Evaluation of the presence of TRPC6 channels in human vessels: A pilot study using immunohistochemistry

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Abstract. The TRPC6 channel is permeable to calcium ions as well as other ions and plays an important role in the physiology and pathophysiology of vessels. Findings from animal and cell culture experiments have shown its involvement in important vascular processes such as the Bayliss effect or endothelial-mediated vasodilatation. Furthermore, the relevance of TRPC6 channels in humans has become apparent based on diseases such as idiopathic pulmonary arterial hypertension, focal segmental glomerulosclerosis and atherosclerosis, amongst others. However, histological evidence that systematically detects TRPC6 channels in human vessels has not been provided to date. In this study, 40 vessel sections from nine body donors were obtained, processed and stained with a knockout-validated antibody against the TRPC6 protein using immunohistochemistry and western blotting. More than half of the samples yielded evidence of TRPC6 channel expression in the intima and adventitia. TRPC6 channels were detected in the tunica media in only one of 40 cases. TRPC6 detection in the human intima confirmed several demonstrated physiological aspects of the TRPC6 channels in the vasculature and may also be involved in associated human diseases. The near absence of TRPC6 channels in the tunica media was in contrast to a view that is primarily based on animal studies, from which its presence was assumed.

Introduction

The transient receptor potential canonical 6 (TRPC6) channel plays an important role in the physiology and pathophysiology of blood vessels. The TRPC6 channel is a membrane-bound, nonselective, calcium-permeable cation channel. As such, it is significantly involved in the regulation of the calcium balance in cells expressing this channel (1). The typical pathway of TRPC6 channel activation is via a G-protein coupled mechanism. Through a ligand on the G-protein, phospholipase C (PLC) is activated. PLC phosphorylates the membrane bound phosphatidylinositol-4,5-bisphosphate and cleaves this into inositol-1,4,5-trisphosphate and diacylglycerol (DAG). DAG can directly activate the TRPC6 channel (2,3). In addition, external DAG analogues can also activate the TRPC6 channel, such as 1-oleoyl-2-acetyl-sn-glycerol (4). The orally bioavailable and selective antagonist, BI-749327 has been shown to be a possible option to modulate the TRPC6 channel (5). Therefore, TRPC6 channels are an interesting target for future pharmacological therapies. Current knowledge regarding the function of the TRPC6 channel in vessels is primarily based on animal models (6) or cell cultures (7). Studies in a mouse model show that the TRPC6 channel has similar functions to the α-adrenoreceptor and is important for regulation of systemic blood pressure (8). Calcium influx directly mediates cellular actions, such as the activation of myosin light chain kinase, which leads to vasoconstriction (9). In relation to animal models, the presence of TRPC6 in the vascular endothelium would be in agreement with the current view; studies have shown that calcium influx via TRP channels is crucial in endothelial cell physiology (10,11). By activating the synthesis of nitric oxide (NO) the TRPC6 channel contributes to vasoactivity. NO leads to vasodilation, thus reducing blood pressure (12,13). The physiological Baylis effect, also termed myogenic autoregulation describes the vasoconstriction triggered by increased blood pressure, or vasodilation caused by an acute drop in blood pressure (14). This mechanism is essential for constant blood flow to the brain and kidney. In mouse models, the TRPC6 channel can be assigned a leading role in the Baylis effect (15,16). TRPC6 is also involved in the hypoxic vasoconstriction (Euler Liljestrand mechanism), which regulates blood flow to capillaries around alveoli based on alveolar ventilation (17,18). In an ischemic brain model, improved circulation in the penumbra with less degraded TRPC6 expression/function was observed, indicating a vasoactive role for TRPC6 and highlighting its potential clinical relevance (19). These examples underpin the vasoactive relevance of TRPC6 channel *in vivo*. Due to the relevance of the

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Abbreviations: DAG, diacylglycerol; HE, hematoxylin-eosin; HRP, horseradish peroxidase; IHC, immunohistochemistry; IPAH, idiopathic pulmonary arterial hypertension; NO, nitric oxide; PLC, phospholipase C; TRPC6, transient receptor potential canonical 6; VSMC, vascular smooth muscle cell

Key words: TRPC6, human vessels, immunohistochemistry

TRPC6 channel in physiological processes, it is hypothesized that it is also involved in certain pathophysiological events.

A well known role of TRPC6 channels in human diseases is focal segmental glomerulosclerosis (20). An alteration of the TRPC6 gene leads to defective formation of podocyte processes and consequently to nephrotic syndrome (21,22).

A disease that affects the vascular system and in which the TRPC6 channel is established to play a role in the development of in humans is idiopathic pulmonary arterial hypertension (IPAH). Pathophysiologically, proliferation of the vessel walls and the microenvironment with a consecutive increase in pressure in the pulmonary vascular system is relevant (23). In IPAH patients, increased expression of the TRPC6 channel in pulmonary vessels may be detected, which could be responsible for the development of the disease (24,25). In addition, inflammation plays an important role in the development of IPAH (26,27). In certain IPAH patients, gene variations of the TRPC6 channel have been detected, which possess special binding sites for NF-κB (a proinflammatory transcription factor), in the promoter region and thus increase the expression of TRPC6 channels through inflammatory processes (28,29). Re-stenosis processes are still feared complications after interventional procedures, such as stent implantation (30). Neointima formation and further fibroblast differentiation play a crucial role in the process of restenosis. Studies have shown that the TRPC6 channel is involved in both neointima formation (31) and fibroblast activation (32,33). Furthermore, this finding raises the hypothesis that the TRPC6 channel is related to intimal proliferation in atherosclerosis. This would suggest etiological involvement of TRPC6 in atherosclerosis and associated diseases, such as coronary artery disease or stroke (34).

