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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.

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

Authors contributions: DG, VIS, YP and OST planned the project, performed experiments, data evaluation, interpretation and manuscript preparation. VIS generated the mouse model. All authors read and approved the final manuscript.

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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

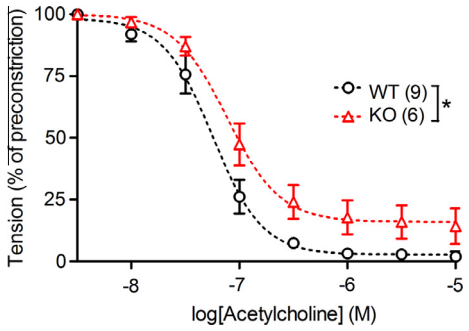

Fig. 1. Concentration–response relationships to ACh in saphenous arteries from WT and Panx1$^{-/-}$ mice. $^{*}P < 0.05$. Numbers in brackets indicate the number of experiments. Error bars for some points are smaller than the symbols.

comparison to WT animals (Fig. 1 and Table 1). We showed as well that Panx1 mRNA localizes predominantly to endothelial cells of the murine saphenous artery [9]. The presence of Panx1 in endothelium was also confirmed by immunohistochemistry in saphenous artery whole mounts (Fig. 2). The Panx1-specific staining was absent in similar preparations from Panx1$^{-/-}$ arteries (Fig. 2). Importantly, genetic ablation of Panx1 did not cause compensatory up-regulation of Panx2 or Panx3 expression in saphenous arteries. Panx2 and Panx3 mRNAs were not found by quantitative PCR in saphenous arteries of either WT or Panx1$^{-/-}$ mice although they were detected in murine brain samples (Panx2) and cartilaginous tissue samples from the ear auricle (Panx3), validating primer functionality.

Next, to explore the role of Panx1 in the endothelium-dependent relaxation, we compared the effects of the combined inhibition of NO and PGI_2 synthesis in WT and Panx1$^{-/-}$ mice. Treatment of the arteries with L-NNA and indomethacin, NO-synthase and cyclooxygenase inhibitors, respectively, has revealed that the combined contribution of NO and PGI_2 to ACh-induced relaxation was greater in Panx1$^{-/-}$ as compared to WT mice (Fig. 3A,B and Table 1).

The part of ACh-induced relaxation resistant to inhibition of NO and PGI_2 synthesis reflects the contribution of EDH-like mechanisms to the endothelium-dependent relaxation. To compare these components in the Panx1$^{-/-}$ and WT mice, we utilized the blockers of endothelial IK_{Ca} and $SK_{Ca,}$ channels TRAM-34 and UCL-1684, respectively. The combined blockade of IK_{Ca} and SK_{Ca} in the presence of L-NNA and indomethacin further reduced ACh-induced relaxations in both WT and Panx1$^{-/-}$ mice (Fig. 3A,B and Table 1). Intriguingly, the portion of the ACh-induced relaxation blocked by the combination of TRAM-34 and UCL-1684 was significantly larger in the arteries of WT vs. Panx1$^{-/-}$ mice (Fig. 3A and B). Combined, these data indicate that the EDH-like mechanism in ACh-induced relaxation is impaired in the absence of Panx1. These conclusions were supported by the experiments, studying the effects of IK_{Ca} and SK_{Ca} blockers in the absence of NO-synthase and cyclooxygenase inhibitors. The sensitivity of saphenous artery to ACh (pD_2 value) was significantly reduced by the blockade of IK_{Ca} and SK_{Ca} channels in WT (Fig. 3C and Table 1), but not in Panx1$^{-/-}$ mice (Fig. 3D and Table 1). Altogether, these results demonstrate that the ablation of Panx1 causes significant reduction of the EDH-like component of endothelium-dependent relaxation in the murine saphenous artery.

3.2. Panx1-mediated purinergic signaling underlies the EDH-like mechanism of endothelium-dependent relaxation

The role Panx1 plays in the regulation of the EDH-like component of endothelium-dependent relaxation raises the question about the underlying molecular mechanism(s). Extracellular ATP released via Panx1 may affect vascular tone directly or indirectly, by conversion into adenosine executed by cell surface ecto-ATPases. Adenosine activates P1-receptors on the vascular wall and leads to vasodilation in an EDH-like manner [16]. To examine the involvement of P1 receptors, we tested their blocker 8-SPT in the presence of L-NNA + indomethacin in arteries of WT (Fig. 4A, blue line) and Panx1$^{-/-}$ (Fig. 4B, blue line) mice. The blockade of the P1 receptors slightly decreased the sensitivity for ACh in WT arteries, as indicated by reduced pD_2 value (Table 1), but did not change the sensitivity for ACh in Panx1$^{-/-}$ vessels (Table 1).

