

Effects of the polyphenol resveratrol on contractility of human term pregnant myometrium

R. Novaković¹, N. Radunović², J. Marković-Lipkovski³, S. Ćirović³,
B. Beleslin-Čokić⁴, B. Ilić⁴, B. Ivković⁵, H. Heinle⁶, V. Živanović⁷, and
Lj. Gojković-Bukarica^{1,*}

¹Institute of Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia ²Institute of Gynecology and Obstetrics, Clinical Center of Serbia, 11000 Belgrade, Serbia ³Institute of Pathology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia ⁴Clinic of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia, 11000 Belgrade, Serbia ⁵Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Belgrade, 11000 Belgrade, Serbia ⁶Institute of Physiology, University of Tuebingen, 72072 Tuebingen, Germany ⁷University Hospital Center 'Dr Dragiša Mišović-Dedinje', 11000 Belgrade, Serbia

*Correspondence address: Tel: +381-11-3643395; Fax: +381-11-3643397; E-mail: l.g.bukarica@sezampro.rs

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ABSTRACT: The ideal agent for prevention and treatment of uterine abnormal contractility has not been found. The polyphenol resveratrol possesses a wide spectrum of pharmacologic properties, but its influence on the contractility of human myometrium is not defined. The present study evaluated the effect of resveratrol on the oxytocin-induced contractions of human term pregnant myometrium *in vitro* and the contribution of different K⁺ channels to resveratrol action. Resveratrol induced a concentration-dependent relaxation of myometrium contractions (pD₂ value and maximal responses were 4.52 and 82.25%, respectively). Glibenclamide, a selective blocker of ATP-sensitive (K_{ATP}), iberiotoxin, a selective blockers of big-calcium sensitive (BK_{Ca}) and 4-aminopiridine, a non-selective blocker of voltage-sensitive (Kv) channels induced a significant shift to the right of the concentration–response curves of resveratrol. Inhibition achieved by 0.1 mM resveratrol was insensitive to all K⁺ channel blockers. A K⁺ channel opener, pinacidil, inhibited oxytocin-induced contractions of pregnant myometrium with comparable potency and efficacy to resveratrol (pD₂ values and maximal relaxation were 4.52 and 83.67%, respectively). Based on K⁺ channel opener/blocker affinities, it appears that the inhibitory response of resveratrol involves different myometrial K⁺ channels. When applied in high concentrations, resveratrol has an additional K⁺-channel-independent mechanism(s) of action. Furthermore, immunohistochemistry staining and western blot analyses detected the presence and distribution of K_{ATP}, BK_{Ca} and Kv channel proteins in pregnant myometrium.

Key words: contraction / K⁺- channels / myometrium / pregnancy / resveratrol

Introduction

Approximately 50% of all preterm deliveries are due to preterm labor which is the cause of death and morbidity of neonates (Di Renzo *et al.*, 2011). It is clear that once initiated, preterm labor is difficult to arrest fully, even though transient suppression by pharmacologic means is possible. Furthermore, preterm labor is more common and serious problem in a specific group of women from *in vitro* fertilization (IVF) programs (Van Voorhis, 2007). Despite little evidence to support improvement in key perinatal outcomes with these drugs, the β₂-adrenoceptor agonists remain the most popular option for the suppression of preterm myometrial contractions.

Previous data have shown that the K⁺ channel family affects cell function and plays a major role in regulation of myometrial contractility (Khan *et al.*, 2001; Brainard *et al.*, 2007). Potassium efflux from myometrial

smooth muscle cells results in membrane repolarization and this is responsible for maintaining the resting membrane potential. Changes in the expression or activity of K⁺ channels can translate into an inadequate repolarization leading to aberrant uterine activity. Thus, K⁺ channels alterations may contribute to certain pathophysiological conditions such as preterm labor. A number of studies have shown that opening of different types of K⁺ channel leads to relaxation of non-pregnant as well as pregnant myometrium (Morrison *et al.*, 1993; Khan *et al.*, 1998; Longo *et al.*, 2003; Novakovic *et al.*, 2007). It seems that function and molecular expression of K⁺ channels are dependent on stage of pregnancy, the mother's age and hormones influences (Song *et al.*, 1999; Knock *et al.*, 2001; Lovasz *et al.*, 2011; Du *et al.*, 2013). For instance, it has been shown that activity of ATP-sensitive K⁺ (K_{ATP}) and big Ca²⁺-sensitive K⁺ (BK_{Ca}) channels are diminished in late pregnancy (Matharoo-Ball *et al.*, 2003; Lovasz *et al.*, 2011). Holdiman *et al.* (2002)

suggest that alternative splicing of the BK_{Ca} channel transcript is regulated by 17β-estradiol and may allow for differential expression of channel isoforms, leading to altered modulation without a change in channel density. Recently, it has been demonstrated that K_{ATP} channels are up-regulated with increasing age of the human myometrium. The myometrium of parturient over 35 years old possess more K_{ATP} channels than the myometrium of younger parturient (Du et al., 2013). It is well known that K⁺ channel family includes more than 100 various protein subunits (Coetzee et al., 1999). The identification of subunits/proteins of K⁺ channels in myometrial smooth muscle cell is important for understanding preterm labor.

