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MECHANISMS OF VASODILATATORY EFFECT OF PERIVASCULAR TISSUE OF HUMAN INTERNAL THORACIC ARTERY

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It has beed showed that perivascular adipose tissue (PVAT) of human internal thoracic artery (ITA) releases adventitia/adipocyte-derived relaxing factor (ADRF). The precise mechanism of vasodilatatory effect of ADRF is still unknown. It was suggested that various potassium channels may be involved in the action of ADRF. The aim of this study was to assess the involvment of potassium channels in the vasorelaxing properties of ADRF in human internal thoracic artery. Human ITA rings were studied *in vitro*. First the ability of perivascular tissue of human ITA to release ADRF to the bath was checked. In subsequent experiments two fragments of skeletonised ITA were used to assess the involvement of various potassium channels in vasorelaxing action of PVAT. Segment of ITA, precontracted with serotonin ($10^{-5.5}$M), was relaxed by adding PVAT to tissue bath, first without and then in the presence of appropriate potassium channel blocker. Second segment served as a control (no addition of PVAT). The magnitude of relaxation was measured and compared between preparations. This protocol was used to analyze the influence of iberiotoxin (100 nM), apamin (1 uM), 4-aminopyridine (1 mM, 5 mM), $BaCl_2$ (100 uM) and glibenclamide (10 uM). The addition of PVAT to precontracted skeletonized ITA caused significant vasorelaxation (54.6±8.03 mN versus 33.7±6.58 mN p=0.03). Similar effect was seen when 5 ml of aliquot from separate incubation of PVAT was added (36.3±5.45 mN versus 20.7±3.02 mN; p<0.001). PVAT dependent relaxation was blocked in the presence of Ca^{+2} dependent potassium channel blocker iberiotoxin (47.4±16.67 mN versus 43.3±14.54 mN; p=0.36) and 4-aminopyridine (5 mM) (59.3±3.54 mN versus 51.6±4.77 mN; p=0.12). We conclude that perivascular adipose tissue of human ITA releases relaxing factor that seems to act with the involvement of Ca^{+2} dependent potassium channels.

Key words: *potassium channels, internal thoracic artery, adipocyte-derived relaxing factor, perivascular adipose tissue*

INTRODUCTION

Left internal thoracic artery (ITA) is a graft of choice in coronary artery bypass surgery. Internal thoracic artery harvested with a pedicle allows for preserving perivascular tissue (PVT) that was found to be a source of potent vasorelaxing factor (1, 2). The pedicled ITA is a functionally different vessel with unlike response to vasoconstrictors (3). Its dissimilar function seems to be primarily related to paracrine activity of perivascular adipose tissue (PVAT) (4).

In 1991 Soltis and Cassis discovered that contractile response in rat aorta is attenuated in the presence of PVAT (5). It was Lohn *et al*. who confirmed that this inhibitory effect of PVAT is most likely mediated by a transferable factor called adipocyte-derived relaxing factor (ADRF) (4). Up to now its existence was proved in the aorta (4) and mesenteric arteries (6) of rat, aorta and mesenteric arteries of mice (7), and also in human ITA (1, 2). We have shown previously that PVAT of human ITA releases anticontractile factor that attenuates ITA contraction to serotonin and angiotensin II (1). The mechanism of PVAT derived anticontraction and relaxation is not fully understood. We have shown that anticontracile activity of ITA's PVAT does not depend on NO or prostacyclin (1). In the study of Lohn *et al*. the PVAT dependent relaxation was proposed to be mediated by opening potassium channels in the plasma membrane of smooth muscle cells (4). However various types of K^+ channels were found to be involved in this relaxation. It depends on the animal species and type of vascular bed analyzed. In the beginning of research on ADRF in rats the first potassium channel proposed to be involved in its action was K_{ATP} (4). Next the other channels were consecutively tested and proposed to be the key role in ADRF dependent relaxation: K_V in rats mesenteric arteries (8) and mice mesenteric vascular bed (9), K_{Ca} in rats and mice aorta (7, 10). Such differences may represent diverse ADRFs or different distribution of K^+ channels in vessels in various species. Very few studies assessed the human vessels' ability to release ADRF and tried to identify its mechanisms of action through various K^+ channels (2). We still do not know if the ability of ITA's PVAT to produce ADRF is of any clinical importance. Preservation of PVT might be crucial for the superb long-term patency of ITA graft. To answer these doubts the clear pathway of relaxation with ADRF should be understood first.

