Testosterone and Blood Pressure

Prenatal Testosterone Exposure Induces Hypertension in Adult Females via Androgen Receptor–Dependent Protein Kinase Cδ–Mediated Mechanism

Chellakkan S. Blesson,* Vijayakumar Chinnathambi,* Gary D. Hankins, Chandra Yallampalli, Kunju Sathishkumar

Abstract—Prenatal exposure to excess testosterone induces hyperandrogenism in adult females and predisposes them to hypertension. We tested whether androgens induce hypertension through transcriptional regulation and signaling of protein kinase C (PKC) in the mesenteric arteries. Pregnant Sprague–Dawley rats were injected with vehicle or testosterone propionate (0.5 mg/kg per day from gestation days 15 to 19, SC) and their 6-month-old adult female offspring were examined. Plasma testosterone levels (0.84±0.04 versus 0.42±0.09 ng/mL) and blood pressures (111.6±1.3 versus 104.5±2.4 mm Hg) were significantly higher in prenatal testosterone–exposed rats compared with controls. This was accompanied with enhanced expression of PKCδ mRNA (1.5-fold) and protein (1.7-fold) in the mesenteric arteries of prenatal testosterone–exposed rats. In addition, mesenteric artery contractile responses to PKC activator, phorbol-12,13-dibutyrate, was significantly greater in prenatal testosterone–exposed rats. Treatment with androgen receptor antagonist flutamide (10 mg/kg, SC, BID for 10 days) significantly attenuated hypertension, PKCδ expression, and the exaggerated vasoconstriction in prenatal testosterone–exposed rats. In vitro exposure of testosterone to cultured mesenteric artery smooth muscle cells dose dependently upregulated PKCδ expression. Analysis of PKCδ gene revealed a putative androgen responsive element in the promoter upstream to the transcription start site and an enhancer element in intron-1. Chromatin immunoprecipitation assays showed that androgen receptors bind to these elements in response to testosterone stimulation. Furthermore, luciferase reporter assays showed that the enhancer element is highly responsive to androgens and treatment with flutamide reverses reporter activity. Our studies identified a novel androgen-mediated mechanism for the control of PKCδ expression via transcriptional regulation that controls vasoconstriction and blood pressure. (Hypertension. 2015;65:683-690. DOI:10.1161/HYPERTENSIONAHA.114.04521.) • Online Data Supplement

Key Words: blood pressure ■ protein kinase C ■ polycystic ovary syndrome ■ testosterone ■ vasoconstriction

A suboptimal intrauterine environment results in impaired fetal growth and leads to increased incidence of cardiovascular, metabolic, and reproductive disorders in adult life.1 An abnormal intrauterine hormonal milieu can influence clearly fetal growth and development and has been identified as a factor known to cause intrauterine programming.2 For instance, excessive prenatal exposure to androgens causes intrauterine growth restriction, hypertension, insulin resistance, reproductive disturbances, and other metabolic abnormalities, simulating the polycystic ovary syndrome (PCOS) phenotype in adult females.3–10

PCOS, which affects ≈10% of women of reproductive age,11 is a multifaceted condition comprising infertility, dysfunctional uterine bleeding, and components of metabolic syndrome, including obesity, diabetes mellitus, dyslipidemia, and hypertension.11 Although prenatal testosterone exposure recapitulates many of these features, including hyperandrogenism with associated hypertension, in adult female rats, it is not clear whether testosterone plays a causative role in the development of hypertension. The contribution of androgens in the development of hypertension in males is straight forward because orchiectomy prevents blood pressure (BP) elevation and testosterone replacement restores hypertension in animal models.4,5,11,14 However, the contribution of androgens to vascular dysfunction and hypertension in females remains controversial.15,16 Several observations suggest that testosterone may amplify hypertension development in females. In genetic hypertensive rat models, such as spontaneously hypertensive rats (SHR)17 and TGR(mRen2)27,18 blockade of the androgen receptor (AR) reduces BP in females, and direct testosterone administration to ovariectomized SHR females increases BP.19 In addition, it has been demonstrated that
adult prenatal testosterone–exposed females have higher testosterone levels, and ovariectomy not only reduces testosterone levels but also reverses BP, suggesting that the presence of increased testosterone levels is essential for increasing BP. These data provide evidences that androgens may contribute to the hypertensive process in females. Available data in humans also indicate that androgens can contribute to BP control in women just as in men because young women with conditions such as PCOS, postmenopausal women, and black women have higher serum testosterone levels, and the frequency of hypertension is greater in these populations. Thus, direct and indirect evidence implicates androgens in hypertension in females. However, the molecular mechanisms underlying androgen-mediated increases in BP remain largely unclear.

Protein kinase C (PKC) is a key regulatory enzyme involved in the signal transduction in vascular health and disease, including cell growth and contractility. Several lines of evidence have implicated the activation of PKC-mediated signaling in pathogenesis of hypertension. For example, PKC-mediated contractile responses are enhanced in aortas of SHR but not Wistar–Kyoto rats, hypertensive models, including the SHR and DOCA-salt hypertensive rats have increased vascular expression/activity of PKC; perfusion of hindlimb of SHR and Wistar–Kyoto rats with the PKC activator, phorbol 12,13-dibutyrate (PDBu) caused prolonged vasoconstriction and increased perfusion pressure; and PKC gene silencing normalized arterial BP in SHR. These studies suggest that increased PKC expression and function may be a key molecule contributing to the development of hypertension. However, the molecular basis of activation of PKCs and the mechanisms that control the expression of PKC isozymes, especially knowledge on the functional elements in the PKC gene promoter or the nature of the factors that control PKC gene transcription is not well characterized.