In the process of aging in humans, dysregulation of calcium hemostasis plays an important role in vascular dysfunction. Co-participation of the TRPC6 channel in these processes is also indicated through altered regulation (35). Studies have determined expression of TRPC6 channels at the gene level in humans (35-37). However, thus far, there are no systematic descriptions of the localization of TRPC6 in human samples of vessels, to the best of our knowledge. TRPC6 channels are interesting targets that can be modulated pharmacologically (37), thus determining their physiological localization may have clinical relevance.

The aim of this pilot study was the investigation of TRPC6 channels in human vessels to assess the translational value of animal and cell culture data. Overall, 40 samples of vessels from different locations from nine body donations were included.

Materials and methods

Immunohistochemistry and controls. Various vessel samples (Table I) were obtained from a total of nine body donors from the Anatomical Institute of Saarland University and immersion-fixed in formaldehyde (Table II). The study was approved by the Permanent Ethics Committee of the Saarland Medical Association, Homburg/Saar, Germany (approval no. 163/20). The time post-mortem was <72 h until the corpses were fixed using Weigners protocol (38). The specimens were then embedded in paraffin and 7 μm thin sections were prepared using a microtome. The specimens were first stained with hematoxylin and eosin (H&E) and assessed to determine the morphology of the vessels and whether they were intact, as a basic requirement for immunohistochemistry (IHC) analysis. The first step of IHC was antigen retrieval using 1%-citrate-buffered-solution for 60 min at 95˚C in heating incubator, after which the samples were allowed to passively cool down for 30 min in the citrate. After washing the samples twice for 2 min in PBS each time, they were incubated with a knockout-validated antibody against TRPC6 channel antigen structures (Alomone Labs; cat. no. ACC-017). The specificity and quality of the antibody was assessed using peptide-blocked control samples (Alomone Labs; cat. no. BLP-CC017). For antibody specificity, 40 μg control peptide was dissolved with 20 μl PBS and then incubated with 40 μl 1:100 diluted TRPC6 primary antibody overnight at 7˚C in tubes. Negative controls incubated with rabbit serum (from an untreated rabbit from the Institute for Biochemistry, Homburg, Germany) instead of TRPC6 antibodies were included in each staining run. The protein concentration in both solutions was identical (0.01 mg/ml). Horseradish peroxidase-conjugated goat anti-rabbit antibodies (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A10547; 1:500) were used as secondary antibodies. These were incubated for 10-12 h at room temperature in a humidity chamber. The dilutions were made with normal goat serum (Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. 01-6201). The added chromogen DAB became visible as brown coloration, which was converted by an HRP catalyzed reaction. The incubation time with the DAB was determined using light microscopy, and was usually 10 min.

Western blotting. As additional evidence of TRPC6 protein expression, western blotting was performed on skeletonized vessel samples from unfixed body donors with a post-mortem interval <48 h. For this, human tissues were collected and frozen until required. Samples were homogenized in RIPA buffer supplemented with proteinase inhibitor Complete® (Roche Diagnostics) using a precellys homogenizer. A total of 80 μg protein extract was loaded on a 10% SDS gel, resolved using SDS-PAGE, transferred to a PVDF membrane (Advansta), and incubated with primary antibodies in 5% non-fat milk in TBS-Tween buffer (0.05% Tween; Merck KGaA) at 4˚C overnight. The following primary antibodies were used for probing: anti-TRPC-6 (1:200; Alomone Labs; cat. no. ACC-017) and anti-β-actin (1:1,000; Sigma-Aldrich; Merck KGaA; cat. no. A5441). Antibody specificity of the anti-TRPC6 antibody was determined by peptide-inhibition using the corresponding blocking peptide (cat. no. BLP-CC017; Alomone Labs) according to the manufacturer's protocol. After washing the membrane in TBS-Tween for 15 min, secondary antibody incubation was performed for 1 h at room temperature using HRP-conjugated goat anti-rabbit (1:7,000; Thermo Fisher Scientific, Inc.; cat. no. A16096) or goat anti-mouse (1:7,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-525409) secondary antibodies. The membrane was washed for 15 min in TBS-Tween. Development of electrochemiluminescent signals was performed by incubation with WesternBright Quantum ECL substrate (Advansta Inc.) for 5 min, and visualized using the ChemiDoc System with the Image Lab Software (both from Bio-Rad Laboratories, Inc.).

Table I. Location of the obtained vessels.