The ACh-induced endothelium-dependent relaxation was reported to be partially associated with the activation of P2Y receptors on endothelial cells by ATP, which provokes the EDH-like relaxation of arteries [12]. Given the large diversity of purinergic receptor subtypes [3] and the lack of truly selective inhibitors for many of them, we tested P2 receptor involvement by depleting extracellular ATP with an externally added apyrase enzyme in the presence of P1 receptors blocker. Apyrase treatment in the presence of 8-SPT + L-NNA + indomethacin significantly impaired the endothelium-dependent relaxations of WT arteries (Fig. 4A) by reducing both pD_2 and E_{max} (Table 1), but didn't alter the relaxatory responses of Panx1$^{-/-}$ arteries (Fig. 4B and Table 1). Therefore, the combined treatment with apyrase and P1-receptor blocker considerably decreases the EDH-like component of ACh-induced relaxation in WT but not Panx1$^{-/-}$ animals.

3.3. The effects of EDH-blockade at the smooth muscle level are reduced in Panx1$^{-/-}$ arteries

To cause vasodilation, the hyperpolarization originating in endothelial cells has to be transmitted to the smooth muscles.

Table 1
pD_2 (the negative logarithm of the EC_{50}) and E_{max} (maximal response) values for ACh for saphenous arteries of WT and Panx1$^{-/-}$ mice under control conditions and in the presence of inhibitors.

	WT		Panx1$^{-/-}$	
	pD_2	E_{max}	pD_2	E_{max}
Control (in the absence of inhibitors)	7.28 ± 0.09	97 ± 2	7.12 ± 0.09	85 ± 7$^{\&}$
TRAM-34 + UCL-1684	6.83 ± 0.20*	95 ± 1	7.17 ± 0.06	86 ± 5
L-NNA + Indo	7.22 ± 0.05	65 ± 10*	6.78 ± 0.11*	39 ± 3*
L-NNA + Indo + TRAM-34 + UCL-1684	6.05 ± 0.21$^{\#}$	14 ± 6$^{\#}$	6.26 ± 0.23	26 ± 8
L-NNA + Indo + 8-SPT	6.60 ± 0.13$^{\#}$	74 ± 7	6.63 ± 0.25	30 ± 11
L-NNA + Indo + 8-SPT + Apyrase	6.09 ± 0.10$^{@}$	42 ± 7$^{@}$	6.34 ± 0.20	28 ± 6
L-NNA + Indo + Ba^{2+} + Ouabain	6.00 ± 0.13$^{\#}$	53 ± 12	6.20 ± 0.08$^{\#}$	19 ± 12
L-NNA + Indo + Ba^{2+} + Ouabain + Carb	6.67 ± 0.05$^{\$}$	22 ± 10$^{\$}$	6.08 ± 0.09	18 ± 7

Indo – indometacine; Carb – carbenoxolone. $^{\&}P < 0.05$ *vs.* WT under control conditions; **vs.* control in the respective animal group; $^{\#}$*vs.* L-NNA + Indo in the respective animal group: $^{@}$*vs.* L-NNA + Indo + 8-SPT in the respective animal group; $^{\$}$vs L-NNA + Indo + Ba^{2+}+Ouabain in the respective animal group.