Because testosterone is known to interact with PKC, upregulating the classical constrictor pathway via upregulation of PKC isoenzymes, we investigated the molecular mechanism by which testosterone transcriptionally modulates PKC expression and determined whether regulation of PKC signaling by testosterone plays an important role in mediating vascular contraction and hypertension.

**Methods**

All experimental procedures were performed in accordance with the National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at the University of Texas Medical Branch. Timed-pregnant Sprague–Dawley rats (Harlan, Houston, TX) were divided into 2 groups on gestational day 14, and 1 group received daily injections of testosterone propionate (Sigma, St. Louis, MO) subcutaneously from gestational days 15 to 19 at 0.5 mg/kg body weight/d (n=8). The other group received vehicle (sesame oil, n=8). This dose and duration of exposure are commonly used to mimic plasma testosterone levels (2-fold increase) observed in preeclamptic women. Dams in both groups were allowed to deliver at term and the birth weights of pups were recorded. The number of pups in the control and testosterone litters were adjusted to 10 pups per dam to ensure equal nutrient access for all offspring (pups with weights at each extreme were euthanized). The ratio of male to female pups remained equivalent after culling, when possible. Pups were weaned at 3 weeks of age, and only females were used for this study. At 6 months of age, arterial pressure was monitored using the telemetry system. After BP measurements, the animals were euthanized, the plasma separated and mesenteric arteries (MAs) were isolated. A portion of the MAs was used for vascular reactivity studies, and the remaining were quickly frozen for RNA/
Prenatal Testosterone Exposure Leads to Hyperandrogenism and Hypertension in Adult Females

Plasma testosterone levels in 6-month-old females were significantly higher by 2-fold in prenatal testosterone–exposed rats (0.84±0.04 ng/mL; n=8) compared with controls (0.42±0.09 ng/mL; n=8; P<0.05). As shown in Figure 1A and 1B, prenatal testosterone exposure significantly increased BP in adult females, as compared with control animals (P<0.05; n=6–8).

Antiangen Treatment and PKCδ Blockade Reverses Increased BP in Hyperandrogenic Prenatal Testosterone–Exposed Rats

Flutamide administration significantly attenuated the increase in BP observed in prenatal testosterone–exposed females (Figure 1A; P<0.05; n=6–8). Flutamide had no significant effect on BP in controls. Stopping flutamide treatment returned arterial pressure to pretreatment levels in prenatal testosterone–exposed adults (Figure 1A and 1B; P<0.05).

Inhibition of PKCδ with rottlerin abrogated increase in BP in prenatal testosterone–exposed females (Figure 1C; P<0.05; n=8). Rottlerin had no significant effect on BP in controls (Figure 1C).

PKCδ Expression Is Increased in the MAs of Hyperandrogenic Prenatal Testosterone–Exposed Females

We next determined the expression profile of PKC isoforms in the MAs. As shown in Figure 2, quantitative real-time polymerase chain reaction shows the expression of at least 6 PKC isoforms, α, β, δ, ε, γ, and ζ in rat MAs. When compared with control rats, vessels in prenatal testosterone–exposed rats showed significantly increased expression levels of only PKCδ (1.5-fold; Figure 2; P<0.05; n=5 in each). Western blotting also showed that PKCδ protein levels were significantly increased (1.7-fold) in the MA from prenatal testosterone–exposed rats (n=6) than in those from the control (n=6; Figure 3; P<0.05). Flutamide administration to prenatal testosterone–exposed rats significantly attenuated the increase in PKCδ protein levels (Figure 3; P<0.05; n=6). PKCδ expression was not significantly different between flutamide-treated prenatal testosterone–exposed rats and flutamide-treated control rats (Figure 3; n=6 in each).

PKCδ-Mediated Vasoconstrictor Responses Were Enhanced in MAs of Hyperandrogenic Prenatal Testosterone–Exposed Females

We next determined the functional implications of increased PKCδ expression in MAs of prenatal testosterone–exposed rats. As shown in Figure 4, stimulation of PKC with PD-Bu significantly increased contractile responses with an increase in maximal responses in MAs of prenatal testosterone–exposed rats (27.2±3.5%; n=6) compared with controls (18.7±1.9%; n=8; P<0.05). To further confirm the primary role of PKCδ toward exaggerated vasoconstriction in prenatal testosterone rats, PD-Bu-induced contractile responses were elicited in the presence of the PKCδ inhibitor, rottlerin. Incubation with rottlerin significantly decreased PD-Bu-induced contractions in MAs of both control and prenatal testosterone–exposed rats (n=8; P<0.05).
**Testosterone Increases PKCδ Gene Expression Levels in Cultured MA Smooth Muscle Cells**

Based on the effects of hyperandrogenism on PKCδ expression and contractions in MAs, we next assessed the mechanism of testosterone stimulation of PKCδ expression. We determined the effects of testosterone on PKCδ mRNA and protein levels in an in vitro cell culture model. Both mRNA and protein levels in cultured MA smooth muscle cells were dose dependently increased by testosterone treatment (Figure 5A and 5B; *P*<0.005; *n*=4), indicating that testosterone induces PKCδ expression.