In the current study we wanted to check whether: 1) human ITA's PVAT releases soluble relaxing factor, 2) proposed in previous animal studies various potassium channels play a role

in the relaxing properties of human PVAT. We hypothesized that PVAT dependent vasorelaxation in human is similarly to animal studies based on activation of potassium channels.

MATERIAL AND METHODS

The study was performed on isolated segments of left human ITA discarded after the conduit had been trimmed to the length necessary for coronary bypass grafting. The Local Research Ethics Committee agreed to the use of waste human tissue for the experimental work and patient informed consent was waived. The study arterial segments were obtained from 53 patients undergoing surgery for stable isolated coronary artery disease. All grafts where harvested pedicled in standard fashion using elecrocautery. The ITA fragments for the experiments where next placed in the cold (4°C) calcium free modified Krebs-Henseleit solution of the following composition: NaCl, 123.0; KCl, 4.70; $MgSO_4$, 1.64; $NaHCO_3$, 24.88; KH_2PO_4, 1.18; glucose, 5.55; sodium pyruvate, 2.0 (mM) (pH 7.4). Than they were transferred immediately to the laboratory. Here the vessels were skeletonized free of surrounding PVAT. The vessels were divided into 3 mm long segments. The arterial rings were suspended on stainless steel wire hooks in the organ bath chamber (20 ml) filled with oxygenated (95%O_2, 5%CO_2) Krebs-Henseleit solution of the following composition: NaCl, 119.0; KCl, 4.70; $CaCl_2$, 1.6; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; glucose, 11.01; sodium pyruvate, 2.0 (mM) (pH 7.4). The temperature was maintained at 37°C. The PVAT was incubated separately in the same conditions. The Schuler isolated organ bath (Hugo Sachs Elektronik (HSE); March-Hugstetten, Germany) was used. Vessel wall tension was measured with isometric force transducer F 30 (HSE), the signal was enhanced with bridge amplifier Type 336 (HSE) and recorded using PowerLab/4SP system and Chart software (AD Instruments, Chalgrove, Oxfordshire, UK). After the short period of initial incubation the vessel wall tension and diameter were normalized in a standardized procedure as described by Mulvany and Halpern (11). This way every vessel ring was set to the 90% of diameter it would have had *in vivo*, when relaxed and under the transmural pressure of 100 mmHg using the Laplace law; P=2T/d. After equilibration the vessel was left for 30 min to stabilize, during which period the tissue was thoroughly washed. Every time before experiment, 60 mM KCl was added to the tissue bath to check for the viability of the artery. Then, the tissue was washed, and the appropriate experimental protocol was applied.

The following substances were used in the study: 5-hydroxytryptamine hydrochloride (serotonin), iberiotoxin, apamin, 4-aminopyridine, $BaCl_2$, glibenclamide (all Sigma-Aldrich Corp., St. Louis, MO).

Experiment 1

We first examined the ability of PVAT from pedicled ITA to release the relaxing factor. ITA transferred from the operating room was skeletonized as described above. Perivascular tissue from skeletonization (mean weight 393±39 mg) was incubated in the separate tissue bath in oxygenated Krebs-Henseleit solution. The PVAT tissue from skeletonization was floating freely in the bath. The two 3 mm ITA segments from one patient were placed in two different tissue bath chambers. After the equilibration and stabilization time both preparations were simultaneously contracted with serotonin ($10^{-5.5}$M). Serotonin was also added to the bath with incubated PVAT. Serotonin concentration used ($10^{-5.5}$M) was chosen based on the results of our previous experiments (1). It produces stable contraction of ITA at the level of EC_{80} in our model. When plateau phase of contraction was achieved 5 ml of aliquots from PVAT incubation was added to one ITA segment. PVAT itself was added to the tissue bath containing another segment of ITA. After reaching stable relaxation, both vessels were thoroughly washed. PVAT was transferred back to separate tissue bath chamber. Next, after time needed for stabilization the experiment was repeated. Additionally in some preparations serving as a control (n=10)