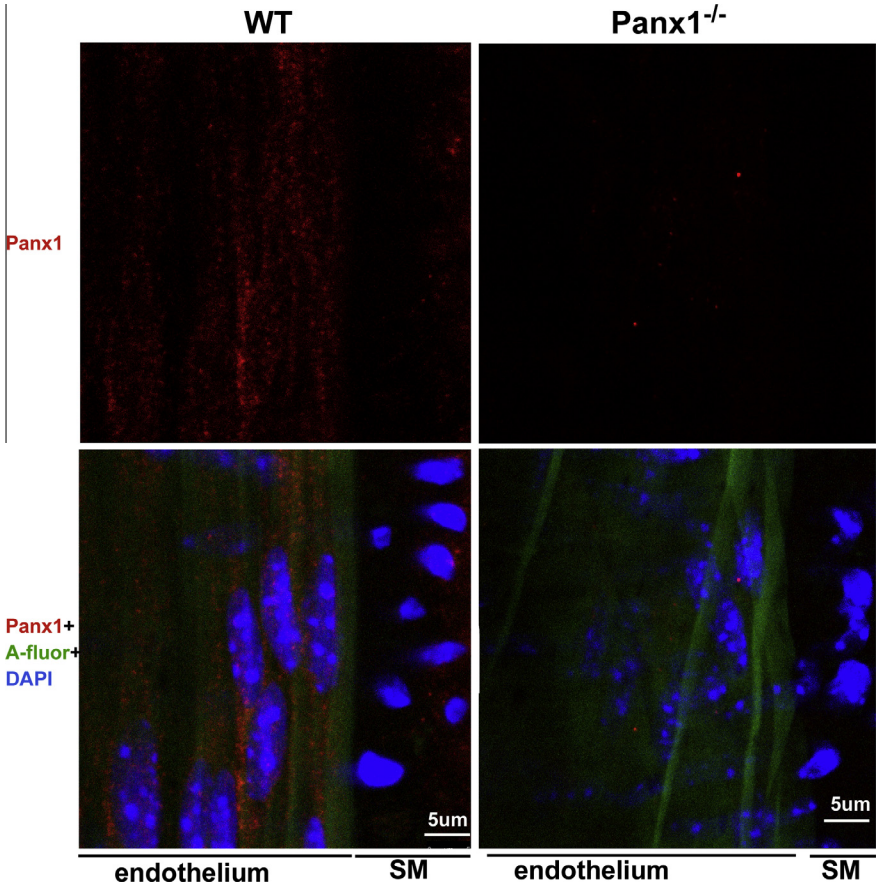

Fig. 2. Panx1 is observed immunohistochemically in the endothelium of saphenous artery in WT mice. Confocal images of merged Z-stacks scanned along the border of endothelium layer fold in a flattened whole mount preparation co-labeled for Panx1 (red) and nucleic acids with DAPI (blue). Autofluorescence of internal elastic lamina (IEL) is shown in green. The thickness of the analyzed section is 5 micron. Punctate Panx1 labeling is abundant in WT arteries and co-localizes with IEL autofluorescence and vertically oriented elliptic-shaped nuclei of endothelial cells. Labeling outside of this region (right side where round-shaped transverse sections of smooth muscle cell nuclei are observed) is significantly reduced. Specific Panx1 labeling is not detected in Panx1$^{-/-}$ arteries. Scale bar: 5 μm.

The conventional mechanism of signal transduction suggests the release of K^+ from endothelial cells via IK_{Ca} channels followed by the activation of Na^+/K^+-ATPase and inwardly rectifying potassium channels (K_{IR}) in the smooth muscles [7,8]. To evaluate the role of this conventional mechanism in EDH-like signal transmission we used ouabain and Ba^{2+}, the blockers of Na^+/K^+-ATPase and K_{IR}, respectively. In the presence of L-NNA and indomethacin, this treatment resulted in a significant decrease in the relaxatory responses to ACh in both WT and Panx1$^{-/-}$ mice (Fig. 5A and B, black lines). The respective change in sensitivity to Ach, however, was smaller in Panx1$^{-/-}$ vs. WT mice (Table 1).

In Panx1$^{-/-}$ animals the EDH-like component of ACh-induced relaxation was almost completely abolished by the combined blockade of Na^+/K^+-ATPase and K_{IR}. In contrast, the part of EDH-like component persisted in WT animals under the same conditions (Fig. 5A and B, black lines). These data suggest the existence of an additional pathway for the EDH-like signal transmission from endothelial to smooth muscle cells in the arteries of WT mice, that is incapacitated in Panx1$^{-/-}$ ones. We also observed, that treatment of arteries with the gap junction blocker carbenoxolone in the presence of ouabain + Ba^{2+} + L-NNA + indomethacin almost completely abolished the relaxatory response to ACh in WT mice, but didn't change the response of Panx1$^{-/-}$ arteries (Fig. 5A,B, blue lines and Table 1). One way to interpret this result is that gap junctions that transmit the EDH-like signal from endothelial to smooth muscle cells in WT animals are lacking in Panx1$^{-/-}$ mice. Importantly, the expression levels of connexin 37 and connexin 43, the isoforms that form myoendothelial gap junctions in the murine saphenous artery [19], did not differ between WT and Panx1$^{-/-}$ arteries (Fig. 5C and D). This result excludes the reduced connexin 37 and 43 expression in Panx1$^{-/-}$ mice as a possible underlying reason for altered EDH-like signal transmission. Connexin 40 expression levels were not different in WT and Panx1$^{-/-}$ arteries (Fig. 5E) and connexin 45 expression level in Panx1$^{-/-}$ arteries was lower compared to WT arteries (Fig. 5F).