Treatment with testosterone for 5 days also increased the protein levels of AR in a dose-dependent manner with significant increases at 50 and 100 nmol/L in MA smooth muscle cells (Figure 6; *P*<0.05; *n*=4).

**AR Binding Sites in PKCδ Gene Promoter and Intron 1 Regions**

Analysis of PKCδ promoter and intron 1 regions showed 6 and 5 androgen response element (ARE)-like sequences, respectively. Putative AREs identified in the promoter region were named p-ARE1 to p-ARE6, and the putative AREs present in intron 1 were named int1-ARE1 to int1-ARE5 (Figure 7; Table S2 in the online-only Data Supplement). We then used the chromatin immunoprecipitation assay to identify if any of these putative AREs interact with AR in the presence of AR-specific ligands. Our results show that putative p-AREs 5,6 and int1-ARE2 interacted with AR in a ligand-dependent fashion. p-AREs 5,6 showed a 2-fold enrichment when treated with testosterone compared with control and flutamide treatment (Figure 8; *P*<0.05; *n*=4). Interestingly, int1-ARE2 (Figure 8; *P*<0.05; *n*=4) showed ~12-fold enrichment in the presence of testosterone compared with vehicle, flutamide, or testosterone+flutamide. However, other predicted putative AREs did not show any ligand-dependent enrichment (data not shown). Thus, our data suggest the presence of functional

**Figure 5.** Testosterone (T) upregulates protein kinase C (PKC) δ expression in cultured mesenteric artery (MA) smooth muscle cells. Concentration-dependent T-induced increase in PKCδ (A) mRNA and (B) protein in cultured MA smooth muscle cells. The primary MA smooth muscle cells were treated with vehicle or T for 5 days with fresh medium along with T replaced every day. Cell extracts were prepared and subjected to PKCδ expression analysis using quantitative reverse transcription polymerase chain reaction and Western blot. Measurement of vascular PKCδ mRNA and protein expression was normalized relative to β-actin. Data represent mean of 4 independent experiments. *P*<0.05 vs controls, **P*<0.001 vs controls.
Results show that the functional enhancer element that binds AR in response to androgen stimulation in the PKCd gene promoter and intron 1, respectively. Regulation of PKCd expression by androgens via the AR has significant functional consequences, as we determined that androgens exert a significant control of PKCd-mediated arterial contraction and hypertension. Therefore, we suggest that increases in vascular PKCd-stimulated responses may mediate the development and maintenance of hypertension induced by hyperandrogenism in adult females.

There is mounting evidence that elevated testosterone levels during pregnancy may have adverse effects on fetal growth and thereby increase the potential for an increased risk of cardiovascular diseases in adult life.3–5,44 Consistent with evidence showing that boys and girls of PCOS mothers are often hyperandrogenic,41,45 we and others using this model of prenatal androgen exposure have shown that adult females tend to produce higher testosterone compared with controls. We previously reported that hyperandrogenism is associated with increased arterial pressure in prenatal testosterone–exposed adult females. In this study, we show that treatment with the selective AR antagonist flutamide abolished the hypertensive response in prenatal testosterone–exposed adult females. This suggests that AR activation contributes, in part, to the increase in BP in prenatal testosterone–exposed hypertensive adults. The observation that cessation of flutamide treatment reverses BP back to hypertensive levels suggests that testosterone actively regulates BP and that testosterone increase may be a key factor in promoting elevation of BP in the adult prenatal testosterone–exposed females. This is consistent with the growing evidence that shows an important role for androgens in the pathophysiology of hypertension.20,25

The question arises as to how androgens provoke increase in BP. Recent studies showed that PKC is a key pathological factor in hypertension.20,46–48 PKC mediates its physiological effects mainly through 13 isoforms, divided into 3 major

Figure 6. Testosterone (T)-induced increase in protein levels of androgen receptor (AR) in cultured mesenteric artery (MA) smooth muscle cells. The primary MA smooth muscle cells were treated with vehicle or T for 5 days with fresh medium along with T replaced every day. Cell extracts were prepared and subjected to AR expression using Western blot. Measurement of vascular AR protein expression was normalized relative to β-actin. Data represent mean of 4 independent experiments. *P<0.05 vs controls, **P<0.01 vs controls.