Vessel	Standardized sampling location	Number of samples
Arteria renalis dextra	2 cm from the outflow from the aorta	7
Arteria renalis sinistra	2 cm from the outflow from the aorta	6
Arteria thoracica interna dextra	Third intercostal space	4
Arteria thoracica interna sinistra	Third intercostal space	2
Arteria radialis dextra	2 cm proximal in hight of Processus styloideus radii	2
Aorta (abdominal/thoracal)	2 cm proximal of the aortic bifurcation, or ascending aorta	6
Vena cava inferior	Paired to the sampling site of the abdominal aorta	2
Vena jugularis interna sinistra	Vagina carotica in hight of larynx	2
Truncus pulmonalis	2 cm distal of Valva trunci pulmonalis	2
Ramus interventricularis anterior	Middle third, beginning after the exit of the ramus circumflexus	2
Arteria iliaca communis sinistra	2 cm distal of bifurcatio aortae	1
Sinus caroticus dextra	Complete sinus caroticus	2
Vena cava superior	Between the junction of the brachiocephalic trunci and the confluence with the atrium dextrum	1
Arteria carotis communis dextra	Ventral of musculus sternocleidomastoideus, in the vagina carotica	1

Statistical analysis. Overall, 40 samples were processed and evaluated using a light microscope (magnification, x10 or x40). The evaluation scheme considered the distinction of the three vessel wall layers: Tunica intima, Tunica media and Adventitia. IHC detected specific staining that was significantly different from the negative control or any background staining. For histological evaluation, the IHC stained specimens were evaluated using a photomicroscope ((magnification, x10, x20 or x40) in a blinded manner by two investigators. The signals were categorized into strongly positive (++), positive (+), uncertain (?) and negative (0). In this study modal values and relative frequencies were determined. Image analyses were not performed due to the complexity of the histological compartments. Stratification was performed into the intima, media and adventitia and its structures, such as the endothelium, subendothelium, elastica interna; stratum musculare and elastica externa, as well as the surrounding connective tissues with nerves, vasa vasorum and other cell types. Absolute and relative frequencies were collected and mathematical modes were determined for comparing the evaluated characteristics (Microsoft Excel 365; Microsoft Corporation). In this descriptive investigation no experimental groups were compared.

Results

The systematic evaluation of positive and specific protein detection of the TRPC6 channel by means of IHC shows differences in its expression in the layers of the vessel wall, as well as in different vessel sections. Overall, 14 different localizations of vessels were assessed based on 40 samples (Table I). In this study the primary focus was the renal artery and aortic samples. The other vessel sections that were evaluated should be seen as random samples. All evaluations of the samples can be seen in Table III. In renal arteries, positive signals were seen in the tunica intima in 77% of cases (10 out of 13), in 0% of cases (0 out of 13) in the tunica media and in 77% of cases (10 out of 13) in the adventitia. Fig. 1A shows a sample of the left renal artery, incubated with the TRPC6 channel antibody. Here, the tunica intima with subendothelium is clearly stained brown. This brown staining is a specific signal for the TRPC6 channel. In addition, the adventitia, also homogeneously positive, possessed a vasa vasorum with erythrocytes displaying clear positive signals. In contrast, no signals were detected in the tunica media, suggesting an absence of the TRPC6 channel in this layer. Fig. 1B shows the included negative control incubated with rabbit serum. The quality of specific staining by the primary antibody used could be confirmed in IHC, as shown in Fig. 1C. Here, the primary TRPC6 antibody was pre-incubated overnight with the control peptide. Similar results were obtained in other renal arteries incubated with the primary antibody. As an example, renal arteries from other body donors are shown in Fig. 2A and B.

In all sections of the aortic samples, positive signals were seen in the tunica intima in 83% of cases (5 out of 6), in 0% of cases (0 out of 6) in the tunica media and in 83% of cases (5 out of 6) in the adventitia. Fig. 3 specifically shows the transition between the tunica intima and media. The subendothelial showed very strong positive signals in contrast to the endothelial cells. The entirety of the tunica media showed negative staining (data not shown).

Of the 40 samples, specific staining was observed in the tunica intima in 50% of cases (20 out of 40). The largest share of these 20 samples were renal arteries and aortic samples. The adventitia showed similar results; positive signals in 55% of cases (22 out of 40). In contrast to the tunica intima, no specific vascular segment was detected in which the TRPC6 channel expression was increased. In all samples in which the vaso vasorum was observable in the adventitia with an intact integrity, the vaso vasorum itself, as well as the erythrocytes contained therein, stained positively. There was less staining in the tunica media, in which vascular smooth muscle cells (VSMCs) were detected, based on H&E staining. The results

Table II. Data on the body donors.

Proband	Ag, years	Sex	Cause of death according to death certificate	Co-morbidities	Special features of the removal
1	90	Female	Dementia as a consequence of arterial hypertension and deep vein thrombosis	Arterial hypertension; deep vein thrombose	
2	89	Male	Decompensated heart failure due to heart failure and CHD	Diabetes mellitus	Severe atherosclerotic changes in the aorta, not directly at the donor site
3	84	Male	Cardiovascular arrest due to heart failure	Condition after STEMI; atherosclerosis; CHD	Partial atherosclerotic changes of the sampling sites; absence of the radial artery dextra
4	78	Female	Respiratory insufficiency due to bronchial carcinoma	Nicotine abuse	Stent of the abdominal aorta
5	70	Male	Sudden cardiac death	Condition after myocardial infarction; arte rial hypertension; Angio sclerosis; Raynaud's disease	Stent of the abdominal aorta with bifurcation aortae
6	69	Male	Tumor hemorrhage due to tumor infiltration of the intrahepatic portal vein system caused by cancer of unknown primary syndrome.	Peritoneal carcinomatosis; incomplete deep paraplegia metastasis related; bone metastases	
7	76	Female	Respiratory insufficiency due to aspiration	Locked-in syndrome; meningioma	
8	64	Female	Suspicion of cardiac arrhythmia due to hyperthyroidism	Graves' disease; Crohn's disease; liver cirrhosis	
9	57	Female	Central respiratory paralysis due to advanced Parkinson's disease	Parkinson's disease	

CHD, cardiovascular disease; STEMI, ST elevation myocardial infarction.

