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CYCLIC NUCLEOTIDE-DEPENDENT RELAXATION IN HUMAN UMBILICAL VESSELS

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Umbilical vessels have a low sensitivity to dilate, and this property is speculated to have physiological implications. We aimed to investigate the different relaxing responses of human umbilical arteries (HUAs) and veins (HUVs) to agonists acting through the cAMP and cGMP pathways. Vascular rings were suspended in organ baths for isometric force measurement. Following precontraction with the thromboxane prostanoid (TP) receptor agonist U44069, concentration-response curves to the nitric oxide (NO) donor sodium nitroprusside (SNP), the soluble guanylate cyclase (sGC) stimulator BAY 41-2272, the adenylate cyclase (AC) activator forskolin, the β-adrenergic receptor agonists isoproterenol (ADRB1), salmeterol (ADRB2), and BRL37344 (ADRB3), and the phosphodiesterase (PDE) inhibitors milrinone (PDE_3), rolipram (PDE_4), and sildenafil (PDE_5) were performed. None of the tested drugs induced a relaxation higher than 30% of the U44069-induced tone. Rings from HUAs and HUVs showed a similar relaxation to forskolin, SNP, PDE inhibitors, and ADRB agonists. BAY 41-2272 was significantly more efficient in relaxing veins than arteries. ADRB agonists evoked weak relaxations (< 20%), which were impaired in endothelium-removed vessels or in the presence of the NO synthase inhibitor L-NAME, sGC inhibitor ODQ. PKA and PKG inhibitors impaired ADBR1-mediated relaxation but did not affect ADRB2-mediated relaxation. ADRB3-mediated relaxation was impaired by PKG inhibition in HUAs and by PKA inhibition in HUVs. Although HUA and HUV rings were relaxed by BRL37344, immunohistochemistry and RT-qPCR analysis showed that, compared to ADRB1 and ADRB2, ADRB3 receptors are weakly or not expressed in umbilical vessels. In conclusion, our study confirmed the low relaxing capacity of HUAs and HUVs from term infants. ADRB-induced relaxation is partially mediated by endothelium-derived NO pathway in human umbilical vessels.

Key words: *nitric oxide donor, soluble guanylate cyclase, adenylate cyclase, human umbilical artery, human umbilical vein, vasorelaxation, β-adrenoceptor, phosphodiesterase inhibitors*

INTRODUCTION

In fetoplacental circulation, regulation of vasomotor tone is important to maintain an adequate blood supply that makes feasible maternofetal gas and solute exchange (1-3). As umbilical blood vessels lack autonomic innervation, control of vascular tone is mainly regulated by circulating and/or locally released vasoactive agents as well as by physical factors, such as flow or oxygen tension (4). Accordingly, constriction and relaxation of human umbilical arteries (HUAs) and veins (HUVs) have been demonstrated in response to several agonists and physical stimuli (5-12). However, when compared to other human vessels, full-term umbilical vessels have a low sensitivity to dilate (5-12). In addition, the results from the few studies that directly compared the reactivity of HUAs and HUVs suggest differences in the responsiveness of the vessels to vasoactive stimuli (7, 13-16). Belfort *et al.* demonstrated that the relaxant potency of the Ca^{2+} channel blocker nifedipine was higher in HUVs than in HUAs (13). Similarly, in a previous study, we observed that the relaxation evoked by H_2S was present in HUVs but not in HUAs (14). In contrast, Krause *et al.* observed that arginase inhibition induced a higher relaxation in HUAs than HUVs (15). Since arginases compete with nitric oxide (NO) synthase (NOS) by their common substrate L-arginine, they speculate that a lower NOS/arginase ratio could be responsible for the higher relaxant activity of arginase inhibition in HUAs (15). Finally, several studies have shown similar responsiveness to exogenous NO in umbilical arteries and veins (7, 16).

Cyclic nucleotides, 3′-5′-cyclic adenosine monophosphate (cAMP) and 3′-5′-cyclic guanosine monophosphate (cGMP) are ubiquitous second messengers that play an essential role in vascular tone regulation (17-19). Intracellular levels of these

second messengers are the result of the balance between the rate of their synthesis and degradation by cyclic nucleotide phosphodiesterases (PDEs) (20-22). Activation of soluble guanylate cyclase (sGC) and generation of cGMP is the main signal-transducing event of the L-arginine-nitric oxide (NO) pathway, whereas cAMP is the main intracellular second messenger of the β-adrenergic receptor (ADRB) and prostanoid-mediated vascular relaxation (23). However, there is overwhelming evidence that a variety of vascular effector mechanisms involve crossover reactivity of cAMP and cGMP signaling pathways (23-25). In this study, we aimed to investigate the differences between HUAs and HUVs on relaxant response to agonists acting through the cAMP and cGMP pathways. We hypothesized that arteries and veins differ in their relaxant capacity due to their different roles in the control of umbilical circulation.

MATERIAL AND METHODS

This study was approved by the local ethics committee (Milan Area 2, Italy. Protocol number 29/2014). Parents gave written informed consent in accordance with the Declaration of Helsinki and by the Medical Research Involving Human Subjects Act (WMO).

Tissue collection

Eighty-one umbilical cords of healthy term newborns (37 to 42 weeks of gestation) born by cesarean section were collected. Infants born from pregnancies complicated by hypertension, pre-eclampsia/eclampsia, diabetes, kidney disease, infection, or other significant medical disorder were excluded. Moreover, the umbilical cords of newborns with congenital disabilities were excluded. Parts of the umbilical cord, 10 cm in length, were excised from the proximal segment of cord (closest to the placental attachment) immediately after cesarean section. Tissue was immediately placed in cold Krebs-Ringer-Bicarbonate (KRB) solution.

Vascular reactivity studies

Human umbilical vessels were dissected free from Wharton's jelly and cut into 3 mm-rings. Rings were suspended in 5 mL organ baths filled with KRB solution maintained at 37ºC and aerated with 95% O_2 – 5% CO_2 (pH 7.4). Changes in isometric tension were measured with force transducers (model PRE 206-4, Cibertec, Madrid, Spain) and recorded with MP100 data acquisition system (BIOPAC System Inc. Santa Barbara, USA). AcqKnowledge III for MP100 software was used for chart recording and data acquisition. Tissues were allowed to equilibrate for 30 min; then, they were stretched to an optimal resting tension of 2 g (19.6 mN). Before starting the experiments, rings were transiently challenged with 62.5 mM KCl to assess the functional state of each vessel and to establish a reference nonreceptor-mediated contraction for standardization of contractile responses. Preparations were washed three times and allowed to recover for 30 minutes.

