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Expression and Function of Ca2+-Activated K+ Channels in Uterine Arteries

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Expression and Function of Ca^{2+}-Activated K^+ Channels in Uterine Arteries

by

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CONTENTS

FIGURES

Figures Page

ABBREVIATIONS

BK_{Ca}	Big conductance calcium-activated potassium channel
$[Ca^{2+}]i$	Intracellular calcium concentration
Ca_V	Voltage-gated calcium channels
cGMP	Cyclic guanosine monophosphate
CTX	Charybdotoxin
DHS	Dihydrosoyasaponin
E2β	Estradiol-17β
EC	Endothelial cells
eNOS	Endothelial nitric-oxide synthase
ERα	Estrogen receptor α
IBTX	Iberiotoxin
IK_{Ca}	Intermediate conductance potassium channel
IUGR	Intrauterine growth restriction
K_{ATP}	ATP sensitive potassium channel
K_{Ca}	Calcium activated potassium channel
Kir	Inward rectifier potassium channel
Kv	Voltage dependent potassium channel
NAC	N-acetylcysteine
NE	Norepinephrine
NPUAs	Nonpregnant uterine arteries
$O_2^{\cdot -}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical

$OONO^-$	Peroxynitrite
P4	Progesterone
PDBu	Phorbol 12,13-dibutyrate
PSS	Physiologic saline solution
PUAs	Pregnant uterine arteries
ROS	Reactive oxygen species
SK_{Ca}	Small conductance calcium-activated potassium channel
SK2	Small conductance calcium-activated potassium channel type 2
SK3	Small conductance calcium-activated potassium channel type 3
Sp	Specificity protein
TEA	Tetraethylammonium
UAs	Uterine arteries
VSMCs	Vascular smooth muscle cells

ABSTRACT OF THE DISSERTATION

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by

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Dr. Lubo Zhang, Chairperson

Chronic hypoxia during pregnancy is one of the most common insults to the maternal cardiovascular system and fetal development, and is associated with increased uterine vascular tone and heightened risk of preeclampsia and fetal intrauterine growth restriction (IUGR). The present study tested the hypothesis that calcium-activated potassium (K_{Ca}) channels play an essential role in uterine vascular adaptation to pregnancy, which is inhibited by chronic hypoxia during gestation. Uterine arteries (UAs) were isolated from nonpregnant ewes (NPUAs) and near-term pregnant ewes (PUAs) that had been maintained at sea level (~300 m) or exposed to high altitude (3,801 m) for 110 days. In normoxic animals, both BK_{Ca} and SK_{Ca} channels were expressed in uterine arterial smooth muscle cells and endothelial cells. Pregnancy selectively enhanced the protein abundances and mRNA levels of BK_{Ca} subunit β1 and SK_{Ca} subtype 2 and 3 in uterine arteries, resulting in enhanced both BK_{Ca} and SK_{Ca} channels activities and their-mediated relaxations, and decreased uterine vascular tone in PUAs as compared with those in NPUAs. Chronic treatment of NPUA with 17β-estradiol (E2β) and progesterone significantly increased BK_{Ca} and SK_{Ca} channel exspression and enhanced both BK_{Ca} activator NS1619- and SK_{Ca} activator NS309-induced relaxations of NPUAs. Chronic

hypoxia during gestation significantly attenuated both NS1619- and NS309-induced relaxations of UAs, which was associated with decreases in BK_{Ca} and SK_{Ca} channel expression and their activities. Chronic hypoxia enhanced the inhibitory role of oxidative stress and PKC on K_{Ca} channel activities and their-mediated uterine arterial relaxation. In addition, chronic hypoxia ettenuated the effect of 17β-estradiol and progesterone in K_{Ca}-mediated relaxations in NPUAs. In conclusion, our results suggest an important role of K_{Ca} channels in the regulation of basal uterine vascular tone. Pregnancy-mediated decrease in uterine vascular tone is associated with an enhanced K_{Ca} channel expression and their activities, which is regulated by steroid hormones. Chronic hypoxia during gestation attenuates the effect of steroid hormone on K_{Ca} channels, resulting in decreased K_{Ca} channel-mediated relaxations and increased uterine vascular tone.

CHAPTER ONE

INTRODUCTION

Pregnancy is associated with a decrease in uterine vascular tone and a significant increase in uterine blood flow that optimizes the delivery of nutrients and oxygen to the developing fetus. The adaptation of the uterine circulation to pregnancy may be mediated, at least in part by vascular remodeling (Osol and Mandala, 2009), enhanced vasodilation (Gangula et al., 1999; Nelson et al., 1998; Ni et al., 1997; Xiao et al., 2001a), blunted vasoconstrictor response (Cooke et al., 2003;Nelson et al., 1995b; Weiner et al., 1991) and decreased vascular tone (Hu et al., 2011; Nelson et al., 1998; Xiao et al., 2001a). However, these adaptive changes in the uterine circulation during gestation are modulated by hypoxia (Jackson et al., 2005; Keyes et al., 1998; Nelson et al., 1995b; Rosenfeld et al., 2009; Xiao et al., 2012). Hypoxia is a pathophysiological condition in which the body as a whole or a region of the body is deprived of adequate oxygen supply. Both short-term (acute) hypoxia and long-term (chronic) hypoxia may result in cardiovascular dysfunction. High altitude is considered above 2500 meters (8000ft) (Martin et al., 1998). High-altitude chronic hypoxia blunted pregnancy-induced reduction of myogenic tone in uterine arteries (Chang et al., 2009; Hu et al., 2012), which in turn attenuated pregnancy-induced increase in uterine blood flow (Julia et al., 2008; Zamudio et al., 1995 a, b). For example, compare to the sea level, pregnancies at high altitude may cause significantly depressed maternal arterial pO2 and changes in placental growth (Zamudio, 2003). In addition, blood pressure in high altitude pregnant women has been

found inversely related with arterial O2 saturation. (Moore et al., 1998; Moore et al., 2001). Previous studies also suggested that women who reside at high-altitude were predisposed to numerous pathological conditions associated with low blood volume or altered vascular reactivity, as well as diabetes, lupus, and vasculitis (Zamudio et al., 1995 a, b). These findings have provided evidence to support that the adverse impact of chronic hypoxia on uterine circulation may be one of the most common insults to the maternal cardiovascular system and fetal development (George et al., 2011; Julian et al., 2008; Khalil et al., 2002; Moore et al., 2011; Palmer et al., 1999; Zamudio et al., 1995a).

Women at high altitude have a lower blood volume than women at moderate-altitude during pregnancy, and have a higher risk of developing preeclampsia and intrauterine growth restriction (IUGR), which have been observed in many regions of the world including Middle East, North and South America and Tibet people in China (Zamudio, 2007). Moreover, the blood volume expansion during pregnancy has been found decreased in women who developed preeclampsia or transient hypertension compared with health women (Longo, 1984; Zamudio, 2003). Therefore, IUGR has been found related with low blood volume in pregnancy (Croall et al., 1978; Gibson, 1973; Goodlin et al., 1981), which is associated with increased risk of premature birth and complications of pregnancy other than hypertension (Goodlin et al., 1981). Furthermore, decreasing uterine artery blood flow has been found in pregnant women at high altitude, which precedes the symptoms of preeclampsia (Zamudio et al., 1995 a, b; Zamudio, 2007). Overall, the susceptibility to develop preeclampsia and IUGR is significantly increased under conditions of maternal hypoxia (Palmer et al., 1992; Zamudio et al., 1993).

K_{Ca} channels and Vasodilation

Ion channels in vascular smooth muscle play an important role in the regulation of microvascular function. Potassium (K^+) channels are highly expressed in the plasma membrane of arteriolar smooth muscle cells and K^+ currents contribute to modulating membrane potential that in turn controls the activity of Ca^{2+} channels in vascular smooth muscle cells and regulates vascular tone (Jackson, 2005; Keyes et al., 1998; Nelso and Quayle, 1995; Rosenfeld et al., 2009). It has been demonstrated that the regulation of potassium channel activities plays an important role during pregnancy to make sure that the myometrial tranquility during gestation is maintained until contractions are necessary in labor (Pierce et al., 2008). In general, the activation of K^+ channels in arterial smooth muscle causes a decrease in vascular tone and an increase in blood flow *via* vasodilation. In contrast, the inhibition of K^+ channels results in vasoconstriction. Pregnancy is associated with increased sex steroids hormones/receptors levels in uterine vasculature. The increased steroid hormones/receptors differentially attenuate protein kinase C (PKC)-mediated signaling in uterine arterial smooth muscle cells (Xiao et al., 2006; Zhang et al., 2006), leading to differential upregulation of K^+ channels expression and/or their activities, which are likely to contribute to the decreased uterine vascular tone and increased uterine blood flow in pregnancy. Exposed to hypoxia during pregnancy attenuates the effects of sex steroid hormones/receptors, leading to enhanced PKC activation in pregnant uterine arteries. The selective inhibition of K^+ channels activities by the increased PKC activation is likely to contribute significantly to the maladaptation of uterine vascular hemodynamics in pregnancy complicated by preeclampsia and fetal intrauterine growth restriction in response to hypoxia. Four types of K^+ channels have

been identified in most arterials smooth muscle: ATP sensitive (K_{ATP}), Ca^{2+} activated (K_{Ca}), voltage dependent (Kv) and inward rectifier (Kir)(Nelson and Quayle, 1995; Standen and Quayle, 1998). As the knowledge of structures and properties of each K^+ channels and their physiological and pathological roles in uterine vasculature continues to grow, it should become possible to develop pharmacologic therapeutic strategies targeting on K^+ channels to prevent or treat uterine vascular dysfunction in pregnancy complications such as diabetes and hypertension in gestation, preeclampsia and fetal intrauterine growth restriction.

Ca^{2+}-activated K^+ (K_{Ca}) channels, which contribute significantly to setting the membrane potential, play an important role in regulating the excitability of vascular smooth muscle cells (VSMCs) (Figure 1) (Ledoux et al., 2006; Jackson, 2005). Based on their conductance, K_{Ca} channels are divided into large-conductance (BK_{Ca}), intermediate-conductance (IK_{Ca}), and small-conductance (SK_{Ca}) channels (Wei et al., 2005). In VSMCs, opening of K_{Ca} channels causes membrane hyperpolarization, leading to closure of L-type voltage-gated Ca^{2+} channels (Ca_V) and subsequent vasodilation. In contrast, closure of K_{Ca} channels triggers membrane depolarization, resulting in opening of Ca_V and vasoconstriction. In addition, hyperpolarization produced by the activation of SK_{Ca} and IK_{Ca} in endothelial cells could be transmitted to VSMCs via the myoendothelial gap junction (Félétou, 2009; Kohler et al., 2010). Activities of K_{Ca} channels are under tight control of external stimuli, such as vasoactive substances and environmental changes (Ledoux et al., 2006; Hu et al., 2012).

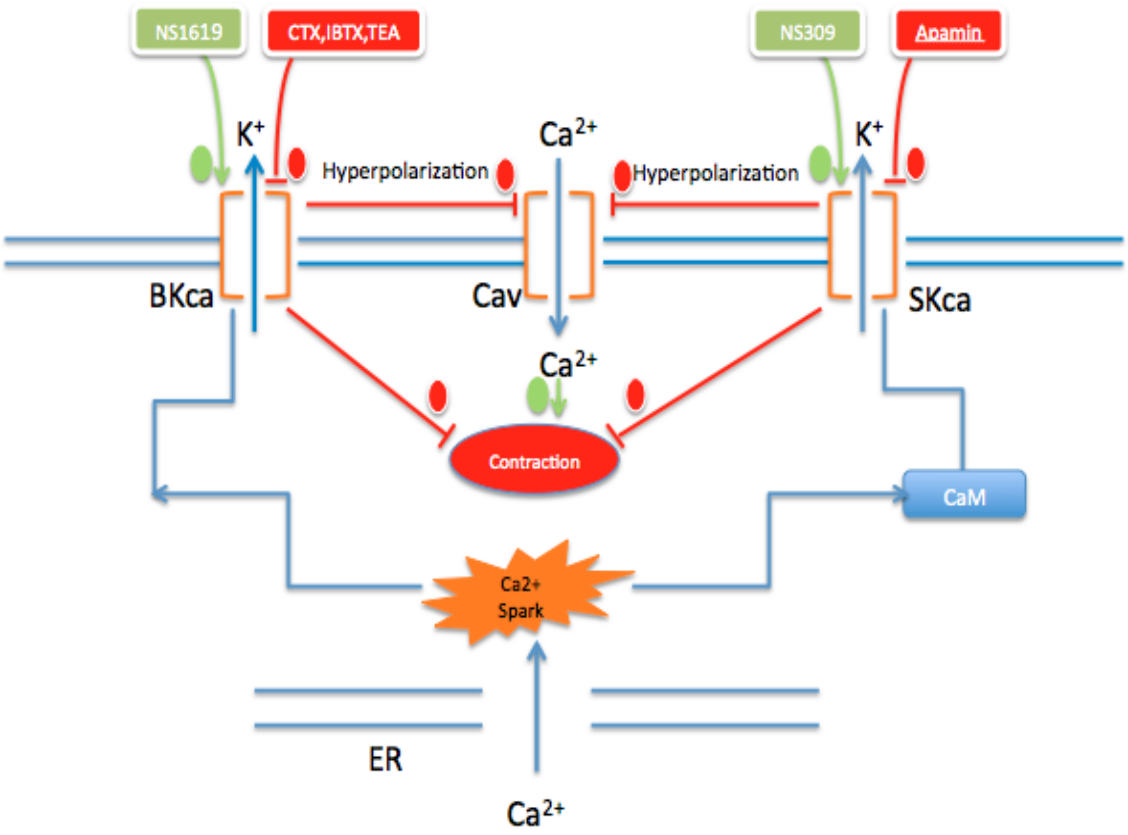

Figure 1. Role of K_{Ca} Channel Regulated Vascular Tone in Smooth Muscle Cell. Activation BK_{Ca} and SK_{Ca} channel by increasing $[Ca^{2+}]i$ in smooth muscle cells enables K^+ efflux, and causing membrane hyperpolarization, leading the voltage-gated Ca^{2+} channel closing and decreasing $[Ca^{2+}]i$, then resulting in relaxation. BK_{Ca} and SK_{Ca} channel also can be opened by their blockers and openers, separately.

Large-conductance (200~250 pS), Ca^{2+}-activated K^+ channels are activated both by changes in intracellular Ca^{2+} concentration and membrane depolarization. The channels have a high single-channel conductance, thus it is also called as "big" K_{Ca} channels (BK_{Ca} channels) (Nelson, 1993). BK_{Ca} channels are comprised of a pore forming α-subunit and four regulatory β-subunits. The α-subunit has seven transmembrane domains (S0-S6) (Nelson, 1993; Nelson et al., 1990; Wellner et al., 1996). In addition, the BK_{Ca} channels have four β-subunit isoforms (β1, β2, β3, β4), each with two transmembrane domains. It has been shown that the β1-subunit predominates in vascular smooth muscle (Ledoux et al., 2006; Tanaka et al., 2004). The major role of β1-subunit is to enhance the apparent Ca^{2+} sensitivity of the channel, modifying the channel's gating properties (Clapp and Jabr, 2003; Cox and Rusch, 2002; Meera et al., 1996; Waldron and Cole, 1999).

BK_{Ca} channels play an important physiologic role in regulating vascular smooth muscle contractility and blood pressure (Ledoux et al., 2006; Tanaka et al., 2004; Korovkina and England, 2002). Studies in BK β1-subunit knockout mice have demonstrated that Ca^{2+} spark-induced BK current is significantly reduced and the mean arterial blood pressure is elevated in the β1-subunit-null mice, leading to left ventricular hypertrophy (Brenner et al., 2000). In addition, BK_{Ca} channels also play a key role in the regulation of myogenic tone. Increased blood pressure induces membrane depolarization and increases $[Ca^{2+}]_i$ leading to the activation of BK_{Ca} channels (Nelson and Quayle, 1995). Activation of BK_{Ca} channels in turn enhances K^+ efflux and counteracts depolarization and constriction-induced by pressure or vasoconstrictors.

BK_{Ca} channels are very effectively blocked by the scorpion peptide toxin

charybdotoxin (CTX), the related peptide iberiotoxin (IBTX) and slotoxin (Wallner et al., 1995; Miller et al., 1985). These blockers bind to the outer vestibule of the channel to physically occlude the pore and prevent ion conduction. Several tremorgenic indole alkaloids molecules such as paxilline, penitrem A and verruculogen are also potent blockers of BK_{Ca} channels. In addition, tetraethylammonium (TEA) is a broad-spectrum K^+ channel blocker. However, relatively low concentrations of TEA ($\leq$ 1 mM) can selectively block BK_{Ca} channel. BK_{Ca} channel openers comprise a large series of synthetic benzimidazolone derivatives such as NS004 and NS1619, biaryl amines, biarylureas, pyridyl amines, 3-aryloxindoles, benzopyrans, dihydropyridines, and natural modulators such as dihydrosoyasaponin-1 (DHS-1) and flavonoids. Both NS004 and NS1619 are known as α-subunit-selective BK_{Ca} openers. NS1619 is the only compound without any effects on other ion channels. Other than benzimidazolone derivatives, a wide structural diversity of drugs such as carbonic anhydrase inhibitors has also been shown BK_{Ca} activation properties. In addition, various drugs such as niflumic, flufenamic, and mefenamic acids, as well as 17-β estradiol, can activate BK_{Ca} channels in a nonselective manner (Archer et al., 1994; Gelband et al., 1993; Hu et al, 1996; Jackson, 2005; Nelson and Quayle, 1995; Wang et al., 1997).

Whereas the BK_{Ca} channel is preferentially expressed in VSMCs, IK_{Ca} and SK_{Ca} channels are initially believed expressed predominantly in endothelial cells (Ledoux et al., 2006; Hu et al., 2012). It was believed that the activation of SK_{Ca} and IK_{Ca} channels indirectly affected smooth muscles through endothelium-dependent mechanisms which associated with generation of nitric oxide in the endothelium and smooth muscle cells (Wei et al., 2005; Kohler et al., 2010). This notion was supported by the finding that a

genetic deficit of SK3 or IK1 channels caused hypertension by abolishing endothelium-derived hyperpolarizing factor-mediated vasodilation (Brahler et al., 2009). However, the presence of SK_{Ca} channels in VSMCs has been detected in several vascular beds by immunohistochemistry (Chen et al., 2004; Potocnik et al., 2009; Sorensen et al., 2011). SK_{Ca} channels are also found expressed in myometrium and regulated by oxygen (Pierce et al., 2010). The SK_{Ca} channels can be activated by NS309 and selectively blocked by apmine (Dalsgaard et al., 2010; Zhu et al., 2013 a).

The Function of K_{Ca} during Pregnancy and Hypoxia

Role of BK_{Ca} Channels in Uterine Vascular Adaptation to Pregnancy and Hypoxia

Both α and β1-subunits of BK_{Ca} channels are expressed exclusively in ovine uterine arterial smooth muscle cells with no evidence of their existence in the endothelium (Rosenfeld et al., 2009; Nagar et al., 2005; Khan et al., 2010). Recent studies have shown a pregnancy-related modification of BK_{Ca} channels gene expression patterns in uterine vasculature (Rosenfeld et al., 2009; Rosenfeld et al., 2001; Rosenfeld, 1984; Rosenfeld, 2005). Three α-subunit species were found in uterine arterial smooth muscle of nonpregnant sheep with 83, 100, and 105 kDa. During pregnancy, there was an absence of the 83-kda protein and a marked decrease in the 105-kDa protein, both reappearing ≥30 days after delivery. The 100-kDa α-subunit rises during pregnancy, but it does not appear to equal the fall in the other two species, suggesting that total channel density may actually fall in pregnancy (Rosenfeld et al., 2009). Other studies showed that the α-subunit of 100 kDa was not significantly different in uterine arteries between nonpregnant and pregnant sheep (Hu et al., 2011). One possible reason for this apparent

difference may be because of different sizes of the vessels used (Rosenfeld et al., 2009; Hu et al., 2011). BK_{Ca} β2-subunits are present in ovine uterine arterial smooth muscle cells, but the levels are low and unchanged throughout the reproductive cycle. However, the β1-subunit expression is increased in pregnant uterine arteries as compared with nonpregnant vessels (Rosenfeld et al., 2009; Hu et al., 2011). The increased β1-subunit expression during pregnancy parallels the rise in uterine blood flow (Rosenfeld et al., 2009; Rosenfeld, 1984; Rosenfeld et al., 2005). Electrophysiological studies demonstrated a greater whole-cell K^+ current density in pregnant, as compared with nonpregnant, uterine arteries. Both of the tetraethylammonium (TEA) and iberiotoxin inhibit K^+ currents to the same extent in uterine arterial myocytes. This suggests that the BK_{Ca} channel current density is significantly increased in uterine arteries of pregnant animals (Hu et al., 2011). Upregulation of β1-subunit expression during pregnancy is likely to enhance the Ca^{2+} sensitivity of BK_{Ca} channels and facilitate the activation of the channel and the consequent reduction in uterine vascular tone in pregnancy. Indeed, previous studies have demonstrated that intra-arterial infusion of TEA into the uterine artery circulation of late-gestation sheep causes a decrease of basal uterine blood flow from 50% to 80% in the absence of systemic effects (Rosenfeld et al., 2001; Rosenfeld, 2005). This is consistent with the recent findings that TEA inhibited K^+ currents by 53% in pregnant uterine arteries, and TEA significantly increased pressure-dependent vascular tone in ovine pregnant uterine arteries and eliminated the difference of the myogenic response between nonpregnant and pregnant uterine arteries (Hu et al., 2011). These observations suggest that the heightened BK_{Ca} channels activity is one of important mechanisms in regulating uterine vascular tone and maintaining uteroplacental blood

flow in pregnancy.

In many vascular beds, hypoxia causes local vasodilation. This response increases blood flow to the affected organ and thus promotes restoration of tissue oxygenation. Many studies suggest that the hypoxia-induced vasodilation and blunted vasoconstriction are associated with an increased BK_{Ca} channels expression and/or their activities in the vasculatures (Long et al., 2002; Gebremedhin et al., 1994; Naik and Walker, 2003; Naik and Walker, 2006; Earleye et al., 2003). However, in the lung, hypoxia causes local vasoconstriction. Paradoxically, the hypoxia-induced pulmonary hypertension is associated with an increased expression of BK_{Ca} channels (Resnik et al., 2006; Ahn et al., 2012), which might suggest an adaptive mechanism counteracting pulmonary hypertension since BK_{Ca} channel activation serves as a feedback modulator of vascular tone when cytoplasmic calcium becomes elevated (Nelson and Quayle, 1995). In the uteroplacental circulation, hypoxia-induced fetoplacental vascular constriction has been well demonstrated (Hampl and Jakoubek, 2009). The hypoxia-induced fetoplacental vascular constriction is largely mediated by hypoxic inhibition of Kv channels rather than its effect on BK_{Ca} channels in smooth muscle of small fetoplacental arteries (Hampl et al., 2002). In pregnant sheep, chronic hypoxia enhances uterine vascular tone (Chang et al., 2009). Although the mechanisms underlying chronic hypoxia-mediated elevation of uterine vascular tone in pregnant animals are not completely understood, the reduction of uterine vascular BK_{Ca} channel activities is a possible mechanism, given a key role of BK_{Ca} channels in the regulation of uterine vascular tone during normal course of pregnancy (Hu et al., 2011; Zhu et al., 2013 b).