Our knowledge regarding the mechanisms that underlie normal physiologic labor has advanced considerably, but the discovery of new drugs that lack the side effects of commonly used tocolytics is not satisfactory. Within the last 10 years, resveratrol is found under spotlight like 'one molecule—many targets' (Pirola and Fröjdö, 2008). Several potential therapeutic applications of resveratrol have come to light (Pirola and Fröjdö, 2008; Li et al., 2012). It has been shown that resveratrol promotes smooth muscle relaxation in non-pregnant rat uterus (Hsia et al., 2011; Novakovic et al., 2013) as well blood vessels (Novakovic et al., 2006a, b; Gojkovic-Bukarica et al., 2008; Protić et al., 2013) and gallbladder (Wang et al., 2008). Moreover, K⁺ channels have been implicated directly or indirectly in the action of resveratrol (Novakovic et al., 2006a, b; Novakovic et al., 2013; Gojkovic-Bukarica et al., 2008; Protić et al., 2013). The relaxant action of resveratrol in non-pregnant rat uterus results in reduced intracellular Ca²⁺ levels and inhibition of different types of contractions: spontaneous rhythmic contractions and contractions induced by oxytocin, prostaglandins, high K⁺ concentration and Ca²⁺ channel activator (Bay K 8644) (Hsia et al., 2011; Novakovic et al., 2013). Resveratrol supplements widely present on the market have been proposed as a potential therapeutic to improve metabolic health (considering their possible cardioprotective, neuroprotective, antidiabetic effects); however, little is known about effects on the contractility of pregnant myometrium.

Thus, the aims of the present study were to elucidate whether resveratrol is able to modulate contractility of pregnant human term myometrium and to investigate a role of K⁺ channels in the action of resveratrol. The expression and distribution of the different K⁺ channels subunits were examined by immunohistochemistry staining and by western blot.

Materials and Methods

Tissue collection and preparation

Patients were recruited in the Institute of Gynecology and Obstetrics, Clinical Center of Serbia. The study was approved by the Ethics Committee of Faculty of Medicine, University Belgrade (No 4211/2), and recruitment was carried out by the provision of information sheets and by obtaining written informed consent. The women were from IVF programs. *In vitro* fertilization program and care during pregnancy have been carried in accordance with the recommendations of the 'Fertility: Assessment and Treatment for People with Fertility Problems' (NICE clinical guideline 11, 2004).

Myometrial samples were obtained from 42 non-laboring women undergoing elective Cesarean section in the third trimester of pregnancy. The maternal demographic details were as follows: mean age 35.46 years (range 27–43); median period of gestation 39 weeks (range 37–40). No tocolytic agents have been administered to the mother 24 h prior to

Caesarean delivery. The reasons for Cesarean section included breech presentation, previous Cesarean section and cephalopelvic disproportion. Biopsies were excised from the midline portion of the upper lip of the incision in the lower uterine segment. Tissue samples for isometric recordings were placed in fresh Krebs–Ringer solution and tissue was stored at 4°C and used within 12 h of collection. Additional tissue samples were rinsed with Krebs–Ringer solution and immediately frozen in liquid nitrogen at 80°C or placed in formalin.

Tissue bath experiments

Longitudinal myometrial strips were dissected, measuring 2 × 2 × 10 mm, and mounted under 2 g of tension in organ tissue baths (TSZ-04/1.2, Experimentia, Budapest, Hungary) for isometric recording as described previously (Longo et al., 2003). Data were recorded via computer using IsoLAB software (Elunit, Belgrade, Serbia). The tissue baths contained 10 ml of Krebs–Ringer solution, which was maintained at 37°C and at a pH of 7.4 and gassed continuously with a mixture of 95% O₂ and 5% CO₂ (Crankshaw, 2001). The strips were allowed to equilibrate in Krebs–Ringer solution for at least 1 h and solution was changed every 15 min during the equilibration period. Following equilibration, contractions were stimulated by the addition of the uterotonic agent oxytocin to achieve a tissue bath concentration of 20 nM for a period of 30 min.

Concentration–response curves were constructed by adding resveratrol (1–100 μM) or the K⁺ channel opener (KCO) pinacidil (0.01–100 μM) directly to the bathing solution in a cumulative way, taking the amplitude of the response immediately before addition of a drug as the control contraction. The results are expressed as the percentage inhibition of the control contraction. Increasing concentrations of resveratrol or pinacidil were added only after the previous concentration had produced an equilibrium response or after 20 min if no response was obtained.

To test the involvement of K⁺ channels in a mechanism of action of resveratrol, the different K⁺ channel blockers were examined. In separate experiments, glibenclamide, iberiotoxin or 4-aminopyridine (4-AP) were added to the bathing solution ~20 min before exposure to resveratrol. The concentration–response curves to resveratrol were obtained in the presence of K⁺ channel blockers. Vehicle-matched control experiments were conducted.

Drugs and solutions

All chemicals were obtained from Sigma-Aldrich Inc., St Louis, MO, USA. Resveratrol was dissolved in 70% v/v ethanol with further dilution in distilled water before use. Working concentrations of ethanol in the bath were <0.01% (v/v). Glibenclamide was dissolved in polyethylene glycol. Stock solution of pinacidil was dissolved in dilute acid solution (0.1 N HCl) with further dilution in water before use. Iberiotoxin, 4-AP and oxytocin were dissolved in distilled water. The Krebs–Ringer solution had the following composition (mmol/l): NaCl 120, KCl 5, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11. All drugs were added directly to the bath in a volume of 50 μl and the concentrations given are the calculated final concentrations in the bath solution.

Immunohistochemistry staining

Thick paraffin sections (5 μm) from the formalin-fixed, paraffin-embedded tissue samples of human myometrium were deparaffinized and dehydrated. For antigen retrieval, citrate buffer (pH 6.0, 20 min in microwave) was used. Primary polyclonal rabbit antibodies Anti-Kir6.1, Anti-Kir6.2, Anti-Kv2.1, Anti-Kv4.2 and Anti-K_{Ca}1.1 (all diluted 1:50, Alomone Labs, Jerusalem, Israel) were incubated for 1 h at room temperature. Sections were then treated with EnVision™ Detection System (Dako, Germany) using 3-amino-9-ethylcarbazole (AEC) as substrate and counterstained with hematoxylin. Negative controls were performed by omitting the primary

antibody and stained by the EnVision™ method (Gojkovic-Bukarica *et al.*, 2011). The slides were evaluated using the light microscope BX53 with DPI2-CCD camera (Olympus, Germany).