Table III. Overview of the scoring results of vessel staining.

Vessel	Tunica intima: stratum endotheliale		Tunica intima: stratum subendotheliale		Tunica media		Adventitia	
Arteria renalis dextra	++	3	++	4	++	0	++	4
	+	2	+	1	+	0	+	0
	?	0	?	2	?	1	?	2
	0	2	0	0	0	6	0	1
Arteria renalis sinistra	++	3	++	3	++	0	++	3
	+	2	+	1	+	0	+	3
	?	0	?	2	?	2	?	0
	0	1	0	0	0	4	0	0
Arteria thoracica interna dextra	++	0	++	0	++	0	++	0
	+	0	+	0	+	0	+	1
	?	1	?	0	?	0	?	0
	0	3	0	4	0	4	0	3
Arteria thoracica interna sinistra	++	0	++	0	++	0	++	0
	+	0	+	0	+	0	+	1
	?	0	?	0	?	0	?	0
	0	2	0	2	0	2	0	1
Arteria radialis dextra	++	0	++	0	++	0	++	0
	+	0	+	0	+	0	+	0
	?	0	?	0	?	0	?	0
	0	2	0	2	0	2	0	2
Aorta abdominal/thoracal	++	4	++	4	++	0	++	1
	+	0	+	1	+	0	+	4
	?	1	?	0	?	0	?	1
	0	1	0	1	0	6	0	0
Vena cava inferior	++	0	++	0	++	0	++	0
	+	0	+	0	+	0	+	1
	?	0	?	0	?	0	?	0
	0	2	0	2	0	2	0	1
Vena jugularis interna sinistra	++	0	++	0	++	0	++	0
	+	0	+	0	+	0	+	0
	?	0	?	0	?	0	?	0
	0	2	0	2	0	2	0	2
Truncus pulmonalis	++	1	++	1	++	0	++	0
	+	0	+	0	+	1	+	1
	?	1	?	1	?	0	?	1
	0	0	0	0	0	1	0	0
10 (Ramus interventricularis anterior)	++	0	++	0	++	0	++	1
	+	0	+	0	+	0	+	0
	?	0	?	0	?	0	?	1
	0	2	0	2	0	2	0	0
Arteria iliaca communis sinistra	++	1	++	1	++	0	++	0
	+	0	+	0	+	0	+	1
	?	0	?	0	?	0	?	0
	0	0	0	0	0	1	0	0
Sinus carotivus	++	0	++	1	++	0	++	1
	+	1	+	1	+	0	+	0
	?	0	?	0	?	0	?	0
	0	1	0	0	0	2	0	1

Table III. Continued.

Vessel	Tunica intima: stratum endotheliale		Tunica intima: stratum subendotheliale		Tunica media		Adventitia	
Vena cava superior	++	0	++	0	++	0	++	0
	+	0	+	0	+	0	+	0
	?	0	?	0	?	0	?	1
	0	1	0	1	0	1	0	0
Arteria carotis communis dextra	++	1	++	1	++	0	++	0
	+	0	+	0	+	0	+	1
	?	0	?	0	?	0	?	0
	0	0	0	0	0	1	0	0

demonstrated specific signals in 2.5% of cases (1 out of 40). All the results are summarized in Fig. 4.

Western blotting detected the presence of TRPC6 protein in skeletonized preparations of the aorta. As shown in Fig. 5, a protein band appeared at the expected ~100 kDa marker, which was absent after peptide inhibition. This is comparable to the results of another study that used the same antibody in western blotting (19,39).

Discussion

The results of the present study show differences in the distribution of TRPC6 expression in the vessels. Strong signals could be detected in the tunica intima of the renal arteries and aorta samples. Signals were also observed in samples of the pulmonary trunk, the carotic sinus dextra and the right common carotid artery. The tunica media showed a negative signal in nearly all samples. The adventitia showed mixed signaling. In certain tissues, TRPC6 signals in the adventitia was observed, whereas in others, the signal was absent. Adventitia showed clustered positive signals in larger arteries compared with the smaller arteries and veins. In this study modal values and relative frequencies were determined. Image analyses were not performed due to the complexity of the histological compartments.

In the tunica intima the endothelium itself did not always exhibit a positive signal. This is indicative of the varied expression of TRPC6 based on the specific vessel segments. However, there may also be a bias in the results; the endothelium became slightly detached during the processes of fixation. This may have led to cell damage and other artefacts that prevented continuous positive staining. Detached cells from the stratum subendotheliale or remnants of blood cells may have resulted in false positive staining. TRPC6 channels were found in erythrocytes in the present, in agreement with a previous study (40).