Role of SK_{Ca} Channels in Uterine Vascular Adaptation to Pregnancy and Hypoxia

Uterine blood flow increases during pregnancy to ensure the optimal growth and development of the fetus. Remodeling of uterine vasculature (Osol and Mandala, 2009), reduced pressure-dependent myogenic reactivity (Hu et al., 2011; Veerareddy et al., 2002; Xiao et al., 2006), blunted vasoconstrictor response (Cooke and Davidge, 2003; Nelson et al., 1995; Weiner et al., 1991) and enhanced vasodilator response (Gangula et al., 1999; Nelson et al., 1998; Ni et al., 1997; Xiao et al., 2001a), all attribute to this hemodynamic change. Vascular tone constitutes the major determinant of the resistance of blood vessels, which regulates blood pressure and the distribution of blood flow between and within tissues and organs. Regulation of vascular tone in uterine arteries contributes to maintain normal pregnancy (Xiao et al., 2006; Xiao et al., 2009). Previous findings demonstrated that BK_{Ca} channels in uterine arteries during pregnancy were vital for decreasing vascular tone and contractility (Hu et al., 2011). Although the function of SK_{Ca} channel in uterine arteries during pregnancy is not clear, recent studies have provided evidence linking pregnancy in regulating SK_{Ca} channel expression in rat and human myometrium (Noble et al., 2010; Mazzone and Buxton, 2003). Moreover, recent studies demonstrated that uterine arteries from nonpregnant transgenic $SK3^{T/T}$ mice overexpressed SK3 channels had larger basal diameters and blunted vasoconstrictor response compared to those from wild-type animals (Rada et al., 2012). Lines of evidence have also implicated SK_{Ca} channels in regulating excitability and contraction of smooth muscle cells from uterus and urinary bladder (Brown et al., 2007; Herrera et al., 2003; Thorneloe et al., 2008). Furthermore, previous studies have shown that sex steroid hormones play a vital role in downregulating uterine artery pressure-dependent myogenic

tone in pregnancy (Xiao et al., 2009). Activation of estrogen receptors can alter gene transcription, which has profound impacts on cardiovascular function (Murphy, 2011). Pregnancy increased the expression of estrogen receptor-α in uterine arteries (Chang et al., 2010). Our group recently demonstrated that 17β-estradiol was responsible for heightened expression and activity of BK_{Ca} channels in uterine arteries of pregnant sheep (Hu et al., 2011). Similarly, the expression of SK3 channels is also regulated by 17β-estradiol in recombinant expression system (Jacobson et al., 2003), hypothalamus (Bosch et al., 2002), and myometrium (Pierce and England, 2010). Therefore, sex steroid hormones play a curial role in the pregnancy-mediated regulations of Kca channel expression and function in the uterine vasculature.

There is a growing number of evidence to suggest that chronic hypoxia abrogate the role of K_{Ca} channels in the regulation of myogenic reactivity in arteries. Previous findings demonstrated that IK_{Ca} channels were downregulated and EDHF-mediated relaxations were impaired in rat pulmonary arteries when expose to chronic hypoxia (Kroigaard et al., 2013). Our recent studies demonstrated that chronic hypoxia abolished the regulatory role of BK_{Ca} in uterine arteries in response to pregnancy (Hu et al., 2012). Moreover, chronic hypoxia during gestation significantly suppressed estrogen receptor-α (ERα) expression in uterine arteries without altering maternal plasma estrogen levels (Chang et al., 2010). More recent studies demonstrated that heightened promoter methylation attributed to chronic hypoxia-mediated downregulation of ERα gene expression (Dasgupta et al., 2012). These findings suggest the ablation of pregnancy-induced upregulation of K_{Ca} channels by chronic hypoxia during gestation may occur at the genomic level.

K_{Ca} Channels and Sex Steroid Hormones

Pregnancy is a state with substantially higher levels of estrogen and progesterone as compared with the nonpregnant state. Growing evidence suggests that the increased levels of sex steroid hormones may regulate uterine vascular tone and uterine blood flow *via* alteration of BK_{Ca} channels-mediated signaling (Hu et al., 2011; Magness et al., 1989; Magness et al., 1998; Rosenfeld et al., 2009; Rosenfeld et al., 2005; Rosenfeld et al., 1976). In ovariectomized sheep or mice, the estrogen treatment enhanced β1-subunit mRNA and protein expression in uterine arteries and myometrial smooth muscle, which suggests a possible role of the steroid hormone in modulating BK_{Ca} channels expression (Nagar et al., 2005; Benkusky et al., 2002). Indeed, the direct treatment of uterine arteries from nonpregnant animals with estrogen and progesterone for 48 hours *ex vivo* significantly enhanced β1-subunit protein expression in uterine arterial smooth muscle (Hu et al., 2011). The expression of β1 subunit was also found higher in the follicular phase as compared with the luteal phase of the ovarian cycle in nonpregnant sheep, probably because of relatively high estrogen levels that were produced endogenously by the ovaries (Khan et al., 2010; Rupnow et al., 2001). Furthermore, TEA had no significant effects on basal uterine vascular resistance and blood flow, but produced a dose-dependent inhibition of the estradiol-17β ($E_2β$)-induced rise in uterine blood flow when infused into the uterine arterial circulation of ovariectomized nonpregnant ewes (Rosenfeld et al., 2000). In addition, $E_2β$-mediated uterine vasodilation is also associated with BK_{Ca} channels activation (Rosenfeld et al., 2009; Rosenfeld et al., 1984; Rosenfeld et al., 2005; Magness et al., 1989; Rosenfeld et al., 2000; Byers et al., 2005; Wellman et al., 1996; Darkow et al., 1997; White et al., 1995). These observations suggest that the

regulation of uterine vascular tone by BK_{Ca} channels is modulated by sex steroids.

As compared with estrogen, the effect of progesterone in the regulation of BK_{Ca} channels is less well established. In contrast to estrogen, progesterone inhibits the BK_{Ca} channel current in Xenopus oocytes (Wong et al.,2008). The inhibitory effect of progesterone on BK_{Ca} channels may partly explain its antagonism against estrogen-mediated vasorelaxation as shown in vitro in porcine coronary arteries (Teoh and Man, 1999). Given the fact that progesterone plays an important role in regulating uterine blood flow during pregnancy (Byers et al., 2005; Perrot-Applanat et al., 1988), whether progesterone-mediated uterine vascular tone is regulated through modulation of BK_{Ca} channels needs to be further investigated.

K_{Ca} Channels and Protein Kinase C

The activation of protein kianse C (PKC) has been shown an inhibition of BK_{Ca} channels in various vascular beds (Lange et al., 1997; Minami et al., 1993). Studies in porcine coronary artery have demonstrated that PKC activators inhibit the BK_{Ca} channels activation by increasing in cytosolic free Ca^{2+} and phosphorylation of the channel protein (Lange et al., 1997; Minami et al., 1993). In addition, PKC-induced phsophorylation of the channel protein inhibits BK_{Ca} channels activities in smooth muscle, and decreases its sensitivity to be activated by cyclic guanosine monophosphate (cGMP)-dependent protein kinas I or protein kianse A (PKA) (Ledoux et al., 2006; Crozatier, 2006). Recent studies have shown that the activation of PKC by Phorbol 12,13-dibutyrate (PDBu) significantly inhibits the whole-cell K^+ current in uterine arterial myocytes. The inhibition of K^+ currents by PDBu is significantly greater in the myocytes of pregnanat sheep than that in nonpregnant animals (Hu et al., 2011). It has been further demonstrated

that the PDBu-induced reduction of K^+ currents is predominately mediated by inhibiting the BK_{Ca} channels. PKC plays an important role in the regulation of vascular smooth muscle contractility (Baraban et al., 1985; Singer and Baker, 1987). The finding that the activation of PKC inhibited BK_{Ca} channels activity and increased pressure-dependent myogenic tone in pregnant uterine arteries provides a functional link between BK_{Ca} channels and PKC-mediated attenuation of myogenic tone of uterine arteries in pregnancy (Zhu et al., 2013 b).

K_{Ca} Channels and Reactive Oxygen Species

Reactive oxygen species (ROS) have been found to play an important role in regulating vascular smooth muscle cell function (Wolin et al., 2005). Enhanced ROS production is associated with pathogenesis of various vascular dysfunctions, such as preeclampsia and pulmonary hypertension (Buetler et al., 2004; Fike et al., 2008; Matsubara et al., 2010). Exposure to hypoxia alters ROS generation in vasculatures (Wolin et al., 2005; Waypa and Schumacker, 2010). Accumulating evidence suggests that ROS regulate vascular tone by altering ion channel function (Faraci, 2006; Paffett and Walker, 2007; Brakemeier et al. 2003). Activities of K_{Ca} channels in VSMCs are modulated by ROS (Brakemeier et al., 2003; Cheranov and Jaggar, 2004; Xiao et al., 2013); and vasorelaxation induced by H_2O_2 is mediated by potassium channels (Faraci 2006; Iidia and Katusic, 2000). Additionally, peroxynitrite ($OONO^-$) inhibits BK_{Ca} channel activity, leading to reduced hyperpolarization-mediated vasodilation (Brzezinska, et al., 2000; Liu et al., 2002). Moreover, we recently demonstrated that heightened oxidative stress in uterine arteries suppresses BK_{Ca} channel activity, resulting in

increased myogenic reactivity during gestational hypoxia (Xiao et al., 2013). These studies suggest that ROS could alter vascular function via altering K_{Ca} channel function in VSMCs. It remains to be determined how ROS exerts its effect on K_{Ca} channels in uterine arteries.

Central Hypothesis

The central hypothesis of our project is that K_{Ca} channels participate in the regulation of uterine artery contractility during pregnancy; and this regulatory role of K_{Ca} channels is impaired by chronic hypoxia. This hypothesis will be tested in uterine arteries from normoxic and long-term high altitude hypoxic sheep with the following three specific aims. Aim 1, to determine the role of K_{Ca} channels in uterine vascular adaptation to pregnancy. Aim 2, to determine the role of K_{Ca} channels in uterine vascular adaptation to chronic hypoxia. Aim 3, to determine the molecular mechanisms underlying K_{Ca}-mediated uterine vascular contractility in response to pregnancy and hypoxia.

Significance

Adverse effects of chronic hypoxia on uterine vascular adaptation in pregnancy are likely to contribute to impaired uteroplacental blood flow associated with chronic hypoxia during gestation, which is a major risk factor of preeclampsia and intrauterine growth restriction (IUGR) (Palmer et al., 1992; Zamudio et al., 1993). However, the mechanisms are not fully understood. Ca^{2+} is essential for excitation-contraction coupling of VSMCs. An elevation in intracellular Ca^{2+} concentrations activates BK_{Ca} and SK_{Ca} channels. Our previous findings suggested that upregulation of BK_{Ca} channels in uterine

arteries during pregnancy was vital for reduced uterine vascular tone and contractility (Hu et al., 2011). Similar changes in SK3 channel expression and function in rat mesenteric arteries have been found in angiotensin II-induced hypertension (Hilgers and Webb, 2007). Moreover, the finding that suppression of SK3 expression elevated blood pressure (Taylor et al., 2003) also provided evidence to support that impairment of SK_{Ca} channel function plays an important role in the pathogenesis of hypertension. However, the role of SK_{Ca} channels and BK_{Ca} channels in regulating uterine vascular function is unknown. Thus, we have high expectation that our study will reveal a novel mechanism of K_{Ca} channels in regulating uterine vascular adaptation to pregnancy, and help improve the understanding of maladaptation of uteroplacental circulation by chronic hypoxia during gestation. Studies on the modulation of K_{Ca} expression and function by steroid hormones and the interaction of PKC and K_{Ca} channel function in response to pregnancy and hypoxia will provide new insights into mechanisms of this regulation. Moreover, K_{Ca} channel mRNA studies will provide future direction for research this regulation from molecular level. Furthermore, the proposed study may help develop new therapeutic strategies that enhance expression and activity of K_{Ca} channels, which should be an attractive tactic for the treatment of hypertension in pregnancy and preeclampsia.

CHAPTER TWO

CHRONIC HYPOXIA INHIBITS PREGNANCY-INDUCED UPREGULATION OF SKCA CHANNEL EXPRESSION AND FUNCTION IN UTERINE ARTERIES.

By

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Abstract

Small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channels are crucial in regulating vascular tone and blood pressure. The present study tested the hypothesis that SK_{Ca} channels play an important role in uterine vascular adaptation in pregnancy, which is inhibited by chronic hypoxia during gestation. Uterine arteries were isolated from nonpregnant and near-term pregnant sheep maintained at sea level (~300 m) or exposed to high-altitude (3801 m) hypoxia for 110 days. Immunohistochemistry revealed the presence of SK_{Ca} channels type 2 (SK2) and type 3 (SK3) in both smooth muscle and endothelium of uterine arteries. The expression of SK2 and SK3 channels was significantly increased during pregnancy, which was inhibited by chronic hypoxia. In normoxic animals, both SK_{Ca} channel opener NS309 and a large-conductance (BK_{Ca}) channel opener NS1619 relaxed norepinephrine-contracted uterine arteries in pregnant but not nonpregnant sheep. These relaxations were inhibited by selective SK_{Ca} and BK_{Ca} channel blockers, respectively. NS309-induced relaxation was largely endothelium-independent. In high altitude hypoxic animals, neither NS1619 nor NS309 produced significant relaxation of uterine arteries in either nonpregnant or pregnant sheep. Similarly, the role of SK_{Ca} channels in regulating myogenic reactivity of uterine arteries in pregnant animals was abrogated by chronic hypoxia. Accordingly, the enhanced SK_{Ca} channel activity in uterine arterial myocytes of pregnant animals was ablated by chronic hypoxia. The findings suggest a novel mechanism of SK_{Ca} channels in regulating myogenic adaptation of uterine arteries in pregnancy, and in the maladaptation of uteroplacental circulation caused by chronic hypoxia during gestation.

Introduction

Vascular tone constitutes the major determinant of the resistance of blood vessels, which regulates blood pressure and the distribution of blood flow among and within tissues and organs. Ca^{2+}-activated K^+ (K_{Ca}) channels contribute significantly to setting the membrane potential, and play a critical role in regulating excitability of vascular smooth muscle cells (VSMCs) (Jackson, 2005; Ledoux et al., 2006). Based on the conductance, K_{Ca} channels are divided into large-conductance (BK_{Ca}), intermediate-conductance (IK_{Ca}), and small-conductance (SK_{Ca}) channels (Wei et al., 2005). K_{Ca} channels have distinct distributions in the vasculature. SK_{Ca} channels are believed to be expressed predominantly in endothelial cells (Hu et al., 2012; Ledoux et al., 2006), and hyperpolarization produced by the activation of SK_{Ca} in endothelial cells may be transmitted to VSMCs *via* the myoendothelial gap junction (Feletou, 2009; Kohler et al., 2010).

During pregnancy, uterine blood flow increases substantially to optimize the supply of oxygen and nutrients to the developing fetus *via* the placenta. Chiefly, this is achieved by adaptive changes such as remodeling of the uterine vasculature (Osol and Mandala, 2009), reduced pressure-dependent myogenic reactivity (Hu et al., 2011; Veerareddy et al., 2002; Xiao et al., 2006), blunted vasoconstrictor response (Cooke and Daridge, 2003; Nelson et al., 1995; Weiner et al., 1991), and enhanced vasodilator response and vasodilator production (Gangula et al., 1999; Ni et al., 1997; Xiao et al., 2001a). Chronic hypoxia during gestation has profound adverse effects on the normal adaptation of uteroplacental circulation to pregnancy (Chang et al., 2009; Chang et al., 2010; Hu et al., 2012; Julian et al., 2008; Zamudio et al., 1995a), leading to a 2 to 4-fold

increase in the incidence of preeclampsia and fetal intrauterine growth restriction (Julian et al., 2008; Keyes et al., 2003; White and Zhang, 2003; Zamudio et al., 1995 a, b).

Previous studies have demonstrated that BK_{Ca} channels participate in the regulation of vascular tone and uterine blood flow during pregnancy (Rosenfeld et al., 2001; Rosenfeld et al., 2005; Rosenfeld et al., 2009). Upregulated expression of the BK_{Ca} channel β1 subunit and enhanced BK_{Ca} channel activity contribute to the attenuated myogenic tone of uterine arteries during pregnancy (Hu et al., 2011). Chronic hypoxia during gestation inhibited pregnancy-induced upregulation of BK_{Ca} channel function in uterine arteries by selectively targeting the β1 subunit (Hu et al., 2012). Although SK_{Ca} channels are predominantly expressed in endothelial cells (Ledoux et al., 2006), apamin-sensitive K^+ currents and positive staining of SK_{Ca} channels have been detected in VSMCs of various vascular beds (Gebremedhin et al., 1996; Guthier et al., 2004; McNeish et al., 2006; Sorensen et al., 2011). Functional roles of SK_{Ca} channels in VSMCs remain elusive. SK_{Ca} channels are also expressed in the myometrium and are regulated by estrogen during pregnancy (Pierce and England, 2010). Of interest, SK_{Ca} channels are subject to regulation by oxygen (Keating et al., 2001; Kroigaard et al., 2013). However, the role of SK_{Ca} channels in the regulation of uterine circulation under physiological and pathophysiological conditions such as pregnancy and chronic hypoxia is unclear. In the present study, we tested hypotheses that SK_{Ca} channels play an important role in regulating the contractility of uterine arteries during pregnancy; and that chronic hypoxia during gestation impairs this regulation. To test these hypotheses, we first determined whether SK_{Ca} channels were expressed in the uterine vasculature and how pregnancy and chronic hypoxia regulated their expression. We then determined

SK_{Ca}-mediated relaxations of uterine arteries and their regulation by pregnancy and chronic hypoxia. Furthermore, we measured the SK_{Ca} channel activity in uterine vascular smooth muscle cells using patch-clamp analysis to see whether pregnancy and chronic hypoxia altered their activities. In addition, we determined the role of SK_{Ca} channels in pressure-dependent myogenic tone of uterine arteries and its regulation by pregnancy and chronic hypoxia.

Materials and Methods

Tissue Preparation and Treatment

Uterine arteries were harvested from nonpregnant and near-term (142-145 days of gestation, the term is about 150 days) pregnant sheep maintained at sea level (~300 m) or exposed to high-altitude (3801 m) hypoxia for 110 days (Chang et al., 2010). For nonpregnant animals, uterine arteries were obtained from the animals with the luteal phase of the ovarian cycle but not the follicular phase. Animals were anesthetized with thiamylal (10 mg/kg, i.v.) followed by inhalation of 1.5% to 2.0% halothane. An incision was made in the abdomen and the uterus exposed. The uterine arteries were isolated and removed without stretching and placed into a modified Krebs solution. All procedures and protocols were approved by the Institutional Animal Care and Use Committee, and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western Immunoblotting

Protein abundance of SK2 and SK3 channels was measured in freshly isolated uterine arteries, as described previously (Hu et al., 2011). Briefly, tissues were

homogenized in a lysis buffer followed by centrifugation at 4°C for 10 minutes at 10,000g, and the supernatants were collected. Samples with equal protein contents were loaded onto 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate, and were separated by electrophoresis at 100 V for 2 hours. Proteins then were transferred onto nitrocellulose membranes. After blocking nonspecific binding sites by dry milk, the membranes were incubated with primary antibodies against SK2 channel (Alomone Ltd, Jerusalem, Israel) and SK3 channel (Santa Cruz Biotechnology, Santa Cruz CA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.

Immunohistochemistry

Uterine arteries were fixed in 10% neutral buffered formalin and embedded in paraffin. Immunohistochemical detection of SK2, SK3, and endothelial nitric-oxide synthase (eNOS) was performed using the Anti-Ig HRP Detection Kit (BD Biosciences PharMingen, San Diego, CA) as described previously (Kougias et al., 2006; Noble et al., 2010). Briefly, tissue slices (10μm thick) were incubated with monoclonal antio-SK2 (1:100), anti-SK3 (1:200), anti-eNOS primary antibody (1:100), respectively, for 60 min at room temperature. After rinsing three times in phosphate-buffered saline for 30 min, slices were incubated with biotinylated secondary antibody (1:100) for 60 min at room temperature. Samples then were exposed to streptavidin-HRP and reacted with diaminobenzidine substrate solution according to the manufacturer's recommendations,

and counterstained with hematoxylin. Endothelial and smooth muscle cell nuclei were stained with hematoxylin. To check whether the staining was specific, negative control staining was performed where the primary antibody was omitted. The slices were viewed with an Olympus BH-2 microscope (Olympus, Tokyo, Japan), and images were captured with an attached SPOT digital camera imaging system.

Contraction Studies

Fourth-generation branches of the main uterine arteries from nonpregnant and pregnant sheep were separated from the surrounding tissue, and cut into 2-mm ring segments. Isometric tension was measured in the Krebs solution in a tissue bath at 37°C, as described previously (Hu and Zhang, 1997; Xiao et al., 2010a). Briefly, each ring was equilibrated for 60 minutes and then gradually stretched to the optimal resting tension, as determined by the tension that developed in response to 120 mmol/L KCl added at each stretch level. After stable responses to KCl were obtained, tissues were rinsed and then contracted with a submaximal concentration of norepinephrine (3 μmol/L) that produced about 50-70% of the maximal contraction. This was followed by a BK_{Ca} channel opener NS1619 or a SK_{Ca} channel opener NS309 added in a cumulative manner, in the absence or presence of BK_{Ca} channel blockers tetraethylammonium (TEA) or iberitoxin (IBTX), or a SK_{Ca} channel blocker apamin, or a BK_{Ca}/IK_{Ca} channel blocker charybdotoxin (CTX), respectively.

Measurement of SK_{Ca} Channel Current

Arterial smooth muscle cells were dissociated enzymatically from resistance-sized uterine arteries, and whole-cell K^+ currents were recorded using an EPC 10 patch-clamp

amplifier with Patchmaster software (HEKA, Lambrecht/Pfalz, Germany) at room temperature, as previously described.[2] Briefly, cell suspension drops were placed in a recording chamber and adherent cells were superfused continuously with HEPES-buffered physiologic salt solution containing (in mmol/L): 140.0 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10.0 HEPES, and 10.0 glucose (pH 7.4). Only relaxed and spindle-shaped myocytes were used for recording. Micropipettes were pulled from borosilicate glass and had resistances of 2 to 5 MΩ when filled with the pipette solution containing (in mmol/L) 140.0 KCl, 1.0 $MgCl_2$, 5.0 Na_2ATP, 5.0 EGTA, 10.0 HEPES (pH 7.2). $CaCl_2$ was added to bring free Ca^{2+} concentrations to 200.0 nmol/L, as determined using WinMAXC software (Chris Patton, Stanford University). Cells were held at –50 mV and whole-cell K^+ currents were evoked by voltage steps from -60 mV to +80 mV by stepwise 10-mV depolarizing pulses (350-ms duration, 10-second intervals) in the absence and presence of 1 μmol/L apamin or 1 μmol/L NS309. K^+ currents were normalized to cell capacitance and were expressed as picoampere per picofarad (pA/pF).

Measurement of Myogenic Tone

Pressure-dependent myogenic tone of resistance-sized uterine arteries was measured as described previously (Chang et al., 2010; Hu et al., 2011; Xiao et al., 2009). Briefly, arterial segments were mounted and pressurized in an organ chamber (Living Systems Instruments, Burlington VT). The intraluminal pressure was controlled by a servo-system to set transmural pressures, and arterial diameter was recorded using the SoftEdge Acquisition Subsystem (IonOptix LLC, Milton MA). Following the equilibration period, the intraluminal pressure was increased in a stepwise-manner from

10 to 100 mmHg in 10-mmHg increments. Each pressure was maintained for 5 minutes to allow vessel diameter to stabilize before the measurement. To determine the maximum passive diameter, the passive pressure-diameter relationship was conducted in Ca^{2+}-free physiologic saline solution (PSS) containing 3.0 mmol/L of EGTA. The following formula was used to calculate percent myogenic tone at each pressure step: % myogenic tone = (D1 – D2)/D1 x 100, where D1 is the passive diameter in Ca^{2+}-free physiologic saline solution (0 Ca^{2+} with 3.0 mmol/L of EGTA) and D2 is the active diameter with normal physiologic saline solution in the presence of extracellular Ca^{2+}.

Data Analysis

Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA). Results were expressed as means ± SEM obtained from the number of experimental animals given. Differences were evaluated for statistical significance ($P < 0.05$) by ANOVA or t test, where appropriate.

