Published in final edited form as:

Reprod Toxicol. 2016 October ; 65: 95–103. doi:10.1016/j.reprotox.2016.07.003.

Roles of ion channels in regulation of acetylcholine-mediated vasoconstrictions in umbilical cords of rabbit/rats

Zhoufeng Zhu^{a,1}, Jiaqi Tang^{a,1}, Xiuwen Zhou^a, Sharon Xiang^{a,b}, Xiaolin Zhu^a, Na Li^a, Ruixiu Shi^a, Yuan Zhong^a, Lubo Zhang^b, Miao Sun^{a,*}, and Zhice Xu^{a,b,*}

^aInstitute for Fetology, the First Affiliated Hospital of Soochow University, Suzhou 215006, China

^bCenter for Perinatal Biology, Loma Linda University, Loma Linda, CA, USA

Abstract

We recently demonstrated that acetylcholine (ACh) produced reliable vasoconstrictions in the umbilical cords. This study investigated the possible mechanisms with different antagonists. ACh-mediated vasoconstrictions were decreased by voltage-operated calcium (Ca^{2+}) channels antagonist nifedipine or inositol-1,4,5-trisphosphate-mediated Ca^{2+} release antagonist 2-aminoethyl diphenylborinate, indicating that both extracellular and intracellular calcium modulated the ACh-stimulated umbilical contraction. Intracellular Ca^{2+} concentrations were increased simultaneously with vasoconstrictions by ACh in the umbilical vessels. Inhibiting large-conductance calcium-dependent potassium (BK) channels enhanced ACh-mediated contraction, whereas inhibiting voltage dependent potassium (K^+), inward rectifier K^+ and ATP-sensitive K^+ channels had no effects. Incubation with specific K^+ channel inhibitors showed that ACh suppressed BK currents rather than 4-aminopyridine-sensitive K^+ channels currents. The results suggested that blood vessels in umbilical cords had special characteristics in response to cholinergic signals. ACh-stimulated umbilical vasoconstrictions were mediated *via* muscarinic receptor subtype 1/3–protein kinase C/cyclooxygenase-BK channel pathways.

Keywords

Acetylcholine; Umbilical cord; Calcium channel; Potassium channel

1. Introduction

The umbilical cord is vital to fetal growth and development. Lacking autonomic innervation, regulation of umbilical vascular tone mainly depends on release of vasoactive substances [1]. Acetylcholine (ACh) is the most common cholinergic chemical in the organs of the body, including the placenta and umbilical vessels [2,3] as well as in other smooth muscle cells [4–6]. Based on others' and our studies on human umbilical arteries and veins, as well as our

*Corresponding authors at: Institute for Fetology, the First Affiliated Hospital of Soochow University, Suzhou 215006, China, miaosunsuda@163.com (M. Sun), xuzhice@suda.edu.cn (Z. Xu).

¹These authors contributed equally to the work.

Conflict of interest

The authors have none to declare.

recent findings in rats and sheep [7], this study tested rabbit and rat umbilical cords in order to determine ACh's special actions on umbilical vessels.

It is well known that ACh mediates vasorelaxation in intact and healthy blood vessels in almost all organs of the body [8]. Our recent work [7] demonstrated that ACh induced reliable vasoconstrictions in human, sheep, and rats. Notably, vascular diameters can control blood and oxygen supply *via* umbilical vessels to fetuses [9]. *In utero* hypoxia has been demonstrated to cause poor development of fetuses as well as low birth weight babies [10,11]. Thus, vigorous vasoconstrictions induced by cholinergic chemicals may induce *in utero* hypoxia and cause toxic consequences to fetuses. The present study determined possible underlying cellular mechanisms involved in the ACh-stimulated umbilical vasoconstrictions.

Responding to the signals transferred from muscarinic receptor (MR) cascades, cytoplasmic calcium concentration ($[Ca^{2+}]_i$) is fundamental in the regulation of vascular tone [12]. ACh induces contraction through vascular L-type voltage-dependent Ca^{2+} channel and intracellular calcium release *via* inositol 1,4,5-triphosphate (IP_3) receptors [13]. In addition, potassium (K^+) channels play an important role in the regulation of vascular tone and membrane potential [14], MR agonists could transiently increase the large-conductance calcium-dependent potassium (BK) channel currents followed by persistent inhibition in different smooth muscle cell types [15,16]. However, it was largely unknown how smooth muscle cells as well as the ion channels reacted to ACh in umbilical blood vessels. Thus, the present study focused on those signaling pathways and determined whether phospholipase C (PLC) or protein kinase C (PKC) [17], as well as calcium and potassium channels might be involved in ACh-mediated umbilical vasoconstrictions. The new information gained should advance further understanding vascular regulations in the umbilical cord-fetal circulation, which is critical to fetal development *in utero*, and benefit for prevention of possible fetal hypoxia induced by toxic effects by cholinergic chemicals.

