Regulation of alpha-1-adrenoceptor-mediated contractions of the uterine artery by PKC

Hongying Zhang

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Regulation of alpha₁-adrenoceptor-mediated contractions of the uterine artery by PKC

by

Hongying Zhang

A Dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Pharmacology

May 2007
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**LIST OF ABBREVIATIONS**

- $[\text{Ca}^{2+}]_{i}$: Intracellular calcium concentration
- CaD: Caldesmon
- p-CaD: Phosphorylation of Caldesmon
- CaP: Calponin
- p-CPI-17: Phosphorylation of CPI-17
- DAG: Diacylglycerol
- ERK: Extracellular signal-regulated kinase
- p-ERK: Phosphorylation of ERK
- IP$_3$: Inositol 1, 4, 5-trisphosphate
- LC$_{20}$: 20-kDa light chain of myosin
- p-LC$_{20}$: Phosphorylation of LC$_{20}$
- MAPK: Mitogen-activated protein kinase
- MEK: MAPK kinase
- MLCK: Myosin light chain kinase
- MLCP: Myosin light chain phosphatase
- NO: Nitric oxide
- PD098059: (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one)
- PDBu: Phorbol 12,13-dibutyrate
- PE: Phenylephrine
- PKC: Protein kinase C
- R$_{f340/380}$: Ratio of fluorescence intensity at wavelength 340 and 380 nm
- TCA: Trichloroacetic acid
ABSTRACT OF THE DISSERTATION

Regulation of alpha₁-adrenoceptor-mediated contractions of the uterine artery by PKC

by

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Doctor of Philosophy, Graduate Program in Pharmacology
Loma Linda University School of Medicine, May 2007
Dr. Lubo Zhang, Chairperson

Pregnancy is associated with a significant decrease in uterine vascular tone and a striking increase in uterine blood flow, which ensures normal fetal development. Multiple mechanisms are involved in the adaptation of uterine artery contractility during pregnancy. Both protein kinase C (PKC) and activation of α₁-adrenoceptors play important roles in the regulation of uterine artery contractility. In addition, PKC mediates the pregnancy-associated decrease in myogenic tone of the uterine artery, and interacts with α₁-adrenoceptors leading to modulation of α₁-adrenoceptor-mediated contractions of vascular smooth muscle. Yet, whether or to what extent PKC regulates α₁-adrenoceptor-mediated contractions of the uterine artery and their adaptation to pregnancy remains unclear. The central hypothesis of the proposed studies is that PKC activation differentially regulates α₁-adrenoceptor-mediated Ca²⁺ mobilization and sensitization in pregnant and nonpregnant uterine arteries. To test this hypothesis, we proposed a series of experiments in the uterine arteries obtained from nonpregnant and near-term (~140 Days) pregnant sheep.
We found that PKC activation has opposite effects on \(\alpha_1\)-adrenoceptor-mediated contractions in pregnant and nonpregnant uterine arteries. In pregnant uterine arteries, PKC activation inhibited \(\alpha_1\)-adrenoceptor-mediated contractions through suppressing \(Ca^{2+}\)-dependent thick-filament regulatory pathway with decreased \(Ca^{2+}\) mobilization and phosphorylation of 20-kDa regulatory light chain of myosin (\(LC_{20}\)). This inhibitory effect of PKC activation was reversed by PKC isozyme-selective inhibitory peptides for PKC\(\beta I\), \(\beta II\) and \(\epsilon\). In contrast, in nonpregnant uterine arteries PKC activation potentiated \(\alpha_1\)-adrenoceptor-mediated contractions through enhancing thin-filament regulatory pathway with increased \(Ca^{2+}\) sensitivity and phosphorylation of extracellular signal-regulated kinase\(_{44/42}\) (ERK\(_{44/42}\)) and Caldesmon-Ser\(^{789}\) without changing the phosphorylation level of \(LC_{20}\). This potentiating effect of PKC activation was reversed by PKC isozyme-selective inhibitory peptides for conventional PKCs, PKC\(\beta I\), \(\beta II\) and \(\epsilon\).

The differential regulation of PKC isozyme expression, basal activity and reactivity, with the up-regulation of PKC\(\beta\) and PKC\(\zeta\), and the down-regulation of PKC\(\alpha\) and PKC\(\epsilon\), is likely to play an important role in the regulation of \(Ca^{2+}\) mobilization and \(Ca^{2+}\) sensitivity in \(\alpha_1\)-adrenoceptor-mediated contractions and their adaptation to pregnancy.
CHAPTER ONE
GENERAL INTRODUCTION

Introduction

Physiological Changes of Uterine Artery during Pregnancy

During pregnancy there are dramatically physiological changes in the maternal cardiovascular system to ensure normal fetal development. The striking alterations in arterial size and function during pregnancy are very unique to the uterine artery. As pregnancy progresses, the uterine blood flow increases around 20 folds in human and ovine (Palmer et al., 1992; Rosenfeld, 1977). In late pregnancy, the uterine circulation carries about one fifth of the maternal cardiac output, with around 80% of the uterine blood flow going to the placenta. The uterine circulation during pregnancy functions as a low-resistance shunt to accommodate the large increase of uteroplacental blood flow, required for normal fetal development. The decreased uterine artery resistance is accomplished by growth and remodeling of vessels, increased endothelial nitric oxide (NO) release, decreased myogenic response, and a reversible sympathetic denervation of the uterine artery.

The marked changes of the uterine artery structure during pregnancy accommodate the substantial increases in uterine blood flow. Pregnancy-related increase in diameter of uterine artery was found in human (Annibale et al., 1990; Palmer et al., 1992), pig (Guenther et al., 1988) and sheep (Fuller et al., 1975). The uterine artery in late-term pregnant sheep significantly enlarged to 5.0 mm or greater in diameter, compared with 2.8 mm in nonpregnant sheep (Fuller et al., 1975). In addition to dilation of vessel, the increase in arterial diameter during pregnancy accompanies with a 2.5-fold increase in
smooth muscle mass per unit length of vessel, in spite of no change in arterial wall thickness (Griendling et al., 1985). Growth in cell length also contributes to the increase in uterine arterial diameter seen in pregnancy, although the number of cells per unit area and the diameter of the cells are unchanged (Annibale et al., 1990). Meanwhile, the biochemical changes in the uterine artery during pregnancy have also been detected. Pregnancy significantly increased protein contents of the uterine arteries from sheep but did not affect the fractional cellular composition of the muscularis, compared with nonpregnant sheep (Annibale et al., 1990). The increase in contractile protein contents might contribute to the increases in stress of uterine arterial smooth muscle associated with pregnancy.

In addition to the growth of new vessels and remodeling of existing vessels during early pregnancy, the increasing nutrient demands of the fetus over the latter part of gestation are met predominantly through decreasing uterine arterial vascular smooth muscle tone, resulting in progressive increases in arterial diameter and uterine blood flow (Rosenfeld, 1984). Despite the extensive studies, the mechanisms of pregnancy-induced decrease in uterine arterial vascular tone are not fully understood. Previous studies have been focused on the adaptation of endothelium to pregnancy and indicated that increased endothelial nitric oxide synthase (eNOS) expression (protein and/or mRNA) and increased NO synthesis/release play important roles in vasodilation of the uterine artery during pregnancy (Kanashiro et al., 2000; Magness et al., 1996 and 1997; Li et al., 1996; Sladek et al., 1997; Weiner et al., 1991; Xiao et al., 2001a and 2001b). The adaptation of contractile mechanisms of uterine arterial smooth muscle to pregnancy is less clear. Studies in Dr. Lubo Zhang’s laboratory have recently demonstrated in ovine uterine
arteries that protein kinase C (PKC) plays an important role in the regulation of uterine artery contractility, and the pregnancy-associated decrease in myogenic tone of the uterine artery is primarily regulated through the PKC signaling pathway (Xiao and Zhang, 2002; Xiao et al., 2004 and 2006). In addition to its regulatory effect of myogenic tone (Davis and Hill, 1999; Xiao et al., 2006), PKC interacts with \( \alpha_1 \)-adrenoceptors and modulates \( \alpha_1 \)-adrenoceptor-mediated contractions of vascular smooth muscle (Ford, 1995; Minneman, 1988; Nishizuka, 1992). Activation of \( \alpha_1 \)-adrenoceptors plays a key role in the regulation of uterine artery smooth muscle contractions (Cox et al., 2004; Ford, 1995; Magness and Rosenfeld, 1986; Stjernquist and Owman., 1990; Zhang et al., 1995). Yet, whether or to what extent PKC regulates \( \alpha_1 \)-adrenoceptor-mediated contractions of the uterine artery and their adaptation to pregnancy remains unclear.

The Role of PKC in the Regulation of Smooth Muscle Contraction

PKC is a key enzyme involved in the regulation of diverse cellular processes such as growth, differentiation, metabolism, secretion, and smooth muscle contraction (Horowitz et al., 1996b; Nishizuka, 1992; Walsh et al., 1996). There are at least 11 PKC isozymes (PKCs), divided into three major groups: 1) classical (conventional) PKCs: \( \alpha \), \( \beta I \), \( \beta II \), and \( \gamma \), which are activated by both diacylglycerol (DAG) and \( \text{Ca}^{2+} \); 2) novel PKCs: \( \delta \), \( \varepsilon \), \( \eta \), and \( \theta \), which are activated by DAG but not \( \text{Ca}^{2+} \); 3) atypical PKCs: \( \zeta \), \( \iota \), and \( \lambda \), which are activated by neither DAG nor \( \text{Ca}^{2+} \). The molecular structures of PKC isozymes are shown in Fig. 1. Phosphatidylserine (PS), DAG and phorbol esters bind to their cysteine-rich C1 domains to activate classical and novel PKCs. C2 domain in classical PKCs is \( \text{Ca}^{2+} \) binding site that does not exist in novelor atypical PKCs. All three
groups of PKCs contain a pseudo-substrate or autoinhibitory domain that binds to the substrate-binding site in the catalytic domain preventing their activation in the absence of cofactors or activators. Phosphorylation of PKCs by themselves and PKC kinase(s) are required for their full activity. Phosphorylation also regulates PKC subcellular distribution and down-regulation. The \( \alpha, \beta I, \beta II, \delta, \epsilon, \) and \( \zeta \) isoymes of PKCs have been detected in vascular smooth muscle (Walsh et al., 1996). PKCs play an important role in regulation of smooth muscle contraction. It has been demonstrated that PKC activation induces slow sustained contractions in many types of vascular smooth muscle (Jiang MJ, 1989; Newton AC, 1995; Singer HA, 1990) and involves in regulation of myogenic tone (Davis and Hill, 1999; Xiao et al., 2006). In addition, activation of PKC by phorbol esters, synthetic analogs of DAG, has been shown to couple to \( \alpha_1 \)-adrenoceptor and regulate \( \alpha_1 \)-adrenoceptor-mediated contractions in vascular smooth muscle. Both PKC-mediated inhibition and potentiation of \( \alpha_1 \)-adrenoceptor-mediated contractions have been reported. Thus, activation of PKC by phorbol esters inhibited norepinephrine-induced contractions in rat aorta, cat and sheep cerebral arteries (Bazan et al., 1993; Cotecchia et al., 1985; Danthuluri and Deth, 1984; Leeb-Lundberg et al., 1985; Longo et al., 2000; McMillan et al., 1986; Salaices et al., 1990). On the other hand, activation of PKC potentiated \( \alpha_1 \)-adrenoceptor-mediated contractions in rabbit aorta, rat mesenteric arteries, femoral arteries, corpora cavernosa, and vas deferens (Amobi et al., 1999; Henrion et al., 1992; Husain et al., 2004; Martinez et al., 2001; Matsumura et al., 2001). The effect of potentiation was abolished with depleting PKC after prolonged treatment with
Fig. 1. PKC kinase family members and their structure. The conventional groups have four conserved regions (C1-C4) and five variable regions (V1-V5). C1 (C1a and C1b): DAG and phorbol-ester binding sites. C2: Ca2+ binding site. C3: ATP binding site. C4: PKC substrate binding sites.
phorbol esters (Henrion et al., 1992). The controversial effects of PKC on $\alpha_1$-adrenoceptor-mediated contractions in different arteries from different species may be due in part to the diversity of PKC isozymes, which have different enzymatic properties, substrates, functions, and different sub-cellular distributions in different blood vessels and species (Horowitz et al., 1996b; Kanashiro and Khalil, 1998 & 2001, Khalil et al., 1992; Liou and Morgan, 1994; Nishizuka Y, 1992).

**Alpha$_1$-adrenoceptor-mediated Contraction of Vascular Smooth Muscle**

Activation of $\alpha_1$-adrenoceptors plays a key role in the regulation of uterine artery smooth muscle contractions (Cox et al., 2004; Ford, 1995; Magness and Rosenfeld, 1986; Stjernquist and Owman., 1990; Zhang et al., 1995). During pregnancy, uterine artery increases acute contractile response to nonsynaptic $\alpha_1$-adrenergic stimulation (Annibale et al., 1989 & 1990; D’Angelo and Osol, 1993 & 1994; Osol and Cipolla, 1993), in spite of the vascular tone is decreased. This paradoxical event is at least partly due to the transient and reversible sympathetic denervation of uterine artery during pregnancy (Klukovits et al., 2002; Naves et al., 1998). As shown in Fig. 2, activation of $\alpha_1$-adrenoceptors leads to hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP$_2$) to inositol 1, 4, 5-trisphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ binds to IP$_3$ receptors and stimulates $Ca^{2+}$ release from intracellular calcium stores, resulting in phosphorylation of 20-kDa regulatory light chain of myosin (LC$_{20}$) and contractions. LC$_{20}$ phosphorylation is regulated through thick-filament regulatory pathway by myosin light chain kinase (MLCK) and myosin light chain phosphotase (MLCP) (Wier and Morgan, 2003). MLCK activation by increased intracellular free $Ca^{2+}$ concentration ([Ca$^{2+}$]) and $Ca^{2+}$/calmodulin
binding leads to an increase in phosphorylated LC$_{20}$ (p-LC$_{20}$) and contraction. Inhibition of MLCP activity increases Ca$^{2+}$ sensitivity of contraction through decreasing dephosphorylation of p-LC$_{20}$. On the other hand, DAG activates PKC that has been suggested to play a key role in $\alpha_1$-adrenoceptor-mediated contraction by regulating Ca$^{2+}$ sensitivity (Buus et al., 1998; Gailly et al., 1997; Kitazawa et al., 2000; Martinez et al., 2000; Nishimura et al., 1990; Sato et al., 2001).
Fig. 2. Signal transduction pathway of $\alpha_1$-adrenoceptor-mediated contraction in vascular smooth muscle cell.
Contractile Mechanisms in Vascular Smooth Muscle

Smooth muscle contraction is regulated through changes in intracellular Ca\(^{2+}\) mobilization and the Ca\(^{2+}\) sensitivity of the contractile apparatus. An increase in [Ca\(^{2+}\)]\(_i\), leads to activation of MLCK and subsequent phosphorylation of LC\(_{20}\) and contraction (Horowitz et al., 1996; Pfitzer, 2001), a mechanism termed Ca\(^{2+}\)-dependent thick-filament regulation. The Ca\(^{2+}\) sensitivity, defined by force production per unit changes in [Ca\(^{2+}\)]\(_i\), is another important mechanism to regulate vascular contractility. Regulation of the Ca\(^{2+}\) sensitivity of myofilaments is through both Ca\(^{2+}\)-independent thick-filament regulatory pathway that involves inactivation of MLCP resulting in an increase in LC\(_{20}\) phosphorylation independent of changes in [Ca\(^{2+}\)]\(_i\) (Somlyo and Somlyo, 2003), and the thin-filament regulatory pathway that leads to an increase in contraction independent of changes in LC\(_{20}\) phosphorylation (Morgan and Gangopadhyay, 2001).

PKC has a dual role in regulation of α\(_1\)-adrenoceptor-mediated Ca\(^{2+}\) mobilization and Ca\(^{2+}\) sensitization. It is well known that α\(_1\)-adrenoceptor induces Ca\(^{2+}\) mobilization in smooth muscle mainly through production of IP\(_3\) leading to Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores (Garcia-Sainz et al., 2000; Suzuki et al., 1990). PKC has been suggested to desensitize α\(_1\)-adrenoceptor resulting in decreases in α\(_1\)-adrenoceptor-mediated PIP\(_2\) hydrolysis, IP\(_3\) production and Ca\(^{2+}\) mobilization (Garcia-Sainz et al., 2000; Leeb-lundberg et al., 1985 and 1987; Lynch et al., 1985; Schimmel et al., 1986; Suzuki et al., 1990; Vazquez-Prado et al., 1996). PKC regulates Ca\(^{2+}\) sensitivity of myofilaments through Ca\(^{2+}\)-independent thick-filament regulatory pathway. CPI-17 is a smooth muscle-specific protein and phosphorylated CPI-17 at Thr-38 (p-CPI-17/Thr\(^{38}\)) becomes an inhibitor of the catalytic activity of MLCP (Wier and Morgan, 2003). PKC
can phosphorylate CPI-17 and enhance its inhibitory potency 1000-fold (Kitazawa et al., 2000). In the meantime, ratio of expression of CPI-17 to MLCP correlates with PKC-induced Ca\(^{2+}\) sensitization (Wier and Morgan, 2003; Woodsome et al., 2001). In addition to the thick filament regulation, PKC also regulates Ca\(^{2+}\) sensitivity through the thin filament mechanisms. It has been suggested that PKC mediates contraction in the uterine artery predominantly through the thin filament regulatory pathway (Xiao et al., 2003).

**Thin Filament Regulation of Smooth Muscle Contraction and the Major Thin Filament-associated Proteins**

Thin filaments are defined as those filaments 6-8 nm in diameter and composed of filamentous actin. The thin filament signal transduction pathway is regulated through both Ca\(^{2+}\) and LC\(_{20}\) phosphorylation independent mechanisms. The existence of thin filament regulation is supported by the following evidences: 1) LC\(_{20}\) phosphorylation levels (myosin or thick filament regulation) do not parallel with tension levels (Dillon PF, 1981); 2) crossbridge cycling rates vary without detectable changes in LC\(_{20}\) phosphorylation (Butler et al., 1986; Haeberle, et al., 1985); 3) contraction may occur without changes in LC\(_{20}\) phosphorylation (Menice et al., 1997). Thin filament regulatory proteins include caldesmon and calponin.

