Alterations in Uterine and Placental Sodium Pump Abundance May Contribute to the Onset of Mouse Labor

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ALTERATIONS IN UTERINE AND PLACENTAL SODIUM PUMP ABUNDANCE MAY CONTRIBUTE TO THE ONSET OF MOUSE LABOR

by

Carlos J. Vance

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Biochemistry

Department of Chemistry and Biochemistry
Brigham Young University
April 2005
This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate’s graduate committee, I have read the thesis of Carlos J. Vance in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

ALTERATIONS IN UTERINE AND PLACENTAL SODIUM PUMP ABUNDANCE MAY CONTRIBUTE TO THE ONSET OF MOUSE LABOR

Carlos J. Vance
Department of Chemistry and Biochemistry
Master of Science

Objective: Reductions in sodium pump (SP) abundance can give rise to increases in contractile force in uterine and vascular smooth muscle as well as an increased secretion in secretory cells, including potentially those of the placenta. To determine whether the mouse might serve as a model for human pregnancy in terms of the SP and to determine whether changes in SP abundance anticipate or follow labor, we studied pregnant mice over the final trimester of their pregnancy.

Study Design: C57Bl6 dams (n=46) were bred and studied during their pregnancy. Animals (n=4) were sacrificed at specific gestational time points. Other mice had labor induced with LPS on Gestational day 15 and were then studied at specific time points after induction. Specimens were studied for mRNA abundance as well as protein abundance using methods such as Real time RT-PCR and Western blot analysis. Data were analyzed by ANOVA with post hoc Duncan’s pair-wise comparisons.
**Results:** Levels of uterine SP α3 isoform mRNA were most abundant on day 14 near the beginning of the third trimester. There was a significant fall in SP α3 mRNA abundance by day 18 with a slightly lower level on the day of birth but an increased SP α3 mRNA abundance by one day post partum. Contrary to the uterus, SP α3 mRNA levels in the placenta increased over the last trimester, from day 14 to the day of birth. Western blot analysis on the two tissues demonstrated a somewhat similar pattern.

In the LPS studies of uterus and placenta, the SP α3 isoform protein abundance appeared to fall when compared to the 2 hour time point. Those animals which were injected with a vehicle control showed very little change in SP α3 abundance after injection. While protein levels were reduced, there was no significant reduction in mRNA for all specimens.

**Conclusion:** Uterine SP α3 isoform protein expression fell late in mouse pregnancy but prior to labor and appeared to be mediated by reductions in its mRNA. These reductions paralleled changes observed in term pregnant women. Such reductions would increase the sensitivity of the uterus to agents causing contraction but may directly increase the force, duration and frequency of contractions. Placental SP α3 isoform protein expression had no significant change over the final trimester. However, unlike uterine protein, the placental protein may not be mediated by its mRNA. Reductions in SP α3 protein abundance were also seen in preterm labor produced by LPS induction. These changes may not be mediated by mRNA. Taken together, changes in the SP α3 isoform may represent a fundamental mechanism in the initiation and/or progression of term labor and in preterm in mouse and potentially in human.
I would like to thank my graduate committee for making it possible to obtain this degree. Most of all I would like to thank Dr. Steven W. Graves, my committee chair, for his patience, guidance and constant support, which made this work possible. In addition I would like to thank Dr. M. Sean Esplin for the use of laboratory supplies, time and support and Steven Hamblin for help with mice and his mentoring in learning new techniques. Lastly I would like to thank members of Dr. Grave’s lab for their help and support with the work.
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INTRODUCTION

The process of human parturition has been extensively studied for many years; however, the mechanisms responsible for the onset and progression of labor are complex and not completely understood. Over the years, several molecular factors have been considered to be the agent causing labor; among these are prostaglandin F\textsubscript{2α} (PGF\textsubscript{2α}) and oxytocin. Although both agents are agonists of uterine smooth muscle contraction, recent studies have suggested that neither is necessary for labor to occur. Among these reports there have been gene knockout studies in mice which have shown that oxytocin was not required for labor to occur and the absence of PGF\textsubscript{2α} only delayed labor but never prevented it (1). Understanding the mechanisms responsible for the onset and progression of labor could have enormous benefit, allowing potentially for efficient induction of labor or conversely in postponing unwanted labor. The ability to regulate or terminate labor is of particular importance in the setting of preterm labor, which continues to be a major public health issue and the number one cause of neonatal death (2).

Preterm Labor

Preterm birth, a considerable maternal and fetal health problem in this nation, has been a major target in research seeking the means of labor. Preterm deliveries occur in 5-9% of pregnancies and are responsible for the majority (~70%) of neonatal deaths and
nearly one half of all cases of congenital neurological disabilities, including cerebral palsy (3-6). Premature infants also have an increased risk of complications such as chronic respiratory illness, blindness and deafness. Although any birth before 37 weeks of gestation is considered preterm, birth before 32 weeks gestation accounts for most neonatal mortality and morbidity, in fact premature babies have a 40-fold increase in neonatal mortality (3).

The causes of preterm birth in general fall into three categories: iatrogenic, where there is demonstrable complication of pregnancy such as preeclampsia or fetal distress that requires obstetrical intervention and delivery of the fetus; premature rupture of the fetal membrane with or without infection; and idiopathic preterm labor. Approximately 30-40% of preterm birth is associated with an underlying infective process and 40-50% of preterm births are idiopathic, i.e. having no known cause (7). Several different approaches of prevention have been studied and include the use of antibiotics, tocolytic drugs and non-steroidal anti-inflammatory drugs (NSAID) (3, 8, 9). These approaches have resulted in varied and modest success; however, we have yet to develop truly efficacious therapies for the treatment or prevention of preterm labor and delivery.

A major obstacle to the development of effective therapies for preterm labor is the limited understanding of the molecular events required to initiate and maintain normal and abnormal labor. Despite many advances in prenatal and neonatal care and substantial investment of resources into research and medical care, the rate of preterm birth in the United States has continued to rise, especially among African American women (6, 10). Along with the increased rate of preterm birth, comes the attendant increased cost of prenatal, neonatal and postnatal care. Annually the cost of treatment of these conditions
nears one billion dollars (10). Understanding the underlying pathophysiology and mechanisms of preterm birth is critical to the prediction and ultimate prevention of this condition.

**Onset of Labor**

Parturition is the process in which the fetus is expelled from the uterus, and to insure optimal survival of the fetus, its timing must be tightly controlled. This process is preceded by a complex interplay of both fetal and maternal molecular factors. In order for parturition to occur, the uterus must change from a quiescent state into a contractile state and the cervical connective tissue along with the cervical smooth muscle must loosen and dilate to allow the passage of the fetus. There is a unique balance in factors that keeps the uterus in a non-contractile state and the cervix closed as opposed to having the uterus active and the cervix ripened (11).

There are several molecular factors that appear to be part of the pathway by which the uterus enters parturition. Estrogen is an important hormone which is involved in the process of parturition. It is essential for uterine growth, development, function and is responsible for the synthesis of several contractile proteins and regulatory enzymes necessary for uterine contractility. Estrogen is responsible for increasing the concentration of receptors for oxytocin and α-adrenergic agents which modulate membrane calcium channels as well as contribute to an increase in the appearance of gap junctions in the uterus. It is also responsible for stimulating the biosynthesis of prostaglandins F$_{2\alpha}$ and E$_2$, which can stimulate uterine contractions (11). Estrogen not
only contributes to uterine contraction, it is also involved in the control of cervical ripening, another process not yet completely understood.