2. Methods

2.1. Animals

Pregnant Sprague–Dawley rats and pregnant New Zealand white rabbits were purchased from the Animal Center of Soochow University. At gestational day (GD) 21, rats were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg; Hengrui Medicine, Jiangsu, China) intraperitoneally [18] and at GD 26, rabbits were anesthetized intravenously with ketamine (30 mg/kg) [19] through an ear vein followed with uterine-incision delivery. Umbilical cords were collected and kept in Ca^{2+} -free physiological salt solution. All procedures were approved by the Institutional Animal Care Committee and conformed to the National Guide for the Care and Use of Laboratory Animals.

2.2. Testing on large blood vessels

Rabbit umbilical vein and carotid artery were isolated, and immediately mounted in an organ bath containing modified Krebs solution (in mM: 115.0 NaCl, 25.0 $NaHCO_3$, 4.6 KCl, 2.5 $CaCl_2$, 1.2 Na_2HPO_4 , 1.2 $MgCl_2$, and 10 D-glucose; pH 7.4), then gassed continuously with

a mixture of 95% O₂ and 5% CO₂. Vascular tension was measured with JZ101 isometric force transducer (Xinhangxingye Technology, Beijing, China). After 60 min of equilibration, each vessel ring was stretched for the optimal resting tension determined by the tension developed in response to 60 mM KCl. Vascular responses to addition of phenylphrine or ACh was monitored and recorded.

2.3. Testing on small blood vessels

Rat umbilical veins were separated and cut into segments. Segments (~3 mm) were mounted on a M series Myograph System (Radnoti Glass Technology, Inc. USA) in a chamber containing Krebs solution with 5% CO₂ in O₂ at 37 °C [20], followed by a 30-min equilibration period, vessels were exposed to an external solution containing 60 mM KCl to assess vessel functional capability. To determine possible signaling pathways mediated the contractile responses to ACh, several inhibitors, including muscarinic receptor antagonist atropine (10⁻⁶ M), muscarinic receptor subtype 1 (MR1) antagonist pirenzepine (10⁻⁶ M), MR2 antagonist methoctramine (10⁻⁵ M), MR3 antagonist pFFHsiD (10⁻⁶ M) [21], phospholipase C (PLC) inhibitor U73122 (5 × 10⁻⁵ M), cyclooxygenase inhibitor indomethacin (10⁻⁵ M), and protein kinase C (PKC) inhibitor GF109203X (10⁻⁶ M) were added into the chambers before application of ACh.

To assess possible involvement of extracellular or/and intracellular calcium in ACh-induced vessel contractions, L-type Ca²⁺ inhibitor nifedipine (10⁻⁵ M) and antagonist 2-aminoethyl diphenylborinate (2-APB, 10⁻⁷ M) [22] for IP₃ were used followed by ACh.

To determine the role of potassium channels in ACh-induced contraction in rat umbilical veins, ACh concentration-response curves were recorded in the presence or absence of the following inhibitors: 4-aminopyridine (4-AP, 3 × 10⁻³ M), charybdotoxin (CTX, 10⁻⁷ M), barium chloride (BaCl₂, 10⁻⁵ M), or glibenclamide (10⁻⁵ M) [23]. Vascular tone was normalized by the maximum contraction elicited with 60 mM KCl. Signals were recorded continuously using Chart 7 with PowerLab system (AD Instruments, Bella Vista, NSW Australia).

2.4. Measurement of [Ca²⁺]_i in rat umbilical veins

The venous segments were mounted in organ chambers (Living Systems, Burlington, USA) as described [24]. Vascular intracellular calcium was monitored and measured using Ca²⁺ indicator fura-2 AM by a Bio-Rad Radiance 2100 confocal system (Zeiss, Oberkochen, Germany) as reported [25]. In brief, the cannulated umbilical venous rings were incubated with 10 μmol/L fura-2 AM-PSS solution for 4 h at room temperature (22–25 °C), and gassed continuously with 95% O₂–5% CO₂. Then rings were incubated in PSS solution for 30 min at 37 °C. Ca²⁺ concentration was calculated qualitatively by fluorescence ratio of fura-2AM at 340 and 380 nm wave lengths (Rf340/380) [26]. KCl (6 × 10⁻² M) or ACh (10⁻⁶ M, 10⁻⁵ M) were added to the chamber respectively. Each venous segment was used for only one drug.