Relaxing agonists were evaluated during contraction induced by the thromboxane prostanoid (TP) receptor 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin F2α (U44069). In previous pilot experiments, we observed that U44069 elicited a stable contraction, allowing the study of relaxant agents. A concentration of 1 μM of U44069 elicited ~80% of the maximal contractile response to the drug in both HUAs and HUVs, as determined in pilot concentration-response curves. When the contraction induced by U44069 (1 μM) reached a plateau, concentration-response curves to the different relaxant agents were performed. Concentration increments of each relaxant agent were made once the response had reached a plateau, or after 5 – 10 min if no response had occurred or a clear plateau was not reached. Following relaxant agents were tested: NO donor sodium nitroprusside (SNP, 1 nM – 0.1 mM), sGC stimulator BAY 41-2272 (1 nM – 0.1 mM), adenylate cyclase (AC) activator forskolin (1 nM – 0.1 mM), non-selective ADRB agonist isoproterenol (1 nM – 0.1 mM), ADRB2 agonist salmeterol (1 nM – 0.1 mM), ADRB3 agonist BRL37344 (1 nM – 0.1 mM), and phosphodiesterase (PDE) inhibitors milrinone (PDE_3 inhibitor,1 nM – 0.1 mM), rolipram (PDE_4 inhibitor, 1 nM – 0.1 mM), and sildenafil (PDE_5 inhibitor, 1 nM – 0.1 mM).

Some experiments were conducted with NO synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 0.1 mM), sGC inhibitor 1H-[1,2,4] oxadiazole [4,3-a] quinoxalin-1-one (ODQ, 10 μM), AC inhibitor 2',3'-dideoxyadenosine (DDA,10 μM) or cyclooxygenase (COX) inhibitor indomethacin (10 μM). Relaxing responses were also studied in endothelium-denuded arteries. For this purpose, the endothelium was removed by gentle rubbing of vessel lumen with a stainless steel wire. In order to better characterize the mechanism of action of ADRB agonists, additional experiments were conducted with atenolol (ADRB1 antagonist, 1 μM), ICI 118,551 (ADRB2 antagonist, 1 μM), or SR 59230A (ADRB3 antagonist, 1 μM), KT5720 (protein kinase A (PKA) inhibitor, 1 μM), and Rp-8-Br-PET-cGMPS (protein kinase G (PKG) inhibitor, 10 μM).

Drugs and solutions

KRB buffer contained (in mmol L^{-1}): NaCl, 118.5; KCl, 4.75; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; $CaCl_2$, 2.5; glucose, 11.1. Solutions containing different concentrations of KCl were prepared by replacing part of the NaCl by an equimolar amount of KCl.

U44069, SNP, isoproterenol, and DDA were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO). All other drugs were obtained from Tocris (Ballwin, MO). Sodium nitroprusside, acetylcholine, isoproterenol, BRL 37344, atenolol, ICI 118,551, DDA, L-NAME, and U44069 were dissolved in distilled deionized water. BAY 41-2272, salmeterol, milrinone, sildenafil, SR 59230A, ODQ, indomethacin, KT5720, and Rp-8-Br-PET-cGMPS were dissolved in DMSO. Forskolin and rolipram were dissolved in ethanol. The final bath concentration of the vehicles did not exceed 0.1% and did not affect mechanical activity.

Immunohistochemistry

Formalin-fixed, paraffin-embedded human umbilical cords were cut into 4 μm sections and placed on electrostatically polarized slides. Immunohistochemistry was performed using the automated system BenchMark XT (Ventana Medical Systems Inc., Tucson, Arizona, USA). Reactions were revealed using UltraView™ Universal DAB, a biotin-free, multimer based detection system, according to the manufacturer's instructions (standard protocol). Immunostaining procedure was accomplished on human umbilical cords using specific antibodies for ADRB1 (SC568), ADRB2 (SC569), and ADRB3 (SC1472) (DBA, Italy). A standard protocol was performed, and primary antibody solutions were diluted at 1/50 in Ventana Diluent. Amplification kit was added for all antibodies (Ventana Medical Systems Inc., Tucson, Arizona, USA). Negative controls were performed by replacement of primary antibody with equal concentrations of nonimmune serum. Images were scanned with Aperio CS2 Scanscope (Leica, Germany).

Human umbilical vein and arterial endothelial cells (HUVEC and HUAEC) isolation and culture

Endothelial cells were obtained from umbilical veins, as previously described (26). HUVEC and HUAEC were isolated, infusing 0.1% collagenase A (Roche, Milan, Italy) for 15 min at 37ºC. Then, the effluent was collected in a sterile 50 ml conical centrifuge tube and sedimented at 463 g for 15 min.

HUVEC and HUAEC pellets were grown in the medium 199 (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS, Sigma, St. Louis, MO), 3% penicillin/streptomycin (200 u/ml, Sigma, St. Louis, MO), 200 µl heparin (25,000 UI/5 ml IV 1 F, HOSPIRA Italy) and 1% L-glutamine (Sigma, St. Louis, MO), at 37ºC in a 5% CO_2 atmosphere. The medium was changed two times weekly. Each culture was used at P0.

RNA extraction and reverse transcription

RNA from HUVEC and HUAEC was extracted using TRIzol Reagent (Life Technologies, Paisley, UK). Residual genomic DNA was removed from all samples using the DNA-free™ DNA removal kit (Ambion, Huntingdon, Cambridgeshire, UK). RNA samples were reverse-transcribed for 2 h at 37ºC in a 20 µL reaction volume using High-Capacity cDNA Archive kit (Life Technologies, Paisley, UK). Reverse transcriptase activity was terminated by heating samples at 85ºC for 5 min. Control samples without reverse transcriptase were included in all experiments to show that all products were RNA-derived and not the result of genomic DNA contamination.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

ABI Prism 7900 fast sequence detection system (Applied Biosystems, Foster City, CA) was used RT-qPCR analysis using HPRT-1 (Hs02800695_m1) as endogenous control. RT-qPCR was performed using specific primers and probes for ADRB1 (Hs02330048_s1), ADRB2 (Hs00240532_s1), ADRB3 (Hs02800695_m1) target genes (Assays-on-Demand Gene Expression Products). Validation experiments were performed using the 1:2 diluted templates. Target and reference genes were amplified in separate wells in duplicate. Reaction conditions included 10 µl of 2X TaqMan Universal PCR Master Mix, 1 µl of primers and probes mixture, 50 ng of template cDNA, and nuclease-free water to a 96-well reaction plate. Total reaction volume was 20 µl. Cycling conditions were as follows: 2 minutes at 50ºC, 10 minutes at 95ºC, and 40 cycles of 15 seconds at 95ºC, followed by 1 minute at 60ºC. Data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. Delta cycle threshold (ΔCt) values from each sample were obtained by subtracting the values for the reference gene from sample Ct. For each experimental sample, the $2^{-\Delta Ct}$ was calculated, and data were graphically indicated as relative expression.

Data analysis

Results are shown as mean ± standard errors of means (SEM) in n cords. The relaxant responses are expressed as the percentage of reduction of the contraction induced by U44069. Sensitivity/potency (expressed as $pEC_{50} = -\log EC_{50}$) and maximal relaxation (E_{max}) to agonists were determined by fitting individual concentration-response data to a non-linear sigmoidal regression curve. Differences between mean values were assessed by Student's *t*-test or one-way ANOVA followed by Bonferroni's *post hoc t*-test. Differences were considered significant at a $P < 0.05$. All analyses were performed using GraphPad Prism (version 7.04 for Windows, GraphPad Software, San Diego CA, USA).