Figure 7. Identification for putative androgen receptor (AR) binding sites in the protein kinase C (PKC) δ gene. Bioinformatic prediction using Consite program shows androgen response element (ARE)–like sites in the promoter and intron 1 of rat PKCδ gene.
groups: (1) classical PKC isoforms (α, βI, βII, and γ) requiring both Ca\(^{2+}\) and diacylglycerol for activation, (2) novel PKC isoforms (δ, ε, η, and θ) requiring only diacylglycerol for activation, and (3) atypical PKC isoforms (λ, μ, and ζ) that are activated by binding phosphatidylinerine but not diacylglycerol or Ca\(^{2+}\). The α, β, δ, ε, and ζ isoenzymes of PKC have been detected in vascular smooth muscles.\(^{50,51}\) Although not all of these seem to be in all vascular smooth muscle cells, our studies show presence of all 6 isoenzymes in the MAs, consistent with previous reports.\(^{27}\) In the present study, we found that only expression of PKCδ was significantly higher in the MAs of hyperandrogenic females. In addition, flutamide treatment reversed the increased PKCδ expression in hyperandrogenic females. These findings suggest that androgens play an important role in stimulating PKCδ expression. This is consistent with the previous findings that coronary PKCδ levels directly correlate with endogenous testosterone levels,\(^{52,53}\) and that testosterone increases PKCδ protein levels in coronary smooth muscle of swine.\(^{52,53}\)

Given the importance of PKCδ in mediating vascular smooth muscle contractile responses,\(^{54-56}\) we investigated the functional impact of elevated PKCδ expression. In the present study, PDBu, an activator of PKC, induced contractile responses that were significantly increased in hyperandrogenic rats. The \(E_{\text{max}}\) were greater in the hyperandrogenic rats and were related to testosterone levels and BP increases. Furthermore, the enhanced vasoconstriction was reversed when the arterial rings were preincubated with rottlerin, suggesting that PKCδ is the primary factor in enhancing vascular contractile responses in hyperandrogenic rats. In fact, previous studies demonstrated that PKCδ located in smooth muscle directly mediates vasocontractile effects.\(^{54,55,57}\) Although PKC isoforms have been implicated in contractile responses in dispersed/cultured smooth muscle cells,\(^{55,56}\) we think this to be the first instance in which individual PKC isoforms have been specifically identified as being involved in a contractile response in intact vessels, at least when challenged with PDBu. Indeed, the present finding that rottlerin a selective PKCδ inhibitor abrogated hypertension observed in hyperandrogenic rats indicates a causative role of the heightened PKCδ-mediated signaling in the pathogenesis of androgen-induced hypertension. Given that flutamide abolished the exaggerated contractile responsiveness to PDBu in hyperandrogenic rats suggests that the enhanced responsiveness to PKC in these rats is testosterone dependent. This reinforces the notion that modulation of the PKCδ by testosterone contributes to hypertension in prenatal testosterone–exposed female rats.

The finding that testosterone exposure to cultured MA smooth muscle cells upregulates PKCδ mRNA suggests that PKCδ is a physiological target for testosterone and that androgens can directly regulate the transcription of PKCδ. The question arises as to how androgens regulate PKCδ expression. Our in silico analysis of a 10-kb region upstream of the PKCδ transcription start site revealed 2 tandem ARE-like sequences (p-AREs 5,6) separated by 9 bp located at \(=\)4.2 kb resembling the ARE consensus sequence. It is known that steroid receptor binding sites can deviate considerably from the consensus high-affinity motif, and steroid receptor recognition by low-affinity sites can be significantly enhanced by flanking sequences and coregulators.\(^{60,61}\) Furthermore, we also found an ARE-like sequence in the intron 1 region of PKCδ (int1-ARE2) at \(=\)2.6 kb from the transcriptional start site, which could potentially act as an enhancer element. The presence of enhancers in various regions including in introns has been well documented.\(^{62}\) Importantly, our chromatin immunoprecipitation studies show that both the p-AREs 5,6 present in the promoter region and int1-ARE2 are efficiently binding to AR in response to testosterone in MA smooth muscle cells, suggesting that it is functionally relevant in vivo. The luciferase reporter assay of int1-ARE2 showed a ligand-dependent activation, indicating that this sequence is responsive to androgens. Androgen-induced upregulation of PKCδ could be mediated

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**Figure 8.** Protein kinase C (PKC) δ gene has an active androgen receptor (AR) binding site. The chromatin immunoprecipitation assay shows AR interaction with putative (A) response and (B) enhancer elements from the promoter region (p-androgen response element [ARE] 5,6) and intron 1 (int1-ARE 2), respectively, when treated with testosterone (T), flutamide (Flu), and both for 5 days. Data represent mean of 4 independent experiments. *\(P<0.05\) vs controls, ***\(P<0.001\) vs controls.

**Figure 9.** Presence of a functional enhancer element in intron 1 of protein kinase C (PKC) δ gene. Reporter assay showing luciferase activity in mesenteric artery smooth muscle cells transfected with reporter plasmid containing int1-androgen response element (ARE) 2 showing an \(=7\)-fold increase when incubated with testosterone (T). This increase was suppressed when treated with T in the presence of flutamide (Flu). Data represent mean of 4 independent experiments. *\(P<0.05\) vs controls.
by a combination of both direct and indirect effects. Direct effects may be mediated by the cis-acting elements present in the promoter and intron-1 regions, acting as possible response and enhancer elements, respectively. Furthermore, we also show that testosterone up-regulates its own receptor, AR in MA smooth muscle cells there by transregulating PKCδ expression via AR levels. Thus, androgen could potentially regulate the expression of PKCδ by a combination of cis- and transregulation.