2.5. Electrophysiology

Umbilical veins in rats were placed in ice-cold physiological salt solution (PSS) containing (in mM) 137 NaCl, 5.6 KCl, 1 MgCl₂, 0.42 Na₂HPO₄, 0.44 NaH₂PO₄, 4.2 NaHCO₃, and 10 HEPES (pH 7.4).

3. Isolation of rat umbilical vein myocytes

Umbilical veins were dissected gently and cut into small fragments (about 1 mm) and placed for 22 min at 37 °C in PSS containing 1 mg/ml collagenase II, 2 mg/ml BSA, and 2 mg/ml DTT. They were then transferred to PSS containing 3 mg/ml papain, 2 mg/ml BSA, and 2 mg/ml DTT for 20 min. Single cells were obtained by gentle trituration with a wide-bore glass pipette, stored at 4 °C and used within 6 h.

4. Whole-cell K⁺ current recording

The isolated myocytes were continuously superfused with a bath solution containing (in mM) 137 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 Glucose; pH 7.4. Patch pipettes (tip resistance, 3–5 M) were pulled by P-97 and filled with pipettes solution containing (in mM) 143 KCl, 1 MgCl₂, 0.5 EGTA, 10 HEPES, and 3 Na₂ATP, pH 7.35. Na₂ATP (3 mM) was included to inhibit ATP-sensitive K⁺ currents and provided a substrate for energy-dependent processes [27]. Seal resistances ranged from 1 to 10 M after seal formation. Protocols were applied using an Axo patch 700 B amplifier with Pclamp 10.2 software. Whole-cell K⁺ currents were evoked by voltage steps delivered from a holding potential of −70 mV to potentials ranging from −60 to +60 mV, in 10 mV increments. Whole cell K⁺ currents were normalized to cell capacitance and expressed as picoampere per pF (pA pF^{−1}). All experiments were performed at room temperature (22–25 °C). Whole-cell K⁺ currents were monitored before or after adding ACh (10^{−5} M), ibertoxin (IBTX, 10^{−7} M), 4-AP (3 × 10^{−3} M), and atropine (10^{−7} M). BK channel currents were tested with 0.1 μmol/L IBTX.

All agonists and antagonists used were purchased from Sigma-Aldrich unless otherwise stated. Drugs were prepared freshly.

4.1. Data analysis

Data were expressed as means ± SEM, where n equals the number of maternal rats from which offspring were taken. A value of P < 0.05 was considered to indicate statistical significance, and determined using *t*-test or two-way analysis of variance. The data was processed with SigmaPlot11 and Graph Pad Prism 5.

5. Results

5.1. The effect of ACh on rabbit and rat umbilical veins

ACh stimulated dose-dependent contraction in the rabbit as well as rat umbilical veins, the responses were reliably repeated (Fig. 1A,C–E), while ACh-induced relaxation in the rabbit carotid artery pre-treated with PE (Fig. 1B). Atropine almost abolished ACh-induced contraction in the umbilical veins from rabbits (Fig. 1A, the maximal vessel response: from

14.94 ± 0.80 to 2.56 ± 0.43, $p < 0.05$) and rats (Fig. 1D, the maximal vessel response: from 239.8 ± 8.03 to 4.82 ± 0.35, $p < 0.05$).

5.2. The possible signal pathway involved in ACh-induced contractile responses in rat umbilical veins

To pharmacologically identify the muscarinic receptor subtypes mediating the contraction, the specific receptor antagonists such as pirenzepine (MR1), methoctramine (MR2), and pFHHSiD (MR3) were incubated. The results showed that MR1 (Fig. 2A, the maximal vessel response: from 237.9 ± 7.43 to 92.54 ± 5.67, and pD2 from 6.98 ± 0.09 to 5.71 ± 0.15, $p < 0.05$) and MR3 (Fig. 2C, the maximal vessel response: from 240.0 ± 7.90 to 146.9 ± 6.34, $p < 0.05$), but not MR2 (Fig. 2B), were involved in the contraction. To investigate possible signal routes, we used inhibitors step by step. The PLC inhibitor U73122 partly suppressed ACh-induced contraction in rat umbilical vein (Fig. 2D, the maximal vessel response: from 237.7 ± 8.17 to 69.39 ± 4.15, $p < 0.05$). In addition, the inhibitor of the PKC (GF109203X, Fig. 2E, the maximal vessel response, from 228.8 ± 8.28 to 121.3 ± 5.22, pD2: from 6.98 ± 0.115 to 6.26 ± 0.119) and cyclooxygenase (indomethacin, Fig. 2F, the maximal vessel response: from 229.2 ± 9.36 to 178.0 ± 7.97, pD2: from 6.76 ± 0.13 to 6.11 ± 0.11) partly attenuated the contraction ($p < 0.05$).

