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Expression of Kv4.2 and Kv4.3 potassium channels in human umbilical veins from normal, diabetic, and hypertensive pregnancies

Ekspresija kalijumovih kanala Kv4.2 i Kv4.3 u humanim pupčanim venama zdravih trudnica, trudnica sa gestacijskim dijabetesom melitusom i trudnica sa gestacijskom hipertenzijom

Vladimir Djokić*, Miloš Gostimirović*, Jovana Rajković*, Jelena Rakočević†, Milica Labudović-Borović†, Svetlana Janković‡, Jelena Stanišić§, Milan Kostić§, Miloš Djurić*, Ljiljana Gojković-Bukarica*

University of Belgrade, Faculty of Medicine, *Institute of Pharmacology, Clinical Pharmacology and Toxicology, †Institute of Histology and Embryology "Aleksandar Dj. Kostić", ‡Department of Obstetrics and Gynecology "Narodni front", Belgrade, Serbia; §Vinča Institute of Nuclear Sciences, Belgrade, Serbia

Abstract

Background/Aim. A substantial line of evidence indicates that Kv4.2 and Kv4.3 channels are the major components of rapid transient-outward potassium currents (A-type currents). It is speculated that those currents may be involved in the maintenance of the membrane potential, as well as in the regulation of propagation and frequency of action potentials. However, very little is known about the presence and function of A-type currents in human vascular smooth muscles such as the human umbilical vein (HUV). Bearing in mind its crucial role in the proper fetal oxygenation, the aim of the study was to determine whether Kv4.2 and Kv4.3 potassium channels are present in HUV smooth muscle and to investigate potential alterations of their expression during maternal pathological conditions such as gestational diabetes mellitus (GDM) and pregnancy-induced hypertension (PIH). **Methods.** Healthy, diabetic, and hypertensive pregnancies were subjects of this investigation. Each group consisted of 6 HUV samples obtained from 6 normal pregnancies, 6 pregnancies with GDM, and 6 with PIH. After pharmacology analysis, immunohistochemistry (IH) and Western blot were performed. **Results.** IH revealed similar expression patterns of both, Kv4.2 and Kv4.3 subunits in HUV smooth muscle in all groups of patients. Results obtained by Western blot were in agreement with IH staining. The expression of Kv4.2 and Kv4.3 subunits were not significantly different between the groups. **Conclusion.** Collectively, this is the first study that demonstrated the presence of Kv4.2 and Kv4.3 potassium channels in the HUV smooth muscle and their preservation during GDM and PIH pregnancies. These channels are most likely major components of rapid A-type currents that may be relevant for maternal-fetus blood flow and hence fetal development. In addition, they may represent sensors for detecting hemodynamic and/or metabolic changes in the local environment.

Key words:
diabetes mellitus; humans; hypertension; pregnancy; umbilical veins.

Apstrakt

Uvod/Cilj. Značajan niz dokaza ukazuje na to da su kanali Kv4.2 i Kv4.3 glavne komponente brzih prolazno-ispravljačkih struja kalijuma (struje tipa A). Pretpostavlja se da bi te struje mogle da budu uključene u održavanje membranskog potencijala ćelije, kao i u regulaciju propagacije i učestalosti akcionih potencijala. Međutim, vrlo malo se zna o prisustvu i funkciji struje tipa A u glatkim mišićima krvnih sudova čoveka, kao što je humana umbilikalna vena (HUV). S obzirom na njenu ključnu ulogu u adekvatnoj oksigenaciji fetusa, cilj rada bio je da se utvrdi da li su podtipovi kalijumovih kanala Kv4.2 i Kv4.3 prisutni u glatkim mišićima HUV-a i da se istraže potencijalne promene njihove ekspresije tokom patoloških stanja majke – gestacijskog dijabetesa melitusa (GDM) i arterijske hipertenzije (AH) izazvane trudnoćom (AHT). **Metode.** U istraživanje su uključene HUV sakupljene posle trudnoće zdravih porodilja, onih čije trudnoće su bile komplikovane GDM i AH. Svaka grupa sastojala se od 6 uzoraka HUV-a dobijenih iz 6 zdravih trudnoća, 6 trudnoća komplikovanih GDM i 6 trudnoća komplikovanih AH. Nakon farmakoloških analiza, urađene su imunohistohemijska (IH) analiza i *Western blot*. **Rezultati.** Primenom IH analize pokazan je sličan obrazac ekspresije

