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Effect of gestational diabetes mellitus and pregnancy-induced hypertension on human umbilical vein smooth muscle K_{ATP} channels

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ABSTRACT

Gestational diabetes mellitus (GDM) and pregnancy-induced hypertension (PIH) can jeopardize mother and/or fetus. Vascular ATP-sensitive potassium (K_{ATP}) channels most likely participate in the processes of diabetes and hypertension. The aim of this research was to examine whether GDM and PIH cause changes in the expression and function of K_{ATP} channels in vascular smooth muscle of human umbilical vein (HUV). Western blot and immunohistochemistry detected significantly decreased expression of Kir6.1 subunit of K_{ATP} channels in GDM and PIH, while the expression of SUR2B was unchanged. In GDM, a K^+ channel opener, pinacidil caused reduced relaxation of the endothelium-denuded HUVs compared to normal pregnancy. However, its effects in HUVs from PIH subjects were similar to normal pregnancy. In all groups K_{ATP} channel blocker glibenclamide antagonized the relaxation of HUV induced by pinacidil without change in the maximal relaxations indicating additional K_{ATP} channel-independent mechanisms of pinacidil action. Iberiotoxin, a selective antagonist of *large-conductance calcium-activated potassium* channels, inhibited the relaxant effect of pinacidil in PIH, but not in normal pregnancy and GDM. Experiments performed in K^+-rich solution confirmed the existence of K^+-independent effects of pinacidil, which also appear to be impaired in GDM and PIH. Thus, the expression of K_{ATP} channels is decreased in GDM and PIH. In GDM, vasorelaxant response of HUV to pinacidil is reduced, while in PIH it remains unchanged. It is very likely that K_{ATP} channels modulation and more detailed insight in K_{ATP} channel-independent actions of pinacidil may be precious in the therapy of pathological pregnancies.

1. Introduction

Human umbilical vein (HUV) is an uncommon blood vessel that provides the delivery of oxygenated blood rich in nutrients from the placenta to the fetus. HUV is located in the umbilical cord together with two human umbilical arteries that are coiling around it in the surrounding Wharton's jelly (Blanco et al., 2011). Fetoplacental circulation is a low pressure and resistance system. The characteristic of fetoplacental blood vessels is that they have neither adrenergic nerves nor vasa vasorum and lymphatics, which indicates an increased influence of mechanical factors, factors of the local environment and vasoactive substances delivered by systemic circulation in the regulation of their vascular tone (Wareing, 2014). Blood pressure of the HUV increases during gestation and amounts 4.5 mmHg in the 18th week, and about 6 mmHg at term. Blood flow through the vein is enabled by pressure gradients caused by fetal heart contractions, fetal respiration and longitudinal distortions of the umbilical arteries, as well as the contraction of the medial layer of the vein itself (Spurway et al., 2015). Additionally, HUV exhibits a vasomotion phenomenon, which also contributes to the maintenance of blood flow (Garcia-Huidobro et al., 2007). Tunica media of human umbilical vein consists of several layers of vascular smooth muscle cells (SMC) oriented in different directions (circular, oblique and longitudinal). This arrangement of SMCs together with the Wharton's jelly properties plays a role in protecting the venous blood flow from stress caused by fetal movements (Koech et al., 2008). It is important to emphasize that HUV is not only passive conduit blood

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vessel and it is able to adjust its vascular tone (e.g., hypoxia-mediated vasodilatation) (Mildenberger et al., 1999).

ATP-sensitive potassium channels, (K_{ATP}) channels were discovered by Noma in cardiomyocytes in 1983. Not long after, these channels have been identified in many other tissues including vascular smooth muscle (Quayle et al., 1997). Even though their name originates from the feature that an increase in intracellular ATP inhibits their activity, it has been demonstrated that their activity can be modulated by a plurality of signaling pathways unbound from their ATP sensitivity. Thus, they are targets in the mechanism of action of many vasoconstrictors and vasodilators (Jackson, 2000; Teramoto, 2006). The predominant type of K_{ATP} channels in vascular SMCs are low conductance channels that are, in the presence of pinacidil, stimulated with micromolar ATP and milimolar UDP or ADP, while higher doses of ATP inhibit their activity. Because of this property, these channels are sometimes referred to as K_{NDP} channels (Shi et al., 2012). K_{ATP} channels in vascular SMCs play an important role in maintaining of resting membrane potential and in the regulation of vascular tone. Acting as metabolic sensors they connect cellular metabolism to excitability (Quayle et al., 1997). The ATP/ADP ratio is the main factor determining the K_{ATP} channel activity. An increase in local metabolism leads to a decrease in ATP/ADP ratio. This enhances the open probability of K_{ATP} channels and induces vascular SMCs relaxation. Conversely, a decrease in the rate of metabolism will reverse the process and increase vascular tone (Akrouh et al., 2009; Flagg et al., 2010). Structurally, K_{ATP} channels are functional heterooctamers constructed from four Kir6.X subunits that form a pore and four regulatory subunits known as sulfonylurea receptors, SURx. Kir subunits are responsible for inhibition by ATP, and SURs for NDP activation. In vascular SMCs, K_{ATP} channels are predominantly heterocomplexes of Kir6.1 and SUR2B subunits (Hibino et al., 2010).

K_{ATP} channels are involved in the processes of diabetes mellitus and hypertension. Studies have revealed alterations in the expression and function of K_{ATP} channels during these pathological conditions in vascular SMCs in other vascular beds and in different species (Tykocki et al., 2017). On the other hand, it has been described that gestational diabetes mellitus (GDM) and pregnancy-induced hypertension (PIH) cause various morphological and ultrastructural abnormalities in HUVs (Pugnaloni et al., 1995). However, to date, influence of GDM and PIH on K_{ATP} channels of HUV smooth muscle is not clarified.