To investigate the modulation of calcium channels, 2-APB significantly inhibited ACh-mediated contraction (Fig. 3B) (the maximal vessel response: from 254.1 ± 8.56 to 21.57 ± 1.35, $p < 0.05$), while nifedipine only partly inhibited the contraction (Fig. 3A) (the maximal vessel response: from 258.6 ± 8.27 to 128.80 ± 5.79, $p < 0.05$). These results suggested that both calcium channels modulated umbilical vein contraction, associated with a ACh-increased $[Ca^{2+}]_i$ shown in Fig. 3C.

5.3. The effects of K⁺ channel blockers on the ACh-mediated contraction

Charybdotoxin (CTX, a potent and selective BK channel blocker) significantly enhanced ACh-induced umbilical vein contractions (Fig. 4A, the maximal vessel response: from 225.9 ± 8.79 to 331.9 ± 10.14, $p < 0.05$). However, 4-AP (non-selective voltage-gated K⁺ channel blocker), barium chloride (inward rectifying K⁺ currents inhibitor), and glybenclamide (K_{ATP} channels blocker) showed no effects (Fig. 4B–D, $p > 0.05$), suggesting that potassium channels modulated ACh-mediated contraction in rat umbilical veins *via* BK channels mainly.

5.4. Whole cell K⁺ currents on smooth muscle cells from rat umbilical vein

To identify if K⁺ channels might contribute to cell K⁺ currents in the smooth muscle cells (SMCs), K_v channel blocker 4-AP showed a partially inhibitory effect on whole-cell potassium currents (from 22.19 ± 1.72 pA/pF at baseline to 17.91 ± 0.70 pA/pF, TP = +60 mv, $p < 0.05$) (Fig. 4H). Application of a specific BK inhibitor, IBTX, suppressed outward currents in the umbilical venous myocytes (from 23.32 ± 1.07 pA/pF at baseline to 8.47 ± 1.04 pA/pF, TP = +60 mv, $p < 0.05$) (Fig. 4G).

6. The effect of cholinergic agonists on the whole cell K⁺ currents

ACh decreased the whole-cell K⁺ currents (from 23.37 ± 1.63 pA/pF at baseline to 14.3 ± 0.76 pA/pF, TP = +60mv, $p < 0.05$) (Fig. 5C). Fig. 5F showed that ACh caused a further decrease in K⁺ currents in the presence of 4-AP (from 17.91 ± 0.70 to 7.65 ± 0.92 pA/pF, TP = +60mv, $p < 0.05$). At the same time, in the presence of IBTX, ACh's effects were almost abolished (from 9.65 ± 1.16 to 8.76 ± 0.67 pA/pF, TP = +60mv, $p > 0.05$) (Fig. 5G), indicating that ACh-decreased activity of whole-cell potassium channels mainly by inhibiting BK channels. In addition, ACh-decreased the whole-cell K⁺ currents were reversed by atropine in the myocytes (Fig. 5C).

7. Discussion

Following previous reports on ACh-mediated umbilical vaso-constriction in human [28], recently, our team not only provided additional new evidence regarding ACh's effects on human umbilical arteries and veins, but also found that ACh-induced umbilical contraction in rats and sheep [7], as the first time to demonstrate ACh-mediated umbilical vasoconstrictions in various animals. The present study extended the study and was the first to show that ACh could reliably cause contraction in umbilical vessels in rabbits too. Based on the finding of ACh-stimulated vasoconstrictions in human, rats, sheep, and rabbits, we would like to propose that blood vessels in umbilical cords have special characters to cholinergic signals, which are very different from healthy blood vessels in other parts of the body where ACh-mediated vasorelaxation was shown. In our experiments during last 6 years, we tested at least one thousand umbilical vessels rings in various species, and observed reliable and repeatable ACh-induced vasoconstriction instead of vasorelaxation [7]. Thus, the accumulated evidence convinces us that it is reasonable to make conclusions now, that the main pharmacological effect of ACh on umbilical blood vessels is to cause vasoconstrictions, no matter in human or animals. Why ACh's effects on umbilical cords are totally different from that in other parts of the body is an interesting question worth further investigation, which is also important to further understanding regulatory mechanisms in controlling blood flow as well as oxygen supply from umbilical cords to fetuses in preventive medicine against toxic hypoxia.

