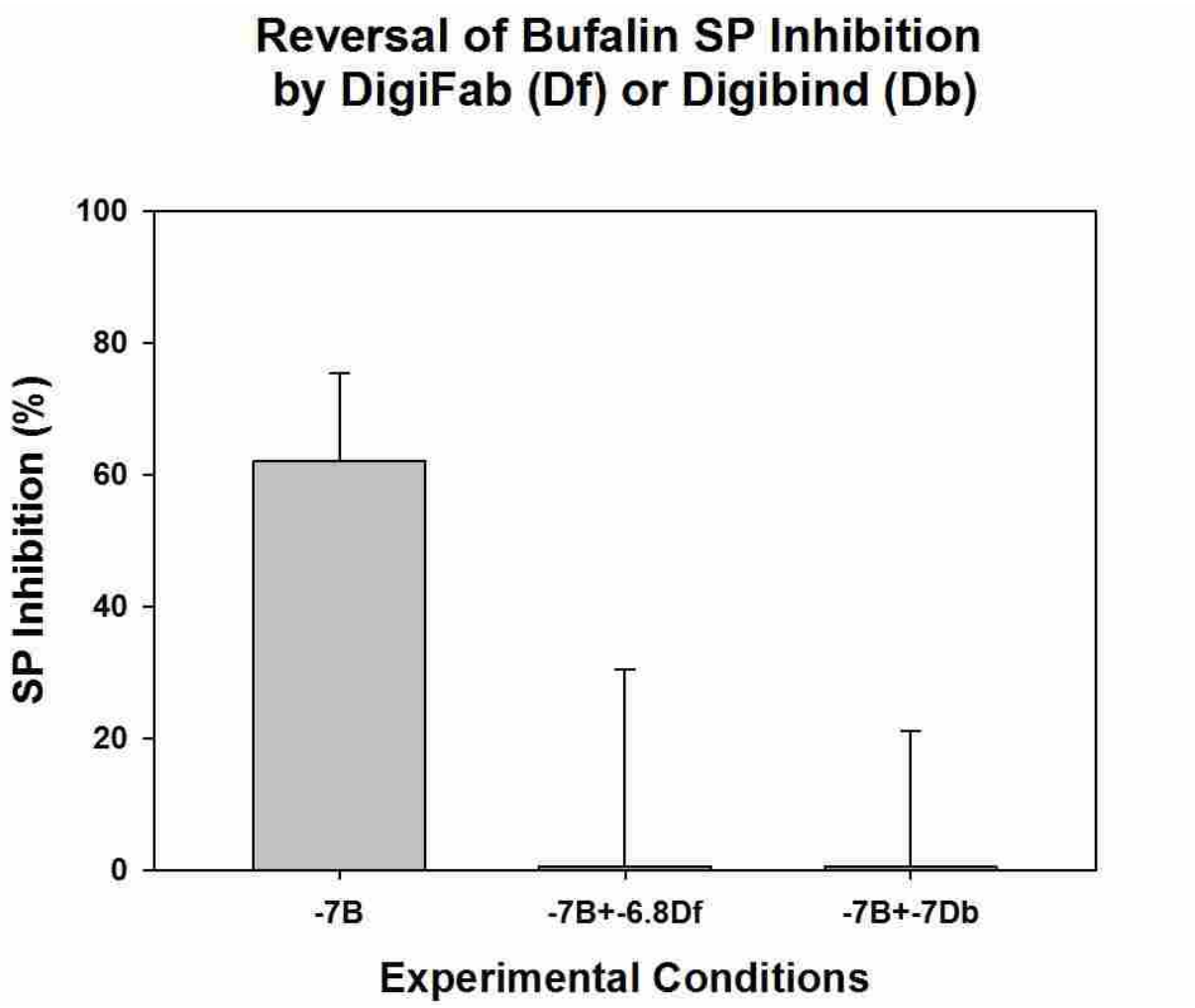


Figure 4-8. Reversal of Bufalin SP inhibition by DigiFab or Digibind.
Effect of DigiFab (Df) or Digibind (Db) to reverse the inhibition of SP mediated Rb ion transport into fresh human red blood cells (RBC). A concentration of 1 x 10^{-7}M bufalin (-7 B) was used to produce inhibition and this concentration was also assayed in the presence of 1.5 x 10^{-7}M DigiFab (-6.8Df) or 1.0 x 10^{-7}M Digibind (-7Db). The reversal was significant ($p<0.05$) and comparable for DigiFab and Digibind for these respective concentrations ($p>0.05$).

The results for bufalin showed that when concentrations of 1x10^{-7}M bufalin were present, 1.5 times as much DigiFab was needed to equal the reversing qualities of Digibind. DigiFab at 1.5x10^{-7}M concentration and 1x10^{-7}M Digibind both showed ~0.00% inhibition.

Serum from Women with Preeclampsia

In these experiments we assessed the ability of Digibind of DigiFab to reverse the inhibition of the RBC SP caused by serum from PE women. We began with concentrations of the digoxin antibody Fab fragments used in the experiments assessing known inhibitors of the SP which are also putative serum EDLFs in PE or in essential hypertension as described above. For some specimens inhibition was completely reversed by either DigiFab or Digibind precluding a differential comparison. Consequently, a somewhat lower final concentration DigiFab or Digibind in the 10^{-7}M range was used for all subsequent studies.

In the first set of studies equimolar DigiFab or Digibind were used and their ability to reverse RBC SP inhibition brought about by PE serum was studied. The results demonstrated substantial reversal by the Digibind with no observable reversal brought about by DigiFab. This is shown in *Fig. 4-9.*

In the second set of studies, sera from PE women were tested in the absence and presence of 1.5 x 10^{-7}M DigiFab and compared with sera treated with 1.0 x 10^{-7}M Digibind. In this case as before, the Digibind was able to reverse most of the SP inhibition brought about by PE serum. The DigiFab also brought about some reversal of inhibition but not as much as with this concentration of Digibind.

In the final set of experiments the concentration of DigiFab was increased to 2.0 x 10^{-7}M. Again serum samples from women with PE were assayed for their ability to inhibit human RBC Rb^{+} uptake. Inhibition of RBC SP mediated Rb uptake was also measured with the PE sera in the presence of either Digibind 1 x 10^{-7}M or DigiFab 2 x 10^{-7}M. In this case the DigiFab caused a significant reversal of inhibition that was as much or slightly greater than that achieved with this concentration of Digibind.

