ONLINE SUPPLEMENT

Pregnancy Upregulates Large-Conductance Ca^{2+}-Activated K^+ Channel Activity and Attenuates Myogenic Tone in Uterine Arteries

By

Xiang-Qun Hu, Daliao Xiao, Ronghui Zhu, Xiaohui Huang, Shumei Yang, Sean Wilson and Lubo Zhang

Center for Perinatal Biology (X.Q.H., D.X., R.Z., X.H., S.W., L.Z.), Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, California; Department of Chemistry and Biochemistry (S.Y.), California State University, San Bernardino, California

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Address correspondence to:
Lubo Zhang, Ph.D.,
Center for Perinatal Biology
Division of Pharmacology
Department of Basic Sciences
Loma Linda University
School of Medicine
Loma Linda, CA 92350

Tel: 909-558-4325
Fax: 909-558-4029
Email: lzhang@llu.edu

Materials and Methods

Tissue Preparation and Treatment

Uterine arteries were harvested from nonpregnant and near-term pregnant (~140 days' gestation with the term at about 145 days) sheep at the same time point in the Spring season. As described previously,[1-3] sheep were anesthetized with thiamylal (10 mg/kg) administered *via* the external left jugular vein. The ewes were then intubated, and anesthesia was maintained on 1.5% to 2.0% halothane in oxygen throughout surgery. An incision was made in the abdomen and the uterus exposed. The resistance-sized uterine arteries (~150 μm in diameter) were isolated and removed without stretching and placed into a modified Krebs solution. For the steroid hormone treatment, uterine arteries from nonpregnant sheep were incubated in phenol red-free DMEM with 1% charcoal-stripped FBS for 48 hours at 37°C in a humidified incubator with 5% CO2/95% air in the absence or presence of 17β-estradiol (0.3 nmol/L, Sigma) and/or progesterone (100.0 nmol/L, Sigma), as reported previously.[1-3] The concentrations of 17β-estradiol and progesterone chosen are physiologically relevant as observed in ovine pregnancy,[4] which have been shown to exhibit direct genomic effects on pressure-dependent myogenic tone in the uterine artery.[1] All of the procedures and protocols were approved by the Institutional Animal Care and Use Committee and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurement of Myogenic Tone

Pressure-dependent myogenic tone of resistance-sized uterine arteries was measured as described previously.[1-3] Briefly, the arterial segments were mounted and pressurized in an organ chamber (Living Systems Instruments, Burlington VT). The intraluminal pressure was controlled by a servo-system to set transmural pressures and arterial diameter was recorded using the SoftEdge Acquisition Subsystem (IonOptix LLC, Milton MA). After the equilibration period, the intraluminal pressure was increased in a stepwise manner from 10 to 100 mmHg in 10-mmHg increments, and each pressure was maintained for 5 minutes to allow vessel diameter to stabilize before the measurement. The passive pressure-diameter relationship was conducted in Ca^{2+}-free physiologic saline solution (PSS) containing 3.0 mmol/L of EGTA to determine the maximum passive diameter. The following formula was used to calculate percent myogenic tone at each pressure step: %myogenic tone = $(D_1 - D_2)/D_1$ x 100, where D_1 is the passive diameter in Ca^{2+}-free physiologic saline solution (0 Ca^{2+} with 3.0 mmol/L of EGTA) and D_2 is the active diameter with normal physiologic saline solution in the presence of extracellular Ca^{2+}.

Measurement of BK_{Ca} Channel Current

Smooth muscle cells were enzymatically dissociated from resistance-sized uterine arteries. Briefly, uterine arteries were minced and incubated in low-Ca^{2+} (0.1 mmol/L $CaCl_2$) HEPES-buffered PSS containing 0.6 mg/ml papain (Worthington Biochemical; Lakewood, NJ), 1 mg/ml bovine serum albumin and 1 mg/ml dithioerythritol for 35 minutes at 37°C. The tissues were then transferred to a new low-Ca^{2+} HEPES-buffered PSS containing 0.75 mg/ml collagenase type IV (Worthington) and 1 mg/ml bovine serum albumin and incubated for 30 minutes at 37°C. Following the enzyme treatment, tissues were washed with low Ca^{2+} PSS. Single smooth muscle cells were released by gentle trituration with a fire-polished glass Pasteur pipette. The cells were kept at 4°C and experiments were conducted within 6 hours of cell isolation. Whole-cell currents were recorded from freshly isolated arterial myocytes using an EPC 10 patch-clamp

amplifier with Patchmaster software (HEKA, Lambrecht/Pfalz, Germany) at room temperature, as previously described.[5,6] The cell membrane is much more fluid at physiological temperature of 37°C, which presents a technical difficulty in maintaining a patch with a good seal for patch-clamp recording. Therefore, most patch-clamp recordings were conducted at room temperature (22−24°C) for the better stability of the patch membrane. Several drops of cell suspension were placed in a recording chamber and the adherent cells were continuously superfused with HEPES-buffered physiologic salt solution containing (in mmol/L): 140.0 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10.0 HEPES, and 10.0 glucose (pH 7.4). Relaxed and spindle-shaped myocytes were used for recording. Micropipettes were pulled from borosilicate glass and had resistances of 2 to 5 MΩ when filled with the pipette solution containing (in mmol/L) 140.0 KCl, 1.0 $MgCl_2$, 5.0 Na_2ATP, 5.0 EGTA, 10.0 HEPES (pH 7.2). $CaCl_2$ was added to bring free Ca^{2+} concentrations to 100.0 nmol/L, as determined using WinMAXC software (Chris Patton, Stanford University). Cells were held at –50 mV and whole-cell K^+ currents were evoked by voltage steps from -60 mV to +80 mV by stepwise 10-mV depolarizing pulses (350-ms duration, 10-second intervals). Whole-cell K^+ currents were normalized to cell capacitance and were expressed as picoampere per picofarad (pA/pF). The BK_{Ca} channel current was determined as the difference between the whole-cell K^+ current in the absence of IBTX or TEA and that in the presence of IBTX or TEA.

Western Immunoblotting Analysis

Protein abundance of BK_{Ca} channel α subunit and β1 subunit was measured in freshly isolated resistance-sized, endothelium-intact uterine arteries and after the hormonal treatment by Western blot analysis, as described previously.[1-3] Briefly, tissues were homogenized in a lysis buffer followed by centrifugation at 4°C for 10 minutes at 10,000g, and the supernatants were collected. Samples with equal proteins were loaded onto 7.5% polyacrylamide gel with 0.1% sodium dodecyl sulfate, and were separated by electrophoresis at 100 V for 2 hours. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites in the membranes were blocked by an overnight incubation at 4°C in Tris-buffered saline solution containing 5% dry milk. The membranes were incubated with primary antibodies against BK_{Ca} channel α and β1 subunit (Santa Cruz Biotechnology, Santa Cruz CA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software. Preliminary studies showed that protein abundance of both alpha smooth muscle actin and GAPDH in uterine arteries was altered by pregnancy. Given the uncertainty of proteins that were not changed by pregnancy in the uterine artery, loading controls were not used and the samples were directly compared by scanning densitometry using arbitrary units.

Data Analysis

Results were expressed as means ± SEM obtained from the number of experimental animals given. Differences were evaluated for statistical significance ($P<0.05$) by ANOVA or *t* test, where appropriate.

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