**Caldesmon**

Caldesmon (CaD) is a thin filament-associated, actin, tropomyosin, myosin and calmodulin (CaM) binding protein and plays an important role in thin-filament regulation. The molecular structure of CaD is shown in Fig. 3. Carboxy terminal
Fig. 3. Domain structure of caldesmon, modified from Lee et al. (J Biol Chem 275:3213-20, 2000) Numbering is according to the chicken gizzard sequence, except that the analogous mammalian Extracellular regulated kinase (ERK) phosphorylation site (759) is given after the ERK gizzard phosphorylation site and the peptide IK29C is from the human sequence. CaM, calmodulin. Tm, tropomyosin; PAK, p21-activated kinase.
domains are responsible for actin binding and inhibition of myosin ATPase activity. The N-terminal half of the molecule has been shown to bind myosin. CaD acts as a cross-linker of actin and myosin. CaD plays an important inhibitory role in the regulation of smooth muscle tone through inhibiting myosin ATPase activity (Katsuyama et al., 1992; Morgan and Gangopadhyay, 2001; Wier and Morgan, 2003; Word et al., 1993). CaD’s endogenous inhibitory effect on myosin ATPase is regulated by the binding of Ca$^{2+}$-CaM and/or the phosphorylation of its C-terminal actin binding domains (Morgan and Gangopadhyay, 2001). It is known that CaD is phosphorylated in vitro by a number of protein kinases including CaM kinase II, casein kinase II, protein kinase A, cdc2 kinase, extracellular signal-regulated kinase (ERK), and PKC. Phenylephrine has been suggested to induce phosphorylation of CaD through activating PKC and/or mitogen-activated-protein kinases (MAPKs) (Dessy et al., 1998; Horowitz et al., 1996a). PKC can directly or indirectly phosphorylate CaD and induces sustain contractions (Horowitz et al., 1996a; Morgan and Gangopadhyay, 2001; Throckmorton et al., 1998; Xiao and Zhang, 2005). In addition, CaD has been suggested to be a good substrate of Ca$^{2+}$-independent isozyme PKCe but not PKC$\zeta$ (Horowitz et al., 1996a). Additionally, phosphorylation of CaD by PKC is exclusively within the C-terminal 35-KDa domain (Tanaka et al., 1990) at different sites at Ser127, Ser587, Ser600, Ser657, Ser686, Ser726, Ser759 and Ser789 (Hedges et al., 2000; Ikebe and Hornick, 1991; Vorotnikov et al., 1994; Yamboliev et al., 2000), which may be due to agonist-, species-, and tissue-specific effects (Morgan and Gangopadhyay, 2001). Nonetheless, the resent studies suggested that, in pregnant uterine artery, phorbol 12, 13-dibutyrate (PDBu)-induced phosphorylation of CaD at ERK specific site of Ser789 may inhibit PKC-mediated phosphorylation of the other sites and
stabilize CaD inhibitory effect on actin-activated myosin ATPase (Xiao et al., 2004). This finding was supported by other reports that phosphorylation of CaD by MAPK had no effects on Ca$^{2+}$ sensitivity in rabbit smooth muscle and cannot induce contractions of smooth muscle (Nixon et al., 1995). Previous study from Dr. Zhang’s laboratory has shown that ERK inhibitor PD-098059 blocked PKC activator PDBu induced phosphorylation of CaD-Ser$^{789}$ in uterine artery (Xiao et al., 2004), which indicated that ERK is a downstream molecule from PKC in the pathway of regulation of Ca$^{2+}$sensitivity (Fig.4.). On the other hand, CaD content was suggested to be different in different types of smooth muscle (Haeberle et al., 1992) and significantly increased in myometrium of pregnant women, which facilitated adaptation of uterus to pregnancy (Word et al., 1993). However, whether or to what extent ERK and CaD involve in PKC-regulated $\alpha_1$-adrenoceptor function and contractions in pregnant and nonpregnant uterine arteries are not defined.
Fig. 4. Model of thin filament regulation, modified from Morgan (J Appl Physiol 91:953-962, 2001)
Calponin

Calponin (CaP) is another family of actin regulatory protein that interacts with actin and inhibits myosin ATPase activity (Morgan and Gangopadhyay, 2001; Gimona et al., 1992). CaP activity is regulated by phosphorylation by PKC or CaMKII, both of which reverse CaP’s inhibitory activity (Winder and Walsh, 1990). In addition to binding to actin, CaP can also bind to calmodulin (Wills et al., 1993), myosin (Shirinsky et al., 1992), desmin (Mabuchi et al., 1997) and phospholipids (Bogatcheva and Gusev, 1995). It has been suggested that CaP play an important role in the regulation of agonist-induced contraction of vascular smooth muscle (Walsh, 200). The role of CaP in smooth muscle contractility is controversial. CaP has been suggested to directly inhibit myosin ATPase activity to regulate contractility of smooth muscle (Winder and Walsh, 1990). On the other hand, it has been proposed that CaP, as a signaling molecule, facilitates PKC and ERK-dependent signaling, leading to smooth muscle contraction (Je et al., 2001; Menice et al., 1997; Morgan and Gangopadhyay, 2001; Leinweber et al., 1999 & 2000). Morgan and Gangopadhyay (2001) proposed that CaP serves as an adaptor protein that binds with PKC and ERK upon stimulation of α1-adrenoceptor agonist or phorbol ester, and cotranslocates with these enzymes to the cell membrane as a complex. At the membrane, ERK is phosphorylated and then releases from the membrane to its target protein CaD. The subsequent phosphorylation of CaD by ERK reverses the inhibitory effect of CaD on myosin ATPase to generate contraction of smooth muscle.
Hormonal Regulation of the Uterine Artery Contractility

Steroid hormone appears responsible for the changes in uterine arterial tone and contractility throughout pregnancy. During pregnancy, vascular tone is strikingly decreased as estrogen concentrations rise. It has been suggested that estrogens directly modulate proliferation and migration of endothelial and vascular smooth muscle cells (Ford SP, 1995; Morales et al., 1995). In addition, estrogens as vasodilators have both genomic and non-genomic actions on vasodilation. The effects of estrogens are mediated by initially interaction with estrogen receptors that are expressed in vascular endothelium and smooth muscle cell (Karas et al., 1994; Suzuki et al., 2003; Venkov et al., 1996). Chronic estrogen exposures alter vascular tone mainly through genomic mechanism by which estrogen receptors regulate transcription of target gene expression. It has been reported that estrogens increase gene expressions of endothelial nitric oxide synthase (eNOS) (Caulin-Graser et al., 1997; Joyce et al., 2002), phospholipase A2 (Rupnow et al., 2002), prostacyclin synthetase (Rupnow HL, 2002) and cyclooxygenase-1 (Jun, et al., Rupnow et al., 2002) but decrease expressions of prostaglandin H synthase (Stewart et al., 1999) and endothelin-1 (Akishita et al., 1988) in endothelium. For many years of in vivo study of the ovarian and pregnant effect on uterine artery vasodilation, Dr. Magness and his colleagues have demonstrated that estrogen increase eNOS gene expression and NO synthesis in uterine artery from sheep (Joyce et al., 2002; Magness et al., 1996, 1997 & 2001; Rupnow et al., 2001; Vagononike et al., 1998). A rapid and direct effect of estrogens was also postulated, which may be the result of non-genomic responses. The regulation of nitric oxide (NO) synthesis and/or release is a major target of estrogen in endothelium to produce vasodilation. Besides chronically increasing its synthesis,
estrogens directly enhance eNOS activity (Caulin-Graser et al., 1997) and inhibit NO degradation, because estrogens are potent antioxidants (Arnal et al., 1996). Meanwhile, estrogens acutely modulate Ca\(^{2+}\) homeostasis in vascular smooth muscle cells resulting in a rapid decrease in vascular tone via endothelia-independent mechanisms. Estrogens have been shown to directly interact with and open smooth muscle cell calcium-activated potassium channels, thus relaxing the cell (Valverde et al., 1999). Furthermore, estrogens have been reported to regulate potential-sensitive calcium channels (PSC). Peroxidase converts estrogens to catechol estrogens, which directly inhibit Ca\(^{2+}\) uptake through PSC, resulting in a decreased PKC-maintained tone of uterine artery (Ford, 1995; Ford et al.). During pregnancy, extremely high concentrations of estrogens and peroxidase are detected in uterine lymphatic fluid that are in close contact with the uterine arterial vasculature and thus may serve to transport catechol estrogens (Ford et al., 1993). It has been indicated that the decreasing PKC activity of uterine artery during pregnancy is associated with catechol estrogen-induced inhibition of calcium uptake through PSC (Farley and Ford, 1992). Progesterone, in contrast to estrogen, inhibits proliferation and migration of endothelia and vascular smooth muscle cells (Lee et al., 1997; Schnaper et al., 2000). Meanwhile, progesterone augments the responsiveness of vascular smooth muscle to norepinephrine related to enhancing PKC activity. During the estrous cycle of ewes, the higher the estrogen:progesterone ratio in systemic blood, the greater is the quantity of blood flowing through the uterine vascular bed. However, after the initial increase in blood flow to the uterus during early pregnancy, changes in uterine blood flow appear to be unrelated to changes in the estrogen:progesterone ratio in fetal and maternal fluids. Since the estrogen:progesterone ratio controls uterine blood flow
through changes in the function of periartrial adrenergic nerves, the disassociation between the estrogen: progesterone ratio and uterine blood flow may relate to a progressive sympathetic denervation observed in the uterus during pregnancy.

**Aim of this project**

The aim of the present project was to examine the main hypothesis that PKC activation differentially regulates $\alpha_1$-adrenoceptor-mediated $\text{Ca}^{2+}$ mobilization and sensitization in pregnant and nonpregnant uterine arteries. To test this hypothesis, we proposed a series of experiments in uterine arteries from nonpregnant and near-term (~140 days) pregnant sheep. Our specific aims were: 1) to test the hypothesis that PKC differentially regulates $\alpha_1$-adrenoceptor-mediated contractions of nonpregnant and pregnant uterine arteries; 2) to test the hypothesis that PKC activation differentially regulates $\alpha_1$-adrenoceptor-mediated $\text{Ca}^{2+}$ mobilization and sensitization in pregnant and nonpregnant uterine arteries through differentially regulations of the thick and thin filament pathways; 3) to test the hypothesis that differential regulation of PKC isozyme activities contributes to the different effect of PDBu on $\alpha_1$-adrenoceptor-mediated contractions between the pregnant and nonpregnant uterine arteries.

**Significance**

The proposed studies are of importance from both basic science and clinical perspectives. From the basic science point of view, the proposed studies of both $\text{Ca}^{2+}$-dependent and $\text{Ca}^{2+}$-independent pathways are especially powerful means of evaluating regulatory mechanisms governing uterine artery contractility, and thereby enhance our
basic understanding of uterine vasculature adaptation to pregnancy. They will provide
exciting novel information from several standpoints. They will be the first to examine
PKC isozyme expression and their functions in the uterine artery, and examine the effect
of pregnancy. They will be the first to offer assessment of the dynamic interactions of
PKC and $\alpha_1$-adrenoceptor-induced signal transduction pathways in the uterine artery, and
to test the novel hypothesis that PKC differentially regulates $\alpha_1$-adrenoceptor function
and contractions in nonpregnant and pregnant uterine arteries. They will be the first
concerted effort to understand the mechanisms underlying the dual role of PKC in the
regulation of contractions in the uterine artery. Not only are these studies important to
understand basic mechanisms underlying adaptation of uterine artery contractile
machinery to pregnancy, but they will also offer novel information in understanding
signaling mechanisms for vascular contraction in general.

The proposed studies are relevant to the mechanisms involved in adjusting
uteroplacental circulation to pregnancy. Given the importance of uterine blood flow in fetal
development and maternal health, failure to make these adjustments is likely to contribute to
many fetal abnormalities, including intrauterine growth restriction, as well as maternal
cardiacovascular disorders. The proposed studies will help provide a mechanistic basis for this
functional adaptation, and thereby improve our understanding of problems associated with
the maladaptation and abnormal pregnancy and permit us to address them in a more
meaningful way.
CHAPTER TWO

REGULATION OF α1-ADRENOCEPTOR-MEDIATED CONTRACTION OF THE UTERINE ARTERY BY PKC: EFFECT OF PREGNANCY

Hongying Zhang, DaLiao Xiao, Lawrence D. Longo and Lubo Zhang

Abstract

Protein kinase C (PKC) plays an important role in the regulation of uterine artery contractility and its adaptation to pregnancy. The present study tested the hypothesis that PKC differentially regulates $\alpha_1$-adrenoceptor-mediated contractions of nonpregnant and pregnant uterine arteries. Phenylephrine-induced contractions of uterine arteries isolated from nonpregnant (NPUA) and near-term pregnant (PUA) sheep were determined in the absence or presence of the PKC activator, phorbol 12, 13-dibutyrate (PDBu). In NPUA, PDBu produced a concentration-dependent potentiation of phenylephrine-induced contractions and shifted the dose-response curve to the left. In contrast, in PUA PDBu significantly inhibited phenylephrine-induced contractions and decreased their maximum response. Simultaneous measurement of contractions and intracellular free Ca$^{2+}$ concentrations ([Ca$^{2+}]_i$) in the same tissues revealed that PDBu inhibited phenylephrine-induced [Ca$^{2+}]_i$ and contractions in PUA. In NPUA, PDBu increased phenylephrine-induced contractions without changing [Ca$^{2+}]_i$. Western blot analysis showed six PKC isozymes, $\alpha$, $\beta_I$, $\beta_{II}$, $\delta$, $\varepsilon$ and $\zeta$ in uterine arteries, among which $\beta_I$, $\beta_{II}$ and $\zeta$ isozymes were significantly increased in PUA. In contrast, PKC$\alpha$ was decreased in PUA. In addition, analysis of sub-cellular distribution revealed a significant decrease in the particulate/cytosolic ratio of PKC$\varepsilon$ in PUA, as compared with NPUA. The results suggest that pregnancy induces a reversal of PKC regulatory role on $\alpha_1$-adrenoceptor-mediated contractions from a potentiation in NPUA to an inhibition in PUA. The differential expression of PKC isozymes and their sub-cellular distribution in uterine arteries appears to play an important role in the regulation of Ca$^{2+}$ mobilization and Ca$^{2+}$ sensitivity in $\alpha_1$-adrenoceptor-mediated contractions and their adaptation to pregnancy.
Introduction

Pregnancy is associated with a significant decrease in uterine vascular tone and a striking increase in uterine blood flow, which ensures normal fetal development. Multiple mechanisms are involved in the adaptation of uterine artery contractility during pregnancy (3, 12, 38, 48, 50). We have recently demonstrated in ovine uterine arteries that protein kinase C (PKC) plays an important role in the regulation of uterine artery contractility, and the pregnancy-associated decrease in myogenic tone of the uterine artery is primarily regulated through the PKC signaling pathway (45, 48, 50). In addition to its regulatory effect of myogenic tone (10, 45), PKC interacts with α₁-adrenoceptors and modulates α₁-adrenoceptor-mediated contractions of vascular smooth muscle (12, 35, 37). Activation of α₁-adrenoceptors plays a key role in the regulation of uterine artery smooth muscle contractions (7, 12, 28, 41, 51). Yet, whether or to what extent PKC regulates α₁-adrenoceptor-mediated contractions of the uterine artery and their adaptation to pregnancy remains unclear.

Activation of α₁-adrenoceptors leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1, 4, 5- trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to IP₃ receptors and stimulates Ca²⁺ release from intracellular calcium stores. On the other hand, DAG activates PKC that has been suggested to play a key role in α₁-adrenoceptor-mediated Ca²⁺ sensitization (4, 13, 24, 31, 36, 40). In addition to its coupling to α₁-adrenoceptors, activation of PKC by phorbol esters, synthetic analogs of DAG, has been shown to regulate α₁-adrenoceptor-mediated contractions in vascular smooth muscle. Both PKC-mediated inhibition and potentiation of α₁-adrenoceptor-
mediated contractions have been reported. Thus, activation of PKC by phorbol esters inhibited norepinephrine-induced contractions in rat aorta, cat and sheep cerebral arteries (2, 6, 9, 25, 27, 34, 39, 53). On the other hand, activation of PKC potentiated α1-adrenoceptor-mediated contractions in rabbit aorta, rat mesenteric arteries, femoral arteries, corpora cavernosa, and vas deferens (1, 14, 18, 32, 33). The effect of potentiation was abolished by depleting PKC after prolonged treatment with phorbol esters (14). The controversial effects of PKC on α1-adrenoceptor-mediated contractions in different arteries from different species may be due in part to the diversity of PKC isozymes, which have different enzymatic properties, substrates, functions, and different sub-cellular distributions in different blood vessels and species (15, 21, 22, 23, 26, 37).