Although we focused on females in this study, it is possible that androgens exert a similar increases in PKCδ-mediated vasoconstriction in male vasculature as well, which needs further investigation. Thus, in this study, we show that prenatal testosterone treatment induces hyperandrogenism, which controls PKCδ expression via transcriptional regulation to cause enhanced vasoconstriction and hypertension in adult female offspring.

**Perspectives**

Evidence indicates that androgens can contribute to BP control in women just as in men because young women with conditions such as PCOS, postmenopausal women, and black women have higher plasma testosterone levels, and the frequency of hypertension is greater in these populations. In this study, higher testosterone levels during adult life significantly increases BP. We have shown that PKCδ plays a role in the hypertensive activity of androgen. This study is the first to show the presence of functional androgen response and enhancer elements in the rat PKCδ gene promoter and intron-1, respectively. Regulation of PKCδ expression by androgens via the AR has significant functional consequences, as we determined that androgens exert a significant increase in PKCδ-mediated vasoconstriction. Altogether, this study sets forth a novel paradigm suggesting that inhibition of PKCδ can be a potential target for diseases that are regulated by androgen-induced hypertension.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- Postnatal androgens dynamically regulate blood pressure in hyperandrogenic females.
- Androgen-mediated mechanism controls the expression of protein kinase C (PKC) δ in mesenteric artery smooth muscle cells by positively regulating PKCδ transcript and protein levels.
- Functional androgen response and enhancer element binds androgen receptor in response to androgen stimulation in the PKCs gene promoter and intron 1, respectively.
- Regulation of PKCδ expression by androgens via the androgen receptor has significant functional consequences, as androgens exert a significant control of PKCδ-mediated arterial contraction and blood pressure.

**What Is Relevant?**

- Sex steroid hormones, including testosterone, have an important influence on blood pressure.
- Hypertension is one of the most common complications during adult life.
- Heightened PKC signaling has been implicated in the development of hypertension.

**Summary**

The present study provides new evidence in an animal model linking hormonal regulation of PKCδ expression and increased risk of hypertension and reveals a mechanistic understanding of the heightened PKCδ-mediated signaling in the pathogenesis of androgen-induced hypertension.
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PRENATAL TESTOSTERONE EXPOSURE INDUCES HYPERTENSION IN ADULT FEMALES VIA ANDROGEN RECEPTOR-DEPENDENT PKC6-MEDIATED MECHANISM

Chellakkan S. Blesson,1,a Vijayakumar Chinnathambi,2,a Gary D. Hankins,2 Chandra Yallampalli1 and Kunju Sathishkumar2,*

1Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX 77030; 2Division of Reproductive Endocrinology Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX 77555

*aContributed Equally

Short Title: Androgens enhance PKCδ mediated vasoconstriction

Supplemental Table: 2

*Corresponding author and reprint requests:
K. Sathishkumar, DVM, PhD
Assistant Professor, Dept. of Obstetrics & Gynecology
University of Texas Medical Branch
301 University Blvd.
Galveston, TX 77555-1062
Phone: (409) 772-7592 Fax: (409) 772-2261
Email: kusathis@utmb.edu
Methods:

Animals

Timed pregnant Sprague-Dawley rats (gestational day, GD 12) were purchased from Harlan Laboratories, Inc. (Houston, TX). Rats were housed in a temperature-controlled room (23°C) with a 12:12-hour light/dark cycle with food and water available *ad libitum*. Plasma levels of testosterone (T) were measured by ELISA (Enzo life sciences, Farmingdale, NY) as previously described.1

Experimental procedures

Measurement of BP and flutamide and rottlerin treatment
Mean arterial pressure in conscious free-moving rats was determined using the telemetry system as previously described.1 Briefly, rats were anesthetized with 2.5% isoflurane, and a flexible catheter attached to a radio transmitter (TA11PA-C10, Data Sciences, Minneapolis, MN) was inserted into the left femoral artery. After surgery, rats were housed in individual cages and allowed to recover for a week. BP measurements obtained with a 10-s sampling period were averaged and recorded every 10 minutes, 24 hours a day, using the software (Dataquest 4.0, Data Sciences, Minneapolis, MN) provided by the manufacturer. A subset of female offspring at 6 months of age was administered with anti-androgen (flutamide, Sigma, 10 mg/kg, s/c) twice daily for 10 days. Before, during, and after flutamide treatment, changes in arterial pressure were recorded as described above.

To another cohort of rats, jugular and carotid arterial catheters were fixed under 2.5% isoflurane anesthesia. After 24 h of recovery, mean arterial pressure was measured through their carotid catheters.2,3 After ensuring that the arterial pressure was maintained at a stable level, rats were injected with rottlerin (0.3 mg/kg through jugular catheter)4,5 and blood pressure changes were assessed.2