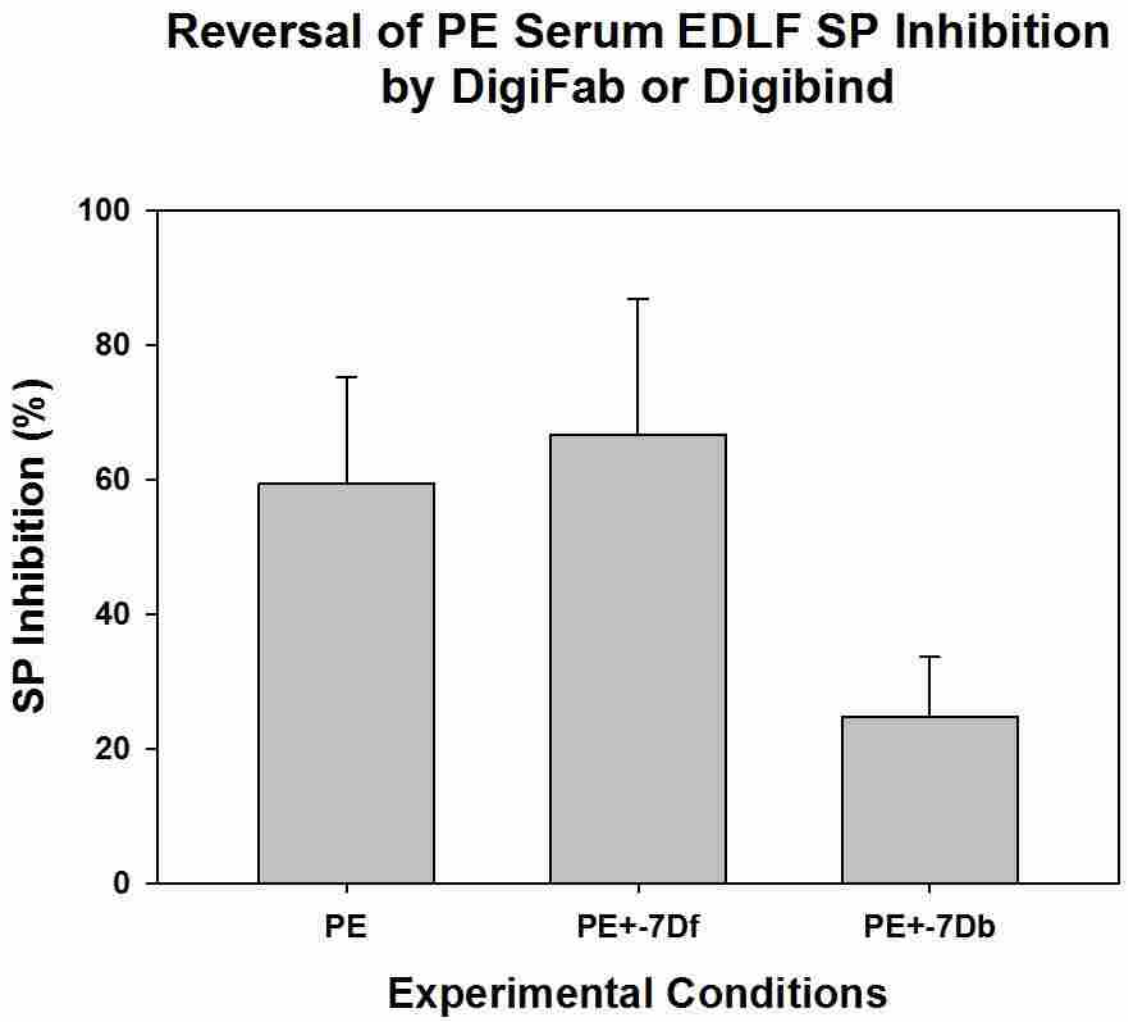


Figure 4-9. Reversal of PE Serum EDLF SP Inhibition by DigiFab or Digibind.
Effect of DigiFab (Df) or Digibind (Db) to reverse the inhibition of SP mediated Rb ion transport into fresh human red blood cells (RBC). Serum from uncomplicated pregnancies was used as the control (CTL) and represented no inhibition, 100% activity. A concentration of 1 x 10^{-3}M ouabain was used to produce 100% inhibition or no activity (-3 Oua). Then previously screened sera from women with PE were assayed (N=9), as were these same sera in the presence of 1.0 x 10^{-7}M DigiFab (-7Df) or 1.0 x 10^{-7}M Digibind (-7Db) (Also N=9). The reversal was significant ($p<0.05$) for Digibind but DigiFab produced no appreciable reversal ($p>0.05$ vs PE serum alone).

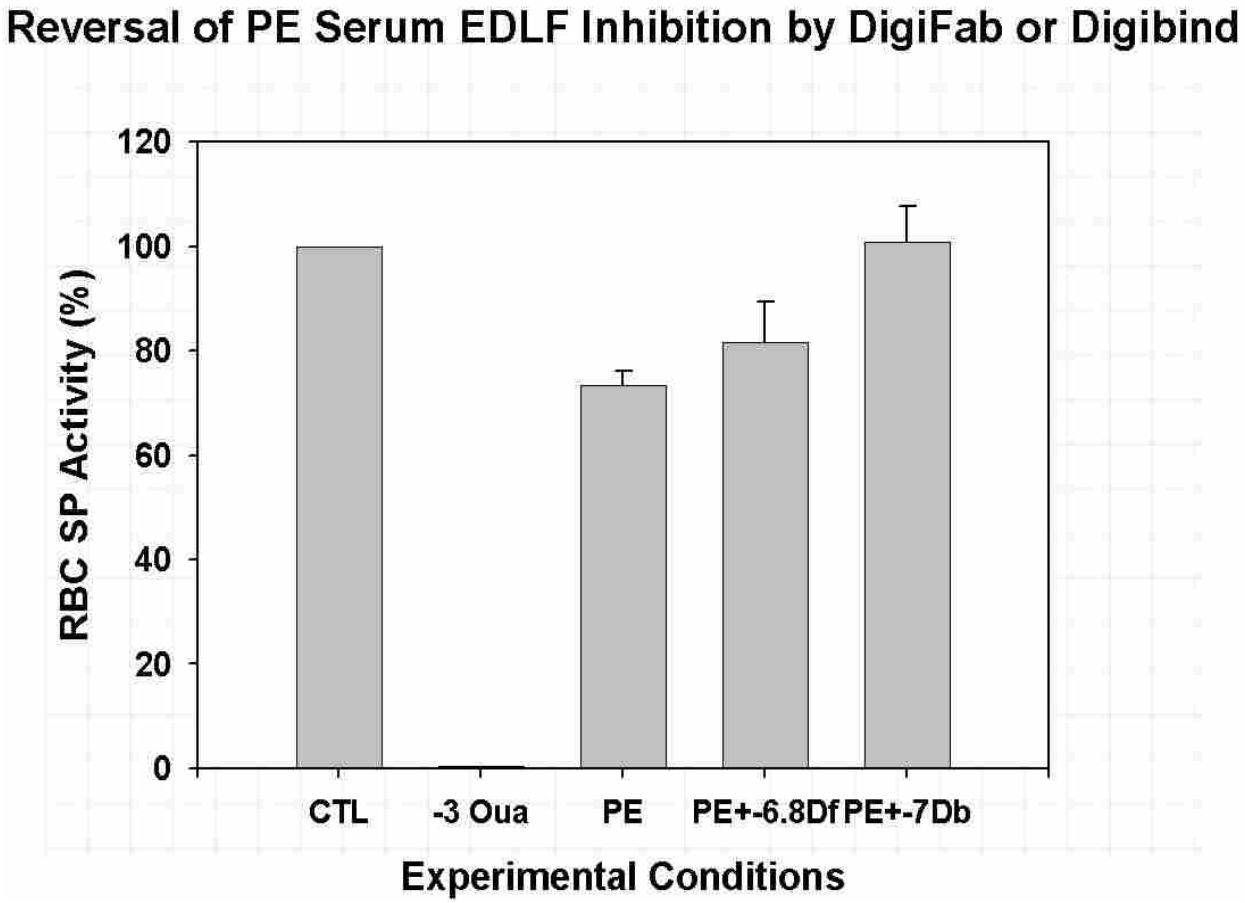


Figure 4-10. Reversal of PE Serum EDLF inhibition by DigiFab or Digibind.
Effect of DigiFab (Df) or Digibind (Db) to reverse the inhibition of SP mediated Rb ion transport into fresh human red blood cells (RBC). Serum from uncomplicated pregnancies was used as the control (CTL) and represented no inhibition, 100% activity. A concentration of 1 x 10^{-3}M ouabain was used to produce 100% inhibition or no activity (-3 Oua). Then previously screened sera from women with PE (PE) were assayed (N=12), as were these same sera in the presence of 1.5 x 10-^{7}M DigiFab (-6.8Df) or 1.0 x 10^{-7}M Digibind (-7Db)(Also N=12). The reversal was significant ($p<0.05$) for Digibind and DigiFab, but Digibind produced significantly more reversal ($p<0.05$ vs Df).