Results

Effect of Pregnancy and Chronic Hypoxia on SK_{Ca} Channel Expression

Protein abundance of both SK2 and SK3 channels was significantly greater in uterine arteries of pregnant sheep than that in nonpregnant animals (Figure 2A). Chronic hypoxia during gestation significantly decreased the expression of SK2 and SK3 channels in uterine arteries of pregnant animals (Figure 2B).

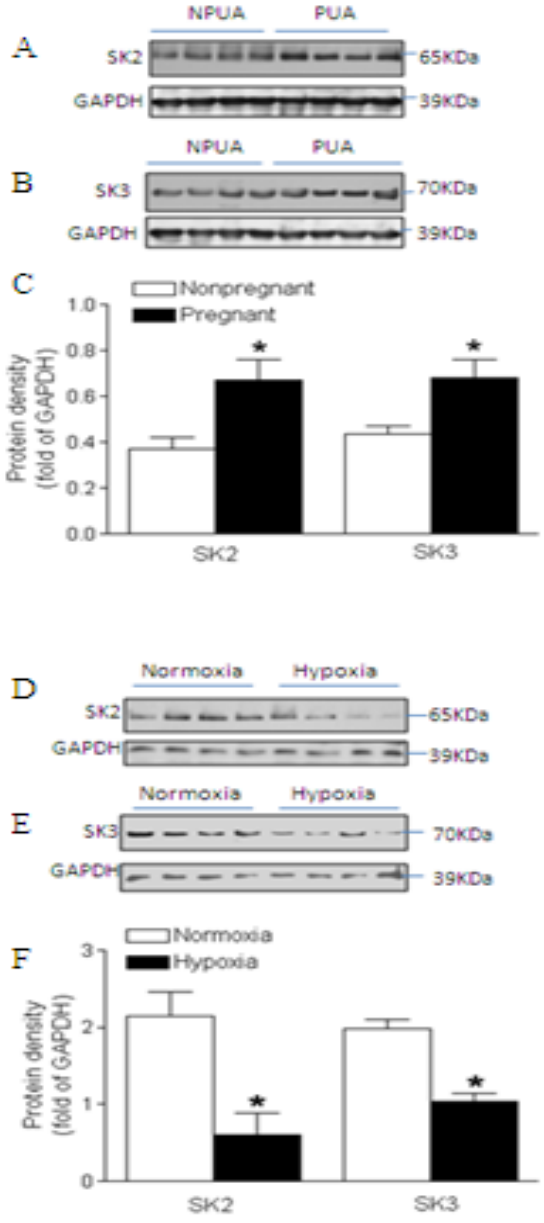

Figure 2. Effect of Pregnancy and Chronic Hypoxia on Small-Conductance Ca^{2+}-Activated K^{+}(SK_{Ca}) Channel Expression. Protein abundance of SK_{Ca} channels type 2(SK2) and type 3 (SK3) was determined by Western blot analyses in uterine arteries of normoxic nonpregnant(NPUA) and pregnant (PUA) animals (**A**-**C**) and in uterine arteries of normoxic and high-altitude hypoxic pregnant animals (**D**-**F**). Data are means ± SEM of tissues from 4 to 6 animals of each group. *$P<0.05$

Effect of Pregnancy and Chronic Hypoxia on SK_{Ca} Channel-Mediated Relaxation

The effect of SK_{Ca}/IK_{Ca} channels on contractility of uterine arteries was examined by exposing norepinephrine-contracted arteries to NS309 (Figure 3). In normoxic animals, NS309 had no effect on uterine artery relaxation in nonpregnant sheep, but produced concentration-dependent relaxations of uterine arteries in pregnant animals, with a maximal relaxation of 64.3 ± 7.5% (Figure 3A). As shown in Figure 3B, blocking of BK_{Ca} channels with IBTX or blocking of BK_{Ca}/IK_{Ca} channels with CTX had no significant effect on NS309-induced relaxation, but the SK_{Ca} channel blocker apamin significantly inhibited NS309-mediated relaxation, suggesting that NS 309-induced relaxation was conferred chiefly by SK_{Ca} channel activation. Long-term, high altitude hypoxia did not change NS309's effect in nonpregnant sheep, but abrogated NS309-mediated relaxations of uterine arteries in pregnant animals (Figure 3C). Similarly, the BK_{Ca} channel activator NS1619-induced relaxations were significantly increased in uterine arteries of pregnant sheep, which was inhibited by chronic hypoxia (Figure 4).

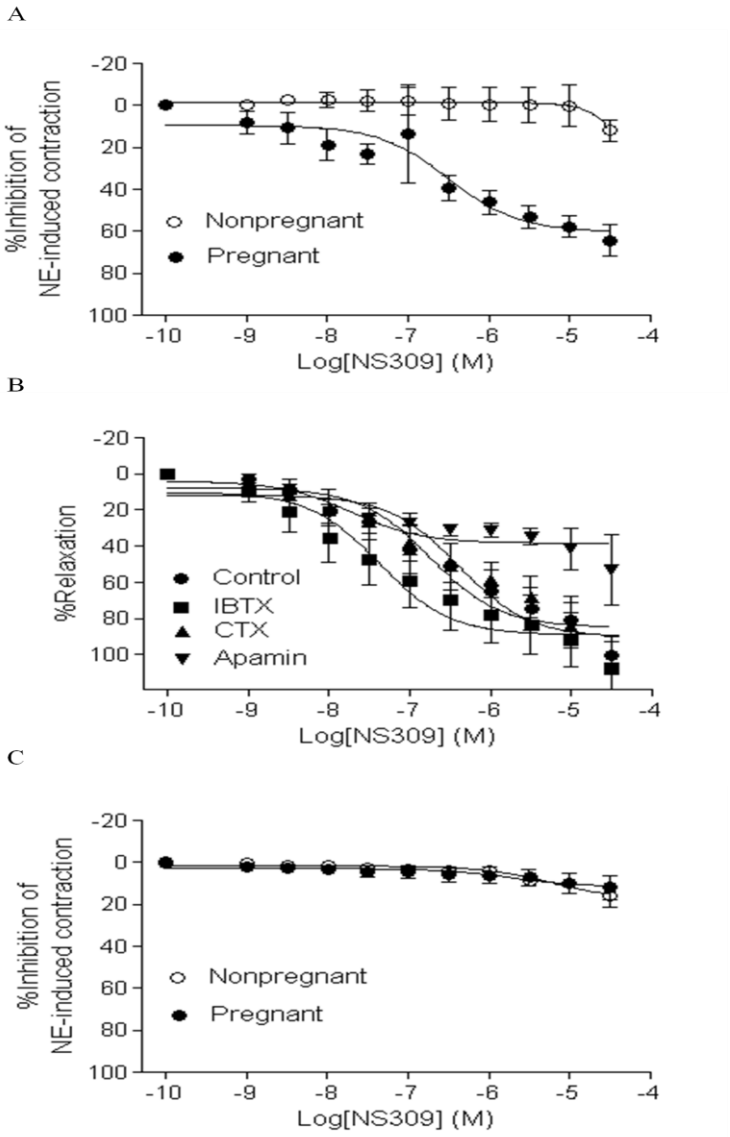

Figure 3. Concentration-Response Curves of NS309-Induced Relaxation. Uterine arteries were contracted with norepinephrine (NE, 3μmol/L) and followed by additions of NS309. **A.** Normoxic animals. **B.** Normoxic pregnant animals in the absence (control) or presence of iberitoxin (IBTX, 100nmol/L), charybdotoxin (CTX, 70 nmol/L), or apamin (500 nmol/L). **C.** High-altitude hypoxic animals. Data are means ± SEM of tissues from 5 to 6 animals in each group.

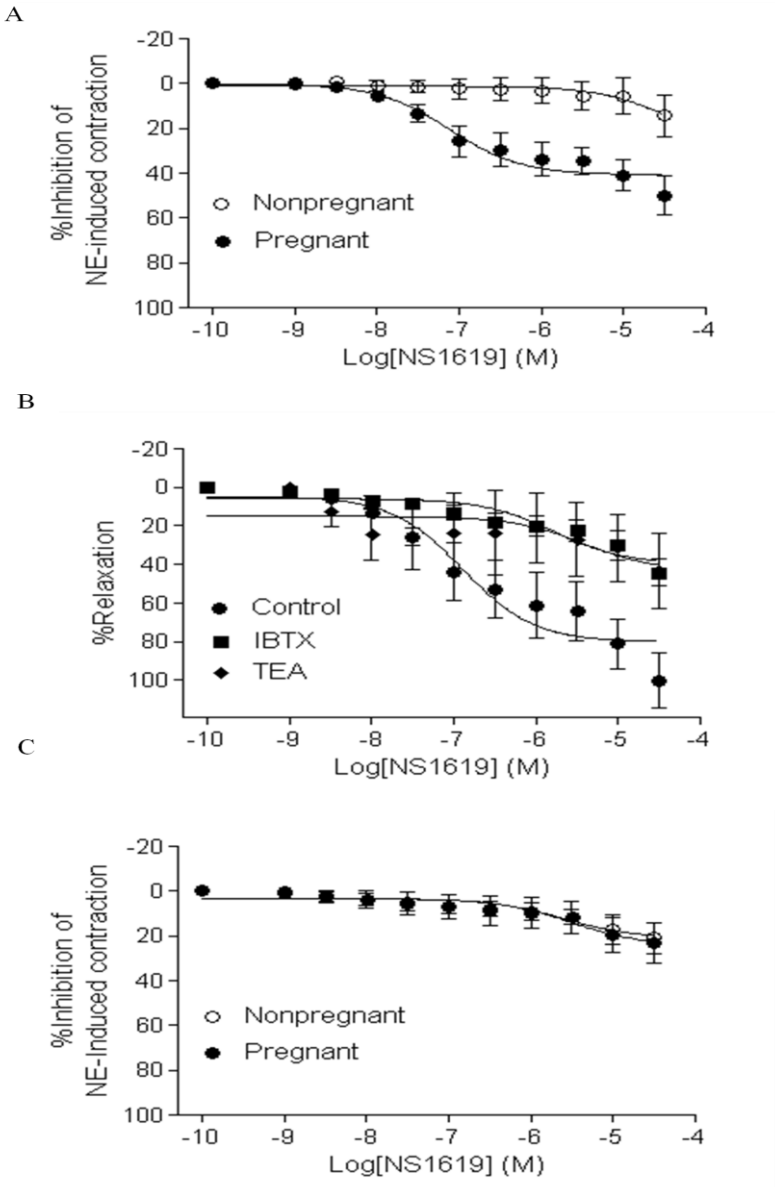

Figure 4. Concentration-Response Curves of NS1619-Induced Relaxation. Uterine arteries were contracted with norepinephrine (NE, 3μmol/L) and followed by additions of NS1619. **A.** Normoxic animals. **B.** Normoxic pregnant animals in the absence (control) or presence of iberitoxin (IBTX, 100 nmol/L) or tetraethylammonium(TEA, 1 mmol/L).**C.** High altitude hypoxic animals. Data are means ± SEM of tissues from 4-8 animals in each group.

Involvement of Smooth Muscle Cells in SK_{Ca} Channel-Mediated Relaxations

Given that SK_{Ca} channels are expressed in endothelial cells, we determined NS309-induced relaxation in endothelium-intact and -denuded uterine arteries. The validity of endothelium removal was confirmed by the absence of eNOS in immunohistochemical staining (Figure 5A). As shown in Figure 5B, endothelial removal did not significantly alter NS309-induced relaxation of uterine arteries (pD_2: endothelium-intact: 6.5 ± 0.3; endothelium-denuded: 6.6 ± 0.2; Emax: endothelium-intact: 64.3 ± 7.5%; endothelium-denuded: 51.3 ± 11.4%, $P > 0.05$). This suggests that NS309-induced relaxation of uterine arteries was mediated mainly by SK_{Ca} channels in vascular smooth muscle cells. Immunohistochemical staining revealed that both SK2 and SK3 channels were expressed in endothelial as well as smooth muscle cells in the uterine artery (Figure 5C).

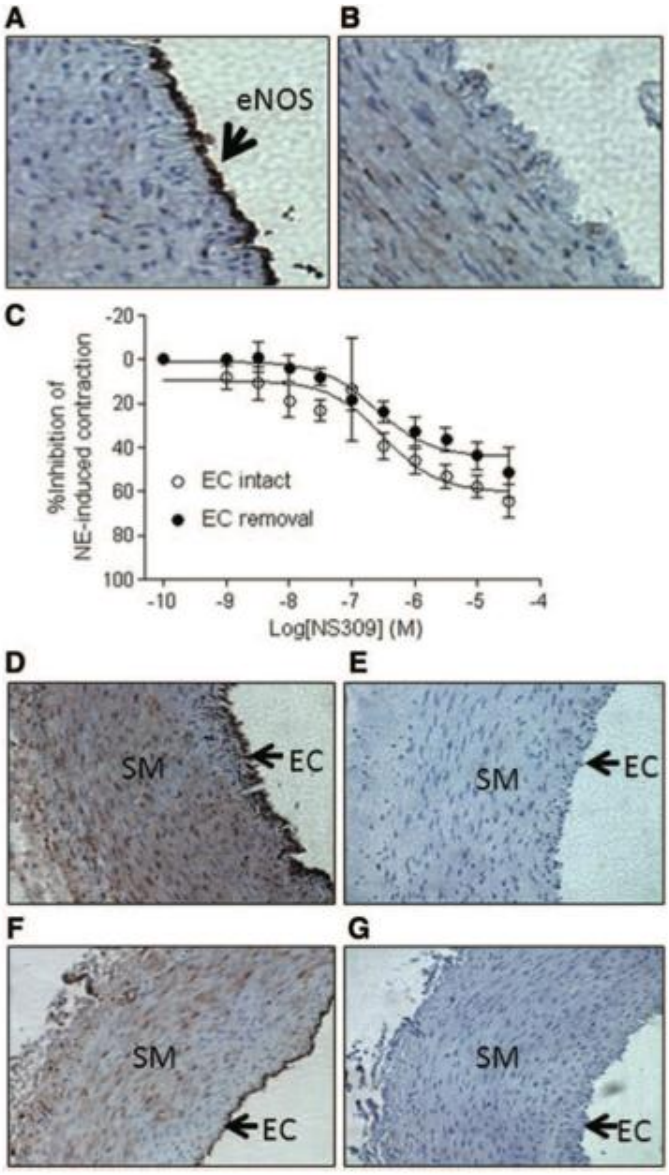

Figure 5. Effect of Endothelial Cells (EC) on NS309-Induced Relaxation. Immunoreactivity of endothelial nitric oxide synthase(eNOS) was present in EC-intact arteries (**A**) but absent in EC-denuded arteries (**B**), demonstrating the effectiveness of EC removal. **C**, NS309-induced relaxation of norepinephrine (NE, 3μmol/L)-contracted pregnant uterine arteries with (EC intact) or without (EC removal) EC. Data are means ± SEM of tissues from 4 to 6 animals in each group. Immunoreactivity of small-conductance Ca^{2+}-activated K^+ channel type 2 (SK2; **D**) and type 3 (SK3; **F**) in EC and vascular smooth muscles (SM) of pregnant uterine arteries. **E** and **G**, Negative controls of SK2 and SK3 staining.

Chronic Hypoxia Inhibited SK_{Ca} Channel Activity in Uterine Arteries

To determine the effect of pregnancy and chronic hypoxia on SK_{Ca} channel activity in uterine arterial smooth muscle cells, whole-cell K^+ currents were recorded in the absence or presence of apamin or NS309 in myocytes freshly isolated from uterine arteries of normoxic control and hypoxic animals. As shown in normoxic pregnant sheep, apamin significantly reduced whole-cell K^+ currents (from 60.7 ± 2.7 pA/pF to 49.6 ± 2.3 pA/pF at +80 mV, $P < 0.05$) in uterine arterial myocytes (Figure 6A). In contrast, in myocytes of hypoxic animals, apamin was without effect on whole-cell K^+ currents (Figure 6B). Similarly, NS309 significantly enhanced whole-cell K^+ currents in myocytes of normoxic pregnant animals (from 57.5 ± 1.3 pA/pF to 72.4 ± 2.9 pA/pF at +80 mV, $P < 0.05$) (Figure 6C) but not in hypoxic animals (Figure 6D). Neither apamin nor NS 309 altered whole-cell K^+ currents in uterine arterial myocytes of nonpregnant sheep in either normoxic or hypoxic animals (data not shown).

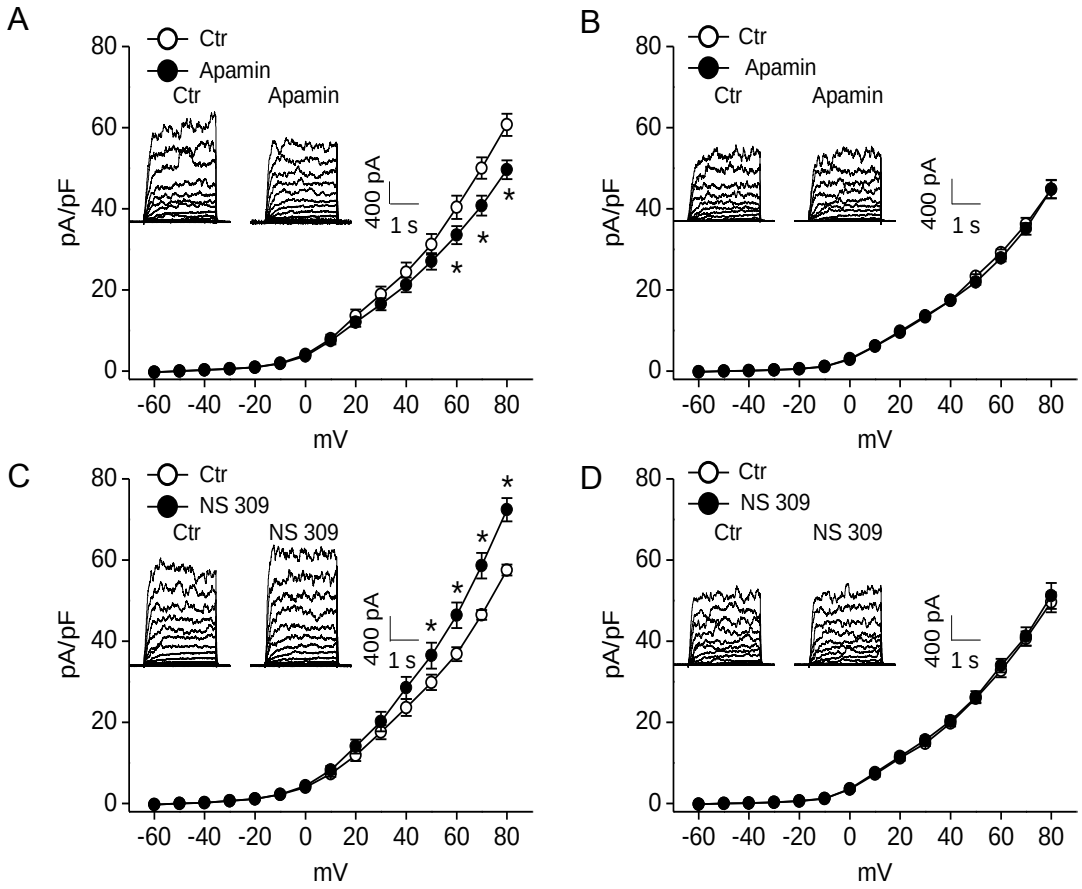

Figure 6. Effect of Chronic Hypoxia on Small-Conductance Ca^{2+}-Activated K^+(SK_{Ca}) Channel Currents in Uterine Arteris of Pregnant Sheep. Arterial myocytes were freshly isolated from uterine arteries of pregnant sheep in normoxic and high-altitude hypoxic animals. Whole-cell K^+ currents were recorded in the absence or presence of apamin (1μmol/L) or NS309 (1 μmol/L). **A** and **C**, Normoxic animals. **B** and **D**, High-altitude hypoxic animals. Data are means ± SEM of cells from 5 to 6 animals of each group. **P<0.05* vs control (Ctr).

Effect of Pregnancy and Chronic Hypoxia on Uterine Artery SK_{Ca} Channel-Mediated Myogenic Tone

As reported previously, pressure-dependent myogenic tone of uterine arteries was significantly reduced in pregnant sheep in normoxic control animals (Figure 5A and 5B). Blockade of SK_{Ca} channels with apamin had no significant effect on pressure-dependent

myogenic reactivity in uterine arteries of nonpregnant animals (Figure 5A), but resulted in a significant increase in myogenic tone in uterine arteries of pregnant animals (Figure 5B). In the presence of apamin, there was no significant difference in myogenic tone of uterine arteries between nonpregnant and pregnant animals (Figure 5A and 5B). In hypoxic animals, apamin had no significant effect on pressure-dependent myogenic tone of uterine arteries in either nonpregnant or pregnant animals (Figure 5C and 5D).

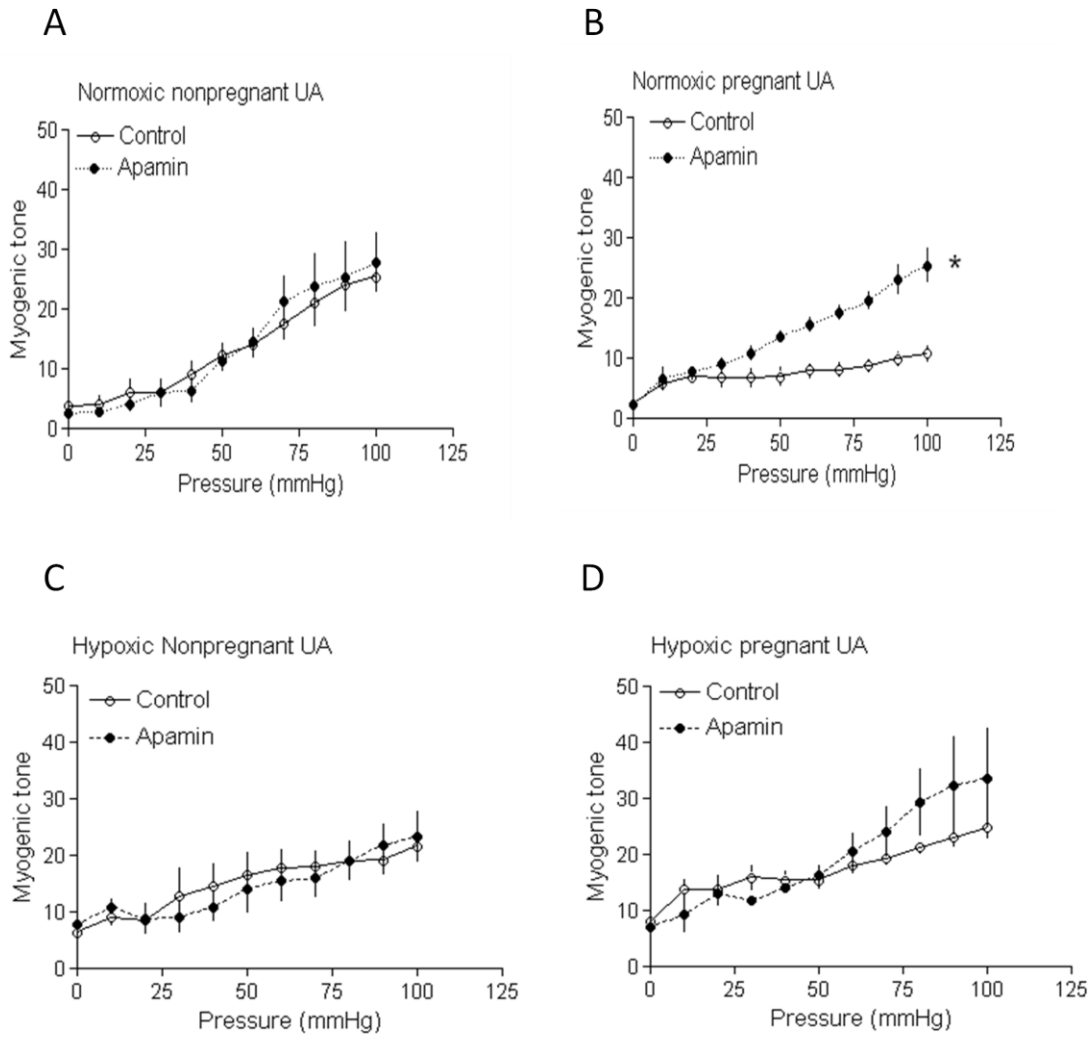

Figure 7. Effect of Pregnancy and Chronic Hypoxia on Small-Conductance Ca^{2+}-Activated K^+(SK_{Ca}) Channel-Mediated Myogenic Tone. Pressure-dependent myogenic tone was determined in the absence or presence of apamin (500 nmol/L) in normoxic nonpregnant uterine artery (UA; **A**), normoxic pregnant uterine artery (**B**), hypoxic nonpregnant uterine artery (**C**), and normoxic pregnant uterine artery (**D**). Data are means ± SEM of tissues from 5 to 6 animals of each group. **P<0.05* vs control.