To determine underlying mechanisms of ACh-mediated umbilical vasoconstrictions, we focused on the rat model due to heterogeneity of human subjects. In the present study, ACh-mediated umbilical vasoconstrictions were abolished by atropine, suggesting roles of muscarinic receptor (MR). Then we used specific receptor antagonists on umbilical vessels before applying ACh, the results showed that MR1 and MR3 antagonists could partially inhibit the vasoconstrictions. Although the contractile response related to MR subtypes was complex, the results in rat umbilical vessels supported the finding from previous studies in human umbilical vessels [28]. It is known that the muscarinic receptor type 3 is the main subtype of muscarinic receptors in endothelium of intact vessels. This study showed, in the endothelium-intact umbilical veins, ACh induced contraction *via* MR3 and MR1. There should be complicated reasons for the differences in vascular responses to ACh between umbilical vessels and non-umbilical vessels observed in the present study, which deserves further investigations.

Muscarinic receptor is G-protein-coupling receptor. When ACh binds to M receptors, phospholipase C is activated, then hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol. IP₃ induces Ca²⁺ mobilization from intracellular stores and diacylglycerol activates protein kinase C (PKC) [5]. Thus, PKC pathway plays critical roles in ACh-mediated vasoconstriction [29]. In the presence of U73122, an antagonist of PLC [30], or GF109203X (a PKC inhibitor), ACh-stimulated vasoconstrictions were attenuated in the present study, indicating M-receptors-PLC-PKC signaling pathway was involved [31]. Previous studies showed that ACh's vascular effects were partly mediated by cyclooxygenase products [32]. In the present study, indomethacin inhibited the umbilical vessel contraction, indicating possible involvement of interaction between M receptors and cyclooxygenase.

Influx of Ca²⁺ or release of Ca²⁺ is important to the control of vasoconstriction. In the present study, intracellular Ca²⁺ levels were significantly enhanced by ACh in the umbilical veins shown by calcium image, with a significant increase of the ratio_{340/380} of calcium flow, simultaneously to an increase of vessel tension, indicating a role of calcium in ACh-mediated umbilical vasoconstrictions. To the best of our knowledge, this was the first to demonstrate that ACh-mediated umbilical vasoconstriction was associated with an increase of intracellular calcium. Then, we used nifedipine (L-type Ca²⁺ inhibitor) and 2-APB (IP₃-mediated calcium release antagonist, putative non-selective cationic channel blocker) [33,34], and found both blockers could suppress ACh-stimulated umbilical vasoconstrictions, while the effect of 2-APB was greater than that of nifedipine. Those data suggested that both extracellular and intracellular calcium contributed to ACh-mediated umbilical vasoconstrictions, while the former may be more critical. However, vessel contraction is not only sensitive to changes in [Ca²⁺]_i, Ca²⁺ sensitivity of the contractile apparatus is also important. The relationship between ACh response and calcium sensitivity is worth of further study.

It is well known that increase or decrease of potassium currents on vascular SMCs will affect vessel contraction or relaxation [35]. In isolated rat umbilical vein SMCs, ACh significantly reduced the basal potassium currents, which could be reversed by adding atropine. The patch clamp study provided new evidence that cholinergic signal-mediated rat umbilical vasoconstriction was related to M receptor-decreased potassium currents.

Vascular potassium channels can be divided into four subtypes, BK, Kv, Kir, and K_{ATP}. BK and Kv channels are two major functional units [36]. This study was the first in demonstration of whole-cell K⁺ currents in rat umbilical venous SMCs. To determine which route of potassium channels may be involved in ACh-reduced outward K⁺ currents in SMCs, we used IBTX or 4-AP separately before adding ACh. The results showed that IBTX could almost abolish ACh-mediated outward K⁺ currents, since BK currents were defined as the 10⁻⁷ M IBTX-sensitive component, indicating that BK channels played major roles in the ACh-mediated effects. The pretreatment with 4-AP did not significantly affect ACh's responses. Furthermore, vessel experiments demonstrated that another BK channel blocker CTX could enhance ACh-stimulated vasoconstrictions in the umbilical veins. Those results showed that BK channels were critical in the vasoconstrictions by ACh in the umbilical veins.