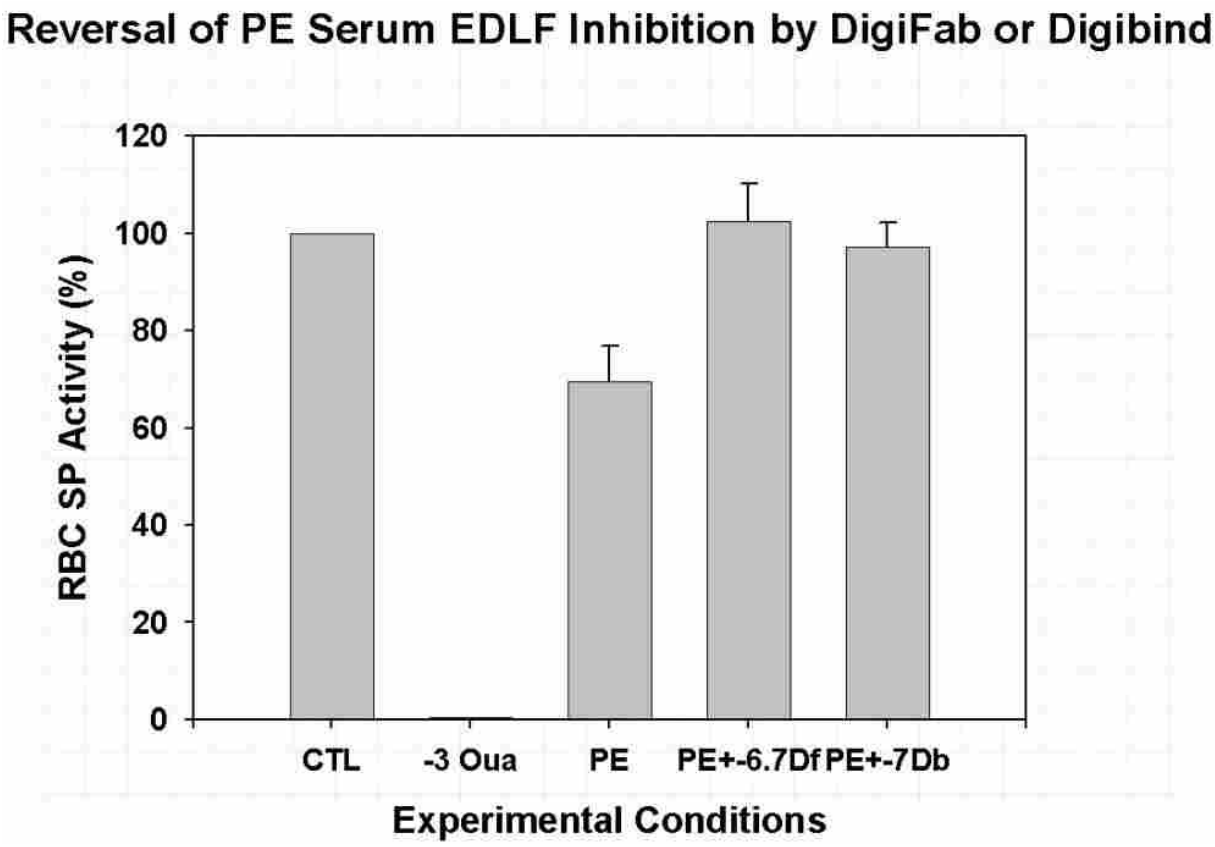


Figure 4-11. Reversal of PE serum EDLF inhibiton by DigiFab or Digibind.
Effect of DigiFab (Df) or Digibind (Db) to reverse the inhibition of SP mediated Rb ion transport into fresh human red blood cells (RBC). Serum from uncomplicated pregnancies was used as the control (CTL) and represented no inhibition, 100% activity. A concentration of 1 x 10^{-3}M ouabain was used to produce 100% inhibition or no activity (-3 Oua). Then previously screened sera from women with PE (PE) were assayed (N=9), as were these same sera in the presence of 2.0 x 10^{-7}M DigiFab (-6.7Df) or 1.0 x 10^{-7}M Digibind (-7Db) (Also N=9). The reversal was significant ($p<0.05$) for Digibind and DigiFab but the extent of reversal was now comparable ($p>0.05$) for both DigiFab and Digibind.

CONCLUSIONS

Digibind and DigiFab were both capable of binding several known inhibitors of the sodium pump. Some evidence suggests that these inhibitors are present in human serum and may represent the EDLFs detected in both essential hypertension and preeclampsia. In general, about one and a half times more DigiFab was required to produce an effect comparable to Digibind and reverse the effects of these factors' inhibition. When serum from women with

established preeclampsia having demonstrable ELDF activity was used, in general, about 1.5 to 2.0 times the concentration of DigiFab was required to produce an effect comparable to Digibind. This demonstrates that DigiFab is an effective therapeutic similar to Digibind in its range of usefulness, but likely requiring somewhat more to be completely effective in treating PE women.

Previously, Digibind had been shown to be an effective agent in binding and reversing the effects of EDLFs. These studies confirmed and extend this finding whether for individual known candidates for EDLF or the EDLF of PE found in serum from women with active disease. In these studies a second commercially available digoxin antibody Fab fragment, DigiFab was evaluated as a potential therapeutic agent.

Also of significance, these studies also indicate, the existence, not only of a SP inhibitor within the serum of women with PE, but that the serum of women with PE contain an inhibitor to the SP that shares the basic structure of several known inhibitors and that the inhibitor binds to both digitalis immune Fab fragments, Digibind and DigiFab. This has implications for treatment of women with PE and also use of these Fab fragments as EDLF specific research tools.

CHAPTER 5: ISOLATION OF THE EDLFS OF PE USING DIGIBIND AFFINITY

INTRODUCTION

Digibind, an anti-digoxin immune Fab, has shown strong binding affinity for not only a broad range of known SP inhibitors (some suggested as EDLFs), but also the EDLFs specific to PE (see previous Chapters). In *Chapter 2* and *3* we show that EDLFs were increased in most women with PE. Since the mechanism of PE remains elusive, this rise in EDLF could prove to be revelatory in determining the mechanism underlying complications of PE or provide a treatment for PE. It has also been shown in this work that Digibind has a strong binding affinity for the EDLFs in PE, so much so that SP inhibition was reversed and symptoms of PE were diminished with Digibind treatment as summaryized in Chapter 3. It was our hypothesis that Digibind can be used as an affinity material to aid in the isolation and purification of the EDLF found in PE. In this chapter, we use the knowledge that Digibind has a known molecular weight of close to 50,000 Daltons and a selective ultrafiltration membrane with a 30,000 Dalton cut off to isolate the EDLF estimated to have molecular weights around 400-700 Daltons.

MATERIALS AND METHODS

Placenta Homogenization

Placentas from women with PE produced the highest percent SP inhibition and were chosen and homogenized as the source for the EDLF to be isolated. The homogenization followed the same protocol as was previously detailed in Chapter 2, but for the readers convenience it has been included here.

Frozen placenta (~1 g) was shaved into flakes using a surgical blade. The frozen flakes were then placed in a stainless steel ball mill cylinder along with 10 to 15 steel balls (Mikro-

dismembrator, Sortorius Stedim, Bohemia, NY) and the entire cylinder, including placenta tissue contents, was submerged in liquid N_2 for 10 min. After thorough freezing of the cylinder, the cylinder was immediately fitted into the Mikro-dismembrator ball mill and set to shake at the maximum setting for 10 minutes. The process of submersion and shaking was repeated at least twice times or until the contents became a well homogenized mixture. The tissue homogenate was removed from the cylinder by adding 1 ml of deionized H_2O to the ball mill to solvent the homogenate, removed by pipette, re-aliquotted in a 50 ml conical tube, and repeated sequentially 5 times. The total of 5 ml reconstituted homogenate could then be stored in freezer until further processing.

To remove large and abundant proteins, the homogenate was treated further with MeOH. Two volumes (10 ml) of HPLC grade MeOH were added gradually in 500 µl increments to the homogenate over 5 min while intermittently vortexing the conical tube. After adding all 10 ml of MeOH, the conical tube containing the MeOH-treated homogenate was centrifuged for 10 min at 2000*x*g at 4ºC. The supernatant was collected and the precipitate discarded. The supernatant was then dried down *in vacuo* to completely remove the MeOH and then resuspended to original volume (5ml) using deionized H_2O. This protein-depleted placenta homogenate was then stored at -80ºC until further processing and assay.

Ultrafiltration

After, depleting the placental homogenate of proteins, the homogenate was passed through an ultrafiltration filter membrane, Amicon® Ultra, Ultracel-30K specifically designed by Millipore (Carrigtwohill, Co. Cork, Ireland) to allow only those molecular species smaller than 30k Daltons to pass through. These filtration devices hold 4 ml of homogenate each. The 4 ml of placental homogenate is transferred to the top chamber of the filter and placed in a fixed angle rotor and centrifuged at 2000*x*g for 10 minutes.