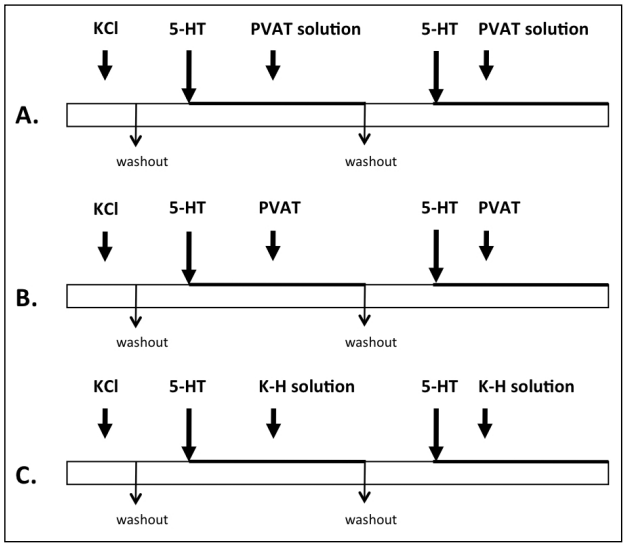

Fig. 1. The protocol of experiment 1. The experiments were always performed in two tissue baths using two 3 mm ITA rings obtained from the same patient. Perivascular adipose tissue (PVAT) remaining from skeletonization was incubated in the separate bath. Five milliliters solution from PVAT bath (panel A) or PVAT (panel B) was added to precontracted with serotonin ($10^{-5.5}$M) artery. In the control (panel C) 5 ml of Krebs- Henseleit solution was added. KCl – potassium chloride, 5-HT – serotonin, PVAT – perivascular adipose tissue, K-H – Krebs Henseleit solution.

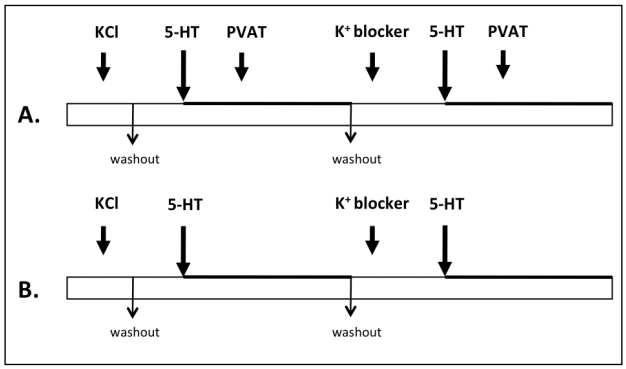

Fig. 2. The protocol of experiment 2. The experiments were always performed in two tissue baths using two 3 mm ITA rings obtained from the same patient. Perivascular adipose tissue (PVAT) remaining from skeletonization was incubated in the separate bath. PVAT was added to precontracted with serotonin ($10^{-5.5}$M) artery, then appropriate potassium channel inhibitor (see text) was added and ITA relaxation to PVAT addition was tested again (panel A). In the control (panel B) no PVAT was added to the tissue bath. KCl – potassium chloride, 5-HT – serotonin, PVAT – perivascular adipose tissue.

5 ml of Krebs-Henseleit solution containing $10^{-5.5}$M serotonin was added instead of PVAT aliquots. The whole experiment was conducted on specimens from 11 patients. The protocol of experiment 1 is shown in *Fig. 1*.

Experiment 2

In this series of experiments we investigated the influence of various potassium channels inhibitors on the relaxing properties of PVAT. ITA transferred from the operating room was skeletonized. PVAT from skeletonization (mean weight 504±43 mg) was incubated in the separate tissue bath chamber with oxygenated Krebs-Henseleit solution. Skeletonized ITA specimen from one patient was divided into two 3 mm segments. The two preparations were placed in two different tissue baths. After the equilibration and stabilization time both preparations were simultaneously contracted with serotonin ($10^{-5.5}$M). Next PVAT was added to one ITA segment with another serving as a control. After reaching plateau phase of relaxation (approx. 30 min) PVAT was removed from tissue bath and transferred back to separate tissue bath. Both vessels were thoroughly washed. Appropriate potassium channel blocker was added to both preparations and serotonin was reapplied (n=7 for each). Again after vessel contraction, PVAT was added once more to the same preparation. The experiment was concluded when stable relaxation was achieved (approx. 30 min). The protocol of experiment 2 is shown in *Fig. 2*.

Statistical analysis

The vessel wall tension above the resting level is measured and expressed in mN. Relaxation is also expressed as the percentage drop of tension from the contraction produced by serotonin. All results are presented as a mean with standard error (S.E.M.).

