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Pannexin 1 facilitates arterial relaxation via an endothelium-derived hyperpolarization mechanism

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ABSTRACT

Pannexin 1 (Panx1) is involved in endothelium-dependent vasodilation in large arteries, but the exact mechanistic role remains poorly understood. We hypothesized that Panx1 facilitates large vessel relaxations regulating endothelium-derived hyperpolarization (EDH)-like mechanisms. The EDH-like component of acetylcholine-induced relaxation of saphenous arteries studied in isometric myograph after inhibition of NO-synthase and cyclooxygenase was significantly impaired in mice with genetically ablated Panx1 (KO) relative to that in the wild type (WT) mice. Application of P1-receptor antagonist and apyrase significantly reduced this component in WT, but not in KO mice, indicating participation of ATP released via Panx1 in the EDH-like relaxation.
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1. Introduction

Recently characterized membrane channel proteins pannexins were shown to participate in numerous physiological and pathological processes [18]. Pannexin family has three members: Panx1, Panx2 and Panx3. Panx1 is represented ubiquitously in mammalian tissues, including brain, heart, skeletal muscles and vasculature. In murine vasculature, the pattern of Panx1 expression and its impact on vascular function depend on the vessel size. Panx1 is abundant in the smooth muscle cells in smaller arteries and arterioles [14]. In such arteries the release of ATP via Panx1 hemichannels was shown to be involved in the incremental contractile response during adrenoceptor activation [1]. Conversely, we have shown that in the saphenous artery (a larger resistance-type vessel) Panx1 is expressed predominantly in the endothelium and Panx1$^{-/-}$ mice lacking this channel have significantly impaired endothelial function [9]. However, the mechanistic understanding of the role of Panx1 in endothelium-dependent relaxation remains to be uncovered.

Currently, three main pathways of endothelium-dependent relaxation are characterized, including those mediated by nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarization (EDH). In contrast to well-defined NO and PGI_2 pathways, the molecular constituents of the EDH mechanism remain controversial. Initially it was described as a factor responsible for smooth muscle hyperpolarization independent of NO or prostacyclin [7]. Currently, a number of diverse factors are suggested to play a role in EDH, including K^+, H_2O_2 and adenosine among them [7,16]. However, regardless of these specifics, the initial step of EDH is the hyperpolarization of endothelial cells following the activation of specific for endothelial cells intermediate (IK_{Ca}) and small conductance (SK_{Ca}) calcium-activated potassium

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channels [7]. This signal is then transmitted to smooth muscle cells, thus leading to relaxation. The influence of EDH-like mechanisms on the arterial dilation can, therefore, be evaluated by the blockade of IK_{Ca} and SK_{Ca} channels.

In this study we focused on the role of Panx1 in EDH mechanisms for the following reasons. First, pannexins are known to form ATP-permeable hemichannels [4,18]. ATP released from endothelial cells can cause vasorelaxation in an EDH-like manner [12]. In addition, ectonucleotidases may hydrolyze ATP to adenosine, the latter demonstrating EDH-like effects in human coronary arteries [16]. Finally, hyperpolarization signal may be conducted from endothelial to smooth muscle cells via myoendothelial gap junctions [5,7]. Notably, pannexins were shown to form functional gap junctions in some cell types [2,11,20,22]. Therefore, it is feasible to suggest that Panx1-formed hemichannels or gap junctions can participate in the EDH-like relaxation. We therefore hypothesized, that Panx1 facilitates endothelium-dependent relaxations via regulation of one or several EDH-mechanisms.

2. Materials and methods

2.1. Animals

The $Panx1^{-/-}$ mouse strain was described previously [6]. All experiments in this study were performed in full compliance with the NIH Guide for the Care and Use of Laboratory Animals and Russian national guidelines for animal research. The protocols were approved by the University of Miami IACUC (protocol #12-051) and Institute for Information Transmission Problems, Russian Academy of Sciences IACUC (protocol #02-2013). Wild type (WT) animals were age-matched (2-3 months old) male mice of the C57BL/6 background. Mice were housed under standard conditions of temperature and humidity, with a 12-h light/dark cycle and free access to food and water.

2.2. Wire myography

We studied the responses of the saphenous artery for which it was previously shown that its relaxation caused by ACh is partially dependent on EDH-mechanisms [10]. For force recording, 2-mm arterial ring preparations were isolated and mounted in wire myograph (DMT, Denmark, Model 620 M). Physiological salt solution contained (in mM): 120 NaCl, 26 NaHCO, 4.5 KCl, 1.6 $CaCl_2$, 1 $MgSO_4$, 1.2 NaH_2PO_4, 5.5 D-glucose, 0.025 EDTA and 5 HEPES was used throughout the experiment. The vessels were stretched to an internal circumference at which they developed maximal active tension [15] and kept at 37 °C, bubbled with a gas mixture (95% O_2, 5% CO_2) for maintaining pH 7.4. Arteries were activated 2 times with 10 μM norepinephrine and the functional activity of the endothelium was checked subsequently using 10 μM acetylcholine (ACh) during 1–3 μM norepinephrine-induced precontraction. Endothelium-dependent relaxation was studied during cumulative addition of ACh in rising concentrations from 0.01 μM to 10 μM after phenylephrine-induced precontraction that averaged 60% of maximal vessel response to norepinephrine. Such level of precontraction was achieved with phenylephrine concentrations ranged from 0.3 μM to 1 μM in both WT and $Panx1^{-/-}$ animals.