The vascular oxidative state is regulated either by physiological stimuli or by pathophysiological stress including hyperglycemia and hypertension (Gutterman et al., 2005). Chronic hypoxia and elevated oxidative stress have been reported in GDM and PIH and can jeopardize the lives of both *mother* and *baby* (Bisseling et al., 2005; Taricco et al., 2009; Toljic et al., 2017; Yang et al., 2018). Many investigators imply that K_{ATP} channels are key players in hypoxia-mediated vasodilatation since hypoxia affects ATP/ADP ratio and because this process is attenuated if K_{ATP} channels activity is impaired (Sorensen et al., 2012; Zhu et al., 2013). Furthermore, impairment of K_{ATP} channels activity by increased oxidative stress in disease states, such as diabetes mellitus, is also demonstrated (Gutterman et al., 2005). Considering this, vascular K_{ATP} channels may be precious pharmacological targets in the therapy of pathological pregnancies.

Therefore, the aim of this research was to examine whether GDM and PIH cause changes in the expression and function of K_{ATP} channels in vascular SMCs of the HUVs.

2. Materials and methods

The study protocol was approved by the Ethics Committee of the Medical Faculty, University of Belgrade, Serbia (No. 2650/VI5). Umbilical cords were obtained from the Clinic for Gynecology and Obstetrics “Narodni front”, Belgrade. “Normal”, diabetic and hypertensive pregnancies were studied. All patients gave their informed, written consent. The segments from the medial area of the umbilical cords were excised after vaginal delivery or Caesarean section, placed in small bottles with cold Krebs-Ringer bicarbonate solution (120 mM NaCl, 25 mM $NaHCO_3$, 11 mM glucose, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1.2 mM KH_2PO_4), transported to the laboratory and stored at 4 °C for ≤24 h (Radenković et al., 2007). Previously, it has been shown that storing of blood vessels under these conditions does not alter its structure and function (Mildenberger et al., 1999). After the isolation and preparation, HUV segments were either immersed in 10% formalin for immunohistochemical staining, frozen at −70 °C for western blot analysis or mounted in the organ bath for pharmacological experiments.

2.1. Clinical terms and definitions

2.1.1. Pregnancy-induced hypertension (PIH)

A term PIH refers to the development of hypertension after 20 weeks of gestation and it includes disorders such as gestational hypertension, preeclampsia and eclampsia. Blood pressure is considered elevated if the systolic blood pressure is ≥140 mmHg or the diastolic blood pressure is ≥90 mmHg, sustained over time (Blanco et al., 2011; Yang et al., 2018).

2.1.2. Gestational diabetes mellitus (GDM)

It is defined as diabetes with initial onset or recognition during pregnancy (Blanco et al., 2011).

2.2. Western blot analysis

Tissue samples of HUV were homogenized on ice in modified RIPA buffer with protease and phosphatase inhibitors. The homogenates were centrifuged at 15000 ×*g*, at 4 °C for 30 min. The BCA method was performed to determine protein concentration. Obtained supernatants were used as a cell lysate for Western blot analysis, after boiling in Laemmli sample buffer. The proteins were fractioned by electrophoresis on a 10% polyacrylamide gel and transferred onto Polyvinylidene difluoride membranes. The membranes were blocked with 5% bovine serum albumin (BSA), and afterwards incubated with primary antibodies (anti-Kir6.1, anti-SUR2B and anti-MaxiK-α; Santa Cruz Biotechnology, Inc., dilution ratio 1:500) at 4 °C, overnight. Then, they were subsequently washed and incubated with secondary anti-rabbit or anti-goat horseradish peroxidase-conjugated antibody. Signals were detected using ECL reagents. β-actin served as a loading control to assess the equal protein loading. The films were scanned and analyzed using ImageJ software (Bundalo et al., 2015; Novaković et al., 2015).

2.3. Immunohistochemistry

Serial 5 μm thick sections were cut from the formalin fixed, paraffin embedded HUV tissue. All sections were dewaxed and rehydrated through graded alcohols to water and heated 30 min in Tris-EDTA buffer at pH 9.0 for antigen retrieval. After cooling, sections were washed with TBS (Tris-buffered saline) and incubated overnight with rabbit polyclonal anti-Kir6.1, goat polyclonal anti-SUR2B and mouse monoclonal anti-MaxiK-α primary antibodies (Santa Cruz Biotechnology, Inc., dilution ratio 1:50). Then they were treated by applying the commercial UltraVision/DAB staining kit (ThermoScientific LabVision TL-060-HD) in case of rabbit and mouse primary antibodies, or with the commercial ImmunoCruz™ goat ABC Staining System (sc-2023, Santa Cruz Biotechnology) for goat primary antibody. Immunoreactions were subsequently developed by using DAB for 10 min. The background staining was performed with hematoxylin in 10 s. Negative controls were produced by omitting the primary antibody. All slides were analyzed using an Olympus BX 41 microscope and photographed with Olympus C–5060 wide zoom digital camera (Rakocevic et al., 2016).

The intensity and distribution of positive staining were evaluated by experienced researcher unaware of the experimental groups using two

Table 1
K^+ channel subunit immunocytochemical scoring system (adapted from Adams et al., 1999).

K^+ channel subunit immunocytochemistry	
Strong	Dark membrane staining that is easily visible and involves > 50% of cells
Moderate	Focal darkly staining areas in < 50% of cells, or moderate membrane staining of > 50% of cells
Weak	Focal moderate staining in < 50% of cells, or pale membrane staining in any proportion of cells not easily seen
Scattered	Dark membrane staining of widely scattered cells
Negative	Smooth muscle that not show non of above

scoring systems. The first scoring system was originally proposed by Fisher and modified by Adams (Fisher et al., 1994; Adams et al., 1999). This scoring system is shown in Table 1 and represents the combination of both, the intensity and distribution of positive staining, as a one score (Adams et al., 1999). The second scoring system, a standard four point scale for intensity, scored slides as negative, +, ++, or +++.

