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Human Endogenous Sodium Pump Inhibitors Measurement, Source, Synthesis and Regulation

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Human Endogenous Sodium Pump Inhibitors: Measurement, Source, Synthesis and Regulation

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ABSTRACT

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The sodium pump (SP or Na^+,K^+-ATPase) is a membrane embedded protein complex that pumps 3 sodium ions out and 2 potassium ions into the cell per cycle and in so doing creates a cell membrane electrochemical potential. The membrane potential is critical for any functional cell. In the vasculature, reduction in the voltage potential causes vascular smooth muscle contraction and a narrowing of blood vessels (vasoconstriction) which can lead to increased blood pressure (hypertension). Substantial research over the past several decades has provided a vast amount of research on SP inhibitors, sometimes called endogenous digitalis-like factors (EDLF). Increased levels of these factors have been implicated in many hypertensive disorders including preeclampsia (PE), a life-threatening complication of pregnancy. It has been demonstrated that EDLF might be a causative factor in the pathophysiology of hypertension in PE. In order to elucidate EDLF production and regulation in PE, We developed a radioimmunoassay (RIA) measuring EDLF that could be applied to serum from pregnant women, placental homogenate and placental tissue culture. This assay employs Digibind, a commercially available Fab fragment derived from polyclonal antidigoxin antibodies that cross reacts with EDLF, as the primary antibody. Using Digibind RIA, we demonstrated that placenta is a source of EDLF production and regulation. Moreover, the identification of an inhibitor, ketoconazole and a substrate, 17-hydroxyprogesterone of the synthetic pathway of EDLF in placenta proved that this pathway shares steps with the steroid synthetic pathway. Some potential regulatory agents which have elevated levels in PE or be associated in PE and thus are thought to mediate PE, such as hydrogen peroxide, tumor necrosis factor-α (TNF-α) and hypoxia have also been demonstrated to be stimuli of EDLF production in placenta. These findings are helpful to the further study on EDLF synthesis and regulation in placenta. Once we elucidate the mechanisms, it could be easier to provide deeper insights into the pathogenesis of PE and subsequently develop earlier diagnosis and effective prevention of or therapeutic approaches to PE.

Keywords: sodium pump, preeclampsia, digoxin (digitalis), endogenous digitalis-like factor (EDLF), Digibind

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Chapter 1: Introduction

The sodium-potassium pump or simply the sodium pump (SP) (also known as the Na^+,K^+-ATPase) is a highly-conserved protein complex embedded in the plasma membrane in all cells of higher organisms. It pumps three sodium ions out and two potassium ions into the cell per cycle and creates a cell membrane electrochemical potential, which is the basis for excitability in nerve and muscle cells (Fig 1). For most animal cells, the SP is responsible for 1/3 of the cell's energy expenditure. For neurons, the SP is responsible for 2/3 of the cell's energy expenditure. In addition, the sodium gradient resulting from export of sodium ions from the cell provides the driving force for several facilitated transporters, which not only import glucose, amino acids and other nutrients into the cell but also export calcium ions out of the cell[1]. For example, SP reduction causes reduction in the cell membrane voltage potential (depolarization) as well as an insufficient driving force for Na^+ entry into the cell through the Na^+-Ca^{2+} transporter, resulting in a Ca^{2+} entry and accumulation of intracellular calcium. In vascular smooth muscle (VSM) cells, increased concentration of calcium leads to contraction with a narrowing of blood vessels (vasoconstriction). Excessive vasoconstriction increases peripheral resistance and then results in high blood pressure (hypertension). In the heart, increased intracellular calcium causes heart muscle contraction[2].

Molecular studies have revealed that the SP, possesses 8 or 10 transmembrane domains, is composed of an α subunit (~113KD) and a glycosylated β subunit (~35KD) which combine to form the functional transporter[1]. The α subunit comprises important functional domains that

undergo a series of conformational changes, bind sodium and potassium ions, and contains the phosphorylation site. The β subunit is critical in facilitating the plasma membrane localization and activation of the α subunit[3]. Several isoforms of both α and β subunits have been identified, including α1, α2, α3, α4, β1 and β2, which have both tissue and species specific distribution as well as functional implications. Northern and western blot analyses carried out by our and other laboratories confirmed that in the kidney, the α1-isoform is the dominant form of the α subunit, in skeletal muscle and vascular smooth, the α2- isoform is prominent, the α3-isoform exists predominantly in fetal brain and adult neural tissues. In the heart, all of the α1-, α2- and α3-isoforms are present, whereas the α4-isoform has only been detected in testis and sperm[4, 5, 6]. These isoforms of the α subunit display distinct sodium affinities as well as sensitivities to cardiac glycosides, such as digoxin and ouabain. For example, the α1-isoform has the highest affinity for sodium ions but has a distinctly lower affinity for ouabain than the other two isoforms, in particular in rat and mouse tissues. Therefore alterations in the SP isoform distribution accompany hypertensive conditions[4, 5, 7].

The mechanism of active SP ion transport is driven by the breakdown of ATP. With bound ATP, the SP binds three intracellular sodium ions. ATP hydrolysis leads to phosphorylation of the SP, and then induces a conformational change in the pump, which translocates the sodium ions across the membrane. The new conformation has a low affinity for sodium ions and a high affinity for potassium ions, so the three bound sodium ions dissociate from the pump and diffuse into the extracellular fluid and two potassium ions bind to the extracellular side of the pump. This causes the dephosphorylation of the SP, reverting it to its original conformation, transporting the potassium ions to the cytoplasm on the inside of the cell. This unphosphorylated

form of the SP has a low affinity for potassium ions, so the two bound potassium ions dissociate from the pump and diffuse into the interior of the cell (Fig 2). The SP then starts a new cycle by binding ATP[1].

The SP can be regulated in several ways and in both acute and chronic timeframes: Its genetic production can be increased or decreased by transcription factors. Its activity can be modified by the SP being phosphorylated or dephosphorylated, often in response to hormones binding to the cell with activation of intracellular kinases. For example, it has been demonstrated that dopamine, a SP inhibitor, inhibits SP activity by stimulating protein kinase C (PKC), and this dopamine-induced SP inhibition requires endocytosis of the subunit into defined intracellular compartments in rat renal tubule cells[8]. We also studied the endocytosis of SP in response to dopamine as well as other regulators in myometrial cells, the main muscle cells of uterine wall, using confocal microscope and immunofluorescence. A final mechanism of regulation, one of particular interest here, is the presence of inhibitors produced by the body which can decrease the activity of the SP[9]. Such inhibitors have also been identified in plants, in particular the common foxglove plant *Digitalis purpurea*, which has been used therapeutically for over 200 years as a cardiac glycoside. Compounds derived from the foxglove leaf, e.g. digoxin (also known as digitalis) and digitoxin, are widely used in the treatment of congestive heart failure, atrial fibrillation, atrial flutter and some heart failure that cannot be controlled by other medication. For example, congestive heart failure is a common disease among the elderly in that the heart demonstrates a reduced ability to compress its chambers which reduces the output of the heart. Digoxin and related compounds have been used to treat this condition effectively by binding to a site on the extracellular aspect of the α-subunit of the SP in heart cells

(myocytes) and inhibiting its SP activity. As described above, inhibition of SP causes a rise in the level of intracellular sodium ions, which in turn slows down the extrusion of calcium ions or even induces depolarization of the cell membrane with calcium entry through voltage dependent calcium channels in the heart and through the Na^{+}-Ca^{2+} transporter that relies on the high sodium gradient in vascular smooth muscle. Increased amount of calcium in the cardiac myocytes leads to increased contractility of the heart and the subsequent increased stroke volume.

However, one of the challenges in using digoxin is that it can be very toxic at concentrations only slightly above those therapeutically needed. Digoxin overdose has been a relatively common problem and has led to the development of an antibody fragment called Digibind® that binds digoxin and eliminates its toxicity[10]. Digibind is a commercially available Fab fragment derived from polyclonal antidigoxin antibodies raised in sheep. It has been shown to be effective and safe in treating digoxin overdose or digitalis glycoside toxicity by binding digoxin and making it unavailable for binding to the SP in the organism generally. The Digibind-digoxin complex accumulates in the blood, from which it is excreted by the kidney. The net effect is to shift the equilibrium away from binding of digoxin to its receptors (SP) in the body, thus reversing its effects.

Because some diseases appear to be related to SP inhibition, substantial research over the past several decades has provided a vast amount of research on SP inhibitors, collectively referred to as endogenous "digitalis-like" factors (EDLF) which have been found in several tissues and body fluids of animals and humans. These EDLFs are the primary research subject here. EDLFs are compounds with biological and immunological properties similar to cardiotonic

drugs, such as digoxin. They cross-react with digoxin antibodies and inhibit SP by binding to the same binding site as used by digoxin. Therefore, the factors may be identical to or structurally related to digoxin or ouabain, but the exact structure of this factor or factors is unknown. Increased levels of these factors have been implicated in many hypertensive disorders. The reasons for these EDLFs being increased is unclear but there is overwhelming data to show that the levels of EDLF are increased in serum in human essential hypertension, many secondary forms of human hypertension and many experimental animal models of hypertension, suggesting that increased levels of EDLF may be a causative factor in the pathogenesis of hypertension[11]. Moreover, in experimental models of hypertension with elevated EDLF levels, Digibind administration has been demonstrated to lower or even normalize blood pressure. Serum from individuals with hypertension has been shown to inhibit the SP in isolated red blood cells (and a variety of other peripheral blood cells and some tissues) and to increase vasoconstriction of isolated blood vessel rings, which effect could be prevented or reversed by the presence of or addition of Digibind[12]. Previous studies also demonstrated that renal arteries from hypertensive animals had increased levels of intracellular sodium ions as well as reduced SP activity, and these effects could also be reversed by Digibind[13]. These observations indicated that EDLFs are involved in hypertension by inhibiting the SP and Digibind can reverse the effect by binding and blocking these factors.

My research has focused on preeclampsia (PE), which is one of the hypertensive disorders related to EDLF. It may now be the number one killer of pregnant women in the world and more often occurs with first pregnancies after 20 weeks gestation. Hypertension, proteinuria, swelling, sudden weight gain, headaches and changes in vision are important symptoms. Less

than one in 100 women with preeclampsia will develop eclampsia or seizures. PE may initially involve generalized damage to the maternal vascular endothelium, sometimes affecting kidneys and liver, with the release of vasopressor factors secondary to the original damage. Additionally, PE is a leading known cause of iatrogenic preterm birth. The unborn baby in preeclamptic pregnancy can suffer prematurity, intrauterine growth restriction (IUGR), acidosis and even death. At present, the causes and pathophysiology of the disease are still poorly understood, partly because that PE is multi-factorial in nature, involving vascular, genetic, immunological and/or environmental factors. In fact it may have several causes all of which lead to a final common pathway. Hence, there is no direct way to test if or when PE will occur, and no treatment to prevent or treat PE. When it is severe, even if it occurs early in the pregnancy, the only cure is to deliver the fetus in order to protect the life of the mother. Therefore more effective therapies to protect the mother while allowing the baby to grow and develop are important[14].

Multiple abnormalities have been reported in PE and as described above, one of the abnormalities is an elevation in the circulating levels of EDLF. Although elevated levels of these factors are found both in normal pregnancy and in pregnancy complicated by PE, the levels in PE are significantly higher than in normal pregnancy. Given its ability to cause vasoconstriction, EDLF might play a role in the pathophysiology of PE. In vitro experiments demonstrated that reduction in SP expression in PE may raise level of intracellular sodium ions or increase pressor sensitivity, which contributes to hypertension in PE[15]. Clinical trials also showed that for preeclamptic women, treatment with Digibind reduced patients' blood pressure dramatically. These observations support the hypothesis that Digibind might improve some of the symptoms

of PE by binding with the SP inhibitor--EDLF, especially hypertension and Digibind could be a potential therapy for women with PE. Therefore, the aim of this study is to elucidate mechanisms of EDLF production and regulation in PE. This work may provide insights into how to interrupt production of EDLF as a possible further therapeutic approach to PE.

It is believed that EDLF is structurally very like the steroidal glycoside digoxin or an analogue with an unsaturated lactone ring and that it is synthesized by the isoprenoid pathway, a very complex route that involves multiple steps (Fig 3) or from cholesterol in the diet. The isoprenoid biosynthetic pathway is a key regulatory pathway generating a high number of end-products essential for cell viability such as cholesterol, sterols, dolichols, coenzyme Q, heme and prenylated proteins[16]. However, because the structures of EDLFs have not been completely identified and the isoprenoid pathway involves diversified metabolites, mechanisms of EDLF synthesis and regulation are not clear. For this research key intermediates of an endogenous digoxin pathway would be of interest.

We hypothesized that some intermediates of the isoprenoid pathway, pregnenolone, progesterone and progesterone derivatives such as 17-hydroxyprogesterone, may be used to generate the EDLF (Fig 4). To verify the hypothesis we tested the effect of 17-hydroxyprogesterone on EDLF production. Increased EDLF levels with the presence of 17-hydroxyprogesterone in placental tissue culture will indicate that this substance contributes to EDLF synthesis and might be a substrate of EDLF biosynthetic pathway.

On the other hand, ketoconazole, an imidazole derivative, and a general inhibitor of P450 dependent enzymes, has been proven to block gonadal and adrenal steroidogenesis in humans[17]. Moreover, it was also found to inhibit cholesterol synthesis by blocking the conversion of methyl sterols to cholesterol[18]. Therefore, ketoconazole would be predicted to be an inhibitor of EDLF synthesis pathway if EDLF synthesis uses enzymes that are part of steroid synthesis. Our experimental data showed that it significantly reduces EDLF levels in placental tissue culture, which is consistent with the hypothesis.

Besides elevation in EDLF levels, there are some other abnormalities reported in PE, including reduced uteroplacental perfusion, hypoxia, higher levels of reactive oxygen species (ROS), defective trophoblast differentiation and invasion of the decidua, altered placental production of cytokines and growth factors, and endothelial dysfunction, which are also considered to be important aspects in the etiology of PE[19]. Studies during the past decade have provided a better pathophysiologic understanding of PE. Currently, etiologic hypotheses of PE focus on maladaptation of immune responses and impaired trophoblast invasion. What is most widely accepted is that reduced uteroplacental perfusion (superficial implantation of the fetus) due to inadequate invasion of the maternal uterine spiral arterioles into the placental trophoblast is an important initiating event in PE. Poor placental perfusion leads to placental and fetal hypoxia. Oxygen plays a central role in human placental pathologies, and hypoxia results in widespread activation and dysfunction of the maternal vascular endothelium via altering production of pro and anti-inflammatory cytokines and cytokine-like angiogenic growth factors. Endothelial dysfunction then enhances formation of vasoconstrictors such as endothelin and thromboxane, increases formation of superoxide and vascular sensitivity to angiotensin II (a

potent hypertensive agent), and reduces formation of vasodilators such as nitric oxide and prostacyclin. These endothelial alterations eventually cause hypertension through increasing vascular resistance and impairing renal function[19, 20, 21, 22]. However, the actual cause of hypertension is unknown. For example, therapeutics that reduced thromboxane while increasing prostacyclin had absolutely no effect of the incidence or severity of PE.

PE is widely believed to be associated with placental hypoxia. During pregnancy, hypoxia induces endothelial dysfunction by activating oxidative stress, which then promotes intrauterine growth retardation, one of the features of PE. Oxidative stress is an excess formation and/or insufficient removal of ROS, such as hydroxyl radical, superoxide anion, hydrogen peroxide and singlet oxygen[23]. There is increasing evidence supporting that hypoxia-induced oxidative stress may be an important contributing factor to the pathogenesis of PE. Compared with healthy pregnant women, preeclamptic women have higher levels of ROS and lower levels of several dietary antioxidants in their blood, including vitamin C and E, lycopene and betacarotene. Elevated levels of markers of oxidative stress, such as lipid peroxides (Fig 5) have also been observed in the placenta and circulation of preeclamptic women. ROS have been demonstrated to play a pathophysiological role in the development of hypertension in PE in some studies. Overproduction of ROS destroys endothelium-derived nitric oxide in vitro and decreases its bioavailability, resulting in impaired endothelium-dependent vasodilatory response and increased vascular resistance, and consequently could cause hypertension[24, 25]. However, there have been several very large studies that have supplemented pregnant women with high intake of both fat soluble and circulating antioxidant vitamins without any effect on the incidence or severity of PE. However, the issue of hypoxia and ROS may be much more localized to the

placenta and its immediate environment. Since both ROS and EDLF may be involved in preeclamptic hypertension, we are interested in their relationship in the pathology of PE. Do ROS induce hypertension through regulating EDLF biosynthesis? Is hypoxia-induced oxidative stress a mediator of EDLF and represent linkage that contributes to the hypertension in PE? Knowing the role of hypoxia or ROS and EDLF synthesis and release would be helpful to elucidate potential pathogenesis of PE and the interplay of different systems that may lead to hypertension. Therefore, we carried on a series of in vitro experiments testing the effects of hypoxia and hydrogen peroxide on EDLF production. The results showed that both hypoxia treatment and addition of hydrogen peroxide into the placental tissue culture lead to increased EDLF levels in the culture, suggesting that hypoxia and ROS might be regulatory factors of EDLF production.

The cytokines produced by the placenta are involved in maternal immune response and also have a role in fetal development. Recently, substantial evidence supports that cytokines and other factors such as lipid peroxides and ROS are potential mediators of endothelial dysfunction[26]. Endothelial tissue is a specialized type of epithelial tissue that is highly active and closely involved in numerous physiological processes. Endothelial cells, lining the entire circulatory system, from the heart to the smallest capillary, are believed to participate in angiogenesis, adaptation of vascular tonus and maintenance of blood fluidity in the microcirculation. It has been demonstrated that endothelial dysfunction has prognostic significance in predicting vascular events, including stroke and heart attacks; in particular it's a central pathophysiological feature in the maternal vascular system in PE[27]. Oxidative stress and altered immune response at the fetal-maternal interface are thought to be contributors of the

development of endothelial and renal dysfunctions in PE. And inflammatory cytokines are believed to link placental hypoxia with maternal endothelial abnormalities and cardiovascular and renal dysfunction. Hence, interactions between placental cytokines and the maternal endothelium might play a central role in the pathogenesis of PE[20, 26, 28].

As described above, shallow trophoblast invasion, the proposed initiating event in PE, may induce an excessive maternal inflammatory response, which results in a chain of events including increased circulating levels of cytokines, such as tumor necrosis factor-α (TNFα), interferon-γ (IFNγ) and interleukin-6 (IL-6). Elevated levels of TNFα and IL-6 have been detected in the circulation of preeclamptic women[29]. Since convincing evidence showed that cytokines have an impact on vascular function and endothelium-derived factors involved in blood pressure regulation, and Digibind attenuates TNFα-induced inflammatory response in endothelial cells, we hypothesized that cytokines, especially TNFα, might be involved in regulation of EDLF synthesis and release[30, 31]. TNFα may influence blood pressure indirectly through regulating EDLF levels, and Digibind reduces these effects by binding with EDLF. In order to verify our hypothesis and elucidate the mechanisms of regulation of EDLF synthesis pathway, we designed experiments to identify the role of TNFα in EDLF production and release. The data indicated that TNFα induce elevation in EDLF levels in placental tissue culture and it might be a regulatory agent that upregulates EDLF biosynthesis pathway.

These studies should greatly clarify the synthetic pathway of EDLF and help us understand the interrelationship of systems thought to play a significant role in the early events leading to the clinical condition we know as PE.

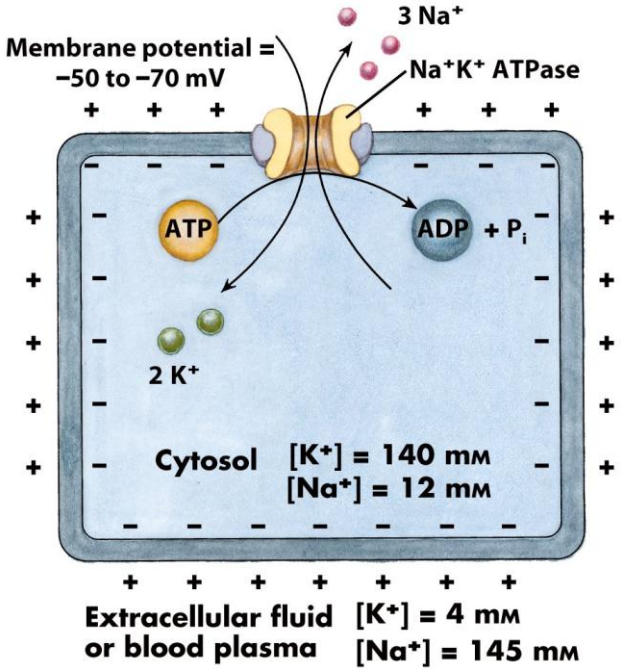

Figure 1. How the SP works. It moves Na^+ out and K^+ into cells and generates the cell membrane potential[32].

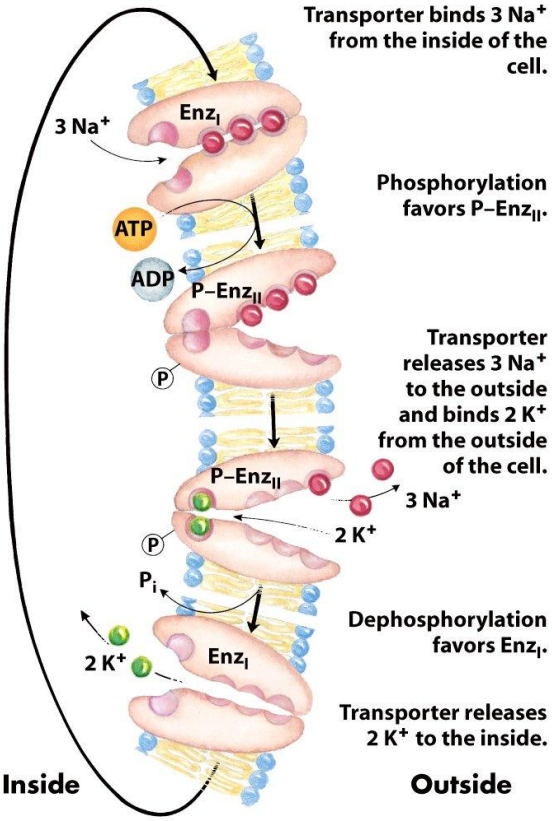

Figure 2. Mechanism of a cycle of active SP. A SP inhibitor, such as ouabain, locks the enzyme in its phosphorylated state and prevents further cycling of the SP. This causes Na^+ to accumulate in the cell and depolarizes the cell membrane leading to increased Ca^{2+} in the cytosol and in turn to VSM contraction[32].

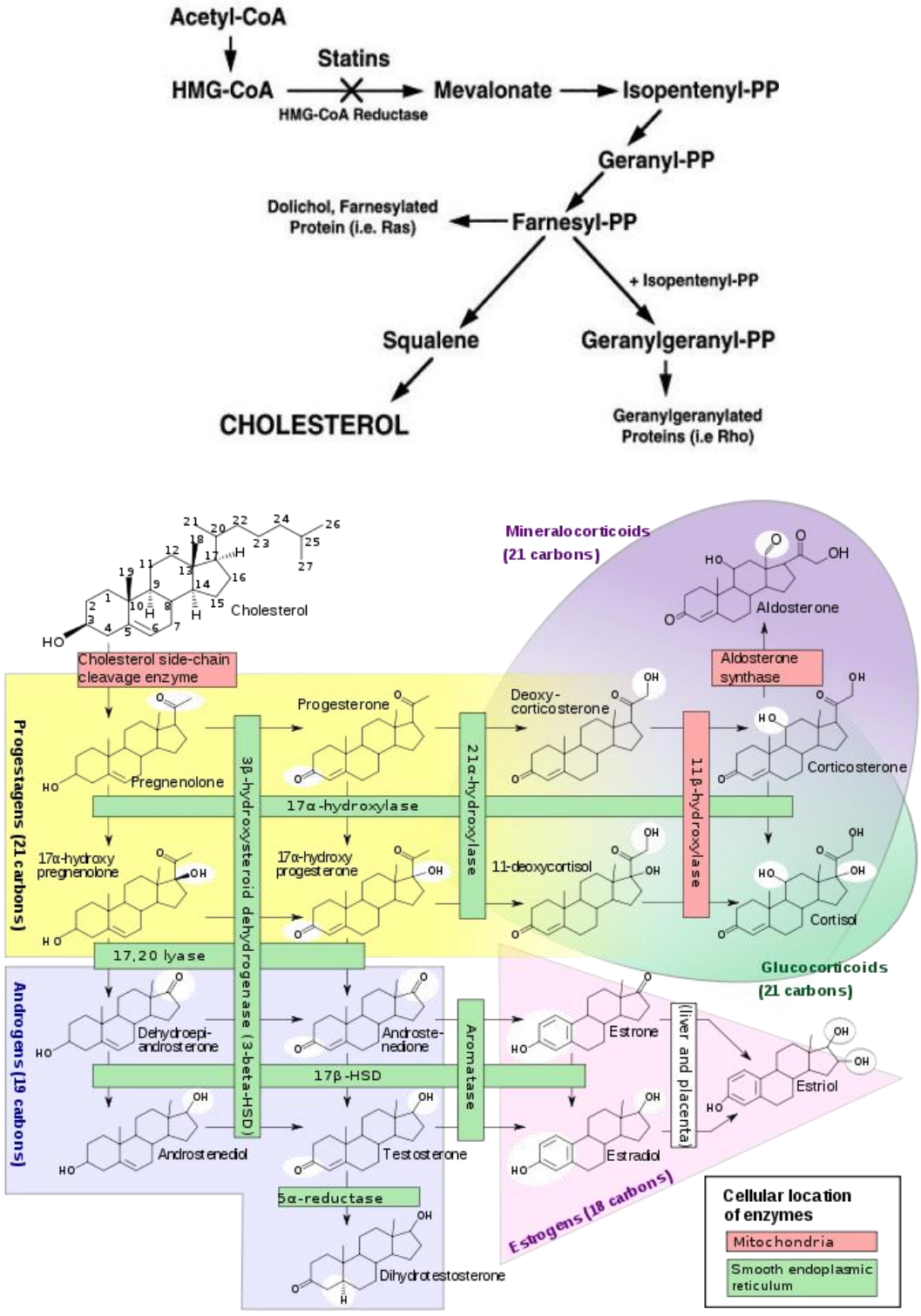

Figure 3. Steroidogenesis by Mikael Haggstrom, showing many enzyme activities of cytochrome P450 enzymes[33].