The present study tested the hypothesis that activation of PKC differentially regulates α1-adrenoceptor-mediated contractions of uterine arteries from nonpregnant and pregnant sheep. Concentration-response curves of phenylephrine-induced contractions of the uterine arteries were conducted in the absence or presence of the PKC activator phorbol 12, 13-dibutyrate (PDBu). To evaluate the role of Ca^{2+} in the PKC-mediated effects, phenylephrine-induced contractions and free intracellular Ca^{2+} concentrations ([Ca^{2+}]_i) were measured simultaneously in the same tissues of uterine arteries. In addition, the differential expression of PKC isozymes and their sub-cellular distributions in nonpregnant and pregnant uterine arteries were determined.
Methods

**Tissue preparation.** Nonpregnant and near-term pregnant (~140 day gestation) ewes were anesthetized with thiamylal (10 mg/kg), administered *via* the external left jugular vein. The ewes were then intubated, and anesthesia was maintained with 1.5–2.0% halothane in O₂ throughout the surgery. An incision was made in the abdomen to expose the uterus. The uterine arteries were isolated and removed without stretching and were placed in a modified Krebs’ solution (pH 7.4) of the following composition (in mM): 115.2 NaCl, 4.7 KCl, 1.80 CaCl₂, 1.16 MgSO₄, 1.18 KH₂PO₄, 22.14 NaHCO₃, 0.03 EDTA, and 7.88 dextrose. The Krebs’ solution was oxygenated with a mixture of 95%O₂-5% CO₂. After the tissues were removed, animals were killed with T-61 euthanasia solution (Hoechst-Roussel; Somerville, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

**Contraction studies.** The third (in nonpregnant ewes) and fourth (in pregnant ewes) branches of the main uterine arteries with similar external diameter were dissected 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 (50). Briefly, each ring was equilibrated for 60 min and then gradually stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. Tissues were then stimulated with cumulative additions of phenylephrine in approximate one-half log increments to generate a concentration-response curve, and contractile tensions were
recorded with an online computer. After washing away phenylephrine, tissues were
relaxed to the baseline and were recovered at the resting tension for 30 min. The second
congestion-response curves of phenylephrine-induced contractions were then repeated
in the absence or presence of PDBu (10, 30, and 100 nM for nonpregnant uterine arteries,
and 0.1, 0.3, and 1 μM for pregnant uterine arteries, for 10 min). The different
concentration ranges of PDBu were chosen in nonpregnant and pregnant uterine arteries,
based on our previous findings that PDBu was 10-time more potent in contracting
nonpregnant than pregnant uterine arteries (pD2: 6.64 ± 0.07 vs. 5.62 ± 0.17) (48). To
determine the effects of PDBu on phenylephrine-induced contractions, concentrations of
PDBu less than EC50 values in nonpregnant and pregnant uterine arteries, respectively,
were utilized in the present study. The concentrations of phenylephrine were chosen to
produce full concentration-response curves in both nonpregnant and pregnant uterine
arteries.

**Simultaneous measurement of [Ca2+]i and tension.** Smooth muscle [Ca2+]i were
measured simultaneously with muscle contractions in the same tissues as described
previously (52). Briefly, the arterial ring was attached to an isometric force transducer in
a 5-ml tissue bath, mounted on a CAF-110 intracellular Ca2+ analyzer (Jasco; Tokyo,
Japan). The tissue was equilibrated in HEPES buffer (in mM, 115.2 NaCl, 4.7 KCl, 1.80
CaCl2, 1.16 MgSO4, 1.18 KH2PO4, 10.0 HEPES, 0.03 EDTA, and 7.88 dextrose, PH7.4)
under a resting tension of 0.5 g for 40 min, followed by stimulation with 120 mM KCl
once and recovery to the resting tension for 30 min. The tissue was then loaded with 5
μM fura-2 AM for 3 h in the presence of 0.02% cremophor EL and 0.25% DMSO at 25
°C. After loading, the tissue was washed with HEPES buffer at 37 °C for 45 min to allow
for hydrolysis of fura-2 ester groups by endogenous esterase. The tissue was then stimulated with 120 mM KCl twice. After recovery for 30 min, the tissue was stimulated with phenylephrine (3 μM for both nonpregnant and pregnant uterine arteries to produce sub-maximal contractions, and 30–200 nM for nonpregnant uterine arteries to produce less than 50% of KCl maximum) in the absence or presence of 0.1 μM PDBu. The experimental protocol was shown in the following diagram:

<table>
<thead>
<tr>
<th>Phenylephrine 1</th>
<th>Wash+Recovery</th>
<th>PDBu</th>
<th>Wash+Recovery</th>
<th>Phenylephrine 3</th>
</tr>
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<tbody>
<tr>
<td>Phenylephrine 2</td>
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Agonist-induced changes in contractile force and fura-2 fluorescence were measured simultaneously at 37 °C in the same tissue. The tissue was illuminated alternatively (125 Hz) at excitation wavelengths of 340 and 380 nm, respectively, by means of two monochromators in the light path of a 75-W xenon lamp. Fluorescence emission from the tissue was measured at 510 nm by a photomultiplier tube. The fluorescence intensity at each excitation wavelength (F340 and F380, respectively) and the ratio of these two fluorescence values (R_{f340/f380}) were recorded with a time constant of 250 ms and stored with the force signal on a computer.

**Measurement of PKC isozymes.** To determine the protein expression of PKC isozymes in uterine arteries, tissues (third or fourth branches of nonpregnant and pregnant uterine arteries, respectively) were homogenized in the lysis buffer containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, 0.2% Triton X-100, β-mercaptoethanol 10 mM, benzamidine 1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, leupetin 50 μM, dithiothreitol 1 mM and aprotinin 2 μg/ml, pH 7.5. Homogenates were centrifuged at 6,000 g for 5 min at 4 °C and the supernatants were collected. Protein
was quantified with a protein assay kit (Bio-Rad). Western blotting was performed as described below.

**Measurement of PKC isozyme distribution and relative activity.** To determine the distribution of PKC isozymes in cytosolic and particulate fractions of uterine arterial smooth muscle, tissues (third or fourth branches of nonpregnant and pregnant uterine arteries, respectively) were homogenized in ice-cold homogenization buffer A containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, β-mercaptoethanol 10 mM, benzamidine 1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, leupeptin 50 μM, dithiothreitol 1 mM and aprotinin 2 μg/ml, pH 7.5. The homogenates were centrifuged at 100,000 g for 20 min at 4 °C, and the supernatants were collected and used as the cytosolic fraction (S). The pellets were re-suspended in homogenization buffer A containing 1% triton X-100 by stirring for overnight at 4 °C, diluted with the buffer A to a final concentration of 0.2% triton X-100, and then centrifuged at 100,000 g for 20 min at 4 °C. The supernatants were collected and referred to as the particulate fraction (P). Protein concentrations were determined with a protein assay kit (Bio-Rad). Immunoreactive bands for PKC isozymes in cytosolic and particulate fractions were determined by Western blotting using specific PKC isozyme antibodies as described below. The ratio of P/S was used to determine the relative activity of PKC isozymes.

**Western immunoblotting analysis.** Protein-matched samples obtained from the tissues or cytosolic and particulate fractions were subjected to electrophoresis on 7.5% sodium dodecylsulfate-polyacrylamide gel, and then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated at room temperature for 1 h in Tris-buffered saline solution (TBS) containing 5% dried milk and 0.5% Tween 20,
followed by incubation with primary anti-PKC isozyme antibodies overnight at 4 °C and secondary antibody for 1 h at room temperature. Polyclonal antibodies to PKCα, βI, βII, δ, ε, ζ, γ were used. Bands were detected with enhanced chemiluminescence (ECL), visualized on Hyperfilm, and analyzed with the Kodak 1D image analysis software. To determine the total PKC levels, the same amount of protein (5 μg) from each sample was loaded to the gel. To normalize the loading variation of each sample, the corresponding actin level presented in each sample was determined by using monoclonal anti-actin as primary antibody. To determine ratio of P/S, equal volume (10 μl) of cytosolic and particulate fractions, respectively, from each sample was loaded to the gel.

**Materials.** Phenylephrine, PDBu, and anti-actin antibody were obtained from Sigma (St. Louis, MO). Anti-PKC isozyme antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Fura-2 AM was from Molecular Probes (Eugene, OR). All electrophoretic and immunoblot reagents were from Bio-Rad. General laboratory reagents were of analytical grade or better and were purchased from Sigma and Fisher Scientific. All drugs were prepared freshly each day and kept on ice throughout the experiment.

**Data analysis.** Data 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. Differences were evaluated for statistical significance (P < 0.05) by one-way ANOVA and Student’s t-test.
Results

Effect of PDBu on phenylephrine-induced contractions. Fig. 1 shows that phenylephrine produced concentration-dependent contractions of uterine arteries from both nonpregnant and pregnant ewes. In nonpregnant uterine arteries, PDBu (10, 30, 100 nM) induced concentration-dependent contractions, and produced a concentration-dependent potentiation of phenylephrine-induced contractions. In the presence of 100 nM PDBu, the concentration-response curve of phenylephrine-induced contractions was markedly shifted to the left with a significant increase in the pD$_2$ value from 6.27 ± 0.10 to 8.86 ± 0.28 (P < 0.001), representing an over 300-fold increase in the potency of phenylephrine-induced contractions in nonpregnant uterine arteries. The maximal responses of phenylephrine-induced contractions were not affected with PDBu (Fig. 1 & Fig. 2). In pregnant uterine arteries, PDBu failed to induce contractions up to 1 μM, which produced a comparable contraction (1.30 ± 0.34 g) as that induced by 100 nM PDBu in nonpregnant uterine arteries (1.38 ± 0.74 g). As shown in Fig. 1, in contrast to its effect in nonpregnant uterine arteries, PDBu produced a concentration-dependent inhibition of phenylephrine-induced contractions in pregnant uterine arteries. Whereas the pD$_2$ values were not affected, the maximal responses of phenylephrine-induced contractions were significantly decreased by 27%, 37%, and 68%, in the presence of 0.1 μM, 0.3 μM, and 1 μM PDBu, respectively (Fig. 1 & Fig. 2). In both nonpregnant and pregnant uterine arteries, KCl-induced contractions were not affected with PDBu (data not shown).
Effect of PDBu on phenylephrine-induced changes of intracellular free Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_i$). Fig. 3 shows the effects of PDBu on phenylephrine-induced increases in [Ca$^{2+}$]$_i$ and contractile tensions, measured simultaneously in the same tissues, in both nonpregnant and pregnant uterine arteries. In pregnant uterine arteries, the sub-maximal concentration (3 μM) of phenylephrine induced significant increases in [Ca$^{2+}$]$_i$ and contractile tension. PDBu (100 nM) produced a minimal contraction in the absence of changes in [Ca$^{2+}$]$_i$. In the presence of PDBu, phenylephrine-induced increases in [Ca$^{2+}$]$_i$ and contractions were significantly inhibited (Fig. 3, the top panel). In nonpregnant uterine arteries, 100 nM PDBu did not affect contractions and [Ca$^{2+}$]$_i$ induced by the sub-maximal concentration (3 μM) of phenylephrine (Fig. 3, the middle panel). However, PDBu significantly increased contractions produced by lower concentrations (30~200 nM) of phenylephrine, in the absence of significant changes in phenylephrine-mediated [Ca$^{2+}$]$_i$ (Fig. 3, the bottom panel). After washing away PDBu from the tissues, phenylephrine produced similar levels of increases in tension and [Ca$^{2+}$]$_i$ as those before the PDBu treatment in both nonpregnant and pregnant uterine arteries (Fig. 3), eliminating the potential time factor that could affect the responses of uterine arteries to phenylephrine during the experiment procedure.

Expression of PKC isozymes in uterine arteries. Fig. 4 shows the expression of at least 6 PKC isozymes, α, βI, βII, δ, ε, and ζ in ovine uterine arteries. PKCγ was not detected in either nonpregnant or pregnant uterine arteries. As compared with nonpregnant uterine arteries, pregnant vessels showed significantly increased expression levels of PKCβI, PKCβII, and PKCζ, but significantly decreased levels of PKCα (Fig. 4). There were no significant differences in the expression levels of PKCδ and PKCε.
between nonpregnant and pregnant uterine arteries (Fig. 4). The distribution of PKC isozymes in cytosolic and particulate fractions in the uterine arteries is shown in Fig. 5. As compared with nonpregnant uterine arteries, the ratio of particulate/cytosolic distribution for PKC isozymes in pregnant uterine arteries was significantly decreased for PKCe, but not significantly different for PKCα, βI, βII, δ, and ζ (Fig. 5).
Discussion

The present study offers the following new findings: 1) activation of PKC potentiated $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine arteries, but inhibited the contractions in pregnant uterine arteries; 2) PKC activation blocked $\alpha_1$-adrenoceptor-mediated increases of $[Ca^{2+}]_i$ in pregnant uterine arteries, but had no significant effect on the $Ca^{2+}$ mobilization in nonpregnant uterine arteries; 3) PKC isozyme expression and sub-cellular distribution were differentially regulated in nonpregnant and pregnant uterine arteries, with significantly increased expression levels of PKC$\beta$ and PKC$\zeta$, but decreased PKC$\alpha$ and the basal activity of PKC$\epsilon$ in pregnant, as compared with nonpregnant, uterine arteries.

It has been suggested that PKC plays an important role in regulating vascular myogenic response (10). Our recent studies have demonstrated that myogenic tone of the uterine artery is reduced during pregnancy, which is primarily regulated through the PKC signal pathway (45). In addition to its role in the regulation of myogenic tone, the present study demonstrated that activation of PKC interacted with $\alpha_1$-adrenoceptors and modulated $\alpha_1$-adrenoceptor-mediated contractions of the uterine artery. To maintain the similar vessel diameters for nonpregnant and pregnant uterine arteries in the study, the third and fourth branches of the main uterine arteries from nonpregnant and pregnant sheep, respectively, were used in the present study. These vessels have been extensively studied in our previous studies (46, 48, 49, 50), and for a comparative purpose they were used in the present study. Similarly, the third and fourth generation uterine arteries from nonpregnant and pregnant sheep were utilized in determining the role of $Ca^{2+}$ and $Ca^{2+}$
channels in regulating basal and angiotensin II-induced prostacyclin production in the uterine artery (29). Although few studies examined the potential cellular and sub-cellular differences between branch generations of small uterine arteries, numerous studies examined structural and signal proteins in pooled all branches of the uterine artery and showed differences between nonpregnant and pregnant uterine arteries, including the studies of PKC (11, 30). In the present study, we have shown that the smaller branches (beyond the 4th generation) of pregnant and nonpregnant uterine arteries utilized in the studies of simultaneous measurement of [Ca^{2+}], and contractions demonstrate the same characteristics of tissue response to PDBu and phenylephrine stimulations observed in the 3rd and 4th branches, i.e. PDBu-mediated potentiation of phenylephrine-induced contraction in nonpregnant uterine arteries, but inhibition of that in pregnant uterine arteries. These findings suggested that the characteristics of PKC-mediated effects were not altered between branches of small uterine arteries. The finding that PKC differentially regulated α1-adrenoceptor-mediated contractions in nonpregnant and pregnant uterine arteries is intriguing, and suggests another important mechanism through which PKC regulates the adaptation of uterine artery contractility to pregnancy. Previous studies have demonstrated that PKC can either inhibit or potentiate α1-adrenoceptor-mediated contractions in different arteries from different species (1, 2, 6, 9, 14, 18, 25, 27, 32, 33, 34, 39). However, to our knowledge, it has not been previously demonstrated that activation of PKC can inhibit and potentiate α1-adrenoceptor-mediated contractions in the same artery of the same species at different physiological states, i.e. nonpregnant and pregnant. Thus, the present finding of a potentiation of α1-adrenoceptor-mediated contractions in nonpregnant uterine arteries, but an inhibition in pregnant uterine arteries
provides a physiological importance of PKC in regulating the adaptation of $\alpha_1$-adrenoceptor-mediated contractions of the uterine artery during pregnancy. The finding that activation of PKC had no effect on KCl-induced contractions suggests that the effect of PKC is selective to $\alpha_1$-adrenoceptor-mediated signal pathways in the uterine artery. Similar findings were obtained in cat cerebral arteries, in which phorbol esters potentiated phenylephrine-, but not KCl-induced contractions (39).

In the present study, activation of PKC significantly enhanced the sensitivity of $\alpha_1$-adrenoceptor-mediated contractions by increasing the pD2 value in nonpregnant uterine arteries. Yet the maximal response was not affected. In contrast, in pregnant vessels, PKC significantly decreased the maximal $\alpha_1$-adrenoceptor-mediated contractions without affecting the sensitivity. These findings suggest that PKC modulates smooth muscle contractile apparatus at different steps of signal transduction pathways responding to $\alpha_1$-adrenoceptor stimulation in nonpregnant and pregnant uterine arteries. In vascular smooth muscle cells, activation of $\alpha_1$-adrenoceptors leads to an increase in free intracellular Ca$^{2+}$ concentrations from intracellular Ca$^{2+}$ stores via IP3 stimulation, resulting in myosin light chain phosphorylation and contractions (35). In the present study, we found that activation of PKC abolished $\alpha_1$-adrenoceptor-induced increase in [Ca$^{2+}]_i$ in pregnant uterine arteries, but had no significant effect on [Ca$^{2+}]_i$ in nonpregnant uterine arteries. The inhibition of [Ca$^{2+}]_i$ was accompanied by decreased contractions, measured simultaneously in the same tissues. This suggests a predominant mechanism of decreased Ca$^{2+}$ mobilization in the PKC-mediated attenuation of $\alpha_1$-adrenoceptor-mediated contractions in pregnant uterine arteries. Consistent with the present finding, previous studies in ovine cerebral arteries demonstrated that PDBu inhibited
norepinephrine-induced increases in intracellular Ca\(^{2+}\) concentrations and contractions (27). Whereas the mechanisms of PKC-mediated inhibition of Ca\(^{2+}\) mobilization in pregnant uterine arteries are not clear at present, it has been demonstrated that activation of PKC decreases agonist binding affinity of \(\alpha_1\)-adrenoceptors, increases phosphorylation of \(\alpha_1\)-adrenoceptors, destabilizes \(\alpha_1\)-adrenoceptor mRNA, down-regulates \(\alpha_1\)-adrenoceptors, and promotes uncoupling of \(\alpha_1\)-adrenoceptors from inositol phospholipid metabolism (2, 5, 6, 19, 25, 27, 34).

In contrast to pregnant uterine arteries, the finding that PDBu had no effect on \(\alpha_1\)-adrenoceptor-mediated Ca\(^{2+}\) mobilization but potentiated the contractions in nonpregnant vessels is intriguing, and provides strong evidence of increased Ca\(^{2+}\) sensitivity in the PKC-mediated potentiation of \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant uterine arteries. In the present studies of simultaneous measurement of [Ca\(^{2+}\)]\(_i\) and contractions, the finding that PDBu had no effect on phenylephrine-induced contractions at a high concentration (3 \(\mu\)M) but potentiated it at low concentrations (Fig. 3, the middle and bottom panels) was consistent with the studies of concentration-dependent contractions that showed the PKC-mediated potentiation occurring in the lower range concentrations of phenylephrine (Fig. 1). These results validated that loading of tissues with fura-2 did not alter characteristics of tissue response to PDBu and phenylephrine stimulation, albeit the tissue size and hence the contractions were much smaller in the studies of simultaneous measurement of [Ca\(^{2+}\)]\(_i\) and contractions. Consistent with the present findings, PKC has been implicated in the regulation of myofilament Ca\(^{2+}\) sensitivity in vascular smooth muscle (10, 15, 43). We have demonstrated that PKC modulates Ca\(^{2+}\) sensitivity in the uterine artery predominantly through the thin-filament
regulatory pathway, *i.e.* independent of changes in phosphorylation of myosin light chain (50). Furthermore, the thin-filament pathway is an important component in $\alpha_1$-adrenoceptor-mediated contractions, particularly in nonpregnant uterine arteries (47, 50). Taken together, we speculate that activation of PKC in nonpregnant uterine arteries produces a permissive and priming effect on the thin-filament pathway, resulting in enhanced $\alpha_1$-adrenoceptor-induced contractions.