For every preparation from experiment 1 the result was the mean of two subsequent relaxations. The change in tension of the same preparation (*e.g.* before and after adding PVAT to tissue bath) was compared using the paired t-test. When comparing to control Student's t-test for unrelated observations was used. In all instances of statistical analysis $p<0.05$ was considered significant. All analyses were performed using SigmatPlot 10.0 and Sigma Stat 3.5 Software (Systat Inc., San Jose, CA).

RESULTS

Experiment 1

The addition of 5 ml of PVAT aliquots to skeletonized ITA resulted in significant 37±6% relaxation of the artery precontracted with serotonin (36.3±5.45 mN versus 20.7±3.02 mN $p<0.001$, n=11). Similar relaxation (40±7%) was achieved when PVAT was added (54.6±8.03 mN versus 33.7±6.58 mN, before and after PVAT, respectively, p=0.03). Time to plateau of relaxation was similar whether PVAT or PVAT aliquots was added: 37±3 min versus 35±3 min, respectively (p=0.7). In the control the addition of K-H solution with serotonin did not change the artery contraction: 50.1±11.15 mN versus 47.5±10.4 mN, before and after respectively, p=0.16, n=9. (*Fig. 3*)

Experiment 2

The effect of various potassium channels blockers on relaxation of ITA segments to PVAT are presented in *Table 1* and *Figs. 4-9*. Precontraction with serotonin ($10^{-5.5}$M) always resulted in stable effect. The tension of ITA did not change significantly when measured thirty minutes after reaching plateau phase of contraction. The change in basal tension of the artery tested after appropriate potassium channel blocker application in presented in *Table 2*. 4-aminopyridine (5 mM) and gibenclamide (10 μM) significantly altered basal tension of the vessel segment.

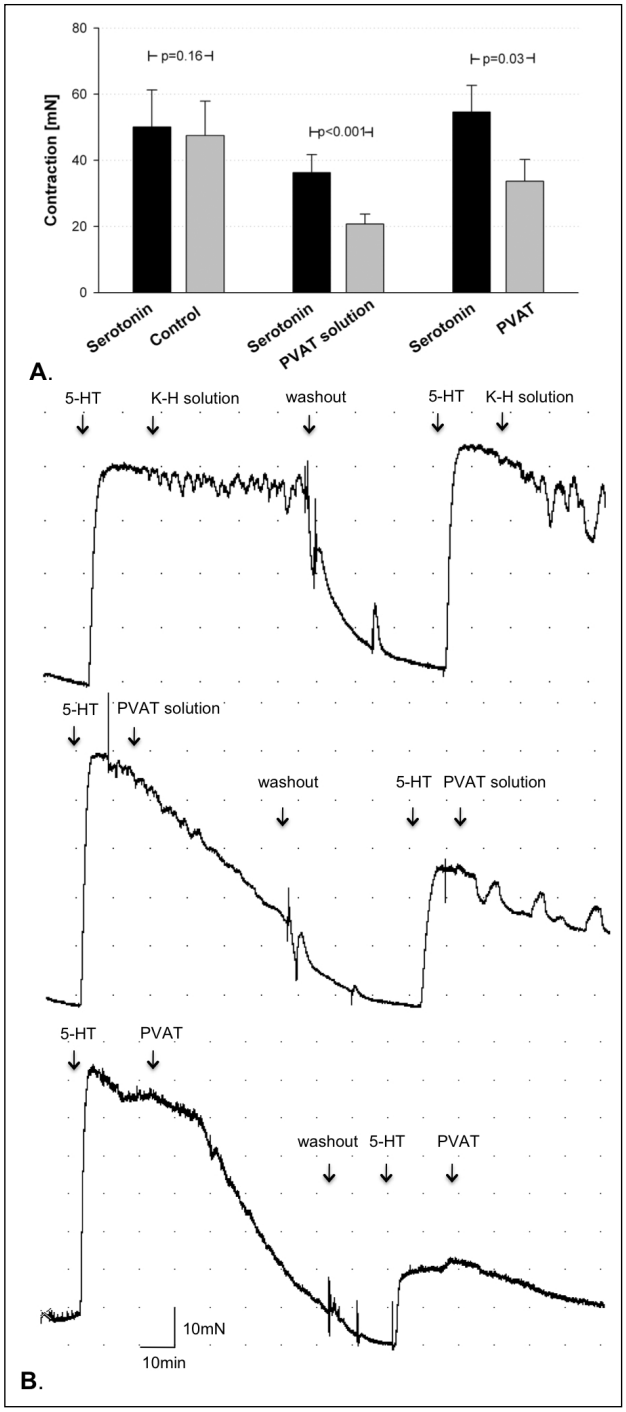

Fig. 3. (*A*) The influence of PVAT solution and PVAT on the relaxation of precontracted with serotonin ($10^{-5.5}$M) human ITA. The contraction response is presented as mean ±S.E.M.; p value from paired t-test. (*B*) The example of original recordings of the experiments. For every preparation the result was the mean of two subsequent relaxations. PVAT – perivascular adipose tissue, K-H – Krebs-Henseleit solution.