In conclusion, this study further demonstrated the umbilical vessels, from human or animals, with specific characteristics in contractile responses to cholinergic agents, which is opposite to ACh's actions on other blood vessels. The underlying mechanism includes: M receptors- PKC signals-intracellular Ca^{2+} and Ca^{2+} dependent K^{+} channels, this signal pathway plays important roles in ACh-stimulated vasoconstrictions in umbilical cords. Considering that the umbilical cord is the only route between mother and fetus in supplying nutrition and oxygen, any factor contributing to the control of its vascular tone should be important to fetal growth, as well as prevention of toxic effects due to *in utero* hypoxia caused by reduced blood flow from the umbilical cord.

Acknowledgments

This study was supported by grants (2012CB947600, 2013BAI04B05), NSFC (81070540, 81320108006, and 81370719). This study was also supported by Jiangsu Key Discipline/Laboratory Funds, and Jiangsu Key Discipline of Human Assisted Reproduction Medicine Funds.

References

1. Fox SB, Khong TY. Lack of innervation of human umbilical cord: an immunohistological and histochemical study. *Placenta*. 1990; 11(1):59–62. [PubMed: 2326237]
2. Tayebati SK, Giannella M, Indraccolo SR, Pignini M, Sabbatini M, Zaccheo D, et al. Muscarinic cholinergic receptors and acetylcholinesterase activity in umbilical artery and vein in pregnancy-induced hypertension (pre-eclampsia). *Clin Exp Hypertens (New York, NY)*. 1993; 19(8): 1205–1217.
3. Wessler I, Michel-Schmidt R, Brochhausen C, Kirkpatrick CJ. Subcellular distribution of choline acetyltransferase by immunogold electron microscopy in non-neuronal cells: placenta, airways and murine embryonic stem cells. *Life Sci*. 2012; 91(21–22):977–980. [PubMed: 22683430]
4. An JY, Yun HS, Lee YP, Yang SJ, Shim JO, Jeong JH, et al. The intracellular pathway of the acetylcholine-induced contraction in catdetrusor muscle cells. *Br J Pharmacol*. 2002; 137(7):1001–1010. [PubMed: 12429572]
5. Ding X, Murray PA. Regulation of pulmonary venous tone in response to muscarinic receptor activation. *Am J Physiol Lung Cell Mol Physiol*. 2005; 288(1):L131–40. [PubMed: 15377494]
6. Eglen RM, Reddy H, Watson N, Challiss RA. Muscarinic acetylcholine receptor subtypes in smooth muscle. *Trends Pharmacol Sci*. 1994; 15(4):114–119. [PubMed: 8016895]
7. Chen N, Lv J, Bo L, Li N, Wu C, Yin X, et al. Muscarinic-mediated vasoconstriction in human, rat and sheep umbilical cords and related vasoconstriction mechanisms. *BJOG*. 2014; 122(12):1630–1639. [PubMed: 25403992]
8. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980; 288(5789):373–376. [PubMed: 6253831]
9. Kaneko M, White S, Homan J, Richardson B. Cerebral blood flow and metabolism in relation to electrocortical activity with severe umbilical cord occlusion in the near-term ovine fetus. *Am J Obstet Gynecol*. 2003; 188(4):961–972. [PubMed: 12712094]
10. Xia S, Lv J, Xu Z, et al. Prenatal exposure to hypoxia induced Beclin 1 signaling-mediated renal autophagy and altered renal development in rat fetuses. *Reprod Sci*. 2015; 22(2):156–164. [PubMed: 24872334]
11. Morton JS, Rueda-Clausen CF, Davidge ST. Flow-mediated vasodilation is impaired in adult rat offspring exposed to prenatal hypoxia. *J Appl Physiol*. 2011; 110 4(April):1073–1082. [PubMed: 21233338]
12. Akata T. Cellular and molecular mechanisms regulating vascular tone: part 2: regulatory mechanisms modulating Ca^{2+} mobilization and/or myofilament Ca^{2+} sensitivity in vascular smooth muscle cells. *J Anesth*. 2007; 21(2):232–242. [PubMed: 17458653]