The apparent opposite effects of PKC on $\alpha_1$-adrenoceptor-mediated contractions in nonpregnant and pregnant uterine arteries may be due in part to the differential expression of PKC isozymes that show different enzymatic properties, substrates, and functions (15, 23, 26). The $\alpha$, $\beta$, $\delta$, $\epsilon$, and $\zeta$ isozymes of PKC have been detected in vascular smooth muscle (44). Although not all of these isozymes appear to be in all vascular smooth muscle tissues, the present study demonstrated their presence in ovine uterine arteries. The finding of significantly increased expression levels of PKC$\beta$ and PKC$\zeta$ in pregnant, as compared with nonpregnant, uterine arteries suggests a potential mechanism for these isozymes in inhibiting $\alpha_1$-adrenoceptor-mediated $\text{Ca}^{2+}$ mobilization and contractions in the pregnant vessels. PKC$\beta$ is a conventional PKC isozyme that is activated by diacylglycerol and phorbol esters in the presence of $\text{Ca}^{2+}$ (15). Previous studies have shown that overexpression of PKC$\beta$ inhibits agonist-induced $\text{Ca}^{2+}$ mobilization, and inhibition of PKC$\beta$ results in a dramatic increase in agonist-mediated $\text{Ca}^{2+}$ mobilization (17, 20, 42). In contrast to PKC$\beta$, inhibition of PKC$\zeta$ by a specific inhibitory peptide did not alter agonist-induced $\text{Ca}^{2+}$ mobilization (17). In addition, PKC$\zeta$ is an atypical PKC isozyme that is not activated by $\text{Ca}^{2+}$, DAG, or phorbol esters (15). These results suggest that the increased PKC$\beta$ in the pregnant uterine arteries is likely to
play an important role in the PKC-mediated inhibition of $\alpha_1$-adrenoceptor-mediated Ca$^{2+}$ mobilization and contractions in the pregnant vessels. Although PKC$\zeta$ may be less likely to be involved in the PDBu-mediated inhibition of intracellular Ca$^{2+}$ mobilization, the increased PKC$\zeta$ in the pregnant uterine arteries may be important in proliferation and remodeling of the uterine artery during pregnancy, as it translocates from perinuclear localization into the nucleus upon activation, and functions in control of gene expression (8, 23, 44). In contrast to increased PKC$\beta$ and PKC$\zeta$, the expression levels of PKC$\alpha$ and the levels PKC$\epsilon$ in the particulate fraction were significantly decreased in the pregnant uterine arteries. Both PKC$\alpha$ and PKC$\epsilon$ have been implicated in contractions of vascular smooth muscle through increasing Ca$^{2+}$ sensitivity (16, 43, 44). Taken together, these findings suggest that the adaptation of the uterine artery to pregnancy is associated with the up-regulation of the PKC isozyme(s) that inhibit intracellular Ca$^{2+}$ mobilization, and the down-regulation of the PKC isozymes that increase Ca$^{2+}$ sensitivity.

In summary, we have shown the opposite effects of PKC on $\alpha_1$-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries. Activation of PKC enhances $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine arteries by increasing Ca$^{2+}$ sensitivity, but inhibits the contractions in pregnant uterine arteries by decreasing intracellular Ca$^{2+}$ mobilization. The differential regulation of PKC isozyme expression, with the up-regulation of PKC$\beta$ and PKC$\zeta$, and the down-regulation of PKC$\alpha$ and PKC$\epsilon$, is likely to play an important role in the adaptation of uterine artery Ca$^{2+}$ homeostasis from pro-Ca$^{2+}$ sensitivity in the nonpregnant vessels to inhibition of Ca$^{2+}$ mobilization in the pregnant vessels. From the physiological perspective, the uterine circulation during pregnancy functions as a low-resistance shunt to accommodate the
large increase of uteroplacental blood flow, required for normal fetal development. In addition to growth and remodeling of vessels, the decreased uterine artery resistance is accomplished by increased endothelial nitric oxide release, decreased myogenic response, and a reversible sympathetic denervation of the uterine artery. Although the decreased sympathetic innervation may sensitize postsynaptic $\alpha_1$-adrenoceptor signal pathways, the present finding of the increased inhibitory effect of PKC on $\alpha_1$-adrenoceptor-mediated contractions in the pregnant uterine artery reveals another important mechanism in maintaining the low uterine vascular tone in pregnancy. The potential effect of steroid hormones in the regulation of differential expression of PKC isozymes in the uterine artery during pregnancy presents an intriguing area for the future investigation.
Acknowledgements

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References


Figure 1. Effect of PDBu on concentration-response curves of phenylephrine-induced contractions in nonpregnant and pregnant uterine arteries. Data are means ± SEM of tissues from 4 animals.
Nonpregnant

Control
PDBu: 10nM

Pregnant

Control
PDBu: 100nM

Control
PDBu: 30nM

Control
PDBu: 300nM

Control
PDBu: 100nM

Control
PDBu: 1μM
Figure 2. Effect of PDBu on the pD2 value and the maximal response (Tmax) of phenylephrine-induced contractions in nonpregnant and pregnant uterine arteries. Data are means ± SEM of tissues from 4 animals. * P < 0.05, control vs. PDBu treatment.
Figure 3. Effect of PDBu on phenylephrine-induced intracellular free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) and contractions in nonpregnant and pregnant uterine arteries. Changes of tension (ΔTension) and [Ca\(^{2+}\)]\(_i\) (ΔR\(_{1340/380}\)) induced by PDBu and phenylephrine before (PE\(_1\)) and after (PE\(_2\)) PDBu treatment were measured simultaneously in the same tissues in the experimental protocol as described in the Methods. PE\(_3\) depicts the recovery of phenylephrine-induced [Ca\(^{2+}\)]\(_i\) and contractions after washing away PDBu from the tissues. **Top Panel:** Pregnant uterine arteries were treated with 3 μM phenylephrine and 0.1 μM PDBu (n = 3). **Middle Panel:** Nonpregnant uterine arteries were treated with 3 μM phenylephrine and 0.1 μM PDBu (n = 4). **Bottom Panel:** Nonpregnant uterine arteries were treated with 30–200 nM phenylephrine and 0.1 μM PDBu (n = 6). Data are means ± SEM. * P < 0.05, PE\(_1\) vs. PE\(_2\).
Figure 4. PKC isozymes in nonpregnant and pregnant uterine arteries. PKC isozymes were detected by Western immunoblotting and expressed as percentage of the standard of each isozymes, blotted in the same membrane. Data are means ± SEM of tissues from 4 animals. * P < 0.05, pregnant vs. nonpregnant.
The diagram shows the expression levels of different types of PKC isoforms in pregnant and nonpregnant states. The horizontal bars represent the standard intensity of each PKC isoform, with the pregnant and nonpregnant states compared.

- **PKCα**: The expression levels in both states are similar.
- **PKCβI**: There is a significant increase in the pregnant state.
- **PKCβII**: The expression level in the pregnant state is higher than in the nonpregnant state.
- **PKCδ**: The expression is significantly higher in the pregnant state.
- **PKCε**: No significant difference is observed between the states.
- **PKCζ**: There is a slight increase in the pregnant state.
- **PKCδ**: A slight increase is also observed in the pregnant state.

The bars with asterisks indicate statistically significant differences between the states.
Figure 5. Subcellular distribution of PKC isozymes in nonpregnant and pregnant uterine arteries. Cytosolic (S) and particulate (P) fractions were prepared from the uterine arteries as described in the Methods. PKC isozymes were detected by Western immunoblotting and expressed as the ratio of particulate/cytosolic fractions. Data are means ± SEM of tissues from 4 animals. * P < 0.05, pregnant vs. nonpregnant.
CHAPTER THREE

REGULATION OF $\alpha_1$-ADRENOCEPTOR-MEDIATED CONTRACTION OF THE UTERINE ARtery BY PKC: ROLE OF THE THICK AND THIN FILAMENT REGULATORY PATHWAYS

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Abstract

Previously we demonstrated that activation of protein kinase C (PKC) enhanced \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant uterine arteries (NPUA) by increasing the \(\text{Ca}^{2+}\) sensitivity, but inhibited the contractions in pregnant uterine arteries (PUA) by decreasing intracellular \(\text{Ca}^{2+}\) mobilization. The present study tested the hypothesis that PKC activation differentially regulated the thick- and thin-filament regulatory pathways in \(\alpha_1\)-adrenoceptor-induced contractions of NPUA and PUA in sheep. Simultaneous measurements of contractions and phosphorylation levels of 20-kDa regulatory myosin light chain (LC\(_{20}\)) in the same tissue revealed that the PKC activator phorbol 12, 13-dibutyrate (PDBu) inhibited phenylephrine-induced phosphorylation of LC\(_{20}\) and contractions in PUA. In NPUA, PDBu significantly potentiated phenylephrine-induced contractions without significantly changing phosphorylation levels of LC\(_{20}\). Further studies in NPUA demonstrated that PDBu-mediated potentiation of phenylephrine-induced contractions was associated with a significant increase in phosphorylation levels of ERK\(_{42/44}\) and caldesmon-Ser\(^{789}\), measured simultaneously with the tension in the same tissue. In addition, the ERK\(_{42/44}\) inhibitor PD098059 and the actin polymerization inhibitor cytochalasin B produced a concentration-dependent inhibition of PDBu-mediated potentiation of phenylephrine-induced contractions in NPUA. The results suggest that activation of PKC inhibit \(\alpha_1\)-adrenoceptor-mediated contractions in PUA through down-regulation of the thick filament pathway and decreased myosin light chain phosphorylation, but enhance the contractions in NPUA through its effect on the thin filament regulatory pathway and activation of ERK/caldesmon and actin polymerization.
Introduction

Smooth muscle contraction is regulated through changes in intracellular Ca\(^{2+}\) mobilization and the Ca\(^{2+}\) sensitivity of the contractile apparatus. An increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) leads to activation of myosin light chain kinase and subsequent phosphorylation of 20-kDa regulatory light chain of myosin (LC\(_{20}\)) and contraction (Horowitz et al., 1996; Pfitzer, 2001), a mechanism termed Ca\(^{2+}\)-dependent thick-filament regulation. The Ca\(^{2+}\) sensitivity, defined by force production per unit changes in [Ca\(^{2+}\)]\(_i\), is another important mechanism to regulate vascular contractility. Regulation of the Ca\(^{2+}\) sensitivity of myofilaments is through both Ca\(^{2+}\)-independent thick-filament regulatory pathway that involves inactivation of myosin light chain phosphatase resulting in an increase in LC\(_{20}\) phosphorylation independent of changes in [Ca\(^{2+}\)]\(_i\) (Somlyo and Somlyo, 2003), and the thin-filament regulatory pathway that leads to an increase in contraction independent of changes in LC\(_{20}\) phosphorylation (Morgan and Gangopadhyay, 2001).

Recently, we demonstrated that activation of protein kinase C (PKC) enhanced \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant uterine arteries by increasing the Ca\(^{2+}\) sensitivity, but inhibited the contractions in pregnant uterine arteries by decreasing intracellular Ca\(^{2+}\) mobilization (Zhang et al., 2006). These findings present an intriguing dichotomy in the mechanisms of PKC in the regulation of uterine artery contractility at different physiological states, i.e., pregnancy and nonpregnancy. It is not known to what extent that the thick- and/or thin-filament regulatory pathways contribute to the dissociate mechanisms of PKC in the regulation of \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries. The previous finding that the PKC activator
PDBu decreased phenylephrine-induced Ca\(^{2+}\) mobilization in pregnant uterine arteries (Zhang et al., 2006) would suggest an inhibition of the thick filament pathway. This hypothesis needs to be tested by measuring phosphorylation levels of LC\(_{20}\) in the uterine arteries.

In addition to the thick filament regulation, previous studies demonstrated the importance of thin filament regulatory pathway in PKC-mediated regulation of the Ca\(^{2+}\) sensitivity and contractions in the uterine arteries (Xiao et al., 2004, Xiao and Zhang, 2005). Among other mechanisms, caldesmon functions as a thin filament regulatory protein inhibiting smooth muscle contractions at given levels of [Ca\(^{2+}\)]\(_i\) and LC\(_{20}\) phosphorylation, and phosphorylation of caldesmon reverses its inhibitory effect (Katsuyama et al., 1992; Matsumura and Yamashiro, 1993; Morgan and Gangopadhyay, 2001; Wier and Morgan, 2003). In intact vascular smooth muscle, extracellular signal-regulated kinase\(_{42/44}\) (ERK\(_{42/44}\)) has been demonstrated as a physiologically relevant caldesmon kinase that mediates caldesmon phosphorylation (Adam et al., 1989). It has been proposed that ERK\(_{42/44}\)-mediated phosphorylation of caldesmon reverses the inhibitory activity of caldesmon on actin-activated myosin ATPase, hence increasing contractions at given levels of [Ca\(^{2+}\)]\(_i\) and LC\(_{20}\) phosphorylation (Horowitz et al., 1996; Morgan and Gangopadhyay, 2001). In the uterine arteries, the activation of PKC produced time-dependent increases in phosphorylation of ERK\(_{42/44}\) and ERK\(_{42/44}\)-dependent phosphorylation of caldesmon at Ser\(^{789}\) (Xiao et al., 2004). Whether and to what extent these thin-filament mechanisms contribute to PKC-mediated enhancement of \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant uterine arteries remain unclear.
The present study tested the hypothesis that the different effects of PKC activation on \( \alpha_1 \)-adrenoceptor-induced contractions observed in nonpregnant and pregnant uterine arteries were due in part to its differential regulations on the thick- and thin-filament regulatory pathways. To test the hypothesis, we first determined the relation between tension and LC20 phosphorylation by measuring tension development and phosphorylation levels of LC20 simultaneously in the same tissue in the presence of PDBu and phenylephrine. We then determined the role of ERK42/44 in the effect of PKC on phenylephrine-induced contractions by measuring phosphorylation levels of ERK42/44 and caldesmon-Ser\(^{789}\) simultaneously with contractions induced by PDBu and phenylephrine. To determine the cause and effect relation between the activation of ERK42/44 and PKC's effect on phenylephrine-induced contractions, we examined the contractions in the presence of the ERK42/44 inhibitor PD098059. Given that actin polymerization plays an important role in the thin filament regulatory pathway, we also examined the effect of cytochalasin B, an inhibitor of actin polymerization, on PKC-mediated enhancement of \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant uterine arteries.
Materials and Methods

**Tissue preparation.** Nonpregnant and near-term pregnant (~140 day gestation) ewes were anesthetized with thiamylal (10 mg/kg), administered via the external left jugular vein. The ewes were then intubated, and anesthesia was maintained with 1.5–2.0% halothane in O₂ throughout the surgery. An incision was made in the abdomen to expose the uterus. The uterine arteries were isolated and removed without stretching and were placed in a modified Krebs’ solution (pH 7.4) of the following composition (in mM): 115.2 NaCl, 4.7 KCl, 1.80 CaCl₂, 1.16 MgSO₄, 1.18 KH₂PO₄, 22.14 NaHCO₃, 0.03 EDTA, and 7.88 dextrose. The Krebs’ solution was oxygenated with a mixture of 95%O₂-5% CO₂. The third (nonpregnant) and fourth (pregnant) branches of the main uterine arteries with similar external diameter were collected and used in the present studies, as previously described (Zhang et al., 2006). After the tissues were removed, animals were killed with T-61 euthanasia solution (Hoechst-Roussel; Somerville, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Simultaneous measurement of tension and LC₂₀ phosphorylation.** LC₂₀ phosphorylation and contractile tension were measured simultaneously in the same tissue of nonpregnant and pregnant uterine arteries, as previously described (Xiao et al., 2004). Briefly, pregnant and nonpregnant uterine arterial rings were equilibrated in the tissue bath and the optimal tension was obtained. The tissues were then treated with phenylephrine in the absence or presence of pretreatment with the PKC activator PDBu
for 10 min. In our previous studies of simultaneous measurement of [Ca\(^{2+}\)], and contractions (Zhang et al., 2006), we demonstrated that pretreatment of PDBu inhibited 3 \(\mu\)M phenylephrine-induced increase in [Ca\(^{2+}\)], and contractions in pregnant uterine arteries. In nonpregnant uterine arteries, PDBu had no effect on phenylephrine-induced contractions at the concentration of 3 \(\mu\)M but potentiated it at lower concentrations of phenylephrine. To be consistent with the previous studies, the concentrations of phenylephrine used in the present study were 3 \(\mu\)M and 1 \(\mu\)M for pregnant and nonpregnant uterine arteries, respectively. As the previous studies demonstrated that 0.1 \(\mu\)M and 1 \(\mu\)M of PDBu produced the maximal effect in nonpregnant and pregnant uterine arteries, respectively (Zhang et al., 2006), these concentrations were used for PDBu in the present study. Tensions developed were continuously recorded with an on-line computer. To measure phosphorylation levels of LC\(_{20}\) simultaneously in the same tissue, arterial rings were snap frozen with liquid N\(_2\)-cooled clamps at the plateau of tension, and were rapidly immersed in a dry ice-acetone slurry that contained a 10% trichloroacetic acid (TCA) and 10 mM DTT mixture. Tissues were then stored at -80°C until analysis of phosphorylated LC\(_{20}\). Tissue LC\(_{20}\) phosphorylation levels were measured as described previously (Xiao et al., 2004). Tissues were brought to room temperature in a dry ice-acetone-TCA-DTT mixture and washed three times with ether to remove the TCA. Tissues were then extracted in 100\(\mu\)l of sample buffer (pH 8.6) that contained 20 mM Tris base and 23 mM glycine, 8.0 M urea, 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue. Samples (20\(\mu\)l) of tissue extraction were electrophoresed at 12 mA for 2.5 h after a 30 min prerun in 1.0 mm mini-polyacrylamide gels that contained 10% acrylamide, 0.27% bisacrylamide, 40% glycerol, and 20 mM Tris base (pH 8.8). Proteins
were transferred to nitrocellulose membranes and subjected to immunoblot with a specific monoclonal anti-LC20 antibody (1:500). Goat anti-mouse IgM conjugated with horseradish peroxidase was used as a secondary antibody (1:2,000). Bands were detected using enhanced chemiluminescence, visualized on films, and analyzed using Kodak electrophoresis documentation and analysis system and Kodak 1D image analysis software. Phosphorylated LC20 were expressed as percentage of the intensity of the phosphorylated LC20 band over the sum of the phosphorylated plus the unphosphorylated LC20 bands.