Progesterone and estrogens play central roles in the maintenance of pregnancy and the initiation of parturition (12). Progesterone, which is considered to be a neurosteroid, supports pregnancy and prevents parturition by promoting uterine quiescence (12, 13). It does this by inhibiting gap junction formation as well as stimulating uterine Nitric oxide (NO) synthase, which appears to be a major contributor to uterine quiescence. NO synthase inhibition leads to preterm birth in mice and is also responsible for down regulating the production of prostaglandins and calcium channel formation (11).

In late pregnancy in women, the major source of circulating progesterone comes from the placenta (11). During pregnancy, progesterone is in dynamic balance with estrogen in the control of uterine activity. In sheep and rodents this progesterone/estrogen balance is very evident (14). Near term there is a sharp decrease in the maternal progesterone/estrogen ratio (14). As progesterone withdrawal takes place, estrogen exerts an increasingly dominate influence (14). Historically it was thought that progesterone withdrawal might be a feature of human parturition by analogy to rodent parturition. However higher primate parturition at term has not been associated with consistent circulating changes in progesterone, estradiol, or in the progesterone/estrogen ratio. Moreover, in human pregnancy maternal progesterone and estrogen levels are high for most of pregnancy and remain elevated during labor and delivery. However, more recent studies have revealed alterations in progesterone receptor type A in uterus. There appears to be an increase in this receptor near term (12). This receptor does not appear to
affect uterine activity but nonetheless reduces the progesterone presented to the other progesterone receptors that are coupled to uterine activity. This then represents a functional withdrawal of progesterone without a change in serum progesterone levels. These results have now been followed up by several studies exploring the use of progesterone as a means of reducing preterm labor and birth and studies to date are promising but have not been fully effective in preventing preterm birth (12).

One molecular factor that was long thought to be the cause of labor is oxytocin. The uterotonic action of the cyclic nonapeptide was demonstrated nearly a century ago (1). It remains one of the most potent uterotonic agents identified and continues to be used in routine clinical practice for augmenting uterine contractions during labor (1). This molecule, primarily produced in the hypothalamic supraoptic and paraventricular nuclei, stimulates contraction of mammary myoepithelial and uterine smooth muscle cells by virtue of its interaction with a specific high-affinity G protein coupled receptor (15). In addition to synthesis within the central nervous system, oxytocin is produced in gravid uterus and fetal membranes, suggesting that it may act by both endocrine and paracrine mechanisms to promote labor (1). Evidence thus far shows that oxytocin plays an important role in labor; however it is not required for parturition to occur in mice. There is also no evidence that there is an increase in the circulating oxytocin levels in late pregnancy or at the onset of labor. Levels only increase once the cervix has reached full dilation. Although the levels of oxytocin do not increase during late pregnancy, the concentration of uterine oxytocin receptors does. The expression of these receptors is increased by estrogen and suppressed by progesterone (11).
Oxytocin is linked to uterine contractions in two ways. First, it stimulates the release of PGE$_2$ and prostaglandin F$_{2\alpha}$ in fetal membranes by activation of phospholipase C, which in turn stimulates uterine contractions (11). It can also directly induce contractions through PLC, which in turns activates calcium channels and increases the release of calcium from intracellular stores (11). Although oxytocin may not be necessary for labor to occur, it has been found necessary for lactation in female mice. These mice failed in milk let down to nourish pups, which led to neonatal mortality (1).

Evidence has shown that prostaglandins play an important role in the final pathway of uterine contractions as well as parturition. Arachidonic acid is converted to prostaglandin H$_2$ by prostaglandin H synthase which is further converted to PGF$_{2\alpha}$ and PGE$_2$, which are the major stimulatory agonists (11). Prostaglandins are produced in the placenta and fetal membranes and levels increase before and during labor in the uterus and membranes in humans (11, 16). They have both direct and indirect effects on the uterus contractility; they stimulate myometrial contractions, further increase uterine sensitivity to uterotonic agents, synchronize myometrial contractions and alter hormone synthesis (4). Although prostaglandins may play an important role in parturition, it has also been shown that they are not necessary for the process to occur in mice. As stated before, gene knockout mice deficient in PLA2, which is responsible for liberating arachidonic acid from the cell membrane, only delayed the onset of labor (~22.3 days) when compared to wild type mice, which deliver at (~19.5 days) (1).

There are several other molecular factors that undoubtedly play important roles in parturition, but the entire process is still unknown. One of these that may be important is the sodium pump because of its unique role in the maintenance of the electrochemical
gradient in the cell as well as its influence on other transport mechanisms. Changes in the membrane potential are essential to control the contractility of the uterus.

**Sodium Pump**

The sodium pump has been studied extensively for many decades. To date much is known about the structure and function of the pump, however a comprehensive understanding of its physiologic role is incompletely understood. The basic function of the Na\(^+\)/K\(^+\) ATPase or sodium pump (SP) is to maintain the high Na\(^+\) and K\(^+\) concentration gradients across the plasma membrane of animal cells, with low Na\(^+\) outside and high K\(^+\) inside (17-20). By using the energy from the hydrolysis of one molecule of ATP, it transports 3 Na\(^+\) out in exchange for 2 K\(^+\), which are taken in (21). This establishes an electrochemical gradient or membrane potential which the SP also maintains. The osmotic balance in turn is coupled to crucial functions of the cell such as cell volume, and uptake of carbohydrates, amino acids and vitamins (22). The resting membrane potential of most tissues and the excitable properties of muscle and nerve cells depend then on the SP (18, 22, 23). It also has an important role in influencing cytoplasmic pH and Ca\(^{2+}\) levels in several cell types through the Na\(^+\)/H\(^+\) and Na\(^+\)/Ca\(^{2+}\) exchangers, respectively, and in driving a variety of secondary transport processes such as Na\(^+\)-dependent glucose and amino acid transport (17, 22).

The sodium pump belongs to a family of enzymes termed, P-type ATPases. These widely distributed enzymes are found in both prokaryotic and eukaryotic cells and are responsible for transporting H\(^+\), Na\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\) and Cd\(^{2+}\) (18). Members of this family include the sarcoplasmic reticulum and plasma membrane Ca\(^{2+}\)-ATPase and
the \( H^+ \), \( K^+ \)-ATPase found in the stomach and colon (17, 18). In a forward mode these enzymes utilize the chemical energy of ATP to form an ionic gradient. In the reverse mode the ionic gradient is used to form ATP (24). The unique characteristic that members of this family share is that they become auto-phosphorylated during the transport by the \( \gamma \)-phosphate group of ATP at an aspartic acid localized with the highly conserved sequence, DKTGS/T (20). Accompanying the phosphorylation-dephosphorylation process, these ATPases transfer cations by rotating between two different cation dependent conformations termed \( E_1 \) and \( E_2 \) (20, 25). The sodium pump is a unique member of this family in that it is specifically inhibited by cardiac glycosides such as ouabain and digitoxin and also low \([K^+]\) (26).