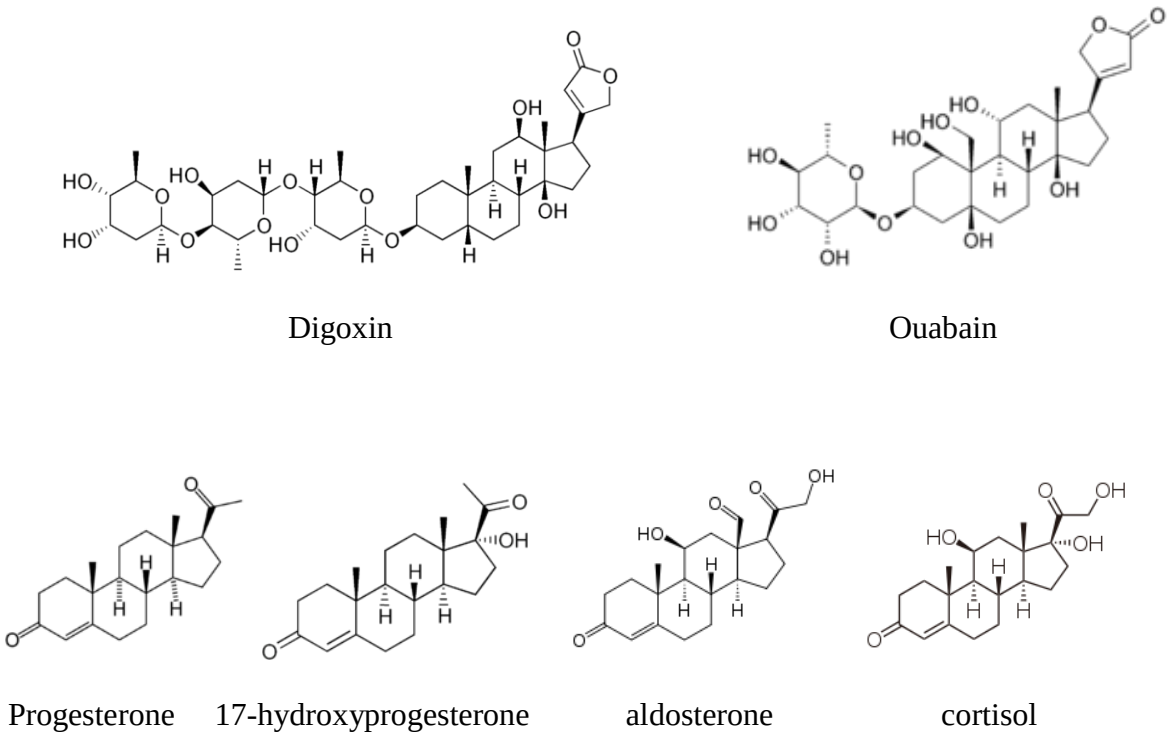

Figure 4. Structures of analogues of EDLF: digoxin and ouabain, and potential substrates of EDLF synthesis: progesterone, 17-hydroxyprogesterone, aldosterone and cortisol.

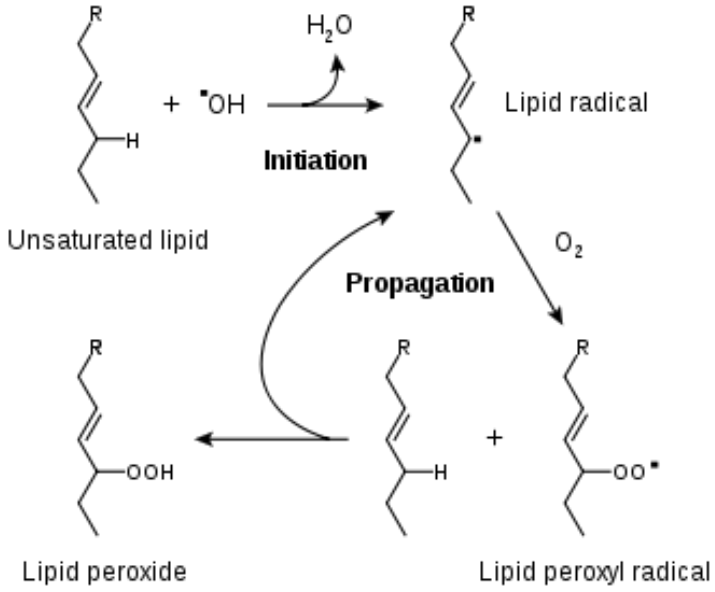

Figure 5. Mechanism of lipid peroxidation. The initiators in living cells are always reactive oxygen species (ROS), such as OH^-, which combines with a hydrogen atom to generate water and a fatty acid radical. The unstable lipid radical reacts readily with oxygen and produces a lipid peroxyl radical, which is also an unstable molecule, reacting with another free fatty acid and generating a different lipid radical and a lipid peroxide if it had reacted with itself. The cycle continues until two radicals react and produce a non-radical species[34].

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Chapter 2. Sodium Pump Endocytosis in Human Myometrial Cells in Response to its Inhibition

Introduction

The sodium pump (SP, also called [Na,K]ATPase) undergoes complex regulation which has been studied for several decades. Several ways are available to govern its abundance and activity. These include effects of the genomic expression of the SP. For example aldosterone, a mineralocorticoid steroid, has present in the cytosol with a nuclear receptor. When aldosterone is increased in the circulation, it can diffuse through cell membranes and combine with its protein receptor. The complex is moved into the nuclear compartment with the complex where it binds to the hormone response elements on the gene(s) coding for the α subunit of the SP thereby increasing its transcription and expression. These events require hours to days to be fully manifest.

In addition, the activity of the SP can be altered by phosphorylation or dephosphorylation of the α subunit protein. Typically, phosphorylation leads to reduced activity and conversely dephosphorylation is typically associated with marked increases in activity. The effect depends on which amino acid is phosphorylated. An example of this is dopamine which binds to a cell surface receptor initiating a cascade of intracellular events typified by the activation of kinases or phosphorylases which add or remove phosphate groups to several proteins, including the SP. These events are rapid, occurring in minutes.

There are other means of regulation. In some cell types some hormones may activate their receptors which in turn can lead to the removal of the SP from the cell membrane, for some in consequence of SP phosphorylation. For example, substantial evidence showed that in renal tubule cells, SP inhibition by dopamine involves endocytosis of SP α- and β- subunits[1], and this inhibition is associated with prior phosphorylation of the α subunit[2].

Another significant means of SP regulation is the presence of specific inhibitors. Structurally, all specific SP inhibitors are sterols with an unsaturated lactone ring coupled to the D ring of the steroid backbone. These are typically circulating inhibitors that bind to an external domain of the SP α subunit with high binding affinity. Not only do these inhibitors produce immediate disruption of the inotropic activity of the SP they also appear to cause SP endocytosis. This has been demonstrated using Western blot analysis of the SP but also using fluorescence imaging and conventional biochemical and biophysical cell separation methods[3].

In these studies efforts were made to clarify and specify the processes that are involved in SP removal from the cell membrane in response to SP inhibition or the application of dopamine, both of which have shown to cause SP removal from the cell membrane. Little information was available about the location of the SP within the cell, in particular the specific organelles used, and whether the pathway was similar to that described for other endocytotic events. Myometrial cells were used to study SP endocytosis in response to the SP inhibitors. The myometrium is the middle layer of the uterine wall which supports stromal and vascular tissue and induces uterine contractions. Myometrial cells are smooth muscle cells rich in the SP. However, primary cultures of myometrial cells have a limited life span. Studies demonstrated that cell culture life

span is related to chromosomal telomere length, and cellular senescence results from progressive telomere shortening and the lack of telomerase expression. Therefore, in order to have continual cell lines, we used telomerase immortalized myometrial cells transfected with human telomerase reverse transcriptase (hTERT) which maintains telomere length and effectively gives normal human myometrial cells an unlimited life span in culture[4].

In order to clarify the changes of SP in response to the inhibitors, we used two approaches. The first used gradient ultracentrifugation to isolate specific organelles before and after exposure to the SP inhibitor ouabain or to dopamine a second agent demonstrated to cause SP removal from the cell membrane. As a second line of evidence we attempted to use confocal microscopy to visualize myometrial cells and potentially subcellular mapping of the SP to organelles treated by ouabain, dopamine or lipopolysaccharide (LPS), another SP inhibitor.

Confocal imaging was invented by Marvin Minsky to overcome some limitations of traditional wide-field fluorescence microscopes in 1957. Conventional fluorescence microscopy images directly and the entire specimen is flooded evenly with light from a lamp. Hence, all parts of the specimen in the optical path are excited at the same time and the resulting fluorescence is detected by the microscope's photodetector or camera, including a large area of unfocused background. In contrast, confocal microscopes use point illumination and a spatial pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal. By scanning one or more focused beams of light, usually from a laser, across the specimen, the point of illumination is brought in focus within the specimen, and laterally scanned under computer control. This technique enables the reconstruction of three-dimensional structures

from the scanned images. Therefore, it is a sophisticated way of analyzing both the inside and outside of cells and has been widely applied in life sciences, semiconductor inspection and material science.

Methods

Cell Culture

Preserved cultures of telomerase immortalized human myometrial cells were taken (~1.5 mL/tube) from the cryo liquid nitrogen, and the tube of cells thawed in 37˚C water bath until there was only a little ice left. Gently, 0.5 mL of the cells were introduced into a culture plate containing 10 mL of warm Dulbecco's Modified Eagle Medium: Nutrient mixture F-12 (DMEM/F12, Invitrogen, Carlsbad, CA, USA) containing 10% Fetal Bovine Serum (FBS, Gibco, Carlsbad, CA, USA) and 0.1% gentamicin (Sigma, St. Louis, MO, USA). The cells were cultured in an incubator at 37˚C and were checked for cell adherence next day. If the cells started to adhere, the medium was exchanged with fresh medium and incubated at 37˚C until cell confluence reached 75-80%, a growing stage which could be passaged with 1:100 trypsin. The culture medium was changed every 2 days to make cells grow well.

Gradient Ultracentrifugation

Sucrose Density Gradient Preparation

5%, 13.75%, 22.5%, 31.25% and 40% of sucrose solution were prepared. 950 μL of each sucrose solution were added in Beckman SW55 Ti rotor tubes sequentially and slowly using a large bore pipette tip (cut off approximately 2 mm from the tip of a 1 mL pipette tip). The tube of sucrose solution layers was kept at 4˚C overnight to form a linear gradient.

Cell lysis and fluorescence label

10 □L of 1 mM ouabain was added to 70-80% confluent myometrial cells and the cells were continued to be incubated in the incubator at 37˚C for 2 hrs or 12 hrs. A 10 μL of sample of miliQ H_2O was added to one plate as a control. After incubation, cells were harvested and lysed in 0.5 mL of homogenization buffer (pH 7.4) containing 250 mM sucrose, 0.5 mM EDTA and 3 mM ididazole at -80˚C overnight. The lysed cells were then stroked in a glass homogenizer for 5 times. The lysate were centrifuged at 1000Xg for 5 min at 4˚C. Supernatant were obtained and incubated with 10 μL of rabbit anti-Rab5 (early endosome antibody, 1:100, Abcam, Cambridge, MA, USA) or goat anti-Rab7 (late endosome antibody, 1:100, Abcam, Cambridge, MA, USA) on a shaker at 4˚C overnight. After incubation, 10 μL of 1:100 Alexa Fluor® goat anti-rabbit IgG (Invitrogen, Eugene, OR, USA) or Alexa Fluor® Donkey anti-goat IgG (Invitrogen, Eugene, OR, USA) was added into the lysates and the mixture was put on ice for 2 hrs.

Ultracentrifugation

100 □L of the crude cellular extract was layered on top of the sucrose gradient solution and centrifuged at 4˚C for 4 hrs at 50,000 rpm. After centrifugation, carefully transfered 300 μL of fractions from the top of the gradient to microcentrifuge tubes with large-bore pipette tips.

Fractions were detected for fluorescent intensities using a fluorometer (ISS, Champaign, IL, USA).

Cell fixation and fluorescence label

Regulatory factors treatment

Confluent myometrial cells were passaged into 4-well Lab-Tek chamber (0.8 mL/well, NUNC, Rochester, NY, USA) and incubated at 37˚C overnight for ~80% confluence. The culture media was exchanged and 2 μL of 0.4 mM ouabain or dopamine solution (final concentration 1 μM) or 1.6 μL of 500 ng/μL LPS was added to the wells respectively and they were incubated at 37˚C for 5 min, 10 min and 15 min. A 2 μL sample of miliQ H_2O was added to one well as a control. The chamber was put on ice to terminate incubation.

Cell fixation

Culture media was removed from the wells and the adherent cells were washed gently with 0.4 mL of PBS per well. 200 μL of 4% para-formaldehyde were added to each well and the whole chamber was incubated on a rocker at speed 2˚ for 30 min at room temperature. Para-formaldehyde was then removed and the wells were washed with 400 μL of PBS for 5-10 min with gentle rocking 3 times.

Blocking

300 μL of PBS-Triton (PBT, 1XPBS, 0.1% Triton x-100) containing 5% FBS was added to each well to block non-specific binding sites. The chamber with blocking solution was incubated on a rocker at speed 2˚ at room temperature for 30 min.

Primary Ab incubation

Mouse monoclonal anti-SP α–3 Ab IgG (ABR, Golden, CO, USA) was used as the primary Ab. 1 μL of 1˚ Ab in 300 μL of PBT containing 2% FBS were added into each well and

the chamber was covered and incubated overnight at 4˚C. After incubation, the 1˚ Ab with PBT was then removed and the wells were rinsed 3X5 min each in PBT.

Secondary Ab incubation

BODIPY® labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) was used as the secondary Ab. 1 μL of the 2˚ Ab in 300 μL of PBT containing 2% FBS were added to each well and the chamber was incubated for 2 hrs at room temperature. After incubation, the 2˚ Ab with PBT was removed and the wells were rinsed 3x for 5 min each with PBS. The wells were air dried. Photos of the fixed cells treated with ouabain/dopamine/LPS were taken by confocal microscope (Olympus FV300 confocal laser scanning biological microscope, 40X). These experiments were carried out ~5 times to find out the optimal concentration and treatment time of the inhibitors.

Besides the above method, we made a lot of attempts to try other Abs and dyes to optimize the assay. For example, we used BODIPY® labeled ouabain (1.6 μL of 500ng/μL per well, Molecular Probes, Eugene, OR, USA) to treat myometrial cells and took images using confocal microscope; we also treated cells with ouabain/dopamine, and then used an early endosome Ab anti-Rab 5B (1 μL of 1 μg/μL, Santa Cruze, CA, USA) and an late endosome Ab anti-MPR (5 μL of 0.2 μg/μL, Chemicon, Billerica, MA, USA) as the primary Ab respectively, and used Alexa Fluor® 546 goat anti-mouse IgG (Invitrogen, Eugene, OR, USA) as the secondary Ab to fluorescence-labeled the endosomes to track their relocation. Similarly, we did ~5 times for each experiment to try different concentrations and treatment time.

Results

The data from sucrose density gradient ultracentrifugation showed that with either 2 hr or 12 hr treatment of ouabain, SP abundance in endosomes increased. Obviously, with 2hr treatment SP in early endosome (EE) were more than in late endosome (LE) (Figure 6), but less than in LE after 12hr treatment (Figure 7). These results indicate that SP relocated in myometrial cells after treated by ouabain and the movement is time dependent.

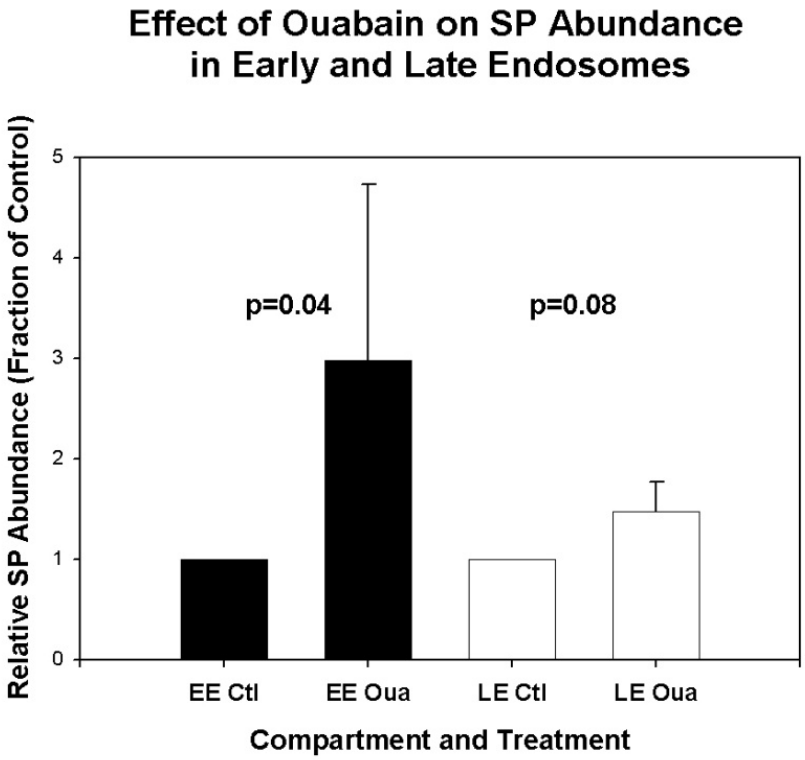

Figure 6. 2hr effect of ouabain on SP abundance in early and late endosomes. Ouabain treated myometrial cells were labeled using early endosome (EE) and late endosome (LE) fluorescent makers and cellular organelles were isolated using sucrose density gradient centrifugation. SP abundance was determined by fluorometer.

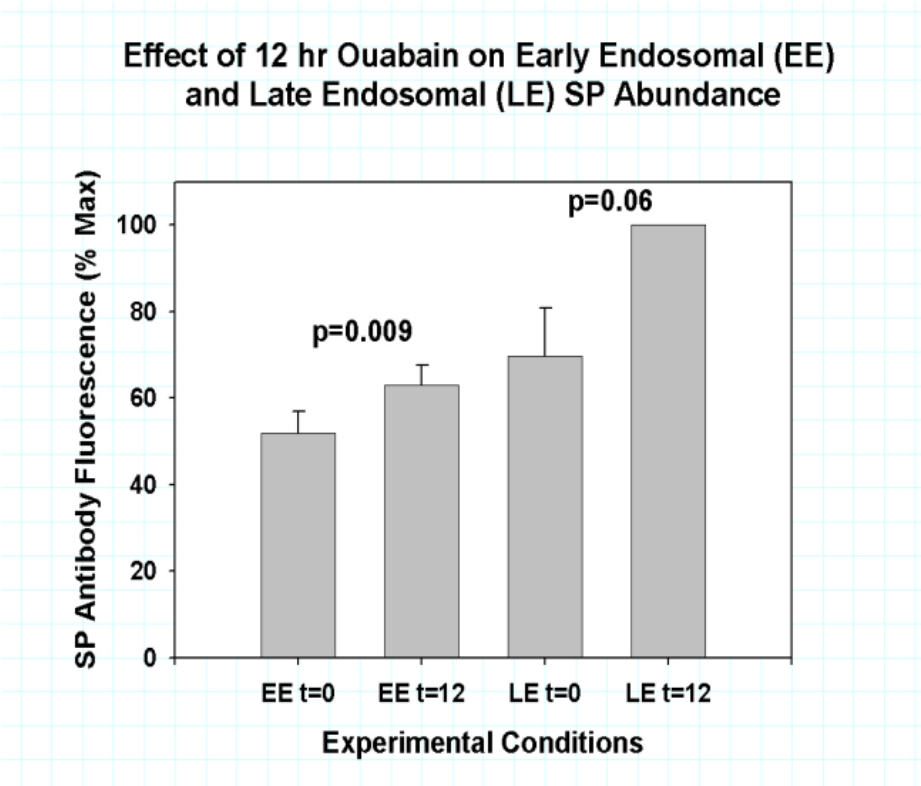

Figure 7. 12hr effect of ouabain on SP abundance in early and late endosomes. Ouabain treated myometrial cells were labeled using early and late endosome fluorescent makers and cellular organelle were isolated using sucrose density gradient centrifugation. SP abundance was determined by fluorometer.

We used dopamine, ouabain and LPS to treat cultured human myometrial cells. In order to reduce the background fluorescence, we fixed the cells in the well of the chamber using para-formaldehyde, and then used fluorescence labeled antibodies to bind with the α-3 subunit of the SP (At the beginning, we carried out some experiments without the cell fixation and blocking steps. This made the background of the images very high and the fluorescence inside the cells was not obvious enough to be identified).

Bodipy labeled antibody (Ab) anti-SP α-subunit appeared to perform better than other Abs and dyes. Images collected by confocal microscopy showed that the addition of both dopamine and ouabain changed cells' shape, specifically caused the elongated uterine smooth muscle cells to

shorten or contract (Figure 8). Notice that cells treated with dopamine (the middle image) and ouabain (the bottom image) became round and short compared with the control cells to which only miliQ H_2O_2 was added. They appear long and narrow in the top image. Moreover, the inhibitors also induced uniform fluorescence intensities, indicating that these inhibitors could result in SP endocytosis. Ouabain appeared to induce more changes in the SP, both in the shape and the relocation of the SP within the cell. LPS did not show any obvious effects on myometrial cells or the SP in myometrial cells using confocal imaging (not shown). This suggests that LPS inhibits SP activity in a different way.

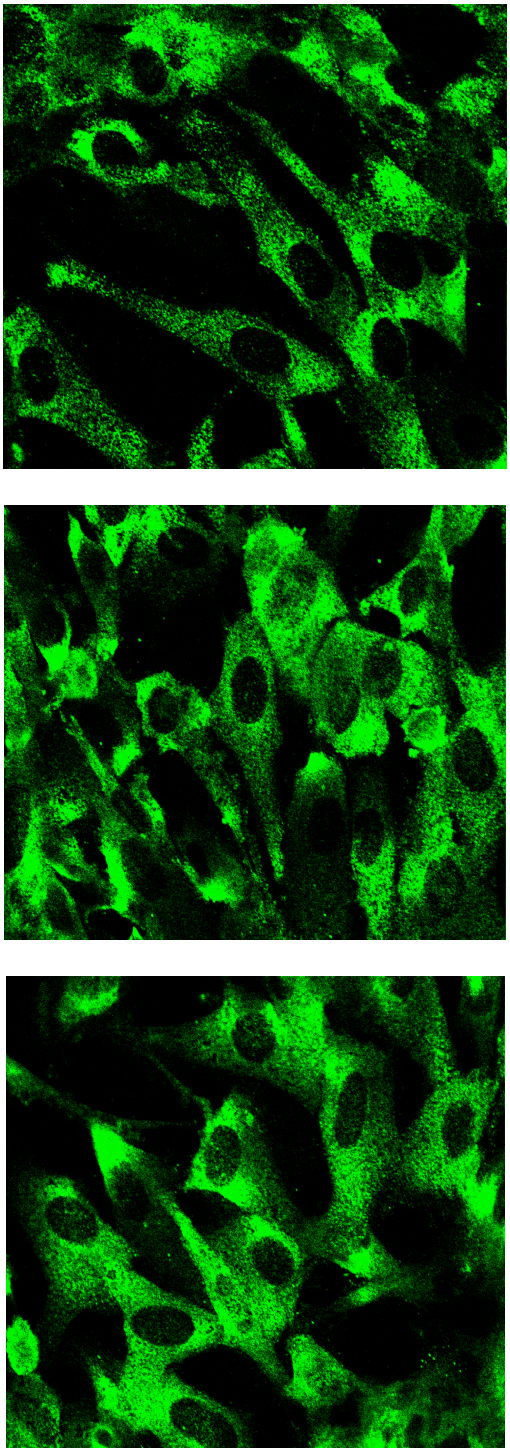

Figure 8. Comparison of effects of dopamine and ouabain on myometrial cells. Pictures were taken by confocal microscope. Top: fixed myometrial cells without treatment; Middle: myometrial cells treated with dopamine for 15 min were fixed and photographed; Bottom: myometrial cells treated with ouabain for 15 min were fixed and photographed.