The filtrate (~4ml) was then incubated with 100 µl of $1x10^{-2}$M Digibind, an amount sufficient to bind any and all EDLF present (in the now filtered, protein-depleted placental homogenate) for one hour at 37ºC in a shaker incubator (Boekel Scientific Model 136400, Feasterville, PA) set to a medium speed. Following the incubation, the EDLF-Digibind complex containing solution was passed through a new Ultracel-30K filter. This time the EDLF-Digibind complex was centrifuged for only 1 min or until the centrifuge reached full speed and then the centrifuge was stopped and allowed to deaccelerate. This allowed smaller uncomplexed compounds to pass through the filter and the EDLF-Digibind complex to remain in the retentate. About 2 ml of retentate were then reserved and treated with 392 µl of 0.2M glycine (Sigma, St.Louis, MO) (pH 3.0) to release the bound EDLF from the Digibind.

The glycine treated placental homogenate was then passed through the Ultracel-30K filter a final time by centrifuging at 2000*x*g for 2 min. In this final filtration step the Digibind remained in the retentate and the EDLF was essentially purified from other endogenous compounds. The pH of the ending filtrate was neutralized by adding ~18 µl of 1 M Tris free base (Fisher Scientific, Fair Lawn, NJ).

HPLC Separation

Prior to this filtration process an HPLC method was developed that effectively separated known candidate EDLFs: ouabain, strophanthidin and bufalin. The linear gradient is shown in Table 5-1 and goes from 100% H_2O to greater organic acetonitrile solvent and eventually elutes the 10 µm

Table 5-1. HPLC SP Inhibitor Gradient

Time(Min)	% H20	% ACN	Curve
0	100.0	0.0	
10	95.0	5.0	Linear
34	80.0	20.0	Linear
45	15.0	85.0	Linear
47	0.0	100.0	Linear
52	100.0	0.0	Immediate

C-18 column with a 100% ACN solvent for 5 minutes. The whole gradient takes 51 minutes. With this established HPLC program, ouabain comes off the column at ~14±1 min, strophanthidin at 24±1 min, and bufalin at 26±1 min. See *Fig. 5-1*.

The purified EDLF (with added glycine and Tris) isolated from the placenta of women with PE was injected onto the HPLC column and eluted using the same gradient that separated the known steroid candidates in *Fig. 5-1*. The Tris and glycine are both very polar and came off in the first 5 minutes in the void volume while the EDLF(s) were expected to elute substantially later in the gradient. Fractions were collected every 1 minute in Eppendorf tubes and then taken to dryness *in vacuo* and eventually reconstituted in 500 µl of deionized H_2O. HPLC fractions from 5 min through 30 min were evaluated for EDLFs found in PE placenta homogenates compared with the elution times of known SP inhibitors. Additional runs were added to the same Eppendorf tubes with the same time fractions as needed be to increase the concentration of the EDLFs.

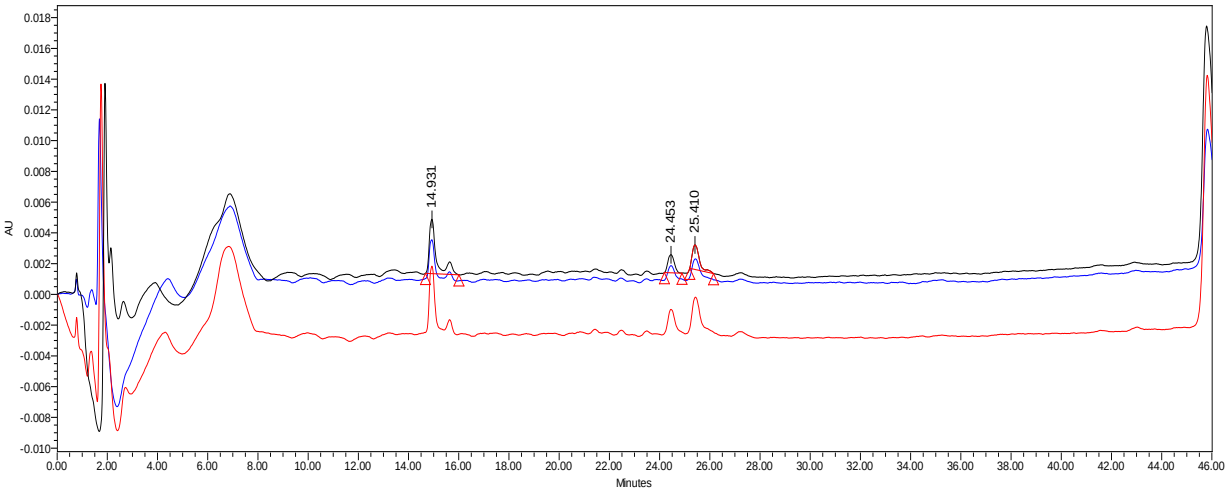


Figure 5-1. Known Inhibitors HPLC Separation.
An overlay of three HPLC runs of three candidate EDLF's, ouabain, strophanthidin, and bufalin. Ouabain is represented by the peak at 11.931 min, strophanthidin at 21.153 min and bufalin at 25.110 min.

Radioactive Immunoassay Using Digibind to Determine EDLF Activity of HPLC Fractions

HPLC fractions 5 through 30 (min) were analyzed for evidence of EDLF using a radioactive immunoassay (RIA) that used Digibind as the primary antibody. The RIA measures the concentration of EDLF present by its competing with ^{3}H-ouabain for binding to Digibind. An Eppendorf tube for each HPLC fraction and for the unlabeled ouabain standards needed to produce a reliable concentration reference curve was prepared. Each Eppendorf tube contained 10 µl of 10mM Tris buffer (pH 7.45), 100 µl deionized H_2O, or HPLC fraction sample with unknown concentrations of EDLF, 50 µl 3H 2.2 x 10^{-8}M ouabain, 300 µl 1.8µg/ml Digibind, and 60 µl of (1:50) secondary antibody (rabbit anti-sheep IgG Fab fragment (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The mixture was incubated overnight at room temperature, and then polyethylene glycol (16%) (PEG)-6000, (Calbiochem, LaJolla, CA) was added to each tube to precipitate out the antibody-Fab-antigen complex, the supernatant was discarded and the pellet resuspended in 500 µl phosphate buffer. The whole solution was added to 4 ml of Ecoscint™ A scintillation cocktail (National Diagnostics, Atlanta, GA) and radioactivity measured. The Perkin Elmer Liquid Scintilation Analyzer Tri-Carb 2910 TR systematically counts each 4 ml vial of scintillation cocktail containing EDLF sample or 3H ouabain for one minute and then reports the counts per min per each vial. An increasing concentration of EDLF found in the HPLC fractions corresponds directly to the lowering of radioactive counts in a given solution. Concentration can be determined from the standard curve created by varying amounts of dH_2O and cold 2.2 x 10^{-8}M ouabain equaling 100 µl.

RESULTS

Ultrafiltration

In order to rule out that the role of glycine or tris on the RIA assay and the concentration of EDLF reported, the effects of glycine (pH 7) and tris (pH 7) were tested in the RIA. No effect was observed. A RIA assay was performed after ultrafiltration in a dose dependent manner. Purified EDLF was added in increments of 10 µl, 30 µl, and 100 µl and the results, pictured in *Fig. 5-2*, showed an increase in relative EDLF concentration. If either the tris or glycine had an effect on the RIA assay, the opposite result would have been evident.