Western blot analysis

The tissue samples of human myometrium were homogenized in RIPA (Radio-Immunoprecipitation Assay) buffer (1 M TRIS-HCl, pH 7.5, 0.5 M EDTA, 10% SDS, 10% sodium deoxycholate, 10% Triton X-100) with protease inhibitors (Roche Applied Science, Mannheim, Germany). Cell lysate was centrifuged for 20 min, 10 000g at 4°C and the supernatant was transferred to a new tube. Protein concentration was measured at 595 nm with spectrophotometer using the Bio-Rad Protein Assay, based on the method of Bradford. Prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, LDS (lithium dodecyl sulfate, pH 8.4) Sample Buffer and Reducing Agent were added to the sample, denatured for 10 min at 70°C and loaded onto a precast 4–12% Bis-Tris gel (Life Technologies, Carlsbad, CA, USA). After electrophoresis, separated proteins were transferred to a nitrocellulose membrane. A membrane was blocked with 5% non-fat dry milk and 0.1% Tween 20 for 1 h at room temperature and probed with primary antibody for Kir6.1 and Kir6.2 subunit of K_{ATP} channels, α -subunits Kv2.1 and Kv4.2 subtype of Kv channels and $K_{Ca}1.1$ subunit of BK_{Ca} channels (Anti-Kir6.1; Anti-Kir6.2; Anti-Kv2.1; Anti-Kv4.2; Anti- $K_{Ca}1.1$; Alomone Labs, Jerusalem, Israel) overnight at 4°C. The membranes were incubated separately with different antibodies. After visualization, the membranes were stripped with 0.2 M NaOH, blocked and reprobred with another antibody. Membranes were probed with horse-radish peroxidase-conjugated antirabbit secondary antibody for 1.5 h at room temperature. Western blotting kit (Roche, Applied Science, Mannheim, Germany) was used for the chemiluminescent detections of proteins. After visualization, the membranes were stripped with 0.2 M NaOH, blocked and reprobred with anti- β -actin antibody (Abcam, Cambridge, UK) and visualized. Protein loading was normalized to β -actin (Beleslin-Cokic *et al.*, 2011). ImageQuant software was used for analysis.

Treatment of data and statistics

The amplitude of the contractions was measured from the baseline to the top of the spike. The mean amplitude during the control period was taken as 100%.

EC_{50} value is defined as the concentration of resveratrol or pinacidil required to produce 50% of the maximum response of contractions, and it was determined for each curve by using a non-linear least square fitting procedure of the individual experimental data, and presented as pD_2 ($pD_2 = -\log EC_{50}$). The results were tested for normality and data are expressed as the mean \pm SEM; n refers to the number of experiments. Statistically significant differences between means were determined by Student's t -test; a value of $P < 0.05$ was considered statistically significant. Data analysis was performed in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

Results

The effects of resveratrol and pinacidil on contractions provoked by oxytocin

Application of nanomolar concentration of oxytocin (20 nM final concentration in the bath solution) produced phasic contractions of constant amplitude (4.95 ± 0.08 g) and frequency (2.65 ± 0.35 contractions per 10 min), as shown in Fig. 1 ($n = 12$). Resveratrol (1–100 μ M) significantly inhibited amplitude of oxytocin-induced contractions in a concentration-

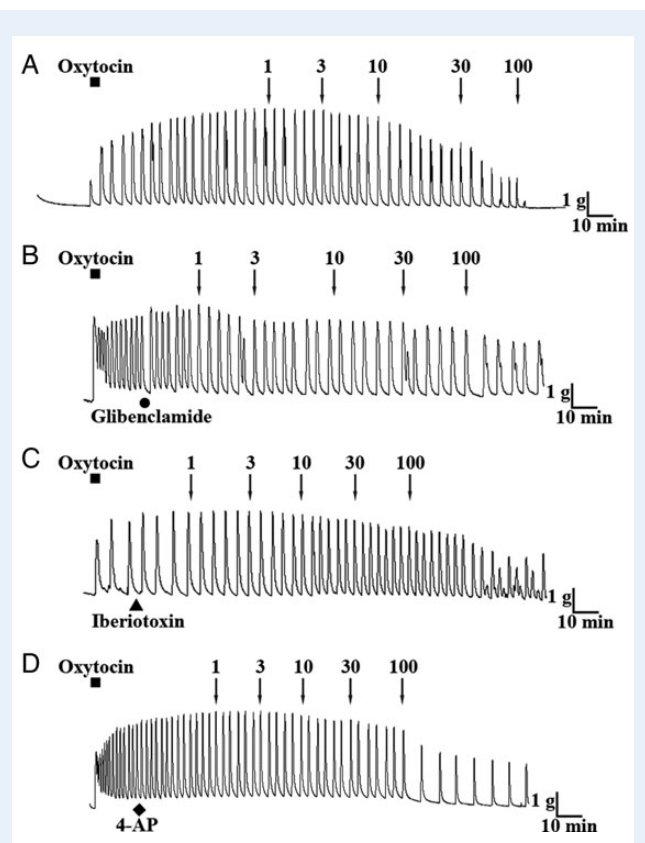


Figure 1 Representative recordings showing the inhibitory effect of resveratrol (1–100 μ M) on oxytocin (20 nM square)-induced contractile activity of human myometrial preparation from term pregnancies in the absence (A) and presence of K^+ -channel blockers: (B) glibenclamide (10 μ M, circle); (C) iberiotoxin (100 nM, triangle); 4-aminopyridine (4-AP, 1 mM, diamond). The plots show tension generated in the preparations (in gram) in real time (in minutes). The dug concentrations given here are the calculated final concentrations in the bath solution.

dependent manner with pD_2 value of 4.52 ± 0.11 , maximal responses: $82.25 \pm 1.50\%$, $n = 12$ (Fig. 1A).