**Simultaneous measurement of tension and phosphorylation of ERK42/44 and caldesmon-Ser**. Phosphorylation levels of caldesmon-Ser and ERK42/44 and contractions were measured simultaneously in the same tissue of nonpregnant uterine arteries. Uterine arterial rings were equilibrated in the tissue bath and the optimal tensions were obtained. Tissues were then subjected to stimulation with 1 µM phenylephrine in the absence or presence of pretreatment with 0.1 µM PDBu. Tensions developed were continuously recorded with an on-line computer. To measure phosphorylation levels of caldesmon-Ser and ERK42/44 simultaneously in the same tissue, arterial rings were snap frozen at the plateau of tension, as described above. Tissues were then homogenized in an ice-cold lysis buffer (pH 7.5) that contained Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, 0.2% Triton X-100, β-mercaptoethanol 10 mM, benzamidine 1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, leupetin 50 µM, dithiothreitol 1 mM and aprotinin 2 µg/ml. Homogenates were centrifuged at 6,000 g for 5 min at 4 °C and the supernatants were collected. Proteins were determined with a protein assay kit (Bio-Rad). Samples with 5 µg protein were subjected to electrophoresis.
on 7.5% (phosphorylated caldesmon-Ser\textsuperscript{789}, p-CaD-Ser\textsuperscript{789}) or 10% (phosphorylated ERK_{42/44}, p-ERK_{42/44}) sodium dodecylsulfate-polyacrylamide gel, and then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated at room temperature for 1 h in Tris-buffered saline solution (TBS) that contained 5% dried milk and 0.5% Tween 20, followed by incubation with primary anti-p-CaD-Ser\textsuperscript{789} or anti-p-ERK_{42/44} antibodies overnight at 4 °C and secondary antibody of anti-rabbit IgG for 1 h at room temperature. Bands were detected using enhanced chemiluminescence (ECL), visualized on Hyperfilm, and analyzed with the Kodak 1D image analysis software.

**Contraction studies.** Nonpregnant uterine arteries were dissected 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 (Zhang et al, 2006). Briefly, tissues were equilibrated for 60 min and then gradually stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. Contractile tensions were recorded with an online computer. Tissues were then stimulated with cumulative additions of phenylephrine in approximate one-half log increments to produce a concentration-response curve. After washing away phenylephrine, tissues were relaxed to the baseline and were recovered at the resting tension for 30 min. The second concentration-response curves of phenylephrine-induced contractions were then repeated in the presence of PDBu (0.1 μM for 10 min) with or without the ERK inhibitor PD098059 (10, 30, 60 μM, for 20 min) or the actin polymerization inhibitor cytochalasin B (5, 10, 30 μM, for 20 min).

**Materials.** Phenylephrine, PDBu, PD098059, cytochalasin B and monoclonal anti-LC\textsubscript{20} antibody were obtained from Sigma (St. Louis, MO). Anti-p-CaD-Ser\textsuperscript{789}
antibody and the secondary antibodies (goat anti-mouse IgM and anti-rabbit IgG conjugated with horseradish peroxides) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-ERK_{42/44} antibody was from Cell signaling Technology (Beverly, MA). All other electrophoretic and immunoblot reagents were from Bio-Rad (Hercules, CA). General laboratory reagents were of analytical grade or better, and were purchased from Sigma and Fisher Scientific. All drug solutions were prepared freshly each day and kept on ice throughout the experiment.

**Data analysis.** Data were expressed as means ± SEM. Differences were evaluated for statistical significance (P < 0.05) using one-way ANOVA followed by Neuman-Keuls post hoc testing. For the analysis of concentration-response curves, data were analyzed by computer-assisted nonlinear regression to fit the data using GraphPad Prism (GraphPad software; San Diego, CA). Values of pD₂ (-log EC₅₀) and the maximal response obtained were used in the statistical analysis.
Results

Effect of PDBu on phenylephrine-induced LC20 phosphorylation and contractions. Fig. 1 shows the effects of PDBu on phenylephrine-induced LC20 phosphorylation and contractions, measured simultaneously in the same tissue, in nonpregnant and pregnant uterine arteries. In both nonpregnant and pregnant uterine arteries, phenylephrine induced contractions with corresponding increases in LC20 phosphorylation in the same tissue. In contrast, PDBu increased contractions in the absence of any significant changes of phosphorylation levels of LC20. These findings were consistent with the previous results that showed LC20 phosphorylation-dependent, and -independent contractions caused by phenylephrine and PDBu, respectively, in the uterine arteries (Xiao and Zhang, 2005). In pregnant uterine arteries, PDBu significantly decreased phenylephrine-induced contractions from 70.9 ± 6.6 to 45.5 ± 6.5% K+ maximum (P < 0.05), with a corresponding reduction of phenylephrine-induced LC20 phosphorylation levels from 30.7 ± 1.8 to 13.5 ± 1.7 % (P < 0.05) (Fig. 1). In contrast, in nonpregnant uterine arteries, PDBu significantly enhanced phenylephrine-induced contractions from 10.5 ± 2.4 to 54.0 ± 15.8 % K+ maximum (P < 0.05), consistent with the previous finding (Zhang et al., 2006). However, phenylephrine-induced phosphorylation levels of LC20, measured simultaneously in the same tissue, were not significantly affected with PDBu (Fig. 1).

Effect of PDBu on phenylephrine-induced contractions and phosphorylation of ERK42/44 and caldesmon-Ser789. The findings that PDBu inhibited phenylephrine-induced Ca2+ mobilization (Zhang et al., 2006) and LC20 phosphorylation (the present finding,
Fig. 1) in the pregnant uterine arteries, indicate that the inhibition of phenylephrine-induced contractions by PDBu is primarily mediated by its down-regulation of Ca\(^{2+}\)-dependent thick-filament regulation in the pregnant uterine arteries. In contrast, the finding in nonpregnant uterine arteries that PDBu enhanced phenylephrine-induced contractions in the absence of changes in LC\(_{20}\)phosphorylation suggests a thin filament mechanism. Thus we further determined the role of ERK activation as a thin filament mechanism in PDBu-mediated potentiation of phenylephrine-induced contractions in nonpregnant uterine arteries. Phenylephrine-induced contractions and phosphorylation of ERK\(_{42/44}\) and caldesmon-Ser\(_{789}\) were measured simultaneously in the same tissue. Fig. 2 shows that 0.1 \(\mu\)M PDBu and 1 \(\mu\)M phenylephrine alone had no significant effects on phosphorylation levels of ERK\(_{42/44}\). However, pretreatment of tissues with PDBu resulted in significant increases in phenylephrine-induced phosphorylation of ERK\(_{44}\) (1.9 ± 0.2 to 4.6 ± 0.7 fold of control, \(P < 0.05\)) and ERK\(_{42}\) (1.7 ± 0.2 to 4.9 ± 0.3 fold of control, \(P < 0.05\)), respectively (Fig. 2). These corresponded with a comparable increase in phenylephrine-induced contractile tension, measured simultaneously in the same tissue (Fig. 2).

Phosphorylation levels of caldesmon-Ser\(_{789}\), measured in the same tissue, were shown in Fig. 3. PDBu and phenylephrine significantly increased caldesmon-Ser\(_{789}\) phosphorylation levels 3.0 ± 0.2 fold (\(P < 0.05\)) and 3.9 ± 0.3 fold (\(P < 0.05\)), respectively. In addition, PDBu significantly increased phenylephrine-induced phosphorylation levels of caldesmon-Ser\(_{789}\) (Fig. 3).

**Effect of inhibition of ERK\(_{42/44}\) and actin polymerization on PDBu-potentiated phenylephrine-induced contractions.** The finding that PDBu significantly increased phenylephrine-induced phosphorylation of ERK\(_{42/44}\) that correlated with the increased
tension development (Fig. 3) suggests a role for ERK_{42/44} as a thin filament mechanism in PDBu-mediated potentiation of phenylephrine-induced contractions. To determine the cause and effect relation, we examined the effect of ERK_{42/44} inhibition on PDBu-mediated potentiation of phenylephrine-induced contractions in nonpregnant uterine arteries. Fig. 4 shows that phenylephrine produced concentration-dependent contractions of nonpregnant uterine arteries. In the presence of 0.1 μM PDBu, the concentration-response curve of phenylephrine-induced contractions was markedly shifted to the left with a significant increase in the \( pD_2 \) value from 5.2 ± 0.1 to 7.7 ± 0.3 (\( P < 0.05 \)), representing an over 300-fold increase in the potency of phenylephrine-induced contractions. Pretreatment of tissues with the ERK inhibitor PD098059 (10, 30, and 60 μM) produced a concentration-dependent inhibition of PDBu-mediated potentiation of phenylephrine-induced contractions (Fig. 4). Given that actin polymerization may play an important role in PKC/ERK_{42/44}-mediated thin filament regulation, we also examined the effect of cytochalasin B, an inhibitor of actin polymerization, on PDBu-mediated enhancement of phenylephrine-induced contractions in nonpregnant uterine arteries. As shown in Fig. 5, pretreatment of tissues with cytochalasin B (5, 10, and 30 μM) produced a concentration-dependent inhibition of PDBu-mediated potentiation of phenylephrine-induced contractions.
Discussion

The present study examined the mechanisms of PKC in its differential regulations of \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries, and offers the following new findings: 1) activation of PKC inhibits \( \alpha_1 \)-adrenoceptor-induced contractions in the pregnant uterine artery through a down-regulation of Ca\(^{2+}\)-dependent thick-filament pathway with decreased phosphorylation levels of LC\(_{20}\); 2) PKC activation enhances \( \alpha_1 \)-adrenoceptor-induced contractions in the nonpregnant uterine artery primarily through thin filament regulatory mechanisms in the absence of changes in LC\(_{20}\) phosphorylation; 3) activation of ERK\(_{42/44}\) is an important thin filament mechanism in PKC-mediated potentiation of \( \alpha_1 \)-adrenoceptor-induced contractions in the nonpregnant uterine artery; and 4) actin polymerization plays an important role in PKC/ERK\(_{42/44}\)-mediated thin filament regulation of \( \alpha_1 \)-adrenoceptor-induced contractions in the nonpregnant uterine artery.

We have previously demonstrated that activation of PKC enhances \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant uterine arteries by increasing the Ca\(^{2+}\) sensitivity, but inhibits the contractions in pregnant uterine arteries by decreasing intracellular Ca\(^{2+}\) mobilization (Zhang et al., 2006). The present follow-up study attempted to elucidate the role of the thick- and/or thin-filament regulatory pathways that contribute to the dichotomy of PKC mechanisms in the regulation of \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries. In the uterine artery, \( \alpha_1 \)-adrenoceptor-mediated contractions are regulated through both thick-filament regulatory pathway (\textit{i.e.} LC\(_{20}\) phosphorylation dependent) and thin-filament regulatory
pathway (i.e. LC20 phosphorylation independent) (Xiao et al., 2004, Xiao and Zhang, 2005). In agreement with the previous findings, the present study showed that phenylephrine induced contractions with corresponding increases in phosphorylation of LC20 in both pregnant and nonpregnant uterine arteries. In contrast, PDBu produced contractions in the absence of significant changes in LC20 phosphorylation in the uterine arteries. This is consistent with the previous studies that demonstrated dissociation between LC20 phosphorylation and tension development in response to phorbol esters in vascular smooth muscle including the uterine arteries (Fujiwara et al., 1988; Sutton and Haeberle, 1990; Laporte et al., 1994; Xiao and Zhang, 2005). Most importantly, the present study showed that PDBu-mediated inhibition of phenylephrine-induced contractions in pregnant uterine arteries was associated with a significant decrease in phosphorylation of LC20, measured simultaneously in the same tissue, but PDBu potentiated phenylephrine-induced contractions in nonpregnant uterine arteries in the absence of any significant changes in LC20 phosphorylation. Given the previous finding that PDBu inhibited α1-adrenoceptor-mediated increases in [Ca2+]i in pregnant uterine arteries (Zhang et al., 2006), the present study suggests that the inhibitory effect of PKC activation on α1-adrenoceptor-induced contractions in pregnant vessels was mediated by suppressing the Ca2+-dependent thick-filament pathway. The finding of the dissociation between LC20 phosphorylation and PDBu-mediated increases in phenylephrine-induced contractions in nonpregnant arteries indicates a thin filament mechanism in the PKC-mediated regulation of α1-adrenoceptor-induced contractions in nonpregnant uterine arteries. The finding that PKC has differential regulatory effects on thick- and thin-filament pathways in α1-adrenoceptor-induced contractions in nonpregnant and pregnant
uterine arteries is intriguing, and provides insights in understanding adaptation of uterine vascular contractile mechanisms in pregnancy. Consistent with the present study, previous studies suggested a transition from thin filament to thick filament regulatory mechanisms in the uterine artery during pregnancy, and that the Ca$^{2+}$-dependent thick-filament pathway, \textit{i.e.} changes in LC$_{20}$ phosphorylation, predominated in $\alpha_1$-adrenoceptor-mediated contractions in pregnant uterine arteries (Annibale et al., 1989, 1990; Xiao et al., 2004; Xiao and Zhang, 2005).

The present finding that PDBu significantly increased phenylephrine-induced phosphorylation of ERK$_{42/44}$ that correlated with the increased tension development, measured simultaneously in the same tissue, suggests a role for ERK$_{42/44}$ activation as a thin filament mechanism in PDBu-mediated potentiation of phenylephrine-induced contractions in nonpregnant uterine arteries. Activation of ERK$_{42/44}$ is dependent on a dual-phosphorylation on Tyr$^{185}$ and Thr$^{187}$ by mitogen-activated/extracellular-regulated kinase kinase or MEK (Anderson et al., 1990). ERK$_{42/44}$ has been proposed to regulate smooth muscle contractions (Adam et al., 1995; Katoch and Moreland, 1995; Watts, 1996; Dessy et al., 1998; Xiao and Zhang, 2002; Zhao et al., 2003). In intact vascular smooth muscle, ERK has been demonstrated as a physiologically relevant caldesmon kinase that mediates caldesmon phosphorylation (Adam et al., 1989). Caldesmon functions as a thin filament regulatory protein and exerts an inhibitory effect on vascular smooth muscle contractions (Nagi and Walsh, 1984; Earley et al., 1998; Morgan and Gangopadhyay, 2001). It has been proposed that ERK-mediated phosphorylation of caldesmon reverses the inhibitory activity of caldesmon on actin-activated myosin ATPase, hence activating the thin filament pathway (Horowitz et al., 1996; Morgan and Gangopadhyay, 2001). The finding
that low concentrations of PDBu (0.1 μM) and phenylephrine (1 μM) alone produced small but not significant increases in ERK_{42/44} phosphorylation, but increased caldesmon phosphorylation at the ERK_{42/44}-specific site Ser^{789} is intriguing, and suggests a typical phenomenon that small signal is amplified through the intracellular signaling cascades. Most importantly, the present study demonstrated that PDBu was able to enhance phenylephrine-induced phosphorylation of both ERK_{44/42} and caldesmon-Ser^{789} and contractions in the same tissue. The cause and effect relation between activation of ERK_{42/44} and the enhancement of contractions in PKC-mediated effect was demonstrated with the present study showing that inhibition of ERK_{42/44} with PD098059 abolished PDBu-mediated increases in phenylephrine-induced contractions. Previous studies demonstrated that PD098059 inhibited phosphorylation of ERK_{44/42} and caldesmon-Ser^{789} in the uterine artery (Xiao et al., 2004). It should be noted that although only phosphorylation of caldesmon-Ser^{789} was determined in the present study because of the availability of the antibody, caldesmon can be phosphorylated at several other sites. It has been shown that PKC phosphorylates sheep aorta caldesmon both in native thin filaments and in the isolated state at multiple sites of Ser^{127}, Ser^{587}, Ser^{600}, Ser^{657}, Ser^{686} and Ser^{726}, and PKC-mediated phosphorylation of both intact caldesmon and of its C-terminal fragment containing 658-756, significantly decreases its ability to inhibit acto-heavy meromyosin ATPase (Vorotnikov et al., 1994). Whether and to what extent these phosphorylation sites contribute to PKC-mediated potentiation of α1-adrenoceptor-induced contractions in the uterine arteries remains an intriguing question for further studies.
In addition to its role in inhibiting actin-activated myosin ATPase, caldesmon has been shown to be important in maintaining actin filament stability and inhibiting Arp2/3-dependent actin polymerization (Galazkiewicz et al., 1989; Matsumura and Yamashiro, 1993; Yamakita et al., 2003; Hai and Gu, 2006). The inhibitory effect of caldesmon on actin polymerization can be reversed by phosphorylation with either ERK_{44/42} or cdc2 kinase (Yamakita et al., 2003; Hai and Gu, 2006). In addition, ERK_{44/42} can phosphorylate an actin-binding protein, cortactin, resulting in activation of the Arp2/3 complex and actin polymerization (Martinez-Quiles et al., 2004). It has been demonstrated that the polymerization of actin filaments from monomeric globular actin (G-actin) to filamentous actin (F-actin), occurring independent of changes in intracellular Ca^{2+} concentrations and LC_{20} phosphorylation, is an important cellular mechanism of thin filament regulation in smooth muscle contraction (Jones et al., 1999; Metha and Gunst, 1999; Gunst and Fredberg, 2003; Ozaki et al., 2004), including contractions induced by PDBu and α_{1}-adrenoceptor agonists (Nunes, 2002; Tang and Tan, 2003; Zhao et al., 2004; Chen et al., 2006). The present finding that inhibition of actin polymerization with cytochalasin B produced a concentration-dependent inhibition of PDBu-mediated potentiation of phenylephrine-induced contractions in nonpregnant uterine arteries suggests a thin filament mechanism of actin polymerization in PKC-mediated regulation of α_{1}-adrenoceptor-induced contractions. It has been demonstrated that the inhibition of contractions by actin polymerization inhibitors does not result from the disruption of thick-filament regulatory pathway (Metha and Gunst, 1999; Obara and Yabu, 1994).