After we knew that ouabain could induce the greatest amount of endocytosis of SP α subunit, we were interested in the sequence of events for SP removal and translocation within the cell. Hence, we carried out time dependent experiments. We treated myometrial cells with ouabain for different times of exposure and then took pictures using the confocal microscope. Figure 9 showed that with longer ouabain exposure, the cells appeared to demonstrate greater changes. The fluorescence moved from the edge of the cells to the inside gradually, making the fluorescent intensities more uniform little by little. It suggested that ouabain-induced SP endocytosis is time-dependent. However, the imaging did not allow us to clarify where the removed SP was then located. The magnification available with this instrument was not sufficient to allow this.

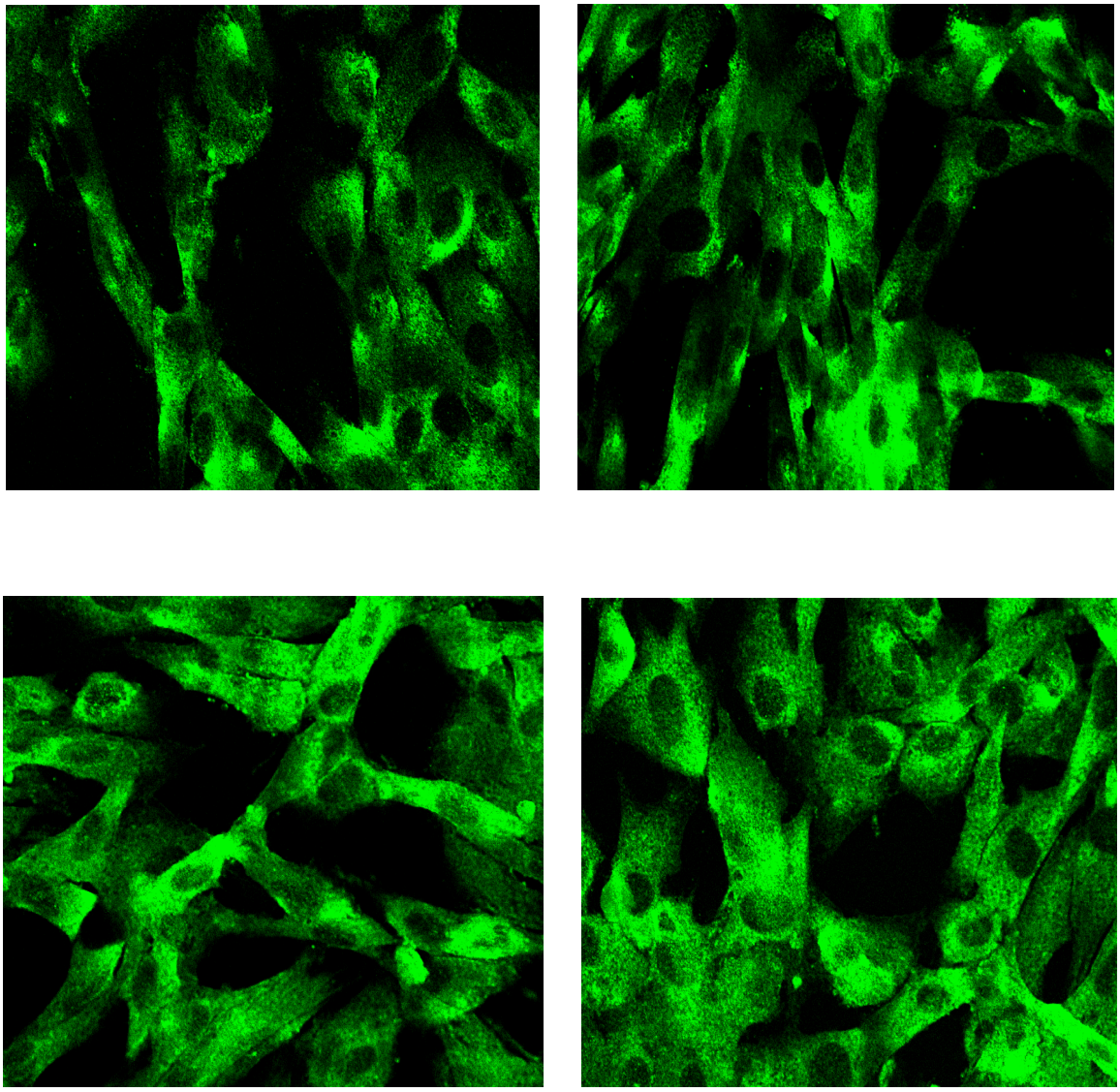

Figure 9. SP regulation in response to ouabain. Pictures taken by confocal microscope showed changes of myometrial cells treated by ouabain with different time (top left: without ouabain, top right: 5 min ouabain treatment, bottom left: 10 min ouabain treatment, and bottom right: 15 min ouabain treatment). Longer time ouabain treatment induced more changes of cells' shape and more uniform fluorescent intensity.

Our results using confocal microscopy were promising. We verified that not only dopamine, but also ouabain could induce SP α subunit endocytosis, and this effect was time-dependent. The changes in cell shape appear to be related to the contraction of the myometrial

smooth muscle cells, consistent with the finding that SP inhibitors lead to uterine contraction. However, due to the poor resolution of the confocal microscope we used, it was hard to further study how the inhibitors induced SP endocytosis, especially the intracellular location of the SP. Eventually, the myometrial cell cultures in our lab were contaminated and additional sample of the immortalized human myometrial cells were unavailable. Finally the project could not be continued.

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Chapter 3: Development of a Theranostic for PE

Introduction

The recent idea of individualized medicine relies on the concept that one can measure biochemicals or genetic information in one patient's body and by knowing this the health care provider can tailor treatment appropriately. This approach has been used successfully for decades for bacterial infections. Culture of specimens then leads to the identification of the specific bacterial organism causing an infection which then dictates which antibiotic to use to insure effective treatment.

More recently, gene analysis has allowed for the identification of specific gene alleles that carry with them a very substantial risk for the later development of breast cancer[1]. Women having this genetic makeup are encouraged to undergo aggressive therapy, including mastectomies, prophylactically to prevent the occurrence of the cancer[2].

It is well accepted that preeclampsia is a syndrome, representing a common clinical endpoint but having more than one cause. In reality none of the proposed etiologies for PE is proven although substantial data suggest the involvement of one or more specific pathologic pathways as the disease progresses. Hence, PE would be an ideal medical target for more specific, rational therapies.

One of the proposed abnormalities in PE that may mediate the increased blood pressure that is a hallmark of PE is the presence of increased levels of a sodium pump inhibitor(s), referred to as endogenous digitalis-like factor or EDLF[3]. The reference to digitalis is based on many studies

indicating similar biological activity and chemical structure of the EDLFs to known plant-derived cardiotonic steroids derived from the digitalis plant. The involvement of these factors in the hypertension of PE has been well enough documented to actually suggest that counteracting EDLFs might improve the maternal features of PE to the extent of prolonging the pregnancy, increasing the viability of the fetus while maintaining a stable maternal condition[4, 5].

Laboratory data have demonstrated that antibodies directed at digoxin, the most commonly used *Digitalis* cardioglycoside, reversed the effects of both known plant derived sodium pump inhibitors and EDLFs[6, 7]. In particular, a digoxin specific antibody Fab fragment named Digibind, used in the treatment of digoxin toxicity, was able to bind and inactivate many, if not most, EDLFs[8, 9]. These laboratory data, which included demonstrations of Digibind having anti-hypertensive effects in experimental animal models of essential hypertension[7], along with a good safety profile for Digibind in individuals treated for digoxin overdose[10], led to the use of Digibind in a few preeclamptic women on a compassionate use basis[4, 5].

These in turn led to a randomized, double blind, clinical trial of Digibind in preeclamptic women in the immediate post partum period[11]. This study demonstrated that women receiving drug required significantly less anti-hypertensive therapy and had faster falling blood pressures than the control preeclamptics receiving placebo.

Based on laboratory data, the incidental use of Digibind in preeclamptics (PE) and the results of the post partum clinical trial led to a FDA approved Phase II clinical trial of Digibind in severe PE[11]. This was designed as a multi-center, randomized, double-blinded clinical study

termed DEEP (Digibind Efficacy Evaluation in Preeclampsia). A total of 51 women were enrolled and 24 received drug for up to 48 hr. The primary outcome measures for the trial were a greater creatinine clearance (a measure of renal function) and reduced use of anti-hypertensive medications in women receiving drug. The first goal was achieved but the second was not. One serious problem with the second goal was that 40% of the women enrolled were already on anti-hypertensive medications and typically such medications would not be discontinued even if blood pressures improved. More information is provided about this particular study for two reasons: Our laboratory provided most of the laboratory experiments that were part of the study and the results of those experiments represent the basis for the research described in this chapter[12].

As mentioned, our laboratory determined the serum levels of EDLFs present in the serum of the severe PE women enrolled in the DEEP trial. The determinations were made using the amount of Rb^+ taken up into human red blood cells over a 45 min period in the presence of serum from non-pregnant individuals, uncomplicated pregnant women and the individual serum from the subjects in the trial. Rb^+ is taken into the cells by the sodium pump, SP or (Na^+,K^+)ATPase, a protein complex that moves 3 sodium ions out of the cell and 2 potassium ions in. Rubidium ion is handled like potassium. If there are inhibitors of the SP present, the amount of rubidium taken up as a function of time is decreased. Also ouabain, a specific SP inhibitor, at 10^{-3} M was used as a control to define 100% SP inhibition. Conceptually, administration of Digibind with its ability to selectively bind EDLFs and block their activity, should cause the SP activity to increase (and the SP inhibition to be relieved), if EDLFs are

present. Likewise, aspects of the disease mediated by EDLFs may also demonstrate improvement.

Indeed, that was observed in the DEEP study. Levels of EDLFs were significantly reduced and SP activity was significantly increased in PE women receiving Digibind compared with PE women receiving placebo (Figure 10). Moreover, creatinine clearance (as a measure of renal function) was significantly higher in PE women receiving Digibind compared with PE women receiving placebo (Data not shown).

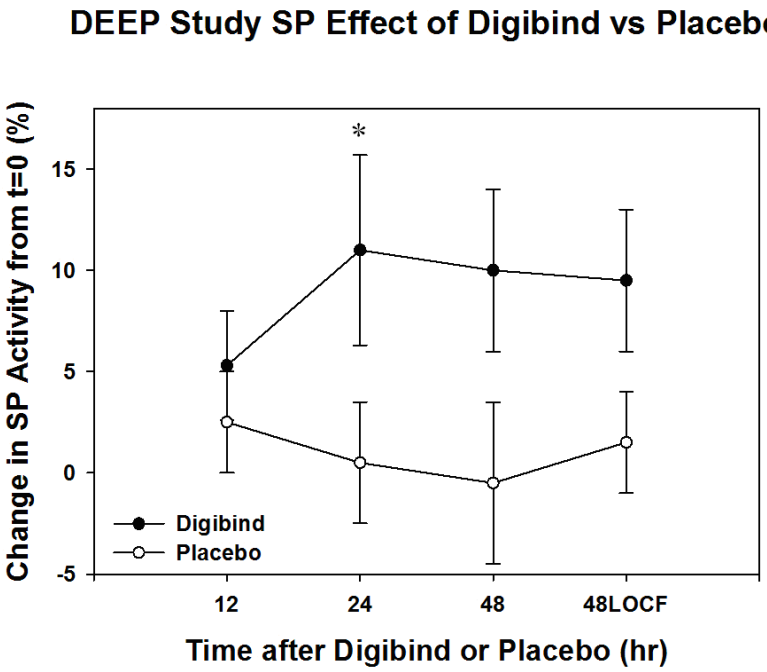

Figure 10. SP activity measured by Rb uptake into human red blood cells. Digibind reduced EDLF levels and hence increased SP activity. 24 hr treatment of Digibind presented a better effect than 48 hr.

As we have continued to analyze the data, we have found two results that suggest that Digibind treatment is a more complex issue than previously thought.

In Fig 11, it is evident that Digibind treatment caused a very significant decrease in SP inhibition in women receiving drug but it did not reverse entirely all of the EDLF produced inhibition. Put another way it would appear that the Digibind dose regimen use in all DEEP subjects was inadequate, at least for some portion of the subjects.

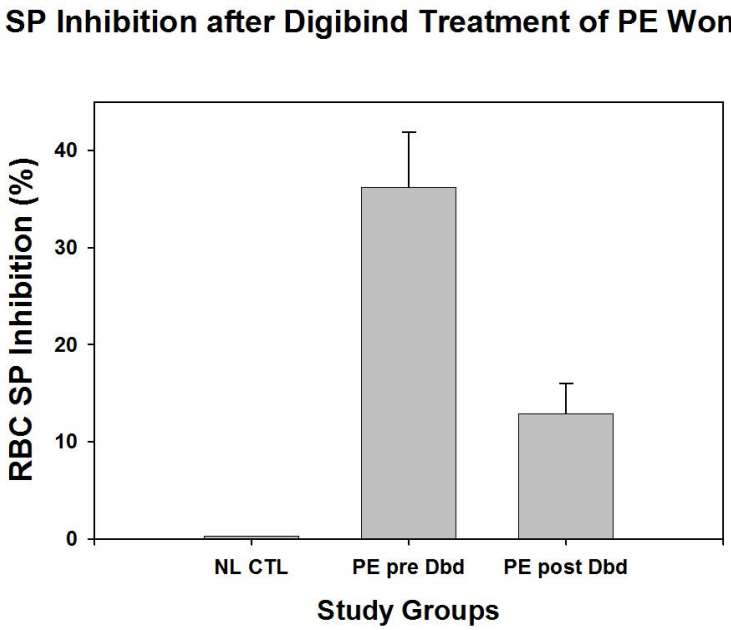

Figure 11. Digibind reversed SP inhibition produced by EDLF. The effect was not thorough but significant.

This leads to the second post hoc analysis that was done which was to determine the distribution of EDLF in the subjects in the study. What was found was that ~20% of the subjects had no measureable EDLF and that many had extremely high levels of EDLF (Figure 12). This is not surprising given the heterogeneity of the disease itself.

This has two implications: First, that Digibind treatment is likely to be ineffective in PE women that are EDLF negative. Clearly, identifying these women would be highly useful to prevent unnecessary and expensive therapy. Second, some women are likely to require a higher dose of Digibind to complex all the EDLF and alleviate its effects. This most likely explains why after treatment that there was not complete normalization of EDLF levels in the treated severe PE women.

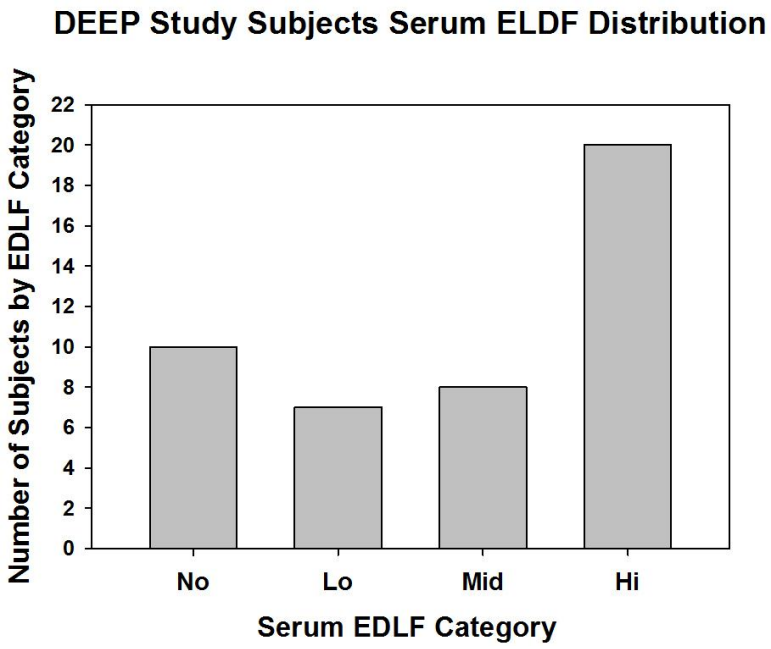

Figure 12. Distribution of EDLF in serum. ~20% of the subjects had no measurable EDLF.

This idea is borne out in part when looking at those severe PE women in the DEEP trial who were EDLF positive. If one considers the data in this way, then one finds that as expected the Digibind effect is more substantial in EDLF positive women and no effect is observed in the EDLF negative women. See Fig 13.

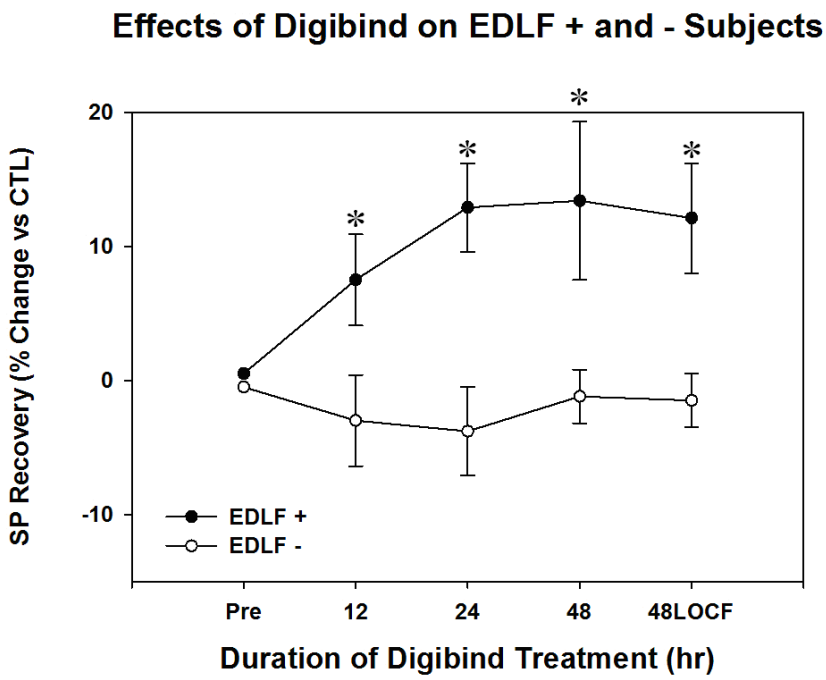

Figure 13. Digibind effects on PE women with positive or negative EDLF. EDLF positive women showed more substantial Digibind effects than EDLF negative women.

Similar results were observed for the creatinine clearances in these women. Specifically, the creatinine clearances were only higher in the Digibind treated EDLF positive women and the Digibind effect was even more pronounced and significant when the EDLF negative subjects were excluded (Data not shown).

Because the DEEP trial experiments suggested that not all preeclamptic women appeared to have appreciable levels of EDLF present in their circulation, the need to identify women likely to respond to therapy and a means to choose an appropriate therapeutic dose became obvious. Consequently, a method to identify women having increased EDLF levels in serum and hence likely to respond to therapy would be of significant value. Because Digibind recognizes and binds the active EDLF in PE, we developed a radioimmunoassay (RIA) using Digibind as the

primary antibody and tritiated ouabain as the tracer. Non-radiolabeled ouabain solutions with graded concentrations were used as standards. The assay then measures the same EDLFs endogenous to PE women which it would bind and inactivate as part of Digibind therapy. Hence, this assay serves as a probe to identify women having observable serum EDLF and which consequently should respond to Digibind treatment, i.e. a theranostic test.

In addition, there appeared to be correlation between this radioimmunoassay employing Digibind and the rubidium uptake Graphite Furnance Atomic Absorption Spectrometry (GFAA) bio assay, which measures inhibition of Rb^+ uptake in human red blood cells. It suggested that this Digibind RIA is a reliable approach to detect EDLF concentrations.

Because Digibind is discontinued and problematically expensive to use as part of a common immunoassay, we sought to identify a comparable monoclonal antibody (Mab) raised against digoxin as the immunogen to replace Digibind. We have screened over 10 different mouse Mabs from different companies with different dilutions and found a few that demonstrate an interaction with the EDLF.

Methods

Placental conditioned medium culture

Placentas were obtained immediately after delivery and 4-5 small tissue pieces (~5mm x 5mm x 5mm) were cut off from the inner section. The tissue pieces were dissected into tiny granules with sterilized scissors. Any visible clots and blood vessels were removed with sterilized tweezers. The remaining villi were washed repeatedly with PBS to remove blood from

the intervillous space. Villous tissue of ~5 mg/well was then patted dry by sterilized paper tower and incubated in a 6-well cell culture plate with 5 mL of serum free DMEM (Gibson, Grand Island, NY, USA) containing 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma, St. Louis, MO, USA) for 48 hours (a general tissue incubation time of placental conditioned media[13]) at 37˚C in an incubator gassed with 95% air and 5% CO_2.

At the end of incubation, samples of the culture medium were collected in 15 ml conical tubes and any residual villi were removed by centrifuging at 4000 rpm for 2 min. The supernatant was stored at -80˚C as conditioned medium until later processing and assay.

Digibind Radioimmunoassay (RIA)

Sera or conditioned media samples were collected and assayed by radioimmunoassay. Digibind (GlaxoSmithKline, Research Triangle Park, NC, USA) was used as the primary antibody interacting with either EDLF or ouabain and rabbit anti-sheep immunoglobulin (IgG) Fab fragment (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used as the secondary antibody. Digibind represents the Fab fragments of an anti-digoxin antisera produced in sheep and used therapeutically to counteract digoxin overdose in humans. Tritiated ouabain (30.0 Ci/mmol, Perkin Elmer, Boston, MA, USA) acted as a tracer. Cold ouabain solutions at graded concentrations were used as standards. A 100 μL aliquot of specimen or standard ouabain solution (50 nM, 0.1 μM, 0.2 μM, 0.5 μM, 1 μM, 3 μM), 50 μL of a 2.22 x 10^{-8} M tritiated ouabain solution, 300 μL of 1.8 μg/mL Digibind solution, 60 μL of 2.12 x 10^{-7} M 2˚ Ab solution and 10 μL of 10 mM pH 7.4 Tris buffer were combined and mixed well, then incubated at room temperature overnight to allow antigen-antibody binding. In conditioned

media assays, because samples contained DMEM medium, 100μL of DMEM were added to standard reaction solutions and 100μL of deionized H_2O were added to specimen reaction solutions to bring them to the same final volume and composition. EDLF in the several conditioned media/homogenate specimens or standard cold ouabain in the calibrating solutions competed with the labeled ouabain for Digibind and then the 2˚ antibody was added to bind to the primary (1˚) antibody-antigen complex and decrease its solubility.

After the overnight incubation (We also tried shorter time incubation such as 2 hrs, 4 hrs and 8 hrs but did not get satisfactory data, so finally chose overnight as the optimal incubation time), 563 μL of 16% polyethylene glycol (PEG)-6000 (Calbiochem, La Jolla, CA, USA) was added to each reaction solution to precipitate the antibody-antigen complex. After centrifugation at 13,200 rpm for 30 min, the supernatant was discarded and the pellet was resuspended in 500 μL of 0.05 M phosphate buffer (pH 7.0). Then 4 mL of Ecoscint™, a scintillation fluid (National Diagnostics, Atlanta, GA, USA) was added to the resuspended solution, and radioactivity in the mixture was measured by scintillation counter to determine EDLF concentration. All individual specimens were assayed in duplicate.

Rubidium (Rb) Uptake Graphite Furnance Atomic Absorption Spectrometry (GFAA)

Conditioned media with EDLF released from cultured placental tissues were assayed by Digibind RIA as previously described to determine EDLF concentration. Simultaneously the samples were assayed by Rb^+ uptake using a graphite furnace atomic absorption instrument (GFAA, model 4100Z, Perkin Elmer, Waltham, MA, USA) that measures [Na^+,K^+]ATPase–dependent Rb^+ transport activity into fresh human red blood cells (RBCs). Ouabain at 10^{-3} M

was used in this assay as a control to achieve complete inhibition of the [Na^+,K^+]ATPase ion transport activity.

Blood was collected from non-pregnant healthy volunteers into EDTA containing tubes, left to stand at room temperature for one hour and centrifuged at 4000 rpm at 4˚C for 10 min to remove the cells from the plasma. The remaining RBCs were washed with two volumes (10 mL) of RbCl buffer (containing NaCl 135 mmol/L, RbCl 6.73 mmol/L, NaH_2PO_4 8.10 mmol/L, Na_2HPO_4 1.27 mmol/L and $MgCl_2$ 1.0 mmol/L, pH 7.45, but omitting K^+) three times vortexing for 5 min before recollecting the cells by centrifuging at 4000 rpm at 4˚C for 10 min.