Glibenclamide (10 μ M) produced a significant rightward shift of the concentration–response curve of resveratrol (pD_2 values: 4.52 ± 0.11 in the absence versus 4.12 ± 0.18 in the presence of glibenclamide, $P < 0.05$, $n = 8$) and suppression of the maximal response ($82.25 \pm 1.50\%$ in the absence versus $56.80 \pm 3.87\%$ in the presence of glibenclamide, $P < 0.05$, $n = 8$, Figs 1B and 2A).

Iberiotoxin (100 nM) produced a significant rightward shift of the concentration–response curve of resveratrol (pD_2 values: 4.52 ± 0.11 in the absence versus 4.05 ± 0.54 in the presence of iberiotoxin, $P < 0.05$, $n = 8$) and suppression of the maximal response ($82.25 \pm 1.50\%$ in the absence versus $54.56 \pm 2.38\%$ in the presence of iberiotoxin $P < 0.05$, $n = 8$, Figs 1C and 2B).

Application of 4-AP (1 mM) produced a significant rightward shift of the concentration–response curve of resveratrol (pD_2 values: 4.52 ± 0.11 in the absence versus 4.02 ± 0.38 in the presence of 4-AP, $P < 0.05$, $n = 8$) with a significant suppression of the maximal response ($82.25 \pm 1.50\%$ in the absence versus $50.79 \pm 2.95\%$ in the presence of 4-AP, $P < 0.05$, $n = 8$, Figs 1D and 2C).

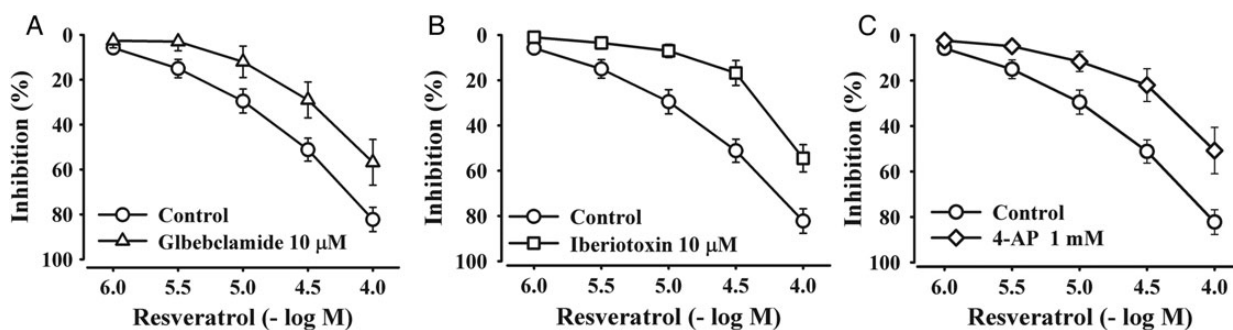


Figure 2 Antagonism of the inhibitory effect of resveratrol by K^+ -channel blockers on contractions provoked by oxytocin on the human term pregnant myometrium. Concentration–response curves for resveratrol in the absence (circle) and presence of (A) glibenclamide (10 μ M, triangle), (B) iberiotoxin (100 nM, square), (C) 4-aminopyridine (4-AP, 1 mM, diamond). The points are the means and the vertical lines show the SEM ($n = 8–12$).

In order to compare the effect of resveratrol with the effect of KCO, pinacidil was used ($n = 8$). Pinacidil (10 nM–100 μ M) inhibited contractions induced by oxytocin in the concentration-dependent manner with pD_2 value of 4.52 ± 0.45 and maximal responses of $83.67 \pm 2.23\%$. There are no statistically significant differences between pD_2 values and maximal responses of resveratrol and pinacidil (4.52 ± 0.11 versus 4.52 ± 0.45 ; 82.25 ± 1.50 versus $83.67 \pm 2.23\%$, respectively).

Detection of K_{ATP} , Kv and BK_{Ca} channels subunit by immunohistochemistry and western blot analyses

Immunohistochemical staining revealed expression of different K^+ channels in human pregnant myometrium. In all analyzed tissues, Kir6.1 (Fig. 3A) and Kir6.2 (Fig. 3C) subunits of K_{ATP} channel, $K_{Ca}1.1$ (Fig. 3E) subunit of BK_{Ca} channel and α -subunit Kv4.2 (Fig. 3G) subtype of Kv channel were detected. Human pregnant myometrium clearly showed expression of Kir6.1, Kir6.2, $K_{Ca}1.1$ and Kv4.2 subunits on smooth muscle cell (Fig. 3). The expression of all four channels on smooth muscle cells was mainly cytoplasmic but with a diffuse extension pattern. The distribution of the Kir6.2 was the most widespread since it was present on all smooth muscle cells (Fig. 3C). However, all other channels were detected on single smooth muscle cells such as Kir6.1 (Fig. 3A), or on groups of smooth muscle cells, such as $K_{Ca}1.1$ and α -subunit of Kv4.2 channels (Fig. 3E and G). In addition to diffuse cytoplasmic expression, channels Kir6.1, Kir6.2 and Kv4.2 revealed strong granular perinuclear localization.

Western blotting analysis detected Kir6.1 and Kir6.2 subunits of K_{ATP} channels, the $K_{Ca}1.1$ subunit of BK_{Ca} channels and α -subunit Kv4.2 subtype of Kv channels in human pregnant myometrium ($n = 5$, each, Fig. 4). Results were normalized to β -actin expression. Immunohistochemical staining and western blotting analyses did not detect expression of Kv2.1 in all analyzed samples.