The \( \alpha \) Subunit

The sodium pump is a critical component in both secretory and absorptive mechanisms (27). It is a heterotrimer, composed of the \( \alpha \), \( \beta \) and \( \gamma \) subunit (23, 27, 28). A number of isoforms of both the \( \alpha \) and \( \beta \) subunits have been identified in various tissues from a number of species, and it has been repeatedly demonstrated that the function of the sodium pump requires the presence of both \( \alpha \) and \( \beta \) subunits (20, 23), but it is the \( \alpha \) subunit that carries out ATP hydrolysis and ion transport and it is the subunit that is phosphorylated (19). The \( \alpha \) subunit has a molecular mass of \(~110-113\) kDa, depending which of the four isoforms is present (18, 19, 20). Structurally the subunit spans the membrane ten times, with both the N-terminus and the C-terminus on the cytosolic side of the membrane (20, 29). The \( \alpha \)-subunit has four known isoforms, each of which is the product of a separate gene (28, 30). It appears that the distribution of the different
isoforms is tissue specific. The α-1 in association with β-1 is found in nearly every tissue (17, 23). The α-2 has been detected in adipocytes, muscle, heart, and brain, skeletal and vascular smooth muscle and α-3 is abundant in nervous tissue, placenta and fetal heart (17, 23, 27). While the first three isoforms are found in a variety of tissues, the α-4 isoform has only been detected in testes (17, 23). There is a high degree of amino acid identity within a species between α-1, 2 and 3, (~87%), with α-4 having only 78% identity with α-1. The greatest structural variability has been seen in the N-terminus, the extracellular ouabain binding site and the cytoplasmic sequence of amino acids 403-503 (31-33). The greatest structural homology is found in the cytoplasmic portion where ATP is bound and the enzyme is phophorylated. This subunit has been termed the catalytic portion of the molecule because it is the site of ATP hydrolysis.

The β Subunit

Like the α subunit, the β subunit has several isoforms, namely β-1, 2 and 3. All β-isoforms share a common basic structure. They consist of a short N-terminus in the cytoplasmic region, a trans-membrane sequence that spans the membrane once and a large extracellular domain (18, 29). Homology of the β-1 and β-2 isoforms across mammalian species is ~95% (34). The trans-membrane domain of the β subunit is the most highly conserved region both among isoforms and species (34). The β subunit is highly glycosylated and has a molecular mass of about 60kDa (17, 20). Recent studies have been able to give a better picture of what the role of this subunit is. It has been shown that the β subunit makes direct contact with the α subunit, thereby stabilizing it and assisting in its transport from the endoplasmic reticulum to the plasma membrane.
(20, 29). The subunit may also have an important influence on the $\alpha$ subunit conformation, and the ion sensitivity, the binding of ouabain and the hydrolysis of ATP (20, 35).

**The $\gamma$ Subunit**

A third subunit of the Na$^+$/K$^+$ ATPase has only recently been identified. The $\gamma$ subunit, which belongs to type I membrane proteins, is a small trans-membrane protein that specifically associates with the Na$^+$/K$^+$ ATPase in a tissue specific manner. Although the peptide was thought to represent a third component of the Na$^+$/K$^+$ ATPase, recent evidence suggests that it is not an integral part of the enzyme complex (17). This small hydrophobic peptide of approximately 7-11 kDa, is specific to the sodium pump because it has been shown to be specifically labeled, along with the $\alpha$ subunit, by a photoactive derivative of ouabain (17, 18). It has a possible interaction with the C-terminus domain of the $\alpha$ subunit (17). Sequence comparisons show strong homology (75%) among different species, with higher homology (93%) when compared among mammalian species (36, 37). Structure analysis has demonstrated that the $\gamma$-subunit contains a single trans-membrane domain, with an N-terminus in the extracellular portion and the C-terminus in the cytoplasmic portion (20). An intriguing feature of the $\gamma$ subunit structure is that it is detected as two species with similar amino acid composition irrespective of the protein separation method used. It was first thought that the two species were results from two single mRNA species. However, it was later shown that the two protein species are due to alternate usage of two distinct start codons in the message (17). Recent mass spectrometry work has shown that they most likely two
splice variants (17). This subunit appears to be present in approximately equimolar amounts compared to the \( \alpha \) and \( \beta \) subunits (17).

The Role of Na\(^+\)/K\(^+\) ATPase in Labor

It is unknown what role the sodium pump has in the onset of term and especially preterm labor. To date there is very little research involving the SP in pregnancy. There have been a few studies involving the distribution of the different isoforms of the SP in pregnant and non-pregnant rat uterus, as well as studies on the effect of LPS on SP isoforms in pregnant rat uterus (38, 39). The role of the sodium pump is of interest because its inhibition results in the release of calcium stores which can promote contraction in the uterus and its inhibition which may increase the release of hormones in the placenta. The SP maintains the cell membrane potential and reductions of SP number and activity will cause an increase in cytosolic-free calcium, resulting in greater muscle tone in mechanically active tissues (40). This has been studied extensively in the heart where cardiac glycosides which are known to inhibit the SP have been shown to increase heart contractility as the SP was inhibited. Decreased active SP units led to greater membrane depolarization, which increased cell calcium, which in turn increased contractions. It is hypothesized that there may be a similar mechanism in the uterus where a depolarization may occur due to lower SP numbers. Indeed, studies by others show that application of ouabain to human uterine smooth muscle causes direct contraction or that after initiation of contractions with prostaglandins, ouabain brings about stronger and more frequent contractions (41).
Previous work from our lab involving the SP during pregnancy has revealed that there is a general increase in all three isoforms in human placenta over the course of gestation up to week 39, followed by a decrease over the sequential weeks. However, these samples were obtained from women who were in active, spontaneous labor. In addition the α-3 protein abundance levels were determined in both women who were in spontaneous labor and those who were not in labor. Protein abundance was determined in both myometrial and placental tissues. There was a substantial reduction in the abundance of the SP α-3 isoform in both tissues in women who were in spontaneous active labor when compared to those who were not in spontaneous labor. This difference was only seen in the SP α-3 isoform, and not in the other two isoforms. This drop in α-3 isoform is of interest as a possible mechanism for the onset or progression of labor (40).

Model for Human Labor

Since tissue collection of human samples throughout the course of pregnancy is ethically unavailable, there is a need for an animal model to test SP involvement in labor. The most attractive candidate for such a model would be primate pregnancy; however the cost and availability of such samples as well as the long gestational period would be impractical for such studies. Consequently, mice may serve as the best available model for human labor. They have the advantage of a short gestation period (~19.5 days), along with a well defined reproductive cycle. One important characteristic that mice have in common with humans is hemochorial placentation. Normal embryonic development and growth in humans and non-human primates is dependent upon successful hemochorial placentation, which requires the transformation of the maternal intramyometrial spiral
arterioles by trophoblast invasion to gain adequate uteroplacental circulation, and establishment and maintenance of a competent fetoplacental vasculature. As a result, a tightly regulated process of trophoblast and endothelial cell differentiation, proliferation, and invasion occurs during placentation (42).