Discussion

In the present study, we have demonstrated for the first time the expression and function of SK_{Ca} channels in uterine arteries. The capacity of activation of SK_{Ca} channels to relax uterine arteries was markedly increased during pregnancy. Additionally, the SK_{Ca} channel blocker apamin significantly increased pressure-dependent myogenic tone in uterine arteries of pregnant sheep and blunted the difference in the myogenic response of uterine arteries between nonpregnant and pregnant animals. These pregnancy-induced changes were accompanied by increased expression of both SK2 and SK3 channels. Consistently, we detected increased activities of SK_{Ca} channels in uterine artery smooth muscle cells of pregnant sheep. The concurrence of those findings suggests that pregnancy-induced upregulation of expression and activity of SK_{Ca} channels contributes to the reduced myogenic reactivity and vascular contractility of uterine arteries during gestation. Decreased myogenic tone and increased vasorelaxing responses of uterine arteries have been implicated in the increase in uterine blood flow during pregnancy (Gangula et al., 1999; Hu et al., 2011; Ni et al., 1997; Xiao et al., 2001a; Xiao et al., 2006; Veerareddy et al., 2002). Hence, our observations provide a novel mechanism of upregulation and heightened activity of SK_{Ca} channels in the adaptation of uteroplacental circulation during pregnancy. Furthermore, the up-regulation of SK3 channels appears to have a role in remodeling of the uterine vasculature. A recent study demonstrated that uterine arteries from nonpregnant transgenic $SK3^{T/T}$ mice that overexpress SK3 channels had larger basal diameters and blunted vasoconstrictor response compared to those from wild-type animals (Rada et al., 2012), although the expression of SK3 channels in uterine arteries was not determined.

At present, the mechanisms responsible for upregulating expression and function of SK_{Ca} channels in uterine arteries during pregnancy are not clear. It is conceivable that sex steroid hormones may contribute to this regulation. Activation of estrogen receptors may alter gene transcription, which has a profound impact on cardiovascular function (Murphy, 2011). Pregnancy up-regulates the expression of estrogen receptor α and β in uterine arteries (Chang et al., 2010; Byers et al., 2005). Moreover, we recently demonstrated the 17β-estradiol-mediated increase in expression and heightened activity of BK_{Ca} channels in uterine arteries of pregnant sheep (Hu et al., 2011). Similarly, SK3 channel expression also was regulated by 17β-estradiol in recombinant expression system (Jacobson et al., 2003), hypothalamus (Bosch et al., 2002), and myometrium (Pierce and England, 2010).

Although SK_{Ca} channels were expressed in uterine arteries of nonpregnant animals, the channel activity was not detected with an electrophysiological approach. Moreover, it appeared that these channels did not participate in regulating myogenic tone and contractility of uterine arteries in nonpregnant animals. One possibility is that in nonpregnant animals SK_{Ca} channels are not expressed in the cell membrane of uterine arteries smooth muscle, but rather are retained inside cells. The incapability of those channels to insert into membrane would prevent them from being activated. It is also possible that in nonpregnant animals despite being present in the myocyte membrane, the efficacy of these channels may be too low to be functional. Similar findings have been reported for both IK_{Ca} and BK_{Ca} channels. Although IK_{Ca} channels were stained at both the plasma membrane and within the cytoplasm (McNeish et al., 2006), the selective IK_{Ca} channel blocker TRAM-34 was unable to alter vascular tone of cerebral arteries

(McNeish et al., 2005). Similarly, BK_{Ca} channels in uterine arteries did not participate in the regulation of relaxation (the present study), myogenic tone, vascular resistance and blood flow in nonpregnant animals (Hu et al., 2011; Rosenfeld et al., 2000).

Initially, SK_{Ca} channels were detected in endothelial cells, but not in VSMCs of $SK3^{T/T}$ mice (Taylor et al., 2003). The regulatory role of SK_{Ca} channels on vascular function is thought to be mediated exclusively by the endothelium. This notion was supported by the finding that a genetic deficit of SK3 and IK1 channels caused hypertension by abolishing endothelium-derived hyperpolarizing factor-mediated vasodilation (Brahler et al., 2009). In the present study, immunostaining demonstrated the expression of SK2 and SK3 channels in both vascular smooth muscle and endothelial cells in uterine arteries. The functional presence of SK_{Ca} channels in uterine arterial smooth muscle cells was confirmed with electrophysiological technique; and a selective SK_{Ca} channel blocker apamin decreased whole-cell K^+ currents by ~20%. This is in agreement with the previous findings in myocytes of rabbit aorta (Guthier et al., 2004) and rat myometrium (Noble et al., 2010) that apamin reduced whole-cell K^+ currents by about 20%. Furthermore, previous studies also have shown the presence of SK_{Ca} channels in other visceral and vascular smooth muscle cells by immunohistochemistry (Sorensen et al., 2011; Chen et al., 2004; Potocnik et al., 2009), although the functional roles of these channels are not known. In uterine arteries, NS309-induced relaxation was largely endothelium-independent, suggesting that SK_{Ca} channels in vascular smooth muscle mediated mainly NS309-induced vasorelaxation. To our knowledge, the present study is the first to demonstrate that SK_{Ca} channels in vascular smooth muscle significantly contribute to the regulation of vascular contractility and tone in this vascular

bed. Lines of evidence have also implicated SK_{Ca} channels in regulating excitability and contraction of smooth muscle cells from the uterus and urinary bladder, which are highly responsive to sex steroids and in particular estrogen (Herrera et al., 2003;Brown et al., 2007; Thorneloe et al., 2008). Our findings thus provide a novel mechanism of SK_{Ca} channels in regulating vascular tone and cardiovascular function.

Previously, chronic hypoxia has been found to abrogate the capacity of BK_{Ca} in regulating myogenic reactivity of uterine arteries in pregnant sheep (Hu et al., 2012). The present findings of diminishment of vasodilator response to NS309 and failure of apamin to alter myogenic tone of uterine arteries in pregnant animals exposed to long-term high altitude hypoxia suggest that chronic hypoxia resulted in a loss of the regulatory role of SK_{Ca} channels in vascular smooth muscle excitability and contractility. Hence, the nullification of the regulatory role of K_{Ca} channels may attribute to chronic hypoxia-induced reduction in uterine blood flow in pregnancy (Julian et al., 2008; Zamudio et al., 1995a). Our data also suggest that the loss of regulatory role of SK_{Ca} channels in uterine arteries of pregnant animals resulted chiefly from reduced channel activities due to suppressed expression of these channels. Similar findings were obtained for BK_{Ca} channels in uterine arteries of pregnant sheep (Hu et al., 2012) and IK_{Ca} channels in pulmonary arteries from animals exposed to chronic hypoxia (Kroigaard et al., 2013). The effect of chronic hypoxia seems to be specific for K_{Ca} channels, as voltage-gated K^+ (K_V) channels were largely unaffected (Hu et al., 2012). Taken together, experimental evidence suggests that targeted suppression of K_{Ca} channels is a major mechanism to alter uterine vascular function by chronic hypoxia and the uterine arteries from chronic hypoxic animals are losing their adaptation to pregnancy. This may account

for the increased incidence of preeclampsia and fetal intrauterine growth restriction associated with chronic hypoxia exposure during gestation. Estrogens have been shown to regulate expression of BK_{Ca} (Hu et al., 2011; Nishimura et al., 2008) and SK_{Ca} (Jacobson et al., 2003; Pierce and England, 2010) channels. Ablation of pregnancy-induced upregulation of SK_{Ca} and BK_{Ca} channels in uterine arteries by chronic hypoxia during gestation likely occurred at the genomic level. Expression of estrogen receptor α in uterine arteries during gestation, but not plasma estrogen levels, was significantly depressed by chronic hypoxia (Chang et al., 2010) due to heightened promoter methylation (Dasgupta et al., 2012). It is possible that chronic hypoxia-mediated suppression of estrogen receptor α expression led to abrogation of upregulation of K_{Ca} channels in uterine arteries during pregnancy. However, Jobe et al has shown there are numerous types of estrogens and estrogen metabolites that are decreased in preeclampsia (Jobe et al., 2013). Therefore, the regulation of K_{Ca} channels by estrogens in VSMCs likely has a significant role in physiological and pathophysiological conditions.

Acknowledgements

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[2] **Disclosures:** None.

CHAPTER THREE

PREGNANCY AND CHRONIC HYPOXIA DIFFERENTIALLY REGULATE THE INTERACTION OF PROTEIN KINASE C AND CALCIUM-ACTIVATED POTASSIUM CHANNELS IN MODULATING UTERINE ARTERIAL CONTRACTILITY

by

Ronghui Zhu, Daliao Xiao and Lubo Zhang

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Abstract

Our previous studies demonstrated that chronic hypoxia during gestation increased uterine artery contractility by upregulating protein kinase C (PKC) activity and downregulating Ca^{2+}-activated K^+ channel (K_{Ca} channel) activity. However, the interaction between PKC activation and K_{Ca} channel activity remains unknown. The present study tested the hypothesis that gestational hypoxia upregulates PKC-induced inhibition of K_{Ca} channel-mediated relaxation of uterine arteries in pregnancy. Uterine arteries were isolated from nonpregnant (NPUA) and pregnant (PUA) (~140 day gestation) sheep maintained at either sea level or high altitude (3,820 m for 110 days, PaO_2: 60 mmHg). Contractions of uterine arteries were determined. In normoxic PUA, selective inhibition of large-conductance K_{Ca} (BK_{Ca}) channels significantly enhanced PKC activator PDBu-induced contractions. This effect was abrogated in PUA of animals treated with chronic hypoxia in gestation. Unlike BK_{Ca} channels, inhibition of small-conductance K_{Ca} (SK_{Ca}) channels had no significant effect on 12, 13-dibutyrate (PDBu)-mediated contractions. In normoxic PUA, activation of both BK_{Ca} with NS1619 or SK_{Ca} with NS309 produced concentration-dependent relaxations, which were not altered by the addition of PDBu. However, in uterine arteries treated with chronic hypoxia (10.5% O_2 for 48 h), both NS1619- and NS309-induced relaxations were significantly attenuated by PDBu. In NPUAs, inhibition of BK_{Ca} channels significantly enhanced PDBu-induced contractions in both normoxic and hypoxic animals. The results suggest that in the normoxic condition BK_{Ca} downregulates PKC activity and uterine vascular contractility, which is selectively attenuated by chronic hypoxia during gestation. In addition, hypoxia induces PKC-mediated inhibition of BK_{Ca} and SK_{Ca} activities and relaxations of uterine arteries in pregnancy.

Introduction

Chronic hypoxia during gestation significantly increases the incidences of preeclampsia and fetal intrauterine growth restriction (IUGR) (Chang et al., 2009; Chang et al., 2010; Hu et al., 2012; Julian et al., 2008; Keyes et al., 2003; White and Zhang, 2003; Zamudio et al., 1995 a, b). However, the mechanisms underlying hypoxia-induced adverse pregnancy outcomes are largely unknown. Recent studies suggest that hypoxia-induced aberration of uteoplacental circulation in pregnancy may be one of the important mechanisms attributing to the pathogenesis of many pregnancy complications (Gerge and Granger, 2011; Julian et al., 2008; Khalil and Granger, 2002; Moore et al., 2001; Palmer et al., 1999; Zamudio et al., 1995b). Indeed, chronic hypoxia during gestation has profound adverse effects on uterine artery contractility and significantly increases uterine vascular tone, leading to attenuation of pregnancy-induced increase in uterine blood flow and increased risk of IUGR and preeclampsia (Change et al., 2009; Moore et al., 2001; Xiao et al., 2006; Zhou et al., 2013).

The molecular mechanisms underlying the adaptation of uterine arterial contractility to normal pregnancy and chronic hypoxia are complex and poorly understood. Pregnancy is characterized by an increase in uterine vascular relaxation and a decrease in uterine arterial constriction and vascular tone. Recent studies have demonstrated that pregnancy-induced decrease in uterine vascular tone is mediated by an increase in Ca^{2+}-activated K^+ (K_{Ca}) channel expression and activity (Hu et al., 2011; Hu et al., 2012). Inhibition of K_{Ca} channels reversed the pregnancy-mediated decrease in uterine vascular myogenic tone and increase in uterine blood flow (Hu et al., 2011; Rosenfeld et al., 2001; Rosenfeld et al., 2005), suggesting that enhanced K_{Ca} channel

function plays an important role in the adaptation of uterine circulation during pregnancy. Chronic hypoxia during gestation inhibited pregnancy-induced attenuation of uterine vascular tone *via* suppressing K_{Ca} channel function (Hu et al., 2012; Zhu et al., 2013 a). These studies showed a novel mechanism of K_{Ca} channels in regulating myogenic adaption of uterine arteries in pregnancy and in maladaptaion of uterine circulation caused by chronic hypoxia during gestation.

In contrast to K_{Ca} channels that are up-regulated by pregnancy and down-regulated by chronic hypoxia, the activity of protein kinase C (PKC) and its mediation of uterine vascular contraction are down-regulated by pregnancy and up-regulated by chronic hypoxia (Farley and Ford, 1992; Magness et al., 1991; Xiao and Zhang, 2002; Xiao and Zhang, 2005). Thus, pregnancy and chronic hypoxia differentially regulate K_{Ca} channel and PKC activities in uterine arteries. The balance between activations of K_{Ca} channels and PKC is likely to play an important role in the adaptation of uterine vascular tone to pregnancy and chronic hypoxia. However, the interaction between K_{Ca} channels and PKC as well as how they orchestrate and integrate to regulate uterine vascular contractility during pregnancy in response to chronic hypoxia remain largely unknown.

The goal of the present study was to investigate the potential effect of K_{Ca} channel inhibitors on PKC-mediated uterine arterial contractions in nonpregnant and pregnant sheep that reside in normoxic sea levels or exposed to long-term high altitude hypoxia. To further determine the interaction of PKC and K_{Ca} channel function and the effect of pregnancy and chronic hypoxia, we also investigated the effect of PKC activation on K_{Ca} channel-mediated relaxations of uterine arteries in nonpregnant and pregnant ewes in the normoxic and hypoxic conditions.

Materials and Methods

Tissue Preparation and Treatment

All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. As previously described (Chang et al., 2010), nonpregnant and time-dated pregnant sheep were obtained from the Nebeker Ranch in Lancaster, CA (altitude: ~300 m; arterial PaO_2: 102 ± 2 mmHg). Uterine arteries were obtained from nonpregnant and near-term (~140 days of gestation) pregnant sheep. Normoxic animals were studied between November and July. For chronic hypoxic treatment, nonpregnant and pregnant (30 days of gestation) animals bred during time period of March to June were transported to the Barcroft Laboratory, White Mountain Research Station, Bishop, CA (altitude, 3,820 m; maternal PaO_2, 60 ± 2 mmHg) and maintained there for ~110 days. Starting from August to October, the animals were transported to the laboratory immediately before the studies. Animals were anesthetized with iv propofol (2 mg/kg), followed by incubated and anesthesia is maintained on 1.5% to 3.0% isoflurane balanced in O_2 throughout the surgery. An incision in the abdomen was made and the uterus exposed. Uterine arteries were isolated and removed without stretching, and placed into a cold physiological salt solution (PSS) containing (in mM): 130 NaCl, 10.0 HEPES, 6.0 Glucose, 4.0 KCl, 4.0 $NaHCO_3$, 1.80 $CaCl_2$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, and 0.025 EDTA, pH 7.4. After removal of the tissues, animals were killed with T-61 (euthanasia solution, Hoechst-Roussel, Somerville, NJ).

Relaxation Studies

The fourth generation branches of the main uterine artery from pregnant sheep were separated from the surrounding tissue, and cut into 2-mm ring segments. For *ex vivo* treatment, the uterine arterial segments were incubated in phenol red-free DMEM with 1% charcoal-stripped FBS for 48 hours at 37 ℃ in both normoxic chamber with 21% O_2 and hypoxia chamber with 10.5% O_2. Isometric tension was measured in the Krebs solution in a tissue bath at 37 ℃, as described previously (Zhu et al., 2013 a). Briefly, each ring was equilibrated for 60 minutes and then gradually stretched to the optimal resting tension, as determined by the tension that developed in response to 120 mmol/L KCl added at each stretch level. After stable responses to KCl were obtained, tissues were rinsed and then contracted with submaximal concentrations of norepinephrine, followed by additions of NS1619 or NS309 in the absence or presence of phorbol 12,13-dibutyrate (PDBu), added in a cumulative manner.

Contraction Studies

The fourth generation branches of the main uterine artery from both pregnant and nonpregnant sheep were isolated, and cut into 2-mm ring segments and mounted in 10-mL tissue baths containing modified Krebs solution equilibrated with a mixture of 95% O_2 and 5% CO_2. Phorbol 12,13-dibutyrate (PDBu, Sigma) or norepinephrine -induced isometric tensions in the absence or presence of large-conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) inhibitor iberoitoxin (IBTX; 100 nmol/L), tetraethylammonium (TEA; 1 mmol/L) or small-conductance Ca^{2+}-activated K^+ channels (SK) blocker apamin (500 nmol/L), as described previous (Gauthier et al., 2004; Xiao et al., 2009; Xiao et al.,

2010b).

Data Analysis

Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA). Results were expressed as means ± SEM obtained from the number of experimental animals given. Differences were evaluated for statistical significance ($P < 0.05$) by ANOVA or t test, where appropriate.

Results

Inhibition of BK_{Ca} Channels Increased PKC-Mediated Contractions

Our recent studies have demonstrated that both BK_{Ca} and SK_{Ca} are expressed in uterine arterial smooth muscle cells (Hu et al., 2011; Hu et al., 2012; Zhu et al., 2013 a). In the present study, we investigated whether both types of K_{Ca} channels play an important role in PKC-mediated uterine vascular contractility. As shown in Figure 8, inhibition of BK_{Ca} channels with IBTX significantly potentiated PKC activator PDBu-induced contractions in uterine arteries of pregnant animals (pD_2 values: 4.5 ± 0.2 in control group *vs.* 5.8 ± 0.3 in IBTX-treated group; $P < 0.05$; E_{max}: 39.7 ± 5.8 % in control *vs.* 88.9 ± 13.1 % in IBTX-treated groups; $P < 0.05$). In contrast, inhibition of SK channels with apamin did not affect PDBu-induced contractions (pD_2: 4.5 ± 0.2 *vs.* 4.5 ± 0.1; $P > 0.05$; E_{max}: 39.7 ± 5.8 % *v*s. 41.7 ± 4.9 %; $P > 0.05$). The data suggest that BK_{Ca}, but not SK_{Ca} channels play an important role in PKC-mediated uterine vascular contractions in pregnancy.

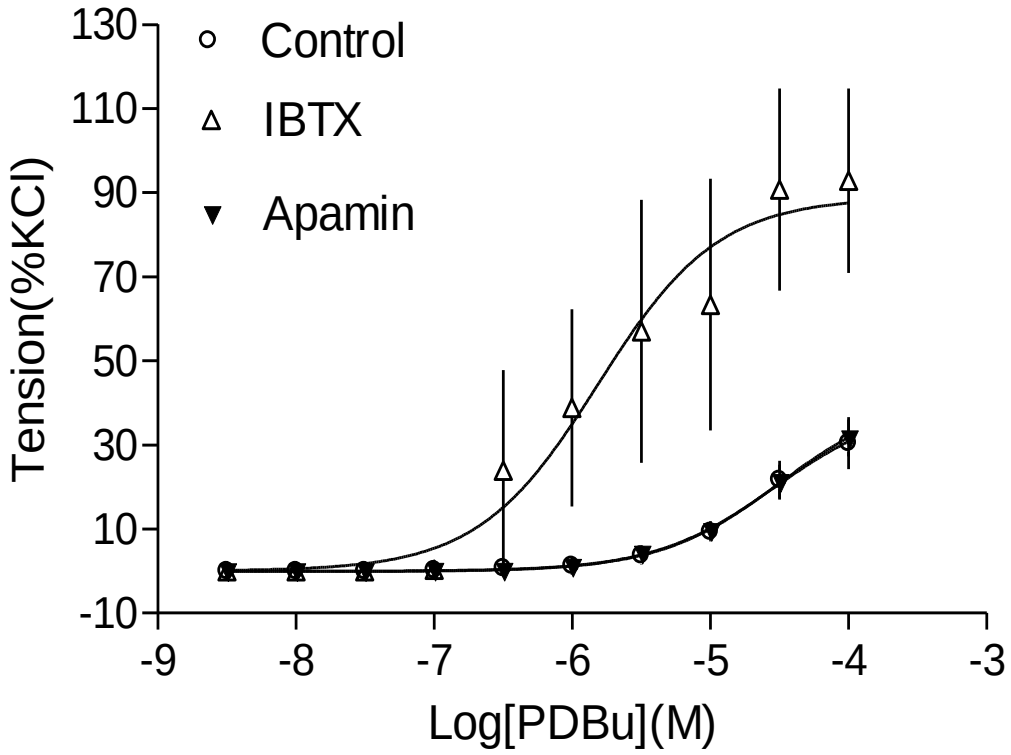

Figure 8. Effect of K_{Ca} Channel Blockers on PDBu-Induced Contractions of Uterine Arteries from Normoxic Pregnant Sheep. PDBu-induced contractions were determined in uterine arteries obtained from normoxic pregnant sheep in the absence (control) or presence of 100 nM IBTX or 500 nM apamin pretreatment for 20 min. Data are means ± SEM of tissues from 4-5 animals in each group.

Gestational Hypoxia Abrogated Inhibitory Effect of BK_{Ca} on PKC-Mediated Contractions

In agreement with the previous findings that chronic hypoxia enhanced PKC-mediated uterine vascular contractions during pregnancy (Chang et al., 2009; Xiao and Zhang, 2002; Xiao and Zhang, 2005), PDBu-induced contractions of uterine arteries of pregnant sheep were significantly greater in hypoxic animals (E_{max}: 83.8 ± 5.4 %) (Figure 9) than those in normoxic animals (E_{max}: 39.7 ± 5.8 %) (Figure 8) ($P < 0.05$). In contrast to the findings in normoxic animals (Figure 8), inhibition of BK_{Ca} channels had no significant effect on PDBu-induced contractions of uterine arteries in pregnant sheep of hypoxic animals (E_{max}: 83.8 ± 5.4% in control group *vs.* 76.9 ± 12.5 % in TEA-treated groups; $P > 0.05$) (Figure 9). As shown in Figure 10, in uterine arteries of nonpregnant sheep, BK_{Ca} channel inhibition significantly enhanced PDBu-induced contractions in both normoxic (upper panel) and hypoxic (lower panel) animals. These data suggest that the inhibitory effect of BK_{Ca} channels on PKC-mediated contractions is selectively abrogated in uterine arteries of pregnant, but not nonpregnant animals acclimatized to long-term high altitude hypoxia.