Concepts based on animal models of TRPC6 channels in the vascular endothelium correspond with the results of the present study. Studies have shown significant calcium influx via TRP channels, and this is involved in the physiology of endothelial cells (10,11). The presence of TRPC6 channels on endothelial cells also fits in with the mechanism of vasodilation involving NO release, and TRPC6$^{-/-}$ mice were shown to have a higher systemic blood pressure than wild type mice (41). This could be explained by decreased NO release from the endothelium due to missing TRPC6 channels. It is possible that future therapeutics may also lead to improved blood flow to the penumbra in acute strokes and thus possibly to an improved outcome (19). The stratum subendotheliale exhibited localized positive signals in renal arteries and aortas. This was evidenced by similar patterns in 69% of the renal arteries. The stratum subendotheliale is delimited by the membrana elastica interna. The staining of the subendothelium followed this border and was clearly demarcated from the tunica media. In aortic samples, subendothelial signals were positive in 83% of cases. In contrast, the demarcation to the tunica media was harder to delineate than in the renal arteries. A cellular component of this layer is connective tissue-producing fibroblasts (42). It has already been demonstrated that TRPC6 channels are expressed on the surface of fibroblasts (32). These results confirm previous hypotheses that certain human fibroblasts possess TRPC6 channels on their surface *in vivo* (32). In the pathogenesis of atherosclerosis, changes often take place in the Tunica intima. Fibrotic remodeling may result from overactivation of fibroblasts (43,44). Fibroblast differentiation through the TRPC6 channel via angiotensin-II and other cytokines has already been demonstrated using mouse models. Upregulated expression of the TRPC6 channel on fibroblasts was observed in wild-type mice vs. TRPC6$^{-/-}$ mice following treatment with angiotensin-II (33). Thus, overactivation of the TRPC6 channel could lead to stenosis in vascular segments. This would explain the increased expression of TRPC6 channels in IPAH patients (24,25). Thus, TRPC6 may also contribute to other diseases in which atherosclerosis is etiologically involved (35). In particular, changes in the renal arteries would allow for the development of renal artery stenosis with consecutive renal (45). Renal artery stenosis is a disease that triggers the renin-angiotensin-aldosterone system through decreased renal artery blood flow, resulting in a systemic increase in blood pressure (46).

The results in the tunica media showed no positive signal in the majority of vessel sections evaluated. In 2.5% of cases TRPC6 could not be detected. Only one sample, the truncus pulmonalis, exhibited TRPC6 staining. The tunica media