Even though mice share this characteristic with humans, there is one primary disadvantage in using this model and that is the difference in the process of parturition. As mentioned, it has been determined that the primary mechanism of parturition in rodents is the withdrawal of progesterone (1). The primary site of progesterone and estradiol production in mice is in the corpus luteum, while in humans these hormones are produced in the placenta. Circulating levels of progesterone in humans do not appear to decrease during parturition, but recent reports suggest the possibility that progesterone withdrawal may in fact be an important component of labor initiation (1). Even though mice may not share every characteristic of pregnancy, labor and delivery with humans, they may be the best and most feasible model to study parturition in humans.

In this study we hypothesize that a reduction of the SP α3 isoform in both the myometrium and placenta would occur in mouse labor and hence may represent a possible mechanism responsible for an increase in uterine contractility and an increase in the secretion of uterotonic factors from the placenta leading to the onset and/or progression of labor. We predicted that there would be a significant decline in SP α3 protein abundance in both tissues in mice, similar to that seen in humans during active labor. To test this hypothesis, we will determine SP α3 protein abundance at specific gestational time points in pregnant mice as well as pregnant mice in spontaneous active labor and one day post partum by Western blot analysis. Along with these protein
studies, we will determine whether there is a substantial reduction in mRNA levels of SP α3 isoform at these time points through the use of real time PCR. These studies may show if there is a decrease in SP α3 as a result of labor or if the decrease is in anticipation of labor. Decreases in SP α3 mRNA may possibly be responsible for the reduction in proteins. Along with gestational age studies, we further hypothesized that the use of lipopolysaccharide (LPS) induction in preterm pregnant mice would lead to a decrease in SP α3 protein abundance in uterus and placenta as well as reduced SP α3 mRNA levels. Again the use of Western blot and real time PCR may determine if there are any reductions. This would be important data to help determine if this same mechanism was used in both term labor and preterm labor in mice.
MATERIALS AND METHODS

Animal Experiments

Female C57Bl mice were used in these studies. A timed breeding procedure was used to control for insemination. Two female mice were placed in a cage with one male for a period of one day. After such time, the females were removed and inspected for seminal plugs. Once a seminal plug was observed, the female was removed from the male cage to ensure accurate gestational timing (2). A visual seminal plug was considered to be day 0 of gestation (43).

Tissue Collection

Pregnant dams were assigned to be sacrificed at certain gestational time points, specifically days 14, 16, 18, during birth and one day postpartum. Dams were euthanized using Halothane (Sigma, St. Louis, MO), an asphyxiation agent. Females were immediately laid on their backs and the abdominal wall was opened. Both horns of the uterus were removed with incisions below the ovaries and near the bladder, leaving the fetuses encased in the uterus. Fetuses were removed from the uterus by cutting along the uterine wall with scissors. Placenta and surrounding membrane were removed with tweezers and placed in a physiologic phosphate buffered saline (PBS) solution. Uterine and placental tissue samples were flash frozen in liquid nitrogen and stored at -80°C until processed.
Females used for the LPS study were injected intraperitoneally with either 500 μl of normal saline solution or 20 μg of LPS (Low dose from *E. coli* 011:B4) (Calbiochem, San Diego, CA) in 500 μL normal saline on day 15 of gestation. Each female received only one injection of either LPS or normal saline. Time of injection was set as 0 hr. Animals were sacrificed at time points of approximately 2, 6, 12, 18 hrs, during birth and one day post partum after injection. Dissection was performed in a similar manner to that described above.

**Tissue Preparation for Western Blot Analysis**

Tissue samples remained frozen at -80°C until homogenized on ice. For homogenization, one placenta or a (~ 3x3 mm or ~0.1g) piece of uterus was used. Each tissue sample was placed into 1 ml of ice cold homogenation buffer (250 mM sucrose, 50 mM Tris-HCl, 1mM ethylenediaminetetraacetic acid (EDTA); pH 7.3). Tissues were homogenized using a polytron homogenizer (Kinematica AG, Littau, Switzerland) at a speed of 6 for 30 seconds. Following homogenization, samples were centrifuged at 11,000 rpm for 30 minutes at 2°C to remove unwanted debris. Protein containing supernatant was subjected to a protein assay.

Protein concentrations were determined by the means of a Bradford assay. Standards of bovine serum albumin (BSA) (initial concentration of 10 mg/ml, R396A, Promega, Madison, WI) ranging in concentration from 0-10 μg/μl were used. Protein was visualized by using the manufacturer’s recommended amount of Bio-Rad protein assay dye reagent (500-006, Bio Rad Laboratories, Hercules, CA). Optical absorbencies were determined using the Ceres UV 900H Di spectrophotometer (Bio-Tek Instruments,
Winooski, VT) at a wavelength of 600 nm. All samples were aliquoted in triplicate into individual wells of a 96 well plate. The average of the three readings was used for comparison to standards to calculate the protein concentration.

For Western blot analysis, 25 μg of placental protein or 25 to 100 μg of uterine protein were separated by 7% SDS-PAGE. Gels were run at 140V for 15 minutes then the voltage was increased to 200V for 45 minutes. The proteins were transferred from the gel to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH) electrophoretically for one hour at 100V. Transfer of proteins was visualized by using Prestained Protein Ladder, ~10-180 kDa, (0501 MBI Fermentas, Hanover, MD). Membranes were then incubated with a non specific binding blocking solution (5% dehydrated milk in 20 ml of Phosphate buffered saline solution plus Tween (PBST) (0.015 mM NaH$_2$PO$_4$, 0.08 mM Na$_2$HPO$_4$, 1.45 mM NaCl, 0.2% Tween 20)) for one hour, with constant shaking on a lab rotator (Lab-Line, Melrose Park, IL) at a speed of 2. Excess milk solution was removed by washing the membranes three times, one 15 minute wash followed by two 5 minute washes with 25 ml of PBS with 2ml of 50% Tween. Membranes were incubated with primary monoclonal antibody raised against Na$^+$/K$^+$ ATPase alpha-3 (MA3-915, Affinity Bioreagents, Golden, CO) for one hour at room temperature or overnight at 4ºC. Antibody dilution was 1:3000 in 10 ml of PBST for placenta and 1:1500 in 10 ml of PBST for uterus. Again excess primary antibody was removed with a series of washes following the same schedule noted above. Following primary antibody incubation, the membranes were incubated with a secondary monoclonal antibody (Anti-mouse IgG tagged with horseradish peroxidase, Sigma, St. Louis, MO) in 10 ml of PBST. Excess secondary antibody was also removed as
previously described. The complex of primary antibody and secondary antibody tagged with horseradish peroxidase then allowed bound protein to be visualized by addition of an appropriate substrate (Enhanced Chemiluminescence Plus Kit, Amersham Bioscience, Piscataway, NJ), which yields a chemiluminescent product. Membranes were overlaid with photographic film (Kodak Scientific Imaging Film XLS, 8x10 in, VWR, West Chester, PA) and exposed for 1-5 minutes.

Resulting gel images were scanned into a computer and digitized by the computer software program UN-SCAN-IT (Silk Scientific, Orem, UT). Relative amounts of SP α3 isoform protein were determined using the earliest time point in the series as a reference value.

Western blot experiments were done with all three SP α isoforms; however SP α1 and SP α2 isoforms were not detectable under these circumstances. This was either due to poor antibodies or the absences of the isoforms in the mouse uterine and placental tissues. Therefore all experiments focused specifically on the SP α3 isoform.