4. Discussion

This study shows that endothelium-dependent relaxation mechanisms are significantly altered in the Panx1$^{-/-}$ mice relative to WT arteries. The EDH-like component of ACh-induced relaxation is impaired in the absence of Panx1, as seen from reduced effects of either IK_{Ca} and SK_{Ca} blockers or blockers of Na^+/K^+-ATPase and K_{IR}. The realization of EDH-like component of endothelium-dependent relaxation in WT saphenous arteries is preferably associated with the activation of P2-receptors and, to a lesser degree, with P1-receptors. However, purinergic signaling is greatly suppressed in Panx1$^{-/-}$ arteries, indicating that Panx1-mediated release of ATP augments the EDH-like component of endothelium-dependent dilations in saphenous arteries.

Large reduction of EDH-like relaxation in Panx1$^{-/-}$ mice was associated with up-regulation of other dilatory pathways, such as NO- and PGI_2-signaling. The factors and mechanisms, responsible for compensatory redistribution of endothelial dilatory pathways

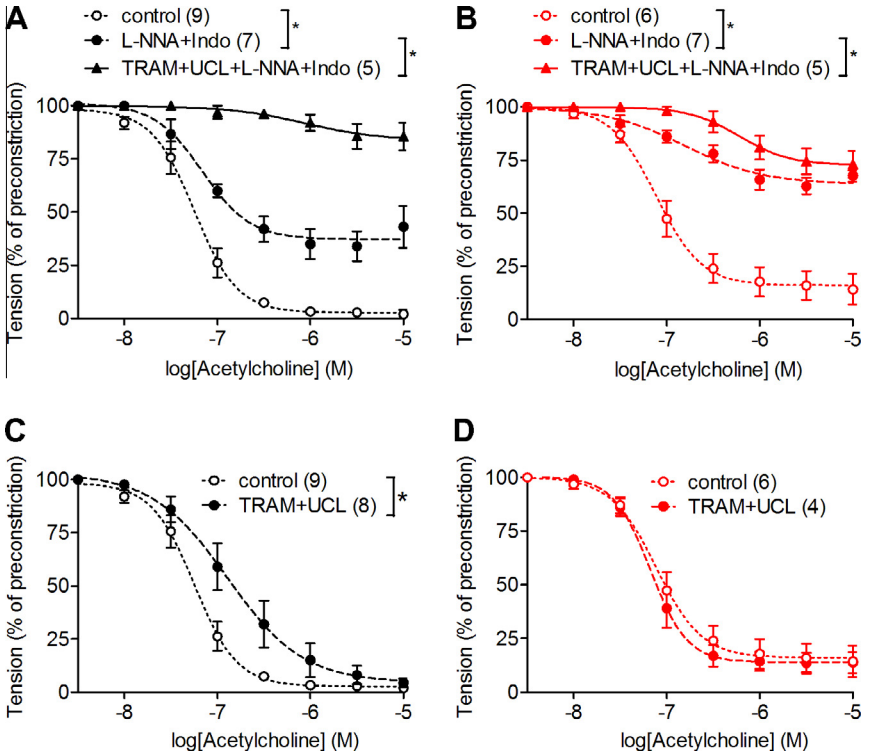

Fig. 3. The effect of Panx1 ablation on the EDH-like component of ACh-induced relaxations of saphenous arteries. (A and B) Concentration–response relationships to ACh of saphenous arteries of WT (A) and Panx1$^{-/-}$ (B) mice in the presence of vehicle (control), or L-NNA + indomethacin or TRAM-34 + UCL-1684 + L-NNA + indomethacin. (C and D) Concentration–response relationships to ACh of saphenous arteries of WT (C) and Panx1$^{-/-}$ (D) mice in the presence of vehicle (control), or TRAM-34 + UCL-1684. *$P < 0.05$. Number in brackets indicates the number of experiments. Error bars for some points are smaller than the symbols.