2.4. Vasorelaxant responses

Isolated tissue bath system is a classical pharmacological method used for > 100 years for evaluating isometric tension and concentration-response relationships of contractile tissues. It has been fully described in a myriad of studies (Jespersen et al., 2015). Preparation and management of the tissue was systematically described in previous papers from our laboratory (Stojnic et al., 2007; Protić et al., 2014). In short, HUVs were cut into approximately 5 mm long ring segments after they had been isolated from the umbilical cord and a Wharton's jelly. This was followed by mechanical deendothelialization gently by scraping with a steal wire. Afterwards, rings were mounted between two stainless steel triangles in a 10-ml organ bath with Krebs-Ringer bicarbonate solution (37 °C, pH 7.4) aerated with 95% O_2 and 5% CO_2. Changes in isometric tensions were registered using transducer (K30, Hugo Sachs, Freiburg, Germany), amplified (model 301, Hugo Sachs amplifier) and recorded on recording system (R60; Rikadenki, Tokyo). All preparations were equilibrated for 60 min and washed every 15 min within this period. Then, vein segments were progressively distended to the optimal resting tension.

Rings were contracted with serotonin (100 μM). Acetylcholine (20 μM) was added to the bath for the assessment of the endothelial function. The lack of vasorelaxation by acetylcholine confirmed the absence of functional endothelium. Thus, possible misinterpretation of the results was excluded (Gojkovic-Bukarica et al., 2011).

In order to study vasodilatator properties of K^+ channel opener pinacidil, HUVs endothelium-denuded segments were precontracted by serotonin (100 μM). When stable plateau was reached, increasing concentrations of pinacidil (0.1–1000 μM) were added and control concentration-response curves were obtained. To test the roles of K_{ATP} channels in pinacidil-induced vasorelaxation, glibenclamide (GLB), a specific blocker of K_{ATP} channels, was added into the bath 20 min prior to the contraction of the ring. In order to check the possibility that other K^+ channel types are involved in pinacidil induced vasorelaxation 4-AP (1 mM), a nonselective blocker of voltage-gated potassium channels (Kv channels), and iberiotoxin (100 nM), a selective blocker of *large-conductance calcium-activated potassium channels* (BK_{Ca} channels), were added to the bath, in the same manner as GLB. In a separate series of experiments, the second contractions were produced by 100 mM K^+ (K^+-rich Krebs-Ringer solution) in order to block the effect of K^+-channels activation and examine K^+-independent actions of pinacidil. Papaverine was used as the general vasodilatator at the end of each experiment to determine maximal possible relaxation of the specimen.

2.5. Drugs and solutions

The following drugs were used: serotonin, acetylcholine, pinacidil, glibenclamide, 4-AP, iberiotoxin, papaverine (Sigma – Aldrich Inc., St. Louis, MO, USA). Pinacidil was dissolved in dilute acid solution (0.01 N HCl) with further dilution in distilled water before use (0.1–1000 μM). GLB was dissolved in 96% *v*/v ethanol. Other drugs were dissolved in distilled water. Composition of Krebs-Ringer solution is previously adduced. K^+- rich Krebs-Ringer solution was prepared by equimolar replacement of 100 mM NaCl with 100 mM KCl. All drugs were added directly into the bath and the concentrations given are the calculated final concentrations in the bath solution.

2.6. Treatment of data and statistics

Data analysis was performed with SigmaPlot (Systat Software Inc., San Jose, CA). Unless otherwise stated, the results are presented as the means ± standard error of the mean (S.E.M.); n = number of experiments. The concentration of pinacidil which produces 50% of maximal response (EC_{50}) was calculated for each curve using regression analyses. It is expressed as pD_2 ($pD2 = -\log EC_{50}$). "Sensitivity" is measured by pD_2 value and describes location of concentration-response curve. "Reactivity" (E_{max}) describes the maximum of relaxations produced by pinacidil. Differences between means were determined by Student's *t*-test; a value of $P < 0.05$ was considered statistically significant.

3. Results

A total of 72 patients were engaged in the study. Forty of them were healthy, 16 had GDM and 16 PIH. Demographic maternal characteristics, clinical findings and therapy during pregnancy are presented in Table 2. Differences between groups in age, delivery methods, conceiving methods and smoking status were not statistically significant. Gestation period in women with PIH was significantly shorter than in normal pregnancy. This is expected because in PIH medical intervention is often required to save the life of a mother or a child (Koech et al., 2008). All patients with GDM were on a diet, two of them used insulin. All women with PIH were treated with methyldopa and nine of them with calcium channel blockers.

3.1. Detection of K^+ channel subunits by western blot and immunohistochemistry

Western blot detected significantly decreased expression of Kir6.1 subunit of K_{ATP} channel in both pathological conditions compared to

Table 2
Sociodemographic and clinical characteristics of patients.

Patient characteristics	NP	GDM	PIH
Age (years ± SD)	31.8 ± 4.8	33.9 ± 6.1	31.8 ± 7.2
Gestation (weeks ± SD)	39.5 ± 1.1	39.7 ± 0.6	37.4 ± 3.0*
Caesarean section	12 (30%)	4 (25%)	8 (50%)
IVF	6 (15%)	1 (6.3%)	3 (18.8%)
Smoking status	4 (10%)	5 (31.3%)	2 (12.5%)
Methyldopa	0 (0%)	0 (0%)	16 (100%)
CCB	0 (0%)	1 (6.3%)	9 (56.3%)
Insulin	0 (0%)	2 (12.5%)	0 (0%)

NP – normal pregnancy; GDM – gestational diabetes mellitus; PIH – pregnancy induced hypertension; IVF – in vitro fertilization; CCB – calcium channel blockers; SD – standard deviation.
* $P < 0.05$ compared to NP.