**RT-PCR**

RNA isolation was accomplished by means of the SV Total RNA Isolation System (Promega, Madison, WI), according to the manufacturers protocol. Samples were stored at -80°C until processed for RNA. All RNA concentrations were determined using a spectrophotometer and obtaining an A_{260}/A_{280} ratio. Equal amounts (2 μg) of isolated RNA were used to produce cDNA by means of the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The protocol for manual transcription was followed except that sample volume requirements were modified. The
sample volume used was 20 μl, instead of the 100 μl called for in the protocol. All reagents employed in the reaction were used at one fifth of the protocol volume. Resulting cDNA was diluted with 80 μl of diethyl pyrocarbonate (DEPC) water and stored at -20ºC.

Real time-polymerase chain reaction was performed on an ABI 7900HT instrument (Applied Biosystems, Foster City, CA). cDNA samples were mixed with the TaqMan Universal PCR Master Mix which included all components necessary for a 5' nuclease assay. Added to this solution was 1.5 μl of gene specific primers. Samples were run with ABI primers specific for the α-3 isoform of the SP, as well as endogenous controls, which were used to account for differences in starting material (3). In this case mouse TATA Binding Protein (TBP) was used (Applied Biosystems, Foster City, CA). Samples were further control by running samples that were not reverse transcribed previous to amplification. Samples were aliquoted in duplicate with 25μl per well. The thermal cycle for all samples was as follows: 50ºC for 2 minutes followed by an increase to 95ºC for 10 minutes, then 40 repeated cycles of 95ºC for 10 seconds then a drop to 60ºC for 1 minute. All samples were analyzed using the manufacturer’s software.

Quantification of the mRNA was done by using a relative quantification method outlined by the manufacturer. This method eliminated the use of a relative standard curve; however, a set of standards, which consisted of previously amplified material, were diluted seven times in 10-fold dilutions to obtain an amplification range for all samples. In order to quantify without a relative standard curve, an endogenous control was used, i.e. mouse TATA Binding Protein. All mouse samples were run separately on the ABI 7900HT with both the SP α3 specific primer and the mouse TBP primer. In
order for the calculation of $\Delta \Delta C_T$ to be valid, the efficiency of the SP $\alpha 3$ and the efficiency of the mouse TBP must be approximately equal. To determine if the efficiencies were equal, template dilutions were amplified and the $\Delta C_T$ were compared.

**Statistical Analysis**

**mRNA Quantification**

Each time point of gestational age and each time point LPS induction was represented by at least four animals for both uterus and placenta. Controls for the LPS inductions were represented by one animal at that time point with the exception of the 18 hour control, which was represented by two different animals. A comparative $C_T$ Method (Separate Tubes) of quantification was used on each of the samples. This method is similar to using a standard curve, but instead uses arithmetic formulas to achieve the same calculation of relative quantification. The average cycle number at a threshold of 0.2 ($\Delta Rn$), from the gene of interest was subtracted from the average cycle number of the endogenous control. This calculation provided the value $\Delta C_T$, which is the difference in cycle number of the gene of interest compared with the control gene. To obtain the $\Delta \Delta C_T$, each sample was normalized to a calibrator, which is a reference sample that all samples were compared to. With regard to the uterus and placenta, each of the LPS induced samples was referenced to the 2 hour LPS injection time. Controls were also referenced to the 2 hour control time. For amplicons designed and optimized according to Applied Biosystems guidelines, the efficiency was close to one. Therefore, the amount of target, normalized to an endogenous reference (mouse TBP) and relative to a calibrator (2 hr LPS time point and control time point) is given by $2^{-\Delta \Delta C_T}$. The $2^{-\Delta \Delta C_T}$ value for each
time point (n= 4) was calculated using the previous equation. Data are expressed as the mean ± SEM. The average of all four animals for each time point was plotted. All gestational age study results were analyzed by analysis of variance (ANOVA) with post hoc Duncan’s pair-wise comparisons. LPS studies were analyzed by AVOVA as well by linear regression analysis. A p-value of 0.05 was considered to be statistically significant.

Protein Quantification

Uterine and placental samples were also subjected to Western blot analysis to determine protein abundance at each gestational time point as well as at time points after LPS injection. Bands on the resulting gels were scanned and digitized to obtain a pixel total. Totals from each animal were averaged and plotted and referenced to day 14 in the gestational age studies or 2 hours post injection in the LPS studies. All studies were analyzed by ANOVA with as post hoc Duncan’s pair-wise comparisons.
RESULTS

In order to study the timing changes in SP α3 abundance we needed a suitable animal model. The suitability of the model required changes in SP α3 isoform abundance comparable or very similar to those observed in laboring and non laboring pregnant women. To this end mice were bred and their pregnancies monitored over the last third of pregnancy.

Uterine SP α3 mRNA Quantity and Protein Abundance as a Function of Gestational Age

Uterine samples were obtained from mice at different gestational time points: day 14, 16, 18, during birth and one day post partum. After isolating total RNA, SP α3 mRNA was analyzed by means of real time-PCR. Each gestational time point represented four separate animals. All samples were quantified using a comparative Cₜ method according to the manufacturer’s protocol and outlined in the Methods section.

The mRNA levels were estimated for each animal at the specified gestational time point and potential changes in mRNA abundance were assessed. During the last third of mouse gestation, mRNA levels in the uterus changed significantly (ANOVA p= 0.004). Day 14 levels of mRNA (1.0±0.0) were selected as the reference point and other values in that same assay were expressed as a fraction of its value. The uterine SP α3 mRNA was most abundant on day 14 (Figure 1). Duncan’s pair-wise analysis showed the following significant changes compared to day 14 levels: SP α3 mRNA was decreased on
day 18 (0.45±.07) and at the time of birth (0.42±0.03). Day 16 SP α3 mRNA levels were (0.76±0.09) decreased when compared with levels during birth. While day 16 showed a

Figure 1. Relative Quantity of Uterine SP Alpha 3 mRNA during Gestation of the Mouse. SP α3 mRNA was obtained from the uterus of four different animals at each of the gestational time points. Day 14 was used as a reference for all other time points. Data were analyzed by ANOVA (p= 0.004) and with post hoc Duncan’s pair-wise comparisons. There was a significant decrease in mRNA levels from day 14 to day 18 and from day 14 to the day of birth. From birth to one day post partum, mRNA levels appeared to rebound, however this difference was not statistically significant. Error bars represent SEM between all four samples. Error bars represent the SEM of all four samples
decrease from day 14 and while there was a continued decrease from day 16 to day 18, these changes were not statistically significant. Interestingly, following birth there was an increase, although insignificant, in SP α3 mRNA levels by the first day post partum day (0.70±0.18), suggesting that levels of mRNA rebound quickly after birth.