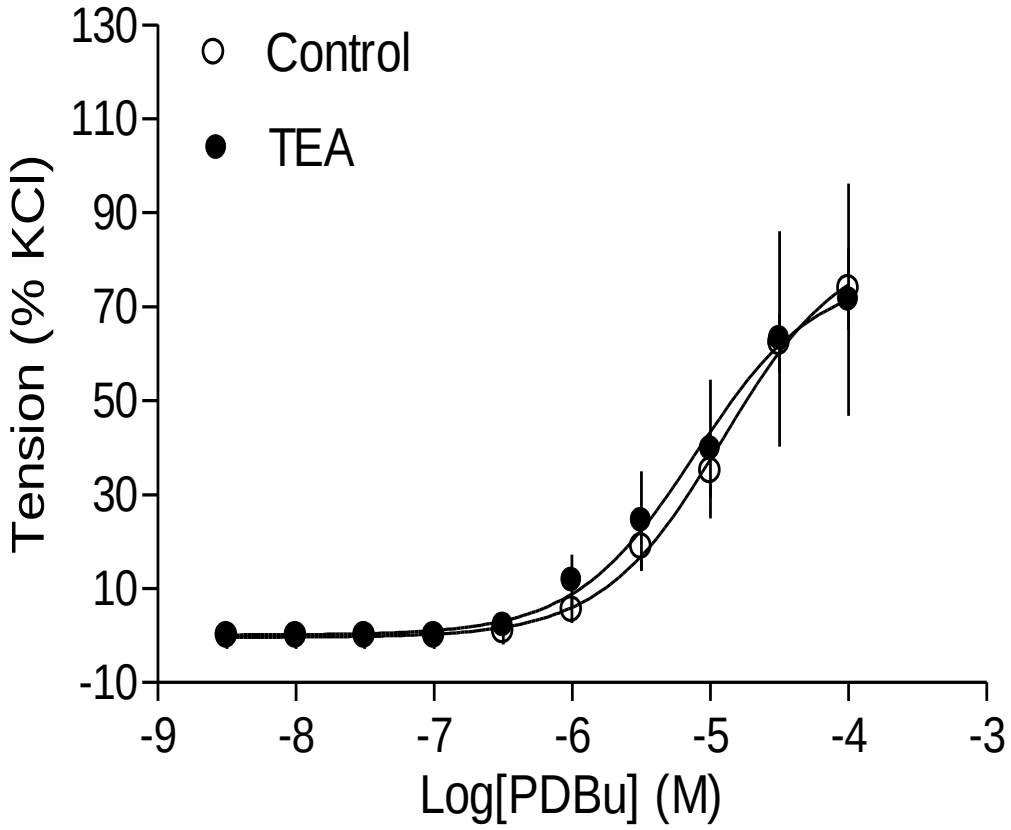

Figure 9. Effect of BK_{Ca} Blocker on PDBu-Induced Contractions of Uterine Arteries from Hypoxic Pregnant Sheep. PDBu-induced contractions were determined in uterine arteries obtained from hypoxic pregnant sheep in the absence (control) or presence of 1 mM TEA pretreatment for 20 min. Data are means ± SEM of tissues from 4 animals in each group.

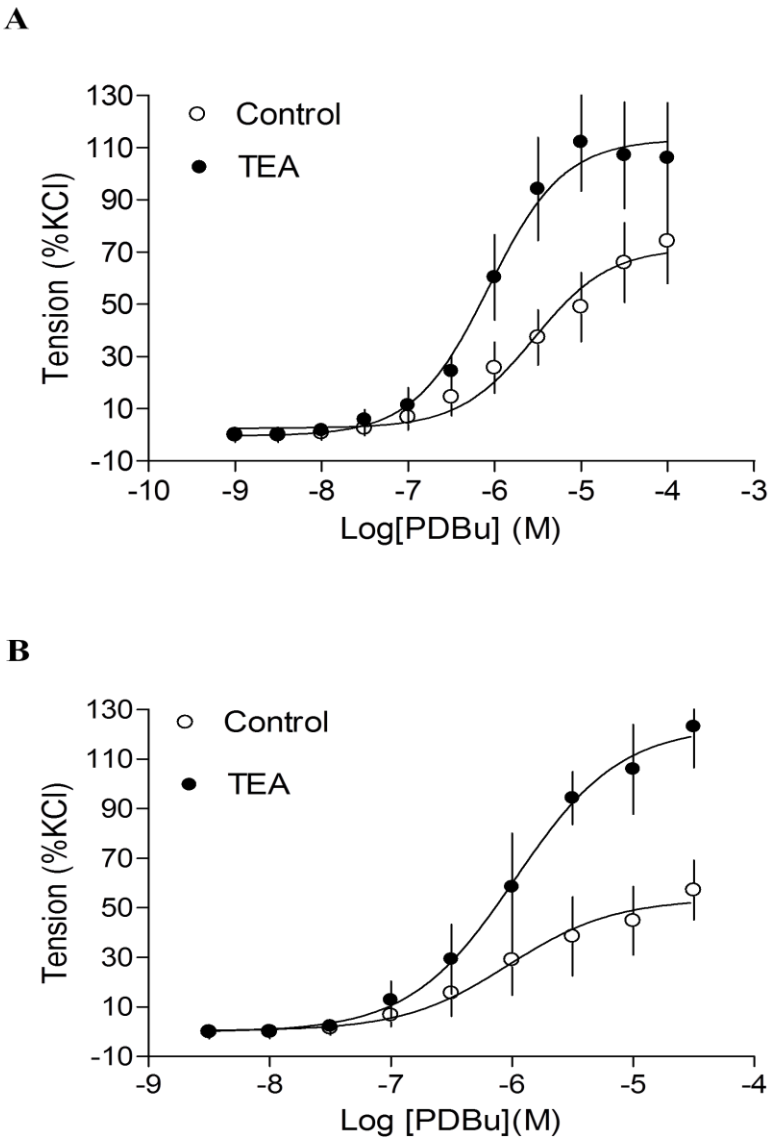

Figure 10. Effect of BK_{Ca} Blocker on PDBu-Induced Contractions of Uterine Arteries from Nonpregnant Sheep. PDBu-induced contractions were determined in uterine arteries obtained from normoxic (panel **A**) and hypoxic (panel **B**) nonpregnant sheep in the absence (control) or presence of 1 mM TEA pretreatment for 20 min. Data are means ± SEM of tissues from 4-5 animals in each group.

Chronic Hypoxia Induced PKC-Mediated Inhibition of K_{Ca} Channel Activity

As shown in Figure 11, both BK_{Ca} channel opener, NS1619 and SK_{Ca} channel opener, NS309 caused concentration-dependent relaxations of uterine arteries from pregnant animals. PDBu had no significantly effect on either NS1619- or NS309-induced relaxations under the normoxic condition (Figure 11). Treatment of tissues with chronic hypoxia (10.5% O_2 for 48 h) significantly reduced both NS1619 (E_{max}: 19.9 ± 1.1 *vs.* 30.6 ± 2.2; $P < 0.05$)- and NS309 (E_{max}: 18.9 ± 1.8 *vs.* 26.5 ± 1.6; $P < 0.05$)-induced relaxations. In addition, under the hypoxic condition, PDBu significantly inhibited both NS1619- and NS309-induced uterine arterial relaxations (Figure 12).

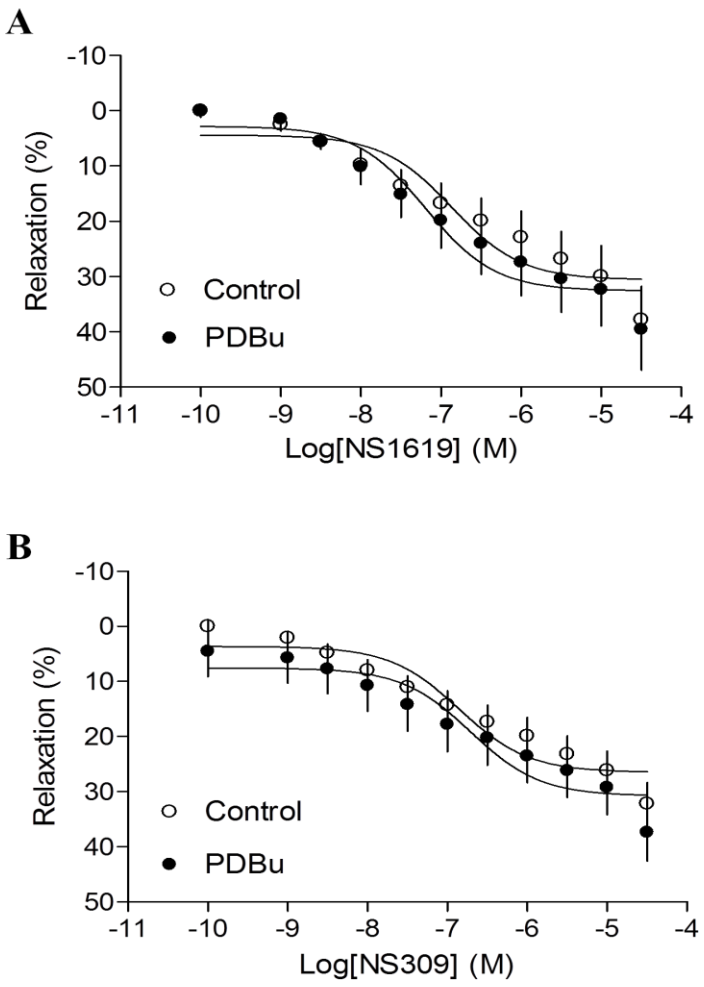

Figure 11. Effect of PDBu on K_{Ca} Opener-Induced Relaxations of Uterine Arteries from Pregnant Sheep under Normoxic Condition. Uterine arteries were isolated from pregnant sheep and incubated under 21% O_2 for 48 h. NS1619 (panel **A**)- and NS309 (panel **B**)-induced relaxations were determined in uterine arteries pre-contracted with norepinephrine (1 μM), in the absence (control) or presence of 1 μM PDBu. Data are means ± SEM of tissues from 8-9 animals in each group.

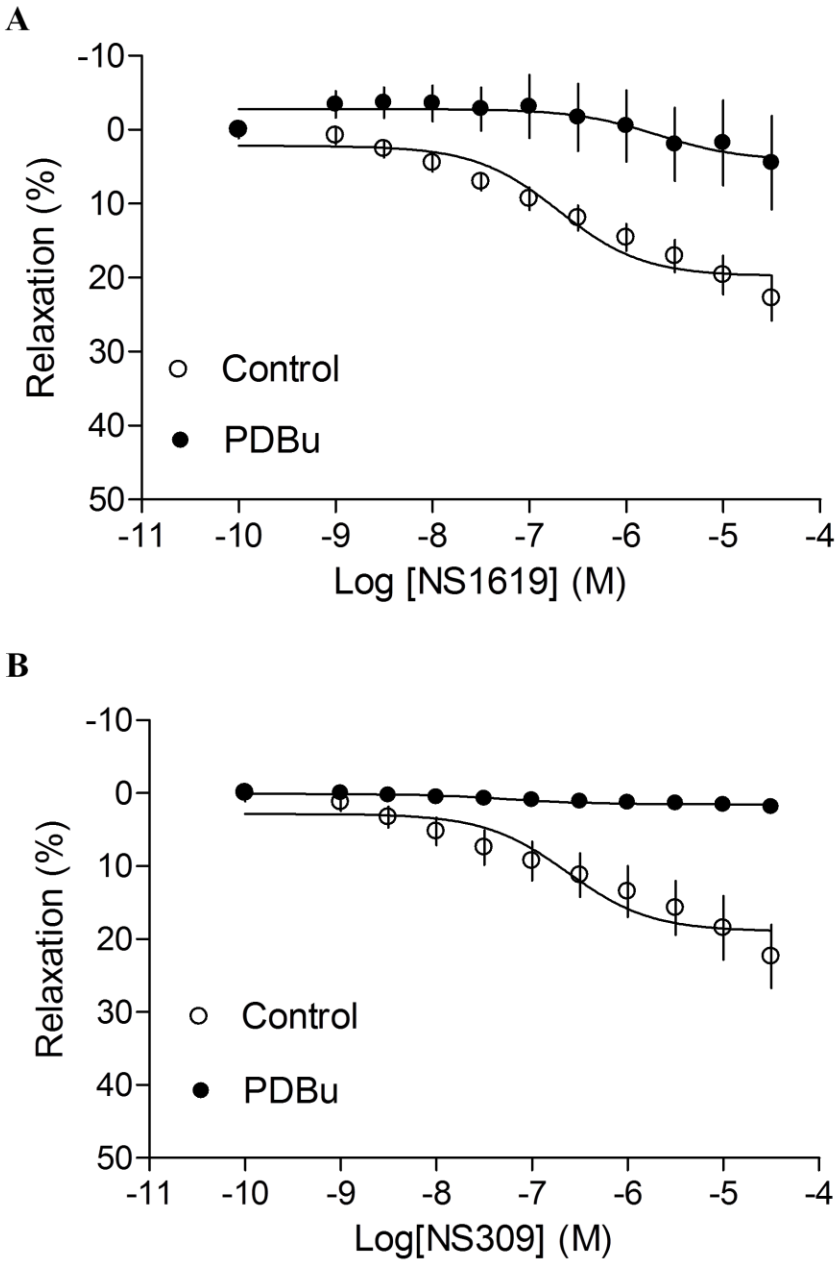

Figure 12. Effect of PDBu on K_{Ca} Opener-Induced Relaxations of Uterine Arteries from Pregnant Sheep under Hypoxic Condition. Uterine arteries were isolated from pregnant sheep and incubated under 10.5% O_2 for 48 h. NS1619 (panel **A**)- and NS309 (panel **B**)-induced relaxations were determined in uterine arteries pre-contracted with norepinephrine (1 μM), in the absence (control) or presence of 1 μM PDBu. Data are means ± SEM of tissues from 5-8 animals in each group.

Inhibition of BK_{Ca} Channels Had no Effect on Norepinephrine-Induced Contractions

To determine the specific interaction of BK_{Ca} channels and PKC-mediated contractions in uterine arteries, the effect of BK_{Ca} channel inhibition on α-adrenoceptor-mediated contractions were examined. As shown in Figure 13, inhibition of BK_{Ca} channels had no significant effects on norepinephrine-induced, concentration-dependent contractions of uterine arteries from nonpregnant or pregnant sheep under either normoxic or hypoxic conditions.

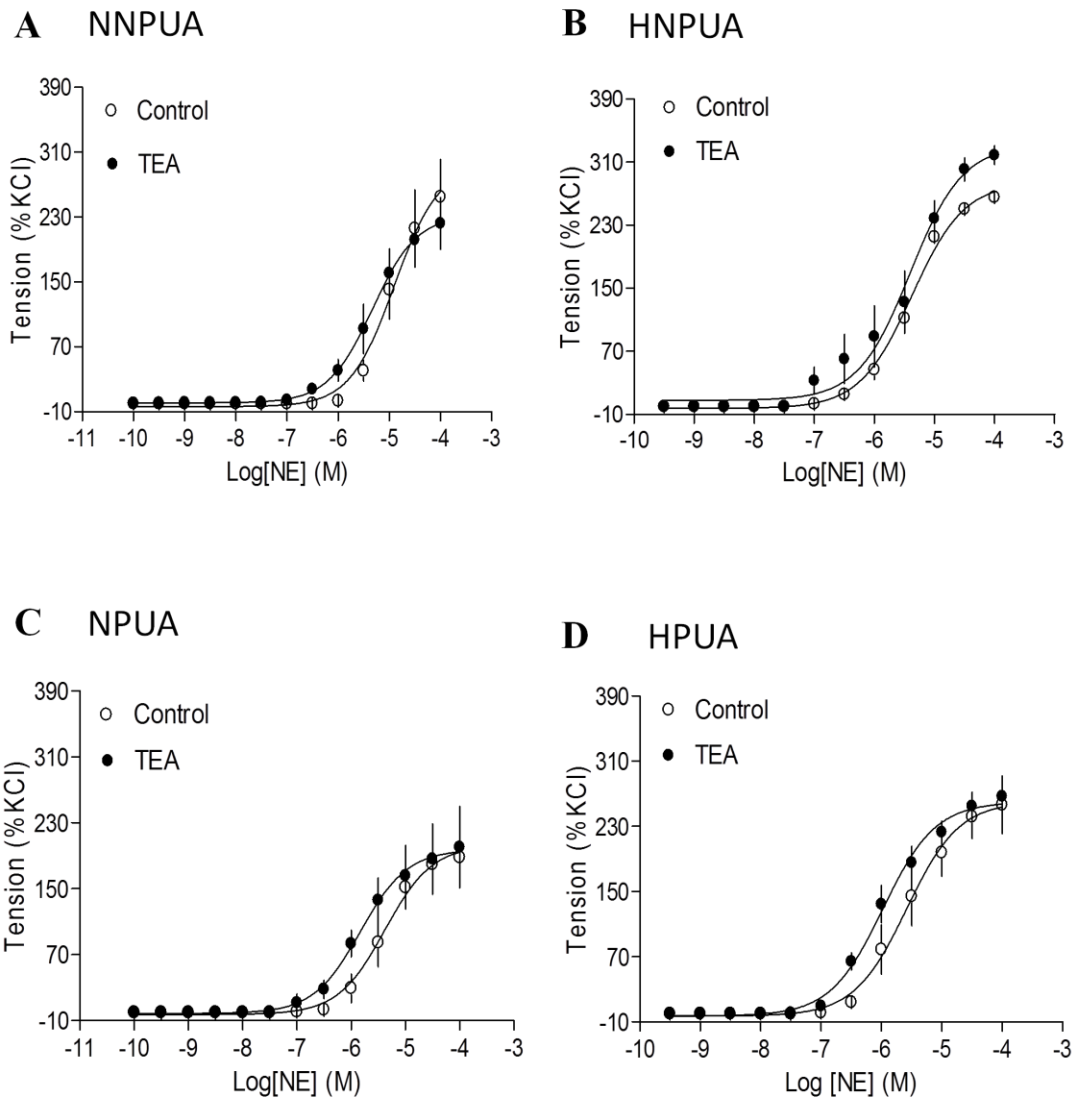

Figure 13. Effect of BK_{Ca} Blocker on Norepinephrine-Induced Contractions of Uterine Arteries. Norepinephrine (NE)-induced contractions were determined in uterine arteries obtained from normoxic nonpregnant (panel **A**), hypoxic nonpregnant (panel **B**), normoxic pregnant (panel **C**) and hypoxic pregnant (panel **D**) sheep in the absence (control) or presence of 1 mM TEA pretreatment for 20 min. Data are means ± SEM of tissues from 4-5 animals in each group.

Discussion

Our previous studies have demonstrated that both PKC and K_{Ca} channels play an important role in uterine vascular adaptation to pregnancy and chronic hypoxia (Chang et al., 2009; Hu et al., 2011; Hu et al., 2012; Xiao et al., 2012; Xiao and Zhang, 2002; Xiao and Zhang, 2005). The present study provides new evidence that PKC and K_{Ca} channels interact each other and integrate to regulate uterine vascular contractility under physiological and pathophysiological conditions. The major findings of the present study are the following: 1) inhibition of BK_{Ca} but not SK_{Ca} channels potentiated PKC-mediated contractions of uterine arteries; 2) chronic hypoxia abrogated the inhibitory effect of BK_{Ca} channels on PKC-induced contractions of uterine arteries from pregnant, but not nonpregnant animals; 3) in uterine arteries of pregnant animals activation of PKC had no significant effects on either BK_{Ca} - or SK_{Ca}-mediated relaxations under the normoxic condition, but significantly inhibited them under the hypoxic condition; 4) inhibition of BK_{Ca} channels had no significant effects on α-adrenoceptor-mediated uterine arterial contractions.

The present finding that inhibition of BK_{Ca} but not SK_{Ca} channels significantly enhanced PDBu-induced contractions suggests that the basal BK_{Ca} channel activity plays a significant role in counteracting PKC-mediated myogenic tone of uterine arteries. BK_{Ca} channels are important in the regulation of resting membrane potential and control of vascular tone (Hu et al., 2011; Rosenfeld et al., 2005). Previous studies demonstrated that PKC played a key role in pressure-dependent myogenic response of uterine arteries (Chang et al., 2009), and inhibition of BK_{Ca} channels significantly increased pressure-dependent myogenic tone in uterine arteries of pregnant sheep (Hu et al., 2011). This is

consistent with the findings that intra-arterial infusion of BK_{Ca} channel inhibitor TEA into the uterine artery circulation of late-gestation sheep caused a significant decrease of basal uterine blood flow from 50% to 80% in the absence of systemic effects or a change in contralateral uterine blood flow (Rosenfeld et al., 2001; Rosenfeld et al., 2005). We have demonstrated that TEA and IBTX inhibit the BK_{Ca} channel currents by the same extent of 53% in uterine arterial myocytes (Hu et al., 2011).

Of importance, chronic hypoxia during gestation abrogated inhibitory effect of BK_{Ca} channels on PKC-mediated contractions of uterine arteries, suggesting that loss of negative regulatory component of basal BK_{Ca} channel activity may be a key signaling mechanism in chronic hypoxia-mediated increase in PKC-induced myogenic contractions of uterine arteries in pregnancy, as demonstrated in the present study as well as the previous studies (Chang et al., 2009; Xiao et al., 2009). This notion is supported by our recent findings that gestational hypoxia downregulated BK_{Ca} channel β1 subunit gene expression and BK_{Ca} channel activity in uterine arteries (Hu et al., 2012). Of interest, this hypoxic-mediated effect is pregnancy-dependent, as inhibition of BK_{Ca} channels enhanced PDBu-induced contractions of uterine arteries of nonpregnant sheep in both normoxic and hypoxic animals. Previous studies demonstrated that pregnancy upregulated BK_{Ca} channel function in uterine arteries *via* the action of 17β-estradiol (Hu et al., 2011). Further studies showed that chronic hypoxia during gestation caused heightened promoter methylation and resultant estrogen receptor-α (ERα) gene repression in uterine arteries (Dasgupta et al., 2012). Taken together, these findings suggest a specific vulnerability of steroid hormone-mediated response in uterine vascular adaptation in pregnancy to gestational hypoxia.

In addition to that basal BK_{Ca} channel activity may be important in regulating PKC-mediated contractions of uterine arteries, several studies have shown a regulatory role of PKC on K_{Ca} channel activation (Barman et al., 2004; Del Carlo et al., 2003; Taguchi et al., 2000). Our previous study showed that activation of PKC inhibited basal BK channel current density in uterine arteries (Hu et al., 2011). In the present study, we found that both BK_{Ca} channel opener NS1619- and SK_{Ca} channel opener NS309-induced relaxations of uterine arteries were not altered by the PDBu treatment. This finding is intriguing and suggests different regulatory mechanisms of basal and activated K_{Ca} channel activities in the uterine artery. Of importance, chronic hypoxia treatment induced PKC-mediated inhibition of NS1619- and NS309-produced relaxations. Our previous demonstrated that chronic hypoxia significantly increased the PKC activity in uterine arteries of pregnant sheep (Chang et al., 2009), suggesting hypoxia-mediated upregulation of PKC activity in inhibiting BK_{Ca} and SK_{Ca} channel-mediated relaxations of uterine arteries in pregnancy. Although the mechanisms of hypoxia-induced upregulation of inhibitory effect of PKC on activated K_{Ca} channel activities remain to be determined, a selective increase in the activity of PKC isozyme PKCε in uterine arteries by chronic hypoxia (Chang et al., 2009) may play a role. Indeed, activation of PKCε causes a stimulation of L-type Ca^{2+} channel through c-Src, resulting in inhibition of K_{Ca} channel activity (Alioua et al., 2002).