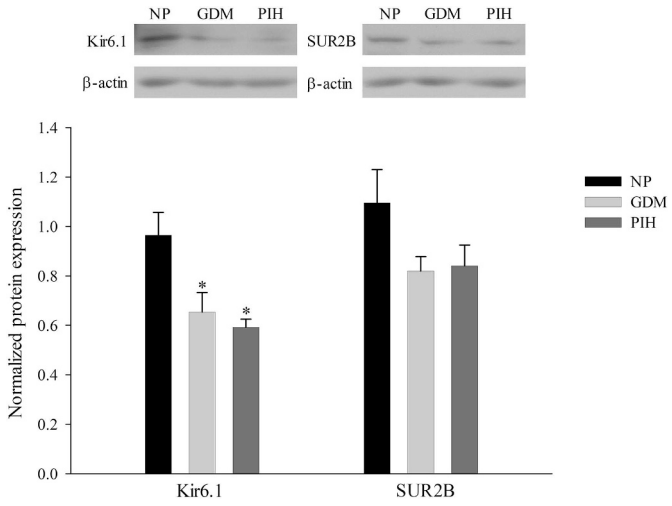

Fig. 1. The expression of Kir6.1 and SUR2B subunits of K_{ATP} channels on human umbilical veins from normal pregnancy (NP, n = 6), gestational diabetes mellitus (GDM, n = 6) and pregnancy induced hypertension (PIH, n = 6). Results are expressed as mean ± S.E.M. * P < 0.05 compared to NP.

normal pregnancy (P < 0.05, Fig. 1). The expression of SUR2B was not significantly different between the groups (Fig. 1). Also, the expression of the pore-forming MaxiK-α subunit of BK_{Ca} channel remained unchanged (Fig. 2).

Immunohistochemistry revealed diffuse sarcolemmal and intracellular expression of Kir6.1 and SUR2B subunits of K_{ATP} channel in the tunica media of HUVs as well as in the SMCs that are occasionally present in the tunica intima. Immunocytochemical scoring results are consistent with western blot analysis. In the vascular SMCs from normal pregnancies (Fig. 3, 1A) Kir6.1 subunit was expressed throughout tunica media (immunocytochemical scoring – moderate/++), but decreased in the vascular SMCs from GDM (Fig. 3, 2A) (immunocytochemical scoring – weak/+) and PIH (Fig. 3, 3A) (immunocytochemical scoring – weak to moderate – +/++). The expression of SUR2B subunit appeared similar in GDM and PIH in comparison to normal pregnancy (Fig. 3, 1B, 2B and 3B) (immunocytochemical scoring – moderate/++). Furthermore, the expression of MaxiK-α subunit of BK_{Ca} channels was similar in all groups (immunocytochemical scoring – moderate/++) (Fig. 4, 1A, 2A, 3A).

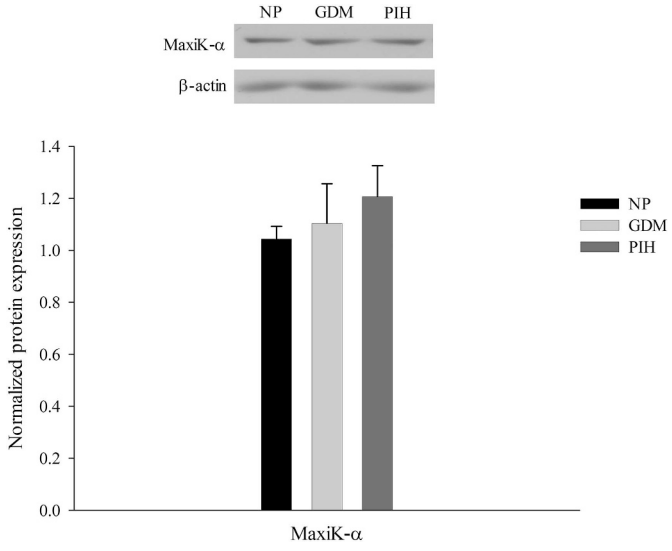

Fig. 2. The expression of MaxiK-α subunit of BK_{Ca} channel on human umbilical veins from normal pregnancy (NP, n = 6), gestational diabetes mellitus (GDM, n = 6) and pregnancy induced hypertension (PIH, n = 6). Results are expressed as mean ± S.E.M.

All results regarding to molecular analysis of K^+ channel proteins are summarized and shown in Table 3.

3.2. Effects of pinacidil on the HUV precontracted with serotonin

Application of pinacidil (0.1–1000 μM) induced relaxation of the HUV rings in a concentration-dependent manner in normal pregnancies (pD_2 value was 4.57 ± 0.06, E_{max}: 98.56 ± 0.36, n = 23), GDM (pD_2 value was 4.29 ± 0.06, E_{max}: 99.21 ± 0.29, n = 18) and PIH (pD_2 value was 4.58 ± 0.05, E_{max}: 98.80 ± 0.25, n = 18) (Fig. 5). GDM produced statistically significant rightward shift of the concentration-response curve to pinacidil in comparison to normal pregnancy (P < 0.05). Maximal relaxations were not significantly different amongst the groups (P > 0.05).

In all groups, GLB (10 μM, n = 6), a selective K_{ATP} channel inhibitor significantly antagonized the relaxation of HUV induced by pinacidil without change in E_{max}. In normal pregnancy pD_2 were 4.61 ± 0.12 in the absence vs. 3.91 ± 0.04 in the presence of GLB, P < 0.05; E_{max}: 98.22 ± 0.82% in the absence vs. 96.92 ± 2.00% in the presence of GLB, P > 0.05 (Fig. 6A). In GDM group pD_2 were 4.27 ± 0.09 in the absence vs. 3.65 ± 0.07 in the presence of GLB, P < 0.05; E_{max} to pinacidil in diabetic pregnancies were 98.32 ± 0.63% in the absence vs. 99.05 ± 0.45% in the presence of GLB, P > 0.05 (Fig. 6B). In PIH values of pD_2 were 4.62 ± 0.09 in the absence and 3.70 ± 0.09 in the presence of GLB, P < 0.05; E_{max}: 99.22 ± 0.36% in the absence and 97.78 ± 0.76% in the presence of GLB, P > 0.05 (Fig. 6C).

4-AP (10 mM, n = 6), a nonselective blocker of Kv channels, produced a significant rightward shift of the concentration-response curve of pinacidil in HUV segments from normal pregnancies, but failed to produce significant change in GDM and PIH. Also, the differences between all of the groups in maximal relaxations were not significant (P > 0.05, Table 4).

Iberiotoxin (100 nM), a selective antagonist of BK_{Ca} channels, significantly modified the vasorelaxant effect of pinacidil in PIH (P < 0.05), but failed to produce the same response in normal pregnancy and GDM. Also, the differences between all of the groups in maximal relaxations were not significant (P > 0.05, Table 4).