RESULTS

Relaxant responses of U44069-contracted human umbilical vessels

Contraction elicited by U44069 (1 µM) was similar in HUA (192.95 ± 13.85% of KCl-induced contraction, n = 110) and HUV rings (207.65 ± 5.88% of KCl-induced contraction, n = 110). AC activator forskolin (*Fig. 1A*), NO donor SNP (*Fig. 1B*), and sGC stimulator BAY 41-2272 (*Fig. 1C*) relaxed U44069-contracted vessels in a concentration-dependent manner. Relaxations induced by forskolin and SNP were similar in HUA and HUV rings (*Fig. 1*, *Table 1*). In contrast, BAY 41-2272 showed a significantly higher efficacy (*i.e.*, higher E_{max}) in HUVs than in

Table 1. Relaxant responses of human umbilical vessels to, sodium nitroprusside (SNP), forskolin and BAY 41-2272.

	Artery			Vein		
	E_{max}	pEC_{50}	n	E_{max}	pEC_{50}	n
SNP	29.62 ± 3.24	–6.27 ± 0.06	10	27.03 ± 1.54	–6.23 ± 0.13	10
Forskolin	24.77 ± 2.90	–6.31 ± 0.08	10	22.44 ± 0.78	–6.45 ± 0.04	11
BAY 41-2272	23.27 ± 0.94	–6.55 ± 0.05	10	32.42 ± 1.90***	–6.50 ± 0.07	10

Values are means ± SEM; n= number of vessels; E_{max} = maximal relaxant effect (% of U44069-induced contraction); $pEC_{50} = -\log EC_{50}$; ***P < 0.001 artery versus vein.

Table 2. Relaxant reponses of human umbilical vessels to PDE inhibitors.

	Artery			Vein		
	E_{max}	pEC_{50}	n	E_{max}	pEC_{50}	n
Milrinone	27.22 ± 1.73§§§	–6.33 ± 0.05	10	30.54 ± 1.92§§§	–6.33 ± 0.04	10
Rolipram	24.57 ± 1.91§§§	–5.95 ± 0.12	10	25.29 ± 1.23§§§	–6.21 ± 0.05	10
Sildenafil	16.42 ± 0.10	–6.17 ± 0.05*	10	15.92 ± 0.72	–5.93 ± 0.09	10

Values are means ± SEM; n = number of vessels; E_{max} = maximal relaxant effect (% of U44069-induced contraction); $pEC_{50} = -\log EC_{50}$; *P < 0.05 artery versus vein; §§§P < 0.001 versus sildenafil in the same vessel type.

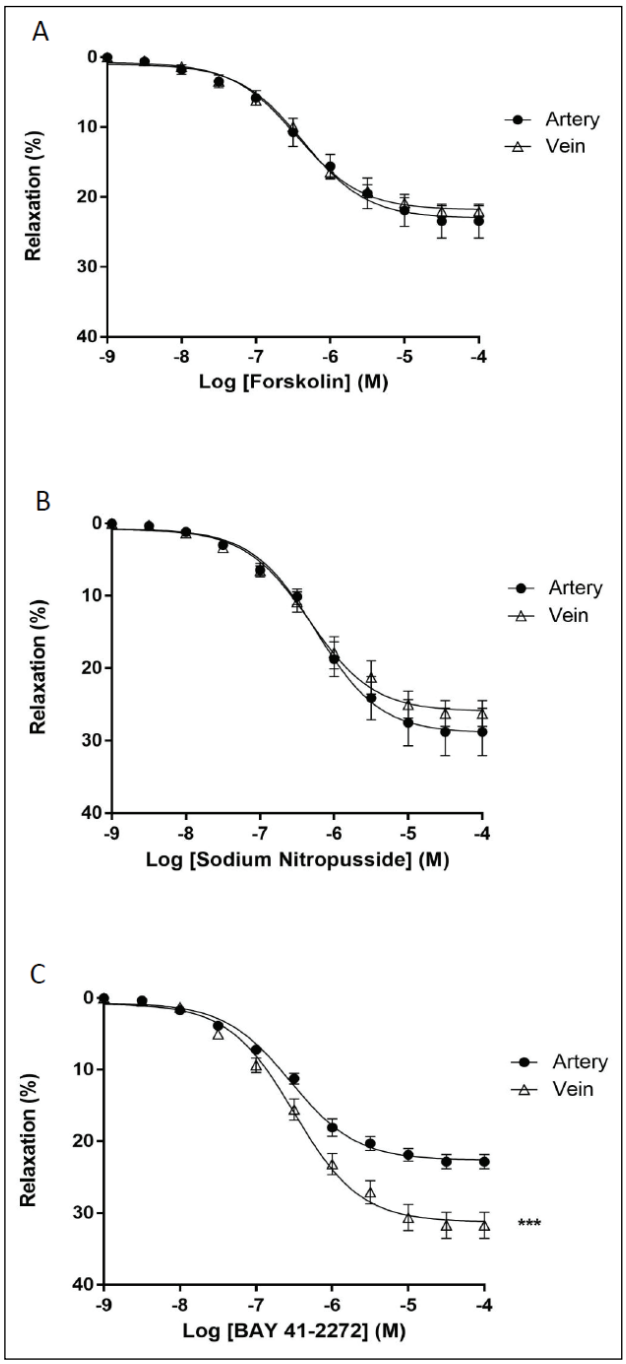

Fig. 1. Cumulative dose-response curves to forskolin (A), sodium nitroprusside (B) 10) and BAY 41-2272 (C) in human umbilical vein and artery rings, contracted with U44069 (1 μM). Each point or bar represents the mean ± SEM of 10-11 experiments. ***P < 0.001 artery versus vein.