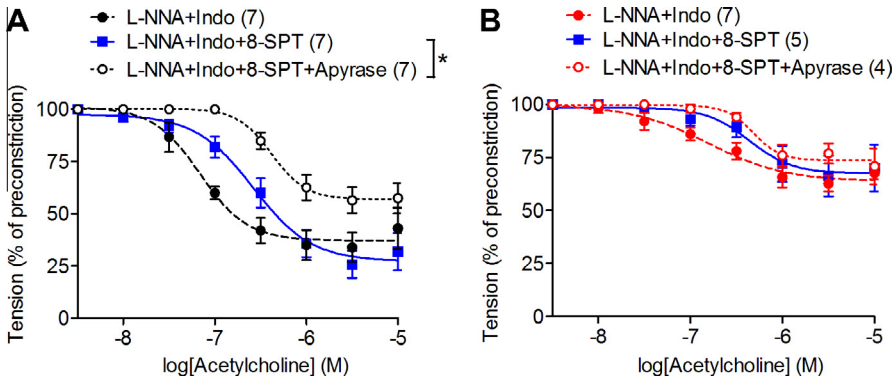

Fig. 4. Purinergic signaling via Panx1-formed hemichannels serves as EDH-like mechanism in murine saphenous artery. Concentration–response relationships to ACh of saphenous arteries of WT (A) and Panx1$^{-/-}$ (B) mice in the presence of L-NNA + indomethacin or 8-SPT + L-NNA + indomethacin or apyrase + 8-SPT + L-NNA + indomethacin. *$P < 0.05$ (repeated measures ANOVA). Number in brackets indicates the number of experiments.

in the absence of functional Panx1 remain to be identified in future studies.

Purinergic signaling (including adenosine and ATP) is responsible for a significant portion of the EDH-like response in saphenous artery, since its inhibition significantly suppressed EDH-like relaxations in WT animals. Consistent with the Panx1 channel function as an ATP-permeable pore in this pathway, the response was smaller and the blockade was not observed in Panx1$^{-/-}$ mice. The increase of cytoplasmic Ca^{2+} concentration in the endothelial cell during ACh stimulation is probably sufficient to cause Panx1 hemichannel activation [13], which allows ATP release into extracellular space. Extracellular ATP can activate P2Y receptors on endothelial cell surfaces, thereby triggering an additional increase in intracellular Ca^{2+} [23] and further potentiating Panx1-hemichannels [13]. An additional mechanism involves adenosine receptor signaling stimulated by the hydrolysis of the released ATP to adenosine that dilates vessels by binding to P1 receptors. Thus, purinergic signaling via Panx1-P2Y pathway may serve as a feedforward mechanism of endothelial activation to provide full realization of EDH-like relaxation. A direct contribution of Panx1-dependent calcium influx in ACh responses is possible as well [22], but the role of this mechanism in mammalian tissues, particularly in the vasculature, has not been explored yet.

Another argument supporting the proposed role of Panx1 in full-manifested endothelium-dependent dilation was obtained by studying the blockade of Na^+/K^+-ATPase and K_{IR} channels. These data demonstrates that the effect of the blockade is significantly reduced in the arteries of Panx1$^{-/-}$ vs WT mice. The part of