Correspondence to: Ljiljana Gojkovic-Bukarica, University of Belgrade, Faculty of Medicine, Department of Pharmacology, Clinical Pharmacology and Toxicology, Dr. Subotića Starijeg 1, 11 000 Belgrade, Serbia. E-mail: lj.bukarica62@gmail.com; bukarica@rcub.bg.ac.rs

obe podjedinice Kv4.2 i Kv4.3 kanala u glatkim mišićima HUV-a u svim grupama trudnica. Rezultati dobijeni *Western blot*-om bili su u skladu sa IH bojenjem. Ekspresija podjedinica Kv4.2 i Kv4.3 nije se značajno razlikovala između grupa trudnica. **Zaključak.** Ovo je prema našim saznanjima prva studija kojom je pokazano prisustvo Kv4.2 i Kv4.3 kalijumovih kanala u glatkim mišićima HUV-a zdravih porodilja, kao i onih porodilja čija je trudnoća bila praćena prisustvom GDM ili AHT. Ti kanali su najverovatnije glavne komponente brzih struja A-tipa, koji mogu biti relevantni za protok krvi majke i ploda i na taj način delovati protektivno za razvoj fetusa. Takođe, oni mogu predstavljati senzitivne pokazatelje hemodinamskih i/ili metaboličkih promena u lokalnoj fetomaternalnoj sredini.

Ključne reči:
dijabetes melitus; ljudi; hipertenzija; trudnoća; v.umbilicalis.

Introduction

The connection between a mother and a child represents the most valuable nature relationship that begins *in utero* and lasts forever. During the antenatal period, the umbilical cord represents an important organ, as it maintains the functionality and integrity of a fetomaternal unit. It has a specific structure – two human umbilical arteries embedded in mucous connective tissue with one human umbilical vein (HUV) [1]. Mucous connective tissue (Wharton's jelly) gives the umbilical cord remarkable elasticity and represents its important feature [2] considering that this structural specificity allows proper fetal supplementation of nutrients and gas. The additional feature of the umbilical cord is represented by the absence of lymphatics, *vasa vasorum*, and innervations, which defines overall umbilical circulation as unique. The properties of the so-called low resistance – low-pressure umbilical circulation lead to amplified effects of systemic vasoactive substances and local inflammatory and mechanical factors in regulating umbilical blood vessel tone [3].

The level of adequate oxygenation and nutritive supply is influenced by the undisturbed blood flow through the umbilical cord [4]. Furthermore, there are several important factors known to have an impact on placental blood flow, including fetal respiration, smooth muscle cell (SMC) contraction, and different pressure gradients caused by the contractility of a fetal heart. Another important factor for the quality of proper supply is the preservation of propagation direction of the umbilical arteries [4, 5] and exhibitions of HUVs autoregulatory vasomotor phenomena, which are additional factors in maintaining the blood flow [6]. The capability of HUVs to regulate their tone is an important fact, considering various pathological conditions that can affect the proper development of the fetus. For instance, hypoxia leads to the activation of the HUVs regulatory mechanisms and consecutive vasodilatation, so it can be concluded that HUV is not just a passive conduit blood vessel [7] and can regulate its diameter depending on the environmental settings. Histologically, vascular SMCs within the *tunica media* of the HUV consist of three types of differently oriented layers: circular, oblique, and longitudinal. The property of those layers, together with the compact structure of the Wharton's jelly, contributes to the maintenance of adequate and undisturbed blood flow through the umbilical cord, which is especially important during fetal movements [8].