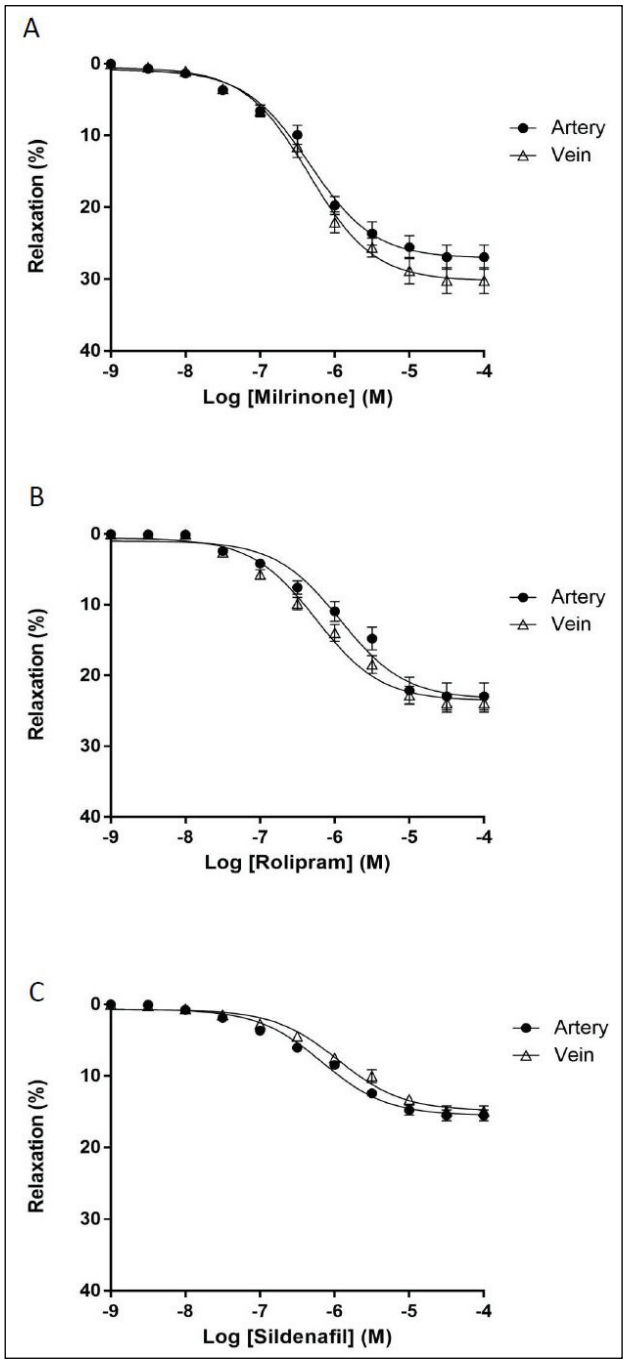

Fig. 2. Cumulative dose-response curves to milrinone (A), rolipram (B) and sildenafil (C) in human umbilical vein and artery rings, contracted with U44069 (1 μM). Each point represents the mean ± SEM of 10 experiments.

HUAs (*Table 1*). In order to assess the possible role of endothelium-derived NO in higher relaxant efficacy of BAY 41-2272 in HUVs, we performed additional experiments with NOS inhibitor L-NAME or in endothelium-denuded vessels. Neither removal of the endothelium nor the presence of L-NAME significantly affected BAY 41-2272-induced relaxation of HUVs (E_{max} endothelium-denuded: 32.35 ± 2.72%, n = 10; E_{max} in L-NAME presence: 28.04 ± 3.12%, n = 10) or HUAs (E_{max} endothelium-denuded: 22.25 ± 2.21%, n = 10; E_{max} in L-NAME presence: 18.40 ± 2.37%, n = 10). Endothelium denudation or L-NAME did not affect the relaxations elicited by forskolin or SNP (data not shown).

The PDE inhibitors milrinone (*Fig. 2A*), rolipram (*Fig. 2B*), and sildenafil (*Fig. 2C*) relaxed U44069-contracted vessels in a concentration-dependent manner. Milrinone and rolipram showed a similar potency and efficacy in HUAs and HUVs (*Table 2*). In contrast, sildenafil was slightly but significantly more potent in relaxing HUAs than HUVs (*Table 2*). As shown in *Table 2*, milrinone and rolipram were more efficient than sildenafil in relaxing HUAs and HUVs.

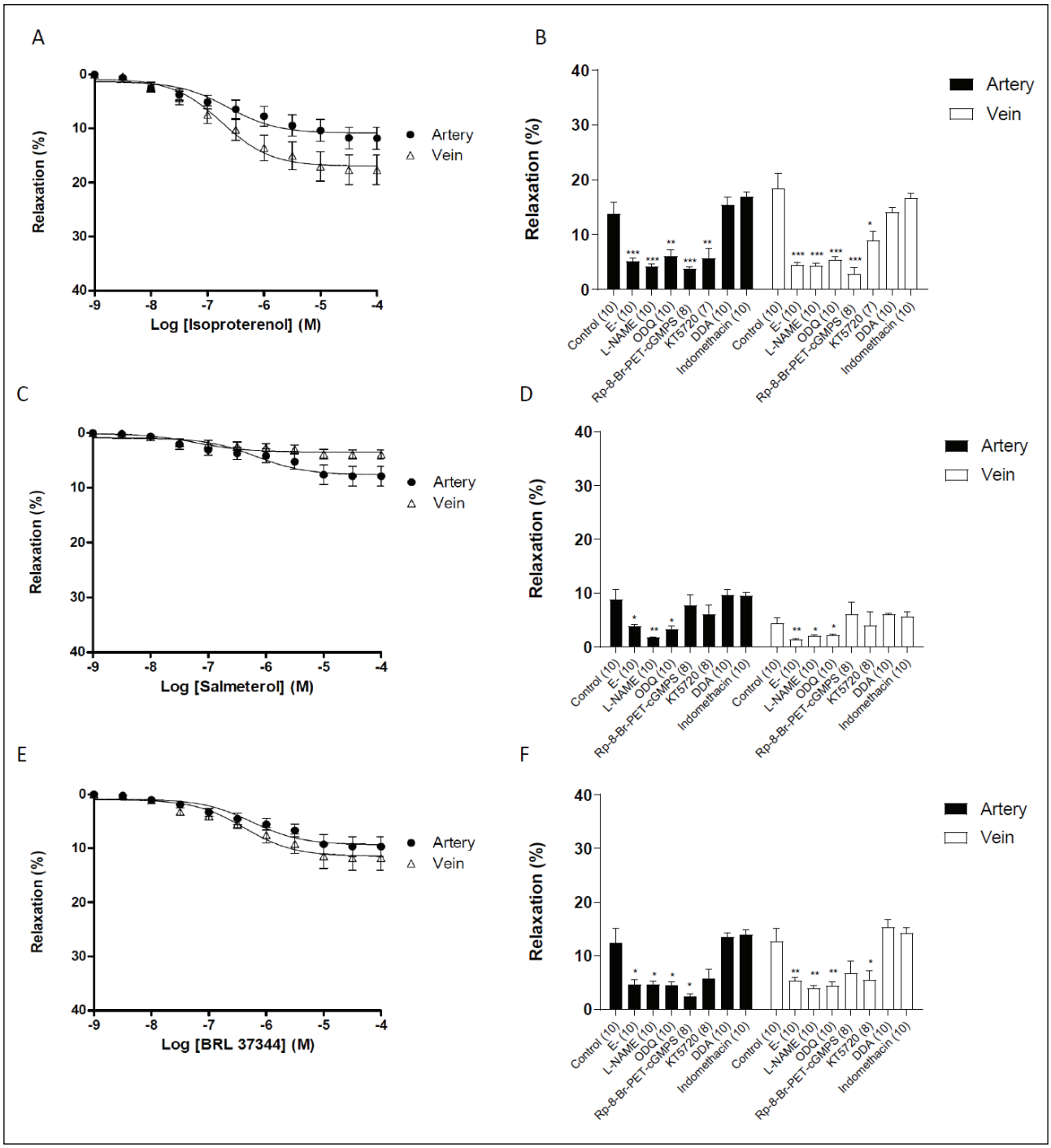

Fig. 3. Cumulative dose-response curves to isoproterenol (A), salmeterol (C) and BRL 37344 (E) in human umbilical vein, and artery rings. Right panels: mechanisms involved in the relaxation induced by isoproterenol (B), salmeterol (D) and BRL 372344 (F) in human umbilical vein and artery rings. Vessels were contracted with U44069 (1 μM). Each point or bar represents the mean ± SEM of experiments. *P < 0.05, **P <0.01, ***P < 0.001 versus control.