3.3. Effects of pinacidil on HUV in the presence of high K^+

Relaxant response to pinacidil (0.1–1000 μM) on HUVs precontracted with K^+-rich solution was significantly inhibited in all of the groups (P < 0.05, Table 4). There were statistically significant differences between maximal responses to pinacidil on HUV rings precontracted with K^+-rich solution between normal pregnancy and GDM as well as between normal pregnancy and PIH (P < 0.05, Table 4).

4. Discussion

To our knowledge, this is the first study which evaluates differences in the expression and function of K_{ATP} channels in HUVs under the influence of GDM and PIH. The major novel findings show that the expression of Kir6.1 subunit of K_{ATP} channels is decreased in both pathological conditions. In GDM, the relaxation of HUV induced by pinacidil was reduced, while in PIH functional response to pinacidil is preserved. The involvement of K_{ATP} channel-independent mechanisms of pinacidil-induced vasorelaxation is also confirmed.

Opening of K^+ channels causes hyperpolarization of the cell membrane, which leads to the closure of the voltage-dependent L-type Ca^{2+} channels, the decrease in the concentration of intracellular Ca^{2+} and vasodilatation. On the contrary, blocking their function leads to the depolarization and vasoconstriction (Ko et al., 2008). Altered K^+ channels function in diabetes mellitus and hypertension may be either a cause (e.g., involvement in the pathogenesis) or a consequence (e.g., compensatory mechanism) of the disease and it is reflected as a change in the number, single conductance and/or open probability of the

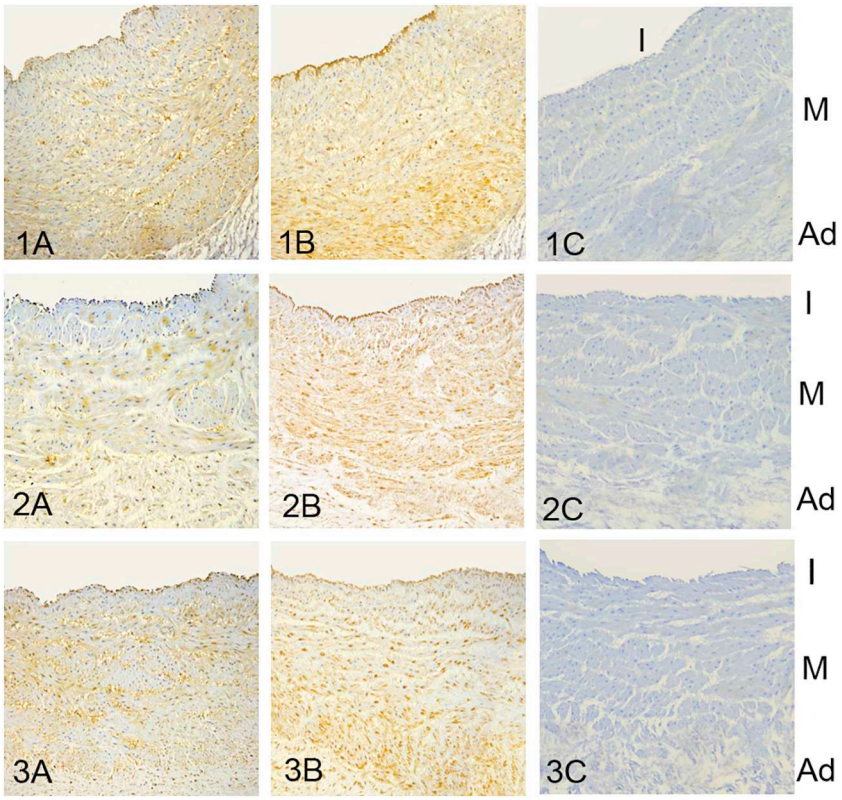

Fig. 3. Immunohistochemical detection of K_{ATP} channel subunits. Normal pregnancy (1); gestational diabetes mellitus (2); pregnancy-induced hypertension (3). Expression of Kir6.1 (brown staining, A); expression of SUR2B (brown staining, B); negative control (C). Tunica intima (I); tunica media (M); tunica adventitia (Ad). Original magnification: 200×. Figure is representative of preparations of four patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

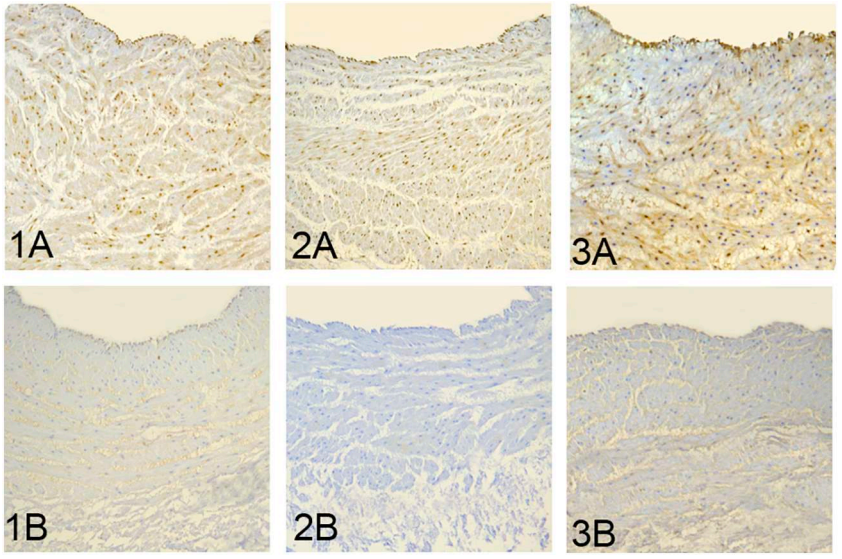

Fig. 4. Immunohistochemical detection of MaxiK-α subunit of BK_{Ca} channel. Normal pregnancy (1); gestational diabetes mellitus (2); pregnancy-induced hypertension (3). Expression of MaxiK-α (brown staining, A); negative control (B). Original magnification: 200×. Figure is representative of preparations of four patients. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Molecular analysis of K^+ channel subunits by immunohistochemistry and western blot.