Taken together, as shown in Figure 14, pregnancy and chronic hypoxia differentially regulate the interaction of PKC and K_{Ca} channels in modulating uterine arterial contractility. Thus, under the normoxic condition, heightened basal BK_{Ca} channel activity in pregnant animals has a negative regulatory effect on PKC-induced contraction

and myogenic tone of the uterine artery. Chronic hypoxia during gestation downregulates basal BK_{Ca} channel activity and abrogates its inhibition of PKC-mediated myogenic response. In addition, chronic hypoxia induces an inhibitory effect of PKC on activated K_{Ca} channel-mediated relaxations of the uterine arteries in pregnant animals. Collectively, these findings demonstrate complex yet integrated effects of pregnancy and chronic hypoxia on the interaction of PKC and K_{Ca} channel activities in the uterine artery, which are important in normal adaptation of reduced uterine vascular tone in pregnancy as well as in maladaptation of increased uterine vascular contractility in response to chronic hypoxia in gestation.

Acknowledgements

[1] **Sources of Funding**: This work was supported by National Institutes of Health Grants HL110125 (LZ), HL089012 (LZ), HD031226 (LZ), DA032510 (DX), and by the Regents of the University of California TRDRP grant 22XT-0022 (to D. Xiao).

[2] **Disclosures:** None

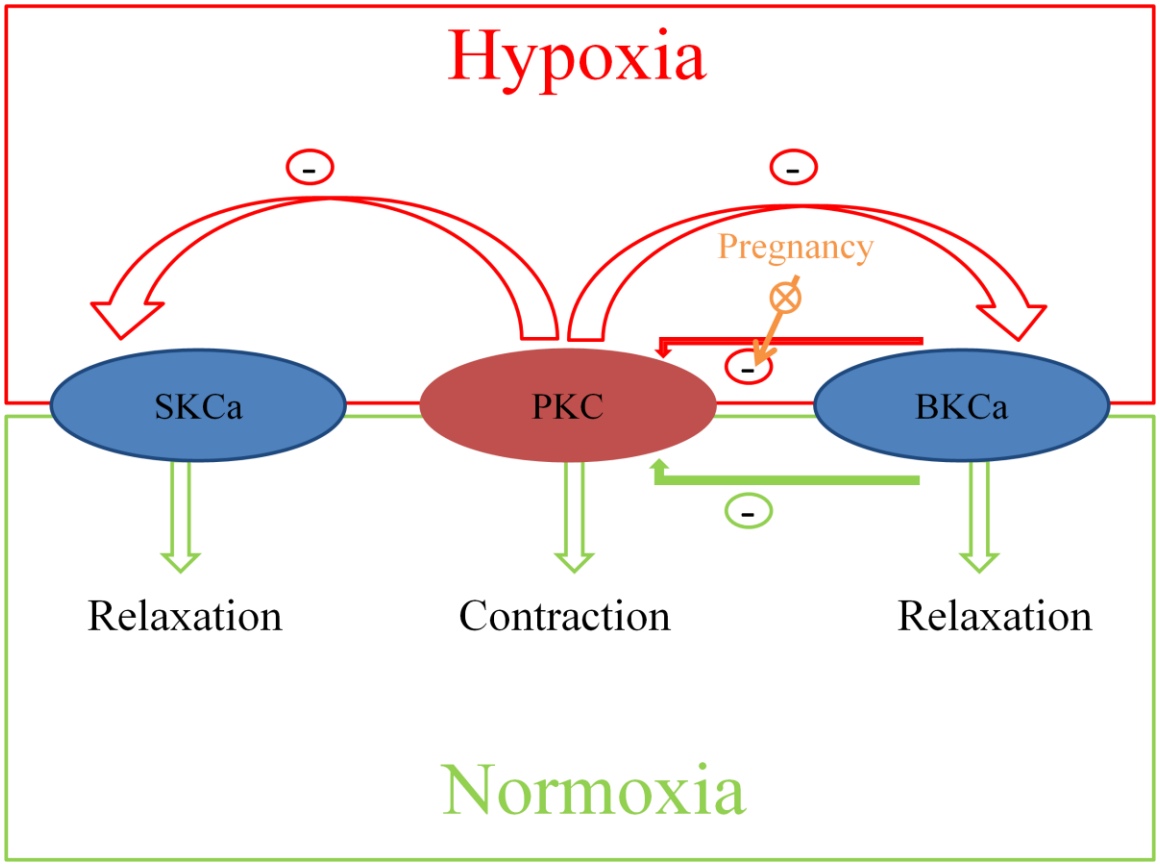

Figure 14. Diagram of the Integrated Effects of Pregnancy and Chronic Hypoxia on the Interactions of PKC and K_{Ca} Channels in Modulating Uterine Arterial Contractility. The diagram show that under the normoxic condition, heightened basal BK_{Ca} channel activity in pregnant animals has a negative regulatory effect on PKC-induced contraction and myogenic tone of the uterine artery. Chronic hypoxia during gestation downregulates basal BK_{Ca} channel activity and abrogates its inhibition of PKC-mediated myogenic response. In addtion, chronic hypoxia induces an inhibitory effect to PKC on activated K_{Ca} channel-mediated relaxations of the uterine arteries in pregnant animals.

CHAPTER FOUR

LONG-TERM HIGH ALTITUDE HYPOXIA INCREASES ROS AND INHIBITS STEROID HORMONE-MEDIATED UPREGULATION OF K^+ CHANNEL ACTIVITY IN UTERINE ARTERIES OF PREGNANT SHEEP

by

Ronghui Zhu, Xiaohui Huang, Xiangqun Hu, Daliao Xiao, Jianjun Zhou, Chiranijib Dasgupta, and Lubo Zhang

This chapter is under review for publication in Hypertension.

Abstract

Ca^{2+}-activated K^+ (K_{Ca}) channels play key role in regulating uterine vascular tone. Previous studies have shown an upregulation of Kca activity and Kca-mediated relaxation of uterine artery in pregnancy, which is inhibited by gestational hypoxia. The present study tested the hypothesis that reactive oxygen species (ROS) play an important role in chronic hypoxia-mediated inhibition of steroid hormone effect in upregulating uterine arterial Kca channel activity in pregnancy. Uterine arteries were isolated from nonpregnant (NPUA) and pregnant (PUA) sheep maintained at either sea-level or high altitude (3,820m, PaO_2: 60mmHg). In PUA, hypoxia significantly decreased large conductance (BK_{Ca}) channel opener NS1619- and small conductance (SK_{Ca}) channel opener NS309-induced relaxations, which were partially abrogated by ROS inhibitor N-acetylcysteine (NAC). Consistently, NAC significantly increased both BK_{Ca} and SK_{Ca} current densities in uterine arterial smooth muscle cells in pregnant animals acclimatized to high altitude. In NPUA, NS1619- and NS309-induced relaxations were diminished as compared with those in PUA. Pregnancy enhanced SK_{Ca} type 2 and 3 channels mRNA levels in uterine arteries, which were alleviated by hypoxia. Treatment of NPUA with 17β-estradiol ($E_2\beta$) and progesterone (P_4) for 48 h increased SK_{Ca} type 3 protein abundance and NS1619- and NS309-induced relaxations, which were inhibited by hypoxia, but this hypoxia-mediated inhibition was reversed by NAC. Consistently, steroid hormones treatment had no significant effects on BK_{Ca} current density in the absence of NAC, but enhanced it in the presence of NAC. The present data suggest an important role of ROS in negatively regulating steroid hormone-mediated upregulation of K_{Ca} channel activity and adaptation of uterine vascular reactivity in pregnancy, which

may contribute to the increased incidence of preeclampsia and fetal intrauterine growth restriction associated with maternal hypoxia.

Introduction

Ca^{2+}-activated K^+ (K_{Ca}) channels are key regulators of vascular tone (Ledoux J, et al., 2006; Hu and Zhang, 2012). Both large-conductance K_{Ca} (BK_{Ca}) and small-conductance K_{Ca} (SK_{Ca}) contribute to the regulation of uterine vascular function (Hu et al., 2011; Hu et al., 2012; Zhu et al., 2013 a). Uterine blood flow increases dramatically during pregnancy to optimize the supply of oxygen and nutrition for the development of the fetus. Steroid hormones such as estrogen and progesterone play an important role in the hemodynamic adaption in part by upregulating K_{Ca} channel (Hu et al., 2011), leading to decreased vascular tone. However, this adaptive change was severely complicated by gestational hypoxia, leading to increased incidence of preeclampsia and fetal intrauterine growth restriction (Zamudio et al., 1995ab; Keyes et al., 2003; Julian et al., 2008). The dysregulation of uterine circulation involves increased vascular tone due to impaired K_{Ca} channel function (Hu et al., 2012; Zhu et al., 2013 a). However, the mechanism underlying the impairment of K_{Ca} channel function remains poorly understood.

Reactive oxygen species (ROS) in the cardiovascular system primarily include superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Increased level of ROS during exposure to hypoxia has been demonstrated in vasculature including uterine arteries from pregnant sheep (Marshall et al., 1996; Rathore et al., 2008; Xiao et al., 2013); and oxidative stress has been implicated in the pathogenesis of various cardiovascular disorders (Wolin et al., 2005; Schnabel and Blankenberg, 2007). Activities

of K_{Ca} channels in VSMCs are subject to modulation by ROS (Brakemeier et al., 2003; Xiao et al., 2013). Considering important roles of K_{Ca} in regulating uterine vascular tone and the fact that increased generation of ROS in uterine arteries from pregnant animals exposed to high-altitude chronic hypoxia, the present studies were investigate whether the hypoxia-mediated heightened ROS altered K_{Ca} channels activities and their-mediated relaxations of uterine arteries in pregnancy. In addition, we were also investigated the effects of steroid hormones on K_{Ca} channels activities and their-mediated uterine vasorelaxations and determined whether this effect of steroid hormones was altered by hypoxia-enhanced ROS.

Materials and Methods

Tissue Preparation and Treatment

Uterine arteries were harvested from nonpregnant and near-term (~140 days' gestation) pregnant sheep maintained at sea level (~300 m) or exposed to high-altitude (3801 m) hypoxia (PaO_2: 60 mmHg) for 110 days (Chang et al., 2010). Animals were anesthetized with Ketamine (10 mg/kg, i.v.) followed by inhalation of 1.5% to 2.0% halothane. An incision was made in the abdomen and the uterus exposed. The uterine arteries were isolated and removed without stretching and placed into a modified Krebs solution. All procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Contraction Studies

The fourth generation branches of main uterine arteries from nonpregnant and pregnant sheep, respectively, were separated from the surrounding tissue, and cut into 2-mm ring segments. Uterine arteries from pregnant sheep were immediately used for contraction studies, whereas uterine arteries from nonpregnant sheep were treated with 17β-estradiol (E2β, 0.3 nmol/L) and progesterone (P4, 100 nmol/L) for 48h in absence or presence of 1 mM N-acetylcysteine (NAC). Isometric tension was measured in the Krebs solution in a tissue bath at 37°C, as described previously (Hu and Zhang, 1997; Xiao et al., 2010c). Briefly, each ring segment was equilibrated for 60 minutes and then gradually stretched to the optimal resting tension, as determined by the tension that developed in response to 120 mmol/L KCl added at each stretch level. After stable responses to KCl were obtained, ring segments were rinsed and then contracted with submaximal concentrations of norepinephrine, followed by NS1619 or NS309 in the absence or presence of NAC, respectively, for tissues from pregnant animals and in the absence of NAC for tissues from nonpregnant animals, added in a cumulative manner.

Western Immunoblotting

Protein abundance of SK2 and SK3 channels were measured in freshly isolated nonpregnant uterine arteries after hormone treatment (Hu et al., 2011; Xiao et al., 2009; Zhu et al., 2013 a). Briefly, tissues were homogenized in a lysis buffer followed by centrifugation at 4°C for 10 minutes at 10,000g, and the supernatants were collected. Samples with equal proteins were loaded onto 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate, and were separated by electrophoresis at 100 V for 2 hours.

Proteins were then transferred onto nitrocellulose membranes. After blocking nonspecific binding sites by dry milk, the membranes were incubated with primary antibodies against SK2 channel (Alomone Ltd, Jerusalem, Israel) and SK3 channel (Santa Cruz Biotechnology, Santa Cruz CA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.

Real-Time RT-PCR

Total RNA was extracted from uterine arteries using TRIzol protocol (Invitrogen, Carlsbad, USA). Then, the SK2 and SK3 mRNA levels were determined by real-time RT-PCR using the iCycler Thermal cycler (BioRad, Hercules, CA). Specific SK2 primers were 5'-ATGGACACTCAGCTGACAAAAAGA-3' (forward) and 5'-GCTTGCAAGAATTTCCGTTGATGT-3' (reverse). Specific SK3 primers were 5'-CCAAGCGGATCAAGAATGCTGC-3' (forward) and 5'-GACGCTCCTCAACTGCAACTGGTGGATA-3' (reverse). Real-time RT-PCR was performed in a 25 μl-reaction mixture according to the instruction of iScript one-step RT-PCR kit (BioRad). RT-PCR was carried out under the following conditions: 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 10 sec, 50 ℃ for 15 sec and 72 ℃ for 15 sec. The results were calculated from the standard curve of 1 to 10^{-13} ng SK2 or SK3 cDNA plasmid run in each assay as previously described (Xiao et al., 2001b).

Measurement of K_{Ca} Channel Current

Arterial smooth muscle cells were enzymatically dissociated from resistance-sized uterine arteries, and whole-cell K^+ currents were recorded using an EPC 10 patch-clamp amplifier with Patchmaster software (HEKA, Lambrecht/Pfalz, Germany) at room temperature, as previously described (Hu et al., 2011). Briefly, cell suspension drops were placed in a recording chamber and adherent cells were continuously superfused with HEPES-buffered physiologic salt solution containing (in mmol/L): 140.0 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10.0 HEPES, and 10.0 glucose (pH 7.4). Only relaxed and spindle-shaped myocytes were used for recording. Micropipettes were pulled from borosilicate glass and had resistances of 2 to 5 MΩ when filled with the pipette solution containing (in mmol/L) 140.0 KCl, 1.0 $MgCl_2$, 5.0 Na_2ATP, 5.0 EGTA, 10.0 HEPES (pH 7.2). $CaCl_2$ was added to bring free Ca^{2+} concentrations to 200.0 nmol/L, as determined using WinMAXC software (Chris Patton, Stanford University). Cells were held at –50 mV and whole-cell K^+ currents were evoked by voltage steps from -60 mV to +80 mV by stepwise 10-mV depolarizing pulses (350-ms duration, 10-second intervals) in the absence and presence of 1 mM tetraethylammonium (TEA) or 1 μmol/L apamin. The K^+ currents were normalized to cell capacitance and were expressed as picoampere per picofarad (pA/pF).

Data Analysis

Concentration-response curves were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad Software, San Diego, CA). Results were expressed as means ± SEM obtained from the number of experimental

animals given. Differences were evaluated for statistical significance ($P < 0.05$) by ANOVA or t test, where appropriate.

Results

Effect of Acute N-acetylcysteine Treatment on K_{Ca} Channel-Mediated Relaxation of Uterine Arteries from Normoxic Pregnant Sheep

As shown in Fig. 15A, the BK_{Ca} channel opener NS1619 induced a concentration-dependent relaxation of uterine arteries in the absence or presence of N-acetylcysteine. However, relaxation-induced by NS1619 was not altered by N-acetylcysteine treatment (pD2: 6.7 ± 0.3 versus 5.8 ± 0.2; Emax: 39.3 ± 3.8 % versus 43.9 ± 5.0 %; $P > 0.05$). Similarly, relaxation induced by the SK_{Ca} channel opener NS309 was also concentration-dependent (Fig. 15B). N-acetylcysteine had no effect on NS309-induced relaxation (pD2: 5.5 ± 0.3 versus 6.5 ± 0.3; Emax: 58.0 ± 11.2 % versus 50.7 ± 11.9 %; $P > 0.05$).

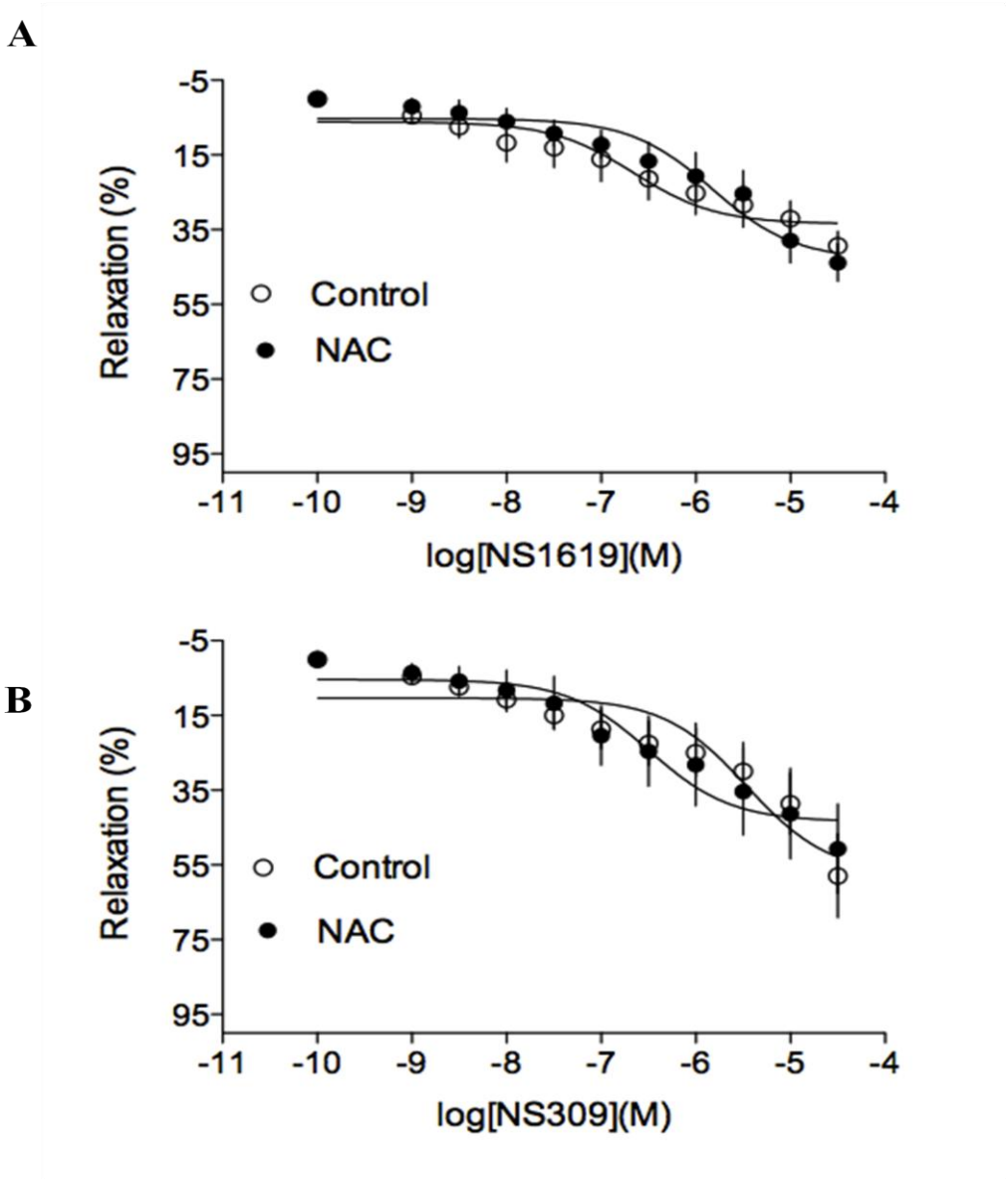

Figure 15. Effect of N-acetylcysteine on K_{Ca} Channel-Mediated Relaxation in Uterine Arteries from Normoxic Pregnant Sheep. **A**. Concentration-response curves of NS 1619-induced relaxation in the absence (Control) or presence of N-acetylcysteine (NAC, 1 mmol/L). **B**. Concentration-response curves of NS 309-induced relaxation in the absence or presence of N-acetylcysteine (1 mmol/L). Uterine arteries were contracted with norepinephrine (NE, 1 μmol/L), and then followed by additions of NS1619 or NS309. N-acetylcysteine was added into organ baths 20 minutes before additon of NS1619 or NS309. Data are means ± SEM from 4 to 5 animals in each group.

Effect of Acute N-acetylcysteine Treatment on K_{Ca} Channel-Mediated Relaxation of Uterine Arteries from Hypoxic Pregnant Sheep

Consistent with previous studies (Zhu et al., 2013 a), relaxations induced by NS1619 and NS309 in uterine arteries were impaired in high-altitude pregnant animals (Fig. 16). As shown in Figure 16A, NS1691-induced maximal response of relaxations of uterine arteries were significantly attenuated in hypoxic sheep (13.7 ± 3.8 %) as compared with those in normoxic animals (39.3 ± 3.8 %, Fig. 15A) ($P < 0.05$). Similarly, NS309-induced maximal response of relaxations of uterine arteries were also significantly attenuated in hypoxic sheep (17.7 ± 3.3 %, Fig. 16B) as compared with those in normoxic animals (58.0 ± 11.2 %, Fig. 1B) ($P < 0.05$). Treatment with NAC significantly enhanced both NS1619-induced maximal relaxation (13.7 ± 3.8 % versus 46.2 ± 9.5 %; $P < 0.05$) (Fig. 16A) and NS309-induced maximal relaxation (17.7 ± 3.3 % versus 34.9 ± 4.6 %; $P < 0.05$) (Fig. 16B).

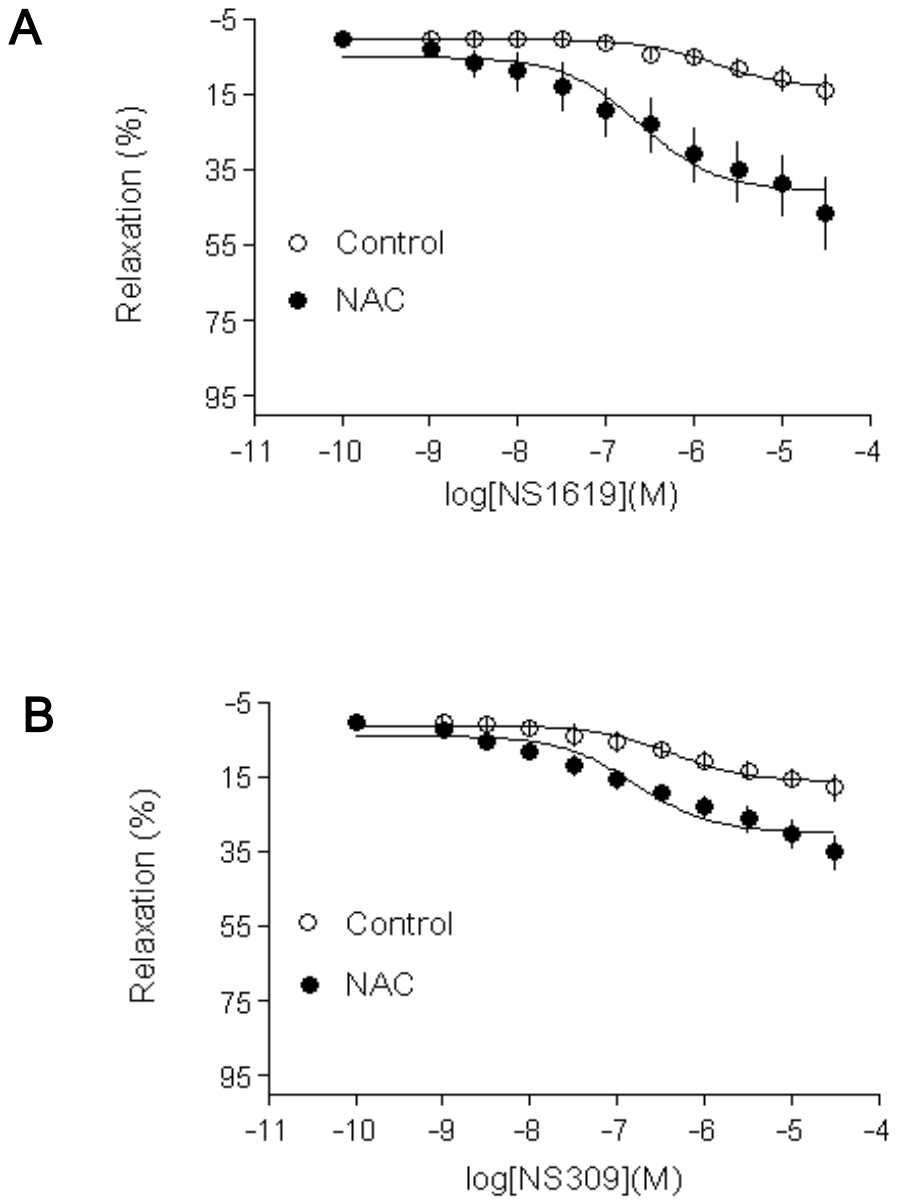

Figure 16. Effect of N-acetylcysteine on K_{Ca} Channel-Mediated Relaxation in Uterine Arteries from Hypoxic Pregnant Sheep. A. Concentreation-response curves of NS1619-induced relaxation in the absence (Control) or presence of N-acetylcysteine (NAC, 1 mmol/L). B. Concentration-response curves of NS309-induced relaxation in the absence or presence of N-acetylcysteine (1 mmol/L). Uterine arteries were contracted with norepinephrine (NE, 1 μmol/L), and then followed by additions of NS1619 or NS309. N-acetylcysteine was added into organ baths 20 minutes before addition of NS1619 or NS309. Data are means ± SEM from 4 to 5 animals in each group.