Table 3. Relaxant responses of human umbilical vessels to β-adrenergic receptor agonists.

	Artery			Vein		
	E_{max}	pEC_{50}	n	E_{max}	pEC_{50}	n
Isoproterenol	16.24 ± 2.09	–6.67 ± 0.33	10	18.37 ± 2.85	–6.66 ± 0.17	10
Salmeterol	8.77 ± 1.89	–6.06 ± 0.29	10	4.45 ± 0.90	–6.51 ± 0.31	10
BRL 37344	12.38 ± 2.73	–6.32 ± 0.18	10	12.76 ± 2.39	–6.85 ± 0.29	10

Values are means ± SEM; n = number of vessels; E_{max} = maximal relaxant effect (% of U44069-induced contraction);); pEC_{50} = –log EC_{50}

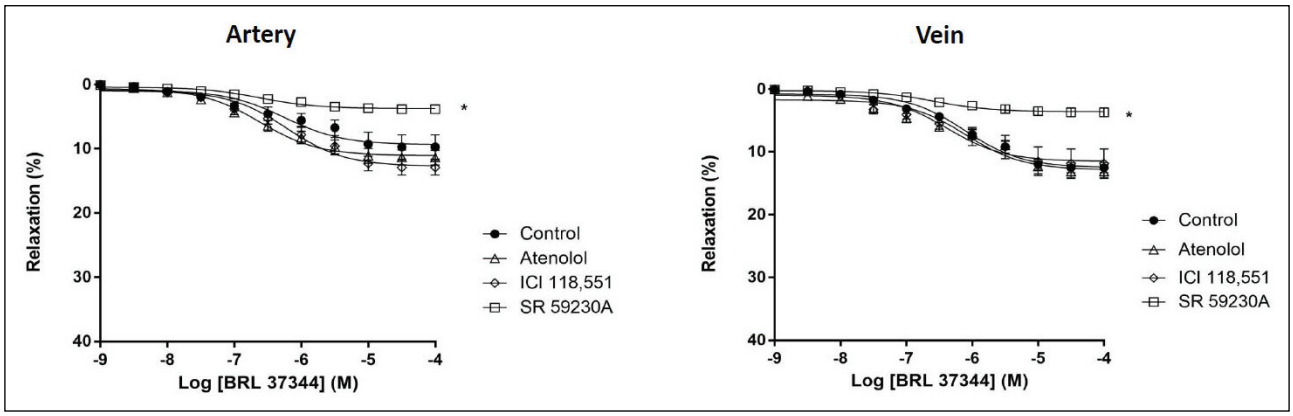

Fig. 4. Cumulative dose-response curves to BRL 37344 in the absence (control) or in the presence of atenolol (1 μM), ICI 118,551 (1 μM), or SR 59230A (1 μM) in human umbilical artery (A), and vein (B) rings. Vessels were precontracted with U44069 (1 μM). Each point or bar represents the mean ± SEM of 10 experiments. *P < 0.05 versus control.

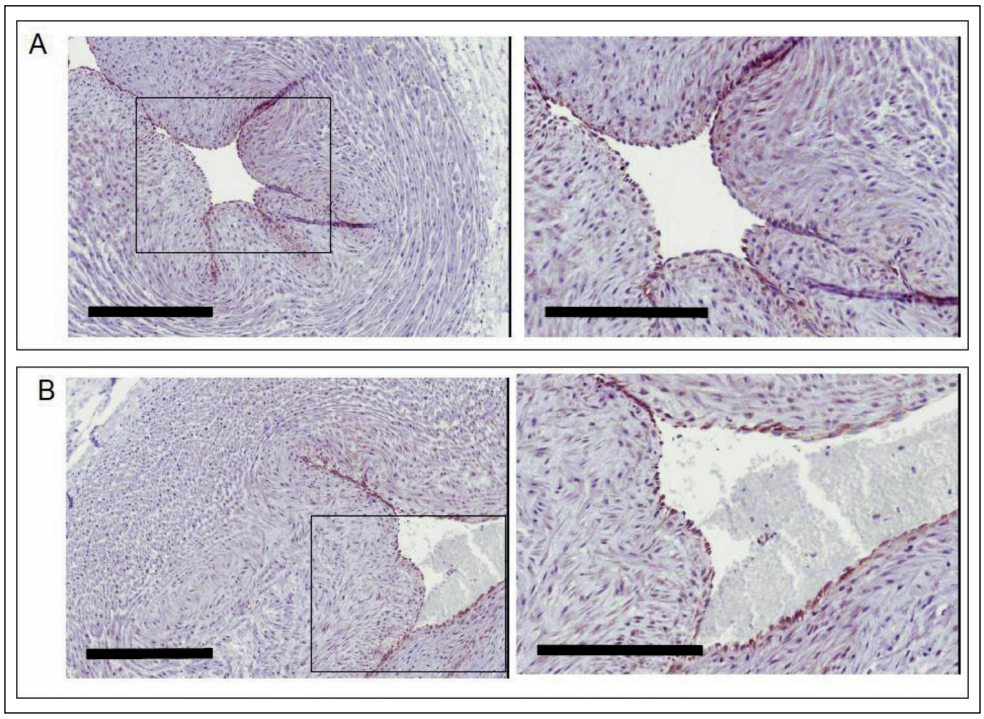

Fig. 5. Immunostaining for β_1-adrenergic receptor (ADRB1) in human umbilical artery (A) and vein (B). Scale bars: left panels, 200 μm; right panels, 60 μm.

Non-selective ADRB agonist isoproterenol (*Fig. 3A*), ADRB2 agonist salmeterol (*Fig. 3C*), and ADRB3 agonist BRL 37344 (*Fig. 3E*) relaxed U44069-contracted vessels in a concentration-dependent manner. However, as shown in *Fig. 3* and *Table 3*, the relaxation elicited by ADRB agonists did not exceed 20% of contraction induced by U44069 and, even with salmeterol relaxation, was below 10%. Efficacy and potency of ADRB agonists were similar in HUAs and HUVs (*Table 3*). Endothelium denudation or pretreatment with L-NAME or ODQ markedly inhibited the relaxant response to isoproterenol (*Fig. 3B*), salmeterol (*Fig. 3D*) and BRL 37344 (*Fig. 3F*). Conversely, DDA, or indomethacin did not affect ADRB-induced relaxation. PKA and PKG inhibitors impaired ADBR1-mediated relaxation (*Fig. 3B*) but did not affect ADRB2-mediated relaxation (*Fig. 3D*). ADRB3-mediated relaxation was impaired by PKG inhibition in HUAs and by PKA inhibition in HUVs (*Fig. 3F*). Relaxant response to BRL37344 was impaired in the presence of ADRB3 antagonist SR 59230, but it was unaffected by ADRB1 antagonist atenolol, and ADRB2 antagonist ICI 118,551 (*Fig. 4*).