After washing and the 10% hematocrit solution prepared, 800 µL of RBC, 100 µL of conditioned media sample or control (ouabain at 1.00 x 10^{-3} M final concentration) and 100 µL of Digibind solution (1.00 x 10^{-6} M final concentration) were mixed in an Eppendorf tube and rocked in an incubator at 37˚C at 220 rpm for 45 min to allow Rb^+ ion uptake into the cells. After incubation, each bioassay solution was centrifuged at 4000 rpm for 10 min to isolate the RBCs from the RbCl buffer. The RBCs were then washed by adding 1 ml of ice cold washing buffer (containing choline chloride 149 mmol/L, $MgCl_2$ 1.0 mmol/L, MOPS 5.88 mmol/L, Tris 2.12 mmol/L, pH 7.40) and centrifuged again to remove any residual extracellular Rb^+. This washing process was repeated 3 times.

Cells were lysed by addition of deionized H_2O, centrifuged to remove cell ghosts and stored at -80˚C overnight. Rb^+ uptake into the cells was then measured by standard operation of a GFAA instrument equipped with a rubidium lamp to determine EDLF ability to inhibit

[Na^+,K^+]ATPase mediated ion transport. Before the GFAA step, 10 μL of the isolated RBCs were diluted to 500 μL with deionized H_2O of which the autosampler of the GFAA injected 10 μL for Rb^+ ion quantitation. All samples were tested in triplicate.

Monoclonal (Mab) Radioimmunoassays

This assay was carried out to screen Mabs from different companies at different dilutions. At this stage of development only cold ouabain standards were used, and sera or conditioned media specimens were not tested. Monoclonal mouse antibody anti-digoxin (Mab) listed below was used as the primary antibody anti-EDLF/ouabain and rabbit anti-mouse immunoglobulin (IgG) whole molecule (Sigma, St. Louis, MO, USA) was used as the secondary antibody. Tritiated ouabain (30.0 Ci/mmol, Perkin Elmer, Boston, MA, USA) acted as a tracer. Cold ouabain solutions at graded concentrations were used as standards. A 100 μL aliquot of one of the standard ouabain solutions (50 nM, 0.1 μM, 0.2 μM, 0.5 μM, 1 μM, 3 μM), 50 μL of a 2.22 x 10^{-8} M tritiated ouabain solution, Mab solution (amounts, concentrations and dilutions listed below), 60 μL of 1.8-2.5 mg/mL (original concentration provided by Sigma) 2˚ Ab (typically 1:50 dilution, exceptions listed below) and 10 μL of 10 mM pH 7.4 Tris buffer were combined and mixed well, then incubated at room temperature overnight to allow antigen-antibody binding. EDLF in the standard cold ouabain competed with the labeled ouabain for the Mab and then the 2˚ antibody was added to bind to the 1˚ antibody-antigen complex and decrease its solubility.

After the overnight incubation, 563 μL of 16% polyethylene glycol (PEG)-6000 (Calbiochem, La Jolla, CA, USA) was added to each reaction solution to precipitate the antibody-antigen complex. After centrifugation at 13,200 rpm for 30 min, the supernatant was

discarded and the pellet was resuspended in 500 μL of 0.05 M phosphate buffer (pH 7.0). Then 4 mL of Ecoscint™, a scintillation fluid (National Diagnostics, Atlanta, GA, USA) was added to the resuspended solution, and radioactivity in the mixture was measured by scintillation counter to determine EDLF concentration. All individual specimens were assayed in duplicate.

Mabs we screened:

300 μL of Mab from OEM concepts (Saco, ME, USA) with original concentration 3.5 mg/mL was tested at 1:500 dilution.

150 μL of Mab from MyBioSource (San Diego, CA, USA) with original concentration 5.78 mg/mL was tested at 1:300, 1:400, 1:500, 1:600 and 1:800 dilutions, and the 2˚ Ab was applied at 1:40, 1:50, 1:60, 1:75 and 1:100 dilutions respectively

200 μL of Mab from Genway (San Diego, CA, USA) with original concentration 1 mg/mL was tested at 1:100 and 1:200 dilutions, and the 2˚ Ab was applied at 1:50 and 1:100 dilutions respectively.

300 μL of Mab from Abcam (Cambridge, MA, USA) with original concentration 0.6 mg/mL was tested at 1:100 dilution.

200 μL of Mab from AbD seroTec (Raleigh, NC, USA) with original concentration 1 mg/mL was tested at 1:100 and 1:200 dilutions, and the 2˚ Ab was applied at 1:50 and 1:100 dilutions respectively.

150 µL of Mab from Biodesign (Saco, ME, USA) with original concentration 5.78 mg/mL was tested at 1:300, 1:400 and 1:500 dilutions, and the 2˚ Ab was applied at 1:40, 1:50 and 1:60 dilutions respectively.

200 µL of Mab from Santa Cruz (Santa Cruz, CA, USA) with original concentration 100 µg/ mL was tested at 1:10 dilution.

300 µL of Mab from Lifespan (Seattle, WA, USA) with original concentration 2.89 mg/mL was tested at 1:400 and 1:500 dilutions, and the 2˚ Ab was applied at 1:50 and 1:60 dilutions respectively.

200 µL of Mab from ARP (Belmont, MA, USA) with original concentration 1 mg/mL was tested at 1:100 dilution.

150 µL of Mab from Thermo Scientific (Rockford, IL, USA) with original concentration 5.78 mg/mL was tested at 1:400 dilution.

300 µL of Mab from Sigma (St. Louis, MO, USA) was tested at 1:100, 1:300, 1:500, 1:800, 1;1000, 1:2000 and 1;5000 dilutions according to the datasheet suggestion (1:10000 for ELISA), and the 2˚ Ab was applied at 1:40, 1:50, 1:60 and 1:100 dilutions for each primary Ab dilution.

300 μL Mab from Fitzgerald (Concord, MA, USA) with original concentration 3.45 mg/mL was tested at 1:250, 1:500 and 1:1000 dilutions, and the 2˚ Ab was applied at 1:30, 1:50 and 1:100 dilutions respectively.

Statistical Analyses

Results are reported as the mean ± 1 SEM. Comparisons of the EDLF RIA with the GFAA Rb^+ ion uptake assay were carried out by Pearson's Product Moment Correlation analysis. Effects of time or concentration on EDLF release from placental tissue were analyzed by ANOVA with post hoc Dunnett's pair-wise comparisons. A p-value of <0.05 was considered significant.

Results

Using Digibind as the primary antibody, rabbit anti-sheep immunoglobulin (IgG) Fab fragment as the secondary antibody, tritiated ouabain as tracer, and cold unlabeled ouabain solutions at graded concentrations as standards, a standard curve of Digibind radioimmunoassay was developed for each assay (a typical standard curve is shown in Figure 14). EDLF concentrations were calculated by reference to the ouabain standards and reported as ouabain equivalent concentrations.

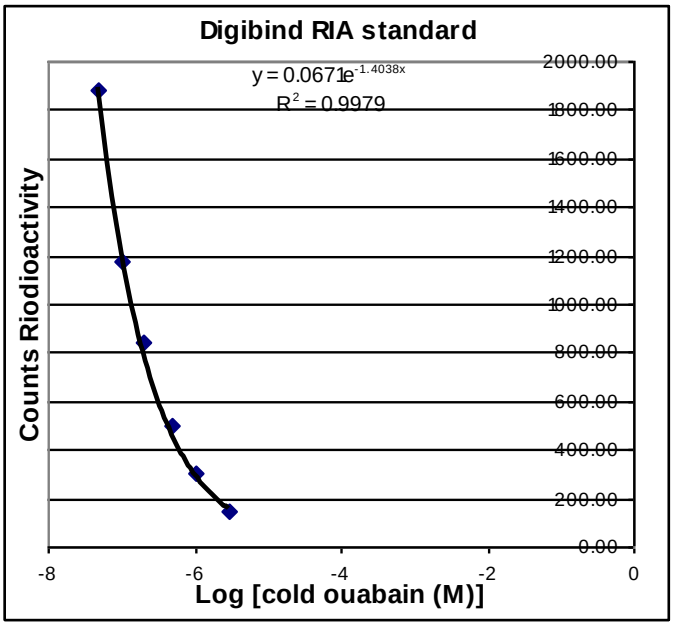

Figure 14. Standard curve for the Digibind RIA. Increasing ouabain concentration (shown as the – log [ouabain]) caused a progressive displacement of [^{3}H]-ouabain from the Digibind and a reduction in counts of radioactivity. The y-axis displays counts of radioactivity measured after the fixed incubation time as a function of ouabain concentration shown on the x-axis

Using the above standard plot, EDLF has been measured by Digibind RIA in the serum of pregnant women with PE (Figure 15). Some women have substantially higher concentrations of EDLF, e.g. serum 2 and 5, than others.

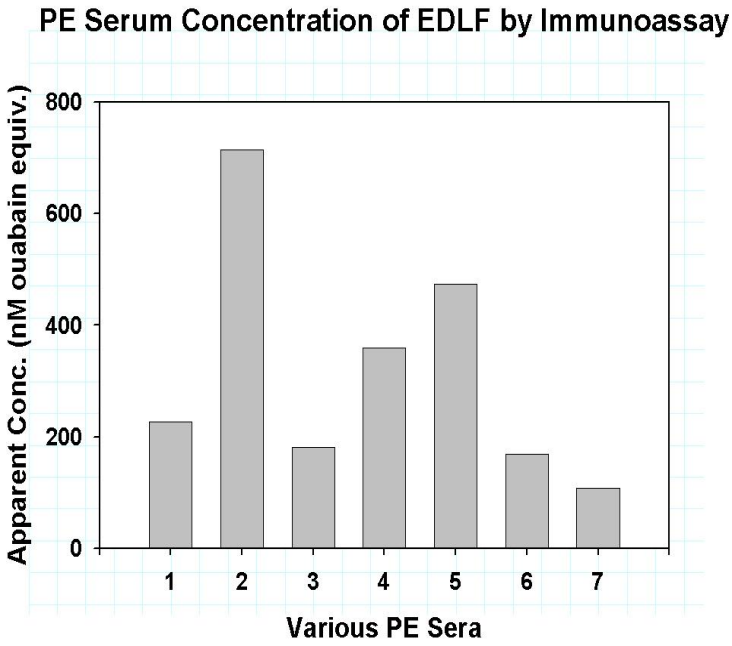

Figure 15. RIA of PE sera. Serum from seven women with PE was assayed using the Digibind RIA. All showed easily detectable levels. Note that some women have substantially higher concentrations of EDLF, e.g. serum 2 and 5, than others.

In addition, this assay was also successfully applied to measure EDLFs secreted in the culture media of freshly explanted human placenta. 11 conditioned media specimens assayed by both Digibind RIA and by SP inhibition Rb uptake using a red blood cell assay demonstrated that there was a significant correlation between these two assays (Figure 16, n=11, p=0.019). The data provided evidence to demonstrate the reliability and comparability of this radioimmunoassay employing Digibind for EDLF and the Rb^+ uptake assay.

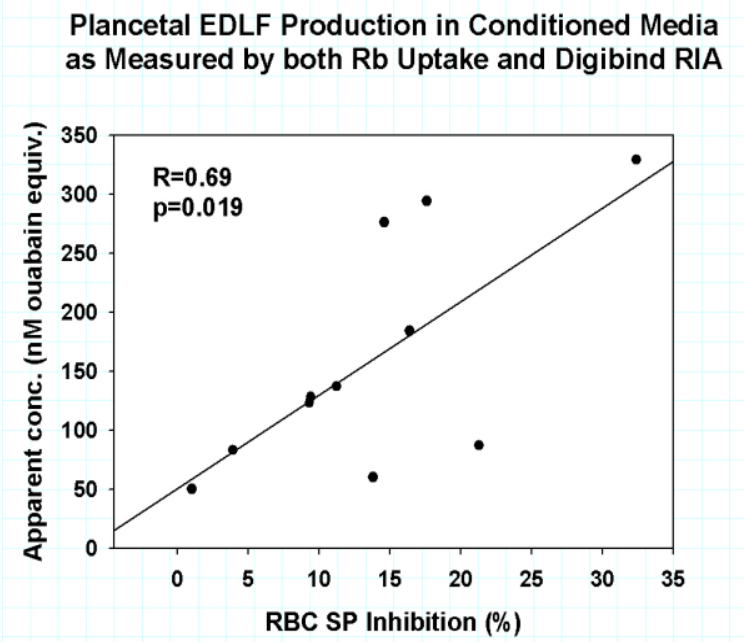

Figure 16. Conditioned media taken from normal human placentas was assayed for EDLF by RIA (y axis) and also for its ability to inhibit the SP in human red cells (x-axis). There was good correlation between the two assays for the several cultured media assayed (n=11, R=0.69, p=0.019).

In an effort to find a monoclonal antibody replacement of Digibind, we screened over 10 Mabs from different companies. Initially we determined the dilution of each Mab according to its original concentration or the manufacturer's datasheet suggestion to make the final concentration close to the concentration of Digibind employed in this RIA. Then additional dilutions were applied if the results showed any promise, i.e. cold ouabain displaced more that 20% of the labeled tracer. Likewise, the concentration of the secondary Ab was also altered if needed. In order to screen more Mabs in a short period, at the beginning of each screen we only used three concentrations of cold ouabain standards instead of the entire set of standards with all graded concentrations, and used a more complete set of ouabain concentrations if the results were promising.

Of all the monoclonal anti-EDLF we screened for replacing Digibind, Figure 17 shows a typical poor performance of a Mab in an RIA assay, which is characterized by very low counts of the radioactivitybeing bound (<500 cpm) or very little change with increasing standard. The low radio-count indicates that the binding between the Mab and ouabain is quite low or even no immuno-binding. Therefore, after the precipitation step, most of free tritiated ouabain was unbound and discarded and the residue tritiated ouabain showed low radio-counts.

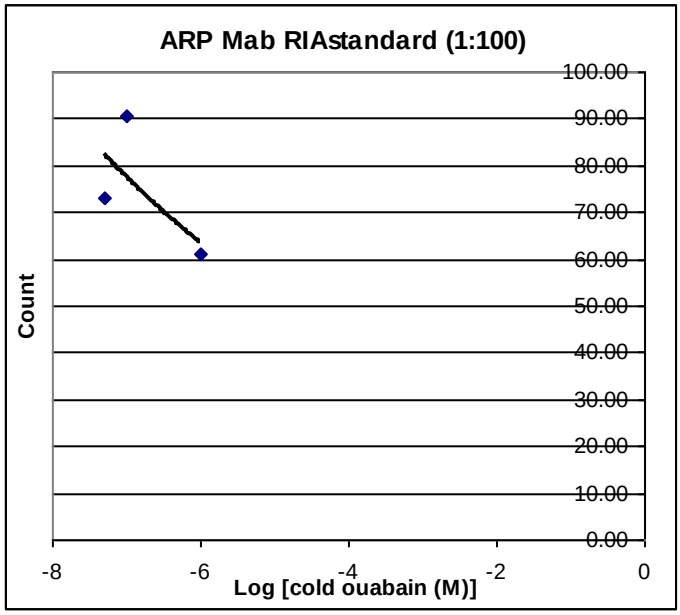

Figure 17. Standard Curve for the monoclonal antibody RIA from ARP. Only three ouabain concentrations (shown as the – log [ouabain]) were tested. The y-axis displays counts of radioactivity measured after the fixed incubation time as a function of ouabain concentration shown on the x-axis.

However, a few Mabs showed higher counts being bound but the trend of the plot was only useful at high (> 10^{-7} M) ouabain concentrations making unlikely that such an assay would measure the relatively low levels of EDLF found in pregnant serum. Figure 18 is a sample of the standard curve from Therm Inc., which is another typical result of the Mabs we screened. With higher cold ouabain concentrations, more ^{3}H ouabain was displaced resulting in a useful change in counts after the precipitation and centrifuge steps, and hence adequate to measure high EDLF. As shown in this figure, we could not see this trend clearly at low ouabain concentration. It means that the binding between ouabain and the Mab might not be strong or stable enough to accomplish the assay comparable to Digibind.

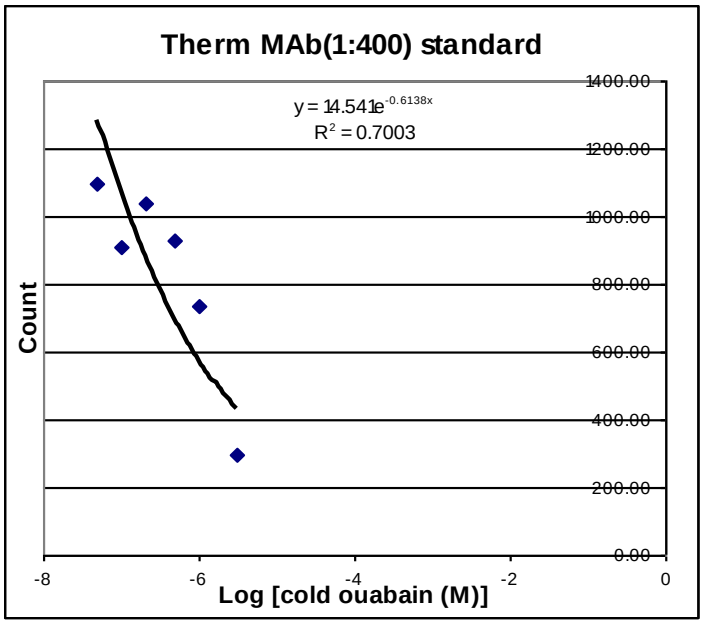

Figure 18. Standard Curve for the monoclonal antibody RIA from Therm. Increasing ouabain concentration (shown as the – log [ouabain]) caused a trendless displacement of [^{3}H]-ouabain from the Mab. The y-axis displays counts of radioactivity measured after the fixed incubation time as a function of ouabain concentration shown on the x-axis.

Nevertheless, Figure 19 shows a sample standard curve of a Mab superior to the others which could be considered for a theranostic. The Mab was from Fitzgerald Industries. Compared with Figure 14, this Mab under the particular assay conditions employed here did not perform as well as Digibind. The trend of the plot is relatively clear compared with other Mabs, but the counts of the radioactivity were not as high as found typically with Digibind, especially the starting point representing the absence of cold ouabain but presence of tritiated ouabain in the reaction mixture, which should show very high radioactivity bound. Improvements in the performance may be achievable with a more extensive optimization of antibody concentration, tracer concentration, etc. However, the intent was to determine the feasibility of a Mab replacement for Digibind and that has been accomplished. Further attempts to identify a better Mab can be carried out as more become commercially available.

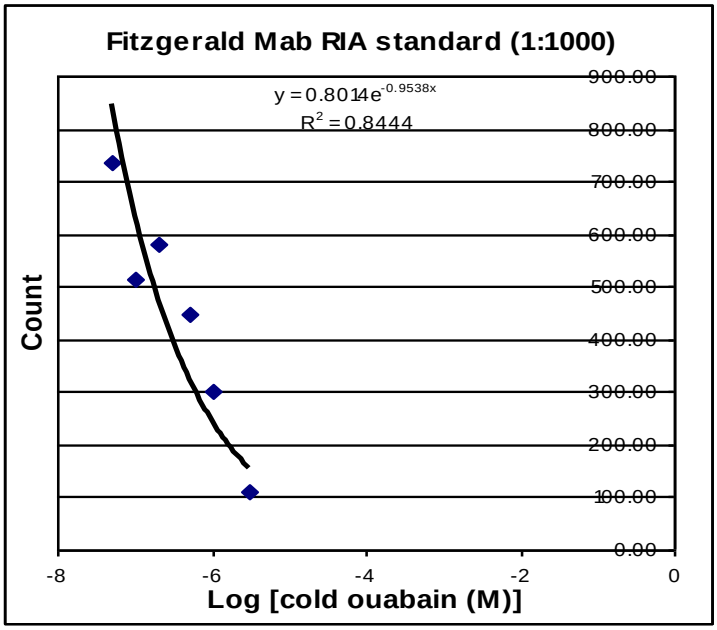

Figure 19. Standard Curve for the monoclonal antibody RIA from Fitzgerald. Increasing ouabain concentration (shown as the – log [ouabain]) caused a progressive displacement of [^{3}H]-ouabain from the Mab. The y-axis displays counts of radioactivity measured after the fixed incubation time as a function of ouabain concentration shown on the x-axis.

Discussion

These studies demonstrate that a theranostic using Digibind was achieved. It appeared able to identify women with varying amounts of serum EDLF. Results using this assay appeared to identify the biologically active SP inhibitor responsible for some abnormalities found in PE as confirmed by Rb^+ uptake assay. This assay provided much more reproducible results compared with the Rb^+ uptake assay (data not shown) and was much easier and simpler to perform. It required only instrumentation that is often found in clinical or hospital laboratories unlike GFAA instruments which are uncommon even at research institutions. Replacement of Digibind with a

digoxin Mab is possible and RIA assays have shown initial promise using existing anti-digoxin Mabs. Using the Fitzgerald Mab may be sufficient to develop a clinically useful RIA. This would require some improvements in assay performance but such optimization is more a commercial undertaking rather than a basic science question.

Additional Mabs can be screened, as they become available, to identify any with greater promise than the Fitzgerald Mab in developing an RIA theranostic. Once the RIA has been developed, it should be possible to adapt the RIA to an ELISA format or a multiplex analyzer format and eliminate the use of radioactive tracer. However, such work is beyond the scope of this project and again reducing the assay to routine practice is now appropriate for companies that specialize in such commercialization practices.

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Chapter 4: Tissue Source and Isolation of EDLF

Introduction

Although there have been many clinical studies indicating that EDLF is involved in essential hypertension and experimental hypertension[1], the chemical identity of the factor or factors present in blood producing the SP inhibition or vasoconstriction, has not been established. Indeed, there have been several compounds isolated from blood, tentatively identified and proposed as the circulating ELDF. All of them are structurally like the steroid glycoside digoxin[2, 3] (Figure 4 in Chapter 1).

Despite the compelling clinical data and experimental animal hypertension data, controversy has surrounded the identity and perhaps even the existence of EDLFs[4, 5]. There have been those who have proposed that these compounds isolated from mammalian sources and touted as EDLF are actually part of the food chain, externally obtained and not produced by the body. For example, ouabain has been proposed as the major EDLF but it contains a rhamnose group attached to the sterol 3-O site and rhamnose has no known synthetic pathway in mammals and is not found as part of native molecules in mammals. Consequently, having clear, unambiguous evidence for tissue synthesis of one or more EDLFs would represent a major milestone in this field.

Moreover, knowing the exact structure of the ELDFs that participates in PE would be very helpful in clarifying EDLF's role in this particular disease and its potential role in one or more of the several actual disease processes that occur in PE. Also, such knowledge would allow for the

development of more accurate measurements of circulating levels of the EDLF or EDLFs that mediate disease in PE women. It would also quite possibly allow for antagonists of EDLF to be developed and used in future therapeutic treatment of women with PE. In the past, the primary obstacle to study EDLF was its low quantities in serum. Consequently, an additional major and unmet need in this field is to identify an easily obtained and maintained tissue source that contains and/or produces useful quantities of one or more EDLFs for use in the laboratory. In order to identify and characterize EDLF in PE, a predictable and enriched source of the factor would seem to be a prerequisite.

Clinical trials revealed that abnormalities of PE, such as hypertension and proteinuria, are reversed when the placenta was delivered[6]. Therefore, we hypothesized that placenta might be a source of EDLF. To test our hypothesis, we developed a method to prepare a consistent homogenate using a ball mill homogenizer and frozen placentas and then deplete intrinsic proteins from homogenates to simplify and purify the specimen and avoid interferences in further assays. EDLF levels in the homogenate specimens were measured by Digibind RIA as described in Chapter 3. Both normal and preeclamptic placentas were assayed and compared.

Furthermore, because progesterone is produced in substantial quantities by the placental, an evaluation of the effect of progesterone on Digibind was designed to determine the crossreactivity of the Fab for progesterone. This was important to insure that the Digibind is specific to and measures only EDLF and hence is an appropriate assay for further studies of EDLF synthesis and regulation.

More importantly we have designed and tested an ultrafiltration method using Digibind as an immuno-affinity agent that can bind the EDLF and extract it. This approach exploits the strong binding between Digibind and EDLF, as well as takes advantage of their distinct molecular weights. Digibind has a known molecular weight of close to 50,000 Daltons, whereas EDLFs' molecular weights are estimated to be around 400-700 daltons. Hence, a specific ultrafiltration membrane with a 30,000 Dalton cut off was considered for use to isolate first free EDLF from large molecular impurities, second the EDLF-Digibind complex from small molecular weight impurities, and finally free EDLF from Digibind. This allowed Digibind to remove EDLF specifically from most of the other materials present in the protein-depleted placental homogenates. Finally the EDLF isolated from placental homogenates or from conditioned media exposed to placenta was examined by Digibind RIA.