SP α3 protein abundance as a function of gestation age followed a pattern somewhat similar to that of the mRNA abundance, again with a marked decrease at birth (ANOVA p= 0.054) (Figure 2). As we had previously done with the mRNA, the mean of protein abundance on day 14 was used as a reference for the comparison of other gestational time points over the final trimester (1.0±0.0). With regards to protein abundance, day 16 demonstrated a somewhat greater SP α3 isoform abundance than the levels on day 14 (1.27±0.37), however, this was not a statistically significant change. There was a drop in SP α3 protein levels from day 14 to day 18 (0.63±0.16) or to the day of birth (0.63±0.09), but these changes did not achieve statistical significance using a Duncan’s test, but were close, however were significant with a t-test (day 14 vs. day 18, p= 0.002). Protein levels did achieve a significant change from during birth to one day post partum (1.6±0.41), as well as from day 18 to one day post partum.
Figure 2. Relative SP Alpha 3 Protein Abundance in Uterus during Mouse Gestation, During Birth and One Day Post Partum. Uterine SP α3 protein abundances were quantified using Western blot analysis. Multiple assays of the same specimen were averaged and considered as a single value. The protein abundance changed (ANOVA p = 0.054). There was a mean significant decrease in protein from day 14 to day 18 and during birth and a significant increase from day 18 or day of birth to the post partum value.

Placental SP α3 mRNA Quantity and Protein Abundance as a Function of Gestational Age

Because of the placenta’s predicted secretory role in labor, we also studied the SP α3 isoform in this tissue as well. Placental samples were harvested at the same time points as uterus. Again each time point was represented by four different animals. Placental
mRNA samples were analyzed by means of real time PCR. Unlike the changes in SP α3 mRNA abundance seen in the uterus, the placental mRNA levels showed an overall progressive increase over the final trimester (ANOVA p = 0.03, Figure 3). Day 14 served as a reference to the other time points during the trimester (1.0±0.01). From day 14 to 16 there was almost no change (1.04±0.2). From day 14 to 18 (1.70±0.33) there was a modest increase, but this was still not significant. The overall trend was an increase, from one time point to another time point, there were significant increases, seen from day 14 to the day of birth (2.28±0.46) and from day 16 to the day of birth.

The placental SP α3 protein abundance from the gestational time points showed slightly different results than those obtained for the mRNA (Figure 4). Again day 14 SP α3 protein abundance levels were selected as a reference point (1.0 ±0.0) and the remainder of the values expressed as ratios. When analyzed by ANOVA (p= 0.76), there were no significant change in placental SP α3 isoform protein abundance overall or between gestational time points. The protein trend looked different from the mRNA trend. There was a suggestion that day 16 decreased from day 14 (0.71±0.24), then increased somewhat by day 18 (2.03±1.47). Samples taken during birth appeared somewhat decreased from day 18, with an overall increase from day 14 (1.62±0.81), but none of the differences were statistically significant due to the substantial variability among the tissues studied.
Figure 3. Relative Quantity of Placental SP Alpha 3 mRNA during the Gestation of the Mouse. Placental SP α3 mRNA was quantified for four different pregnant animals for each time point. There was a significant increase in placental SP α3 mRNA form day 14 to the day of birth ANOVA (p= 0.03).
Figure 4. Relative Protein Abundance of the Placental SP α3 during Mouse Gestation. Placental SP α3 protein was quantified using Western blot analysis. All results for a single specimen were averaged and considered as a single value. There were four specimens analyzed for each time point. There was no significant change in levels of α3 protein (ANOVA p= 0.77).

Uterine SP α3 mRNA Quantity and Protein Abundance Post LPS Injection

Agents such as LPS (endotoxin) are associated with a substantial portion of human preterm birth. This same agent can also induce mouse labor, even in preterm animals. Hence, LPS can be considered a representative preterm labor initiator. It was of
interest to see if changes in SP α3 abundance might also participate in preterm labor and delivery. Moreover, LPS allows for a much more careful way of examining the timing of SP changes. Consequently, pregnant mice were induced to labor by means of a single LPS injection on day 15 of gestation, delivering ~ 18 to 22 hours later. Induced animals were dosed with 20 μg of LPS in 500 μL of normal saline solution. Control animals were injected with 500 μL of normal saline. In each study the animal was injected in the morning, and the time of injection was counted as time 0. Uterus and placenta from one animal were collected with animals sacrificed at time points of approximately 2, 6, 12, 18 hours, during birth and one day post partum. The actual times were noted and used for analysis. After isolation, mRNA was quantified as described above for the gestational age studies of uterine mRNA. Each time point for mRNA represents four different animals raised under the same conditions with the exception of 18hr and during birth which were represented by two and five animals respectively. Quantified mRNA values were generated for each animal and levels from the same time point were averaged and plotted as the mean and standard error. Samples from 18 hr and during birth were combined for ANOVA assessment because of small numbers of viable specimens obtained at 18 hours. All specimens were calibrated to the 2 hour control and LPS specimens respectively. Control animals’ uterine mRNA levels showed a slight but insignificant decrease from 2 hours to 18 hours post injection. LPS injected animals did not demonstrate a significant change in mRNA between injection and labor (ANOVA p=0.58, Figure 5). From 2 hours to 6 hours there was little change (105±23). At 12 hours there was the suggestion of a decrease in SP α3 isoform mRNA in response to LPS (52±19). The specimens obtained at 18 hours and during birth (58±21) suggested that in
response to LPS the amounts of SP α3 mRNA may have been reduced. Interestingly, one day post partum (90±22) α3 mRNA levels appeared to have rebounded to almost what they were 2 hour after injection. This raises the possibility that SP α3 mRNA rebounds quickly after birth.

Uterine samples were also analyzed by Western blot analysis, using a SP α3 isoform specific monoclonal antibody. All samples used in the mRNA quantification study were also use in the protein abundance study. Additionally, several other samples from other LPS injected animals were obtained at various time points from animals studied prior to the institution of the mRNA arm of the studies. Methods of quantification used are described in the Methods section. Replicate results for the same animal were averaged together and plotted as a single point; hence each point represents a single animal.

SP α3 protein levels showed a significant drop in abundance as a function of time after LPS injection when analyzed by linear regression analysis (R= -0.55, p= 0.002, Figure 6) or by ANOVA (p= 0.047, Figure 6). ANOVA post hoc comparisons of LPS induced uterine specimens found significant changes from 2 hours to 20 hours post injection and from 20 hours to one day post partum. Control samples were referenced to the 2 hour time point. Controls showed very little change in abundance from 2 hour to 18 hours (R= -0.42 p= 0.3).
Figure 5. Relative Quantity of Uterine SP α3 mRNA Post LPS Injection. Mice were injected with 20 μg of LPS in 500 μl normal saline on day 15 of gestation. Control mice were injected with 500 μl of normal saline. Mice were sacrificed at specific time points after LPS injection. Control samples were calibrated to the 2 hour time point, likewise the LPS induced samples were calibrated to the 2 hour induced sample. Control samples showed a slight but insignificant decrease from 2 hours to 18 hours post injection. LPS induced samples demonstrated a slight rise in mRNA levels at 6 hours, then a marked drop at 12 hours. There were no significant changes (ANOVA p= 0.58).
Figure 6. Relative Abundance of Uterine SP α3 Protein Post LPS Injection. Uterine samples were analyzed by Western blot analysis, a α3 specific monoclonal antibody. Data from the same animal were averaged and plotted as a single point. SP α3 protein levels showed a significant drop in abundance from 2 hours to 24 hours after injection (R= -0.55, p= 0.002). Control samples showed very little change in abundance from 2 hour to 18 hours. Closed

Placental SP α3 Quantification of mRNA and Protein Abundance Post LPS Injection

Placenta samples were collected using the same procedure as detailed above in the uterine LPS induced animal study section. All placental tissues were isolated at the same
time as the uterine tissues. Each time point was represented by four animals with the previously mentioned exceptions. Mean values were plotted and data analyzed by ANOVA (p = 0.30) (Figure 7). All control samples were referenced and calibrated to the 2 hour time point. The LPS induced animal specimens referenced and calibrated to the 2 hour post injection time point. The individual runs were highly variable. From the 2 hour time point (100±17), mRNA levels were somewhat, but not significantly increased at 6 hours (126±14). From 6 hour to 12 hour (73±14) levels decreased, but this change was not significant. Levels of SP α3 mRNA during birth (76±28) were not statistically different from those seen at 2 hours. Overall there was no significant change in mRNA levels.