Immunohistochemical detection of ADRB1, ADRB2, ADRB3 in human umbilical vessels and ADRB3 in HUVEC

We analyzed the protein expression of ADRB1, ADRB2, and ADRB3 through immunohistochemistry to verify the presence and localization of receptors. Immunohistochemistry showed that ADRB1 (*Fig. 5*), and ADRB2 (*Fig. 6*) were abundantly expressed in endothelial and smooth muscle cells of both HUAs and HUVs, while ADRB3 expression was not detectable (*Fig. 7A* and *7B*). However, a weak expression of ADRB3 was shown by Western blot analysis in primary HUVEC cultures (*Fig. 7C*).

ADRB1, ADRB2, ADRB3 mRNA expression in HUVEC and HUAEC

ADRB1, *ADRB2*, *ADRB3* mRNA expression was investigated in HUVEC (n = 7) and HUAEC (n = 7) cultures by RT-qPCR analysis. In HUVEC, there was no significant difference between *ADRB1* and *ADRB2* mRNA levels, while

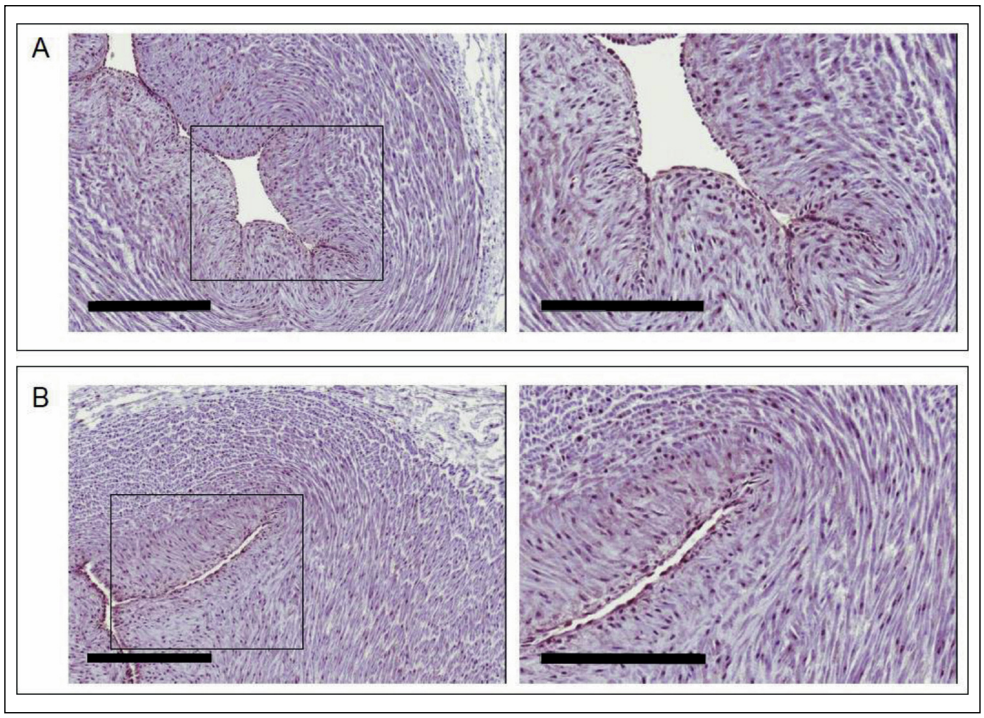

Fig. 6. Immunostaining for β_2-adrenergic receptor (ADRB2) in human umbilical artery (A) and vein (B). Scale bars: left panels, 200 μm; right panels, 60 μm.

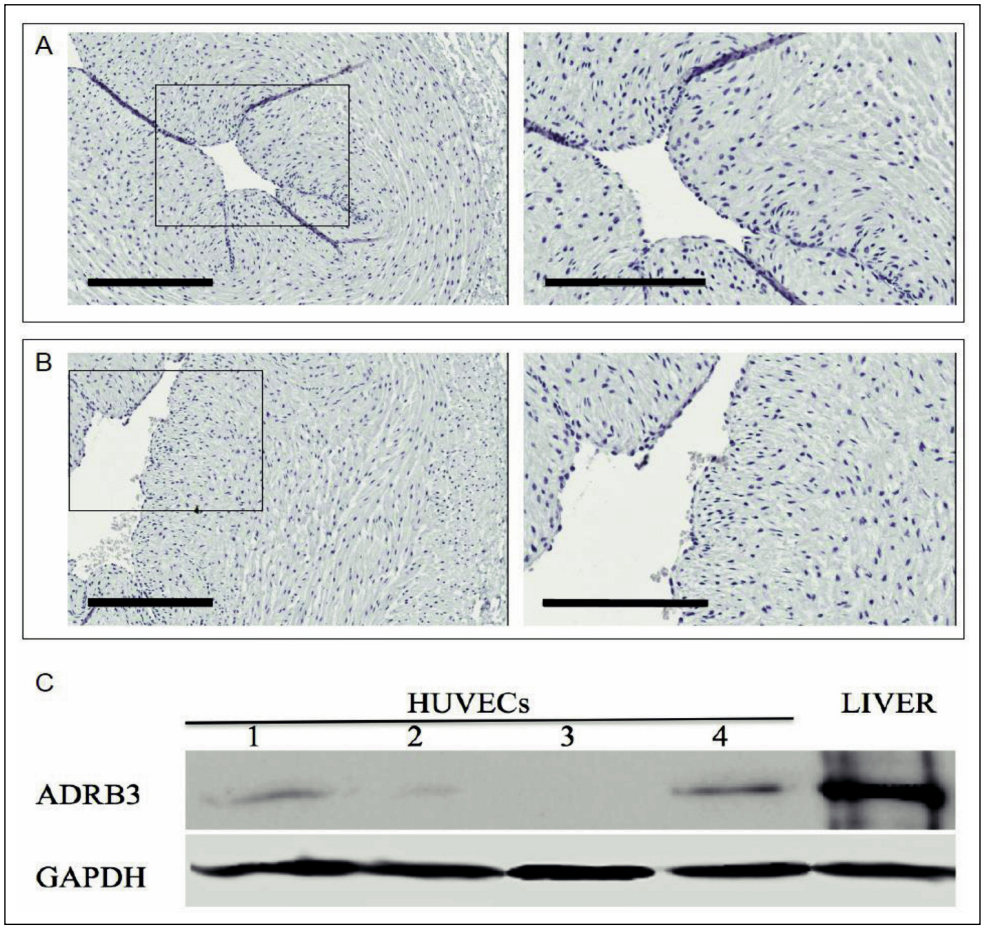

Fig. 7. Lack of immunostaining for β_3-adrenergic receptor (ADRB3) in human umbilical artery (A) and vein (B). Scale bars: left panels, 200 μm; right panels, 60 μm. (C): ADRB3 in primary HUVEC cultures. Protein lysates were prepared from primary HUVEC cultures and analyzed by Western blotting using the ADRB3 antibody. Representative Western blots for ADRB3 and GAPDH (internal control gene) are shown.

ADRB3 mRNA was significantly reduced (*Fig. 8*). In contrast, the expression of *ADRB2* was significantly higher than *ADRB1* and *ADRB3* ($P < 0.05$) in HUAEC (*Fig. 8*).