Methods

Placental homogenate preparation

Placentas were obtained from both normal pregnancies and preeclamptic pregnancies immediately after delivery and a full thickness cut (~2cm x 2cm x 2cm) was removed, snap frozen in liquid nitrogen and stored at -80˚C until later processing and assay. Placental pieces were shaved into flakes using a surgical blade and tissue flakes were placed into a Sartorius Mikro Dismembrator stainless steel cylinder along with 15 stainless steel balls. The entire cylinder, including contents, was submerged in liquid nitrogen for 4-5 minutes. After the thorough freezing of the cylinder contents, the cylinder was placed in a Sartorius ball mill (Sartorius, Bohemia, NY, USA) and shaken at 2000 rpm for 10 minutes. The process of submersion and shaking was repeated until the contents became a fine powder.

The placental homogenate was transferred from the cylinder to a 50 mL conical tube and the volume was brought up to 5 mL by adding deionized H_2O. To remove protein, two volumes of methanol (10 mL) were added gradually to the homogenate while the mixture was vortexed continuously for 5 minutes. The placental sample mixture was then centrifuged for 10 min at 4000 rpm to remove the precipitated proteins.

The supernatant was transferred to a new conical tube and dried down overnight in vacuo to remove residual methanol. Then the volume was brought back to the original volume of 5 mL using deionized H_2O. The placental homogenate was store at -80˚C for further processing and assay.

Placental conditioned medium culture

As previously described in Chapter 3, placentas were obtained immediately after delivery and 4-5 small tissue pieces (~5mm x 5mm x 5mm) were cut off from the inner fetal side and outer maternal side. The tissue pieces were dissected finely with sterilized scissors. After removing clots and blood vessels, the villi were washed repeatedly with phosphate-buffered saline (PBS) to remove blood. Fetal and maternal tissues of ~5 mg/well were patted dry by sterilized paper tower and incubated in a 6-well cell culture plate with 5 mL of serum free DMEM (Gibson, Grand Island, NY, USA) containing 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma, St. Louis, MO, USA) for up to 48 hours (a general tissue incubation time of placental conditioned media[13 in Chapter 3]) at 37˚C in an incubator gassed with 95% air and 5% CO_2.

During the course of the incubation, samples of the culture medium were collected in 15 ml conical tubes and any residual villi were removed by centrifuging at 4000 rpm for 2 min. The supernatant was stored at -80˚C as conditioned medium until later processing and assay.

Radio immunoassays of cultured media used standards in the assay that contained the same amount of media as was contained in the unknown conditioned media exposed to placental tissue.

Progesterone crossreactivity experiment

In order to determine if results of Digibind RIA determinations represent EDLF levels and not the presence of steroid compounds that weakly crossreact, we tested the effect of progesterone, the main placental sterol, on the assay. Graded concentrations of progesterone (20μL) at final concentrations of 1.00×10^{-9} M, 1.00×10^{-8} M and 1.00×10^{-7} M, 100μL of deionized H_2O, 100 μl of conditioned medium sample, 50 μL of tritiated ouabain solution, 300 μL of Digibind solution, 60 μL of 2˚ Ab solution and 10 μL of 10 mM pH 7.4 Tris buffer were combined and mixed well. A reaction solution containing 20μL of deionized H_2O instead of progesterone solution was used as the negative control. All reaction solutions were incubated, precipitated and analyzed as described in Digibind RIA procedure.

EDLF isolation by ultrafiltration

After depleting proteins and drying off residual organic solvent from the placental homogenate samples, 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 filter devices hold 4 mL of homogenate each. The 4 mL of protein-depleted, placental homogenate were added to the top of the filter and placed in a fixed-angle rotor and centrifuged at 2000*X*g for 10 minutes.

The filtrate (~ 4 mL) was then incubated with 100 µL of $1x10^{-2}$ M Digibind, an amount sufficient to bind all EDLF present in the 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. After incubation, the EDLF-Digibind complex containing solution was passed through a new ultrafitration filter (Ultracel – 30K) again. This time we only centrifuged the EDLF-Digibind for 1 min or until the centrifuge reached full speed and then the centrifuge was turned off. This allowed most of the smaller compounds to pass through the filter and prevented the EDLF-Digibind complex in the retentate to avoid adsorption on the filtration membrane. About 1-2 ml of retentate were then reserved and treated with 392 µL of 0.2 M 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 30K filter a final time followed by centrifuging at 2000*X*g for 2 min. With this final filtration step the Digibind remained in the retentate or on the membrane and the EDLF in the filtrate was essentially purified from other endogenous compounds. The pH of the ending filtrate was then neutralized by adding ~ 18 µL of 1 M Tris (Fisher Scientific, Fair Lawn, NJ, USA).

Statistics

Data are presented as the mean ± 1 standard error. Comparisons of two groups were accomplished by Student's t test if the data were normally distributed or by Wilcoxon Rank Sum Test if not normally distributed. A p-value of <0.05 was considered statistically significant.

Results

Efforts to study the chemical nature of EDLFs have been difficult due to a lack of a readily available, high abundance source of these factors. Research in this area has also suffered from the inability to unequivocally demonstrate that EDLFs are actually produced by human tissues and are not present in the body because of their ingestion and accumulation from food. To this end preliminary data suggesting the presence of one or more EDLFs in placenta led to the hypothesis that this might be a source for EDLFs and might explain their apparent increased presence in serum during pregnancy, especially preeclamptic pregnancies.

Placental homogenate specimens were prepared as described in Methods and assayed by Digibind RIA as described in Chapter 3 to determine EDLF concentrations in homogenates. Figure 20 indicates that protein-depleted placental homogenates from women with PE often appeared to have much higher EDLF levels than found in PE sera (compare with Figure 15 in Chapter 3).

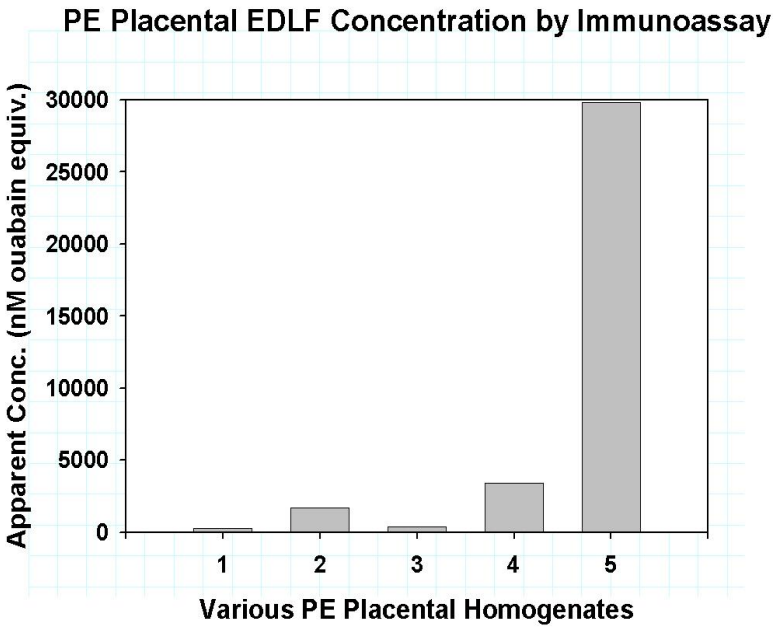

Figure 20. RIA of PE placenta. Protein depleted placental homogenate from five women with PE was assayed using the Digibind RIA. All showed easily detectable levels. Homogenate 4 and 5 have substantially higher placental concentrations of EDLF than other specimens. In general placental concentrations were much greater than serum concentrations.

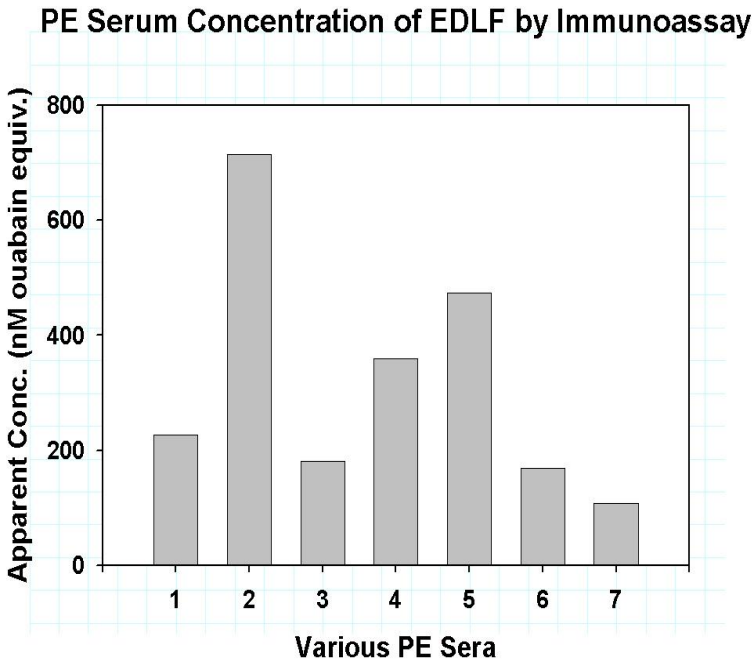

Figure 15. RIA of PE sera. Serum from seven women with PE was assayed using the Digibind RIA. These results show lower EDLF levels in PE sera than in PE placental homogenates (notice the different scales shown in Fig 20), but having comparable levels to those secreted EDLF levels from normal placentas (Compare with Fig 21).

As was found with PE serum, some women have substantially higher placental concentrations of EDLF than others, e.g. homogenate 4 and 5. In addition, with this assay, we detected levels of the EDLF factor or factors secreted in the culture media of freshly explanted normal human placenta (Figure 21). The concentrations of secreted EDLF in the media from normal placental culture were in the range of those found in PE sera (Figure 15).

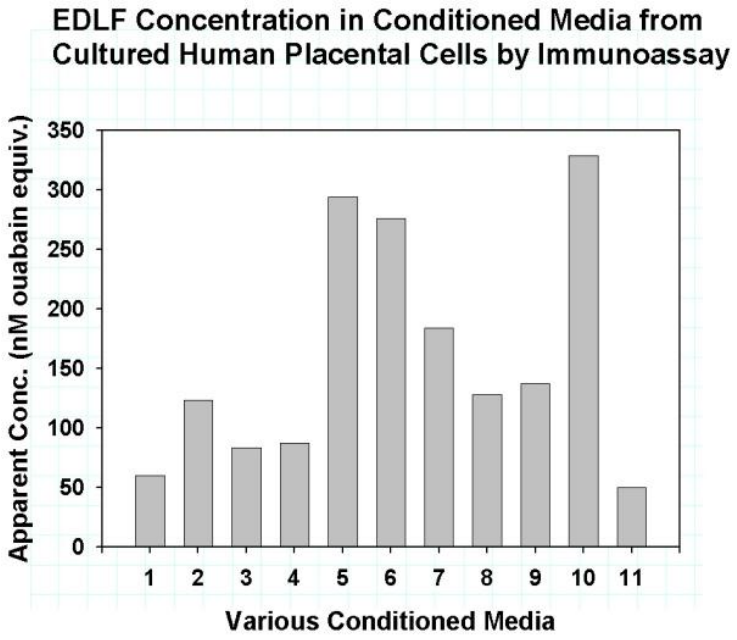

Figure 21. RIA of EDLF produced by cultured normal human placenta and accumulated over 48 hr. Media used to culture fresh human placenta was protein depleted and assayed by RIA. All placentas elaborated EDLF from PE women. In general media concentrations were in the range of those found in serum.

When more carefully examined, it was found that after 48 hr in culture, the fetal placental membranes produced significantly higher levels of EDLF than did comparable amounts of maternal placental tissue. See Figure 22 (n=5, fetal: 178 ± 14.9 vs n=5, maternal: 12.3 ± 6.3 nM EDLF equiv, p=0.04). The EDLF was also shown to accumulate as a function of time in the culture medium (Figure 23, n=5, 6hr: 87.6 ± 2.56, 12 hr: 237.0 ± 6.92, 24 hr: 393.2 ±14.76; 36 hr: 675.9 ± 26.89, 48 hr: 813.8 ± 18.77 nM ouabain equivalents EDLF; $p<0.05$) indicating EDLF production. Based on these observations, and Figure 16 in Chapter 3, which showed that the concentrations of the secreted factors detected by Digibind RIA appear to be the same as the secreted placental circulating factors with the ability to inhibit the SP activity in red blood cells,

we concluded that the factors secreted in the culture media were EDLF and placenta appeared to be a source of EDLF.

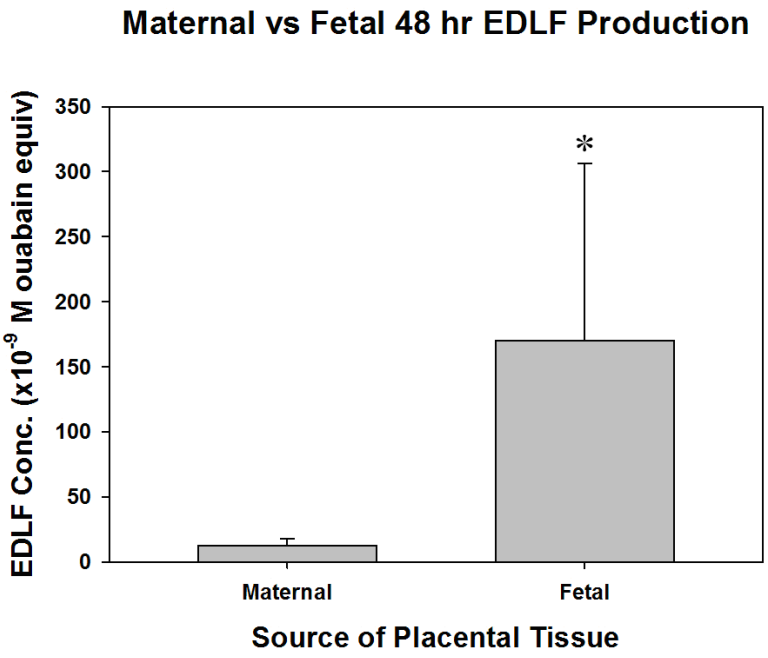

Figure 22. Comparison of human placental maternal membrane and fetal membrane EDLF levels released after 48 hr culture. Concentrations of EDLF secreted from fetal side of placentas were significantly higher than maternal side (n=5, p=0.04).

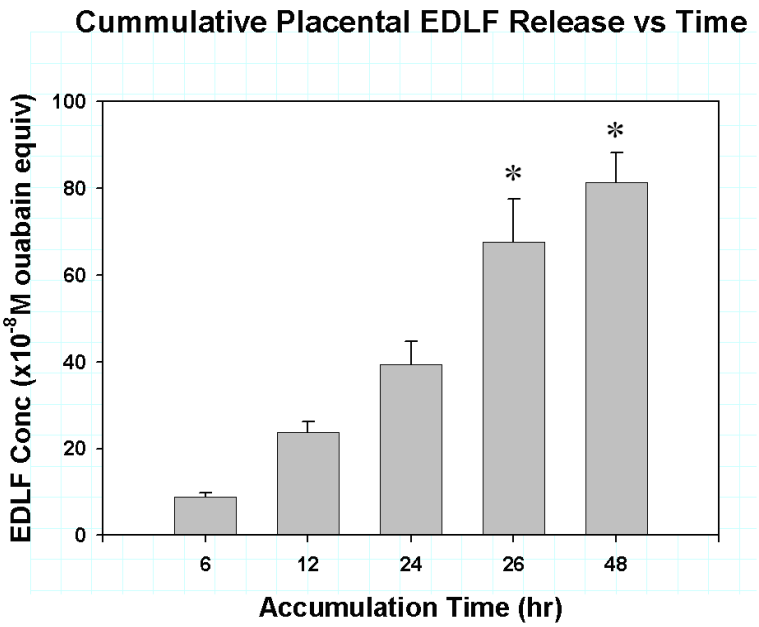

Figure 23. EDLF accumulation in culture medium as a function of time. Longer incubation times produced significantly more EDLF secreted into the medium (n=5, $p<0.05$).

Given that the placenta produces substantial quantities of steroid hormones, particularly progesterone, it was important to insure that the factor(s) detected by the Digibind RIA was EDLF and that the observed levels of EDLF did not represent progesterone. Experiments were conducted to determine progesterone crossreactivity with the RIA. EDLF levels could not be accounted for by progesterone being measured by the RIA. Even adding enough progesterone to produce a final concentration of $1.2X10^{-7}$ M of progesterone, which is substantially higher than reported levels of placental progesterone in the literature[7], the concentrations of EDLFs secreted into the media were not altered appreciably. Also, progesterone does not inhibit the RBC SP.

In addition, experiments were conducted to demonstrate that the concentration profile of EDLFs measured on dilution showed the anticipated proportional effect unlike non-specific interferents with RIAs which often have a somewhat constant negative interference with the

assay at most concentrations. Digibind RIA data of EDLF concentrations in placental homogenates from women with PE and those from women with uncomplicated pregnancies showed that PE placentas had increased EDLF levels and this difference between normal and PE homogenates were still significant even with sequential dilutions of the specimens (Figure 24, n=8, ctl: 74.4 ± 4.1 vs n=8, PE: 329 ± 52 nM EDLF equiv, p=0.0002). These data further verified that RIA employing Digibind as the antibody is a reliable approach to evaluate EDLF levels, human placentas could be used as an enriched source of EDLF and the placental EDLF secreted in the culture media could be analyzed for the study of EDLF synthesis and regulation.

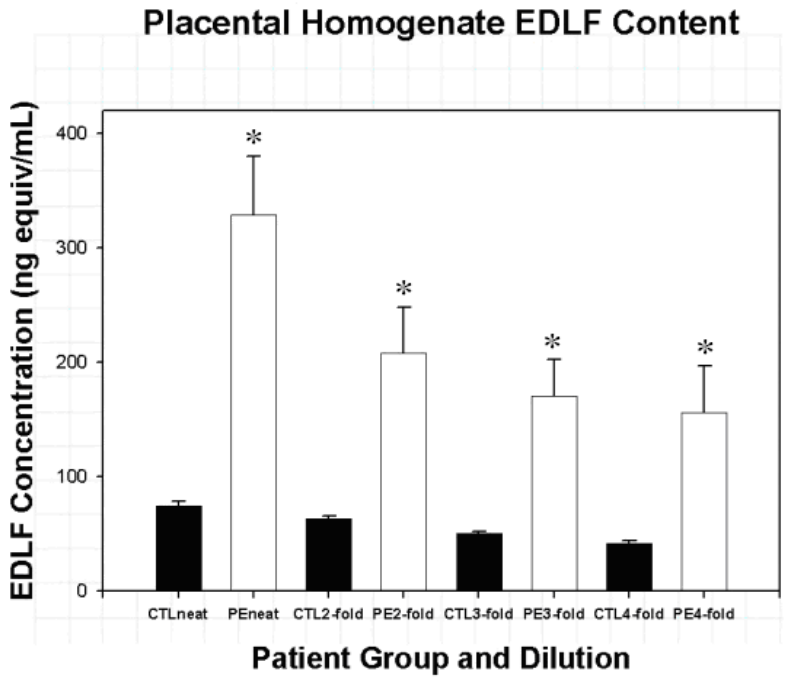

Figure 24. Comparison of the EDLF concentration in protein free placental homogenates from women with preeclampsia (n=8, PE) versus women with uncomplicated pregnancies (n=8, CTL) by radioimmunoassay. Differences were statistically significant for undiluted homogenate (neat, p=0.0002) or with sequential dilutions (1:2 dilution p=0.002, 1:3 dilution p=0.002, 1:4 dilution p=0.02)

In order to further verify the presence of an EDLF in placental homogenates, EDLF isolation from other components of placental homogenates was accomplished using an affinity approach employing Digibind coupled with ultrafiltration. The assay was applied to measure free EDLF levels (none containing Digibind) at different stages of the purification process: first in the protein-depleted homogenate, then after its removal from the homogenate by the Digibind and finally the EDLF after both release from the Digibind and after ultrafiltration (Figure 25). The protein-depleted homogenate reconstituted in buffer (Tissue, left bar) showed substantial amounts of ELDF measured by RIA. Then this protein-depleted homogenate was incubated with Digibind. The EDLF, which is small, was bound by the Digibind, which is large. When the mixture was ultrafiltered (UF) through a 30 kD ultrafilter, the EDLF was mostly retained by the Digibind Fab with very little appearing in the ultrafiltrate (Filtrate, middle bar). RIA measurement of EDLF showed significant ($p<0.05$, Wilcoxon Rank Sum Test, $n=4$) reductions in EDLF levels after Digibind treatment and UF. Then glycine at pH 3 was used to release the EDLF from Digibind and this solution was ultrafiltered. EDLF was found to be released and then to pass through the UF membrane. The solution was then neutralized and assayed by RIA for EDLF (Released, right bar) which demonstrated significantly more EDLF ($p<0.05$ Wilcoxon, $n=4$) present than in EDLF depleted solution shown in the middle column, providing solid evidence that EDLF was retained by Digibind and then released.

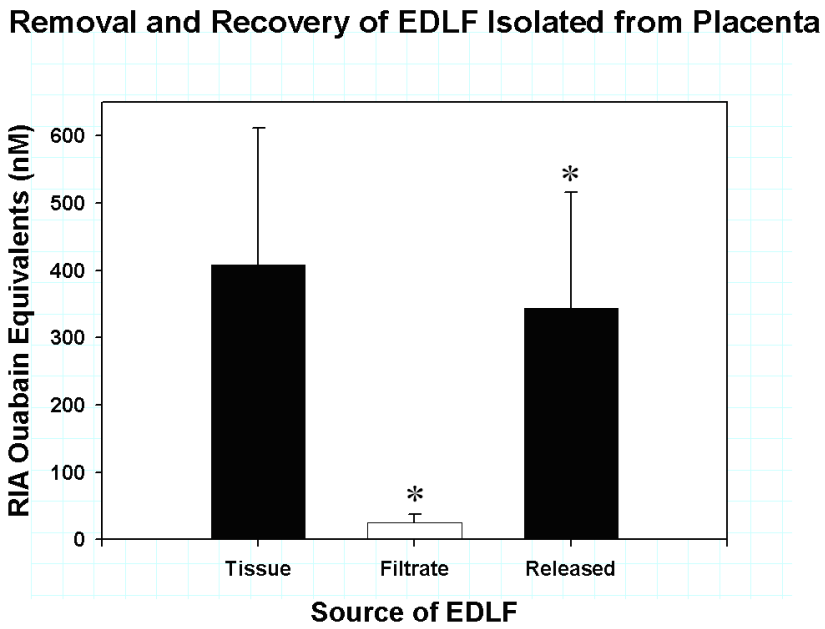

Figure 25. Use of Digibind as an affinity material. Columns showed free EDLF concentrations measured by Digibind RIA. Tissue, left bar: the protein-depleted homogenate; Filtrate, middle bar: homogenates incubated with Digibind and then ultrafiltered through a 30 kD ultrafilter; RIA measurement of EDLF showed significant ($p<0.05$, Wilcoxon Rank Sum Test, n=4) reductions in EDLF levels after Digibind treatment and UF; Released, right column: pH 3 glycine treated solution followed by another ultrafiltration; RIA data demonstrated significantly more EDLF ($p<0.05$ Wilcoxon, n=4) present than in the filtrate.

Discussion

One of the most fundamental limitations of research on EDLFs has been the lack of a clear demonstration of its secretion from a mammalian tissue source. Critics have contested the very existence of endogenous cardiac sterols in humans proposing that the SP inhibitory activity observed in the serum of individuals with hypertension may arise from external sources. Indeed ouabain administered to animals is actively taken up by the adrenal[1, 8]. Digoxin and digitoxin are dispersed into lipophillic tissues and small amounts equilibrate slowly with the vascular

compartment[1, 8]. Hence, plant-derived cardiac glycosides could be partitioned into animal tissue albeit in very small amounts.

Our results collectively strongly support that human placenta produces and secretes one or more EDLFs. This presents compelling evidence for a synthetic pathway being present in at least one human tissue. This pattern of secretion was not uniform throughout the placenta, but high levels were clearly shown to come from the fetal membranes. It is known that the trophoblast cells of the placenta have the ability to synthesize sterol compounds[9]. However, this EDLF secreted by the placenta cannot be explained by the high levels of progesterone produced by the placenta. Experiments described in the next chapter extend these findings by exploring regulation of EDLF systhesis.

The need for an enriched source of EDLF has been a long standing need in this field of research. These results demonstrated compellingly that we have a reliable and enriched source of the EDLF of PE --- placenta. Not only that but the evidence strongly suggests that human placenta produces and releases substantial amounts of EDLF, quantities similar to those found in the circulation of pregnant women. In addition, we have a sensitive and specific approach to detect EDLF levels in placenta --- a radioimmunoassay employing Digibind as primary antibody. Finally, we have developed ways of isolating EDLFs using Digibind as affinity agent and using a selected ultrafiltration step to isolate EDLF from placental specimens or from conditioned media. These results make the further study of EDLF structure, its synthesis and regulation promising.