In 1949, the squid giant axon served as an animal model for Hodgkin and Huxley to report a discovery – electrical currents through Kv channels. After that, around three decades had passed before that statement on vascular SMCs was confirmed [9]. Now it is known that vascular SMCs contain Kv currents that can be divided into two main types, regarding their time-dependency property: slow "delayed-rectifier" and rapid "transient-outward" K^+ currents (also known as A-type currents) [10, 11]. As for delayed-rectifier K^+ current, it shows delayed activation and slow inactivation and has been detected in the majority of vascular SMCs. Furthermore, those currents are responsible for the repolarization and setting of the resting membrane potential. The threshold for the activation in SMCs is about -30 mV [12–14]. On the contrary, A-type K^+ currents show very rapid activation, as well as inactivation. This current is less frequently detected in vascular SMCs; it has a relatively small contribution to the total outward Kv currents and is mainly inactivated at the resting membrane potential. A-type currents are activated at negative (subthreshold) membrane potentials, typically between -45 and -60 mV. Additional properties typical for these types of currents are inactivation caused by voltage-dependence, strong steady state, and repolarization to the potentials negative to -50 mV, typically required for regeneration of channel function [15]. Since the discovery of A-type of K^+ currents in the human SMCs, detailed studies that have been examining its function and presence in human HUVs are limited [16]. In recent years, studies that have been using molecular techniques of Kv proteins in other tissues have demonstrated that the Kv4 subfamily of Kv channels (Kv4.1, Kv4.2, and Kv4.3) contributes to the overall A-type currents. Moreover, newer studies provide a substantial line of pieces of evidence specifically indicating that particularly Kv4.2 and Kv4.3 channels are the major components of A-type currents in many SMC, including vascular SMCs [15–17].

The aim of this study was to reveal whether Kv4.2 and Kv4.3 potassium channels are present in the SMCs of HUV and to investigate potential alterations of their expression during pathological conditions such as gestational diabetes mellitus (GDM) and pregnancy-induced hypertension (PIH) that can alter blood flow to the fetus.

Methods

Ethics statement

The permission to conduct this research was obtained from the Ethics Committee of the Faculty of Medicine,

University of Belgrade, Serbia (permission No 2650/VI-5, from June 26, 2018).

Collecting of the tissues and their manipulation

Samples of umbilical cords were collected from the Clinic for Gynecology and Obstetrics "Narodni front", Belgrade, Republic of Serbia. All women have agreed to participate in the study by signing the written consent. The objects of investigation (umbilical cords) were collected immediately after vaginal delivery or Caesarean section, no later than 2 hrs after labor. Afterward, they were transported to the laboratory in small vials filled with cold Krebs-Ringer bicarbonate [18]. Samples were divided into three groups based on the health status of a pregnant woman: "normal" (healthy, i.e., having no known or apparent active disease) and two "experimental" groups: diabetic and hypertensive. After the preparation, HUVs were immersed in fixative (10% formaldehyde) for immunohistochemical staining and frozen at -70 °C for the Western blot analysis.

Clinical terms and definitions

GDM was implied as diabetes mellitus developed or recognized during pregnancy [2].

Elevated blood pressure developed after 20 weeks of gestation with sustained values of systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg was termed PIH. It is a wide entity and includes the following disorders: gestational hypertension, preeclampsia, and eclampsia [2, 19].