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)
Total RNA was isolated from mesenteric arteries by using TRIzol reagent (Invitrogen, Grand Island, NY). All RNA isolates were made DNA free by treatment with DNase and further purification with RNeasy clean-up kit (QIAGEN Inc., Valencia, CA). Total RNA concentration and purity were determined using an ND-1000 model Nanodrop spectrophotometer (Thermo Fisher Scientific, Newark, DE). Two micrograms of total RNA were reverse transcribed using a modified Maloney murine leukemia virus-derived reverse transcriptase (New England Biolabs Inc., Ipswich, MA) and a blend of oligo(dT) and random hexamer primers (Invitrogen). The reaction was carried out at 28°C for 15 min and then at 42°C for 50 min, and the reaction was stopped by heating at 94°C for 5 min, followed by 4°C, before storage at −20°C until further analysis. One microliter of the resulting cDNA was amplified by real-time RT-PCR using SYBR Green (Bio-Rad, Hercules, CA) as fluorophore in an CFX96 model real-time thermal cycler (Bio-Rad). Specific pairs of primers from published literature were used for each gene amplification (Table S1). PCR conditions used were 10 min at 95°C for 1 cycle, 15 sec at 95°C, 30 sec at 60°C, and 15 sec at 72°C for 40 cycles, with a final dissociation step (0.05 sec at 65°C and 0.5 sec at 95°C). Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold-level increases or decreases the expression of genes of interest in prenatal exposed T offspring vs
those in control rats. All reactions were performed in duplicate, and β actin was used as an internal control.

**Western Blotting**
Protein extract (20 µg) from each sample was resolved on 4%–12% precast gradient polyacrylamide gels (NuPAGE® Bis-Tris Gels, Invitrogen). Proteins from the gel were transferred to a PVDF membrane (Millipore, Billerica, MA) by electroblotting. After blocking the membranes in 5% BSA in TBS containing 0.1% Tween for 1 hour at RT, they were incubated overnight at 4°C with primary antibodies (PKCδ: cat# 2058 and β-actin: cat# 3700 from Cell signaling, Danvers, MA and Androgen receptor: cat # ab74272 from Abcam, Cambridge, MA) diluted 1:1000 in 5% BSA. Membranes were washed and incubated for 60 min at RT with HRP-conjugated secondary antibodies (1:1000, anti-rabbit). Membranes were washed and incubated in Pierce Western Blotting detection reagents (Thermo Scientific, Rockford, IL) for 1 min. and exposed to film and developed. Densitometric analyses of the films were performed using AlphaView software (Alpha Innotech, Santa Clara, CA).

**Ex Vivo Vascular Reactivity Studies**
Freshly excised third-order mesenteric arteries from 6-month-old female offspring were placed in an ice-cold modified Krebs bicarbonate solution (KBS) of the following composition (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, and 11 dextrose. The mesenteric arteries were cleaned of adherent connective tissue and precisely cut into rings of same length (2 mm). Two to 4 rings from 1 rat were used for 1 experiment. The n presented with each figure represents the number of animals studied. Endothelium was denuded by gently rubbing it with tungsten wires. Two 25-µm tungsten wires were threaded through the lumen, and the rings were mounted in an isometric wire myograph system (model 610M wire myography, Danish Myotechniques, Aarhus, Denmark). The rings were bathed in 6 mL KBS, gassed with 95% oxygen and 5% carbon dioxide, maintained at a temperature of 37°C, and allowed to equilibrate for 30 minutes before normalization to an internal diameter of 0.9 of L13.3kPa by using a normalization software package (Myodata, Danish Myotechnologies). This corresponds to a transmural pressure of approximately 90 mmHg. Following normalization, rings were repeatedly exposed to KCl (80mM) to test their viability and to determine a standard contractile response for each of them. The rings were contracted with phenylephrine (3µM, Sigma), and when responses were stable, endothelium-denudation was confirmed by an absence of relaxation to acetylcholine (10µM, Sigma). The rings were then allowed to recover for 60 minutes, after which cumulative concentration-response curves were generated with the PKC activator, PDBu (10⁻⁸ to 3x10⁻⁵ M, Sigma). Concentration-response curves of PDBu were obtained by the cumulative addition of the agonist in approximate one-half log increments. Additionally, contractile responses to PDBu were assessed in the presence of rottlerin (3 µM, selective PKCδ inhibitor, Sigma). Arterial rings were incubated with rottlerin for 30 min prior to and during PDBu dose-response. Contractile responses to PDBu were expressed as percent of 80 mM KCl constriction.

**Vascular Smooth Muscle Cell isolation and Culture**
Mesenteric artery smooth muscle cells were isolated from adult female rats as described earlier. Isolated cells were assessed for their purity by α-actin staining and were found to be >95% pure.
Cells were routinely cultured in DMEM containing 4.5g/L glucose supplemented with 25mM HEPES, 2mM L-glutamine, 10% FBS, and antibiotics without sodium pyruvate. Cells were changed to serum and phenol red-free media 48 hours prior to treatments. Cells were treated with T (1–100nM) for 5 days with fresh media changes every day. After treatments, cells were washed with PBS and lysed for RNA or protein preparation.

Bioinformatic analysis
A bioinformatic analysis was performed on the rat PKCδ gene (Gene ID: 170538) at the promoter and intron 1 region using ConSite data base for the presence of androgen response element- (ARE) like sequences with 75% transcriptional factor cut off score. The analysis was performed up to 10 kb upstream to the transcriptional start site to cover the promoter region and the entire intron 1 region, which is in the 5’ un-translated region directly upstream to the initiation codon. The predicted ARE-like sequences present on the positive DNA strands were considered for further analysis by chromatin immunoprecipitation ChIP assay.