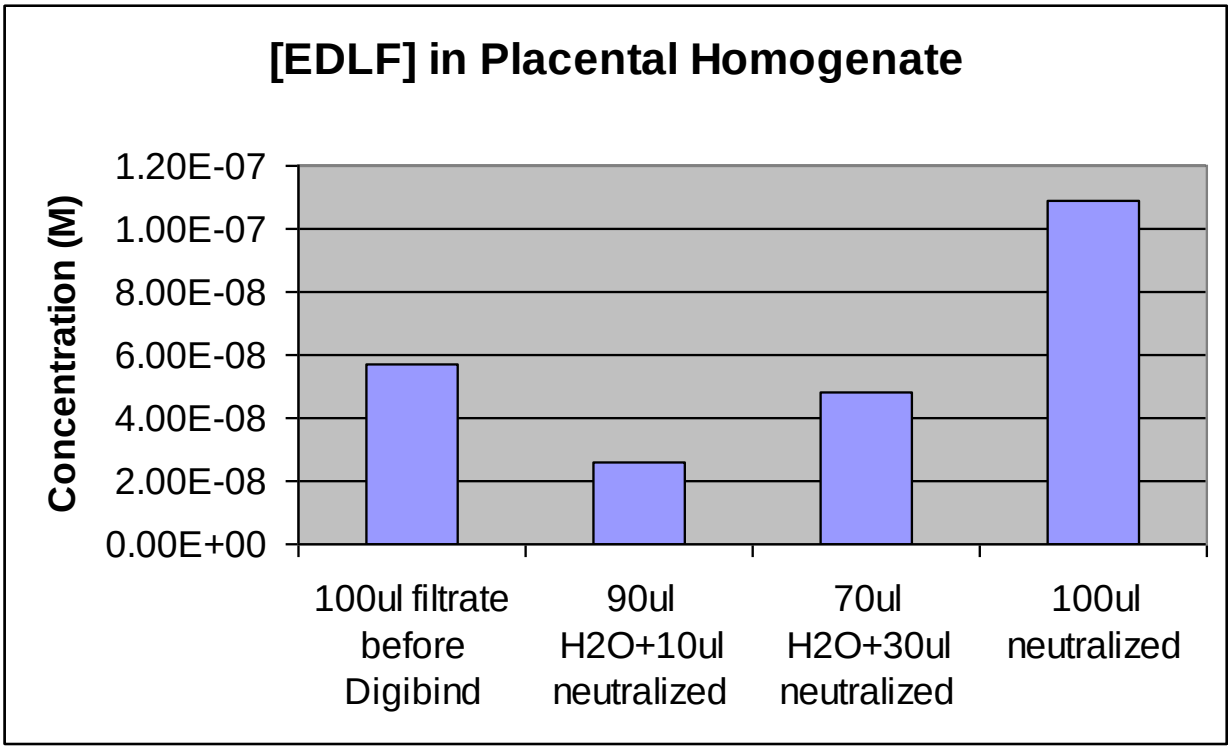


Figure 5-2. RIA Relative EDLF Concentration Dose Dependency.
This figure shows the results of a RIA assay where dose dependency of EDLF purification and ultrafiltration is evident. The results are measured in relative EDLF concentration as compared to ouabain concentration. The first column represents a control where the original placental homogenate is added to the RIA. Subsequent columns represent 10 µl, 30 µl, 100 µl additions of purified EDLF are added to the RIA. Increased aliquots of purified EDLF show increased concentration as measured by RIA using Digibind as the secondary antibody.

The ultrafiltration process using Digibind as an affinity material was successful in binding the EDLF found in placenta from women with PE. *Fig. 5-3* shows that before the placental homogenate went through the filtration process, there was EDLF activity with an apparent concentration of ~$1.4x10^{-7}$M. When Digibind was incubated with the placental homogenate and then bound to the EDLF, evidence that the filtration process removed the EDLF is illustrated in *Fig. 5-3*. The figure shows that after a reduction in relative EDLF concentration when EDLF was bound to Digibind, then the concentration of EDLF returned again after the release and removal of EDLF from the Digibind. This demonstrates that the Digibind remained in the retentate and the EDLF was released from the Digibind-EDLF complex.

The figure also demonstrates that not all the EDLF was bound by the Digibind visualized by the EDLF concentration in the second column of *Fig 5-3*. It is probably that a higher Digibind concentration can be used to bind more EDLF. This experiment was actually performed and the results can be seen in *Fig. 5-4*. In the first mentioned figure the EDLF activity did return to its original concentration after the interruption of the Digibind-EDLF complex release and filtration of the released EDLF. In fact in *Fig. 5-3* we see an apparent increase of EDLF concentration. This is due to a final volume half of the original volume that doubling the EDLF concentration in the retentate. The final filtrate is not only substantially more pure but also more concentrated than the original unpurified placental homogenate. This is a benefit to the isolation process and the next HPLC step to yield a more concentrated sample.

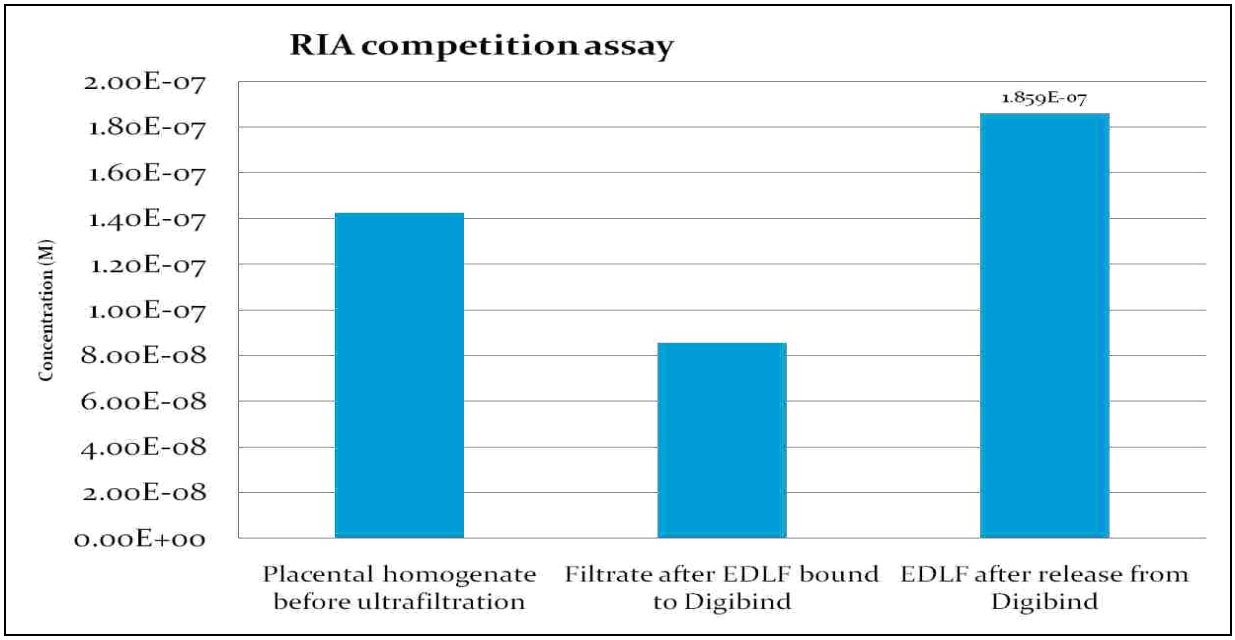


Figure 5-3. RIA Results Showing Effectiveness of Ultrafiltration Process.
The far left column shows the concentration of EDLF measured before any ultrafiltration had been performed and represents the protein-depleted placental homogenate. The middle column represents the EDLF concentration after the first pass through the filter and after the incubation with Digibind. The Digibind-EDLF complex is in the retentate, and so this column shows what EDLF is not bound by Digibind and ends up in the filtrate after ultracentrifugation. The far right column represents the finished product after glycine is added to release Digibind bound EDLF and allowed to pass into the filtrate. This solution is pH neutralized before the RIA assay performed and represents a more concentrated final solution. The Digibind-affinity ultrafiltration process proved successful.

Fig. 5-4 shows the results of presentation of an excess of Digibind ($1x10^{-2}$M) to the placental homogenate allowing most of the EDLF to bind. The majority of the EDLF did bind to Digibind and remained in the retentate, but after glycine was added to the Digibind-EDLF complex, the EDLF is released while Digibind is retained by the 30K membrane. Some EDLF remained in the retentate with the Digibind.