2.3. Gene expression

Gene expression levels were measured by quantitative PCR, performed in Rotor Gene 6000 (Corbett Research, Australia) with SYBR Green master mix (Syntol, Russia) and the following protocol: 95 °C for 10 min, followed by 40 cycles, consisting of three phases: 30 s at 95 °C, 30 s at 62 °C (for Panx2) or 60 °C (for Panx3, Cx43, Cx40 and Cx45) or 64 °C (for Cx37 and GAPDH) and 60 s at 72 °C. Primer sequences (5′-3′) for Panx2: forward TCACC AAGAACTTCGCAGAGGA, reverse GGAAGTTGAGCTCCGAGGTGA; for Panx3: forward CAGGAGTTCTCATCAGGGTCT, reverse AGAGA GTAGGGAAGAGCCTTGT; for connexin 37: forward CGGGCAAGCA GGCGAGAGAG, reverse CCGGCTGGGCTGTGTTACACT; for connexin 43: forward CACTTTCATTAAGTGAAAGAGAGGT, reverse GGGTTGTT GAGTGTTACAGCGA; for GAPDH (reference gene): forward ATGGA GAAGGCCGGGGCCCA, reverse GATGGCATGGACTGTGGTCATG; for connexin 40: forward GGAGGAGGAAAGGAAGCAGGTG, reverse GCCCAGGACCAGCATGCGGA; for connexin 45: forward CAATCG AGGAACTCAAGAGAGAAT, reverse ATAGCATATCCCAGGTACATCA CA. To avoid possible amplification from genomic DNA, primers were designed to span the intron and to continue to the adjacent exon by 3 nucleotides on the 3′ prime end.

2.4. Immunohistochemistry

After transluminal perfusion by 4% paraformaldehyde in PBS and saphenous arteries were dissected, cleaned from adjacent tissues and cut into 0.5–1.0 mm long segments. Staining was performed in whole mounts. In brief, the segments were permeabilized by 0.5% Triton X-100 in PBS for 1 h and incubated in blocking buffer with 5% rabbit serum, 5% BSA, 0.3% Triton X-100 in PBS for 3 h with shaking at room temperature. Staining with primary anti-mouse Panx1 CT-395 antibodies ([17]; 1:800 dilution) was performed at room temperature in blocking solution overnight with shaking to ensure luminal penetration of antibodies. After washing, secondary goat anti-rabbit Alexa-546 conjugated antibodies were added and incubated overnight in the same conditions. After washing and incubation with DAPI, arterial segments were mounted on slides, coverslipped without longitudinal cutting (resulting in flattened samples) and imaged at Leica SP5 confocal microscope using ×40 objective.

2.5. Drugs

Acetylcholine, phenylephrine, 8-(p-sulfophenyl)-theophylline (8-SPT; 100 μM), apyrase (10 U/ml), carbenoxolone (100 μM), $BaCl_2$ (30 μM), indomethacin (10 μM) and TRAM-34 (1 μM) were obtained from Sigma. L-NNA (100 μM) was obtained from Alexis Biochemicals, UCL-1684 (0.1 μM) from Tocris and ouabain (10 μM) from MP Biomedicals. The numbers in brackets show the concentrations of substances used in the experiments.

2.6. Statistical analysis

All data are expressed as means ± S.E.M.; n represents the number of animals tested. The differences between concentration–response relationships to ACh were evaluated using Repeated Measures ANOVA, followed by Bonferroni correction for repeated measurements to define the effects of inhibitors at certain ACh concentrations. In addition, individual concentration–response relationships were fitted to a sigmoidal dose–response with variable slope using GraphPad Prizm 5.0 Software (La Jolla, CA, USA) for calculation of pD_2 (the negative logarithm of the EC_{50} value) and E_{max} (maximal response value). Statistically significant differences for pD_2 and maximal response values, as well as for relative mRNA expression were determined using unpaired Student's t-test. Statistical significance was reached at $P < 0.05$.

3. Results

3.1. EDH-like component of relaxation is impaired in $Panx1^{-/-}$ mice

In line with our previous findings [9], endothelium-dependent relaxations to ACh were impaired in arteries of $Panx1^{-/-}$ mice in

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