Placental SP α3 protein abundance was determined and changes in levels as a function of time after LPS injection assessed. Again all replicate data from one animal was averaged and considered as a single data point compared with other animals at that time. Initially, all the control and LPS injected animal placental SP α3 protein data were plotted and analyzed by linear regression. Placental SP α3 isoform protein abundance control samples were calibrated to the 2 hour control time point, and the LPS induced samples were calibrated to the LPS 2 hour time point. When SP α3 protein abundance was analyzed for the control tissues by linear regression, no significant changes in protein abundance as a function of time post injection was found. The line was nearly flat. In contrast to the controls, placental SP α3 protein abundance fell dramatically post LPS injection. Placental α3 showed a significant inverse relationship with time as shown by linear regression (R = -0.76, p = 3.12x 10^-8). Because there was the suggestion of a small increase in α3 protein abundance at 6 hours, potential differences from one sampling time
to another were assessed by ANOVA with post hoc pair-wise comparisons. When samples were analyzed by ANOVA, there were significant decreases in protein abundance from either 1 or 2 hours to 20 hours or time of birth (ANOVA $p=3.26 \times 10^{-5}$). There were also significant changes from 4 hours to 12, 18, 20 hours and during birth and from 6 hours to during birth.

**Figure 7.** Relative Abundance of the Placental SP $\alpha_3$ mRNA Post LPS Injection. Placenta mRNA samples were studied by RT-PCR. All time points were represented by four animals, with the exception of 18 hour and during birth, which were represented with 2 and 5 animals respectively. Data from animals at the same time points were averaged and plotted. Results in placental SP $\alpha_3$ mRNA were similar to those seen the uterus. Control samples were calibrated to the 2 hour time point and showed very little change from 2 to 18 hours. LPS induced samples showed a decrease in levels after LPS injection. There was no significant change in SP $\alpha_3$ mRNA abundance for controls or LPS induced animals.
Figure 8. Relative Abundance of Placental SP α3 Protein Post LPS Injection. Placental samples were analyzed by Western blot analysis. Multiple data determination from the same animal was averaged together and plotted as a single point. The results were analyzed by linear regression. Control samples showed very little change from 2 to 18 hours. Placental SP α3 protein abundance showed a significant decrease from 2 to 18 hours.
Validation Experiment for Real Time PCR

When using the comparative CT method for mRNA quantification described by Applied Biosystems, it is vital to perform a validation experiment to determine if the primer to the gene of interest along with the primer to the endogenous control is amplifying at a similar rate. The change in cycle number from the endogenous control and SP α3, were plotted versus the log of the input concentrations (Figure 10). In order for the experiment to be valid the slope of the trend line must be less than 0.10. Diluted samples of previously amplified cDNA were used to cover the change in cycle number range of all samples which were amplified by both primers. The slope of the line was (-0.053) and an $R^2$ value of (0.26) and hence amplification rates were considered to be similar.
**Relative Efficency Plot of SP Alpha 3 and Mouse TATA Binding Protein**

\[ y = -0.053x + 2.652 \]

\[ R^2 = 0.2588 \]

**Figure 9.** Validation Experiment to Determine the Primer Amplification Efficiency. Previously amplified cDNA specimens were diluted 10 fold, and amplified with both primers for the gene of interest and for the endogenous control. The change in cycle number of four concentrations that span the range in which both genes were amplifying all samples were plotted versus the log of the total RNA used. In order for the experiments to be valid, the slope of the line must be less than 0.1. The span of concentrations for the two primer exhibits a slope of less than 0.1, indicating that the amplification rates are similar.
DISCUSSION

Previously it was determined that the expressed protein levels of the α3 isoform of the SP in both placenta and uterus were substantially reduced in women who were in spontaneous, active labor compared with women at comparable gestational age who had not experienced labor (40). All three of the SP α isoforms were studied, but the other two α isoforms showed small insignificant differences between labor and non labor states. Because reductions of SP number can increase or even initiate uterine smooth muscle contraction, the changes observed in these pregnant women raised the question of whether the SP might play an active role in human labor. However, for the SP to have a role in labor it was important to know whether the timing of these reductions occurred prior to or during labor, and not merely a response observed after labor was largely completed.

As part of these earlier studies, there were no clear data available about the natural course of SP α3-isoform expression in human gestational tissues throughout pregnancy. There was, however, the suggestion that placental SP α isoform protein levels increased during gestational up to week 39, then declined. These results were confounded by the fact that these women were already in active labor. The ability to study gestational tissues such as the uterus and placenta in a predetermined, timed manner is nearly impossible in humans. As a consequence, we sought an animal model and decided to evaluate the C57Bl6 mouse to determine if it modeled changes as seen in
human parturition. The pregnant mouse would have to display similar reductions in SP α3 protein abundance toward the end of gestation as was seen in human subjects. We hypothesized that there would be such a decline in SP α3 mRNA and protein abundance during the time approaching labor in both uterine and placental tissues in the mouse. This had not previously been studied nor had any other model of the SP in labor been proposed or evaluated. If indeed the SP α3 isoform was involved in a possible mechanism responsible for the onset of labor, we further hypothesized that the use of LPS induction, which represented preterm, would constitute a decline in the SP α3 protein abundance.

Our results demonstrated that the pregnant C57Bl6 mouse is an adequate model. The pregnant mice did in fact show similar declines in uterine SP α3 protein abundance as those seen in women in spontaneous active labor. Placental protein data were too variable to know if there were SP α3 isoform reductions there. Using the pregnant mouse as a model, it was possible to study both mRNA levels as well as protein abundance levels as a function of gestational age. In addition to the protein data in uterus, we found that SP α3 isoform mRNA levels decreased significantly during the final trimester in anticipation of labor. An interesting note is that levels rebounded one day post partum. Protein levels of SP α3 seemed to decline somewhat later than mRNA levels but still in anticipation of labor, with the lowest levels seen at day 18 and during birth. Again a rebound in protein levels was seen one day post partum. Consequently the data strongly suggest that SP α3 protein levels during the latter point of mouse pregnancy are mediated by a reduction of its mRNA. This leads us to believe that there is transcriptional regulation of the gene responsible for producing the SP α3 isoform in anticipation of
delivery. What regulates this change in mRNA is of considerable interest but is beyond the scope of these studies. It also appears that there is an up regulation of the gene after birth has taken place to possibly restabilize the electrochemical gradient in the uterus and to diminish contractions.