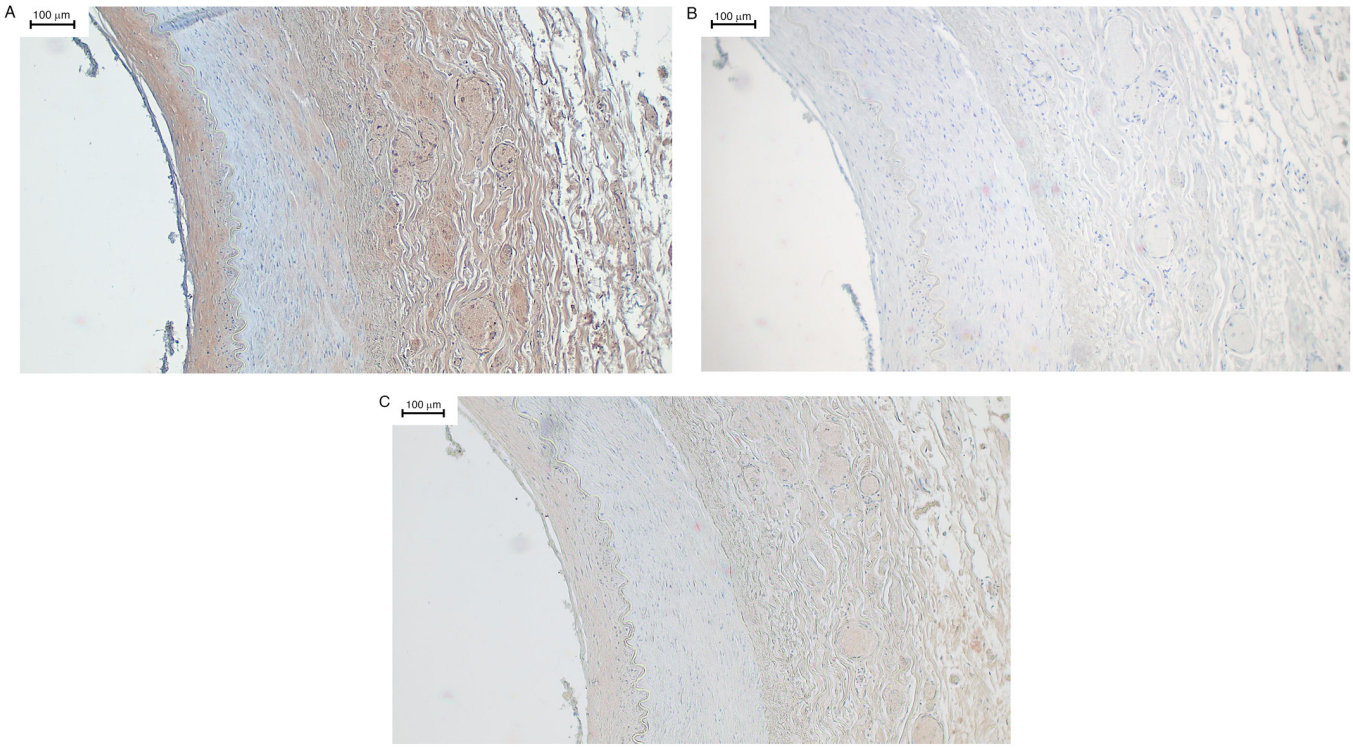

Figure 1. (A-C) Left renal artery. Left renal artery, immunohistochemistry using a primary antibody against TRPC6 followed by DAB staining. The stratum endothelial, in the picture on the left, is artefactually lifted in places, but still well recognizable by the flat cell nuclei. The underlying stratum subendotheliale can be clearly distinguished from the tunica media by the membrana elastica interna. Due to the loss of some fibrin fibers, the elastic fibers of this membrane have contracted as expected and the membrana elastica interna also appears artefactual. The tunica media is relatively unchanged and shows no staining. After the membrana elastica externa, which is clearly visible, the adventitia is located at the right of the membrana elastica externa. Its is stroma slightly loosened and impresses with several vasa vasorum. In these, erythrocytes can be identified. Apart from these two entities and the surrounding connective tissue, there are no assessable structures here. (B) The corresponding negative control did not display any non-specific staining. Here, all wall layers were negative. The same artefacts that are present in the positive control can be detected. The erythrocytes in the vaso vasorum can also be approximated, but there is no brown staining. (C) The corresponding sections of the vessel in Panels A and B in the same shape. The staining was performed using primary antibodies, which were previously incubated with a control peptide. The staining pattern is the same as in the positive control, although the intensity of the staining is clearly reduced. This corresponded with the expected staining and showed the quality of the antibody in the test series. Magnification, x10. TRPC6, transient receptor potential canonical 6.

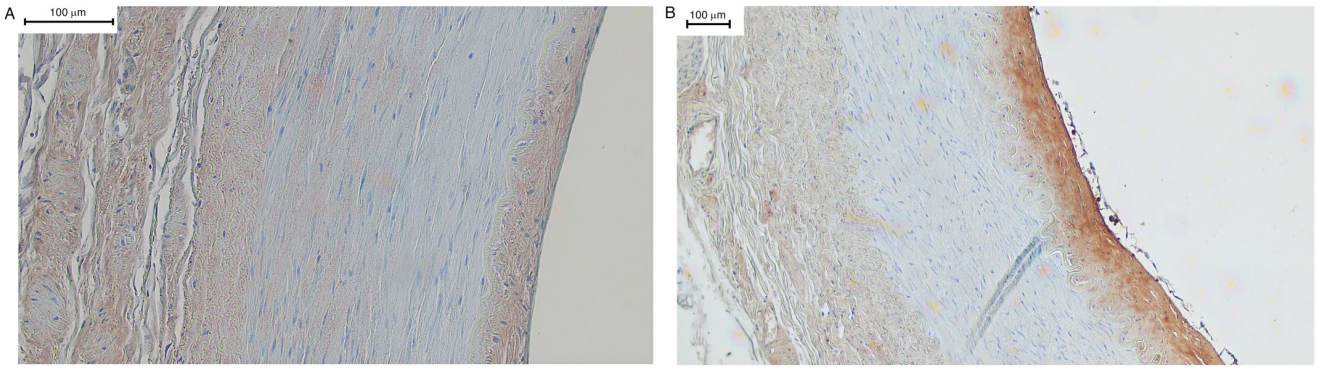

Figure 2. (A) Right renal artery. Right renal artery, IHC analysis with the primary antibody against TRPC6 (positive control) and DAB staining. Magnification, x20. From the same body donor as Fig. 1A. (B) The individual entities in this image correspond to those discussed in Fig. 1A as they are from the same series of sections. The only difference was the staining. Magnification, x20. This is the corresponding right renal artery from the same body donor describes in Fig. 2A. The description begins on the right side of the image. The endothelial stratum is positive (+) and the subendothelial stratum is clearly positive (++). The endothelial cells themselves are attached here and are not artefactually detached. The tunica media is also well demarcated again and shows a questionable positive signal (?) on the side facing the adventitia. These questionable stains are found only focally and not in the entire specimen. This staining pattern in the tunica media could also only be observed in the renal arteries of this body donor. The adventitia is roughly homogeneously stained since there are no special entities and was therefore evaluated as clearly positive (++). Magnification, x20. IHC, immunohistochemistry; TRPC6, transient receptor potential canonical 6.

consists primarily of smooth muscle cells (47). These results contradict current research assumptions that the TRPC6 channel is present on human VSMCs. Supporting a potential false negative result of these results, and thus a presence of TRPC6 channel on the cell surface of VSMCs highlight a candidate carrying a relevant calcium influx in VSMCs. This influx causes contraction of the muscle cells and leads to vasoconstriction with a consecutive increase in blood pressure (48).