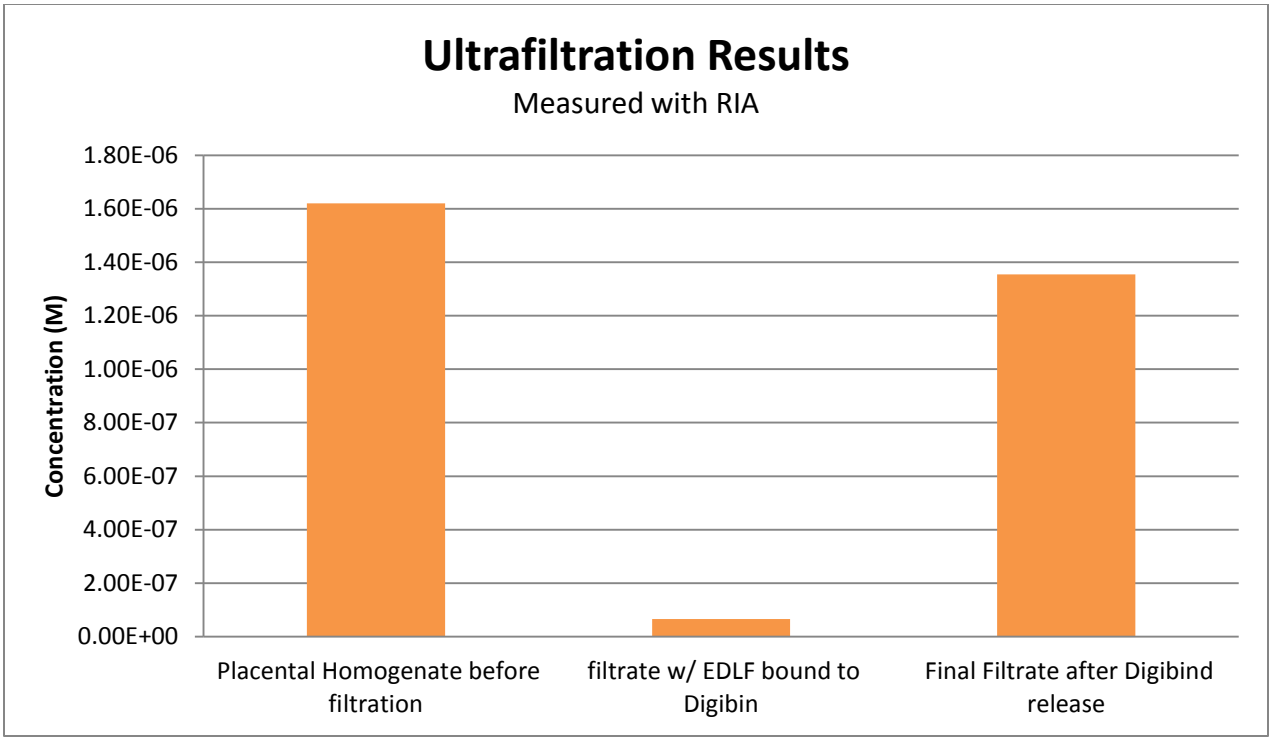


Figure 5-4. RIA Shows Effective Concentration of EDLF.

RIA results showing effectiveness of assay using 1x10-2M Digibind in excess. As in Figure 5-2 the columns represent the concentration of EDLF found in each sample. The furthest left column is the EDLF concentration before any filtration or incubation with Digibind. The middle column shows more EDLF bound to the higher concentration (1x10-2M) of Digibind and a decrease in the amount passed through the filter. In the right column the EDLF concentration represents after EDLF is released from Digibind. The lower concentration exists due to the extra Digibind blocking the filtration membrane and more EDLF remains in the retentate here.

HPLC Separation of EDLFs in Placental Homogenates

Processing the affinity-purified EDLF solution to the HPLC was effective in resolving EDLFs to determine how many EDLFs there may have been in the post ultrafiltration solution as well as preparing individual EDLFs for possible later identification. If the EDLFs present behaved chromatographically like known candidates already characterized using the same HPLC gradient program, then this would strongly point to the EDLF being the known inhibitor. In *Fig. 5-5* three different EDLF purifications were applied to the HPLC in 75 µl aliquots, allowed to run through the HPLC solvent gradient, eluate collected in 1 minute intervals into Eppendorf tubes, dried down *in vacuo*, and then the same process repeated collecting into the same Eppendorf tubes, allowing a more concentrated sample to be produced until all purified placental homogenate had been applied to the HPLC (~ 4 ml). A single RIA assay was then done for each affinity purified EDLF solution. The fourth and largest chart shows the data from these 3 purifications compiled to show the evidence of EDLF activity in individual HPLC fractions.

The results from the HPLC RIA assays do show more than 1 EDLF which is a separation based on the polarity of the compounds. In each run we see increased EDLF activity in fractions collected around 15 minutes which corresponds closely to our ouabain standard peak shown in *Fig. 5-1*. We also see increased activity in our fractions from around 24 minutes which could also correspond somewhat to bufalin, though perhaps 1-2 min before, but close to the strophanthidin peak in *Fig. 5-1*. Strophanthidin has never been proposed as an EDLF, but other close analogues of bufalin have. This is not conclusive evidence that the EDLFs in placental homogenates from PE are these two known EDLFs but it does lead us to believe that we can detect actual EDLFs present in placental homogenate found in women with PE after purification.

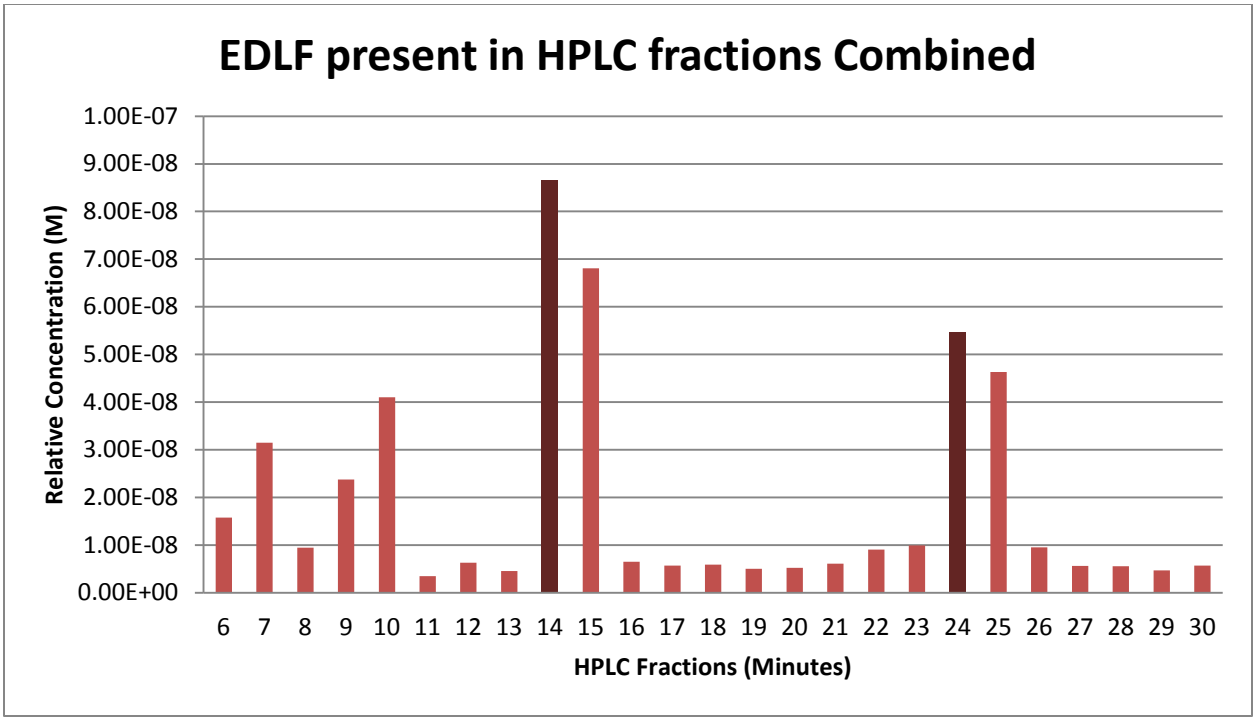


Figure 5-5. EDLF found in HPLC fractions with RIA.
Graphs show the EDLF activity evident in each fraction collected from the HPLC in which 75 µl of purified EDLF from placental homogenates was added sequentially to HPLC gradient method, dried down in vacuo, and reconstituted in ionized H_2O. Graph is the compiliation of three separate RIA assay results which combines 10 HPLC fractions into each numbered sample. Numbered fractions represent the minute that the HPLC fraction was collected from the HPLC. Void volume of HPLC has been removed from this figure. Relative concentration is measured by a standard curve using ouabain as the EDLF. Strong presence of EDLF is shown in fractions 14 15 and 24 and 25.