Immunohistochemistry

The procedures of molecular technique and staining methods used in this study after collecting and preparation of the samples have been published earlier [5, 20]. In short, after the isolation from the umbilical cords, HUV samples were fixed in 10% formaldehyde and embedded in paraffin. All sections were deparaffinized and rehydrated through solutions with decreasing alcohol concentrations to distilled water and heated for 30 min in Tris-ethylenediaminetetraacetic acid (EDTA) buffer at pH 9.0 for antigen retrieval. Sections were washed with Tris-buffered saline and incubated overnight with anti-Kv4.2 and anti-Kv4.3 goat polyclonal primary antibodies (Santa Cruz Biotechnology, Inc., dilution ratio 1 : 50). Then they were treated by applying the commercial ImmunoCruz™ goat ABC Staining System (sc-2023, Santa Cruz Biotechnology). Diaminobenzidine as chromogen served as a developing tool for immunoreactions for 10 min. More detailed procedures can be found in the paper by Djokic et al. [5].

The experienced researcher evaluated the intensity and the distribution of positive staining using two scoring systems. The scoring system for immunocytochemical analysis used in this paper was initially introduced by Fisher et al. [21] and was later adapted by Adams et al. [22].

This scoring system unites the intensity and distribution of positive staining into one score and is shown in Table 1 [22]. The other scoring system was a semi-quantitative four-point scale that scored the intensity of slides as negative and positive (+, ++, or +++) [21, 22].

Western blot analysis

HUV rings were homogenized on ice in modified RIPA buffer (50 mmol/L Tris/HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.2% Na-deoxycholate, 0.2% SDS, 1 mmol/L EDTA, and protease and phosphatase inhibitors). The homogenates were centrifuged at 15,000 × g for 30 min at +4 °C. The BCA method was performed to determine the concentration of proteins. Obtained supernatants represented a cell lysate for Western blot after being boiled in the Laemmli sample buffer. The proteins were separated by electrophoresis on a 10% polyacrylamide gel and moved onto Polyvinylidene difluoride membranes. The type of membrane, blockers signal detecting, and visualization techniques are described in the author's previous paper [23].

Statistical analysis

Data analysis was conducted with SigmaPlot (Systat Software Inc., San Jose, CA). The results are presented as the means ± standard error of the mean (S.E.M.); n = number of experiments. The Student's *t*-test was used to determine the significance of differences for Western blot. After statistical analysis, *p*-values < 0.05 were considered statistically significant.

Results

Each group consisted of 6 HUV samples obtained from 6 normal pregnancies, 6 pregnancies with GDM, and 6 pregnancies with PIH.

Immunohistochemistry

Immunohistochemistry analysis indicated membranous and cytoplasmic expression of Kv4.2 and Kv4.3 subunits in SMCs of HUVs. The expression of Kv4.2

Table 1

Scoring system of Kv channel subunit for immunocytochemical analysis (adapted from Adams et al. [22])

Strong	More than 50% of cells are involved. Black/opaque staining that is clearly visible.
Intermediate	Less than 50% of cells are involved (with dark/opaque staining that is clearly visible) or more than 50% of cells (with intermediate membrane staining).
Weak	More than 50% of cells are involved (with intermediate focal staining that is clearly visible) or an unidentified percent of cells with light membrane appearance.
Sporadic	Unidentified percent of randomly distributed cells (with black/opaque membrane staining).
None	This group is represented by smooth muscle cells that show none of the aforementioned.

showed a similar staining pattern in GDM and PIH compared to normal pregnancy (immunocytochemical scoring – moderate/++). The expression of the Kv4.3 subunit was similar through *tunica media* in all HUVs regardless of the sample group: normal pregnancies, GDM, or PIH. The expression pattern was also similar in all experimental groups (immunocytochemical scoring – moderate/++) (Figures 1A-1C).

Western blot analysis

The Western blot results were in agreement with immunohistochemical staining. There were no detected differences in the expression of Kv4.2 and Kv4.3 subunits between the groups (Figure 2).

In the end, all results referring to Kv4.2 and Kv4.3 subunits distribution and expression are shown in Table 2.