In summary, the present study demonstrated that PKC activation by PDBu differentially regulated α_{1}-adrenoceptor-mediated thick- and thin-filament pathways and
resulted in the opposite effects on α₁-adrenoceptor-mediated contractions in pregnant and nonpregnant uterine arteries. The findings of a transition from a thin- to thick filament mechanism in PKC-mediated regulation of α₁-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries and a predominant inhibitory effect of PKC on α₁-adrenoceptor-mediated, Ca²⁺-dependent thick-filament regulatory pathway in the pregnant uterine arteries, provide insights into the adaptation of uterine artery contractile mechanisms in pregnancy. Further studies are needed to investigate the mechanisms of steroid hormones in PKC-regulated α₁-adrenoceptor signaling pathways in the uterine arteries and their adaptation to pregnancy.
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References


Figure 1. Effect of PDBu on phenylephrine-induced LC$_{20}$ phosphorylation and contractions in nonpregnant and pregnant uterine arteries. PDBu and/or phenylephrine (PE)-induced LC$_{20}$ phosphorylation and contractions were measured simultaneously in the same tissues as described in the Methods. **Top panel and middle panels:** Phosphorylated (p-LC$_{20}$) and unphosphorylated (unp-LC$_{20}$) LC$_{20}$ were detected by Western immunoblotting and levels of p-LC$_{20}$ are expressed as percentage of p-LC$_{20}$/ (p-LC$_{20}$ + unp-LC$_{20}$). **Bottom Panel:** Simultaneously measured contractions are expressed as percentage of 120 mM KCl-induced contraction (% K$^+$). Data are means ± SEM of tissues from 4 animals. a: P < 0.05, vs. control. b: P<0.05, vs. phenylephrine treatment alone.
Figure 2. Effect of PDBu on phenylephrine-induced phosphorylation of ERK$_{42/44}$ and contractions in nonpregnant uterine arteries. PDBu and/or phenylephrine (PE)-induced phosphorylation of ERK$_{42/44}$ and contractions were measured simultaneously in the same tissue as described in the Methods. **Top and middle panels:** Phosphorylated ERK$_{42}$ and ERK$_{44}$ (p-ERK$_{42}$ and p-ERK$_{44}$) were detected by Western immunoblotting. The levels of p-ERK$_{42}$ and p-ERK$_{44}$ induced by PDBu and/or phenylephrine are expressed as fold of control. **Bottom Panel:** Simultaneously measured contractions are expressed as percentage of 120 mM KCl-induced contraction (% K$^+$). Data are means ± SEM of tissues from 4 animals. a: P < 0.05, vs. control. b: P < 0.05, vs. PDBu plus phenylephrine treatment.
Cont. PDBu PE PE&PDBu

p-ERK_{44} -
p-ERK_{42} -

Net Intensity (fold of control)

Cont. PDBu PE PDBu&PE Cont. PDBu PE PDBu&PE

p-ERK44  p-ERK42

Tension (% K')

Cont. PDBu PE PDBu&PE
Figure 3. Effect of PDBu on phenylephrine-induced phosphorylation of caldesmon-Ser789 in nonpregnant uterine arteries. PDBu and/or phenylephrine (PE)-induced phosphorylation levels of caldesmon-Ser789 were measured in the same tissues collected in Fig. 2. Phosphorylated caldesmon-Ser789 (p-CaD-Ser789) was detected by Western immunoblotting and expressed as fold of control. Data are means ± SEM of tissues from 4 animals. a: P < 0.05, vs. control. b: P < 0.05, vs. PDBu plus phenylephrine treatment.
$p$-CaD-Ser$^{789}$
(fold of control)

![Bar chart showing the fold of control for different conditions: Cont, PDBu, PE, and PDBu&PE. The chart indicates significant differences between the conditions, with PDBu&PE showing the highest fold of control.](image)
Figure 4. Effect of ERK inhibition on PDBu-potentiated phenylephrine-induced contractions in nonpregnant uterine arteries. Panel A. Effect of PDBu and/or ERK inhibitor PD098059 (10, 30, 60 μM) on concentration-response curves of phenylephrine-induced contractions. Panel B. Data analysis of the pD₂ values and the maximal responses (T max) of phenylephrine-induced contractions. Phenylephrine-induced contractions are expressed as percentage of 120 mM KCl-induced contraction (% K⁺). Data are means ± SEM of tissues from 5–9 animals. a: P < 0.05, vs. control. b: P < 0.05, vs. PDBu alone.
Figure 5. **Effect of inhibition of actin polymerization on PDBu-potentiated phenylephrine-induced contractions in nonpregnant uterine arteries.** Panel A. Effect of PDBu and/or actin polymerization inhibitor Cytochalasin B (5, 10, 30 μM) on concentration-response curves of phenylephrine-induced contractions. Panel B. Data analysis of the pD₂ values and the maximal responses (Tₘₐₓ) of phenylephrine-induced contractions. Phenylephrine-induced contractions are expressed as percentage of 120 mM KCl-induced contraction (% K⁺). Data are means ± SEM of tissues from 5–9 animals. a: P < 0.05, vs. control. b: P < 0.05, vs. PDBu alone.
CHAPTER FOUR

ROLE OF PKC ISOZYMES IN THE REGULATION OF $\alpha_1$-ADRENOCEPTOR - MEDIATED CONTRACTIONS IN THE UTERINE ARTERIES

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Abstract

Previously we demonstrated that activation of protein kinase C (PKC) enhanced $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine arteries (NPUA), but inhibited the contractions in pregnant uterine arteries (PUA). The present study tested the hypothesis that differential regulation of PKC isozyme activities contributes to the different effects of PDBu on $\alpha_1$-adrenoceptor-mediated contractions between the pregnant and nonpregnant uterine arteries. Phenylephrine-induced contractions of ovine NPUA and PUA were determined in the absence or presence of the PKC activator phorbol 12, 13-dibutyrate (PDBu), and/or conventional and novel PKC isozymes inhibitor GF109203X, PKC isozyme-selective inhibitory peptides for conventional PKCs, PKC$\beta$I, PKC$\beta$II and PKC$\epsilon$. GF109203X produced a concentration-dependent inhibition of phenylephrine-induced contractions in both NPUA and PUA, and reversed the PDBu-mediated potentiation and inhibition of phenylephrine-induced contractions in NPUA and PUA, respectively. In addition, PKC$\beta$I, PKC$\beta$II and PKC$\epsilon$ inhibitory peptides blocked the PDBu-mediated responses in both NPUA and PUA. Western blot analysis showed that PDBu induced a membrane translocation of PKC$\alpha$, $\beta$I, $\beta$II and $\epsilon$ in PUA, and PKC$\beta$I, $\beta$II and $\epsilon$ in NPUA. The results disprove the hypothesis that the dichotomy of PKC mechanisms in the regulation of $\alpha_1$-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries is caused by the activation of different PKC isozymes, and suggest down-stream mechanisms of differential subcellular distributions for the distinct functional effects of PKC isozymes in the adaptation of uterine arteries to pregnancy.
Introduction

During pregnancy, the uterine artery maintains low resistance to accommodate the large increase in uteroplacental blood flow to ensure normal fetal development. Several mechanisms are involved in the adaptation of uterine artery contractility during pregnancy (Ford, 1995; Rosenfeld, 2001; Xiao and Zhang, 2002; Bird et al., 2003; Xiao and Zhang, 2005). Activation of $\alpha_1$-adrenoceptors is an important mechanism in the regulation of uterine artery smooth muscle contractions (Magness and Rosenfeld, 1986; Stjernquist and Owman, 1990; Ford, 1995; Zhang et al., 1995; Cox et al., 2004). Protein kinase C (PKC) plays a key role in $\alpha_1$-adrenoceptor-mediated contractions of vascular smooth muscle (Minneman, 1988; Nishizuka, 1992; Ford, 1995) and pressure-dependent myogenic tone of the uterine artery (Xiao and Zhang, 2002; Xiao and Zhang, 2005; Xiao et al., 2006). Recently we have demonstrated that activation of PKC by phorbol-12, 13-dibutyrate (PDBu) potentiates $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine arteries, but inhibits the contractions in pregnant uterine arteries (Zhang et al., 2006). In addition, we have shown that differential regulations of the thick and thin filament pathways play an important role in the dichotomy of PKC mechanisms in the regulation of $\alpha_1$-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries (Zhang and Zhang, under review). However, it remains unknown whether and to what extent activation of differential PKC isozymes contributes to these pregnancy-specific effects of PDBu observed in the uterine arteries.

PKC, a serine/threonine kinase family, consists of at least 11 isozymes that are further classified into three subfamilies: the conventional isozymes (PKC$\alpha$, $\beta$, $\gamma$), the novel isozymes (PKC$\delta$, $\varepsilon$, $\eta$, $\theta$), and the atypical isozymes (PKC$\zeta$, $\iota$, $\lambda$). We have recently
demonstrated that PKCα, β, δ, ε, and ζ isozymes express in ovine uterine arteries, with significantly increased expression levels of PKCβ and PKCζ, but decreased PKCα and the basal activity of PKCε in pregnant arteries, as compared with nonpregnant vessels (Zhang et al., 2006). Each PKC isozyme has unique enzymatic properties, substrates, functions, and sub-cellular distributions in different blood vessels and species (Khalil et al., 1992; Nishizuka, 1992; Liou and Morgan, 1994; Horowitz et al., 1996b; Kanashiro and Khalil, 1998; Kanashiro and Khalil, 2001). Despite extensive studies, the physiological role of each individual PKC isozymes in the regulation of vascular contractility, including the uterine artery, remains unclear, which is likely due to a lack of highly selective pharmacological agents to either inhibit or activate the isozymes. The majority of available pharmacological compounds act on more than one PKC isozyme and on other protein kinase(s). Synthesized aminoalkyl bisindolylmaleimide, GF109203X has been demonstrated to be a potent and selective inhibitor of PKC over other protein kinases and has been widely used as a tool to explore the role of PKC in particular signal transduction pathway (Toullec et al., 1991). GF109203X selectively inhibits activity of conventional and novel PKC isozymes (Gailly et al., 1997; Damron et al., 2002; Nakano et al., 2004) and decreases their agonist-induced translocation (Giardina et al., 2001; Sirous et al., 2001; Chu et al., 2003). More recent discovery of PKC isozyme-selective inhibitory peptides (Csukai and Mochly-Rosen, 1999) allows determining the function of particular individual PKC isozyme. Following activation, each PKC isozyme translocates to its unique subcellular sites and binds to isozyme-specific anchoring proteins, receptors for activated C-kinase (RACKs). Each isozyme has a specific RACK-selective binding site that is exposed only after the activation of PKC. PKC isozyme-selective inhibitory
peptides, containing isozyme-specific RACK-binding sites, have been demonstrated to inhibit translocation of the corresponding isozymes and consequently inhibit their isozyme-unique function in cardiac myocytes and cerebral arteries (Csukai and Mochly-Rosen, 1999; Dorn and Mochly-Rosen, 2002; Bright and Mochly-Rosen, 2005).

The present study tested the hypothesis that differential regulation of PKC isozyme activities contributes to the different effects of PDBu on α1-adrenoceptor-mediated contractions between the pregnant and nonpregnant uterine arteries. Concentration-response curves of phenylephrine-induced contractions of the uterine arteries were conducted in the absence or presence of the PKC activator PDBu and/or the PKC inhibitor GF109203X, PKC isozyme-selective inhibitory peptides. In addition, the PDBu-mediated PKC isozyme translocations were determined in the uterine arteries.
Methods

*Tissue preparation.* Nonpregnant and near-term pregnant (~140 day gestation) ewes were anesthetized with thiamylal (10 mg/kg), administered via the external left jugular vein. The ewes were then intubated, and anesthesia was maintained with 1.5–2.0% halothane in O₂ throughout the surgery. An incision was made in the abdomen to expose the uterus. The uterine arteries were isolated and removed without stretching and were placed in a modified Krebs' solution (pH 7.4) of the following composition (in mM): 115.2 NaCl, 4.7 KCl, 1.80 CaCl₂, 1.16 MgSO₄, 1.18 KH₂PO₄, 22.14 NaHCO₃, 0.03 EDTA, and 7.88 dextrose. The Krebs’ solution was oxygenated with a mixture of 95%O₂-5% CO₂. After the tissues were removed, animals were killed with T-61 euthanasia solution (Hoechst-Roussel; Somerville, NJ). All procedures and protocols used in the present study were approved by the Animal Research Committee of Loma Linda University and followed the guidelines in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

*Contraction studies.* The third (nonpregnant) and fourth (pregnant) branches of the main uterine arteries with similar external diameter were used in the present studies, as previously described (Zhang et al., 2006). Uterine arteries were dissected 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 (Zhang et al., 2006). Briefly, each ring was equilibrated for 60 min and then gradually stretched to the optimal resting tension as determined by the tension developed in response to 120 mM KCl added at each stretch level. Tissues were then stimulated with cumulative additions of phenylephrine in
approximate one-half log increments to generate a concentration-response curve, and contractile tensions were recorded with an online computer. After washing away phenylephrine, tissues were relaxed to the baseline and were recovered at the resting tension for 30 min. The second concentration-response curves of phenylephrine-induced contractions were then repeated in the absence or presence of GF109203X (0.1, 0.3, and 1 μM for 20 min), PKC isozyme-selective inhibitory peptides for conventional PKCs, PKCβI, βII or ε (3 μM for 20 min) and/or PDBu (30, 100 nM for nonpregnant uterine arteries, and 0.3, 1 μM for pregnant uterine arteries, for 10 min).

**Measurement of PKC isozymes translocation.** PDBu-stimulated PKC isozymes translocation and contractions were measured simultaneously in the same uterine arteries. Pregnant and nonpregnant uterine artery rings were equilibrated in the tissue bath and the optimal tensions were obtained. Tissues were then subjected to stimulation with PDBu (0.2 μM for nonpregnant, 1 μM for pregnant) for 10 min. At the end of treatment, the tissues were quickly frozen in liquid N2 and cytosolic and particulate fractions were prepared as described previously (Zhang et al., 2006). Briefly, the tissues were homogenized in ice-cold homogenization buffer A containing Tris-HCl 20 mM, sucrose 250 mM, EDTA 5 mM, EGTA 5 mM, β-mercaptoethanol 10 mM, benzamidine 1 mM, phenylmethylsulfonyl fluoride (PMSF) 1 mM, leupetin 50 μM, dithiothreitol 1 mM and aprotinin 2 μg/ml, pH 7.5. The homogenates were centrifuged at 100,000 g for 20 min at 4 °C, and the supernatants were collected and used as the cytosolic fraction. The pellets were re-suspended in homogenization buffer A containing 1% triton X-100 by stirring for overnight at 4 °C, diluted with the buffer A to a final concentration of 0.2% triton X-100, and then centrifuged at 100,000 g for 20 min at 4 °C. The supernatants were collected and
referred to as the particulate fraction. Protein concentrations were determined with a protein assay kit (Bio-Rad). Protein samples (5 μg) of particulate fractions were subjected to electrophoresis on 7.5% sodium dodecylsulfate-polyacrylamide gel, and then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated at room temperature for 1 h in Tris-buffered saline solution (TBS) containing 5% dried milk and 0.5% Tween 20, followed by incubation with primary anti-PKC isozyme antibodies overnight at 4 °C and secondary antibody for 1 h at room temperature. Polyclonal antibodies to PKCa, βI, βII and ε were used. Bands were detected with enhanced chemiluminiscence (ECL), visualized on Hyperfilm, and analyzed with the Kodak 1D image analysis software. To normalize the loading variation of each sample, the corresponding actin level presented in each sample was determined by using monoclonal anti-actin as primary antibody.

**Materials.** Phenylephrine, PDBu, GF109203X and anti-actin antibody were obtained from Sigma (St. Louis, MO). Anti-PKC isozyme antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The PKC isozyme-selective inhibitory peptides for conventional PKCs, PKCβI, βII and ε were from KAI pharmaceuticals (South San Francisco, CA). All electrophoretic and immunoblot reagents were from Bio-Rad. General laboratory reagents were of analytical grade or better and were purchased from Sigma and Fisher Scientific. All drugs were prepared freshly each day and kept on ice throughout the experiment.

**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. Differences were evaluated for statistical significance (P < 0.05) by one-way ANOVA and Student’s t-test.
Results

Effect of GF109203X on phenylephrine-induced contractions. Fig. 1 shows that phenylephrine produced concentration-dependent contractions of uterine arteries from both nonpregnant and pregnant ewes. In both nonpregnant and pregnant uterine arteries, GF109203X (0.1, 0.3, 1 μM), a selective inhibitor for conventional and/or novel PKC isozymes, produced a concentration-dependent inhibition of phenylephrine-induced contractions. In the presence of 1 μM GF109203X, the concentration-response curve of phenylephrine-induced contractions in nonpregnant uterine artery was shifted to the right with a significant decrease in the pD₂ value from 5.48 ± 0.15 to 4.79 ± 0.10 (P < 0.05). The maximal responses of phenylephrine-induced contractions were not affected with GF109203X (Fig. 1 & Fig. 2). In pregnant uterine arteries, 0.3, 1 μM GF109203X shifted the concentration-response curve of phenylephrine-induced contractions to the right with a significant decrease in the pD₂ values from 6.26 ± 0.07 to 5.77 ± 0.02, and from 6.20 ± 0.08 to 5.40 ± 0.02, respectively (P < 0.05). The maximal responses of phenylephrine-induced contractions were significantly decreased from 185.20 ± 5.73 to 172.33 ± 1.72 % K⁺ maximum (P < 0.05) in the presence of 1 μM GF109203X (Fig. 1 & Fig. 2).