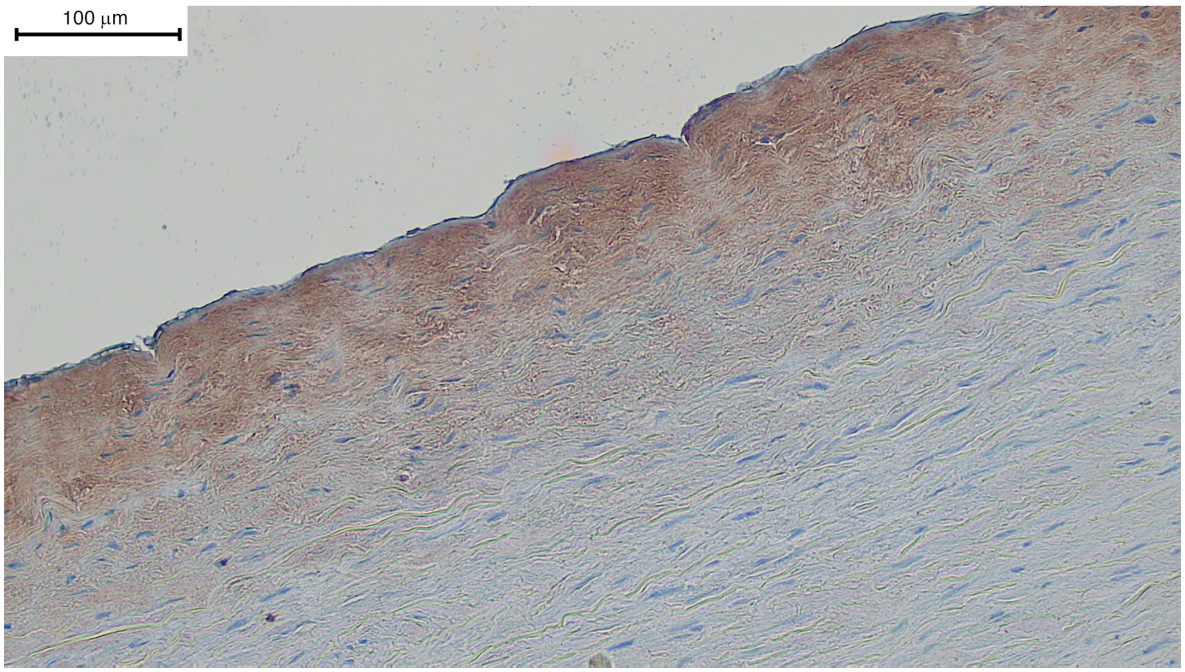

Figure 3. Arteria thoracica. Aorta thoracica, immunohistochemical staining with a primary antibody against the TRPC6 channel followed by DAB staining. The ascending aorta possessed very flat nuclei on the luminal side (top left of the picture), which can be interpreted as endothelial cells. The underlying subendothelium showed clear brown staining as evidence of the TRPC6 channel. In contrast to the renal arteries, the membrana elastica interna was hardly visible here and thus a demarcation to the tunica media is blurred. The trend showed a definite weakening brown signal from the endothelial side towards the adventitia. Thus, the tunica intima was evaluated as clearly positive (++). The histologically determined tunica media did not show brown staining at all, and was thus assessed as negative (0). The adventitia, which is not visible in the picture, should be mentioned here; it displayed a positive (+) staining pattern. Magnification, x20.

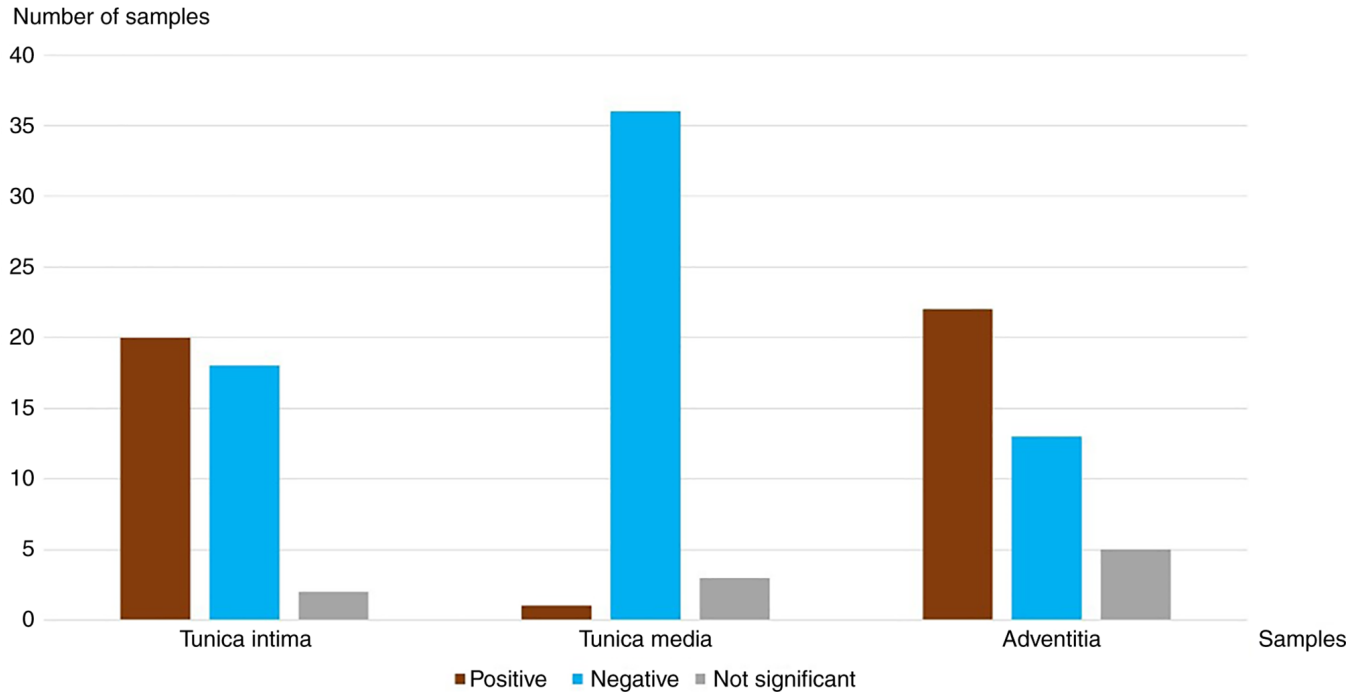

Figure 4. Summary of findings. A summary of the findings of the present study. The individual vessel wall layers are plotted on the x-axis. The y-axis shows the number of samples stained positive in the immunohistochemical staining.