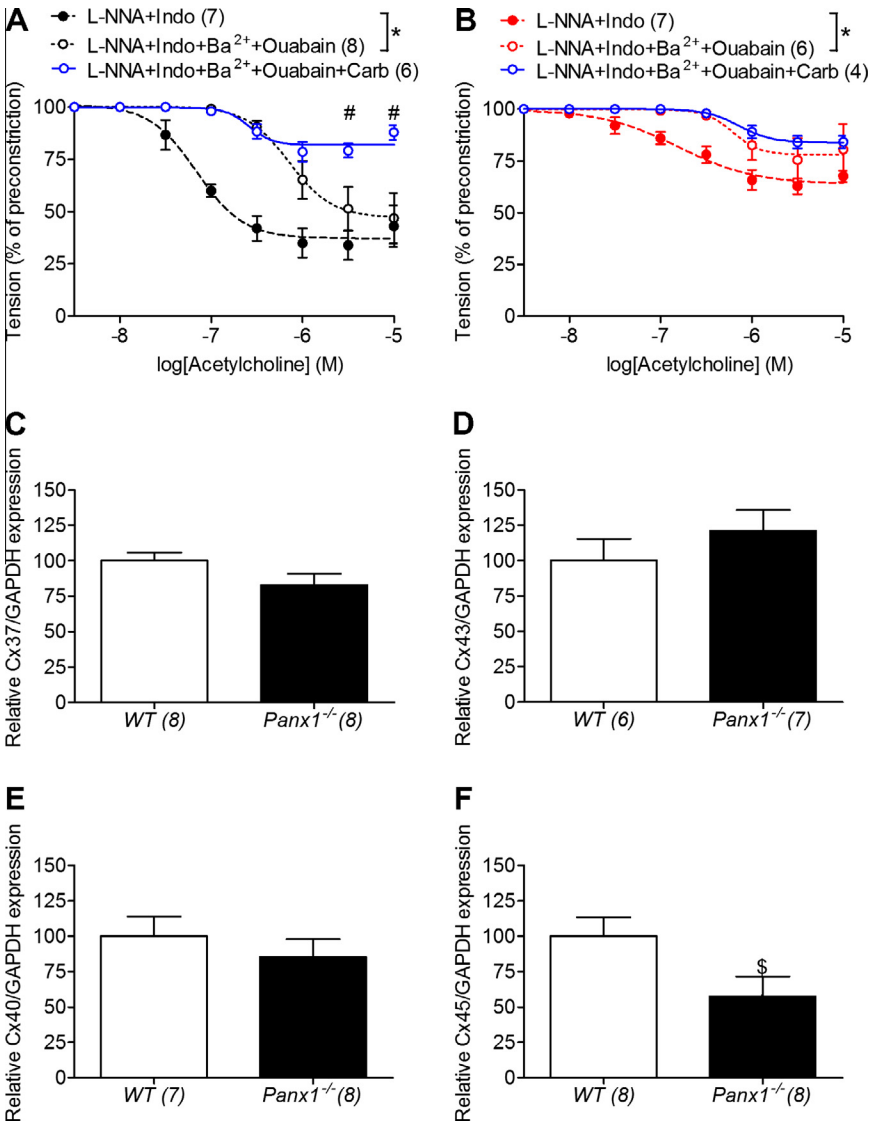

Fig. 5. Panx1 is essential for transmission of EDH-like signal from endothelial to smooth muscle cells. (A and B) Concentration–response relationships to ACh of saphenous arteries of WT (A) and Panx1$^{-/-}$ (B) mice in the presence of L-NNA + indomethacin or L-NNA + indomethacin + Ba^{2+} + ouabain or L-NNA + indomethacin + Ba^{2+} + ouabain + carbenoxolone. $^{*}P < 0.05$ (repeated measures ANOVA); $^{\#}P < 0.05$ (ANOVA followed by followed by Bonferroni correction for repeated measurements). (C – F) Relative to GAPDH mRNA expression levels of connexin 37 (C), connexin 43 (D), connexin 40 (E) and connexin 45 (F) in saphenous arteries of WT and Panx1$^{-/-}$ mice. $^{\$}P < 0.05$ (Student's *t*-test). Number in brackets indicates the number of experiments.

EDH-like relaxation resistant to Ba^{2+} and ouabain may reflect possible involvement of gap junctions in mediating signal transduction from endothelial to smooth muscle cells [8]. The existence of such alternative mechanism of EDH-like signal transduction was confirmed in the experiments with gap junction blocker carbenoxolone, which significantly reduced the endothelium-dependent relaxation in WT, but not in Panx1$^{-/-}$ arteries. Connexins 37 and 43 were previously shown to form myoendothelial gap junction in the murine saphenous artery [19]. However, our analysis showed that the levels of the connexin 37, 40 and 43 gene expression did not differ in saphenous arteries of WT and Panx1$^{-/-}$ animals. Despite the expression level of connexin 45 was reduced in arteries of Panx1$^{-/-}$ mice, we assume that this connexin isoform does not play a role in the EDH-like signal transduction, because it is expressed in smooth muscle, but not in endothelial cells [21]. Thus, the difference between the strains in responses to carbenoxolone may be assigned to the activity of Panx1. This hypothesis will be tested directly in our future studies.