Effect of GF109203X on PDBu-affected phenylephrine-induced contractions. Our previous study demonstrated that PDBu suppressed phenylephrine-induced contractions in pregnant uterine arteries, but potentiated the contractions in nonpregnant uterine arteries (Zhang et al., 2006). To determine whether the effect of PDBu on phenylephrine-induced contractions in uterine arteries is through the activation of PKC, the PDBu-mediated responses were determined in the absence or presence of GF109203X in nonpregnant and pregnant uterine arteries. Consistent with the previous
findings, Fig. 3 shows that in pregnant uterine arteries, PDBu (0.3 and 1 μM) dose-dependently inhibited phenylephrine-induced contractions, with marked decreases in the maximal response. GF109203X significantly inhibited the PDBu-mediated response and reversed its effect on phenylephrine-induced maximal response in the pregnant uterine arteries (Fig. 3). In the presence of 1 μM GF109203X and 0.3 μM or 1 μM PDBu, respectively, the pD2 values of phenylephrine-induced contractions were not significantly different from each other (5.03 ± 0.25 vs. 4.98 ± 0.25, P > 0.05), and were not significantly different from the pD2 value of phenylephrine-induced contractions in the presence of 1 μM GF109203X alone (5.40 ± 0.02, P > 0.05), shown in Fig. 1. In nonpregnant uterine arteries, also consistent with the previous findings (Zhang et al., 2006), PDBu (30 and 100 nM) produced a concentration-dependent potentiation of phenylephrine-induced contractions with significant increases in the pD2 values but no significant effects on the maximal responses (Fig. 4). In the presence of 100 nM PDBu, the concentration-response curve of phenylephrine-induced contractions was markedly shifted to the left with a significant increase in the pD2 value from 5.30 ± 0.08 to 8.45 ± 0.34 (P < 0.05), representing a 3-order of magnitude increase in the potency of phenylephrine-induced contractions. As shown in Fig. 4, in the presence of 1 μM GF109203X and 0.3 μM or 1 μM PDBu, respectively, the pD2 values of phenylephrine-induced contractions were not significantly different from each other (4.81 ± 0.19 vs. 5.05 ± 0.22, P > 0.05), and were not significantly different from the pD2 value of phenylephrine-induced contractions in the presence of 1 μM GF109203X alone (4.79 ± 0.10, P > 0.05), shown in Fig. 1. The maximal responses of phenylephrine-induced
contractions were not significantly different among the treatments in nonpregnant uterine arteries.

**Effect of PKC isozyme-selective inhibitory peptides on PDBu-affected phenylephrine-induced contractions.** To determine the cause and effect relations between individual PKC isozymes and the PDBu-mediated responses, we determined the effects of PKC isozyme-selective inhibitory peptides for conventional PKCs (cPKCs), PKCβI, PKCβII and PKCε on PDBu-mediated effects on phenylephrine-induced contractions in pregnant and nonpregnant uterine arteries. Because these peptides themselves cannot cross biological membranes, they are modified with conjugation of peptide carriers via Cys-Cys bonds to facilitate their transportation through biological membranes into cells. Once in the cells, the Cys-Cys bonds are reduced to enable the exit of the carriers but trapping the peptides inside the cells (Dorn and Mochly-Rosen, 2002). As shown in Fig. 5, in both nonpregnant and pregnant uterine arteries, carrier alone had no significant effects on PDBu-mediated responses on phenylephrine-induced contractions. In nonpregnant uterine arteries, PDBu with the peptide carrier alone significantly increased the pD2 of phenylephrine-induced contractions from 5.22 ± 0.07 to 7.39 ± 0.20 (P < 0.05), which was significantly inhibited by PKC isozyme-selective inhibitory peptides for cPKCs (6.02 ± 0.18), PKCβI (5.47 ± 0.20), PKCβII (5.33 ± 0.31), and PKCε (5.44 ± 0.36), respectively (P < 0.05 for all, as compared with PDBu plus carrier alone). In the presence of PKCβI, PKCβII, and PKCε inhibitory peptides, respectively, the pD2 values of phenylephrine-induced contractions were not significantly different in the absence or presence of PDBu (Fig. 6). In addition, the maximal responses of phenylephrine-induced contractions were not significantly different among the
treatments in nonpregnant uterine arteries (Fig. 6). In pregnant uterine arteries, there were no significant differences in the pD2 values of phenylephrine-induced contractions among the treatments (Fig. 5 and 6). PDBu with the peptide carrier alone significantly decreased the maximal response of phenylephrine-induced contractions to 22.5% of control, which was partially blocked by specific PKCβI (55.3%), PKCβII (43.8%), and PKCε (50.9%) inhibitory peptides, respectively (P < 0.05 for all, as compared with PDBu plus carrier alone) (Fig. 5 and 6). In contrast, cPKCs inhibitory peptide had no significant effect on PDBu-mediated responses in the pregnant uterine arteries.

**PDBu-induced translocation of PKC isozymes in uterine arteries.** In nonpregnant uterine arteries, PDBu significantly increased the levels of PKCβI, PKCβII, and PKCε in membrane particulate fractions to 174.1 ± 23.97%, 358.4 ± 96.86%, and 252.9 ± 84.98% of control (P < 0.05 for all), respectively, without affecting PKCa levels, suggesting that PDBu induced translocation and activation of PKCβI, PKCβII, and PKCe, but not PKCa (Fig. 7). In pregnant uterine arteries, PDBu significantly increased the levels of PKCa, PKCβI, PKCβII, and PKCe in membrane particulate fractions to 237.35 ± 33.0%, 329.03 ± 33.47%, 122.29 ± 7.76%, and 666.12 ± 167.70% of control, respectively (P < 0.05 for all), suggesting that PDBu induces translocation and activation of all these four PKC isozymes in pregnant uterine arteries.
Discussion

The present study demonstrated that PKC activation involved in α₁-adrenoceptor-mediated contractions in ovine uterine arteries, and was responsible for the effects of PDBu on α₁-adrenoceptor-mediated contractions of nonpregnant and pregnant uterine arteries. More importantly, individual isozymes of conventional PKCs, PKCβ and PKCε were identified to be involved in the PDBu-mediated responses.

It has been suggested that PKC play a key role in α₁-adrenoceptor-mediated smooth muscle contractions (Nishimura et al., 1990; Gailly et al., 1997; Buus et al., 1998; Kitazawa et al., 2000; Martinez et al., 2000; Sato et al., 2001). In agreement with the previous findings, the present study showed that GF109203X, an inhibitor for conventional and novel PKC isozymes, concentration-dependently inhibited phenylephrine-induced contractions in both nonpregnant and pregnant uterine arteries, suggesting a role of conventional and novel PKC isozymes in α₁-adrenoceptor-mediated contractions in the uterine artery. The concentrations of GF109203X used in the present study are within the range shown to inhibit PKC in the previous studies (Toullec et al., 1991; Damron et al., 2002; Chu et al., 2003).

Our previous study demonstrated that PDBu inhibited α₁-adrenoceptor-mediated contractions in pregnant uterine arteries (Zhang et al., 2006). In the present study, we found that PDBu-mediated responses were blocked by GF109203X, suggesting a cause-effect relation between PKC activation and PDBu-mediated inhibitory effects in the pregnant uterine arteries. The finding that inhibition of PKC has a dual effect on α₁-adrenoceptor-mediated contractions, i.e., inhibition of α₁-adrenoceptor-mediated contractions and blockade of PDBu-mediated inhibitory effects on α₁-adrenoceptor-
mediated contractions, is intriguing and suggests a two-compartment model of PKC in the regulation of α₁-adrenoceptor-mediated contractions in pregnant uterine arteries. One compartment of PKC may be tightly associated with α₁-adrenoceptors and forms a "signalsome", which is activated by phenylephrine and participates in α₁-adrenoceptor-mediated contractions. The other more diffusely distributed compartment locates distal to α₁-adrenoceptors, which is activated non-selectively by phorbol esters and mediates an inhibitory effect on α₁-adrenoceptor-mediated contractions. It has been well demonstrated that the function of each PKC isozyme depends on the subcellular location and the availability of protein substrates that can be phosphorylated by the isozyme at the site of anchoring (Dorn and Mochly-Rosen, 2002).

The other possibility is that different PKC isozymes may be involved in the regulation of α₁-adrenoceptor-mediated responses. GF109203X is a nonselective PKC inhibitor that blocks both conventional and novel PKC isozymes. We have demonstrated the presence of PKCα, βI, βII, ε, but not γ, isozymes in the uterine arteries (Zhang et al., 2006). In the present study, we have used the selective inhibitory peptides for PKCα, βI, βII, and ε isozymes to determine the role of PKC isozymes in the PDBu-mediated responses. The inhibitory activities of these peptides are obtained at an intracellular concentration of 5-50 nM (Dorn and Mochly-Rosen, 2002). In pregnant uterine arteries, we found that the inhibitory effect of PDBu on α₁-adrenoceptor-induced contractions was partially, but significantly reversed by inhibition of PKCβI, βII and ε, respectively, suggesting an involvement of these isozymes in the PDBu-mediated inhibitory effect. Previous studies have shown that overexpression of PKCβ inhibits agonist-induced Ca^{2+} mobilization, and inhibition of PKCβ results in a dramatic increase in agonist-mediated
Ca^{2+} release (Hua et al., 1993; Vuong et al., 1998; Kang et al., 2001). In addition, it has been demonstrated that PKCe inhibits L-type Ca^{2+} current and PKCe inhibition increases phenylephrine-induced maximal contractions (Hu et al., 2000; Grimm et al., 2006). Given our previous finding that PDBu significantly inhibited α1-adrenoceptor-mediated increases in intracellular Ca^{2+} concentrations in pregnant uterine arteries (Zhang et al., 2006), the present results suggest that the inhibitory effect PDBu on α1-adrenoceptor-mediated contractions in pregnant uterine arteries is caused by activation of PKCβI, βII and ε, resulting in a decrease in α1-adrenoceptor-mediated Ca^{2+} mobilization. This is supported with the finding that PDBu induced a membrane translocation of PKCβI, βII and ε in the uterine artery. It should be noticed that none of these PKC-isozyme selective peptide inhibitors fully reversed the PDBu-mediated inhibition of phenylephrine-induced contractions in pregnant uterine arteries. This is possibly due to either not high enough inhibitor concentrations, or presence of an additive/synergistic effect of PKC isoforms in the PDBu-mediated response. In contrast to PKCβI, βII, and ε inhibitor peptides, the inhibitor peptide for conventional PKCs, including PKCa, β and γ, had no significant effect on PDBu-inhibited phenylephrine-induced contractions. We have demonstrated previously that PKCγ does not express in ovine uterine arteries (Zhang et al., 2006). This suggests that PKCa does not contribute to the inhibitory effect of PDBu on α1-adrenoceptor-mediated contractions in pregnant uterine arteries. Indeed, the finding that the conventional PKC isoform-selective inhibitor peptide that blocks both PKCa and β had no effect, but PKCβ inhibitor peptides partially reversed PDBu-mediated response, would suggest that PKCa activation by PDBu appears to increase the contraction. This is supported by the previous findings that PKCa activation induced contractions of vascular
smooth muscle (Singer et al., 1992; Walsh et al., 1994; Walsh et al., 1996). Given our previous finding of increased expression levels of PKCβ and decreased PKCα in pregnant, as compared with nonpregnant, uterine arteries (Zhang et al., 2006), the present results suggest that the adaptation of the uterine artery to pregnancy is associated with the upregulation of the PKC isozymes that inhibit α₁-adrenoceptor-mediated contractions, and the down-regulation of the PKC isozyme(s) that increase α₁-adrenoceptor-induced contractions.

In nonpregnant uterine arteries, it appears that PKC activation has a consistent and positive regulatory role in α₁-adrenoceptor-mediated contractions. GF109203X inhibited phenylephrine-stimulated contractions and blocked PDBu-mediated potentiation of phenylephrine-induced contractions, suggesting a single functional compartment of PKC isozyme(s) in the regulation of α₁-adrenoceptor-mediated contractions. Unlike pregnant uterine arteries, PKCβ and ε inhibitor peptides produced a near complete inhibition of PDBu-mediated potentiation of α₁-adrenoceptor-induced contractions in nonpregnant uterine arteries, suggesting that activation of PKCβ and ε contributes in parallel to the PDBu-mediated effect. Consistent with this finding, PDBu increased the membrane translocation of PKCβI, βII and ε in nonpregnant uterine arteries. The role of PKCα is not clear at the present, given that PDBu did not increase its membrane translocation. We have recently demonstrated that PDBu potentiates α₁-adrenoceptor-induced contractions in nonpregnant uterine arteries by increasing the Ca²⁺ sensitivity (Zhang et al., 2006). Given the previous findings that PKCε and β isozymes regulated vascular smooth muscle contractions through increasing the Ca²⁺ sensitivity (Bitar et al., 1991; Singer et al., 1992; Walsh et al., 1994; Horowitz et al., 1996a; Walsh et al., 1996;
Dessy et al., 2000), the present study suggests that PDBu activates PKC\(\beta\) and \(\varepsilon\) and increases the Ca\(^{2+}\) sensitivity, resulting in the potentiation of \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant uterine arteries.

The finding that activation of PKC\(\beta\) and \(\varepsilon\) participates in apparent opposite effects of PDBu on \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries, is intriguing and suggests that the dichotomy of PKC mechanisms in the regulation of \(\alpha_1\)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries is not caused by the activation of different PKC isozymes. Although previous studies have shown that different PKC isozymes have unique enzymatic properties, substrates, functions in different blood vessels and species (Khalil et al., 1992; Liou and Morgan, 1994; Horowitz et al., 1996b; Nishizuka, 1992; Kanashiro and Khalil, 1998; Kanashiro and Khalil, 2001), the present study is the first one to show that activation of the same PKC isozymes, \(i.e.\) PKC\(\beta\) and \(\varepsilon\), exhibit opposite effects in the same vessel in different physiological states, \(i.e.\) pregnancy and nonpregnancy. This finding suggests the down-stream mechanisms of PKC isozymes are involved. Because the function of each PKC isozyme requires localization to the specific subcellular sites such as plasma membrane, cytoskeletal filaments, or myofilaments, and ability of the isozyme to phosphorylate substrates co-localized to the sites, the distinct functional effects of the same PKC isozymes with essentially identical catalytic activity can be achieved by their binding and proximity to a particular set of substrates (Dorn and Mochly-Rosen, 2002). It is possible that pregnancy alters the subcellular distribution of PKC isozymes, resulting in regulating different sets of substrates in the uterine arteries. We have demonstrated that activation of PKC enhances the contractions in nonpregnant uterine arteries through its
effects on the thin filament regulatory pathway and activation of ERK/caldesmon and actin polymerization, but inhibits \( \alpha_1 \)-adrenoceptor-mediated contractions in pregnant uterine artery through down-regulation of the thick filament pathway and decreased myosin light chain phosphorylation (Zhang and Zhang, under review). Taken together, our results suggest a transition of subcellular localization of PKC\( \beta \) and \( \epsilon \) from thin filaments to thick filaments in the uterine arteries during pregnancy. Given the finding that steroid hormones induced modulation of cytosolic and membrane-bound regulatory proteins including PKC, and regulate their function (Simoncini et al., 2000; Kelly and Levin, 2001; Simoncini et al., 2002; Tostes et. al, 2003; Orshal and Khalil, 2004), future studies are needed to investigate the mechanisms of steroid hormones in pregnancy-adaptation of PKC isozyme subcellular distribution and its role in the regulation of \( \alpha_1 \)-adrenoceptor signaling pathways in the uterine arteries.
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References


Figure 1. Effect of GF109203X (GF) on concentration-response curves of phenylephrine-induced contractions in uterine arteries. Phenylephrine-induced contractions were expressed as percentage of 120 mM KCl-induced response (% $K^+$). Data are means ± SEM of tissues from 4~10 animals.
Figure 2. Effect of GF109203X on the pD₂ values and the maximal response (T_{max}) of phenylephrine-induced contractions in uterine arteries. Data are means ± SEM of tissues from 4–10 animals. *, P < 0.05, control vs. GF109203X treatment.
Figure 3. Effect of GF109203X on PDBu-inhibited phenylephrine-induced contractions in pregnant uterine arteries. Panel A and B show the effect of PDBu (A: 0.3 μM; B: 1 μM), in the absence or presence of 1 μM GF109203X, on concentration-response curves of phenylephrine-induced contractions. The pD2 values and the maximal response (Tmax) are shown in Panel C and D, respectively. Phenylephrine-induced contractions were expressed as percentage of 120 mM KCl-induced contraction (% K+). Data are means ± SEM of tissues from 4 animals. a, P < 0.05, vs. control; b, P < 0.05, PDBu vs. PDBu plus GF109203X.
Figure 4. Effect of GF109203X on PDBu-inhibited phenylephrine-induced contractions in nonpregnant uterine arteries. Panel A and B show the effect of PDBu (A: 30 nM; B: 100 nM), in the absence or presence of 1 μM GF109203X, on concentration-response curves of phenylephrine-induced contractions. The pD₂ values and the maximal response (Tₘₐₓ) are shown in Panel C and D, respectively. Phenylephrine-induced contractions were expressed as percentage of 120 mM KCl-induced contraction (% K⁺). Data are means ± SEM of tissues from 4 animals. a, P < 0.05, vs. control; b, P < 0.05, PDBu vs. PDBu plus GF109203X.
Figure 5. Effect of PKC isozyme-selective inhibitory peptides on PDBu-affected phenylephrine-induced contractions in uterine arteries. Phenylephrine-induced contractions were expressed as percentage of 120 mM KCl-induced contraction (% K⁺). Data are means ± SEM of tissues from 4 animals.
Figure 6. Effect of PKC isozyme-selective inhibitory peptides on pD2 values and the maximal response (Tmax) of PDBu-affected phenylephrine-induced contractions in uterine arteries. Data are means ± SEM of tissues from 4 animals. a, P < 0.05, vs. control; b, P < 0.05, PDBu plus carrier vs. PDBu plus PKC isozyme-selective inhibitory peptide.
Figure 7. PDBu-induced membrane translocation of PKC isozymes in uterine arteries. Membrane translocation of PKC isozymes was measured with Western blotting, as described in Methods. The results were expressed as percentage of control of each isozyme, blotted in the same membrane. Data are means ± SEM of tissues from 4 animals. *, P < 0.05, vs. control.
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![Graph showing % Control for Nonpregnant and Pregnant groups](image_url)

*Significant difference from control group.*
CHAPTER FIVE

GENERAL SUMMARY

Differential Regulatory Effect of PKC on $\alpha_1$-adrenoceptor-induced Contractions in the Nonpregnant and Pregnant Uterine Artery

The present research project examined the mechanisms of PKC activation effect on $\alpha_1$-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries, and offers the following new findings: 1) activation of PKC inhibits $\alpha_1$-adrenoceptor-induced contractions in the pregnant uterine artery through a down-regulation of Ca$^{2+}$-dependent thick-filament pathway by decreasing Ca$^{2+}$ mobilization and phosphorylation levels of LC$_{20}$; 2) PKC activation enhances $\alpha_1$-adrenoceptor-induced contractions in the nonpregnant uterine artery primarily through increasing Ca$^{2+}$ sensitivity via thin filament regulatory mechanisms; 3) the expression and activity of PKC isozymes were differentially regulated in pregnant and nonpregnant uterine arteries, and individual isozymes of conventional PKCs, PKC$\beta$ and PKC$\epsilon$ were identified to be involved in the PDBu-mediated opposite effects on $\alpha_1$-adrenoceptor-induced contractions in those vessels.