CONCLUSIONS

The EDLFs present in PE placenta can be effectively purified or isolated using Digibind as an affinity material. The binding of Digibind to the EDLF present in PE is obvious and with the newly designed ultrafiltration process outlined here EDLFs can be isolated and enriched. The HPLC can further assist in separating the affinity isolated EDLFs based on polarity and help to further isolate and purify each EDLF so that further investigation and identification of these EDLFs can be accomplished with little interference from impurities.

CHAPTER 6: CONCLUSIONS AND DISCUSSION

EDLF

EDLF Increased in Women with PE

The experiments in *Chapter 2* show strong evidence that in general women with PE show an a decrease in SP activity or an increase in inhibition as compared to women who experience normal pregnancy. Evidence that this inhibition is caused by a circulating compound within the serum or placental homogenate of women with PE is an EDLF can be concluded from the evidence that Digibind binds these EDLFs and prevents them from inhibiting the SP. The placenta also presents itself as a possible source for this EDLF as seen by the statistically significant increase in SP inhibition with placental homogenates from women with PE as compared to plasma from women with PE.

Further investigation as to the placenta being a source of PE or EDLF is needed. Studies by this lab have been done to investigate the mechanism of EDLF production in the placenta and strong evidence exists that supports the production of EDLF in the placenta (Ma, 2011).

EDLF Positive Women Improve with Digibind

Overall, women with severe PE involved in the DEEP study, and that received Digibind, saw improvements in their condition. As defined in *Chapter 2*, the SP inhibition can be caused by an EDLF. Secondary analysis of the results that grouped women by degree of inhibition, further defined the role of EDLF in PE. Creatinine clearance, pulmonary edema, SP activity, IVH and NEC, all improved in women or infants who showed initial SP inhibition, i.e. increased EDLF activity and received treatment, but the data also showed that those women who were EDLF negative or had no initial SP inhibition did not improve from the Digibind treatment.

Evidence that all women with severe PE may not have increased EDLF does give rise to important questions as to the cause of PE, but there is enough evidence here that EDLF does play

a role in PE. The previous multi-system definition of PE, is supported with these results, but of great significance is the more in depth look at EDLF as a major participant in the complications of PE and possibly even more significant as the cause of PE complications.

EDLF in PE Similar to Other Known SP inhibitors

Candidates for human EDLFs were investigated here in *Chapter 4* and the EDLF found in women with PE behaves very similar to these other well known SP inhibitors. This is seen by the way that Digibind and DigiFab bound all investigated SP inhibitors as well as the EDLF found in women's plasma and placenta that had PE. This binding was evident due to the reversal of SP inhibition when Digibind or DigiFab was added to the EDLF candidates or the EDLF found in the plasma or placenta of women with PE. The specificity of the digoxin immune Fabs leads us to believe that the structure of the EDLF found in these women with PE is very similar to one or more of these candidate EDLFs.

EDLF in Women with PE can be Purified for Identification

The unique procedure outlined in *Chapter 5* provides a powerful means to isolate the EDLF found in serum or placental homogenates. The small concentration of EDLF found in serum has originally lent to the difficulty in isolation and identification in the past, but the evidence seen here that EDLF concentration is increased in placental tissue of those women with PE provides us with an excellent source of EDLF. Again Digibind not only verifies that the EDLF found in PE performs and acts like other known SP inhibitors, but here it is used as an affinity material to isolate the EDLF.

Further work is needed to identify the EDLFs found in women with PE. Confidently the protocol outlined here can be used to purify larger amounts of the EDLF so that extensive analysis and identification of this EDLF can be performed. Mass Spectrometry, chemical

analysis, and NMR can all potentially be used as a means to identify this compound when enough EDLF is obtained.

DIGIBIND

Digibind, an anti-digoxin Fab fragment, has been used here as an effective means to bind EDLFs in serum and placenta of women with PE and deactivate their SP inhibiting activity. It has been used here in an affinity ultrafiltration purification method to isolate EDLFs found in the placenta of women with PE, and also in a radioimmunoassay to quantify and detect the concentration of these EDLFs. Of applicable importance, Digibind was also used *in vivo* as a therapeutic to treat women with severe PE and a reduction in symptoms related to PE was seen. All of these notable cases show Digibind as an effective and novel tool in diagnostics, therapeutics, and sequestering of EDLFs in PE.

Digibind Reduces Inhibition of EDLFs Found Raised in Women with PE

PE's pathology still remains undefined, but perhaps the information contained here has led us closer to an actual cause of this severe pregnancy condition. Not only have we identified that the level of EDLFs is increased in serum of women with severe PE, but we have also seen an even greater increase in placental EDLFs of women with severe PE. This significant increase in placental EDLFs points to the placenta as a possible source for the production or release of these EDLFs. This supports previous research and theory that placental factors are released with the improper placentation or failed arterial remodeling that occurs in earlier stages of pregnancy leading to PE. A role for EDLFs in the hypertension of pregnancy is also supported by the findings here. EDLFs found in serum and placental homogenates of women with PE did show an inhibition of the SP which has been previously proven to have an effect on hypertension and high blood pressure involved with PE.

Digibind Therapeutically Reduced Symptoms of Severe PE

The significance of Digibind being able to capture and bind these circulating EDLFs is paramount. Additions of Digibind to the serum and placental homogenates did effectively reverse the previous inhibition associated with EDLFs. This finding supports the current trials that an antibody Fab fragment raised against digoxin or Digibind could act as an effective therapeutic for women who have PE.

Arguments have been raised, as they have with all new drugs, as to the safety of administering the Fab fragment therapy, but it is currently administered as an effective overdose control agent in human patients who receive digoxin for congestive heart failure. Digibind as a therapeutic is not the end all of PE, but with the significant improvement in creatinine clearance observed with the administering of Digibind to women with severe PE, the data strongly suggests that Digibind may be beneficial in handling some of the symptoms and immunological responses associated with PE.

Digibind Proves Effective in Comparison with Other Digoxin Immune Fab Fragment

Digibind and DigiFab were both compared for their ability to bind and inactivate 4 known EDLFs as well as any EDLFs found in serum from women who had PE. Digibind and DigiFab both effectively bound all of the EDLFs investigated as well as those found in serum. We know this because usually a concentration of 10^{-6}M was sufficient to reverse >20 % of the inhibition caused by the inhibitor of the same concentration. Theoretically, Digibind has a molar ratio of 2 to 1 for the known inhibitors investigated and this fits in to our findings as well.

Digibind Effectively Verifies the Existence of EDLF in PE Serum and Placenta

Verifying that the inhibition caused by the addition of serum from women with PE was actually from an EDLF and not from another interfering compound or factor was accomplished by the addition of Digibind to the Rb uptake assay. Digibind specifically binds digoxin or

ouabain-like structures that contain the 5 or 6 member lactone ring. The reversal of SP inhibition using the Rb uptake assay, when Digibind was added, shows that there are indeed EDLFs in the serum and also the placental homogenates.

Digibind also effectively verifies the presence of EDLFs in the affinity ultrafiltration purification process. After HPLC separation, the radioactive immunoassay used Digibind as its primary antibody to bind any EDLFs present in the HPLC fractions. The RIA was able to detect EDLF's in more than one fraction and was also effective in helping to determine the concentration of the EDLF contained in serum and placental homogenates from women with PE. Therefore, this adds an additional use for the Fab fragment.

Digibind Affinity for EDLF Enables Affinity Ultracentrifugation Purification

Finally, Digibind was used as an affinity material to pull out the EDLF found in placental homogenates from women who had PE. This novel protocol used an ultrafiltration membrane specific to particles smaller than 30K daltons, so that when EDLFs are bound by 50K dalton Digibind, they do not pass through the filter, but when they are released with low pH glycine, they will pass through the membrane. This allows us to remove most impurities except the specific EDLFs which we are interested in. The results of this process show through the RIA that we were successful in purifying enough EDLF to separate on the HPLC.

Purification, in the future will enable us to identify these EDLFs present in these HPLC fractions using other means such as mass spectrometry. With the right concentration of EDLF the Orbitrap Mass Spectrometer has a strong enough resolution that we may be able to identify the molecular mass and elemental composition of the EDLFs as well as additional chemical structure information by using fragmentation and software designed to calculate the different functional groups that might exist. Knowledge of the other known EDLF candidates will help narrow this process.