Discussion

This is the first report showing the inhibitory effects of resveratrol on contractility of human term pregnant myometrium. Resveratrol significantly decreased the amplitude of phasic contractions induced by oxytocin. This sensitivity was in line with sensitivity of oxytocin-induced

contractions of non-pregnant rat uterus as well as uterine arteries (Naderali et al., 2000; Novakovic et al., 2013). There are differences between the pD_2 value of resveratrol obtained here and the pD_2 values in the investigations on the different types of human and rat blood vessels (Novakovic et al., 2006b; Nagaoka et al., 2007; Gojkovic-Bukarica et al., 2008) and the guinea pig gallbladder smooth muscles (Wang et al., 2008). This discrepancy in the results obtained in the various experimental models suggests tissue and species selectivity of resveratrol.

In order to analyze the contribution of K^+ channels to the inhibition of myometrial contractions produced by resveratrol, we used agents that are known to possess a K^+ channel-blocking/opening activity.

In the present study, glibenclamide, a highly specific K_{ATP} channels blocker, partly antagonized the effect of resveratrol on the oxytocin-induced contractions, indicating the involvement of K_{ATP} channels in resveratrol mechanism of action. These data are in agreement with results obtained on the rat non-pregnant myometrium and heart, where resveratrol depressed contraction due to activation of K_{ATP} channels (Buluc et al., 2007; Novakovic et al., 2013). Structurally, K_{ATP} channels are composed of pore-forming inward rectifiers channels, Kir6.1 or Kir6.2, and regulatory subunits, SUR1, SUR2A or SUR2B that assemble to form an octameric complex, and the presence of all five subunits was confirmed in human pregnant myometrium (Du et al., 2013). Previous papers have shown two combinations of K_{ATP} channels subunits, namely Kir6.1/SUR2B and Kir6.2/SUR1, in the smooth muscle of non-pregnant rat and human uterus at the transcription level (Chien et al., 1999; Curley et al., 2002). Also, the immunohistochemical study presented here has confirmed existence of both Kir6.1 and Kir6.2 subunits in the pregnant human myometrium. These findings are in line with the research of Xu et al. (2011). The K_{ATP} channels composed of Kir6.1 and SUR2B subunits are expressed at higher levels in human non-pregnant myometrium compared with late gestation (Curley et al., 2002). It is possible that resveratrol may produce relaxation of pregnant myometrium, in part, by activation of Kir6.2/SUR1 channels.

According to the results with iberiotoxin, a highly specific BK_{Ca} channel blocker, and resveratrol, it is reasonable to conclude that BK_{Ca} channels are involved in the mechanism of action of resveratrol on human pregnant myometrium. Similarly, iberiotoxin antagonized the response to resveratrol on the spontaneous rhythmic contractions and phasic contractions induced by oxytocin, but did not antagonize