13. Weirich J, Dumont L, Fleckenstein-Grun G. Contribution of capacitative and non-capacitative Ca^{2+} -entry to M3-receptor-mediated contraction of porcine coronary smooth muscle. *Cell Calcium*. 2005; 38(5):457–467. [PubMed: 16099040]
14. Jackson WF. Ion channels and vascular tone. *Hypertension*. 2000; 35(1 Pt 2):173–178. [PubMed: 10642294]
15. Nakamura T, Kimura J, Yamaguchi O. Muscarinic M2 receptors inhibit Ca^{2+} -activated K^{+} channels in rat bladder smooth muscle. *Int J Urol*. 2002; 9(12):689–696. [PubMed: 12492954]
16. Semenov I, Wang B, Herlihy JT, Brenner R. BK channel $\beta 1$ subunits regulate airway contraction secondary to M2 muscarinic acetylcholine receptor mediated depolarization. *J Physiol*. 2011; 589(Pt 7):1803–1817. [PubMed: 21300746]
17. Tobin G, Giglio D, Lundgren O. Muscarinic receptor subtypes in the alimentary tract. *J Physiol Pharmacol*. 2009; 60(1):3–21.
18. Li S, Fang Q, Zhou A, Wu L, Shi A, Cao L, et al. Intake of high sucrose during pregnancy altered large-conductance Ca^{2+} -activated K^{+} channels and vessel tone in offspring's mesenteric arteries. *Hypertens Res*. 2013; 36(2):158–165. [PubMed: 23013887]
19. Zhang X, Xiao Z, Yao J, Zhao G, Fa X, Niu J. Participation of protein kinase C in the activation of Nrf2 signaling by ischemic preconditioning in the isolated rabbit heart. *Mol Cell Biochem*. 2013; 372(1–2):169–179. [PubMed: 23010891]
20. Parajuli SP, Petkov GV. Activation of muscarinic M3 receptors inhibits large-conductance voltage- and Ca^{2+} -activated K^{+} channels in rat urinary bladder smooth muscle cells. *Am J Physiol Cell Physiol*. 2013; 305(2):C207–14. [PubMed: 23703523]
21. Wu J, Takeo T, Suga S, Kanno T, Osanai T, Mikoshiba K, et al. 2-aminoethoxydiphenyl borate inhibits agonist-induced Ca^{2+} signals by blocking inositol trisphosphate formation in acutely dissociated mouse pancreatic acinar cells. *Pflugers Archiv Eur J Physiol*. 2004; 448(6):592–595. [PubMed: 15197598]
22. Callaghan B, Koh SD, Keef KD. Muscarinic M2 receptor stimulation of $\text{Cav}1.2b$ requires phosphatidylinositol 3-kinase, protein kinase C, and c-Src. *Circ Res*. 2004; 94(5):626–633. [PubMed: 14739158]
23. Medina P, Segarra G, Mauricio MD, Vila JM, Chuan P, Lluch S. Modulation of adrenergic responses of human vas deferens by K^{+} channel inhibitors. *Urology*. 2010; 76(6):1518. e7–1518. e12.
24. Medina P, Segarra G, Mauricio MD, Vila JM, Chuan P, Lluch S. Modulation of adrenergic responses of human vas deferens by K^{+} channel inhibitors. *Urology*. 2010; 76(6):1518. e7–1518. e12.
25. Xiao D, Dasgupta C, Li Y, Huang X, Zhang L. Perinatal nicotine exposure increases angiotensin II receptor-mediated vascular contractility in adult offspring. *PLoS One*. 2014; 9(9):e108161. [PubMed: 25265052]
26. Bo L, Jiang L, Zhou A, Wu C, Li J, Gao Q, et al. Maternal high-salt diets affected pressor responses and microvasoconstriction via PKC/BK channel signaling pathways in rat offspring. *Mol Nutr Food Res*. 2015; 59(6):1190–1199. [PubMed: 25737272]
27. Yang Y, Murphy TV, Ella SR, Grayson TH, Haddock R, Hwang YT, et al. Heterogeneity in function of small artery smooth muscle BKCa: involvement of the $\beta 1$ -subunit. *J Physiol*. 2009; 587(Pt. 12):3025–3044. [PubMed: 19359368]
28. Pujol Lereis VA, Hita FJ, Gobbi MD, Verdi MG, Rodriguez MC, Rothlin RP. Pharmacological characterization of muscarinic receptor subtypes mediating vasoconstriction of human umbilical vein. *Br J Pharmacol*. 2006; 147(5):516–523. [PubMed: 16444291]
29. Ding X, Murray PA. Acetylcholine activates protein kinase C- α in pulmonary venous smooth muscle. *Anesthesiology*. 2007; 106(3):507–514. [PubMed: 17325509]
30. Jin W, Lo TM, Loh HH, Thayer SA. U73122 inhibits phospholipase C-dependent calcium mobilization in neuronal cells. *Brain Res*. 1994; 642(1–2):237–243. [PubMed: 8032885]
31. Andersson KE, Holmquist F, Fovaeus M, Hedlund H, Sundler R. Muscarinic receptor stimulation of phosphoinositide hydrolysis in the human isolated urinary bladder. *J Urol*. 1991; 146(4):1156–1159. [PubMed: 1654463]