DISCUSSION

Our findings confirm low responsiveness of umbilical vessels to relaxant agonists acting through cAMP and cGMP

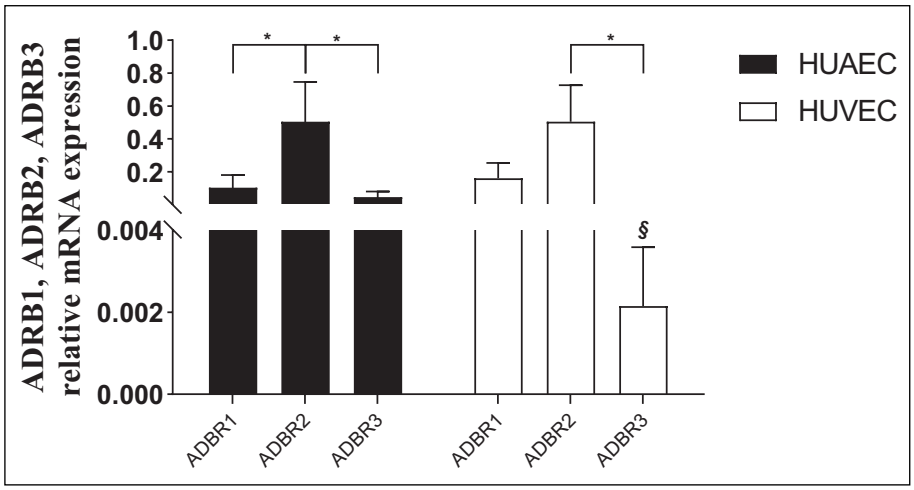

Fig. 8. ADRB1, *ADRB2*, and *ADRB3* gene expression in HUVEC and HUAEC, evaluated by *RT-qPCR*. ADRB1, ADRB2, ADRB3 mRNA derived from 7 HUVEC and HUAEC cultures were quantified by RT-qPCR analysis using the HPRT-1 as an endogenous control. Data were analyzed using the comparative Ct method and are expressed as mean ± SEM of ADRB1, ADRB2, ADRB3 relative expression, which corresponds to the $2^{-\Delta Ct}$. *P < 0.05; §P < 0.05 ADBR3 vein versus artery.

pathways. Maximal relaxation that was achieved did not exceed 30% of the precontractile stimulus. HUVs and HUAs showed a similar relaxation to forskolin, SNP, PDE inhibitors, and ADRB agonists. In contrast, sGC stimulator BAY 41-2272 was significantly more efficient in relaxing HUVs than HUAs.

The study of relaxant agonists in isolated blood vessels requires the presence of an active tone induced by a contractile agonist (27). A consequence of this fact is that the efficacy of the relaxant agonist will depend on the mechanisms by which the contractile agonists induce its response, and the role of a given mechanism may vary with species, vascular bed, and age (28-32). Thromboxane A2 mimetics (TP receptor agonists), such as U46619 or U44069, and serotonin (5-HT) are frequently used to contract umbilical vessels in vascular reactivity studies because they induce a stable tone (7, 13-16, 33). In addition, both thromboxane A2 and 5-HT have been proposed to play a physiological role in the physiological regulation of umbilical vascular tone (33-35). In the present study, umbilical vessels were contracted with the TP receptor agonist U44069. The signaling pathway for TP receptor-induced vasoconstriction involves a variety of protein kinases such as protein kinase C (PKC) and Rho kinase (29, 30). TP receptor agonists inhibited (*via* PKCζ) voltage-gated K^+ channels (K_V channels), leading to membrane depolarization, activation of L-type Ca^{2+} channels, increase in intracellular Ca^{2+} concentration and contraction (29, 30). In addition, the RhoA/Rho kinase pathway of Ca^{2+} sensitization also contributes to TP receptor agonist-induced contractions in pulmonary and systemic vessels (29, 30). Interestingly, Cogolludo *et al.* showed that the relative contribution of PKCζ-K_V-Ca^{2+} pathway and the RhoA/Rho kinase pathway could vary during development in porcine pulmonary arteries (30). Thus, in newborns, which showed larger K_V currents, the PKCζ-K_V-Ca^{2+} pathway seems to play a major role, whereas in older animals, showing smaller K_V currents and increased expression of RhoA, the RhoA/Rho kinase pathway appears to be the main contributing mechanism (30). The exact contribution of these mechanisms to TP receptor-mediated contraction of human umbilical vessels has not been fully characterized and is beyond the scope of the present study. However, it is possible that some of the differences that we observed between HUAs and HUVs are related to tissue-specific differences in the transduction mechanisms of the contractile and/or relaxant agonists. In addition, it should be noted that the use in our experimental setting of a different contractile agonist, such as 5-HT, could have produced somewhat different results than those reported here. TP receptor agonists and 5-HT share some common transduction mechanisms (29, 30, 36), and even endogenous production of thromboxane A2 is involved in 5-HT-induced contraction in HUAs (34). Nevertheless, transduction mechanisms such as activation of tyrosine kinase, and a caveolae pathway appear to be particularly relevant in 5-HT-mediated contraction (36, 37).

BAY 41-2272 is a drug that directly stimulates sGC and sensitizes it to low levels of bioavailable NO (38-42). Soluble GC consists of an α- and β-subunits, the latter of which contains a binding site for a prosthetic heme moiety (38, 43). Organic nitrates and other nitrovasodilators such as SNP release NO or NO-related compounds that bind to the heme of sGC and thus activate the enzyme (38). In contrast, BAY 41-2272 binds to a regulatory site on α-subunit of sGC and stimulates the enzyme synergistically with NO (38-42). Therefore, endogenous basal production of NO contributes to the relaxation induced by BAY 41-2272. Interestingly, previous studies have shown similar responsiveness to exogenous NO in umbilical arteries and veins but higher endothelial NOS (eNOS) expression and/or activity in HUVs when compared with HUAs (7, 44-47). However, a higher release of endothelium-derived NO was not the underlying mechanism for the higher relaxant efficacy of BAY 41-2272 in our HUVs, since the relaxation was not affected by endothelium denudation. An alternative explanation may rely on the endogenous production of NO by the neuronal NOS (nNOS), which is expressed in HUVs but not in HUAs (48). Nevertheless, BAY 41-2272-induced relaxation was not affected by the presence of the non-specific NOS inhibitor L-NAME. Altogether, our results suggest that a higher endogenous NO production, either by eNOS or nNOS, was not responsible for the higher relaxant efficacy of BAY 41-2272 in HUVs when compared with HUAs. In addition, several studies showed a cGMP-independent component of the vascular effects of BAY 41-2272 (40, 49-51). These cGMP-independent mechanisms include cAMP activation (51), stimulation of the Na^+-K^+-ATPase (51), or blockade of Ca^{2+} influx (40) and remain to be investigated in umbilical vessels.

Cyclic nucleotides cAMP and cGMP are degraded by a family of enzymes known as phosphodiesterases (PDEs) (52, 53). Eleven different PDE families are currently known to be expressed in mammalian tissues. PDEs families differ in their regulation and substrate specificity (cAMP or cGMP) (52-54). Depending on the species, the main PDEs present in VSMCs are PDE1, PDE3, PDE4, and PDE5 (20, 21, 31). PDE1 is a Ca^{2+}-calmodulin-activated PDE which hydrolyzes cAMP and cGMP (52, 54). PDE3 is a cGMP-inhibited PDE which hydrolyzes cAMP with a rate 10-fold greater than cGMP hydrolysis. PDE4 selectively hydrolyzes cAMP, whereas PDE5 selectively hydrolyzes cGMP with high affinity (52-54). Expression and activity of PDE1, PDE3, PDE4, and PDE5 in human umbilical vessels were previously demonstrated (35, 55, 56).