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Chapter 5: EDLF Synthesis and Regulation in Placenta

Introduction

Having developed a specific immunoassay to measure EDLF concentrations and having identified a promising tissue source of EDLF production, it was then possible to attempt to provide a greater understanding of EDLF tissue synthesis and to identify factors that may regulate that production. Such a demonstration of specific synthetic steps and regulated secretion of EDLFs would represent a marked advance in this research. It would help to integrate EDLF into the overall context of PE and other hypertensive diseases.

Current evidences all suggest that EDLF is a sterol having an unsaturated lactone ring attached to the 17-C of the steroid backbone[1, 2]. Consequently, the hypothesis is that its synthetic pathway should share steps with the steroid synthetic pathway. There are known substrates of these enzymatic steps and also some agents that can block the enzymes involved in the pathway. In this chapter, we applied these known agents to placental tissues in culture and tried to elucidate mechanisms of EDLF synthesis and regulation using the Digibind radioimmunoassay.

First of all, we needed to verify the hypothesis that synthetic pathway of EDLFs share steps of the steroid synthetic pathway. It is known that ketoconazole is an agent that generally blocks steroid synthesis by inhibiting several cytochrome P-450-dependent enzymes. These kinds of enzymes are widely involved in steroidogenesis, such as cholesterol side-chain cleavage enzyme, hydroxyl steroid dehydrogenase (HSD), 17α-hydroxylase, 17-20 lyase and 11β-

hydroxylase (shown in Chapter 1, Figure 3)[3]. Therefore, we hypothesized that ketoconazole would inhibit EDLF synthesis. If the EDLF biosynthetic pathway shares steps with steroidogenesis, ketoconazole should be an inhibitor of EDLF synthesis, and consequently, addition of ketoconazole into placental tissue culture would decrease EDLF concentrations.

It was also hypothesized that if the EDLF synthetic pathway shared steps with steroidogenesis one or more precursor compounds such as 17-hydroxyprogesterone could have an effect on EDLF production in placental tissue culture. As shown in Fig 3 and Fig 4, 17-hydroxyprogesterone is in the center of the steroidogenesis pathway and may contribute the sterol backbone to EDLF structure. If its presence has an impact on EDLF production, it would be consistent with its being an intermediate or substrate of EDLF synthesis. In order to further clarify the role of 17-hydroxyprogesterone in the pathway, time course experiments were carried out. Steady accumulation of placental EDLF with 17-hydroxyprogesterone treatment by time would indicate that it is potentially a substrate of EDLF synthesis.

Besides EDLF synthesis, it is of considerable interest to understand more about EDLF regulation. There are a several regulatory factors that are thought to be important in mediating features of PE. Specifically, PE is thought to be a state of reduced oxygen perfusion leading to increased oxidative stress with elevated levels of reactive oxygen species (ROS), including hydrogen peroxide[4]. Also, it is thought to be a state of vascular endothelial damage with increased levels of inflammatory cytokines[5]. One of the best documented of these in the setting of PE is tumor necrosis factor α (TNFα). In this chapter, we hypothesized that there would be effects of hypoxia, ROS, and cytokines on EDLF levels produced by placental tissue in culture.

We also sought to determine if one of these pathways was responsible for the other, e.g. hypoxia leading to increased cytokine production, to make clear if any or all of these factors contribute directly to EDLF synthesis and release. Identifying the regulatory factors and elucidating the mechanisms of their actions in PE would be very helpful to construct a more complete story of EDLF synthesis and regulation, and hence provide deeper insights into the pathogenesis of PE and subsequently develop earlier diagnosis and effective prevention of or therapeutic approaches to PE.

It is believed that a decrease in oxygen to placenta tissue is a hallmark of PE. Placental hypoxia resulting from reduced uteroplacental perfusion then leads to maternal endothelial dysfunction, which in turn leads to later development of hypertension or impaired renal function, important features in PE[6, 7]. Given that EDLFs can be causative factors of hypertension, knowing the relationship between hypoxia and EDLF release would be helpful to understand the pathogenesis of PE. In this Chapter we hypothesize that hypoxia is a condition that stimulates greater EDLF levels in placenta. Hypoxia may induce endothelial dysfunction which stimulates EDLF or perhaps it leads to increased EDLF which may contribute to endothelial dysfunction[8].

There is increasing evidence supporting the possibility that oxidative stress is an important contributor to the pathogenesis of PE. Compared with healthy pregnant women, preeclamptic women have higher levels of ROS and lower levels of several dietary antioxidants in their blood, including vitamin C and E, lycopene and betacarotene[9]. Elevated levels of markers of oxidative stress, such as hydrogen peroxide, have also been observed in the placenta and circulation of preeclamptic women. Overproduction of ROS destroys endothelium-derived nitric oxide in vitro

and decreases its bioavailability, resulting in impaired endothelium-dependent vasodilatory response and increased vascular resistance, which consequently could contribute to hypertension[10]. Therefore, we hypothesized that oxidative stress could also regulate EDLF levels in placenta.

Endothelial dysfunction, including altered levels of inflammatory cytokines, has been thought to be a central pathophysiological feature in the maternal vascular system in PE[11]. Also, increased circulating levels of cytokines, such as TNFα and IL-6, have been found in preeclamptic women[12]. Hence, PE could be considered as a state of exaggerated inflammatory response. Convincing evidence suggested that inflammatory cytokines link placental hypoxia with maternal endothelial, cardiovascular and renal dysfunction, all of which are abnormalities that occur in PE[11]. Furthermore, our collaborator at Louisiana State University, Dr. Yuping Wang, found that TNFα-induced inflammatory response in endothelial cells could be reduced by Digibind[8]. Consequently, we hypothesized that cytokines are regulatory factors of EDLF production, that they induce an inflammatory response in PE by up-regulating EDLF release from endothelial cells, and Digibind reverses this effect by binding with EDLF.

The results of the experiments presented here should be helpful in elucidating the mechanisms and regulation of EDLF synthesis and should allow for more focused ideas and studies on both the EDLF synthetic pathway and a possible therapeutic.

Methods

Placental tissue culture with specified treatment

Placentas were obtained fresh shortly after delivery and processed as described in the section "Placental conditioned media culture" in Chapter 3. Dissected placental tissues of 5 mg/ well were placed in a 6-well cell culture plate with serum free DMEM (Gibson, Grand Island, NY, USA) of 5 mL/ well containing 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma, St. Louis, MO, USA). Before incubation, specified treatments were carried out following the descriptions provided below respectively:

Effects of a general steroid synthesis inhibitor, ketoconazole, on placental EDLF production.

According to earlier published in vitro experimental data from other labs, which suggested that 2-100 μM of ketoconazole could reduce cholesterol synthesis, we chose 1 μM, 2 μM, 5 μM, 10 μM and 20 μM as the concentrations of ketoconazole for these experiments[13, 14, 15]. The graded concentrations of ketoconazole (Sigma, St. Louis, MO, USA) were added to the medium which contain dissected placental tissues respectively and mixed well. Tissue culture without ketoconazole was used as a control. In addition to 48 hr, the general placental tissue culture incubation time, we also did 24 hr experiments to explore the effects of ketoconazole on EDLF production with shorter time incubation. This was repeated 5 times.

Effects of a possible steroid synthesis precursor, 17-hydroxyprogesterone, on placental EDLF production.

Previous clinical research reported that human serum contains17-hydroxyprogesterone (17-OHP) at levels around 2 nM, and that this level of 17OHP is 100- to 1000-fold too low to be candidate for EDLF[16, 17]. Thus we carried out tissue culture experiments to assess the role of 17-hydroxyprogesterone at concentrations of 200 nM, 500 nM, 1 μM and 2 μM on EDLF synthesis. The graded concentrations of 17-hydroxyprogesterone (Sigma, St. Louis, MO, USA) were added to the culture plate with medium as well as dissected placental tissues respectively and mixed well. Tissue culture without 17-hydroxyprogesterone was used as a control. Because it has been demonstrated that fetal placental tissues produce significantly higher EDLF levels than maternal tissues, or maybe only fetal tissues produce EDLF. This is based on earlier studies reported in Chapter 4, Figure 22 and on the results of ketoconazole experiments reported later in this Chapter which showed that maternal tissues produced too little EDLF to affect the integrated EDLF from the entire placenta (details in the following "Results" section). Consequently, we used whole placenta cuts instead of attempting to separate the two sides for all following experiments. 48 hr incubations were carried out. This was repeated 6 times.

Because our earlier data have shown that EDLFs were accumulated as a function of time in placental tissue culture (Figure 23 in Chapter 4), we were interested if there were similar or enhanced accumulation of EDLF production with the treatment of 17-hydroxyprogesterone as a function of time. Hence, time course experiments (6 hr, 12 hr, 24 hr, 36 hr and 48 hr) were then performed 5 times to study the effect of the potential substrate.

Effects of hypoxia on placental EDLF production

In order to clarify if hypoxia could affect EDLF levels in placenta, we placed the culture plates with dissected placental tissues in a portable air chamber (Billups-Rothernberg, Del Mar, CA, USA) flushed daily with a gas containing 2% oxygen, 5% carbon dioxide, 93% nitrogen (Airgas AcuGrav^{TM}, Salt Lake City, UT, USA) following hypoxic experiment conditions reported by our collaborator of LSU[18]. The chamber was also housed in a regular incubator gassed with 95% air and 5% CO_2 to maintain 37˚C for 48 hr. Culture plate with similar media and tissues placed directly in the regular incubator was used as a control. Also, we did 24 hr incubations. This was repeated 6 times.

Effects of reactive oxygen species on placental EDLF production

We chose a known and also commercially available ROS, hydrogen peroxide, to elucidate the effects of ROS on placental EDLF production. Given that high concentrations of hydrogen peroxide are damaging to tissues, we started these experiments with hydrogen peroxide concentrations of 1 nM, 5 nM, 10 nM and 20 nM. The graded concentrations of H_2O_2 (Fisher Scientific, Fair Lawn, NJ, USA) were added to the culture plate with medium as well as dissected placental tissues respectively and mixed well. Tissue culture without H_2O_2 was used as a control. Both 24 hr and 48 hr incubations were performed. At each time the experiments were performed 5 times.

Effects of cytokines on placental EDLF synthesis and release

TNFα, a cytokine often involved in inflammatory processes including PE, was used to verify our hypothesis that cytokines are agents capable of up-regulating EDLF release. Due to the finding that high TNFα levels causes tissue damage, we chose very low concentrations such

as 1 nM, 2 nM, 5 nM, 10 nM and 20 nM to carry out these experiments. The graded concentrations of TNFα (Sigma, St. Louis, MO, USA) were added to the culture plate along with medium as well as dissected placental tissues respectively and mixed well and incubated for 48 hr. Tissue culture without TNFα was used as a control. The experiments were carried out 6 times.

After incubation at 37˚C in an incubator gassed with 95% air and 5% CO_2 (except the hypoxic experiments), aliquots of the conditioned culture medium (~4-5 mL) were collected in 15 ml conical tubes and any residual villi were removed by centrifuging at 4000 rpm for 2 min. The supernatant was stored at -80˚C as conditioned medium until later Digibind radioimmunoassay.

Lipid hydroperoxide and TNFα determination assay

After identifying some agents that modify the rate of EDLF synthesis in and release from placenta, we were also interested in answering additional questions regarding such regulation. More specifically, does hypoxia induce increased oxidative stress? Does hypoxia / oxidative stress alter cytokine levels in placenta? In order to answer these questions, we used commercially available kits to assay TNFα and lipid hydroperoxide, a product of ROS mediated free radical reactions and clear evidence of oxidative stress[19]. Answers to these questions should allow a clearer picture of which processes are primary and which secondary to help understand more precisely what may be directly responsible for changes observed.

Conditioned media cultured with hydrogen peroxide or under low O_2 conditions as well as controls were collected and assayed by Lipid Hydroperoxide (LPO) Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) and Human TNF-alpha Quantikine ELISA Kit (R&D Systems, Mineapolis, MN, USA). Data were analyzed following the instructions provide by the companies. The experiments and assays were done 5 or 6 times, up to the amounts of conditioned media collected from specified treatment described above.

Statistical Analyses

Results were reported as the mean $\pm$ 1 SEM. Comparisons of just two conditions were made by student's t-test if the data were normally distributed or by Wilcoxon Rank Sum Test if they were not. Effects of time or concentration on EDLF release from placental tissue were analyzed by ANOVA with post hoc Dunnett's pair-wise comparisons. A p-value of <0.05 was considered statistically significant.

Results

Effects of a general steroid synthesis inhibitor, ketoconazole, on placental EDLF production.

To test the effects of the steroid synthesis inhibitor, ketoconazole, after freshly delivered placenta was dissected and placed in incubation, we added graded concentrations of ketoconazole into the culture medium of individual cultured placental tissues. Samples of conditioned media were collected as a function of ketoconazole concentration and EDLF levels were measured by RIA after 24 hr and 48 hr incubation respectively. Data showed that ketoconazole caused inhibition of EDLF production and release into the culture media in a dose dependent manner. Reductions were suggested by 24 hr but were more pronounced after 48 hr

of exposure. In more detailed studies involving dissection of fresh placenta into maternal and fetal tissues, a more elaborate different set of profiles was obtained. In the fetal tissues there was a marked decrease in EDLF production in response to ketoconazole (Figure 26, n=5, 48 hr control no ketoconazole: 178 ± 14.8 nM EDLF equiv compared with the progressive effects of graded increasing concentrations of ketoconazole respectively, 48 hr 1 μM ketoconazole: 79.9 ± 13.21, 48 hr 2 μM ketoconazole: 55.8 ± 8.91, 48 hr 5 μM ketoconazole: 16.0 ± 1.62, 48 hr 10 μM ketoconazole: 14.7 ± 1.64, 48 hr 20 μM ketoconazole: 12.5 ± 6.4 nM EDLF equiv; $p=0.04$) whereas the maternal tissues showed little change or a possible small increase in EDLF production compared with untreated tissue after 48 hr of ketoconazole treatment (Figures 27, n=5, 48 hr control no ketoconazole: 12.3 ± 6.3 nM EDLF equiv compared with the progressive effects of graded increasing concentrations of ketoconazole respectively, 48 hr 1 μM ketoconazole: 19.0 ± 2.05, 48 hr 2 μM ketoconazole: 17.9 ± 1.96, 48 hr 5 μM ketoconazole: 18.3 ± 2.03, 48 hr 10 μM ketoconazole: 17.3 ± 1.82, 48 hr 20 μM ketoconazole: 24.6 ± 2.60 nM EDLF equiv; $p<0.05$).

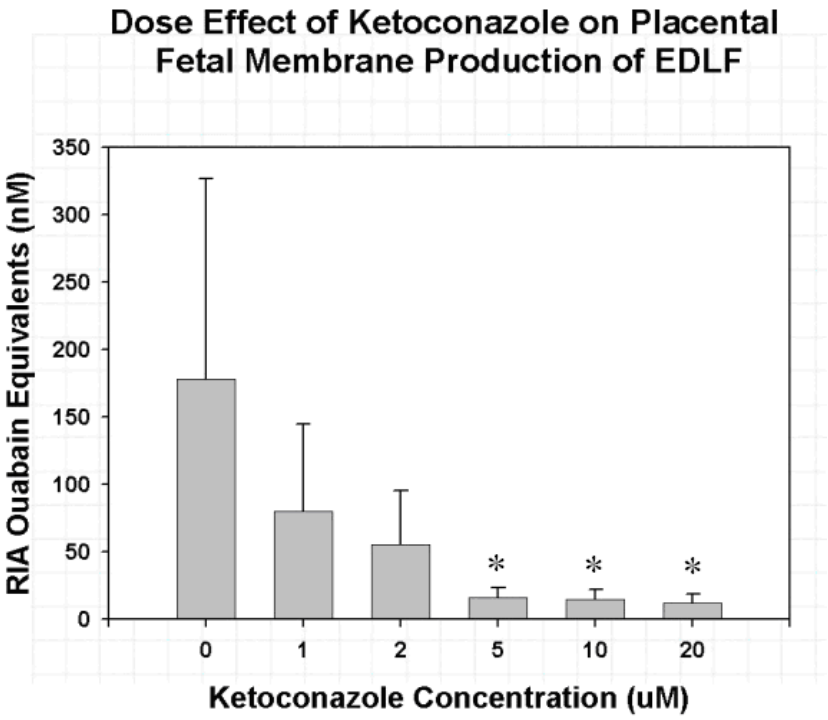

Figure 26. Concentration effect of ketoconazole on human plancental fetal side production of EDLF. Freshly collected human placenta was dissected into fetal and maternal tissues. These were incubated in buffered culture media for 48 hr in the absence or presence of graded concentrations of ketoconazole. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show a significant and progressive decrease in EDLF production with higher ketoconazole concentration (n=5, p=0.04, Wilcoxon Rank Sum Test). EDLF release was significantly suppressed by 5, 10 and 20 μM ketoconazole compared with no ketoconazole as assessed by post hoc pair wisc comparisions (*, $p<0.05$).

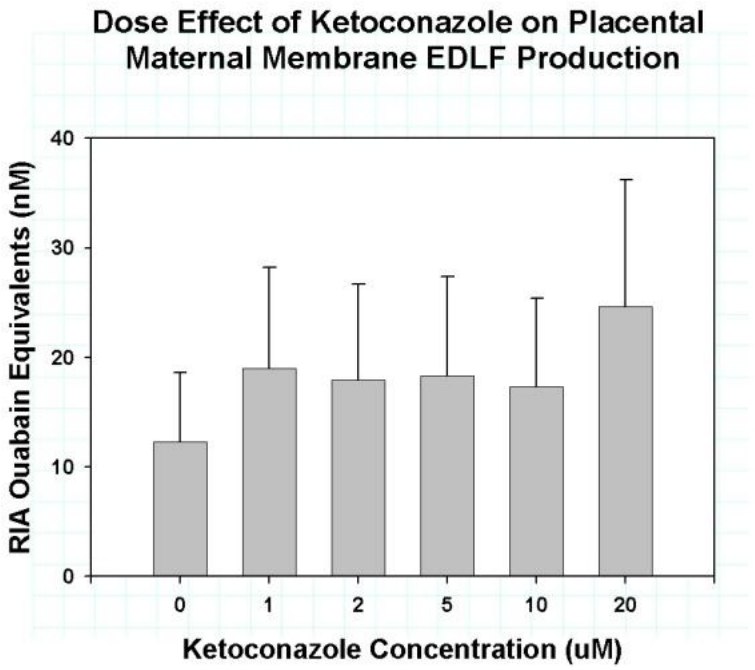

Figure 27. Concentration effect of ketoconazole on human plancental maternal side production of EDLF. Freshly collected human placenta was dissected into fetal and maternal tissues. These were incubated in buffered culture media for 48 hr in the absence or presence of graded concentrations of ketoconazole. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show little change with a small increase in EDLF released in response to ketoconazole ($n=5$, $p<0.05$, Wilcoxon Rank Sum Test, '0' vs 20 uM ketoconazole).

As shown in Figure 28, placenta has both fetal membranes and maternal membranes. The results suggested a very robust and obvious effect of ketoconazole on fetal tissue EDLF production. The results for maternal EDLF production are ambiguous, i.e. EDLF release was very low initially and if anything seemed to increase slightly with increasing ketoconazole. This may simply reflect that there was very little or no basal EDLF production and hence ketoconazole would not have an effect or maybe there is a small amount of EDLF that is present but not produced by these tissue basally. There is clearly complexity in EDLF production and it is likely that only certain cells have the synthetic machinery to produce steroids or EDLF.

Placenta is made up of particular layers of tissue with several cell types, each unique in its function. The placenta connects the developing fetus and the maternal circulation. Recent evidence showed that most of the placental cholesterol which is important for fetal development is synthesized by the fetus, in other words, by the cells on the fetal side of the placenta instead of transporting cholesterol from the maternal side. Moreover, the placental barrier was found to be impermeable to low-density lipoprotein (LDL) and other lipoprotein particles. Therefore, offspring of hypercholesterolemic mothers generally have normal cholesterol levels at term birth, whereas maternal cholesterol still remains at a high level[20]. According to these observations we conclude that placental EDLF syntheses on fetal and maternal sides, if it exists on the maternal side, are relatively independent. Also, because PE is a multi-factorial disorder involving vascular, genetic and immunological factors and ketoconazole inhibits various cytochrome P-450-dependent enzymes which participate in multiple bioactivation and metabolism steps, it is hard to explain clearly why ketoconazole may induce a small increase of EDLF production on the maternal side of the placenta at this point. However, the integrated effect of ketoconazole on placental EDLF production is down-regulation if one combines the data of Figure 26 and 27. It means that ketoconazole is an inhibitor of placental EDLF synthesis, and the synthetic pathway of EDLF very likely shares some steps with steroidogenesis involving the cytochrome P-450-dependent enzymes.

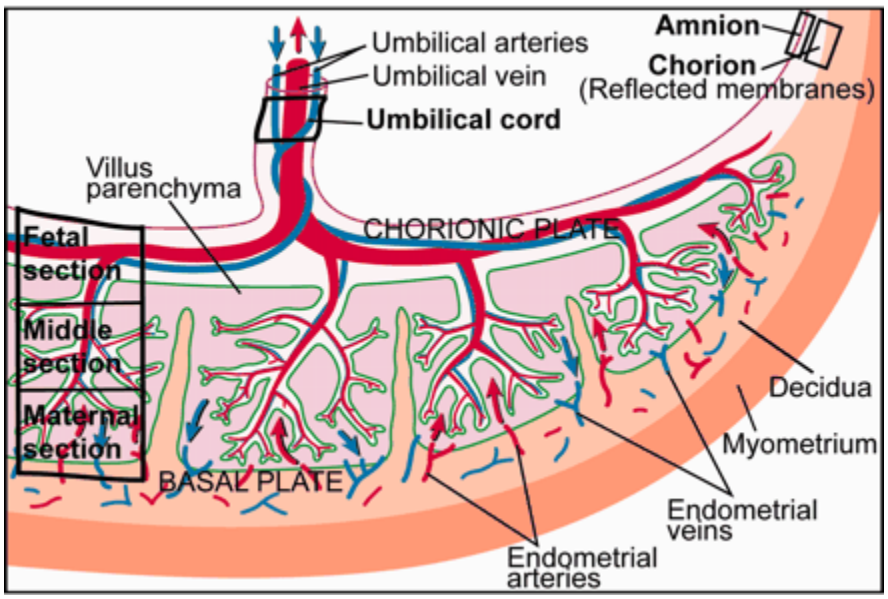

Figure 28. Schematic representation of a human term placenta, showing fetal side and maternal side[21].

These results suggest strongly that the synthetic pathway for EDLF production can be further elucidated. In addition, it may provide insights into how to interrupt EDLF production as one considers possible future therapeutic approaches.

Effects of a potential steroid synthesis intermediate, 17-hydroxyprogesterone, on placental EDLF production.

In addition to inhibition of EDLF production, one would predict that increasing the availability of synthetic precursor molecules could increase synthesis, especially in comparison with placental cultures that lack that precursor. If such a factor could be found whose presence can increase EDLF production, this would strongly reaffirm that EDLFs are produced in the placenta and may share synthetic steps with steroid synthesis. Increased synthesis may also

support and enhance efforts to isolate and identify the EDLF of PE. Therefore, we were interested in identifying at least one substrate that participates in EDLF synthesis. To this end, we applied graded concentrations of 17-hydroxyprogesterone to placental tissue culture, which is a potential steroidogenesis intermediate. There is evidence that placenta lacks the 17-hydroxylase and 17-OH progesterone then bypasses that deficiency[22]. Figure 29 shows that 48 hr of 17-hydroxyprogesterone treatment resulted in increased levels of EDLF released into the culture media in a dose dependent manner (n=6, 48 hr control no 17-OH progesterone: 29.6 ± 4.3 nM EDLF compared with the progressive effects of graded increasing concentrations of 17-OH progesterone respectively, 48 hr 0.2 μM 17-OH progesterone: 36.0 ± 1.06 nM, 48 hr 0.5 μM 17-OH progesterone: 37.6 ± 0.84 nM, 48 hr 1 μM 17-OH progesterone: 44.0 ± 5.3 nM, 2 μM 17-OH progesterone: 49.1 ± 1.52 nM ouabain equivalents EDLF; p=0.03). In order to further identify its role in the pathway, we did time-dependent experiments using the optimal concentration (2 μM). Control placental tissues maintained in the absence of 17-hydroxyprogesterone showed that EDLF release increased with increasing incubation time (Also shown in Chapter 4, Figure 23, n=5, 6hr: 87.6 ± 2.56 nM, 12 hr: 237.0 ± 6.92 nM, 24 hr: 393.2 ±14.76 nM; 36 hr: 675.9 ± 26.89 nM, 48 hr: 813.8 ± 18.77 nM ouabain equivalents EDLF; $p<0.05$). Moreover, addition of 17-hydroxyprogesterone into the culture media enhanced the accumulation of EDLF and the effect was time dependent (Figure 30, n=5, 6 hr of 2 μM 17-OH progesterone: 126.0 ± 3.81 nM, 12 hr of 2 μM 17-OH progesterone: 283.0 ± 6.30 nM, 24 hr of 2 μM 17-OH progesterone: 553.9 ± 33.62 nM, 36 hr of 2 μM 17-OH progesterone: 950.0 ± 40.44 nM, 48 hr of 2 μM 17-OH progesterone: 1324.3 ± 74.37 nM ouabain equivalents EDLF; $p<0.05$).