It has been suggested that PKC plays an important role in regulating vascular myogenic response (Davis and Hill, 1999). The recent studies in Dr. Lubo Zhang’s laboratory have demonstrated that myogenic tone of the uterine artery is reduced during pregnancy, which is primarily regulated through the PKC signal pathway (Xiao et al., 2006). In addition to its role in the regulation of myogenic tone, the study in chapter two demonstrated that activation of PKC interacted with $\alpha_1$-adrenoceptors and differentially modulated $\alpha_1$-adrenoceptor-mediated contractions in the pregnant and nonpregnant
uterine arteries. Previous studies have demonstrated that PKC can either inhibit or potentiate $\alpha_1$-adrenoceptor-mediated contractions in different arteries from different species (Amobi et al., 1999; Bazan et al., 1993; Cotecchia et al., 1985; Danthuluri et al., 1984; Henrion et al., 1992; Husain et al., 2004; Leeb-Lundberg et al., 1985; Longo et al., 2000; Martinez et al., 2001; Matsumura et al., 2001; McMillan et al., 1986; Salaices et al., 1990). However, it has not been previously demonstrated that activation of PKC can inhibit and potentiate $\alpha_1$-adrenoceptor-mediated contractions in the same artery of the same species at different physiological states, i.e. nonpregnant and pregnant. Thus, the present finding of a potentiation of $\alpha_1$-adrenoceptor-mediated contractions in nonpregnant uterine arteries, but an inhibition in pregnant uterine arteries provides a physiological importance of PKC in regulating the adaptation of $\alpha_1$-adrenoceptor-mediated contractions of the uterine artery during pregnancy.

$\alpha_1$-adrenoceptor-mediated smooth muscle contraction is regulated through changes in intracellular Ca$^{2+}$ mobilization and the Ca$^{2+}$ sensitivity of the contractile apparatus. Ca$^{2+}$ mobilization is regulated through Ca$^{2+}$-dependent thick-filament regulation (i.e. Ca$^{2+}$ and LC$_{20}$ phosphorylation dependent) (Horowitz et al., 1996b; Pfitzer, 2001). Regulation of the Ca$^{2+}$ sensitivity of myofilaments is through both Ca$^{2+}$-independent thick-filament regulatory pathway (i.e. Ca$^{2+}$ independent but LC$_{20}$ phosphorylation dependent), and the thin-filament regulatory pathway (i.e. LC$_{20}$ phosphorylation independent) (Morgan and Gangopadhyay, 2001; Somlyo and Somlyo, 2003; Xiao et al., 2004, Xiao and Zhang, 2005). In the studies of chapter two and three, activation of PKC was found to abolish $\alpha_1$-adrenoceptor-induced increase in [Ca$^{2+}$], and LC$_{20}$ phosphorylation in pregnant uterine arteries, which were accompanied by decreased contractions, measured simultaneously in
the same tissues. These suggest that the inhibitory effect of PKC activation on $\alpha_1$-adrenoceptor-induced contractions in pregnant vessels was mediated predominantly by suppressing the $Ca^{2+}$-dependent thick-filament pathway with decreased $Ca^{2+}$ mobilization and consequently decreased $LC_{20}$ phosphorylation. Consistent with the present finding, previous studies in ovine cerebral arteries demonstrated that PDBu inhibited norepinephrine-induced increases in intracellular $Ca^{2+}$ concentrations and contractions (Longo et al., 2000). On the other hand, in nonpregnant uterine artery, PKC activation by PDBu had no effect on $\alpha_1$-adrenoceptor-mediated $Ca^{2+}$ mobilization and $LC_{20}$ phosphorylation but potentiated the simultaneously measured contraction. The dissociation between $LC_{20}$ phosphorylation and PDBu-mediated increases in phenylephrine-induced contractions in nonpregnant arteries indicates a thin filament mechanism of increased $Ca^{2+}$ sensitivity in the PKC-mediated regulation of $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine arteries.

Among other thin filament mechanisms, caldesmon functions as a thin filament regulatory protein inhibiting smooth muscle contractions at given levels of $[Ca^{2+}]_i$ and $LC_{20}$ phosphorylation, and phosphorylation of caldesmon reverses its inhibitory effect (Katsuyama et al., 1992; Matsumura and Yamashiro, 1993; Morgan and Gangopadhyay, 2001; Wier and Morgan, 2003). ERK$_{42/44}$, as a physiologically relevant caldesmon kinase that mediates caldesmon phosphorylation (Adam et al., 1989), has been proposed to phosphorylate caldesmon and reverse its inhibitory effect on actin-activated myosin ATPase, hence activating the thin filament pathway (Horowitz et al., 1996; Morgan and Gangopadhyay, 2001). In the uterine arteries, the activation of PKC produced time-dependent increases in phosphorylation of ERK$_{42/44}$ and ERK$_{42/44}$-dependent
phosphorylation of caldesmon at Ser^{789} (Xiao et al., 2004). In the studies in chapter three, the finding that PDBu significantly increased phenylephrine-induced phosphorylation of ERK_{42/44}, caldesmon-Ser^{789} and consequently increased tension development in the same tissue suggests a role for ERK_{42/44} activation as a thin filament mechanism in PDBu-mediated potentiation of phenylephrine-induced contractions in nonpregnant uterine arteries. Meanwhile, the cause and effect relation between activation of ERK_{42/44} and the enhancement of contractions in PKC-mediated effect was demonstrated with the studies in chapter three showing that inhibition of ERK_{42/44} with PD098059 abolished PDBu-mediated increases in phenylephrine-induced contractions. Previous studies demonstrated that PD098059 inhibited phosphorylation of ERK_{44/42} and caldesmon-Ser^{789} in the uterine artery (Xiao et al., 2004).

The polymerization of actin filaments from monomeric globular actin (G-actin) to filamentous actin (F-actin), occurring independent of changes in intracellular Ca^{2+} concentrations and LC_{20} phosphorylation, plays an important role in the thin filament pathway to regulate smooth muscle contraction (Jones et al., 1999; Metha and Gunst, 1999; Gunst and Fredberg, 2003; Ozaki et al., 2004), including contractions induced by PDBu and a_{1}-adrenoceptor agonists (Nunes, 2002; Tang and Tan, 2003; Zhao et al., 2004; Chen et al., 2006). The studies of chapter three showed that inhibition of actin polymerization with cytochalasin B produced a concentration-dependent inhibition of PDBu-mediated potentiation of phenylephrine-induced contractions in nonpregnant uterine arteries. Given previous finding that the inhibition of contractions by actin polymerization inhibitors does not result from the disruption of thick-filament regulatory pathway (Metha and Gunst, 1999; Obara and Yabu, 1994), the present studies suggested a
thin filament mechanism of actin polymerization in PKC-potentiated $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine artery. The increase in actin polymerization might be related to PDBu-enhanced $\alpha_1$-adrenoceptor-induced phosphorylation of ERK$_{44/42}$ and caldesmon-Ser$^{789}$, because 1) caldesmon has been shown to play and important role in maintaining actin filament stability and inhibiting Arp2/3-dependent actin polymerization (Galazkiewicz et al., 1989; Matsumura and Yamashiro, 1993; Yamakita et al., 2003; Hai and Gu, 2006); 2) ERK$_{44/42}$ can phosphorylate caldesmon reverse its inhibitory effect on actin polymerization (Yamakita et al., 2003; Hai and Gu, 2006); 3)ERK$_{44/42}$ can phosphorylate an actin-binding protein, cortactin, resulting in Arp2/3-dependent actin polymerization (Martinez-Quiles et al., 2004).

**Role of PKC Isozymes in the Differential Regulation of $\alpha_1$-adrenoceptor-mediated Contractions in the Uterine Arteries**

The apparent opposite effects of PKC on $\alpha_1$-adrenoceptor-mediated contractions in nonpregnant and pregnant uterine arteries may be due in part to the differential regulation of PKC isozymes that show different enzymatic properties, substrates and functions (Horowitz, 1996b; Khalil, 1992; Liou, 1994). In chapter two, PKC isozyme expression and sub-cellular distribution were suggested to be differentially regulated in nonpregnant and pregnant uterine arteries, with significantly increased expression levels of PKC$\beta$ and PKC$\zeta$, but decreased PKC$\alpha$ and the basal activity of PKC$\epsilon$ in pregnant, as compared with nonpregnant, uterine arteries.

The study in chapter four suggested that there are two possible mechanisms to explain the inhibitory effect of PDBu on $\alpha_1$-adrenoceptor-mediated contractions in the
pregnant uterine arteries. One of the mechanisms is an existence of tow compartments of PKC in the regulation of α₁-adrenergic receptor-mediated contractions. One compartment of PKC may be tightly associated with α₁-adrenergic receptors and participate in α₁-adrenergic agonist-induced contractions. The other compartment locates distal to α₁-adrenergic receptors and more diffusely in cytosol, which is activated non-selectively by phorbol esters and mediates an inhibitory effect on α₁-adrenergic receptor-mediated contractions. This proposed two-compartment model of PKC is supported with previous finding that the subcellular location and the availability of protein substrates of PKC determine the function of each PKC isozyme (Dorn and Mochly-Rosen, 2002). The other proposed mechanism to regulate PKC inhibitory effect in the pregnant uterine arteries is that different PKC isozymes may be involved in the regulation of α₁-adrenergic receptor-mediated responses. The results in chapter four suggested that PDBu-mediated inhibition of α₁-adrenergic receptor-mediated contractions in pregnant uterine arteries is caused by activation of PKCβI, βII and ε, resulting in a decrease in α₁-adrenergic receptor-mediated Ca²⁺ mobilization. In agreement with this finding, previous studies have shown that PKCβ and ε inhibits agonist-induced Ca²⁺ mobilization, and their inhibition results in a respective increase in agonist-mediated Ca²⁺ release or maximal contractions (Hu et al., 2000; Hua et al., 1993; Grimm et al., 2006; Kang et al., 2001; Vuong et al., 1998). Nevertheless, the role of PKCα activation by PDBu appears to increase the α₁-adrenergic receptor-mediated contraction. Taken together with the finding in chapter two, the present research project suggests that the adaptation of the uterine artery to pregnancy is associated with the upregulation of the PKC isozymes (eg. PKCβI and βII) that inhibit α₁-adrenergic receptor-mediated contractions,
and the down-regulation of the PKC isozyme (e.g. PKCa) that increases \( \alpha_1 \)-adrenoceptor-induced contractions.

In contrast, a single functional compartment of PKC isozyme(s) in the regulation of \( \alpha_1 \)-adrenoceptor-mediated contractions in nonpregnant uterine arteries was suggested in the study of chapter four, since PKC inhibitor reduced phenylephrine-stimulated contractions and blocked PDBu-mediated potentiation of phenylephrine-induced contractions. Meanwhile, the results in chapter four suggested that PDBu activates PKC\( \beta \) and \( \epsilon \) and increases the \( Ca^{2+} \) sensitivity, resulting in the potentiation of \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant uterine arteries. This finding is supported by previous suggestion that PKCe and \( \beta \) isozymes regulated vascular smooth muscle contractions through increasing the \( Ca^{2+} \) sensitivity (Bitar et al., 1991; Singer et al., 1992; Walsh et al., 1994; Horowitz et al., 1996a; Walsh et al., 1996; Dessy et al., 2000). However, the role of PKCa is not clear at the present, given that PDBu did not increase its membrane translocation in nonpregnant uterine arteries.

The finding that activation of PKC\( \beta \) and \( \epsilon \) participates in apparent opposite effects of PDBu on \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries, disproves the hypothesis that the dichotomy of PKC mechanisms in the regulation of \( \alpha_1 \)-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries is caused by the activation of different PKC isozymes. The study in chapter four was the first one to show that activation of the same PKC isozymes, i.e. PKC\( \beta \) and \( \epsilon \), exhibit opposite effects in the same vessel in different physiological states, i.e. pregnancy and nonpregnancy. This finding suggested the down-stream mechanisms of PKC isozymes are involved. Because the distinct functional effects of the same PKC isozymes
with essentially identical catalytic activity have been suggested to be achieved by their binding and proximity to a particular set of substrates (Dorn and Mochly-Rosen, 2002), it is possible that pregnancy alters the subcellular distribution of PKC isozymes, resulting in regulating different sets of substrates in the uterine arteries. For example, PKCβ and ε have been suggested to decrease agonist-induced Ca\(^{2+}\) mobilization resulting in reduction of contractions (Hua et al., 1993; Vuong et al., 1998; Hu et al., 2000; Grimm et al., 2006; Kang et al., 2001), or regulate contractions through thin filament pathway with increased Ca\(^{2+}\) sensitivity (Bitar et al., 1991; Singer et al., 1992; Walsh et al., 1994; Horowitz et al., 1996a; Walsh et al., 1996; Dessy et al., 2000). In addition, the study in chapter three demonstrated that activation of PKC enhances the contractions in nonpregnant uterine arteries through its effects on the thin filament regulatory pathway and activation of ERK/caldesmon and actin polymerization, but inhibits α\(_1\)-adrenoceptor-mediated contractions in pregnant uterine artery through down-regulation of the thick filament pathway and decreased myosin light chain phosphorylation. Taken together, the results suggest a transition of subcellular localization of PKCβ and ε from thin filaments to thick filaments in the uterine arteries during pregnancy. Given the finding that steroid hormones induced modulation of cytosolic and membrane-bound regulatory proteins including PKC, and regulate their function (Simoncini et al., 2000; Kelly and Levin, 2001; Simoncini et al., 2002; Tostes et. al, 2003; Orshal and Khalil, 2004), steroid hormones might play an important role in this thin-thick filament transition and the regulation of α\(_1\)-adrenoceptor signaling pathways in the uterine arteries.
Conclusion and Implications

In conclusion, the present research project has demonstrated the opposite effects of PKC on $\alpha_1$-adrenoceptor-induced contractions in nonpregnant and pregnant uterine arteries. Activation of PKC potentiates $\alpha_1$-adrenoceptor-induced contractions in nonpregnant uterine arteries by increasing $Ca^{2+}$ sensitivity through the thin filament regulation pathway, but inhibits the contractions in pregnant uterine arteries by suppressing the $Ca^{2+}$-dependent thick filament regulation pathway. The findings of up-regulation of the PKC isozymes that inhibit intracellular $Ca^{2+}$ mobilization, down-regulation of the PKC isozymes that increase $Ca^{2+}$ sensitivity, and a transition of subcellular localization of PKC$\beta$ and $\epsilon$ from thin filaments to thick filaments in the uterine arteries during pregnancy provide insights into the adaptation of uterine artery contractile mechanisms in pregnancy. Steroid hormones might play an important role in this contractile adaptation to pregnancy, since estrogen has been suggested to decrease vascular tone through modulating $Ca^{2+}$ homeostasis in vascular smooth muscle cell (Duckles SP, 1996; Orshal JM, 2004; Suzuki T, 2003). In addition, steroid hormones induced modulation of cytosolic and membrane-bound regulatory proteins including PKC, and regulate their function (Simoncini et al., 2000; Kelly and Levin, 2001; Simoncini et al., 2002; Tostes et. al, 2003; Orshal and Khalil, 2004). From the physiological perspective, the uterine circulation during pregnancy functions as a low-resistance shunt to accommodate the large increase of uteroplacental blood flow, required for normal fetal development. In addition to growth and remodeling of vessels, the decreased uterine artery resistance is accomplished by increased endothelial nitric oxide release, decreased myogenic response, and a reversible sympathetic denervation of the
uterine artery. However, at the same time, uterine artery increases acute contractile response to nonsynaptic $\alpha_1$-adrenergic stimulation throughout gestation (Annibale DJ, 1989 & 1990; D'Angelo G, 1993 & 1994; Osol G, 1993), and retains its ability to phasically contract to shunt blood away from the uterus to other vascular beds during times of acute maternal stress to facilitate maternal survival. Nevertheless, when stress is prolonged, myogenic tone is continuously increased with activated PKC. This PKC activation is suggested in the present studies to reduce $\alpha_1$-adrenoceptor-mediated contractions in pregnant uterine artery. The finding of increased PKC-mediated inhibition on $\alpha_1$-adrenoceptor-mediated contractions reveals another important mechanism in maintaining the low uterine vascular tone during chronic stress, such as pregnancy, to facilitate fetal survival and normal development. The present studies enhance our basic understanding of uterine vasculature adaptation to pregnancy, help provide a mechanistic basis for this functional adaptation, and thereby improve our understanding of problems associated with the maladaptation and abnormal pregnancy and permit us to address them in a more meaningful way.
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