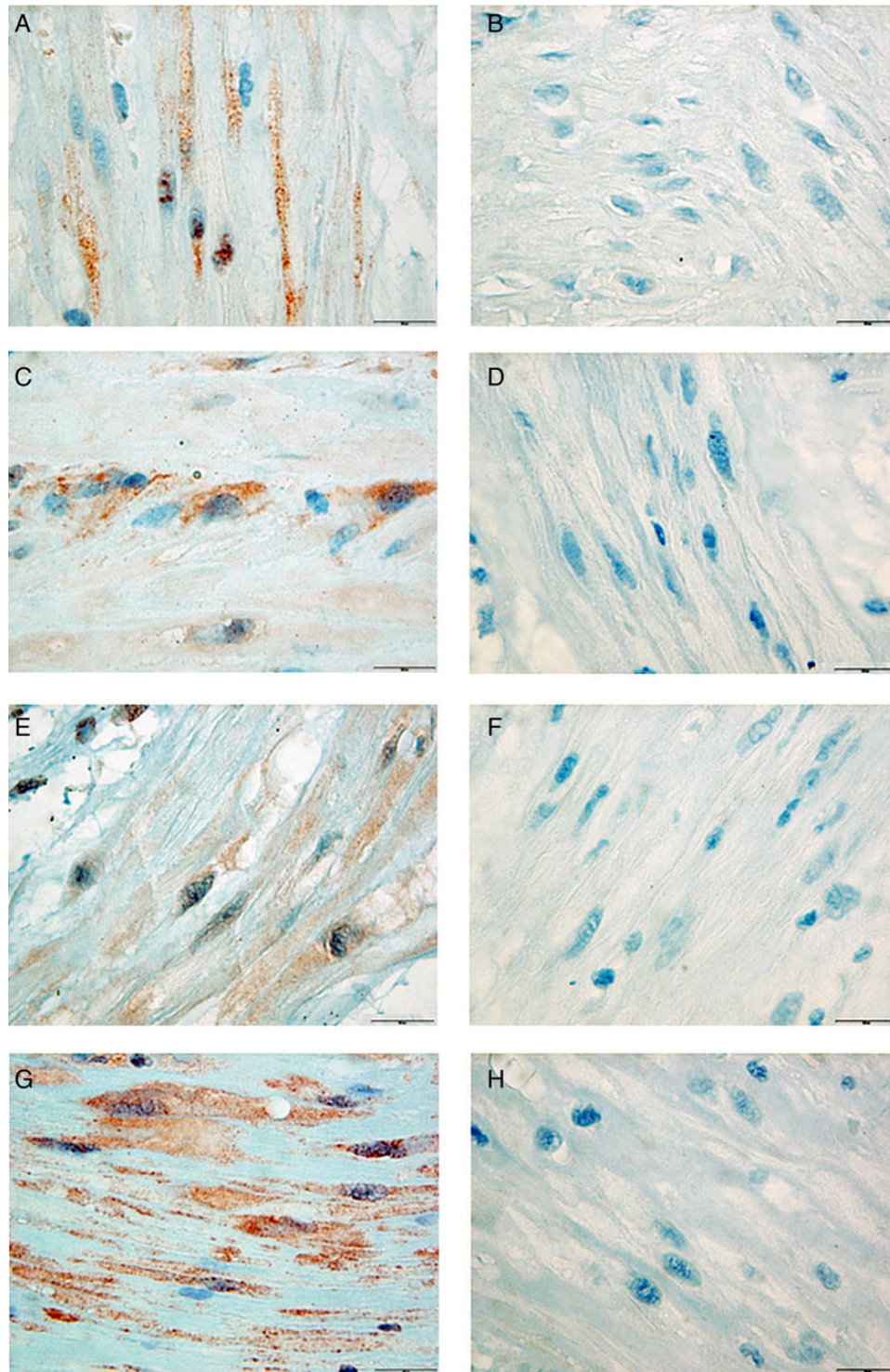


Figure 3 Detection of four different K^+ channels on the human term pregnant myometrium by immunohistochemistry staining. Expression of Kir6.1 (brown staining) on single smooth muscle cells (**A**); diffuse expression of Kir6.2 (brown staining) on smooth muscle cells (**C**); expression of $K_{Ca}1.1$ (brown staining) on smooth muscle cells (**E**); expression of Kv4.2 (brown staining) on smooth muscle cells (**G**). In all photos, nuclei were stained with hematoxylin (blue color). Negative controls (**B**, **D**, **F** and **H**). Original magnification $400\times$ (A–H).

the effect of resveratrol on the tonic contractions induced by oxytocin in non-pregnant rat myometrium (Novakovic *et al.*, 2013). In the pancreatic β cells, Chen *et al.* (2007) have suggested that resveratrol could activate

BK_{Ca} channels through an increase in intracellular Ca^{2+} . Similarly, it has been shown in the portal vein that BK_{Ca} channels partly mediate the inhibitory effect of resveratrol by affecting the smooth muscle Ca^{2+}

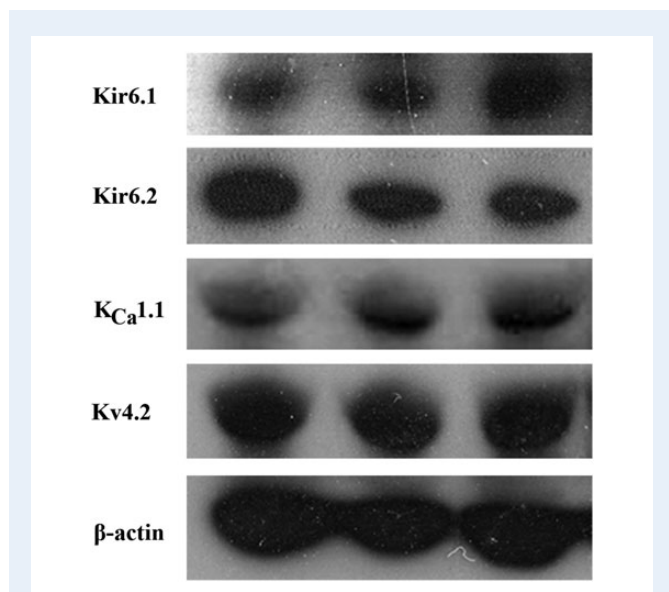


Figure 4 Western blot analysis of K^+ channels in the human term pregnant myometrium. The expression of Kir6.1 and Kir6.2 subunit of K_{ATP} channels, α -subunits of Kv4.2 channels and α -subunit ($K_{Ca1.1}$) of BK_{Ca} channels protein relative to β -actin. The three lanes show samples from different women.

channels and/or Ca^{2+} mobilizing through cells (Protić et al., 2013). It is possible that resveratrol modulates intracellular Ca^{2+} homeostasis and in turn indirectly regulates potassium efflux. Our data suggest the involvement of BK_{Ca} channels in the relaxant effect of resveratrol, but according to relative resistance of this effect to iberiotoxin, it seems that resveratrol exerts inhibition of the pregnant myometrium by acting on additional sites. The results of experiments from immunohistochemistry and western blotting analysis showed the presence of BK_{Ca} channels. Despite being encoded by a single gene, BK_{Ca} channel diversity is high. The expression of α -subunits is highly tissue-specific. The presence of α -subunits was confirmed at transcript and protein level in non-pregnant rat as well as non-pregnant and pregnant human myometrium (Chanrachakul et al., 2003; Curley et al., 2004; Novakovic et al., 2013) and results present here correspond to these findings.

The Kv subtype of K^+ channels is a large group with a remarkably diversity of channels that are activated by voltage. The data presented here showed that 4-AP antagonized the effect of resveratrol on the oxytocin-induced contractions of pregnant myometrium. Previous studies have confirmed that Kv channels participate in the resveratrol effects in the different blood vessels and phasic contractions of non-pregnant rat myometrium (Novakovic et al., 2006a, b; Novakovic et al., 2013; Gojkovic-Bukarica et al., 2008). However, the relaxant effects of resveratrol on the tonic oxytocin-induced contractions of non-pregnant rat myometrium and tonic contraction of the retinal arterioles were not blocked by 4-AP (Nagaoka et al., 2007; Novakovic et al., 2013). In contrast, it has been shown that resveratrol inhibits Kv channels in the pancreatic β cells (Chen et al., 2007). Based on electrophysiological studies, three separate Kv currents have been identified in myometrial cells: 4-AP insensitive, 4-AP sensitive rapidly inactivating current and 4-AP sensitive slowly inactivating current (Smith et al., 2007). These various Kv current components have different gating properties and

sensitivities to drugs. Until recently, only evidence from animal models for the Kv4.x pore-forming subunit of Kv channels has been provided (Suzuki and Takimoto, 2005). The findings of molecular expression of Kv4.x channels in human myometrium were not established yet. A few studies on mouse and rat uterus have shown that Kv4.3 subtype expression is reduced during pregnancy (Suzuki and Takimoto, 2005; Smith et al., 2007). However, the fact that both immunohistochemistry staining and western blot confirmed the presence of α -subunit of Kv4.2 channels in human pregnant myometrium suggests that this type of channel was not down-regulated during pregnancy. This assumption is in line with the research on term-pregnant mice (Smith et al., 2007). Taken together, these findings suggest a possible role for Kv4.2 channels in the action of resveratrol.