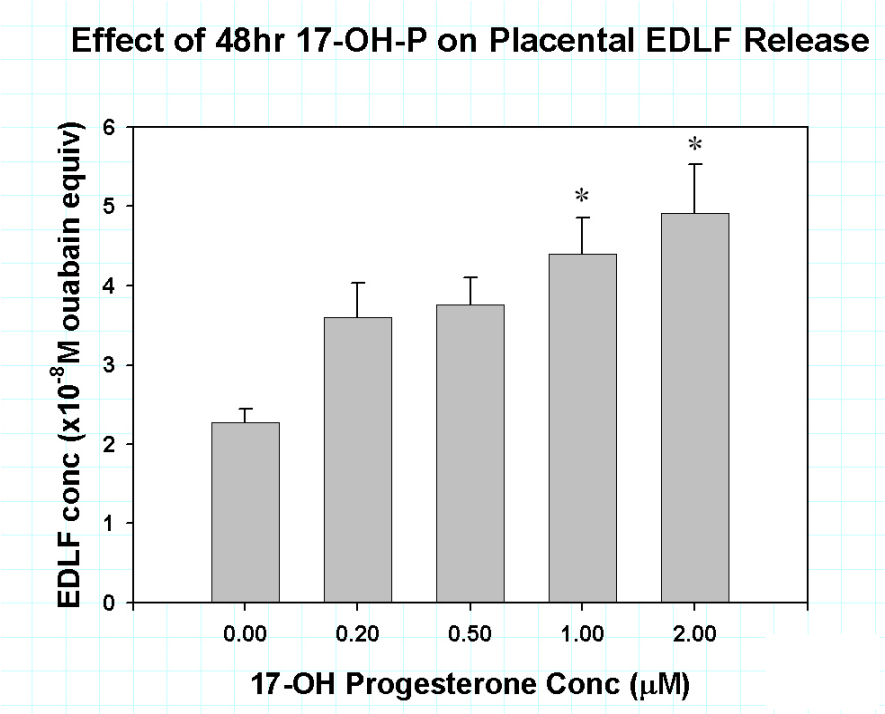

Figure 29. Effect of 17-hydroxyprogesterone on human plancental EDLF production. Freshly collected human placenta was dissected and incubated in buffered culture media for 48 hr in the absence or presence of graded concentrations of 17-hydroxyprogesterone. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show a progressive increase in EDLF production with higher 17-hydroxyprogesterone concentration (n=6, p=0.03).

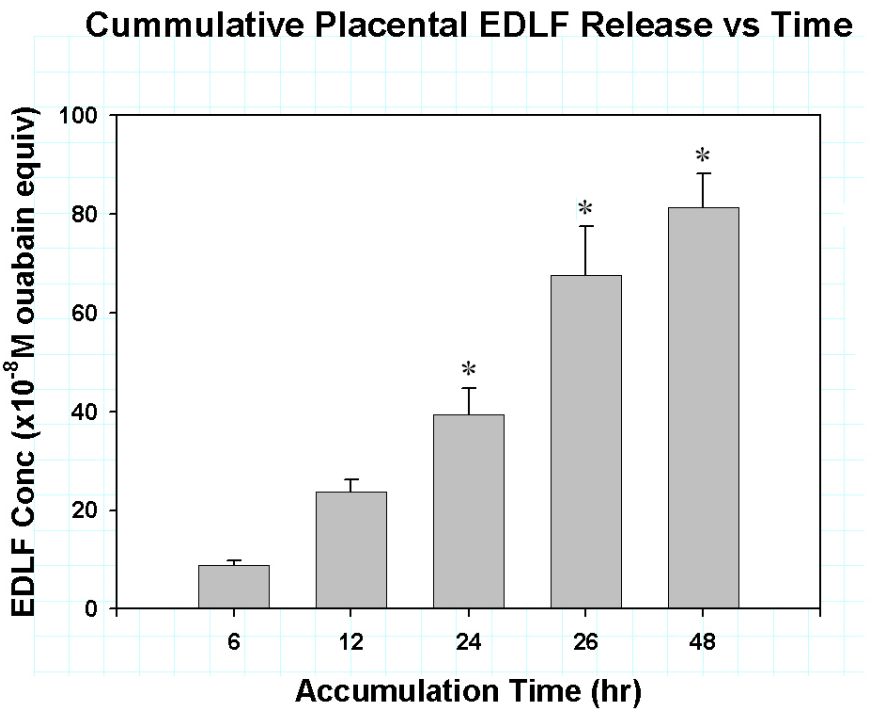

Figure 23. Human plancental EDLF accumulation. Freshly collected human placenta was dissected and incubated in buffered culture media for increasing time. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show a significant and progressive increase in EDLF production with longer incubation time ($n=5$, $p<0.05$).

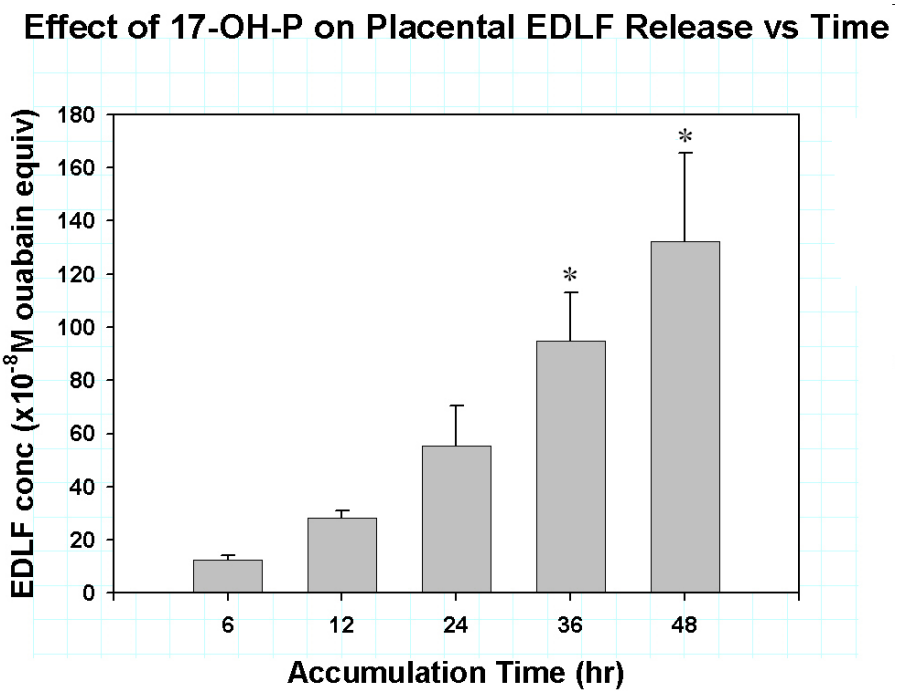

Figure 30. Effect of 17-hydroxyprogesterone on human plancental EDLF production vs time. Freshly collected human placenta was dissected and incubated in buffered culture media for increasing time in the presence of 2μM 17-hydroxyprogesterone. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show a significant and progressive increase in EDLF production with longer treatment time ($n=5$, $p<0.05$).

These results suggest strongly that 17-hydroxyprogesterone has a strong influence on EDLF production and is possibly a substrate of the EDLF synthetic pathway or is converted into a substrate of that pathway and EDLFs are potentially synthesized through the steps involving 17-hydroxyprogesterone (shown in Figure 3 in Chapter 1).

Effects of hypoxia on placental EDLF production

PE is attended by many changes. Among those considered mechanistically important are placental hypoxia, increased production of ROS, and greater levels of proinflammatory cytokines. Consequently, there was interest in knowing the effects of such factors on EDLF production and release. Because previous studies suggested that hypoxia contributes to the development of PE or is an early feature of PE, we cultured placental tissues under low O_2 conditions and then measured EDLF levels released in the media by Digibind RIA. Figure 31 and 32 showed that hypoxia minimally stimulated EDLF production and release into the culture media after both 24 hr and 48 hr incubation (n=6, 24 hr control normal O_2: 59.4 ± 4.4 nM vs 24 hr low O_2: 70.0 ± 6.6 nM ouabain equivalents EDLF; p=0.028, and n=6, 48 hr control normal O_2: 29.2 ± 2.4 vs 48 hr low O_2: 34.6 ± 2.7 nM ouabain equivalents EDLF; p=0.13). While the changes were statistically significant at 24 hr, they were not at 48 hr.

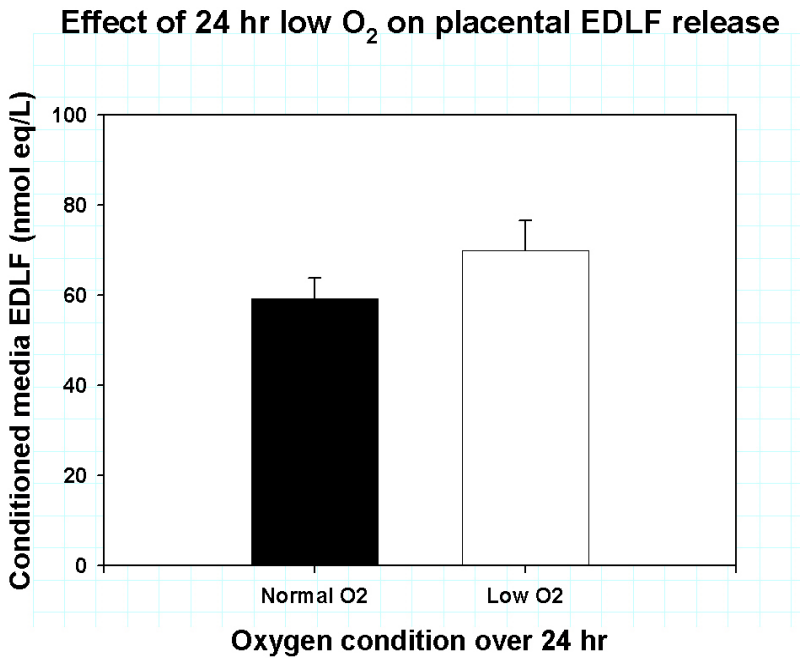

Figure 31. Effect of 24 hr hypoxia on human placental EDLF production. Freshly collected human placenta was dissected and incubated in buffered culture media under normoxia (21% O_2: 5% CO_2/95% air) and hypoxia conditions (2% O_2/5% CO_2/93% N_2) for 24 hr. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show increased EDLF amount released in response to low O_2 tension (n=6, p=0.028).

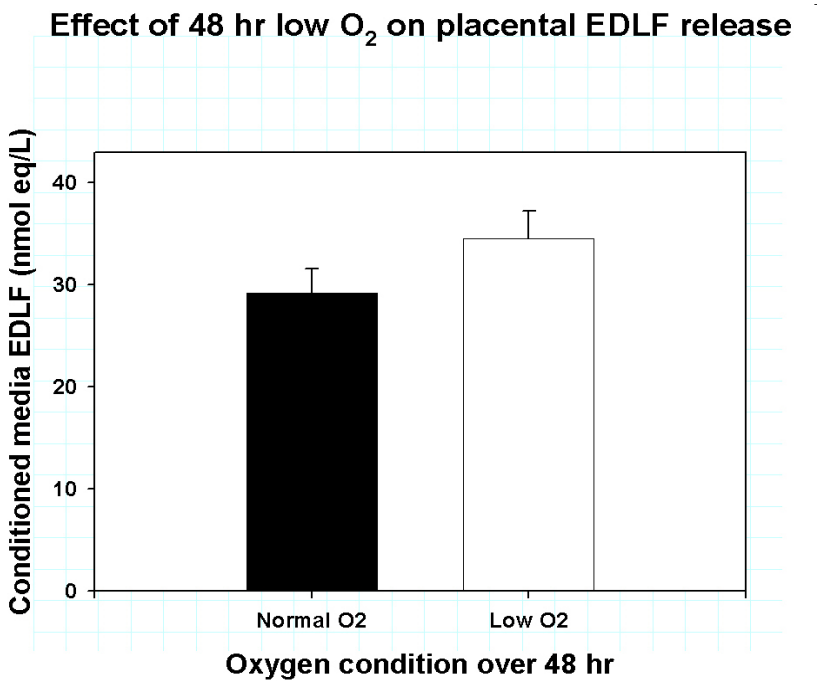

Figure 32. Effect of 48 hr hypoxia on human placental EDLF production. Freshly collected human placenta was dissected and incubated in buffered culture media under normoxia (21% O_2: 5% CO_2/95% air) and hypoxia conditions (2% O_2/5% CO_2/93% N_2) for 48 hr. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show increased EDLF amount released in response to low O_2 tension (n=6, p=0.13).

These data indicated that placental hypoxia may be a weak stimulus of EDLF synthesis and release. Despite the somewhat different statistical results between 24 hr and 48 hr incubation, both showed EDLF increases, though modest, in human placental tissue culture under hypoxic conditions. This is consistent with the finding that hypoxia stimulates a marked release of a sodium pump inhibitor called hypothalamic inhibitory factor (HIF) from midbrain and adrenal in Wistar rats[23].

Effects of hydrogen peroxide as a model ROS on placental EDLF production

Because oxidative injuries have been shown to be increased in PE and oxidative stress continues to be thought to contribute to the pathogenesis of hypertension in PE, we hypothesized that oxidative stress may be a potential stimulus of EDLF synthesis and release. Therefore, we used hydrogen peroxide as a representative ROS that mediates oxidative stress, to treat placental tissue in culture and assayed the media by Digibind RIA to reveal its effect on EDLF concentrations. By analyzing specimens from five individual placentas treated with graded concentrations of H_2O_2, we found that the effects of 24 hr H_2O_2 treatment on placental EDLF production did not show a clear trend whereas 48 hr H_2O_2 treatment appeared to increase placental EDLF levels. However, 5 nM of H_2O_2 appeared to be the optimal concentration to induce EDLF production and release. This concentration resulted in approximately double the quantity of the released EDLF compared with untreated culture media (Figure 33, n=5, 48 hr control: 29.2 ± 2.4 vs 48 hr of 5 nM H_2O_2: 49.3 ± 5.8 nM EDLF equiv; p=0.009). In the media treated with higher concentrations (10 nM, 20 nM) of H_2O_2, EDLF levels were equal or even slightly, though not significantly, lower than those produced with 5 nM of H_2O_2 (n=5, 48 hr of 10 nM H_2O_2: 35.3 ± 4.6 nM EDLF equiv, p=0.001, and n=5, 48 hr of 20 nM H_2O_2: 45.5 ± 5.3 nM EDLF equiv; p=0.003).

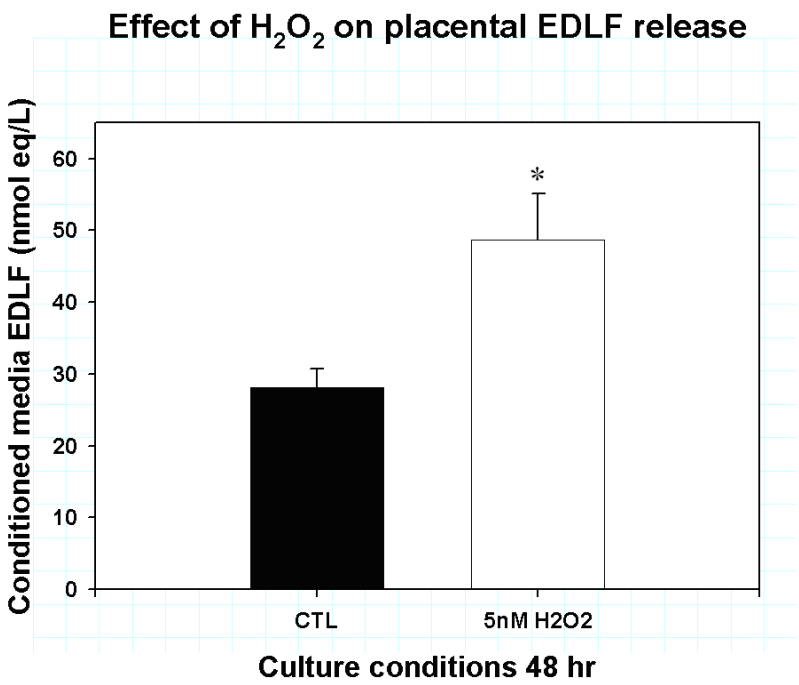

Figure 33. Effect of H_2O_2 on human placental EDLF production. Freshly collected human placenta was dissected and incubated in buffered culture medium for 48 hr in the absence or presence of 5 nM of hydrogen peroxide. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show increased EDLF levels released in response to 5 nM H_2O_2 (n=5, p=0.009).

These observations suggested that as what we predicted, H_2O_2 stimulates EDLF synthesis and release. Its effects on EDLF production may be in a parabolic manner, or a saturating manner, and concentrations higher than 5 nM of H_2O_2 may actually result in a slight fall off of EDLF levels.

Effects of a PE related cytokine TNFα on placental EDLF production

Because research suggests that cytokines may play an important role in the pathogenesis of preeclampsia and/or may be a result of generalized endothelial dysfunction, it was of interest to

know if cytokines might be regulators of EDLF production. Therefore, we carried out experiments using TNFα, a cytokine highly studied and previously shown to be increased in PE[12], to test its effect on placental EDLF production. RIA data show that after 48 hr TNFα treatment, levels of EDLF released into the culture media increased and this effect was also TNFα dose dependent (Figure 34, n=6, 48 hr control: 19.8 ± 2.7 vs 48 hr of 20 nM TNFα: 44.7 ± 4.0 nM EDLF equiv, p=0.0004).

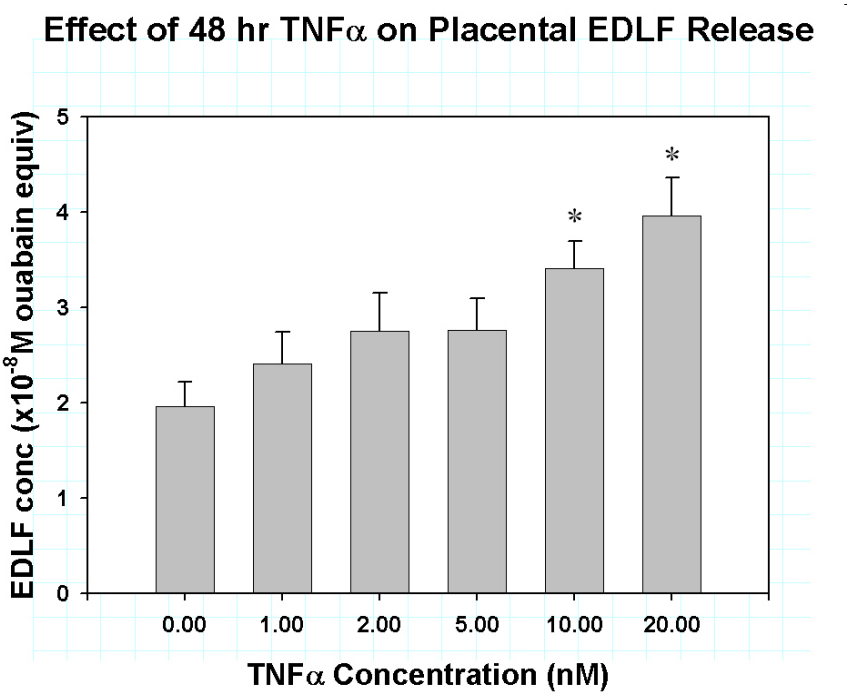

Figure 34. Effect of TNFα on human plancental EDLF production. Freshly collected human placenta was dissected and incubated in buffered culture media for 48 hr in the absence or presence of graded concentrations of TNFα. The culture media was collected and the amount of EDLF released from the tissue was measured using an RIA. The data show a progressive increase in EDLF production with higher TNFα concentration (n=6, ANOVA p=0.0004).

These results suggested that TNFα could increase levels of EDLF synthesis and release. In addition, this finding is consistent with the observations that TNFα causes systemic endothelial activation and therefore may participate in several main symptoms of PE, such as hypertension, renal dysfunction and a further exaggerated inflammatory response.

Placental ROS release

As demonstrated above, low O_2 tension potentially and low concentrations of H_2O_2 significantly induced elevated EDLF levels from human placental tissue culture. In order to further assess the role of these oxidative stresses on placental tissue modification in PE, we used a lipid hydroperoxide immunoassay to quantify lipid peroxidation in culture media treated with low O_2 or H_2O_2. These lipid peroxides represent products of ROS and are considered to be a clear demonstration of oxidative damage, thus indicating ROS activity at increased levels. Figure 35 (n=6, control normal O_2: 5.85 ± 3.11 vs 48 hr low O_2: 10.30 ± 5.72 μM lipid hydroperoxide; p=0.01) showed that hypoxia conditions induced higher levels of ROS lipid in the media than normoxia conditions. The increase was ~2 fold. As expected with higher H_2O_2 concentrations the higher the levels of lipid hydroperoxides (Figure 36, n=5, control no H_2O_2: 5.05 ± 2.69, 1 nM H_2O_2: 6.62 ± 3.31, 5 nM H_2O_2: 9.17 ± 3.18, 10 nM H_2O_2: 11.43 ± 3.67, 20 nM H_2O_2: 13.13 ± 5.04 μM lipid hydroperoxide; $p<0.05$), suggesting that addition of H_2O_2 in placental tissue culture resulted in oxidative injuries. Moreover, lipid peroxidation for 2% O_2 and 5 nM H_2O_2 were very comparable, the levels being slightly higher for low O_2 than for 5 nM H_2O_2.

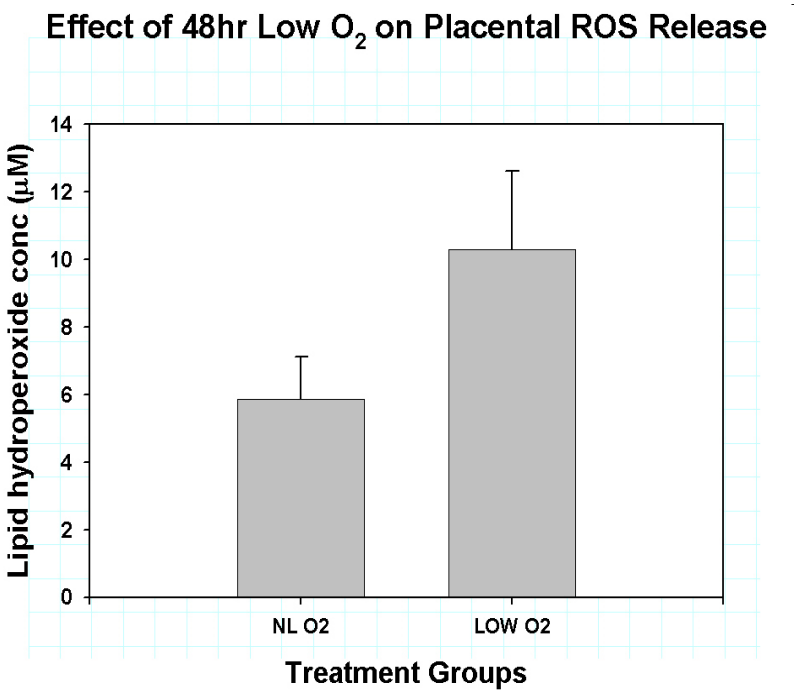

Figure 35. 48hr hypoxia effect on placental ROS release. Freshly collected human placenta was dissected and incubated in buffered culture media under normoxia (21% O_2: 5% CO_2/95% air) and hypoxic conditions (2% O_2/5% CO_2/93% N_2) for 48 hr. The culture media was collected and the amount of lipid hydroperoxide released from the tissue was measured using a kit. The data show significantly increased LPO amount released in response to low O_2 tension (n=6, p=0.01).