Moreover, PDE5-inhibitor sildenafil is one of the most promising approaches to treat fetal growth restriction and preeclampsia (54, 57). Our study is the first comparing the relaxant effects of PDE inhibitors in HUAs and HUVs. We did not observe any differences between arteries and veins on the relaxant efficacy of PDE3-inhibitor milrinone, PDE4 inhibitor rolipram, or PDE5-inhibitor sildenafil. We did not observe any differences between arteries and veins on the relaxant efficacy of the PDE3 inhibitor milrinone, the PDE4 inhibitor rolipram, or the PDE5 inhibitor sildenafil. Of note, milrinone and rolipram were more efficacious than sildenafil in relaxing umbilical vessels. This suggests a higher activity of cAMP- than of cGMP-degrading PDEs in the umbilical circulation and warrants further investigation.

Classically, the final response of blood vessels to catecholamines was thought to depend on the relative expression levels of vasoconstrictive α1-adrenergic receptors (ADRA1) and vasorelaxant ADRB2 in the VSMCs and effectiveness of their signaling pathways (58, 59). However, it is now accepted that three ADRB subtypes, β_1, β_2, and β_3, are expressed in vascular endothelium and VSMCs, managing vascular relaxation (58, 59). In human umbilical and placental vessels, previous studies have demonstrated that ADRB-induced relaxation is mediated by a mixed population of ADRB2 and ADRB3 (10, 59-62). In the present study, we observed that the relaxant efficacy of ADRB2 and ADRB3 agonists is similar in HUAs and HUVs. However, it should be taken into account that ADRB agonists elicited weak relaxations in HUAs and HUVs.

As mentioned in the introduction, the adenylate cyclase/cAMP pathway is considered the main mechanism for ADBR-mediated relaxation (23, 63-65). Nevertheless, our study confirmed previous findings on the role of the NO/cGMP pathway in the vascular relaxation mediated by ADRB agonists (10, 59, 61, 66). Removal of endothelium or the presence of inhibitors of NOS (L-NAME) or sGC (ODQ) markedly impaired the relaxation induced by ADRB agonists in HUAs and HUVs, suggesting that at least part of the relaxation is mediated by endothelium-derived NO. In contrast to ADRB-mediated relaxation, the relaxation mediated by the adenylate cyclase activator forskolin was not affected by endothelium denudation or the presence of L-NAME. Interestingly, previous studies showed that both ADRB agonists and forskolin enhanced NOS activity and cGMP accumulation in HUVECs (10, 67). Nevertheless, our results suggest that this endothelial effect of forskolin did not affect its relaxant activity in umbilical vessels. In contrast, there is evidence from other vascular tissues, such as rat aorta, that forskolin elicits an endothelium-dependent relaxation (68, 69). This endothelium-dependent component of forskolin/cAMP-induced relaxation appears to be partially mediated by an increase in endothelial NO release due to an enhanced eNOS activity through PKA activation in endothelial cells (69).

The main mechanism involved in cyclic nucleotide-mediated relaxation is the activation of cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively). Interestingly, Santos Silva *et al.* showed that the relaxation induced by cAMP and cGMP in HUAs was not totally mediated by the activation of their respective kinases, and they suggest the participation of PKA- and PKG-independent mechanisms as described in other vascular tissues (70, 71). Notably, Kim *et al.* demonstrated that NO production and NO/sGC/cGMP/PKG pathway is stimulated by activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway both in HUVECs and rat thoracic aorta rings, relaxed by aqueous extract of Mantidis oothecal (72). Moreover, PI3K/Akt pathway has been involved in ADRB-mediated vascular relaxation and NO formation (67, 73). We tested the effect of the inhibition of PKG and PKA on ADRB-mediated relaxation. PKA and PKG inhibitors impaired ADBR1-mediated relaxation but did not affect ADRB2-mediated relaxation. ADRB3-mediated relaxation was impaired by PKG inhibition in HUAs and by PKA inhibition in HUVs. These results are difficult to interpret because, in our experimental setting, ADRB2 and ARB3 agonists induced very weak relaxations, and there was a high inter-experiment variability. Therefore, additional experiments are required to further elucidate the different mechanisms involved in ADRB-mediated relaxation in human umbilical vessels.

Our immunohistochemistry study confirmed that ADBR1 and ADBR2 proteins are expressed in the endothelium and VSMCs of HUA and HUVs. In contrast, immunohistochemistry could not demonstrate any expression of ADBR3 protein. Nonetheless, we observed that umbilical vessels expressed *ADRB3* mRNA, and, in some samples, a low level of ADRB3 protein was detected in Western blot analysis. However, this mRNA expression was markedly lower than the mRNA expression of *ADRB1* and *ADRB2* in HUVEC and the mRNA expression of *ADRB1* in HUAEC. Previous studies have not found a reduced expression of *ADRB3* mRNA in human umbilical or placental vessels (62, 74). We have not an explanation for the low *ADRB3* expression and the lack of correlation with the functional experiments since we observed that HUAs and HUVs relaxed to the ADRB3 agonist BRL 37344 and this relaxation was markedly impaired by the presence of the ADRB3 antagonist SR 59230A. Moreover, BRL 37344-induced relaxation was not affected by the simultaneous presence of atenolol and ICI 118,551, suggesting the lack of involvement of ADRB1 or ADBR2. Altogether our data suggest that ADRBs are present and/or functional in HUAs and HUVs, but their possible role in the control of umbilical circulation seems to be limited by the low relaxant efficacy.

In conclusion, our study confirmed the low relaxing capacity of HUAs and HUVs from term infants. It has been speculated that this low relaxing capacity of umbilical vessels has physiological implications (5-7). An inappropriate or excessive relaxant response to physiological agents may increase umbilical blood flow to placenta and therefore shunt the blood away from the fetal body (8, 75). In addition, maintenance of a high vasomotor tone is also considered as an essential factor for support of placental circulation on the fetal side during uterine contractions (8). Previous studies show that the contractile phenotype of umbilical vessels undergoes marked developmental changes during the last trimester of gestation and suggest that the sensitivity of umbilical vessels to relaxant agents decrease with the progress of gestation (76, 77). Therefore, whether the umbilical vessels of preterm infants show a different pattern of cGMP- or cAMP-mediated relaxation warrants further investigation.

Authors' contributions: G.C., L.P., G.R., I.A., S.G., A.T., and A.G. performed functional experiments. D.L. and A.T. performed HUVEC and HUAEC culture, RNA extraction, reverse transcription, and RT-qPCR. G.E. performed Immunohistochemistry. G.C., L.P., G.R., D.L., A.G., and E.V. conceived and wrote the manuscript. All authors contributed to the revision of the manuscript and approved the final draft.

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