Likewise, the sympathetic influence on vascular position is hypothesized to be modulated by TRPC6 channels. Relevant calcium influxes were shown in experiments comparing the effects of the α1-adrenoreceptor in mediating vasoconstriction via transmitters with TRPC6 ligands (49). It appears that α1-adrenoreceptor mediated calcium influx causes upregulation of TRPC6 channel density at the cell membrane (50). The physiological Bayliss effect also argues for a mechanosensitive

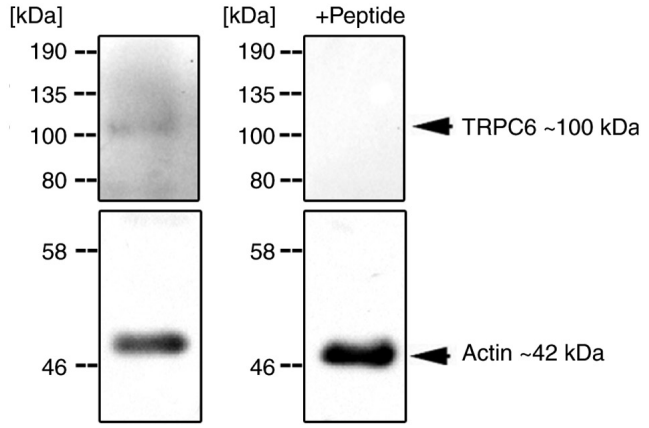

Figure 5. Western blots of TRPC6. Western blot of TRPC6 expression in the aorta ascendens. Western blot analysis using anti-TRPC6 antibodies showed an immunosignal corresponding to a ~106 kDa protein in the aortic tissue sample (n=1). Specificity was determined by pre-absorption of primary antibodies with a specific blocking peptide (+ peptide). Both membranes were incubated with antibodies against actin as a loading control. TRPC6, transient receptor potential canonical 6.

component that could mediate constriction of vascular muscle cells through the involvement of TRPC6 channels (51,52). In support of a valid negative result observed in the present study however, and thus an absence of TRPC6 channels at the cell surface of vascular muscle cells, a previous study showed higher blood pressure in TRPC6$^{-/-}$mice compared with wild-type mice (42). This may be explained by an inferior vasodilatory effect due to reduced NO release from the endothelium and a relatively minor effect of TRPC6 on smooth muscles. It should be noted that none of these studies replicate situations observed in humans. The majority of these assumptions are based on research results from mouse models (6,9) or cell cultures with human embryonic cells, so-called HEK cells (53). For this section, it can be summarized that there are studies that have systematically detected TRPC6 expression in samples taken directly from humans previously. This is complicated by the challenges posed when working with body donors and the limited number of samples available at any given time. The present study is the first work in this field, and the results indicate absent TRPC6 channel expression in human vascular muscle cells, in contrast with the current research opinion based on animal models. Further studies are required to verify or refute this result.

Of all the layers evaluated, the adventitia was the one with the most artefacts. The loose connective tissue, which forms the majority of the adventitia, enables its anchoring and displaceability in the tissue (54). In the present study, it may have been destroyed by various mechanical stimuli during dissection. In further processes, this layer may have been further progressively destroyed, or become detached, by chemical stimuli. Large artefacts were already partially visible in the H&E sections. Scattering of other tissue types or cells into the adventitia cannot be excluded. Thus, in addition to resident fibroblasts, macrophages, erythrocytes or other cells may have also migrated in. The results show in some sections clear positive staining of well preserved entities, such as the vaso vasorum with erythrocytes. In some cases, the entire adventitia also showed a homogeneous positive signal. The morphological appearance did not allow an assessment of the type of cells. Most of the sections showed only unspecific staining, and the tissues in the adventitia could not be identified correctly in many cases. Most of the results were too unspecific and single specific positive signals should only be considered as a rough indication for protein detection of the TRPC6 channel. In the future, other methods should be considered to identify cells showing positive protein detection of TRPC6 channels. For example, immunofluorescence could be used to visualize TRPC6 using cell specific markers (e.g. for fibroblasts).

In conclusion, the systematic evaluation of TRPC6 channel expression using IHC showed differences in its expression in the layers of the vessel wall as well as in the different vessel sections. The subendothelial stratum in the tunica intima shows localized and clear protein detection of the TRPC6 channel in only a few of the vascular sections examined. Here reproducible patterns of positive signals was shown in several samples from the left and right renal artery as well as the aorta. The endothelial cells themselves also showed evidence of TRPC6 channel expression in some preparations. Both are in agreement with current research opinion. The tunica media was negative in almost all cases, which suggests that the TRPC6 channel is not expressed on cells in this layer. As some research findings point to vasodilatation rather than vasoconstriction of vessels following TRPC6 induced NO release, this result is only partially consistent with current research findings. However, this result contrasts the results of animal models, which have shown the TRPC6 channel is present on VSMC in humans, too. The adventitia showed no clear staining pattern in a comparison between different vessel sections. Only individual structures in the adventitia, such as the vasa vasorum, were labeled with the antibody against TRPC6. Understanding the pathogenesis of cardiovascular diseases is of importance due to the high prevalence and mortality of those affected by diseases of the cardiovascular system. The possibilities of pharmacological modulation of the TRPC6 channel may open up novel therapeutic options for certain patient groups.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JA and TT conceived the study. DS, TJ and AB designed the study. JA, AB and TJ performed the experiments. JA wrote the draft of the manuscript. All authors have read and approved

the manuscript. All authors confirm the authenticity of the raw data.

Ethics approval and consent to participate

The study was approved by the Permanent Ethics Committee of the Saarland Medical Association, Homburg/Saar, Germany (approval no. 163/20).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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