Subunit	Immunohistochemical staining			Western blot
	NP	GDM	PIH	
Kir6.1	moderate ++, m/cp, diffuse	weak +, m/cp, focal	weak to moderate +/++, m/cp, diffuse	reduced in GDM and PIH, $P < 0.05$
SUR2B	moderate ++, m/cp, diffuse	moderate ++, m/cp, diffuse	moderate ++, m/cp, diffuse	no statistically significant difference
MaxiK-α	moderate ++, m/cp, diffuse	moderate ++, m/cp, diffuse	moderate ++, m/cp, diffuse	no statistically significant difference

NP – normal pregnancy; GDM – gestational diabetes mellitus; PIH – pregnancy induced hypertension; m/cp – membranous/cytoplasmic.

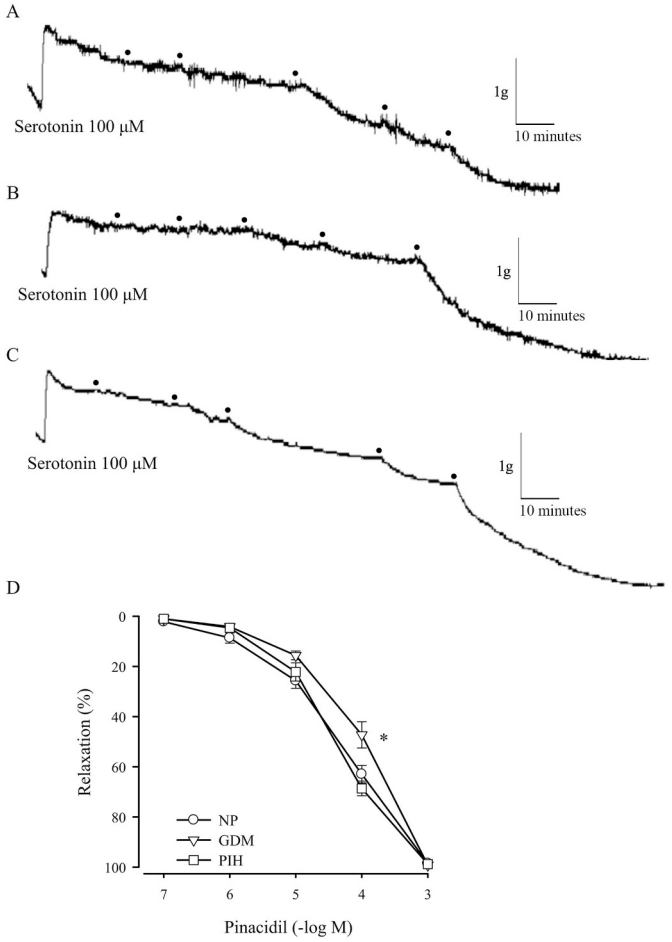

Fig. 5. Original recordings of the vasorelaxant effect of pinacidil in human umbilical vein (HUV) precontracted with serotonin. A – normal pregnancy (NP); B – gestational diabetes mellitus (GDM); C – pregnancy-induced hypertension (PIH); D – concentration-response curves for pinacidil on the HUVs precontracted with serotonin in NP (n = 23, circle), GDM (n = 18, triangle) and PIH (n = 18, square). Data points represent the means and the vertical lines are the S.E.M. * P < 0.05 compared to NP.

channels (Sobey, 2001). It has been reported that cellular metabolism participates in the regulation of gene expression of the K_{ATP} channels and that alterations of metabolism (e.g., during diabetes) will lead to a change in channels number (Zhuo et al., 2005). Coexpression of both K_{ATP} channels subunits in a 4 to 4 ratio is required for their function (Hibino et al., 2010). Therefore, significantly reduced expression of the pore-forming Kir6.1 subunit in GDM and PIH obtained in our study, with similar expression of SUR2B, is the evidence of the decreased expression of the K_{ATP} channels in the vascular SMCs of the HUV. These results are in agreement with the results obtained in different models of diabetes and hypertension in other vascular beds (Tykocki et al., 2017). The short exposure time of HUV to diabetic and hypertensive environment, as well as the application of therapy that maintains glycemia and vascular tension at relatively normal levels during pregnancy are possible explanations of the conserved expression of the SUR2B subunit. Moreover, in aortic vascular smooth muscle of spontaneously hypertensive rats (SHR) already noted decreased expression of Kir6.1 and SUR2B subunits in 16 weeks old animals was further reduced with the passage of time. Subsequently, in the group of 49 weeks old SHRs expression of K_{ATP} channel subunits was much more reduced compared to 16 weeks old (Liu et al., 2016).

The reduced response to pinacidil in GDM is concordant with the results of molecular analysis of K_{ATP} proteins. GLB, a selective inhibitor of the K_{ATP} channels, at a dose of 10 μM antagonized the HUV response to pinacidil in GDM. Recently, Li et al. (2018) have shown that diminished vasodilatator response to pinacidil in human umbilical arteries was mainly a consequence of the suppressed expression of these channels in GDM. Previously, Bisseling et al. (2005) detected impaired K_{ATP} channels function in the fetoplacental circulation of women with diabetes mellitus type 1. On the model of insulin-resistant rats overproduction of reactive oxygen species (ROS) and increased oxidative stress reduced pinacidil-induced vasorelaxation in cerebral arteries, but relaxant effect was completely restored after treatment with superoxide dismutase and catalase (Erdos et al., 2004). The long-term hypoxia in uterine arteries of pregnant sheep also decreased K_{ATP} channels activity (Xiao et al., 2010). Furthermore, high glucose-induced protein kinase C, independent from ROS, is another of the proposed mechanisms of suppression of the K_{ATP} channel function in vascular SMCs during diabetes (Tykocki et al., 2017). Randomized trials and studies, performed so far, have provided evidence that oral hypoglycemic agents, including GLB, could be acceptable alternatives for insulin in the treatment of GDM (Ryu et al., 2014). Decreased sensitivity to pinacidil, in the presence of GLB, on HUVs from GDM, noted in the present study, is something that might be relevant in the case of clinical use of these drugs, since relative insensitivity of HUV to vasodilatators could compromise the venous blood flow and delivery of oxygen and nutrients to the fetus.