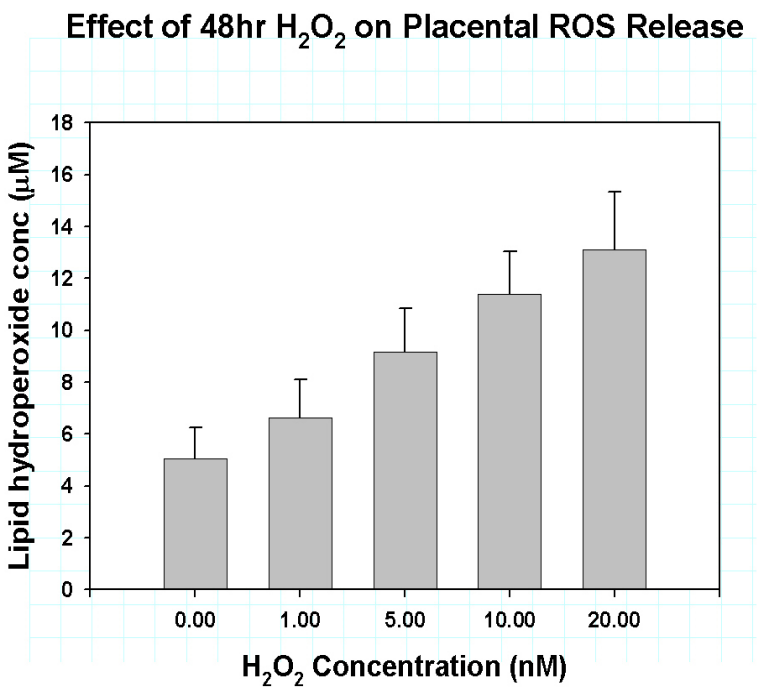

Figure 36. 48hr H_2O_2 effect on placental ROS release. Freshly collected human placenta was dissected and incubated in buffered culture for 48 hr in the absence or presence of graded concentrations of hydrogen peroxide. The culture media was collected and the amount of lipid hydroperoxide released from the tissue was measured using a kit. The data show a progressive increase in LPO levels with higher H_2O_2 concentration (n=5, $p<0.05$).

Placental TNFα release

In addition, we were also interested in studying the interaction of hypoxia and oxidative stress on placental TNFα production. Given that TNFα seemed to represent the strongest stimulus for EDLF production, we wanted to determine if the effect of hypoxia or hydrogen peroxide on EDLF were actually mediated by their generating higher levels of TNFα. Therefore, we used a TNFα immunoassay to measure TNFα concentrations in culture media of human placental tissues treated with either 48 hr low O_2 or 48 hr H_2O_2 at graded concentrations. Figure 37 indicated that 48 hr low O_2 treatment induces more TNFα release than media incubated under

normoxic conditions (n=6, control normal O_2:19.01 ± 10.16 vs 48 hr low O_2: 126.80 ± 249.6 pg/ml TNFα; p=0.03) whereas in Figure 38 there is not a significant increase of TNFα release with increasing graded concentrations of H_2O_2 treatment (n=5, control no H_2O_2: 15.13 ± 3.98, 1 nM H_2O_2: 18.61 ± 13.60, 5 nM H_2O_2: 14.95 ± 6.39, 10 nM H_2O_2: 15.83 ± 6.63, 20 nM H_2O_2: 13.50 ± 7.67 pg/ml TNFα; p=0.90).

These data suggested that under hypoxia conditions, placental tissues release significantly higher levels of TNFα, maybe also other cytokines, and that H_2O_2 treatment of the tissue culture under the concentrations used in the EDLF experiments did not significantly stimulate TNFα release.

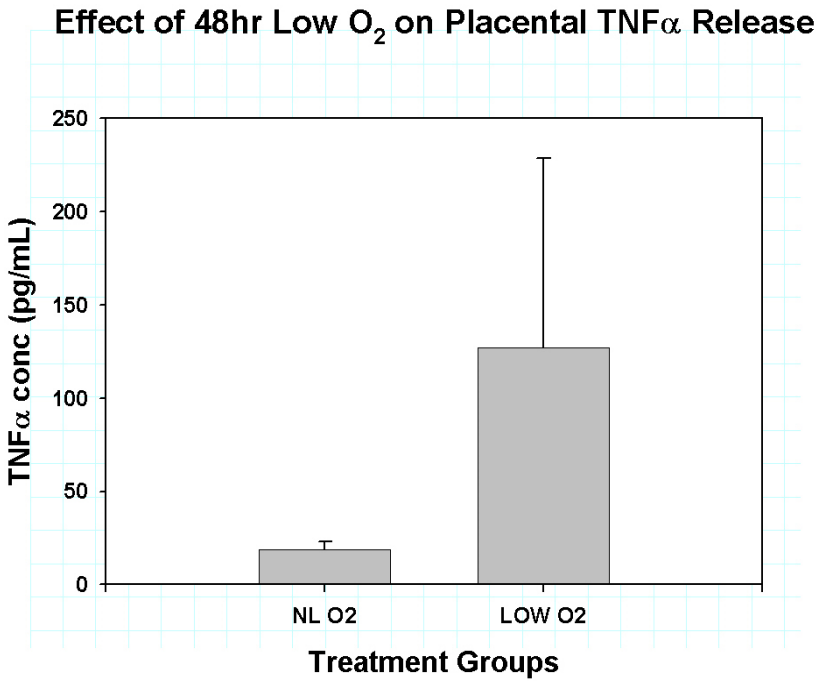

Figure 37. 48hr hypoxia effect on placental TNFα release. Freshly collected human placenta was dissected and incubated in buffered culture media under normoxia (21% O_2: 5% CO_2/95% air) and hypoxia conditions (2% O_2/5% CO_2/93% N_2) for 48 hr. The culture media was collected and the amount of TNFα released from the tissue was measured using a kit. The data show increased TNFα amount released in response to low O_2 tension (n=6, p=0.03).

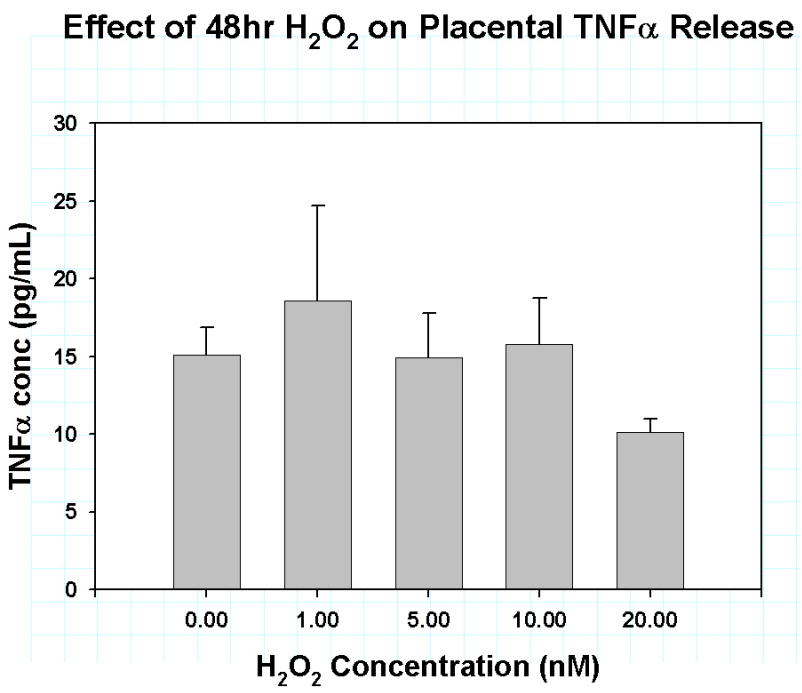

Figure 38. 48hr H_2O_2 effect on placental TNFα release. Freshly collected human placenta was dissected and incubated in buffered culture for 48 hr in the absence or presence of graded concentrations of hydrogen peroxide. The culture media was collected and the amount of TNFα released from the tissue was measured using a kit (n=5, p=0.90). There was no significant change in placental TNFα release with H_2O_2 exposure.

Discussion

With an enriched source of EDLF-placenta- and a sensitive approach which could measure EDLF levels in placenta- the radioimmunoassay employing Digibind, we achieved initial experimental evidences to further verify that EDLFs were synthesized in placenta and this synthetic pathway shared some steps with steroidogenesis pathway.

Ketoconazole, a general inhibitor of steroid synthesis, was demonstrated to reduce EDLF placental production in a dose-dependent manner. Despite the slight increase of EDLF levels presenting in maternal tissues, ketoconazole appeared to inhibit EDLF production in whole

placenta. Also, the addition of 17-OH progesterone in the placental tissue culture media, which is a potential intermediate of steroidogenesis, was demonstrated to result in an elevation of EDLF production. This effect was dose-dependent and time-dependent. Moreover, the accumulation of EDLF in the placental culture by time was enhanced by the treatment of 17-OH progesterone. These findings suggested that EDLF synthesis and steroidogenesis share some steps involving 17-OH progesterone and enzymes inhibited by ketoconazole. Again, those are reliable evidences supporting that EDLF biosynthetic pathway occur in the placenta. However, 17-OH progesterone may affect EDLF product indirectly, e.g. it could act as a regulator rather than a substrate or be converted into something that acts as a regulator and not a substrate.

In order to know more about EDLF synthetic pathway, more intermediates of steroid pathway need to be identified, such as pregnenolone and/or progesterone. Once we obtain sufficient data to determine or exclude candidates for EDLF synthetic intermediates / substrates, the pathway could be established step by step.

In addition to EDLF synthesis, the regulation of EDLF in placenta was another consideration of my research. Hypoxia, oxidative stress and cytokines, which are established features of PE or perhaps even participants in PE, were hypothesized to be regulatory agents of EDLF synthesis and release. Experimental data showed that hypoxia conditions of appropriate time, addition of low concentrations of hydrogen peroxide, a represent of ROS, and TNFα, a represent of cytokines, could stimulate increase of EDLF placental production. Thus, we concluded that hypoxia, hydrogen peroxide and TNFα are regulatory agents up-regulating EDLF synthesis and release. Probably we also could deduce that not only H_2O_2 and TNFα, but other

ROS and cytokines are stimuli of EDLF release. It should be noted that in the hypoxia experiments, the control conditions were using 21% O_2 which is its concentration in air. However, diffusion of O_2 into placental tissue would have been limited and consequently 21% O_2 may also represent a hypoxic state compared with in vitro placenta being perfused by blood.

Immunoassays indicated that hypoxia treatment of the placental tissue resulted in higher ROS levels released into the culture medium. It means that hypoxia might regulate EDLF levels by inducing oxidative stress in PE. Also, the observations that hypoxia conditions led to higher TNFα levels released into the placental tissue culture suggested that hypoxia might be responsible for the alteration of TNFα, or even other cytokines levels, in PE. However, addition of hydrogen peroxide did not elevate TNFα levels significantly, indicating that ROS and cytokines might regulate EDLF productions through independent pathways.

Although we have identified the roles of H_2O_2 and TNFα in EDLF synthesis and release in placenta, the effects of other ROS and cytokines have still to be determined. Such studies would provide more insights to understand the regulation of EDLF in placenta.

However, because preeclampsia is a multi-systemic disorder, our research only revealed limited aspects of the disease. EDLFs are regulated by several agents and have impacts on several factors as well. The in vitro placental tissue experiments could not reflect the whole story occurred in PE. For example, endothelial dysfunction including alteration of cytokine levels has an effect observed in maternal vascular system in PE and EDLFs likely play an important role in vascular events in PE. Questions naturally arise: Do EDLFs mediate endothelial dysfunction and

hypertension? Or are there independent pathways in the two systems-the vascular system and the immune system? Since maternal vascular system is involved in EDLF events, why were very low levels of these factors measured in maternal tissues in placenta? It is possible that factors such as ROS or cytokines might stimulate maternal production. Perhaps PE placentas behave differently. If placental maternal tissues cannot produce EDLF, considered as a possibility earlier in this Chapter according to the data of ketoconazole experiments, how were EDLF translated from fetal layer to maternal layer? Or are there any other sources of EDLF that exist? If so, do the sources produce more EDLF than placenta? If so, probably we could isolate and identify EDLFs more easily. Further work is needed to elucidate these questions.

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Chapter 6: Summary and Future Studies

During the past few decades, a large number of studies on preeclampsia have been performed worldwide. However, the mechanisms responsible for its causes and pathogenesis, as well as the prevention and treatment of the disorder, remain unclear due to the complexity of the syndrome. According to current understanding of the pathogenesis of PE, it can be simplified to a two-stage process. The placenta being predisposed to hypoxia is the highly variable first stage, and the release of soluble factors resulting in many of the other observed symptoms is the following stage. Multiple systems have been implicated in the development of PE, such as the immune system, the cardiovascular system, the central nervous system and the renin-angiotensin system[1]. Currently, many hypotheses have attempted to explain why PE occurs and have linked the syndrome to the presence of various disorders, including endothelial cell injury, immune rejection of the placenta, compromised placental perfusion, altered vascular activity, imbalance between prostacyclin and thromboxane, decreased intravascular volume, disseminated intravascular coagulation, uterine muscle stretch (ischemia), vitamin deficiency, increased central nervous system irritability, genetic factors, etc. These indicate that PE is likely a multi-factorial disease process or set of diseases and hence the study of PE is complicated.

As the most visible and consistent sign of PE, hypertension plays an important role in the pathophysiology of PE as well as being the product of other earlier pathophysiologic processes. For the studies of PE in our lab, EDLF, which was found to be at increased levels in the circulation most preeclamptic women and thus potentially an important causative factor of the hypertension in PE, was considered to be the main research focus. We were interested in its

synthetic pathway, regulation and isolation, knowing these would be of great importance. Such insights would be helpful in understanding the actual processes underlying PE, allow for the development of antagonists of EDLF, and quite possibly improve current measurement and therapy of PE. In order to clarify the mechanisms of EDLF synthesis and regulation in PE, we needed an accurate approach to the measurement of EDLF levels in specimens and a rich source of these factors to provide sufficient amounts to perform structural characterization and molecular composition experiments in vitro.

Currently, my research in conjunction with other studies from our laboratory have provided substantial evidence for increased serum concentrations of ELDF in essential hypertension, animal models of hypertension and, most importantly for my work, in women with PE. Substantial work, much of it described here, provides consistent evidence for the existence and source of EDLF. In addition, we have developed a sensitive theranostic assay employing Digibind to measure EDLF levels in women with PE that should allow for the determination of which women will respond positively to Digibind therapy. This same assay also provided a sensitive and selective tool and has been used here to measure EDLF levels in placental tissue culture, placental homogenates and sera. More importantly, we have carried out initial experiments to explore the regulation and synthetic pathway of EDLF, and obtained sufficient data to begin to have an outline of the story.

Convincing experimental and clinical evidence support that Digibind effectively binds to EDLF and blocks its activity in PE. For our research, Digibind was used as a tool to measure and isolate EDLF. The radioimmunoassay we developed applies Digibind as the primary antibody to

selectively bind EDLF or ouabain, and the content of EDLF in human sera, placental homogenates or placental tissue culture could be determined through competition of EDLF with a tritiated ouabain tracer. This method is critical because it is a fast and accurate way to quantify EDLF content in *in vitro* specimens and it makes further studies of EDLF synthesis and regulation possible. Moreover, it also provides a means to identify preeclamptic women who will respond to the therapy using Digibind and facilitates determination of an appropriate therapeutic dose of Digibind.

However, due to the discontinuation and high expense of Digibind, the exploitation of a monoclonal substitute is needed. Although we have found one or more monoclonal anti-digoxin antibodies which have relatively good affinity for EDLF, their standard curves using cold ouabain with known graded concentrations showed that these Mabs did not as yet provide a high enough change in counts of radioactivity to make them highly accurate. In their current form they may not have the sensitivity for the measurement of serum EDLF content at least in normal pregnant women. Therefore, further optimization of these antibodies or screening of additional Mabs is still in process in our lab. Based on previous experimental data, the discovery of a monoclonal replacement for Digibind is promising. The process of making such a Mab usable in the clinical setting would require substantially greater work, including having all components standardized and the overall assay compatible with automated instrument platforms as used in hospitals and clinics. This becomes a commercial undertaking once a suitable antibody is demonstrated.

Finally, these Mabs have all been developed against digoxin. Clearly, Mabs specific to one or more EDLFs would represent a marked improvement in having a true theranostic assay and having accurate concentration determinations of specific EDLFs. This awaits unequivocal chemical identification of the relevant EDLF or EDLFs.

Besides an effective approach to measure EDLF levels, we also need an enriched source of EDLF to perform later studies. According to previous clinical data which demonstrated that placenta has higher EDLF levels than serum, placenta was hypothesized to be a source of EDLF. Our in vitro experimental data also reaffirmed that placental homogenates from women whose pregnancies were complicated with PE have much higher EDLF levels than sera from PE women. The levels of EDLF secreted from normal placental tissue culture were even comparable with those in sera from PE women. Hence, we concluded that placenta is a source of EDLF and can be used to study the synthetic pathway and regulation of the factors. Also, having a rich source and/or the ability to accumulate EDLF should facilitate efforts to isolate sufficient quantities of one or more of these factors to carry out more structurally definitive instrumental analyses including MS, NMR and perhaps even x-ray crystallography. These latter two techniques require substantial amounts of material and consequently it may require a long time to acquire this much pure factor.

In addition, a method using Digibind to isolate EDLF from placenta was developed. RIA data showed that an appropriately chosen ultrafiltration filter could isolate Digibind-bound EDLF from placental homogenates and most EDLF could be released from its affinity binding by altering pH. This opens up the possibility to isolate EDLF quite cleanly and then identify its

actual structure. Knowing the structure of EDLF would also facilitate research into its synthetic pathway and regulation, and hence the future development of antagonists for use in anti-hypertensive or PE therapy.

Since we already verified that placenta is a source of EDLF, the mechanisms of its synthesis in placenta were then of considerable interest. We carried out placental culture experiments using ketoconazole, a general inhibitor of steroidogenesis, and found that it also inhibited EDLF synthesis in placenta. In addition, results from placental tissue culture experiments using 17-hydroxyprogesterone indicated that it may be an intermediate or a substrate or converted into an intermediate or substrate of the EDLF synthetic pathway in placenta. The cumulative EDLF concentrations as a function of time were enhanced by the treatment of 17-hydroxyprogesterone. These findings reaffirmed that EDLFs are synthesized in placenta. Furthermore, these also provide evidence supporting that the EDLF synthetic pathway share steps of steroid synthetic pathway, which may involve 17-hydroxyprogesterone and cytochrome P-450-dependent enzymes. Hence, an outline of EDLF synthetic pathway could be drawn. However, one cannot rule out that the effect of 17-OH progesterone was indirect. For example, 17-OH progesterone is a known substrate for cortisol and consequently the tissue may convert the progesterone to cortisol and cortisol may regulate the EDLF production. This will require other types of experiments where the 17-OH-progesterone is labeled and shown to result in labeled EDLF or experiments involving the direct application of cortisol to placental tissue and its effects determined.

Although these experiments support that EDLF is produced by the placenta and shares synthetic features with the steroid synthetic pathway, the individual steps in the synthesis are still unknown. Additional experiments that use agents that are specific inhibitors of individual enzymes are needed for all possible known steroid synthetic enzymes. Where such agents do not exist or as confirmatory experiments, the use of small inhibiting RNAs can transiently knock down specific genes and their products. Such an approach could be used in this tissue placental culture to probe the involvement of any of several enzymes.

If EDLF is going to be a contributor to the disease process of PE, then its relationship to other factors considered currently to be very important in the onset and/or maintenance of PE is essential. As mentioned above, a predisposing placenta is held to represent the first stage of disease. The problem seems to arise by the placenta failing to produce syntyciotrophoblasts or adequate quantities of these to remodel the maternal vasculature to provide an adequate blood supply to the intervillous space. This results in a low oxygen, low nutrient environment for the fetus and is thought to result in the production of ROS. These factors lead to vascular endothelial damage and release of proinflammatory cytokines including TNFα. Do these factors, representing potentially early events in PE, have effects on EDLF production, potentially leading to its exerting a hypertensinogenic effect on the mother?

It has been known that hypoxia and oxidative stress are features of PE and many contend contribute to PE. Therefore, we hypothesized that they induce clinical aspects of PE by upregulating EDLF. Placental tissue culture and RIA experimental data verified that hypoxia, possibly, and hydrogen peroxide, a typical ROS, lead to elevation in EDLF production and

secreted levels. Interestingly, the increase of EDLF levels under 24 hr hypoxia conditions was more pronounced than 48 hr. Also, 5 nM hydrogen peroxide treatment of cultured placental tissues may have resulted in more increase of secreted EDLF levels than 10 nM and 20 nM. While the differences were not statistically different, both of these higher concentrations registered somewhat lower levels of EDLF in these experiments. We do not have an explanation for either of these observations but perhaps the somewhat lower EDLF levels at 48 hr or with higher hydrogen peroxide for 48 hr may compromise the integrity of the tissue.

The observations of the effects of hypoxia and ROS on EDLF release were consistent, suggesting that they upregulate EDLF production. Only appropriate treatment of hypoxia and oxidative stress could induce most EDLF release. Too long a time of hypoxia or too high a dose of hydrogen peroxide may not lead to yet higher EDLF production. Furthermore, immunoassays indicated that ROS release in placental tissue culture was elevated by hypoxia treatment. It proved that in vitro hypoxia treatment of placental tissue culture we utilized cause additional oxidative stress of the tissue with release of molecules modified by oxidative stress. Also, higher levels of know products of ROS were measured in the placental tissue culture treated with higher concentrations of hydrogen peroxide, confirming that hydrogen peroxide added into the placental tissue culture represents an appropriate oxidative stress. These results verified the hypothesis that hypoxia contributes to PE by inducing oxidative stress, and in turn increases EDLF release according to our current results. There are of course other ROS. The exact combination of factors present in the maternal circulation and their timing or more particularly the exact combination of ROS and duration of each in the immediate vicinity of the placenta are still open questions.

Cytokines are another important set of candidates thought to mediate several aspects of PE. It was of interest to know if they regulate PE synthesis. Our data showed that TNFα is a significant stimulus of EDLF secreted from placental tissue in culture. This effect occurred in a dose-dependent manner. Hence, the presence and increase in EDLF is also consistent with previous findings that levels of cytokines were increased in PE perhaps as a consequence of vascular endothelial dysfunction, which is another feature of PE. Elevated levels of cytokines then could induce an exaggerated inflammatory response, another PE symptom. However, there has never been an exhaustive evaluation of all cytokines in PE and certainly we have not carried out experiments to determine if all inflammatory cytokines increase EDLF or to the same degree.

The primary obstacle for the studies on EDLF in PE has been their low quantities. After indentifying regulatory agents which upregulate EDLF synthesis and release, we could use these agents to increase EDLF production and make the identification of their structures more feasible.

PE is a syndrome and thus the effects of the agents discussed above may not be independent. For example, recent evidence suggested that cytokines stimulate NADPH oxidases in the vessel and kidney, an enzyme contributing to ROS production, which then directly or indirectly promote vasoconstriction[2]. Some hypertensive stimuli, such as high salt and angiotensin II, also promote ROS production. Hence vasoconstriction may lead to higher ROS. In order to elucidate the relationship of the regulatory agents, we also tested TNFα production in tissue culture treated with hypoxic conditions or with hydrogen peroxide. The results showed that hypoxia induced substantially higher release of TNFα in the culture than from the normoxic control, whereas

hydrogen peroxide did not cause significantly increased TNFα release in placental culture. These results suggest strongly that the effects of hydrogen peroxide on EDLF release are not due to the intervening release of TNFα. Hypoxia clearly elevated TNFα but had little or no effect on placental EDLF output. Again this suggests that both TNFα and hydrogen peroxide have independent pathways for the increase in EDLF production. Neveretheless, while having this provided important insight into EDLF regulation, the complete set of regulators and their hierarchical importance remains to be determined.

The studies on EDLF in our lab have provided substantial evidence supporting that EDLF exist in most PE women and are present at elevated levels, and that they are synthesized in placenta but also potentially in other maternal tissues. We also obtained initial data to elucidate the regulation and synthetic pathway of these factors. Future work is promising because we already developed an accurate method to detect EDLF and also found an enriched source of the factors. TNFα is a typical cytokine. More cytokines will be tested to confirm the role of the entire species in regulating EDLF production, such as IFNγ and IL-6. More candidates of the regulatory agents will also be tested in the future, such as anti-angiogenic factors and thromboxane, which were found to be at increased levels in the serum of women with PE and thus were thought to contribute to the pathogenesis of the disease[3, 4]. The regulatory factors we already identified also brought out some questions we need to figure out in the future. For example, since we've already known that TNFα and hydrogen peroxide upregulate EDLF production in placenta in individual pathways, based on the findings that oxidative stress promotes endothelial dysfunction by several mechanisms[5], and that TNFα is a contributor to endothelial dysfunction[6], we are interested that if these pathways have some relationship directly

or indirectly? If they do, how? Furthermore, as proposed above, in order to elucidate individual steps in the synthetic pathway of EDLF in placenta, specific inhibitors of individual enzymes and / or siRNA inhibiting specific intermediate or substrate could be used. In addition, our laboratory has carried out some initial experiments in analyzing the structures of EDLFs using mass spectrometry. Once the structures of the factors have been identified, or even partially identified, the screening of the specific Mab (s) anti-EDLF(s) which could replace Digibind and provide good performance in the RIA might be easier. Obviously, a theranostic of PE would be developed as well. Even though there remain many questions of the study on EDLF, with the tools and the extremely encouraging results we have, we believe that the isolation, identification, regulation and synthesis of EDLF will be elucidated in the near future.

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