ChIP assay and qPCR
ChIP assay was performed to screen for AR binding sites in the promoter and intron 1 region of the PKCδ gene. The assay was performed using a SimpleChIP® Chromatin Immunoprecipitation kit (Cell Signaling Technologies), following the manufacturer’s instruction. Briefly, MASM cells (approximately 3X10⁷ cells per group) were cultured in the presence of T (50nM), flutamide (1µM), T+flutamide, and vehicle for 5 days. Cells were washed and cross linked by incubating them with 1% formaldehyde for 10 min at room temperature. Crosslinking was stopped by the addition of 1X glycine for 5 min. After they were washed with ice-cold PBS, the cells were scrapped and lysed to prepare chromatin. The chromatin was then fragmented by enzymatic digestion using micrococcal nuclease for 20 min at 37°C with frequent mixing. The digestion was stopped by adding 0.5 M EDTA on ice. Next, the nuclei were pelleted and sonicated for 20s twice with 40% power to break the nuclear membrane. The lysates were then clarified by spinning at 10,000 rpm for 10 min at 4°C. The supernatant contained the required cross-linked chromatin. The chromatin was then treated with RNase A and then incubated with Proteinase K for 2 hours at 65°C. DNA from the digested chromatin was isolated using spin columns, quantified, and run on a 1% agarose gel to verify the enzymatic digestion of chromatin. Chromatin (5µg of DNA in 500µl volume for each immunoprecipitation) was then subjected to immunoprecipitation with an AR antibody (25µl per reaction, Abcam ab74272) along with positive (10µl anti-histone antibody; Cell Signaling Technologies #4620) and negative (1µl of rabbit IgG, Cell Signaling Technologies # 2727) controls. Immunoprecipitation was performed overnight at 4°C with constant rotation. ChIP-grade Protein G magnetic beads were then added to each reaction and incubated for 2 h at 4°C with rotation. Next, the pellets were precipitated using a magnetic rack. Cross links were then reversed by adding 5mM NaCl and proteinase K and incubating at 65°C for 2 h. DNA was purified using spin columns, quantified, and stored for further analysis. Purified DNA from ChIP assays along with their respective 2% inputs were subjected to qPCR to identify the ligand-dependent AR binding to putative AR binding sites in PKCδ gene. Primer sets were synthesized to flank the predicted putative ARE sites (Table S2). PCR conditions used were 10 min at 95°C for 1 cycle, 15 sec at 95°C, 30 sec at 60°C, and 15 sec at 72°C for 40 cycles, with a final dissociation step (0.05 sec at 65°C and 0.5 sec at 95°C).
Percentage Input was calculated by calculating $2\% \times 2^{(C[T] \cdot 2\%\text{Adjusted Input Sample} - C[T] \text{ IP Sample})}$ ($C[T] = $ Threshold cycle of PCR reaction).
**Reporter Assay**

The intron 1 region (approximately 7 kb) of the rat PKCδ gene was cloned and sequenced (GenBank: KF573407.1), and a reporter construct was made by inserting it into pGL3 basic vector (Promega, Madison, WI). Mesenteric artery smooth muscle cells were transiently transfected using Amaxa reagent for human aortic smooth muscle cells (VPC-1001, Lonza) using program number U-025. Five transfections were performed with each transfection containing 5µg of pGL3-int1 ARE vector and 0.5 pRL µg DNA with ~ 5x10^5 mesenteric artery smooth muscle cells. After transfections, cells were pooled from all cuvettes and were seeded onto a 12 well plate. Control transfections were also performed with pGL3 and pmaxGFP® vectors to check for background and transfection efficiency, respectively. After 24 hours, cells were washed and were cultured in the presence of T (50nM), flutamide (1µM), T+flutamide, and vehicle (ethanol) for 5 days in DMEM containing 4.5g/L glucose, supplemented with 25mM HEPES, 2mM L-glutamine, and antibiotics without sodium pyruvate and FBS at 37°C with 5% CO2. Fresh media containing T, F, and vehicle were replaced every day. The cells were then washed with ice-cold PBS thrice and were lysed by adding 250 µl of passive lysis buffer per well (Promega). A luciferase assay was performed using the Dual-Luciferase® reporter assay system (Promega), following the manufacturer’s instruction. Cell lysates (25µl) were transferred on to a 96 well plate and 100µl of Luciferase Assay reagent II was dispensed, and firefly luciferase activity (activity of inserted ARE like sequences) was measured. Stop & Glo® reagent was then added to measure the renilla luciferase activity. Luminescence was measured using Fluorstar Omega (BMG Labtech, Cary, NC) with 1s readout per well. The firefly luciferase activity was normalized with the renilla luciferase activity, and the results were expressed as relative luminescence.

**Statistics**

All values are given as mean ± SEM. Sigmoidal functions were modeled to individual dose–response curves (Prism, GraphPad Software, San Diego CA), and statistical comparisons of Emax and BP values were made by ANOVA with Bonferroni correction for multiple comparisons. For statistical comparison of single parameters, an independent t test was used. Statistical significance was assumed if \( P < 0.05 \).

