Vascular Pharmacology 83 (2016) 66–77

Contents lists available at ScienceDirect

Vascular Pharmacology

journal homepage: www.elsevier.com/locate/vph

TRPV3 expression and vasodilator function in isolated uterine radial arteries from non-pregnant and pregnant rats

Timothy V. Murphy [a,*], Arjna Kanagarajah [a], Sianne Toemoe [a], Paul P. Bertrand [a], T. Hilton Grayson [a], Fiona C. Britton [a], Leo Leader [b], Sevvandi Senadheera [a], Shaun L. Sandow [a,c]

[a] Department of Physiology, School of Medical Sciences, University of New South Wales, Sydney, NSW 2052, Australia
[b] School of Women's and Children's Health, University of New South Wales, Sydney, NSW 2052, Australia
[c] Inflammation and Healing Cluster, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore, QLD 4558, Australia

ARTICLE INFO

Article history:
Received 21 March 2016
Accepted 4 April 2016
Available online 9 April 2016

Keywords:
TRPV3
Carvacrol
Uterus
Radial artery
Pregnancy

ABSTRACT

This study investigated the expression and function of transient receptor potential vanilloid type-3 ion channels (TRPV3) in uterine radial arteries isolated from non-pregnant and twenty-day pregnant rats. Immunohistochemistry (IHC) suggested TRPV3 is primarily localized to the smooth muscle in arteries from both non-pregnant and pregnant rats. IHC using C′ targeted antibody, and qPCR of TRPV3 mRNA, suggested pregnancy increased arterial TRPV3 expression. The TRPV3 activator carvacrol caused endothelium-independent dilation of phenylephrine-constricted radial arteries, with no difference between vessels from non-pregnant and pregnant animals. Carvacrol-induced dilation was reduced by the TRPV3-blockers isopentenyl pyrophosphate and ruthenium red, but not by the TRPA1 or TRPV4 inhibitors HC-030031 or HC-067047, respectively. In radial arteries from non-pregnant rats only, inhibition of NOS and sGC, or PKG, enhanced carvacrol-mediated vasodilation. Carvacrol-induced dilation of arteries from both non-pregnant and pregnant rats was prevented by the IK_{Ca} blocker TRAM-34. TRPV3 caused an endothelium-independent, IK_{Ca}-mediated dilation of the uterine radial artery. NO-PKG-mediated modulation of TRPV3 activity is lost in pregnancy, but this did not alter the response to carvacrol.

1. Introduction

Intracellular $[Ca^{2+}]$ and Ca^{2+} entry into the endothelial and smooth muscle cells of arteries are vital in regulating arterial diameter. In addition, changes in cellular Ca^{2+} handling occur during vascular cell growth and proliferation, and in arterial remodelling which occurs during pregnancy and disease. In the endothelium, nitric oxide synthase (NOS) activity and endothelium-derived hyperpolarization (EDH) and subsequent relaxation of the overlying smooth muscle depend upon an increase in endothelial cell intracellular $[Ca^{2+}]$ [1]. The key role of voltage-sensitive Ca^{2+} channels in vascular smooth muscle cell function is established and knowledge of other pathways for Ca^{2+} entry into smooth muscle and endothelial cells is increasing [1,2]. The emergence of the transient receptor potential (TRP) super-family of voltage-insensitive cation channels has allowed identification of new mechanisms for controlling Ca^{2+} entry and homeostasis in vascular cells [3]. The physiological roles of the various TRP channel subtypes remain to be fully established, but evidence is emerging that such channels play a vital role in several and varied vascular functions. For example, members of the TRPC (canonical) class including TRPC1, TRPC3 and TRPC6 mediate Ca^{2+} entry into both smooth muscle and endothelial cells induced by Ca^{2+} store depletion or G-protein-coupled receptors [3], whilst other studies suggest that TRPC and TRPV4 (vanilloid type-4) are mediators of endothelium-dependent vasodilation stimulated by G-protein-coupled receptors and the shear-stress of blood flow [3–5].

The TRPV family offer an interesting target for investigation due to their sensitivity to vanilloid compounds including capsaicin, resiniferatoxin, camphor and eugenol, suggesting a range of selective ligands [6]. TRPV are also osmo- and thermo-sensitive [7]. Of the six TRPV channels present in mammals, TRPV1–4 have been identified in the vasculature, but relatively few studies have examined potential vascular roles of TRPV3 compared to TRPV1, V2 and V4 [7,8]. Like TRPV4, vascular TRPV3 may also stimulate vasodilation, although very few studies have examined this potential outside cerebral arteries [7,9,10]. Indeed remarkably little is known or understood regarding the function of vascular TRPV3.