32. Schuurman MJ, Villamor E. Endothelium-dependent contraction induced by acetylcholine in the chicken ductus arteriosus involves cyclooxygenase-1 activation and TP receptor stimulation. *Comparative biochemistry and physiology Part A. Mol Integr Physiol.* 2010; 157(1):28–34.
33. Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P₃-induced Ca²⁺ release. *J Biochem.* 1997; 122(3): 498–505. [PubMed: 9348075]
34. Rae MG, Hilton J, Sharkey J. Putative TRP channel antagonists, SKF 96365, flufenamic acid and 2-APB, are non-competitive antagonists at recombinant human $\alpha 1\beta 2\gamma 2$ GABA(A) receptors. *Neurochem Int.* 2012; 60(6):543–554. [PubMed: 22369768]
35. Brayden JE, Nelson MT. Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science (New York NY).* 1992; 256(5056):532–535.
36. Martin P, Rebolledo A, Palomo AR, Moncada M, Piccinini L, Milesi V. Diversity of potassium channels in human umbilical artery smooth muscle cells: a review of their roles in human umbilical artery contraction. *Reprod Sci.* 2014; 21(4):432–441. [PubMed: 24084522]

Fig. 1.

(A,C) Concentration to acetylcholine (ACh) in the rabbit umbilical venous rings or pre-treated with atropine. (B) The representative trace of ACh-induced relaxation in the carotid artery pre-treated with phenylephrine (PE) while ACh-mediated contraction in the rabbit umbilical veins. (D,E) Concentration response to ACh in the rat umbilical venous rings pre-treated with atropine and representative trace of the contraction. *, $P < 0.05$ vs. the control, $n = 5$ for rabbit, $n = 6$ for rat.

Fig. 2.

(A–C) The effects of pirenzepine (MR1 inhibitor), methoctramine (MR2 inhibitor), or pFHHSiD (MR3 inhibitor) on ACh-mediated contraction in RUV. (D–F) The effects of PLC antagonist U73122, cyclooxygenase inhibitor indomethacin, or PKC antagonist GF109203X on ACh-mediated contraction in RUV. Venous rings were pretreated with U73122 for 30 min and then subjected to the cumulative addition of ACh in Krebs solution. *, $P < 0.05$ vs. the control, $n = 5-6$. RUV, rat umbilical vein.

Fig. 3. (A,B) Voltage-operated calcium channels inhibitor nifedipine reduced the ACh-induced contraction while IP_3 receptor antagonist 2-APB reduced the ACh-induced contraction. (C) Intracellular calcium levels in response to ACh in the rat umbilical veins. Typical recordings of ACh-increased $[\text{Ca}^{2+}]_i$ in the rings, $n = 6-7$.

Fig. 4. (A–D) The effects of charybdotoxin(CTX), 4-AP, barium chloride(BaCl₂), glybenclamide (Gly) on the ACh-induced contraction. (E, F) Representative traces show the inhibition of total K⁺ currents by iberiotoxin(IBTX) or 4-AP. (G, H) Whole cell K⁺ currents on rat umbilical venous myocytes treated with K⁺ channel antagonisms. *, $P < 0.05$ vs. the control, $n = 6$.

Fig. 5. (A, B) Representative traces of baseline of K^+ currents and in the presence of ACh, (C) Summarized $I-V$ relationships showed an inhibition by ACh on the whole cell K^+ currents that were reversed by atropine. (D,E) Representative traces of K^+ currents in the 4-AP + ACh and IBTX + ACh. (F,G) Pre-treatment with IBTX inhibited ACh-induced K^+ currents, however, the ACh-inhibited in K^+ currents was not affected by 4-AP; *, $P < 0.05$ vs. the control, $n = 6-7$.