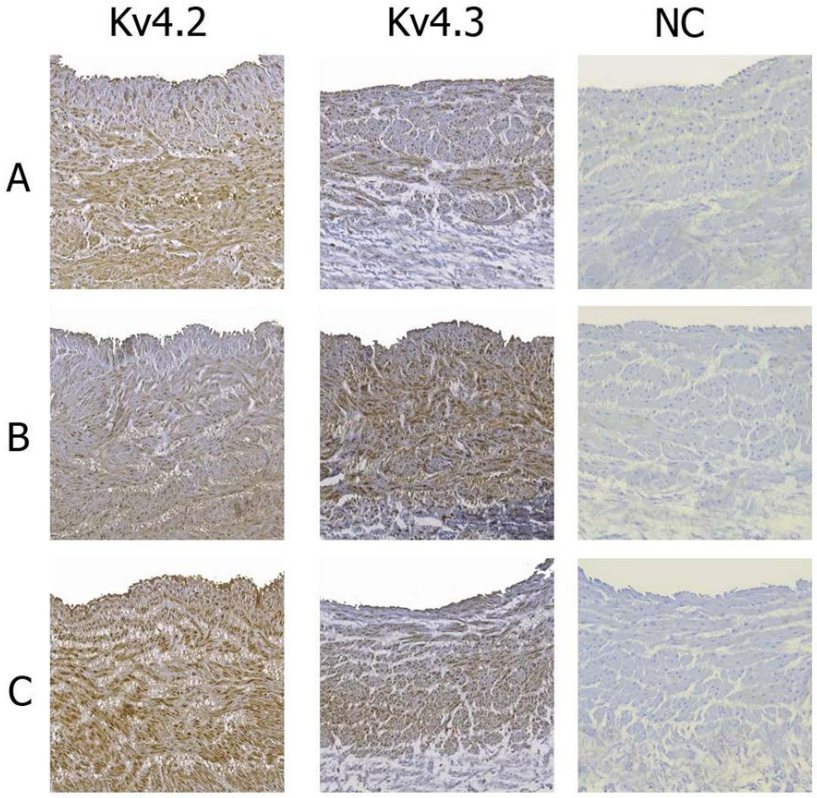

Fig. 1 – Immunohistochemical staining of Kv4.2 and Kv4.3 subunits. Normal pregnancy (A); gestational diabetes mellitus (B); pregnancy-induced hypertension (C). Expression of Kv4.2 and Kv4.3 (brown staining); negative control (NC). Original magnification: 200x. The figure is representative of the preparations of four patients.

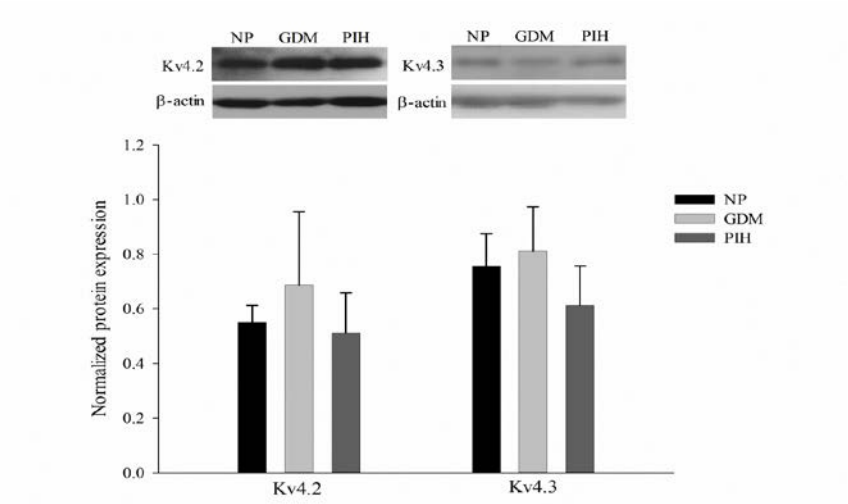

Fig. 2 – The expression of Kv4.2 and Kv4.3 subunits on human umbilical veins from normal pregnancy (NP, n = 6), gestational diabetes mellitus (GDM, n = 6), and pregnancy-induced hypertension (PIH, n = 6). Results are expressed as mean ± standard error of the mean.