Abbreviations: TRPV3, transient receptor potential vanilloid type-3 channel; IHC, immunohistochemistry; NO, nitric oxide; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase; PKG, cGMP-dependent protein kinase; BK_{Ca}, IK_{Ca}, SK_{Ca}, large-, intermediate- and small-conductance Ca^{2+}-activated K^+ channel (respectively); L-NAME, Nω-nitro-L-arginine methyl ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PE, phenylephrine.

* Corresponding author.
E-mail address: tim.murphy@unsw.edu.au (T.V. Murphy).

http://dx.doi.org/10.1016/j.vph.2016.04.004
1537-1891/

TRPV3 is a generally non-selective cation channel, but does display moderately selective permeability for Ca^{2+} over Na^{+} [11]. Utilising the TRPV3 activator carvacrol, the current study examined the expression, distribution and function of TRPV3 in uterine radial arteries from age-matched non-pregnant and late (20-day) pregnant rats. Uterine radial vessels undergo extensive alteration in pregnancy, with changes in Ca^{2+} signalling fundamental to such adaptations [12–14]. Previous studies from our laboratory identified a role for TRPV4 channels in modulating uterine artery function during pregnancy [14]. Therefore, the hypothesis of the present study was that changes in the expression of the related TRPV3 contributed to pregnancy-induced functional changes in uterine radial arteries.

2. Materials and methods

2.1. Animals and tissue collection

Female non-pregnant Sprague–Dawley rats (280 ± 8 g; n = 18) in the oestrus of their cycle were used along with age-matched 20 day pregnant rats (428 ± 13 g; n = 17; noting that full gestation in rats is 21–22 days). Rats were anaesthetised using sodium thiopentone (100 mg/kg, i.p.) and third-order radial arteries collected as described previously [14]. All dissections were performed in dissection buffer composed of (concentrations in mM): 3 MOPS, 145 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 0.02 EDTA, 2 pyruvate, 5 D-glucose and 1% BSA. All studies were performed in accordance with the guidelines of the National Health and Medical Research Council of Australia and the UNSW Animal Ethics and Experimentation Committee (AEEC #09/43B).

2.2. Immunohistochemistry

Arteries were fixed in 2% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) for 10 min. After being washed, tissues were incubated at room temperature for 2 h in blocking buffer (1% BSA and 0.2% Triton in PBS), washed and incubated overnight with the primary antibody at 4 °C. The primary antibodies for TRPV3 were rabbit anti-human N′ (1:100; Santa Cruz (H-150), sc-50414, lot #E2507) or goat anti-human C′ (1:100; Santa Cruz (K-16), sc-23372, lot#B1907), and IK_{Ca} as rabbit anti-human to N′ amino acids 2–17 (IK1, 1:100, Chen, M20; see [15]). The tissue was washed again and incubated with the secondary antibody for 2 h at room temperature. Anti-rabbit IgG Alexa Fluor 633 (1:200; Molecular Probes/Invitrogen, A21070; lot #50728A) and anti-goat IgG Alexa Fluor 633 (1:200; Molecular Probes/Invitrogen, A21082; lot #399683) were used as secondary antibodies. Following final washing the tissue was mounted on slides in anti-fade mounting media, with the media of selected preparations also containing 0.002% propidium iodide to clarify cell layer patency. An FV1000 confocal microscope (Olympus, North Ryde, Australia) was used to examine the tissue under uniform settings. Controls involved peptide block of primary antibody in a 1:10 (v/v) excess of the immunizing peptide.

For the estimation of TRPV3 and IK_{Ca} channel density, the fluorescence level of confocal signals was determined using CellR software (Olympus). The mean fluorescence density of 4 randomly selected 100 mm^2 regions of interest, each of 4 different preparations from a different animal, was determined; with secondary only taken as baseline fluorescence. Control images for TRPV3 and IK1 antibodies are shown in the Supplementary Fig. A

2.3. Western blot

Pooled third-order radial arteries from non-pregnant and pregnant rats (arteries from 4 animals per sample) and corneal tissue from non-pregnant rats were ground in liquid nitrogen and resuspended in PBS (pH 7.4) containing a protease inhibitor cocktail (Roche, Castle Hill, NSW). This was centrifuged at 3000*g* at 4 °C for 5 min. The supernatant was collected, and cooled on ice. The pellet was snap frozen in liquid nitrogen. This process was then repeated and the supernatants combined and centrifuged at 25,000*g* at 4 °C for 1 h. The resulting pellet was resuspended in PBS containing 0.1% Triton X-100 and protease inhibitor cocktail, aliquoted, snap frozen and stored at −80 °C. Bradford assay (Bio-Rad, Gladesville, NSW) was used to determine protein concentration in samples. Lysates of HEK293T cells overexpressing TRPV3 cDNA (containing an in-frame FLAG epitope) and control HEK293T cells containing empty vector were purchased from OriGene (Rockville, MD, USA).