The model summarizing possible involvement of the Panx1 channel in EDH-like mechanisms is shown in Fig. 6. First, ACh binds to the receptor on endothelial surface, leading to the increase in cytosolic Ca^{2+} concentration, which in turn, activates Panx1 hemichannels and causes the release of ATP from the cell. Extracellular ATP activates P2 receptors or is hydrolyzed to adenosine, which activates P1 receptors. Signaling pathways downstream of these receptors cause the additional rise of cytoplasmic Ca^{2+} concentration thereby augmenting IK_{Ca} and SK_{Ca} channels activation and hyperpolarization of endothelial cell. In our opinion, this feedforward loop represents the major mechanism of Panx1 involvement in the EDH-response. Under the conditions of full-manifested endothelial cells activation, the hyperpolarizing signal is transmitted to neighboring smooth muscle cell by either K^+ released through IK_{Ca} channels or via myoendothelial gap junctions, formed by connexins and probably by Panx1. Therefore, Panx1 may facilitate endothelium-dependent arterial relaxation via regulation of one or two EDH-mechanisms.

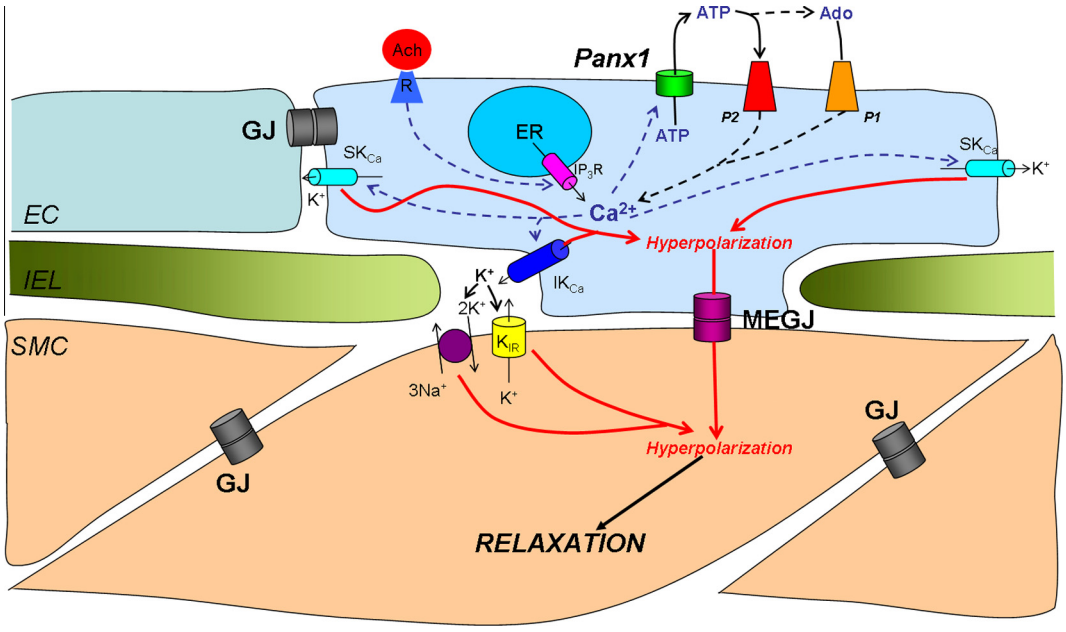

Fig. 6. Possible mechanisms of Panx1 involvement in the functioning of EDH-like mechanism during ACh-induced stimulation followed by smooth muscle relaxation. For further explanation see text. IK_{Ca} – endothelial intermediate conductance calcium-activated potassium channels; SK_{Ca} – small conductance calcium-activated potassium channels; R – receptor; ACh – acetylcholine; Ado – adenosine; ER – endoplasmic reticulum; IP_3R – 1,4,5-inositol trisphosphate receptor; EC – endothelial cell; IEL – internal elastic lamina; SMC – smooth muscle cell; MEGJ – myoendothelial gap junctions; GJ – gap junctions; K_{IR} – inwardly rectifying potassium channels.

Conflicts of interest

None declared.

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