Table 2

Distribution and expression of Kv channel subunits according to immunohistochemistry (IH) and Western blot (WB)

Subunit	NP (IH)	GDM (IH)	PIH (IH)	WB
K_v4.2	moderate/++ m/cp, diffuse	moderate/++ m/cp, diffuse	moderate/++ m/cp, diffuse	n.s.
K_v4.3	moderate/++ m/cp, diffuse	moderate/++ m/cp, diffuse	moderate/++ m/cp, diffuse	n.s.

NP – normal pregnancy; GDM – gestational diabetes mellitus; PIH – pregnancy induced hypertension; m/cp – membranous/cytoplasmic, n.s. – no statistically significant difference.

Discussion

Based on our information so far, this is the first study to evaluate the expression of Kv4.2 and Kv4.3 channels in the HUV SMC obtained from healthy, diabetic, and hypertensive pregnancies. The principal new findings show that Kv4.2 and Kv4.3 channels are expressed in all experimental groups. The expression of Kv4.2 and Kv4.3 subunits remains preserved in both pathological conditions in comparison to normal pregnancy. The distribution of both channel subtypes appeared similar in HUV SMC from all groups. Activating voltage-gated potassium (Kv) channels hyperpolarizes and relaxes vascular SMCs by decreasing the activity of L-type voltage-gated Ca^{2+} channels. Conversely, inhibition of Kv channels induces vasoconstriction [24]. Vascular SMCs have been reported to express members of the Kv1, Kv2, Kv3, Kv4, Kv6, Kv7, Kv9, and Kv11 subfamilies of Kv channels [25].

Kv channels are complexes of several subunits constructed of membrane-integrated α-subunits and accessory subunits. The α-subunits form a tetrameric complex that represents the core of the Kv channel, which consists of a pore domain with activation/inactivation gates and a selectivity filter connected to peripheral voltage-sensor domains. Auxiliary subunits may increase the diversity of Kv channels by changing the function and expression of the α-subunits and control membrane trafficking [14, 26].

HUV responds very actively to different *stimuli*, especially to ones expected to be abnormal, often seen in diabetic and hypertensive pregnancies. Previously, we have shown that the ability of pinacidil (recognized as a potassium channel opener) to produce vasorelaxation of HUVs from healthy pregnancies in the presence of nonspecific Kv1-4 channels inhibitor, 4-aminopyridine (4-AP), is decreased [26]. Compared to HUVs obtained from GDM and PIH, these effects were absent [5]. Furthermore, in order to obtain more details about the Kv channel subtypes involved in the relaxation produced by pinacidil on HUVs, we have used margatoxin (MgTx), a specific blocker of Kv1.2 and Kv1.3 channels. In the presence of MgTx, pinacidil-induced vasorelaxation of HUVs from normal pregnancies was significantly reduced, confirming the involvement of Kv1.2 and/or Kv1.3 in the pinacidil response [27].

These findings made us conduct additional experiments in order to check the presence of Kv4.2 and Kv4.3 channels in HUV SMC, bearing in mind that the inhibition by 4-AP is considered one of the pharmacological hallmarks of A-type currents [15]. Our assumption was that their expression could be reduced in GDM and PIH.

A-type currents were first described in molluscan neurons [28]. It was suggested that they have roles in controlling neuronal action potential threshold, frequency, and duration [17]. A-type currents have also been detected in a myriad of non-neural tissues, including atrial and ventricular cardiomyocytes and visceral and vascular SMCs. In contrast to neuronal A-type currents, currents found in cardiomyocytes were available at resting membrane potentials and were predominantly responsible for the initial repolarization (phase 1) of the cardiac action potential. In SMCs, A-type currents may be related to the maintenance of membrane potential. In addition, they may regulate propagation and frequency of action potentials [29, 30].