Studies performed on genetically modified animals have confirmed the importance of K_{ATP} channels in blood pressure control. The key role

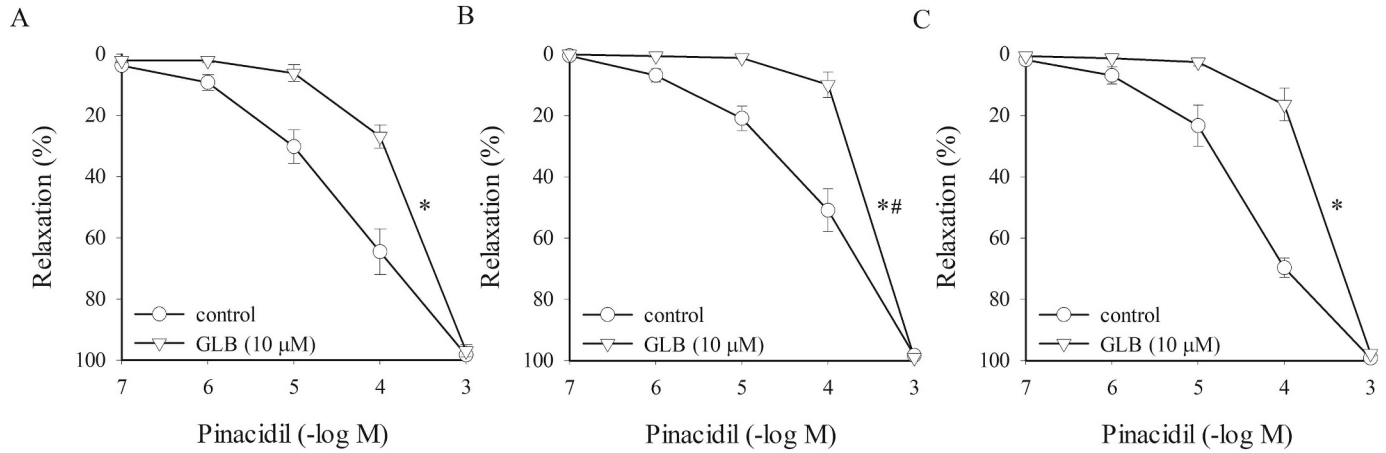

Fig. 6. Antagonism of the relaxant effect of pinacidil by glibenclamide on the human umbilical veins precontracted with serotonin. A – normal pregnancy (n = 6); B – gestational diabetes melittus (GDM, n = 6); C – pregnancy-induced hypertension (PIH, n = 6). Concentration-response curves in the absence (circle) and the presence of glibenclamide (GLB, triangle). Data points present the means and the vertical lines are the S.E.M. * P < 0.05 compared to control; # P < 0.05 compared to normal pregnancy.

Table 4
Vasorelaxant effects of pinacidil on the human umbilical vein in the absence and the presence of 4-AP, iberiotoxin (IBTX) and 100 mM K^+ with marked statistically significant differences.

	NP		GDM		PIH	
	pD_2	E_{max} (%)	pD_2	E_{max} (%)	pD_2	E_{max} (%)
Control	4.66 ± 0.08	98.48 ± 0.35	4.35 ± 0.10#	98.53 ± 0.69	4.61 ± 0.10	98.75 ± 0.45
4-AP (1 mM)	4.25 ± 0.13*	96.32 ± 1.56	4.18 ± 0.09	98.67 ± 0.30	4.30 ± 0.14	98.78 ± 0.56
Control	4.55 ± 0.10	98.43 ± 1.08	4.23 ± 0.10#	98.87 ± 0.72	4.58 ± 0.12	98.70 ± 0.51
IBTX (100 nM)	4.57 ± 0.20	97.94 ± 0.31	4.12 ± 0.12	98.18 ± 0.70	4.18 ± 0.20*	98.10 ± 0.46
Control	4.50 ± 0.08	99.22 ± 0.33	4.27 ± 0.10	99.10 ± 0.45	4.51 ± 0.05	99.18 ± 0.34
100 mM K^+	3.73 ± 0.04*	97.80 ± 0.80	3.38 ± 0.03*#	90.26 ± 0.99*#	3.51 ± 0.10*	91.46 ± 2.26*#

NP – normal pregnancy; GDM – gestational diabetes mellitus; PIH – pregnancy induced hypertension; Control – respective control for each experiment, precontracted with serotonin. Results are presented as the mean ± S.E.M; n = 6–7.
* $P < 0.05$ compared to respective control.
# $P < 0.05$ compared to NP.

of Kir6.1 subunit in blood pressure control is pointed out since the mice with deletion of this subunit exhibited hypertension (Aziz et al., 2014). On the other hand, the gain of function of Kir6.1 subunit with K_{ATP} channels hyperactivity led to hypotension. Hyperactivity of the K_{ATP} channels with systemic hypotension has also been confirmed in people with Cantu syndrome (Li et al., 2013). According to our findings, there was no change in pinacidil-induced relaxation of HUVs in PIH despite the decrease in the expression of the K_{ATP} channels. GLB caused significant inhibition of pinacidil-induced relaxation in PIH, without any differences compared to normal pregnancy. These results are somewhat unexpected. However, Blanco-Rivero et al. (2008) got similar results on the SHRs aorta where they demonstrated that reduced expression of Kir6.1 and SUR2B subunits did not affect the blood vessel response to pinacidil. Furthermore, in the similar setup, Liu et al. (2016) described that vascular response to diazoxide, but not to pinacidil, is decreased in SHRs. This emphasizes extreme chemical diversity of K^+ channel openers and great complexity of their mechanism of action which may, at least in part, provide explanations for our findings.