In order to compare the pharmacologic profile of resveratrol to KCOs, we tested the effects of pinacidil in the same experimental model. Under the experimental conditions described here, pinacidil produced inhibition of oxytocin-induced contractions with the same pD_2 value as resveratrol.

It can be concluded that resveratrol exerts a potent inhibitory effect on human pregnant myometrial contractility. Based on K^+ channel blocker/opener affinity, it appears that resveratrol exerts its effect on induced contractions by modulating K^+ channel, but it has an additional K^+ -channels-independent mechanism(s) of action and further studies are needed to identify the intracellular targets of resveratrol in human myometrial cells. The present study demonstrated the presence of K_{ATP} , Kv and BK_{Ca} channel proteins in myometrial cells of the human term pregnant uterus.

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Authors' roles

R.N. designed the research, carried out the pharmacologically experiments, analyzed data, wrote the paper, coordinated the study; N.R.: conducting Cesarean section, provided clinical data and the samples of myometrium; J.M.-L.: interpretation of IHH data; S.Ć. carried out the IHH research, analyzed the IHH data; B.B.-Č. and B.I. carried out the WB experiments and analyzed data; B.I., V.Ž. and H.H.: interpretation of data, contributed to discussion; Lj.G.-B. designed the research, analyzed the data, wrote the paper and coordinated the study.

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Conflict of interest

None declared.

References

Beleslin-Čokić BB, Cokić VP, Wang L, Piknova B, Teng R, Schechter AN, Noguchi CT. Erythropoietin and hypoxia increase erythropoietin