Placenta protein levels in the mice did not obviously follow a pattern similar to that seen in human subjects. In those human studies, the placenta, like the uterus demonstrated a significant decline in SP α3 protein abundance in women in active spontaneous labor compared to those women not in labor. Unlike the drop seen in humans, mouse placenta protein levels did not show a significant change. The results must be considered inconclusive given the variability found for these specimens. However, we were able to determine SP α3 mRNA abundance over the final trimester of gestation. The mRNA results showed that there was a significant increase in levels from day 14 or day 16 to the day of birth. Because the mRNA levels increase over the last trimester up to the time of birth, it might be anticipated that additional protein studies will also demonstrate an increase. The general pattern in placental protein SP α3 abundance suggested a possible increase. Such a result would be distinct and different than the findings in women.

A model of preterm birth was also studied using the C57Bl6 mouse as a model. Induction was initiated by using LPS (endotoxin) through an intraperitoneal injection, which is believed to be a cause of preterm birth in the human (39). It has been shown previously in a rat model that intraperitoneally administration of LPS has led to increased myometrial contractions (39). Pregnant mice induced on day 15 with LPS were studied to determine SP α3 mRNA levels as well as SP α3 protein changes in both uterine and
placental tissues. Compared to control animals injected with vehicle, animals induced with LPS showed a modest decline in uterine SP α3 mRNA around 12 hours post injection. Levels fell and remained at that level at 18 hours and during birth. Like the SP α3 mRNA levels observed in the gestational age studies, there was an increase in uterine levels one day post partum, again suggesting a rapid rebound after birth. In contrast SP α3 protein abundance also manifests a marked decline after LPS injection. Control samples had insignificant changes from 2 to 18 hours. Although mRNA levels decreased somewhat post injection, they don’t seem to completely explain the decreases seen in protein abundance. They occurred later and were of smaller magnitude, even if real. Hence, these studies suggest that there is another mechanism responsible for the reduction seen in SP α3 protein after LPS is injected.

These SP changes suggest strongly that the SP probably plays an important role in LPS induced labor in mouse. This level of SP reduction is known to increase contractility of uterine smooth muscle and would also be expected to increase uterine sensitivity to other uterotonic agents. If such changes occurred in preterm human labor, it would explain how the contractions of labor progress to stronger, longer and more frequent events. A similar mechanism might also occur in term pregnancy. How LPS causes the observed changes is therefore of exceptional interest to understand this interaction mechanistically. There is some published experimentation describing such an action for LPS on the SP activity but these mechanisms linking LPS and SP activity have not been considered or studied in the setting of pregnancy (39).

Placenta from LPS induced mice showed similar results to those seen in the uterus. Here too there appeared to be a marked decline in SP α3 isoform protein
abundance, but there was no significant decrease in the mRNA. Like the uterus, the changes in SP α3 protein abundance do not seem to be mediated by the mRNA.

A reduction of SP α3 protein in the uterus may play an important role to the overall contractile status of that tissue during pregnancy because changes in the myometrial membrane potential are fundamental to the control of uterine activity (44). It is well established that both preterm delivery and spontaneous abortion are associated with an increased uterine activity. With LPS induction in mice, there appears to be a reduction in the abundance of SP α3 in the uterus. This reduction may lead to depolarization of the membrane, which in turn leads to the activation of L-type Ca$$^{++}$$ channels. Activation of these channels in turn leads to the increased contractions of the uterus. LPS studies on these L-type channels have been previously performed in rat uterus, in which it was determined that LPS led to increased uterine contractions as well as a decrease in SP activity probably through inhibition (39). It is also possible that loss of active SP units leads to depolarization and activation of Na, Ca exchange with more sustained increases in cell calcium and increased basal contractile tone.

The consequence of reduction or increase of SP α3 in placenta after LPS induction is not fully understood. However, in the placenta a reduction in SP activity would be anticipated to increase secretory function. It is known that the placenta is responsible for the production of prostaglandins. In fact during late pregnancy in women the placenta is the major source of circulating progesterone. Corticotrophin releasing hormone, which is the principle mediator of stress-induced preterm birth is also produced in the placenta. The placenta is also essential with regards to progesterone in humans, because it is the site where progesterone is synthesized from acetate and cholesterol.
Reduction of SP activity has been shown to increase secretion of prostaglandins and other hormones from a variety of cell types (45). Of course the SP status in the placenta is still unclear. Protein levels of SP α3 isoform may not adequately describe the functional status of SP activity which can be modified by inhibitors or by phosphorylation/dephosphorylation events of the SP protein by hormone mediated processes independent of SP autophosphorylation.

Limitations in these studies include the variability in the placental gestational age data. Potential sources of variation might be due to placental variation within a given animal. In the future it would be useful to look at the difference in SP α3 mRNA and protein abundance as found in each of the several different placentas that come from the same animal. The average pregnant mouse produces between 7 and 11 individual placentas per pregnancy. Total RNA for real time PCR and homogenized samples for Western blot were generally taken from one or two placentas for a given animal. If there is variation between different placentas, that might explain the variability that we found. Other limitations include the Western blot analysis of the uterus gestational and LPS specimens. Homogenization of the uterus is very difficult, often leaving large pieces which were not fully homogenized. As a result, detection and determination of the SP α3 by Western blot analysis was very difficult and often the quantity was too little to be visualized after SDS-PAGE. This explains why we obtained fewer results from uterine specimens. Also included in the limitations is the significant variation of signal intensity when using Western blot analysis. This made gel to gel comparison impossible. Because there were so many samples, a large number of gels had to be run, resulting in significant
variation in protein abundance between gels. This also meant that not all specimens from a given experimental series could be analyzed on the same gel.

Although uterine SP $\alpha_3$ protein appears to be mediated by the mRNA in the course of gestation, the placental and uterine protein abundance after LPS seems to be mediated by other means. There have been other mechanisms that could possibly explain the reduction in protein without the reduction of mRNA. Recent studies have shown a reduction in SP abundance through a process similar to endocytosis (46). Methods to study this mechanism have been developed which would enable the quantity of SP, which has been removed from the cell membrane and conveyed to other membrane compartments within the cell to be determined. This transfer has been found to occur for the SP after a specific phosphorylation event or after its interaction with an extracellular inhibitor (46). Another possible mechanism to explain the reduction in protein involves the $\beta$ subunit of the SP. It is known that this subunit is responsible for the insertion of the SP in the cell membrane. If there are reductions in a specific $\beta$ subunit, this might preclude the $\alpha_3$ isoform from being inserted into the membrane. Future work can be envisioned using Western blot analysis to establish the abundance of different $\beta$ subunits in both the uterus and placenta of the pregnant mouse after LPS injection. If there are changes it would then be critical to determine if there is a significant decrease in the relevant mRNA for that subunit.

In conclusion, progressive reductions in SP $\alpha_3$ protein abundance in uterus tissue may lead to uterine contractility alterations. Alterations in SP $\alpha_3$ protein abundance may also increase secretions from the placenta. Along with data from gestational age studies, data from the LPS induction studies, which also showed a marked reduction in SP $\alpha_3$
protein abundance in both uterus and placenta, suggest that the SP, and specifically its α3 isoform may, represent a major fundamental and previously unrecognized mechanism by which uterine contractions become more intense, more frequent and of longer duration which in turn contribute to delivery in mouse and possibly in humans.
REFERENCES