**Reference List**


Table S1: Primers used to amplify PKC isoforms

<table>
<thead>
<tr>
<th>PKC</th>
<th>Forward 5’- 3’</th>
<th>Reverse 5’- 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>GTGCAAGGAACACATGATGG</td>
<td>GTAATCCGGAGTCCCACACAG</td>
</tr>
<tr>
<td>Beta</td>
<td>CTCAGAGCGGAAGGTACAG</td>
<td>GTGCACTCCACATCGTCATC</td>
</tr>
<tr>
<td>Delta</td>
<td>TACCGGGCTACGGTTTAT</td>
<td>ACATCCCGAAAGTCAGCAATC</td>
</tr>
<tr>
<td>Epislon</td>
<td>ACGGTGGAACCTCATGTTC</td>
<td>TCACTCAGTGTTGGTGGAGA</td>
</tr>
<tr>
<td>Gamma</td>
<td>TTGATGGGAAGATGGAGGAG</td>
<td>CGGGAGATGGACTTGAGGATA</td>
</tr>
<tr>
<td>Iota</td>
<td>CGGCATGTGATAAGGAAGGAT</td>
<td>CAGTCAAGCCTGAAGGCCATA</td>
</tr>
<tr>
<td>Theta</td>
<td>TAGAAGGGAGGCAAAGGAT</td>
<td>GCGGAGTCTCTGCTCTGACTC</td>
</tr>
<tr>
<td>Zeta</td>
<td>CCAGGACTGCTGTGAGGAG</td>
<td>CCTCAGTGCCCCACTT</td>
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Table S2: Primers used to amplify putative AREs from promoter and intron 1 regions of PKCδ gene after ChIP assay

<table>
<thead>
<tr>
<th>Primers used in Promoter Region</th>
<th>Putative AR binding sites</th>
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<tbody>
<tr>
<td><strong>Prkcd-P-ARE 1</strong></td>
<td>5’TGAGGCTCGATGTGTGCCACAT3’</td>
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<tr>
<td>TCATAGTGGGACTTCCCAG-3’</td>
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<tr>
<td>Reverse: 5’-</td>
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<tr>
<td>TGTAGAGGGGAAGCCAGTGA-3’</td>
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</tr>
<tr>
<td><strong>Prkcd-P-ARE 2</strong></td>
<td>5’GCAAGGACACACGCGTCTGACC3’</td>
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<tr>
<td>Forward: 5’-</td>
<td></td>
</tr>
<tr>
<td>CCACCATAACCATGGTCTTC-3’</td>
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</tr>
<tr>
<td>Reverse: 5’-</td>
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<tr>
<td>CCAAATTCTTGGGAACCA-3’</td>
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</tr>
<tr>
<td><strong>Prkcd-P-ARE 3</strong></td>
<td>5’CCCAGTACATTTCTGGCCGTA3’</td>
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<tr>
<td>Forward: 5’-</td>
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</tr>
<tr>
<td>CTTCGAGACTCGTTGCTTC-3’</td>
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<td>Reverse: 5’-</td>
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<tr>
<td>CAGTCTTCTGAGCTCAGGG-3’</td>
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<tr>
<td><strong>Prkcd-P-ARE 4</strong></td>
<td>5’CAGGAAACTCCCTGTTCGCCC3’</td>
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<tr>
<td>GTGTGTGTGTGAGAATCAGG-3’</td>
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<tr>
<td>Reverse: 5’-</td>
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<tr>
<td>CTGAGGAAACTCCTCAACGCC-3’</td>
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<td><strong>Prkcd-P-ARE 5-6</strong></td>
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<tr>
<td>ACACAGGGAGACAAAGCCAA-3’</td>
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<tr>
<td>GGACATTCGCAGAAAGAAGC-3’</td>
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* Putative AREs were very close and hence two were covered by the same set of primers

<table>
<thead>
<tr>
<th>Primers used in Intron 1 Region</th>
<th>Putative AR binding sites</th>
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<tr>
<td><strong>Prkcd-Int1-ARE1</strong></td>
<td>5’GAAAGCCACAGCCTCCTTGAC3’</td>
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<tr>
<td>TGGACCTATCTCCTGGTGTT-3’</td>
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<tr>
<td>Reverse: 5’-</td>
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<tr>
<td>CTGTGACTGACTGGTGGA-3’</td>
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<tr>
<td><strong>Prkcd-Int1-ARE2</strong></td>
<td>5’CTGTGCCACCTTGCTCCACGG3’</td>
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<tr>
<td>GCTGAGGAAGCTGACTCAC-3’</td>
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</tr>
<tr>
<td>Reverse: 5’-</td>
<td></td>
</tr>
<tr>
<td>GGCCTTTGTCCAGATACCAA-3’</td>
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</tr>
<tr>
<td><strong>Prkcd-Int1-ARE3&amp;4</strong></td>
<td>5’CTCATCCACATTTACTGGCC3’</td>
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<tr>
<td>Forward: 5’-</td>
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</tr>
<tr>
<td>TGAAACTCCTGCAGTGACCA-3’</td>
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<tr>
<td>Reverse: 5’-</td>
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<tr>
<td>ACCAGCAACAGGACTCACC-3’</td>
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<tr>
<td><strong>Prkcd-Int1-ARE5</strong></td>
<td>5’CTAAGACAGTCTTGTTCGACA3’</td>
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<tr>
<td>TGGAGTCTGTTGTGGGTG-3’</td>
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