Protein extracts in lithium dodecyl sulphate (LDS) sample buffer (Life Technologies, Mulgrave, VIC) were heated at 70 °C for 10 min, electrophoresed on Bis-Tris denaturing polyacrylamide gels (Life Technologies) and electro-blotted onto polyvinylidene difluoride (PVDF) membranes overnight at 4 °C. Blots were blocked for 2 h with Invitrogen purified casein Tween 20 blocker, and incubated with primary antibody at 4 °C overnight. Primary antibodies were TRPV3 N′ and C′ (as above; 1:1000) and mouse monoclonal anti-FLAG (F3165, Sigma; 1:2000). Following subsequent washes in TNT buffer, membranes were incubated with appropriate secondary antibodies either conjugated with alkaline phosphatase or horseradish peroxidase, (1:5000 in 5% milk-TNT), for 2 h at 4 °C. Antibody binding was visualised either by chemiluminescence or using NBT/BCIP reagent (Pierce Biotechnology, Rockford, IL, USA). Blots were stripped and re-probed with rabbit anti-actin antibody (1:1000; A2066, Sigma). Digital densitometry was used to quantify TRPV3 band intensities relative to the intensity of actin staining.

2.4. Quantitative and analytical RT-PCR

Radial arteries from pregnant or non-pregnant animals (n = 5 for each) were pooled for RNA isolation. Total RNA was extracted from homogenized vessels using RNeasy Micro Kit (Qiagen, Doncaster, VIC). RNA quantity and purity were measured by 260/280 nm absorbance using a NanoDrop™ ND-1000 spectrophotometer (ThermoFisher Scientific, Mulgrave, VIC). RNA samples (1 μg each) were reverse transcribed using the VILO™ cDNA reaction kit (Invitrogen, Mulgrave, VIC) in 20 μL reactions using an ABI 2720® thermal cycler (Applied Biosystems, Mulgrave, VIC) at 25 °C for 10 min, 42 °C for 60 min, 85 °C for 5 min. Following reverse transcription, cDNA samples were diluted 5 fold for quantitative analysis. cDNA quality was evaluated by PCR amplification of glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

Real-time PCR reactions were performed on a Mastercycler realplex^2 (Eppendorf, North Ryde, Australia) using pre-designed FAM™ or VIC® dye-labelled TaqMan Gene Expression assays (Applied Biosystems, Mulgrave, VIC) optimized for the detection of rat TRPV3 (assay ID: Rn01460303_m1) and 18S ribosomal RNA (assay ID: Rn03928990_g1). Reactions were 20 μL volumes, consisting of TaqMan Universal PCR master mix, specific TaqMan assay and 2 uL cDNA. Each assay was run in triplicate and repeated in a duplicate PCR experiment. Control PCRs were carried out substituting RNase-free water. RT-PCR was also performed using primer set specific for rat TRPV3 mRNA [GenBank: NM_001025757]. Details of the primer sets used are provided in Table 2. PCR was performed using AmpliTaq Gold® PCR reagent (Applied Biosystems, Mulgrave, Australia) with cDNA from pregnant and non-pregnant animals (see above). PCR reactions included rat kidney cDNA as a control for primer efficiency and a template-free negative control. Reactions were performed in a ABI 2720® thermal cycler consisting of 95 °C for 10 min, then 35 cycles of 95 °C for 15 s, 55 °C for 25 s and 72 °C for 25 s, followed by a final step at 72 °C for 3 min. PCR products were resolved on 2% agarose gels. TRPV3 amplification products were purified on QIAquick columns (Qiagen, Doncaster, VIC) and subsequently sequenced at the Garvan Institute Molecular Genetics facility, Sydney. Nucleotide sequences were analysed using Vector NTI v.11 software (Invitrogen, Mulgrave, VIC) and BLASTN v.11 software [http://www.ncbi.nlm.nih.gov/BLAST/].