It has been reported that A-type current coexists with the delayed-outward current in vascular SMCs of the rabbit (portal vein, pulmonary artery, aorta), rat (pulmonary artery, renal resistance artery), and human mesenteric artery. In all mentioned blood vessels, Kv4.2 and Kv4.3 were detected as major or particularly important components of A-type currents in vascular SMC [12, 15]. For the first time, we detected in the present study the expression of Kv4.2 and Kv4.3 subunits throughout the *tunica media* of the HUVs from normal, diabetic, and hypertensive pregnancies. That confirmed the presence of A-type currents in the HUV SMC. Moreover, the differences in the expression of the aforementioned channels between the groups were not statistically significant, confirming that the expression of Kv4.2 and Kv4.3 subunits in diabetic and hypertensive veins remains preserved in pathological pregnancies. That, for instance, is in contrast with our previous research conducted on ATP-sensitive K channels, where reduced Kir6.1 expression in vascular SMCs of the HUV during GDM and PIH was found [5]. Observations that A-type currents in vascular SMCs often coexist with delayed-outward K^+ currents and that 4-AP is a nonspecific Kv1-4 inhibitor [12, 31] lead to the conclusion that alterations of some other members of the Kv channel family are probably responsible for the loss of antagonism to 4-AP during GDM and PIH in our previous study. This statement will probably be the basis of our further research.

Diminished expression of Kv4.2 and Kv4.3 subunits have been reported in cardiac myocytes from spontaneously hypertensive rats alongside lower A-type currents density [32, 33]. Moreover, downregulation of the Kv4.2, but not

Kv4.3 channels, have been reported in diabetic rat ventricle and human diabetic cardiomyopathy [34, 35]. However, by reviewing the literature, we have not found any research that has studied alterations of these channels in vascular SMC during diabetes mellitus and hypertension in other vascular beds and other species.

To date, the precise physiological role of A-type currents in vascular SMCs has not been completely clarified. Some reports have suggested that the primary role of the A-type current in vascular SMCs is to suppress membrane excitability. In retinal microvascular SMCs, the application of 4-AP to current-clamped vessels has caused membrane depolarization alongside increased cell contractility. Thus, it has been revealed that regulation of A-type currents in retinal vascular SMCs may be of great importance for the control of local tissue perfusion [11]. Bearing in mind that HUV is able to adjust its vascular tone influenced by various factors of the local microenvironment, we can speculate that A-type channels in HUV may be sensors for detecting hemodynamic and/or metabolic changes in the local environment. Furthermore, another possibility is that A-type currents play a role in the genesis of myogenic oscillations of the vein. Namely, in rabbit portal vein, it has been demonstrated that A-type currents tend to be a feature of phasic SMCs [15]. In HUV, these currents and hence Kv4.2 and Kv4.3 channels may be involved in the vasomotion phenomena. In that way, by producing myogenic oscillations, A-type currents might be significant for umbilical venous blood flow and may contribute to fetal development [6].

Limitations of the study

A limitation of this research is that A-type currents were not measured and recorded using methods of electrophysiology or real-time polymerase chain reaction. However, it should be highlighted that, in all previous publications, Kv4.2 and Kv4.3 channel subunits were, without exception, identified as major constituents of A-type currents.

Conclusion

To the best of our knowledge, this study was the first that demonstrated the presence of Kv4.2 and Kv4.3 potassium channels in the HUV SMCs and their preservation during the course of GDM and PIH. These channels are most likely major components of A-type currents that may be relevant for mother-fetus blood flow and hence fetal development. Additionally, they may represent sensors for detecting hemodynamic and/or metabolic changes in the local environment. Further investigations in this field may be of great importance for understanding the regulation of umbilical circulation and may provide new strategies for reducing the risk of negative pregnancy outcomes.

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Conflict of Interest

The authors declare no conflict of interest.

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