Of considerable noteworthiness, is the fact that EDLF was found increased in the placenta. Placenta, as mentioned in the Introduction, has been hypothesized as the origin of PE. Additional studies, by Jie Ma, another graduate student within this lab, have shown that by inducing hypoxic conditions, or oxidative stress, placental cells will secrete or produce EDLFs. This was verified by the assays above. Conversely, it was mentioned previously that symptoms of PE disappear with the removal of the fetus and placenta post partum. The remodeling of spiral arteries and development of the placenta as the interface between maternal and fetal physiology is such an intricate and multistep endeavor that interruption of this process could lead to stresses both maternal and fetal. The question next to be answered may be the source of signaling for these factors being released and whether they originate with the fetus or the woman.

Digibind, although not originally designed for the purposes implemented here, has proven to be an effective tool in diagnosing the existence of EDLFs in PE. A future diagnostic practice may be to use Digibind or other digoxin immune Fab fragments in assays to alert obstetricians of ensuing PE. As with any disease or medical condition, earlier detection could be the difference between life and death for mother or fetus.

Digibind proved to be a useful tool in the scientific research of PE. Here it was used to bind and inactivate harmful EDLFs *in vivo* and help to manage symptoms, which may cause permanent damage of women with PE. Digibind effectively assisted in identifying the increase of EDLFs found in the serum and placenta of women with PE. It was used as the probe of a radioimmunoassay to detect and quantify EDLF concentrations and it was also used effectively as an affinity material to isolate and purify EDLFs found in the placental homogenates of women with PE. This antibody will hopefully continue to enable continued research into the pathology and treatment of PE to better the lives of thousands of women and their infants.

CONCLUSIONS

Although PE is a multisystem disorder, EDLF is shown here to be one of the controllable circulating factors increased in PE. Management of this EDLF was accomplished with the use of an anti-digoxin immune Fab, as seen here with Digibind. DigiFab also proved to be sufficient in accomplishing the same results. EDLF is increased in the serum and placenta of women with PE, and Digibind or a similar anti-digoxin immune Fab demonstrates itself as an effective tool in controlling the levels of EDLF *in vitro* as well as *in vivo*. Within the narrow scope of the DEEP study, Digibind showed no side effects but benefit to both mother and child in controlling the symptoms of PE. Further, DigiFab shows comparable results for its comparable effectiveness in binding known SP inhibitors as well as the EDLF found in PE. Finally of great importance Digibind provides a means to identify the presence of EDLF in blood and tissue and can be used to isolate these EDLFs for further investigation.

WORKS CITED

Adair CD, Buckalew VM, Kipikasa J, Torres C, Stalling SP, Briery CM. Perinatal/Neonatal Case Presentation: Repeated dosing of digoxin-fragmented antibody in preterem eclampsia. *Journal of Perintology* **2009**, 163-165.

Adair CD, Buckalew VM, Taylor K, Ernest JM, Frye AH, Evans C et al. Elevated endoxin-like factor complicating a multifetal second trimester pregnancy: treatment with digoxin-binding immunoglobin. *Am J Nephrol* **1996**, 529-531.

Adair CD, Busckalew VM, Graves SW, Lam GK, Johnson, MD, Saade G, Lewis DF, Robinson C, Danoff TM, Chauhan N, Hopoate-Sitake ML, Porter KB, Humphrey RB, Trofatter KF, Amon E, Ward S, Kennedy L, Mason L, Johnston JA. Digoxin Immune Fab Treatment for Sever Preeclampsia. *Am J Perinatol* **2010**, 27, 655-662.

D'Urso, G, Frascarelli S, Balzan S, Zucchi R, Montali U. Production of ouabain-like factor in normal and ischemic rat heart. *J Cardiovasc Pharm* **2004**, 43:5, 657-62.

Duckitt K and Harrington D. Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *BMJ* **2005**, 330, 565.

Effereth T, Davey M, Olbrich A, Rucker G, Gebhart E, Davey R. Activity of Drugs from traditional Chinese medicine toward sensitive and MDR1- or MRP1-overexpressing multidrug resistant human CCRF-CEM Leukemia cells. *Blood Cells, Molecules and Disease* **2002**, 160-168.

Gao, J et all. Isoform-specific stimulation of cardiac Na/K pumps by nanomolar concentrations of glycosides. *J Gen Physiol* **2002**, 119, 297-312.

Hamlyn JM, Hamilton BP, and Manunta P. Endogenous ouabain sodium balance and bloodpressure: a review and a hypothesis. *J Hypertens* **1996**, 14, 169-171.

Hopoate-Sitake M, Adair CD, Mason, LA Torres C, Kipikasa J, and Graves, SW. Digibind reverses inhibition of cellular Rb^+ uptake caused by endogenous sodium pump inhibitors present in serum and placenta of women with preeclampsia. *Repr Sci* **2011**, 18:2. 190-199.

Jing Y, Ohizumi K. Selective inhibitory effect of bufalin on growth of human tumor cells *in vitro*: association with the induction of apoptosis in leukemia HL-60 cells. *Jpn J Cancer Res* **1994**, 85:6, 645-51.

Kelly R, O'Hara D, Mitch W, Steinman T, Goldszer R, Solomon H, and Smith T. Endogenous digitalis-like factors in hypertension and chronic renal insufficiency. *Kidney Inter* **1986**, 30, 723-729.

Laredo, J. et al. Ouabain is secreted by bovine adrenocortical cells. *Endocrinology* **1994**,135, 794-797.

Levine R, Maynard S, Qian C, Lim K, England L, Yu K, Schisterman E, Thadhani R, Sachs B, Epstein F, Sibai B, Suhkatme V, Karumanchi A. Circulating angiogenic factors and risk of preeclampsia. *N Engl J Med* **2004**, 350, 672-83.

Ma, J. Human endogenous sodium pump inhibitors measurement source, synthesis and regulation. PhD Thesis, Brigham Young University, Provo, UT, April 2011.

Martinell J, Jodal U, Lidin-Janson G. Pregnancies in women with and without renal scarring after urinary infections in childhood. *BMJ* **1990**, 300, 840–4.

Murrell, JR. Endogenous ouabain: upregulation of steroidogenic genes in hypertensive hypothalamus but not adrenal. *Circulation* **2005**, 112:9, 1301-8.

Ogawa, H. *PNAS* **2009**, 13742-7.

Goodlin RC. Antidigoxin antibodies in eclampsia. *N Engl J Med* **1988**, 518-519.

Redman. CW, Sargent IL. Latest Advances in Understanding Preeclampsia. *Science* [Electronic version] **2005**, *308*, 1592-1594.

Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* **2000**, 183:1. S1-S22.

Rhorer, DC. Bufalin. *Crys Aceta* **1982,** 1865-1868.

Schneider, R, Wray V, Nimtz M, Lehmannpar WD, Kirch U, Antolovic R, Schoner W. Bovine Adrenals Contain, in Addition to Ouabain, a Second Inhibitor of the Sodium Pump. *J Biol Chem* **1998**, 273:2, 784-92.

Solomon CG, Seeley EW. Preeclampsia—searching for the cause. *N Engl J Med* **2004**, 350, 641-642.

Therien AG, Blostein R. Mechanisms of sodium pump regulation. *Am J Physiol Cell Physiol* **2000**, C541-566.

Vu, H, Ianosi-Irimie M, Pridjian C, Whitbred J, Durst J, Bagrov AY, Federova O, Pridjian G, Puschett J. Involvement of marinobufagenin in a rat model of preeclampsia. *Am J Nephr* **2005**, 25:5, 520-8.

Wallis AB, Saflas AF, Jsia J, Atrash HK. Secular treands in the rates of preeclampsia, eclampsia and gestational hypertension, United States, 1987-2004. *Am J Hypertens* **2008**, 521-526.

Yeh, Jiun-Yih, Huang W, Kan S, Wang P. Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. *The Prostate* **2002**, 54:2, 112-124.

Zhen Y, Franz KB, Graves SW. A novel assay of cell rubidium uptake using graphite furnace atomic absorption application to rats on a magnesium-deficient diet. *J Nutr Biochem* **2005**, 16, 291-296.