Pinacidil is a representative of the group of cyanoguanidines. It was synthesized in 1970. However, it was identified as the K^+ channel opener only in 1987. Pinacidil was used as a peripheral vasodilator, but today, it is used only for experimental purposes. The binding site for pinacidil is well characterized and it is located on the SUR2B subunit of smooth muscle K_{ATP} channel (Bray et al., 1987; Rubaiy, 2016). The ability of pinacidil to completely relax HUV in the presence of GLB support the notion that pinacidil partially acts through K_{ATP} channel-independent mechanisms. There are studies suggesting that pinacidil and his analogues, especially in high doses, in addition to K_{ATP} channels open other subtypes of potassium channels, including BK_{Ca} and Kv channels (Stockbridge et al., 1991; Khan et al., 1998; Thorne et al., 2002; Novakovic et al., 2012).

BK_{Ca} (also known as MaxiK) channels are constructed from pore-forming α subunits and auxiliary β or γ subunits. Four MaxiK-α subunits can form a functional channel by themselves. Auxiliary subunits act as potent regulators of the majority of the channel properties, including Ca^{2+} sensitivity and sensitivity to pharmacological modulators. The predominant auxiliary subunit in vascular SMCs is most likely β-1 subunit (Nimigean and Magleby, 2002; Lee et al., 2010; Li and Yan, 2016). In order to analyze the contribution of BK_{Ca} channels in the vasorelaxant response to pinacidil, iberiotoxin was used. Iberiotoxin is a selective blocker of the BK_{Ca} channels. In our study it caused a statistically significant shift of the concentration-response curve for pinacidil to the right in the PIH, but not in normal pregnancy and GDM. This result suggests that hypertension induces upregulation of the BK_{Ca} channels which is known compensatory mechanism against blood vessels hyperreactivity. In various animal models of hypertension treatment with BK_{Ca} channel blockers provoked exaggerated constrictor responses and thus revealed augmented BK_{Ca} channel activity (Sobey, 2001; Hu and Zhang, 2012). Additionally, by using whole-cell and single channel patch clamp techniques, it has been demonstrated that iberiotoxin-sensitive K^+ current was elevated in vascular SMCs from hypertensive animals in relation to controls (Joseph et al., 2013). In the present study, an unaltered exspression of MaxiK-α subunit in PIH indicates that noted upregulation is not caused by an increase in the BK_{Ca} channels number but it is rather functional. It most likely occurs due to post transcriptional modifications of MaxiK-α subunits and/or changes in interaction with auxiliary subunits. These alterations affect single conductance and/or open probability of the channels (Joseph et al., 2013; Li and Yan, 2016). Using these observations we can, at least in part, explain the preservation of the vasorelaxant response of HUV to pinacidil in PIH despite the decrease in the expression of the K_{ATP} channels. However, further detailed research of the BK_{Ca} channel functional expression in PIH is needed.

In order to examine the involvement of the Kv channels in pinacidil-induced vasorelaxation of HUV, we used 4-AP, which administered at a concentration of 1 mM represents a non-specific blocker of these channels. Antagonism of pinacidil-induced relaxation by 4-AP in normal pregnancy confirmed the involvement of these channels in its effect. The absence of inhibiting the functional response to pinacidil in both pathological conditions implies that in the course of gestational diabetes and hypertension the function of Kv channels in vascular SMCs was damaged. Results obtained in chorionic plate arteries and veins from pregnancies complicated by intrauterine growth restriction provide similar conclusions. Herein, authors demonstrated that venous but not arterial contractility was increased. Also, 4-AP was less effective in inducing contraction in both vascular compartments in comparison to normal pregnancy, suggesting reduced Kv channels activity (Wareing et al., 2006; Wareing, 2014). All of these K_{ATP} channel-independent results require further investigation.

In addition to the effects described so far, it has been shown that pinacidil if applied at high doses (≥ 100 μM) also exhibits mechanisms of action independent of the K^+ channels. To date, these mechanisms have not been completely clarified, and there are some assumptions that they include: stimulation of Na^+-K^+ pump or plasmalemmal Ca^{2+} extrusion mechanism, stimulation of the forward mode Na^+-Ca^+-exchanger, direct inhibition of plasmalemmal Ca^{2+} channels, inhibition of intracellular Ca^{2+} mobilisation, decreasing of Ca^{2+} sensitivity of contractile proteins, inhibition of a calcium-independent chloride conductance, activation of cyclic nucleotide-dependent signaling pathways and/or modulation of protein kinase C-mediated contraction (Barber and Henderson, 1996; Tsang et al., 2003; Stojnic et al., 2007). To distinguish these K^+-independent actions of pinacidil we used K^+-rich solution. In normal pregnancy, the vasorelaxant response to pinacidil in HUVs precontracted with K^+-rich solution was significantly inhibited while the maximal response remained unchanged; hence existence of K^+-independent actions of pinacidil was demonstrated. In GDM and PIH, K^+-rich solution also led to significant inhibition of the vasorelaxant response to pinacidil, but with significant suppression of the

maximal responses. These results lead us to conclude that the K^+-independent mechanisms of the action of pinacidil are also affected during GDM and PIH. The increased activity of the protein kinase C and Rho-kinase, described in preeclampsia, with the enhancement of sensitivity of contractile elements to Ca^{2+} is one of the speculations by which we can interpret these results (Zhu et al., 2013). However, the exact identification of K^+-independent mechanisms of the action of pinacidil, as well as the examination of the possibility of impaired function of other intracellular signaling pathways involved in the modulation of K_{ATP} channels on vascular SMCs requires further detailed research.

In summary, this study found that the expression of Kir6.1 subunit of K_{ATP} channels was significantly decreased in GDM and PIH, while the expression of SUR2B was similar in both pathological conditions compared to normal pregnancy. The impaired functional response to pinacidil in GDM correlates with reduced expression of K_{ATP} channels. On the other hand, in PIH, the functional response to pinacidil remains preserved, despite the decrease in the expression of the K_{ATP} channels. This can at least be partially explained by the compensatory increase in the functional expression of the BK_{Ca} channels. Experiments performed in the K^+-rich solution indicate the existence of K^+-independent effects of pinacidil which also appear to be impaired in GDM and PIH. Precise identification of these mechanisms of actions, as well as a clearer insight into the function and the expression of other subtypes of potassium channels in the GDM and PIH requires further investigations, and may provide new tools in the therapy of pathological pregnancies.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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