- receptor and nitric oxide levels in lung microvascular endothelial cells. *Cytokine* 2011;**54**:129–135.
- Brainard AM, Korovkina VP, England SK. Potassium channels and uterine function. *Semin Cell Dev Biol* 2007;**18**:332–339.
- Buluc M, Ayaz M, Turan B, Demirel-Yilmaz E. Resveratrol-induced depression of the mechanical and electrical activities of the rat heart is reversed by glyburide: evidence for possible K(ATP) channels activation. *Arch Pharm Res* 2007;**30**:603–607.
- Chanrachakul B, Matharoo-Ball B, Turner A, Robinson G, Broughton-Pipkin F, Arulkumaran S, Khan RN. Immunolocalization and protein expression of the alpha subunit of the large-conductance calcium-activated potassium channel in human myometrium. *Reproduction* 2003;**126**:43–48.
- Chen WP, Chi TC, Chuang LM, Su MJ. Resveratrol enhances insulin secretion by blocking K (ATP) and K (V) channels of beta cells. *Eur J Pharmacol* 2007;**568**:269–277.
- Chien EK, Zhang Y, Furuta H, Hara M. Expression of adenosine triphosphate-sensitive potassium channel subunits in female rat reproductive tissues: overlapping distribution of messenger ribonucleic acid for weak inwardly rectifying potassium channel subunit 6.1 and sulfonylurea-binding regulatory subunit 2. *Am J Obstet Gynecol* 1999;**180**:1121–1126.
- Coetzee WA, Amarillo Y, Chiu J, Chow A, Lau D, McCormack T, Moreno H, Nadal MS, Ozaita A, Pountney D *et al*. Molecular diversity of K⁺ channels. *Ann N Y Acad Sci* 1999;**868**:233–285.
- Crankshaw DJ. Pharmacological techniques for the in vitro study of the uterus. *J Pharmacol Toxicol Methods* 2001;**45**:123–140.
- Curley M, Cairns MT, Friel AM, McMeel OM, Morrison JJ, Smith TJ. Expression of mRNA transcripts for ATP-sensitive potassium channels in human myometrium. *Mol Hum Reprod* 2002;**8**:941–945.
- Curley M, Morrison JJ, Smith TJ. Analysis of Maxi-K alpha subunit splice variants in human myometrium. *Reprod Biol Endocrinol* 2004;**2**:67.
- Di Renzo GC, Roura LC, Facchinetti F, Antsaklis A, Breborowicz G, Gratacos E, Husslein P, Lamont R, Mikhailov A, Montenegro N *et al*. Guidelines for the management of spontaneous preterm labor: identification of spontaneous preterm labor, diagnosis of preterm premature rupture of membranes, and preventive tools for preterm birth. *J Matern Fetal Neonatal* 2011;**24**:659–667.
- Du Q, Jovanović S, Tulić L, Slijvančanin D, Jack DW, Žižić V, Abdul KS, Tulić I, Jovanović A. KATP channels are up-regulated with increasing age in human myometrium. *Mech Ageing Dev* 2013;**134**:98–102.
- Gojkovic-Bukarica L, Novakovic A, Kanjuh V, Bumbasirevic M, Lesic A, Heinle H. A role of ion channels in the endothelium-independent relaxation of rat mesenteric artery induced by resveratrol. *J Pharmacol Sci* 2008;**108**:124–130.
- Gojkovic-Bukarica L, Savic N, Peric M, Markovic-Lipkovski J, Cirivic S, Kanjuh V, Cvejic J, Atanackovic M, Lesic A, Bumbasirevic M *et al*. Effect of potassium channel opener pinacidil on the contractions elicited electrically or by noradrenaline in the human radial artery. *Eur J Pharmacol* 2011;**54**:266–273.
- Holdiman AJ, Fergus DJ, England SK. 17beta-Estradiol upregulates distinct maxi-K channel transcripts in mouse uterus. *Mol Cell Endocrinol* 2002;**192**:1–6.
- Hsia SM, Wang KL, Wang PS. Effects of resveratrol, a grape polyphenol, on uterine contraction and Ca²⁺ mobilization in rats in vivo and in vitro. *Endocrinology* 2011;**152**:2090–2099.
- Khan RN, Morrison JJ, Smith SK, Ashford ML. Activation of large-conductance potassium channels in pregnant human myometrium by pinacidil. *Am J Obstet Gynecol* 1998;**178**:1027–1034.
- Khan RN, Matharoo-Ball B, Arulkumaran S, Ashford ML. Potassium channels in the human myometrium. *Exp Physiol* 2001;**86**:255–264.
- Knock GA, Tribe RM, Hassoni AA, Aaronson PI. Modulation of potassium current characteristics in human myometrial smooth muscle by 17beta-estradiol and progesterone. *Biol Reprod* 2001;**64**:1526–1534.
- Li H, Xia N, Förstermann U. Cardiovascular effects and molecular targets of resveratrol. *Nitric Oxide* 2012;**26**:102–110.
- Longo M, Jain V, Vedernikov YP, Hankins GD, Garfield RE, Saade GR. Effects of L-type Ca²⁺-channel blockade, K⁺(ATP)-channel opening and nitric oxide on human uterine contractility in relation to gestational age and labour. *Mol Hum Reprod* 2003;**9**:159–164.
- Lovasz N, Ducza E, Gaspar R, Falkay G. Ontogeny of sulfonylurea-binding regulatory subunits of K(ATP) channels in the pregnant rat myometrium. *Reproduction* 2011;**142**:175–181.
- Matharoo-Ball B, Ashford ML, Arulkumaran S, Khan RN. Down-regulation of the alpha- and beta-subunits of the calcium-activated potassium channel in human myometrium with parturition. *Biol Reprod* 2003;**68**:2135–2141.
- Morrison JJ, Ashford ML, Khan RN, Smith SK. The effects of potassium channel openers on isolated pregnant human myometrium before and after the onset of labor: potential for tocolysis. *Am J Obstet Gynecol* 1993;**169**:1277–1285.
- Naderali EK, Doyle PJ, Williams G. Resveratrol induces vasorelaxation of mesenteric and uterine arteries from female guinea-pigs. *Clin Sci (Lond)* 2000;**98**:537–543.
- Nagaoka T, Hein TW, Yoshida A, Kuo L. Resveratrol, a component of red wine, elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and potassium channels. *Invest Ophthalmol Vis Sci* 2007;**48**:4232–4239.
- National Collaborating Centre for Women's and Children's Health (UK). Principles of care. *Fertility: Assessment and Treatment for People with Fertility Problems*. London, UK: RCOG Press, 2004.
- Novakovic A, Gojkovic-Bukarica L, Peric M, Nezic D, Djukanovic B, Markovic-Lipkovski J, Heinle H. The mechanism of endothelium-independent relaxation induced by the wine polyphenol resveratrol in human internal mammary artery. *J Pharmacol Sci* 2006a;**101**:85–90.
- Novakovic A, Gojkovic-Bukarica L, Kanjuh V, Heinle H. Potassium channels-mediated vasorelaxation of rat aorta induced by resveratrol. *Basic Clin Pharmacol Toxicol* 2006b;**99**:360–364.
- Novakovic R, Milovanovic S, Protic D, Djokic J, Heinle H, Gojkovic-Bukarica L. The effect of potassium channel opener pinacidil on the non-pregnant rat uterus. *Basic Clin Pharmacol Toxicol* 2007;**101**:181–186.
- Novakovic R, Ilic B, Beleslin-Cokic B, Radunovic N, Heinle H, Scepanovic R, Gojkovic-Bukarica L. The effect of resveratrol on contractility of non-pregnant rat uterus: the contribution of K⁺ channels. *J Physiol Pharmacol* 2013;**64**:795–805.
- Pirola L, Frjödö S. Resveratrol: one molecule, many targets. *IUBMB Life*, 2008;**60**:323–332.
- Protić D, Beleslin-Čokić B, Novaković R, Kanjuh V, Heinle H, Šcepanović R, Gojković-Bukarica L. Effect of wine polyphenol resveratrol on the contractions elicited electrically or by norepinephrine in the rat portal vein. *Phytother Res* 2013;**27**:1685–1693.
- Smith RC, McClure MC, Smith MA, Abel PW, Bradley ME. The role of voltage-gated potassium channels in the regulation of mouse uterine contractility. *Reprod Biol Endocrinol* 2007;**5**:41.
- Song M, Zhu N, Olcese R, Barila B, Toro L, Stefani E. Hormonal control of protein expression and mRNA levels of the MaxiK channel alpha subunit in myometrium. *FEBS Lett* 1999;**460**:427–432.
- Suzuki T, Takimoto K. Differential expression of Kv4 pore-forming and KChIP auxiliary subunits in rat uterus during pregnancy. *Am J Physiol Endocrinol Metab* 2005;**288**:E335–E341.
- Van Voorhis BJ. Clinical practice. In vitro fertilization. *N Engl J Med* 2007;**356**:379–386.
- Wang LD, Qiu XQ, Tian ZF, Zhang YF, Li HF. Inhibitory effects of genistein and resveratrol on guinea pig gallbladder contractility in vitro. *World J Gastroenterol* 2008;**14**:4955–4960.
- Xu C, You X, Gao L, Zhang L, Hu R, Hui N, Olson DM, Ni X. Expression of ATP-sensitive potassium channels in human pregnant myometrium. *Reprod Biol Endocrinol* 2011;**9**:35.