Effect of Acute N-acetylcysteine Treatment on K_{Ca} Channel Activities in Uterine Arterial Smooth Muscle Cells

To determine the effect of ROS on K_{Ca} channel activities in uterine arterial smooth muscle cells, whole-cell K^+ and K_{Ca} currents were obtained in the absence or presence of N-acetylcysteine in myocytes freshly isolated from uterine arteries of normoxic and hypoxic pregnant animals. N-acetylcysteine was without effect on whole-cell K^+ currents in myocytes of normoxic animals (50.8 ± 2.0 pA/pF versus 52.8 ± 1.7 pA/pF at +80 mV; $P > 0.05$). However, N-acetylcysteine significantly increased whole-cell K^+ (from 42.4 ± 0.8 pA/pF to 50.4 ± 2.1 pA/pF; $P < 0.05$) and BK_{Ca} (from 18.5 ± 1.4 pA/pF to 26.6 ± 2.4 pA/pF; $P < 0.05$) currents in uterine arterial myocytes of hypoxic animals (Fig. 17). Consistent with previous finding (Zhu et al., 2013 a), no apamin-sensitive K^+ current was detected in uterine arterial myocytes of hypoxic animals; and N-acetylcysteine treatment failed to upregulate SK currents (data not shown).

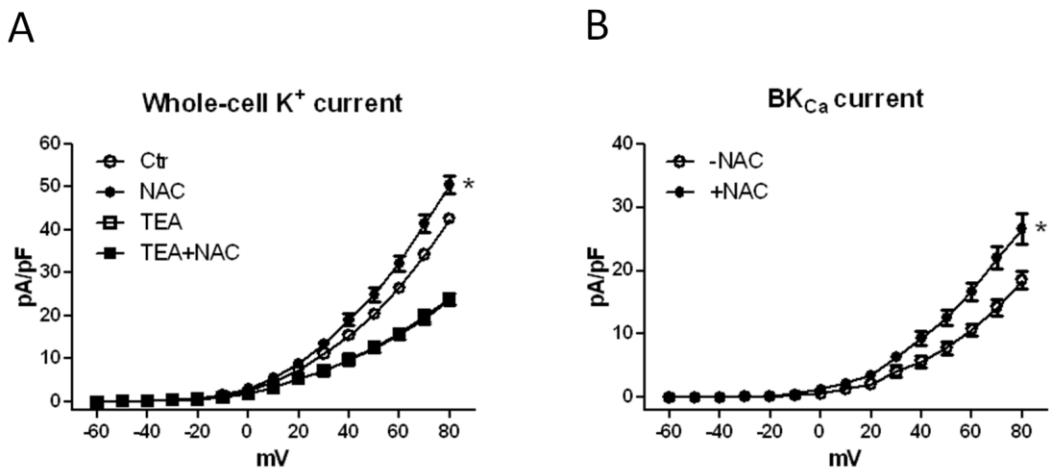

Figure 17. Effect of N-acetylcysteine on K_{Ca} Channel Activities in Myocytes of Uterine Arteries from Hypoxic Pregnant Sheep. **A.** Effect of chronic hypoxia on whole-cell K^+ currents in myocyte isolated from uterine arteries of hypoxic pregnant sheep in the presence of N-acetylcysteine (NAC, 1 mmol/L). Whole-cell K^+ currents were recored in the absence or presence of tetraethylammonium (TEA, 1 mmol/L). *$P<0.05$ vs control (Ctr). **B.** Effect of chronic hypoxia on BK_{Ca} currents in myocytes isolated from uterine arteries of hypoxic pregnant sheep. BK_{Ca} currents were determined as the difference between the whole-cell K+ current in the absence of tetraethylammonium (TEA) and that in the presence of TEA. Mocytes were exposed to N-acetylcysteine 10 min before applying the voltage-step protocol. Data are means ± SEM of cells from 5 animals of each group. *$P<0.05$ vs in the presence of N-acetylcysteine (NAC).

Effect of Pregnancy and Chronic Hypoxia on SK_{Ca} Channel mRNA Levels of Uterine Arteries

Our previous studies have demonstrated that chronic hypoxia during gestation down-regulate both BK_{Ca} and SK_{Ca} channels protein expressions in uterine arteries (Hu et al., 2012; Zhu et al., 2013 a). In this study, we further determined the mRNA levels of SKca channels in uterine arteries. As shown in Fig. 18A, the mRNA levels of SK2 channels in uterine arteries were significantly higher in pregnant animals than those in nonpregnant animals. In addition, chronic hypoxia significantly attenuated the mRNA levels in pregnant but nonpregnant animals. Similarly, the mRNA levels of SK3 channels were also significantly increased in pregnant animals as compared with nonpregnant animals (Fig. 18B), but chronic hypoxia significantly decreased the mRNA levels in both pregnant and nonpregnant animals.

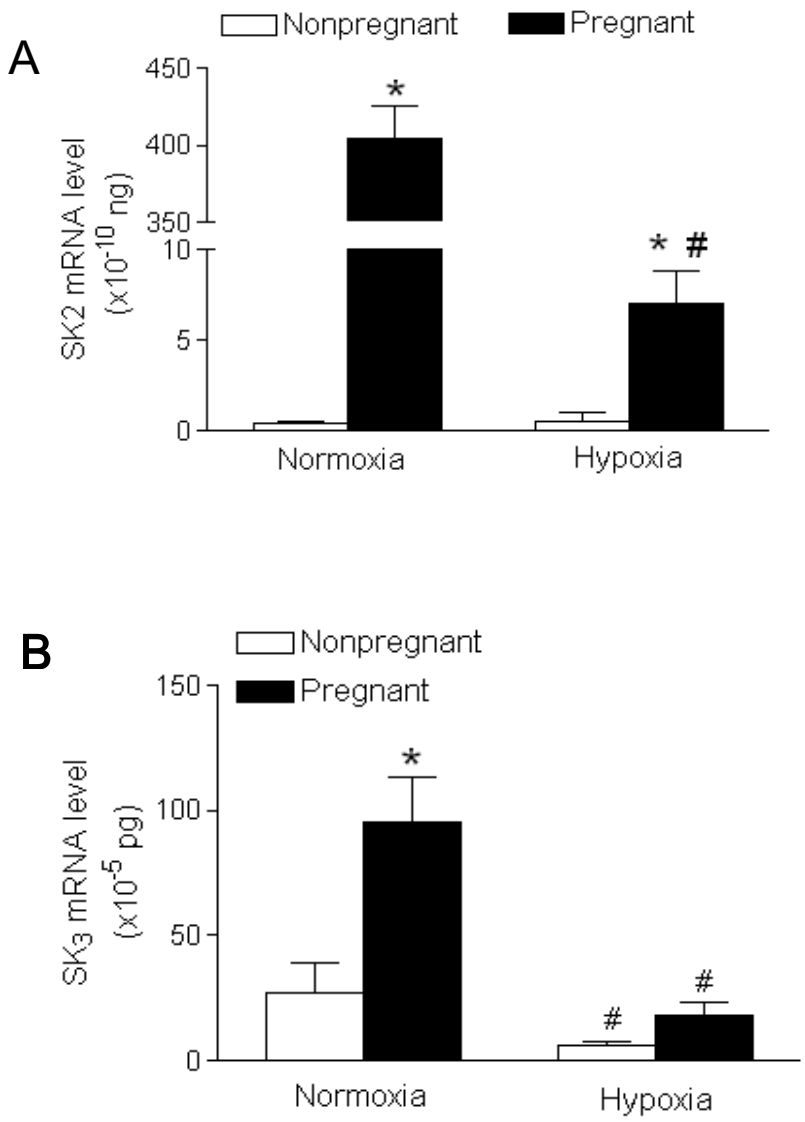

Figure 18. SK_{Ca} Channels mRNA Level in Uterine Arteries. SK_{Ca} channels type 2 and 3 mRNA levels were quantified by RT-PCR, as described in MATERIALS AND METHODS, in freshly isolated uterine arteries from nonpregnant and pregnant sheep in both normoxic and hypoxic groups. The results were calculated from the standard curve of SK2 and SK3 cDNA plasmid run in each assay. Data are means ± SEM of 5 nimals in each groups. *$P<0.05$, pregnant vs. nonpregnat; #$P<0.05$, hypoxia vs. normoxia.

Effect of Sex Steroid Hormones on K_{Ca} Channel-Mediated Relaxation of Uterine Arteries from Normoxic Nonpregnant Sheep

As shown in Figure 19A, NS1619-induced maximal response of relaxations of uterine arteries in normoxic nonpregnant animals (12.67 ± 2.1%) were significantly lower than those in normoxic preganant animals (39.3 ± 3.8 %, $P < 0.05$; Fig. 15A). Similarly, NS309-induced maximal response of relaxations of uterine arteries in normoxic nonpregnant animals (10.35 ± 1.0%; Fig 19B) were significantly lower than those in normoxic preganant animals (58.0 ± 11.2 %, $P < 0.05$; Fig. 15B)

We recently demonstrated upregulation of BK_{Ca} channel activity and expression in uterine arteries during pregnancy was mediated by actions of 17β-estradiol and progesterone (Hu et al., 2011). In this experiment, we further examined the effect of steroid hormones on K_{Ca} channel-mediated uterine arterial function. As shown in Fig. 19A, hormonal treatment significantly enhanced the potency (pD2: 5.4 ± 0.3 versus 7.4 ± 0.4; $P < 0.05$) but not the maximal response (12.67 ± 2.1% versus 18.84 ± 2.42%) of relaxation-induced by NS1619. However, both the potency (5.6 ± 0.2 versus 6.4 ± 0.2; $P < 0.05$) and the maximal response (10.35 ± 1.0% versus 24.18 ± 2.14%) of relaxation-induced by NS309 were significantly increased by the hormonal treatment (Fig. 19B).

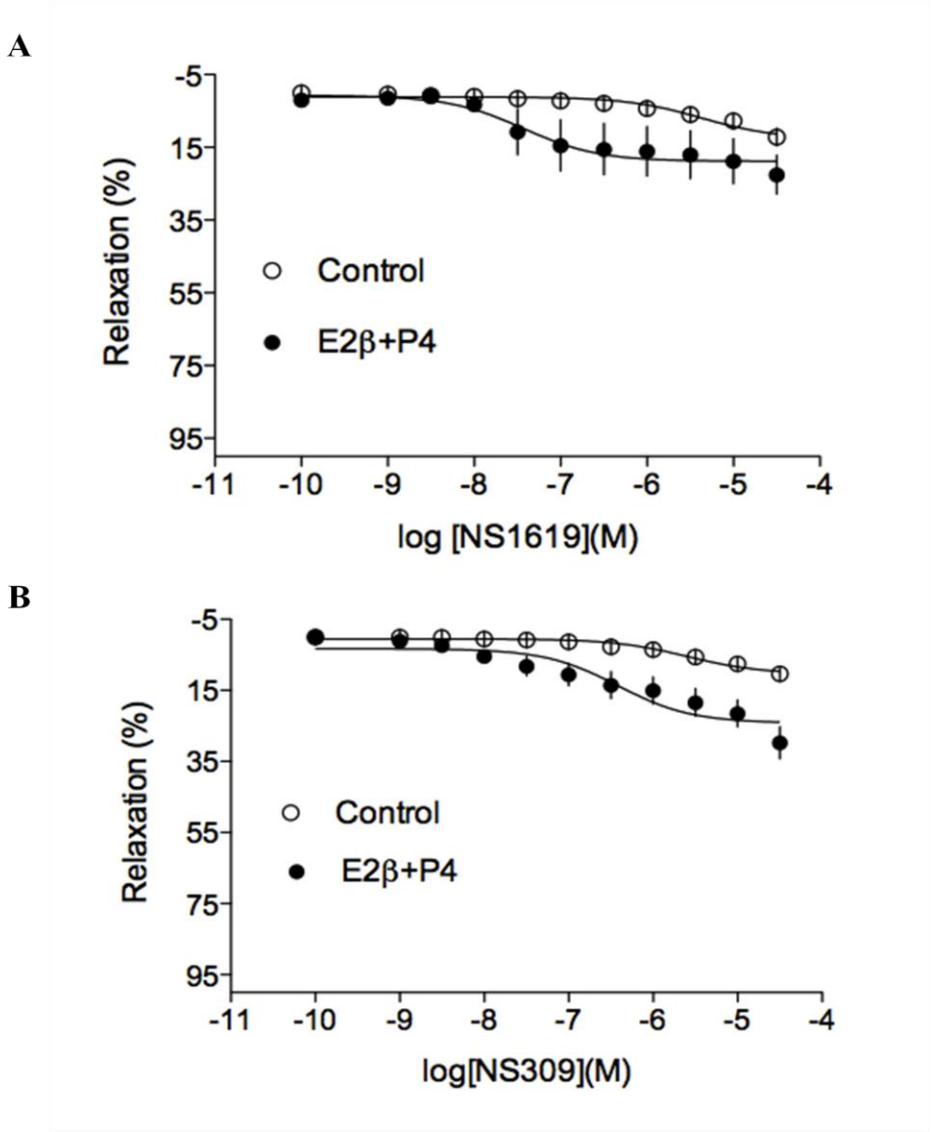

Figure 19. Effect of Ex Vivo Hormonal Treatment on K_{Ca}-Mediated Relaxation in Uterine Arteries from Normoxic Nonpregnant Sheep. Uterine arteries isolated from normoxic nonpregnant sheep were treated ex vivo with 17β-estradiol (E2β; 0.3 nmol/L) plus progesterone (P4; 100.0 nmol/L) under 21% O_2 for 48 hours. Tissues were then contracted with norepinephrine (NE, 1 μmol/L) and followed by additions of NS1619 or NS309. **A.** Concentration-response curves of NS1619-induced relaxation in uterine arteries treated without (Control) or with steroid hormones (E2β+P4). **B.** Concentration-response curves of NS309-induced relaxation in uterine arteries treated without (Control) or with steroid hormones (E2β+P4). Data are means ± SEM from 4 to 6 animals in each group.

Effect of Sex Steroid Hormones on SK_{Ca} Channel Expression in Uterine Arteries

Our recent study revealed that *ex vivo* hormonal treatment of uterine arteries from normoxic nonpregnant sheep resulted in increased expression of BK_{Ca} channel β1 subunit (Hu et al., 2011). To determine whether steroid hormones upregulate expression of SK_{Ca} channel, uterine arteries from normoxic nonpregnant sheep treated *ex vivo* with 17β-estradiol (0.3 nmol/L) and progesterone (100.0 nmol/L) for 48 hours. As shown in Fig. 20, the hormonal treatment significantly increased SK3 channel, but not SK2, protein abundance in uterine arteries.

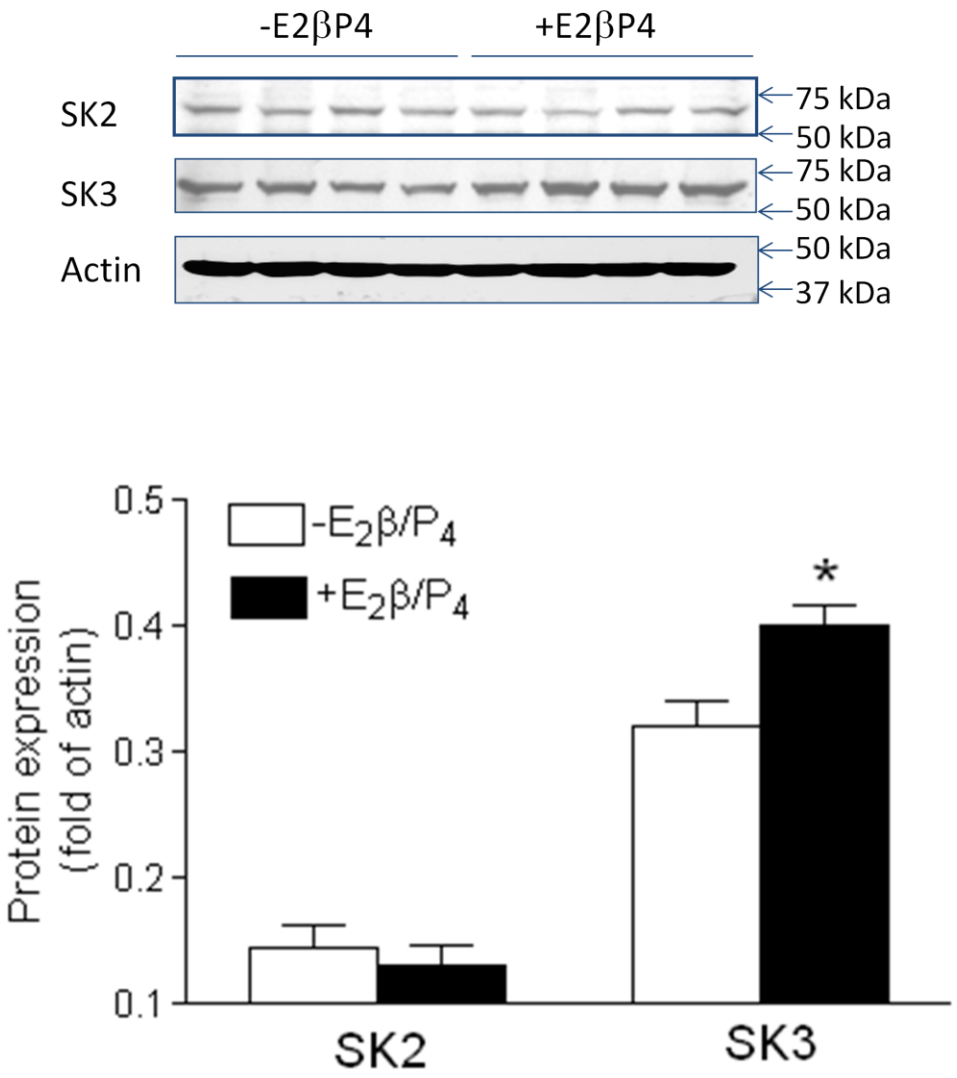

Figure 20. Effect of Sex Steroid Hormones on SK2 and SK3 Protein Expression in Uterine Arteries from Normoxic Nonpregnant Sheep. Uterine artereis from nonpregnant sheep of normoxic animals were treated ex vivo with 17β-estradiol (E2β; 0.3 nmol/L) plus progesterone (P4; 100.0 nmol/L) under 21% O_2 for 48 hours. Protein abundance of SK2 and SK3 were determined by western blot analyses. Data are means ± SEM of tissues from 4 animals of each group, *$P<0.05$, +E2β/P4) vs. -E2β/P4.

Effect of Chronic N-acetylcysteine Treatment on Steroid Hormones-Mediated K_{Ca} Channel-Mediated Relaxation of Uterine Arteries in Hypoxic Nonpregnant Sheep

In contrast to the effect of steroid hormones on Kca-mediated relaxations of uterine arteries in normoxic animals (Fig. 19), hormonal treatment did not alter both NS1619-induced relaxations (Emax: 11.17 ± 1.03 % versus 12.89 ± 1.14 %; $P > 0.05$, Fig. 21A) and NS309-indiced relaxations of uterine arteries (Emax: 11.02 ± 0.54 % versus 10.34 ± 1.4 %; $P > 0.05$, Fig. 21B) in hypoxic animals. However, hormonal treatment in the presence of N-acetylcysteine significantly enhanced both NS1619-induced maximal relaxations (17.17 ± 1.29 % versus 29.98 ± 2.57 %; $P<0.05$, Fig. 21A) and NS309-induced maximal relaxations of uterine arteries (12.7 ± 0.86 % versus 33.10 ± 4.02 %; $P<0.05$, Fig. 21B).

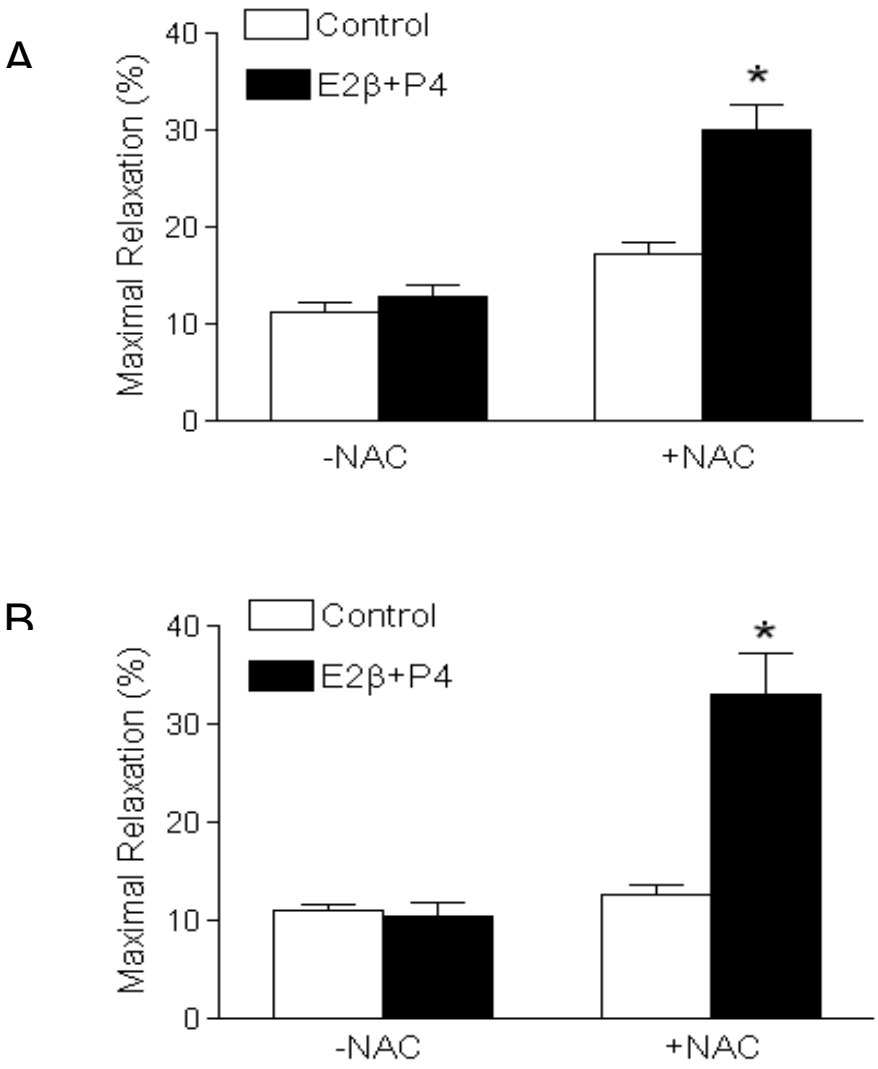

Figure 21. N-acetylcysteine (NAC) Reverses Chronic Hypoxia-Induced Impairment of K_{Ca} Channel-Mediated Relaxation of Uterine Arteries from Nonpregnant Sheep Treated with Steroid Hormones Ex Vivo. Uterine arteries isolated from hypoxic nonpregnant sheep were treated ex vivo with 17β-estradiol (E2β; 0.3 nmol/L) plus progesterone (P4; 100.0 nmol/L) under 10.5% O_2 for 48 hours in the absence and presence of NAC (1 mmol/L). Tissues were then contracted with norepinephrine (NE, 1 mmol/L) and followed by additions of NS1619 or NS309. A. NS1619-induced maximal response of relaxation in uterine arteries. B. NS309-induced maximal response of relaxation in uterine arteries. Data are means ± SEM from 4 to 6 animals in each group. *$P < 0.05$, E2β+P4 vs. control.

Effect of Chronic N-acetylcysteine Treatment on Regulation of K_{Ca} Channel Activities in Uterine Arteries from Hypoxic Nonpregnant Sheep by Steroid Hormones

We also examined K_{Ca} channel activities in uterine arteries from hypoxic nonpregnant sheep treated *ex vivo* with steroid hormones in the presence of N-acetylcysteine for 48 hrs. As shown in Fig. 22, whole-cell K^+ and BK_{Ca} current densities in uterine arteries treated with N-acetylcysteine alone were similar to those observed in arteries treated without N-acetylcysteine (Hu et al., 2012). However, in the presence of N-acetylcysteine, estrogen and progesterone were able to enhance both whole-cell K^+ and BK_{Ca} current densities. Whole-cell K^+ currents in uterine arteries treated with N-acetylcysteine were not sensitive to apamin. Furthermore, enhanced whole-cell K^+ currents by the hormonal treatment in the presence of N-acetylcysteine were also not inhibited by apamin (data not shown).

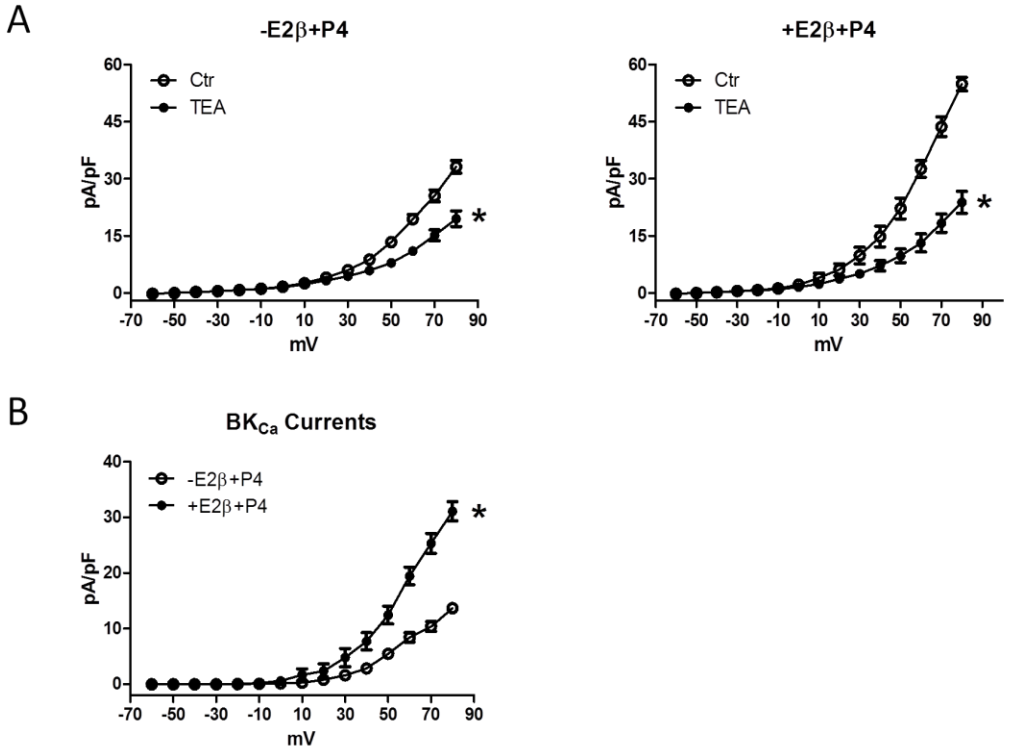

Figure 22. N-acetylcysteine Reverses Chronic Hypoxia-Induced Impairment of K_{Ca} Channel Activities in Myocytes of Uterine Arteries from Hypoxic Nonpregnant Sheep Treated with Steroid Hormones Ex Vivo. Uterine arteries isolated from hypoxic nonpregnant sheep were treated ex vivo with 17β-estradiol (E2β; 0.3 nmol/L) plus progesterone (P4; 100.0 nmol/L) under 10.5% O_2 for 48 hours in the absence and presence of N-acetylcysteine (1 mmol/L). **A.** Effect of N-acetylcysteine on whole-cell K^+ currents in myocytes isolated from uterine arteries of hypoxic nonpregnant sheep ex vivo treated without or with steroid hormones. Whole-cell K^+ currents were recorded in the absence or presence of tetraethylammonium (TEA, 1 mmol/L). **P<0.05* vs control (Ctr). **B.** Effect of N-acetylcysteine on BK_{Ca} currents in myocytes isolated from uterine arteries of hypoxic nonpregnant sheep ex vivo treated without or with steroid hormones. BK_{Ca} currents were determined as the difference between the whole-cell K^+ current in the absence of tetraethylammonium (TEA) and that in the presence of TEA. Data are means ± SEM of cells from 5 animals of each group. **P<0.05*, +E2β/P4 vs −E2β/P4.

Discussion

In the present study, we have demonstrated that the functions of both BK_{Ca} and SK_{Ca} channel in uterine arteries are suppressed by heightened oxidative stress during chronic hypoxia. Our results suggest that chronic hypoxia-induced elevated oxidative stress exerts its adverse impact on K_{Ca} channel through suppressing steroid hormone-induced up-regulation of K_{Ca} channels. Our study provides strong evidence that ROS is a common mediator to impair BK_{Ca} and SK_{Ca} channel function in uterine arteries and contributes to the dysfunction of uterine circulation caused by chronic hypoxia during gestation.

In consistent with our previous studies (Zhu et al., 2013 a), our current finding that both NS1619 and NS309-induced relaxations of uterine arteries were significantly attenuated by chronic hypoxia in pregnant animals, further suggests that chronic hypoxia downregulate both BK_{Ca} and SK_{Ca} channels activities and their gene expressions. In deed, our finding that mRNA levels of SK_{Ca} type 2 and 3 channels in uterine arteries were significantly enhanced by pregnancy, which was attenuated by chronic hypoxia, suggests hypoxia during gestation downregulates SK_{Ca} gene expression. However, the molecular mechanisms underlying chronic hypoxia-mediated downregulation of BK_{Ca} and SK_{Ca}-mediated uterine arterial relaxation remain unclear. The present findings that treatment with NAC, an antioxidant to scavenge free radicals (Sun, 2010), significantly enhanced both NS1619- and NS309-induced relaxations in hypoxic but not normoxic animal, suggest that hypoxia-mediated heightened ROS may be one of the key mechanisms in attenuation of both BK_{Ca} and SK_{Ca}-mediated uterine vascular relaxations during gestation. ROS plays an important role in pathogenesis of cardiovascular dysfunctions

(Wolin et al., 2005; Schnabel and Blankenberg, 2007). ROS has been shown to inhibit BK_{Ca} channel activity (Brakemeier et al., 2003; Liu Y et al., 2002; Soto et al., 2002; Tang et al., 2004). We recently demonstrated that gestational hypoxia increased ROS production in uterine arteries (Xiao et al., 2013). Therefore, heightened ROS likely attribute to the suppressed function of BK_{Ca} and SK_{Ca} channels in uterine arteries from pregnant sheep. In the present study, our finding that NAC could partially reverse the impairment of BK_{Ca} channel activity, suggests that ROS could directly alter BK_{Ca} channel activity, resulting in regulation of BK_{Ca}-mediated uterine vascular contractility in response to hypoxia exposure. These observations are consistent with previous findings that the NADPH oxidase inhibitor apocynin partially reversed the suppression of BK_{Ca} channel function by chronic hypoxia (Xiao et al., 2013). Similarly, impaired BK_{Ca} channel-mediated relaxation of cerebral arteries from insulin resistant rats was restored by superoxide dismutase plus catalase which catalyze the breakdown of free radicals (Erdös B et al., 2004).

A novel and interesting finding in the present study is that the heightened ROS regulate SK_{Ca} channel-mediated uterine vascular contractile function but without alteration of SK_{Ca} channel activity. Our data indicated that hypoxia-mediated impairment of SK_{Ca} channel-mediated relaxation of uterine arteries was alleviated by N-acetylcysteine. However, N-acetylcysteine treatment failed to restore SK_{Ca} channel activity in VSMCs of uterine arteries. It is likely the N-acetylcysteine enhanced SK_{Ca}-mediated relaxation of uterine arteries via an endothelium-dependent mechanism. In deed, SK_{Ca} channels are present in both VSMCs and endothelial cells of uterine arteries (Zhu et al., 2013 a). N-acetylcysteine treatment might increase SK_{Ca} channel activity in

endothelial cells, which in turn could cause vasorelaxation via releasing vasodilators and transmitting hyperpolarization into vascular smooth muscle via myoendothelial gap junctions (Feletou, 2009). Given the fact that the increased blood pressure in the rat model with reduced uterine perfusion pressure was alleviated by N-acetylcysteine (Chang et al., 2005), the up-regulation of K_{Ca} function by N-acetylcysteine may have therapeutic implications.

We have demonstrated that BK_{Ca} channel function can be directly regulated by estrogen and progesterone through a genomic effect (Hu et al., 2011). Similarly, 17β-estradiol and progesterone also selectively up-regulated the expression of SK_{Ca} type 3 channels in uterine arteries. This finding mimicked enhanced SK_{Ca} channel activity seen in pregnant uterine arteries form normoxic animals (Zhu et al., 2013 a). Estrogen receptor-α (ER-α) is the predominant estrogen receptor in the uterine arteries (Chang et al., 2010). Estrogen has been shown to control SK_{Ca} channel expression in human myometrial cells via the specificity protein (Sp) family of transcription factors (Pierce and England, 2010). Hence, the up-regulation of SK_{Ca} channel expression in uterine arteries by steroid hormones likely occurs at genomic level.

In contrast to the upregulation of K_{Ca} channel activities and K_{Ca} channel-mediated relaxation in uterine arteries of normoxic animals by estrogen and progesterone, the effect of steroid hormones on regulation of K_{Ca} channels activities and their-mediated relaxation was diminished in hypoxic animals. These findings suggest that hypoxia-mediated downregulation of K_{Ca} channels activities and their-mediated relaxations may be regulated through steroid hromones-mediated signaling. These observations are not surprising since ER-α receptor is down-regulated during gestational hypoxia via

increased methylation of the receptor gene (Chang et al., 2010; Dasgupta et al., 2012). Down-regulation of ER-α, in turn, suppresses K_{Ca} channel expression, leading to reduced channel activities and relaxation mediated by K_{Ca} channels.

Off most interesting findings that hormonal treatment in the presence of N-acetylcysteine restored K_{Ca} channel-mediated relaxation in uterine arteries from hypoxic animals, suggest that heightened oxidative stress during chronic hypoxia may diminish the ability of steroid hormones to regulate of K_{Ca} channel functions. In deed, ROS has been shown to induce post-translational modifications of ERα, leading to ERα down-regulation in human breast cancer cells (Weitsman et al., 2009). Scavenging free radicals by N-acetylcysteine removed inhibitory effects of ROS, allowing steroid hormones to up-regulated K_{Ca} channel expression and function. Correspondingly, BK_{Ca} channel activity in uterine arterial VSMCs was also restored by N-acetylcysteine after hormonal treatment. Although co-treatment of uterine arteries from hypoxic animals with steroid hormones and NAC failed to up-regulate SK_{Ca} channel activity in VSMCs, it could up-regulate SK_{Ca} channel function in endothelial cells, which in turn results in enhanced SK_{Ca} channel-mediated relaxation via releasing vasodilators and hyperpolarizing VSMCs through myoendothelial gap junctions as aforementioned. Indeed, it has been shown that N-acetylcysteine supplement improves human coronary and peripheral endothelium-dependent vasodilation (Andrews et al., 2001).

In conclusion, heightened ROS-induced by chronic hypoxia attenuated steroid hormones-mediated signaling, which leads to downregulation of K_{Ca} channels activities and their gene expression and results in decreased relaxations of uterine arteries during gestation. The attenuation of K_{Ca} channels-mediated relaxation may contribute to the

enhanced uterine vascular tone and increased incidence of preeclampsia and fetal intrauterine growth restriction associated with maternal hypoxia.

Acknowledgements

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[2] **Disclosures:** None

CHAPTER FIVE

DISCUSSION

Discussion

Our recent studies suggested that upregulation of BK_{Ca} channel expression and activities attributed to attenuated myogenic tone of uterine arteries in pregnancy, and chronic hypoxia inhibited the steroid hormone-mediated upregulation of the β1 subunit and BK_{Ca} channel activity in uterine arteries (Hu et al., 2011; Hu et al., 2012). However, the role of SK_{Ca} in the regulation of uterine vascular reactivity under physiological and pathophysiological conditions such as pregnancy and chronic hypoxia is unclear. My research project has provided the evidence of K_{Ca} channels in uterine vascular adaptation to pregnancy and in response to chronic hypoxia during gestation and the mechanism associated with sex steroid hormone regulation.

In chapter 2, our focuse was to determine if SK_{Ca} channel expression and function in uterine arteries have been changed in response to pregnancy and hypoxia. We demonstrated that pregnancy upregulated SK_{Ca} channel expression and function. However, the mechanisms are unknown. Based on findings that activation of estrogen receptoe may alter gene transcription (Murphy, 2011), and pregnancy up-regulates the expression of estrogen receptor α and β in uterine arteries (Byers et al., 2005; Chang et al., 2010), as well as the findings that 17β-estradiol-mediates an increase in expression and heightened activity of BK_{Ca} channels in uterine arteries of pregnant sheep (Hu et al., 2011). We proposed that sex steroid hormones may contribute to the regulation of SK_{Ca}

channel activity in the uterine artery. Our studies in chapter 4 have supported this hypothesis. In Chapter 4, we demonstrated that 17β-estradiol and progesterone also upregulated the expression of SK_{Ca} channel in uterine arteries. Previous studies have shown that estrogen receptor-α (ER-α) is the predominant estrogen receptor in uterine arteries (Chang et al., 2010). Estrogen was shown to control SK_{Ca} channel expression in human myometrial cells via the specificity protein (Sp) family of transcription factors (Pierce and England, 2010). Therefore, the upregulation of SK_{Ca} channel expression in uterine arteries by steroid hormones is likely to occur at the genomic level. Furthermore, in chapter 4, we also found that hormonal treatment ex vivo under 10.5% O2 failed to upregulation of K_{Ca} channel activities and K_{Ca} channel-mediated relaxation in uterine arteries of hypoxic animals. This finding suggests that chronic hypoxia inhibits the ability of 17β-estradiol and progesterone to modulate K_{Ca} channel function. The explaination of this finding may be that ER-α is down-regulated during gestational hypoxia due to increased methylation of the receptor gene (Chang et al., 2010; Dasgupta et al., 2012). Down-regulation of ER-α may in turn suppress K_{Ca} channel expression, leading to reduced channel activities and relaxation mediated by K_{Ca} channels.

In chapter 2, we demonstrated the expression of SK2 and SK3 channels in both vascular smooth muscle and endothelial cells in uterine arteries. This is in agreement with the previous findings in other visceral and vascular smooth muscle cells by immunohistochemistry (Chen et al., 2004; Potocnik et al., 2009; Sorensen et al., 2011). The functional role of SK_{Ca} channels in vascular smooth muscle was comfirmed with our electrophysiological and contractility studies. Previous studies showed the expression and function of SK_{Ca} channels in endothelial cells (Brahler et al., 2009; Taylor et al.,

2003). The chapter 2 is the first to demonstrate that the vascular tone and contractility may also be regulated by SK_{Ca} channels in smooth muscle cells. Hence, the present study provides a novel mechanism of SK_{Ca} channels in regulating vascular tone and function.

Similar to previous findings on BK_{Ca} channels in uterine arteries of pregnant sheep (Hu et al., 2012) and IK_{Ca} channels in pulmonary arteries from animals exposed to chronic hypoxia (Kroigaard et al., 2013), chapter 2 demonstrated that chronic hypoxia impaired the regulatory role of SK_{Ca} channels in vascular smooth muscle excitability and contractility. This impairment was due to decreased expression and reduced channel activities of these channels. Our previous studies have shown that voltage-gated K^+ (Kv) channels were largely unaffected by chronic hypoxia, and suggested that chronic hypoxia may be specific for K_{Ca} channels in the uterine artery (Hu et al., 2012). These findings provide evidence of K_{Ca} channels attributing to changes in uterine vascular function by chronic hypoxia and provide a possible explanlation for increased incidence of preeclampsia and fetal intrauterine growth restriction under chronic hypoxia exposure during pregnancy. The mechanism of chronic hypoxia in regulating K_{Ca} function is unknown. Our previous study demonstrated that chronic hypoxia changed the expression of estrogen receptor α in uterine arteries during gestation, without affecting the plasma estrogen levels (Chang et al., 2010; Dasgupta et al., 2012). Expression of BK_{Ca} (Hu et al., 2011; Nishimura et al., 2008) and SK_{Ca} (Jacobson et al., 2003; Pierce and England, 2010) are changed by hormone treatment. Hence, estrogen plays an important role in regulating the adaptation of K_{Ca} channel function in the uterine artery during pregnancy and chronic hypoxia. Our studies in chapter 4 were based on this hypothesis.

In chapter 3 we demonstrated that inhibitory effect of BK_{Ca} but not SK_{Ca} channels on PKC-mediated contractions of uterine arteries and this inhibitory effect was impaired by chronic hypoxia during pregnancy, suggesting that chronic hypoxia-mediated increase in PKC-induced myogenic contractions of uterine arteris in pregnancy (Chang et al., 2009; Xiao et al., 2012) by the modulation of BK_{Ca} channel activity. Gene expression and BK_{Ca} channel activity studies support this conculation (Hu et al., 2012). In addition, this chapter demonstrated that chronic hypoxia-mediated effect was pregnancy-dependent. Taken together with previous findings that BK_{Ca} channel expression and function were regulated by 17β－estradiol (Hu et al., 2012), these studies provide new insights of mechanisms of chronic hypoxia-mediated vascular tone by estrogen in chapter 4. On the other hand in chapter 3, an inhibitory effect of PKC on activated K_{Ca} channel-mediated relaxations and channel activities of the uterine arteries in pregnant animals were induced by chronic hypoxia. Although the mechanism is unknow, it may be associated with the finding that the activity of PKC isozyme PKCε is selectively increased by chronic hypoxia (Chang et al., 2009). Without suprise, activation of PKCε inhibited K_{Ca} channel activity by stimulation of L-type Ca^{2+} channel via c-SRC (Alioua et al., 2002).

In chapter 4, we demonstrated two mechanisms in the changes of K_{Ca} expression and function in response to pregnancy and chronic hypoxia including direct inhibition of channel activity and indirect inhibition of channel activities via suppressing steroid hormone-induced up-regulation of K_{Ca} channels. This chapter provides evidence that ROS impair BK_{Ca} and SK_{Ca} channel function in uterine arteries contributing to the maladpatation of uteroplacental circulation caused by chronic hypoxia during pregnancy.

Consistent with chapter 2 that BK_{Ca} and SK_{Ca} function and protein expression have enhanced by pregnancy and chronic hypoxia suppressed this enchancement, chapter 4 has shown that mRNA levels of type 2 and type 3 in uterine arteries are significantly increased by pregnancy, which is decreased by chronic hypoxia. The mechanisms may be complex. Our finding that ROS inhibitor N-acetylcysteine siginificantly increased chronic hypoxia-mediated NS1619- and NS309-induced relaxation in high altitude hypoxia animals, suggests that the mechanism of decreasing BK_{Ca} and SK_{Ca}-mediated uterine vascular relaxation during pregnancy may be associated with enhanced ROS production caused by chronic hypoxia. This is consistent with the previous study showing that BK_{Ca} channel activity was inhibited by ROS (Brakemeier et al., 2003; Liu Y et al., 2002; Soto et al., 2002; Tang et al., 2004). Consistent with our recently findings that apocynin partially reversed the impaired BK_{Ca} channel function by chronic hypoxia (Xiao et al., 2013), this project demonstrated that N-acetylcysteine altered BK_{Ca} channel activity, attributing to BK_{Ca}-mediated vascular contractility in response to hypoxia.

The most interesting finding in chapter 4 is that the impaired K_{Ca} channel-induced relaxation in uterine arteries of hypoxic animals was restored by hormonal treatment in the presence of N-acetylcysteine, providing evidence that the regulatory role of steroid hormones on K_{Ca} channel functions was impaired by heightened oxidative stress during chronic hypoxia. Consistent with the contractility study, the electrophysiological study in the present project showed that BK_{Ca} channel activity in uterine arterial VSMCs was restored by N-acetylcysteine after hormonal treatment. Surprisingly, restoration of SK_{Ca} channel activity in VSMCs has not been found in hypoxic animals in the same treatment. One explaination is that this treatment upregulates SK_{Ca} channel function in endothelial

cells, which releasing vasodilators and hyperpolarizing VSMCs through myoendothelial gap junctions to enhance SK_{Ca} channel-mediated relaxation. This is consistent with previous finding of vascular endothelium-dependent vasodilation was increased by N-acetylcysteine supplement (Andrews et al., 2001).

Conclusions and Future Directions

The present and previous studies (Hu et al., 2011; Hu et al., 2012) have demonstrated that BK_{Ca} and SK_{Ca} channels are expressed in uterine arterial smooth muscle cells and playing an important role in the regulation of vascular contractility and basal tone. Consistent with these studies, the present studies demonstrated that pregnancy significantly upregulated BK_{Ca} and SK_{Ca} channel expression by selectively targeting on BK_{Ca} β1 subunits and SK2 and SK3 channels. In addition, BK_{Ca} and SK_{Ca} channel activities and these channel-mediated relaxations were enhanced by pregnancy, and myogenic tone of uterine arteries was decreased. In constrast, chronic hypoxia during gestation decreased BK_{Ca} and SK_{Ca} channel expression and impaired their function in the regulation of contractility and myogenic tone. Hence, these studies demonstrated the role of K_{Ca} channels in uterine vascular adaptation to pregnancy and chronic hypoxia. Moreover, we demonstrated that pregnancy and chronic hypoxia differentially regulated the interaction of protein kinase C and calcium-activated potassium channels in modulating uterine arterial contractility. Futhermore, steroid hormones play an important role in the hemodynamic adaption by upregulating K_{Ca} channel, causing decreased vasculat tone. Our present project demonstrated that chronic hypoxia inhibited this adaptive changes in uterine arteries of pregnant sheep via increased ROS production. This study provides new insights of mechanisms in the dysfunction of uterine circulation

caused by chronic hypoxia during gestation. In general, the present project may attibut to improving our understanding of the pathophysiological mechanisms underlying the maladaptation of uteroplacental circulation and pregnancy complications including preeclampsia and fetal growth restriction associated with chronic hypoxia during gestation.

Our project determined a reduction in SK2 and SK3 mRNA and protein abundance in the uterine artery of high altitude chronic hypoxic animals, providing new insights of future research in further investigation of possible epigenetic mechanisms involved in uteroplacental circulation adaption mechanims. This will provide further evidence that chronic hypoxia-mediated adaptation may encompasse epigenomic levels.

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