Table 1. Perivascular adipose tissue dependent relaxation of ITA during various K^+ channels inhibition.

	Tested (%)	Control (%)	p
Iberiotoxin 100 nM (n=7)			
serotonin ($10^{-5.5}$M)	100	100	
relaxation	50±11.6	10±5.9	0.008
serotonin + iberiotoxin	100	100	
relaxation	0±6.3	–6±3.7	0.5
Apamin 1 μM (n=7)			
serotonin ($10^{-5.5}$M)	100	100	
relaxation	53±7.4	0±8.4	<0.001
serotonin + apamin	100	100	
relaxation	51±5.6	19±4.7	<0.001
4-aminopyridine 1 mM (n=7)			
serotonin ($10^{-5.5}$M)	100	100	
relaxation	46±7.1	-9±16.6	0.009
serotonin + 4-AP	100	100	
relaxation	48±10.3	6±4.4	0.003
4-aminopyridine 5 mM (n=7)			
serotonin ($10^{-5.5}$M)	100	100	
relaxation	67±4.9	4±13.8	0.001
serotonin + 4-AP	100	100	
relaxation	12±5.8	10±3.3	0.7
$BaCl_2$ 100 μM (n=7)			
serotonin ($10^{-5.5}$M)	100	100	
relaxation	49±7.1	–15±8.5	<0.001
serotonin + $BaCl_2$	100	100	
relaxation	62±8.7	3±8.13	<0.001
Glibenclamide 10 μM (n=7)			
serotonin ($10^{-5.5}$M)	100	100	
relaxation	49±7.1	–15±8.5	<0.001
Serotonin + glibenclamide	100	100	
relaxation	62±8.7	3±8.13	<0.001

Tested – PVAT added, Control – no PVAT. p value from t-test in comparison to the control. PVAT – perivascular tissue, 4-AP – 4 aminopyridine.

Table 2. The effect of potassium channels inhibitors on basal tension of human ITA.

	Tension change (mN)	p
Iberiotoxin 100 nM (n=14)	0.14±0.14	0.3
Apamin 1 μM (n=14)	0.1±0.3	0.8
4-aminopyridine 1 mM (n=14)	12.3±6.25	0.07
4-aminopyridine 5 mM (n=14)	2.5±0.88	0.01
$BaCl_2$ 100 μM (n=14)	1.7±2.65	0.5
Glibenclamide 10 μM (n=14)	–0.7±0.21	0.01

P value from paired t-test in comparison to pretreatment; positive value indicates contraction, minus value indicates relaxation. Data pooled from every application of proper K^+ channel inhibitor (protocol 2A and 2B).

The ITA relaxation induced by PVAT was inhibited with large conductance Ca^{+2} dependent K^+ channels (BK_{Ca}) blocker - iberiotoxin (100 mN) (*Fig. 4*). Neither the inhibition of small conductance Ca^{+2} dependent K^+ channels (SK_{Ca}) with 1 μM of apamin (*Fig. 5*), voltage dependent K^+ channels (K_v) with 1 mM of 4-aminopyridine (*Fig. 6*), ATP dependent K^+ channels (K_{ATP}) with 10 μM of glibenclamide (*Fig. 9*) or inward-rectifier K^+ channels (K_{ir}) with 100 μM of $BaCl_2$ (*Fig. 8*) influenced the ITA relaxation to PVAT. When 4-aminopyridine was used in the concentration of 5 mM it significantly diminished PVAT dependent relaxation (*Fig. 7*).

DISCUSSION

Results of the present study firstly show that the human ITA's PVAT releases soluble potent relaxing factor. This is concordant with the results of our previous work when we proved that PVAT releases a substance that significantly attenuates contractile response to serotonin and angiotensin II (1). Comparing to our previous study when we transferred only the tissue here we clearly demonstrated that also the solution from incubation with human ITA's perivascular tissue is a potent vasorelaxant. The relaxing response is similar regardless of transferred tissue or

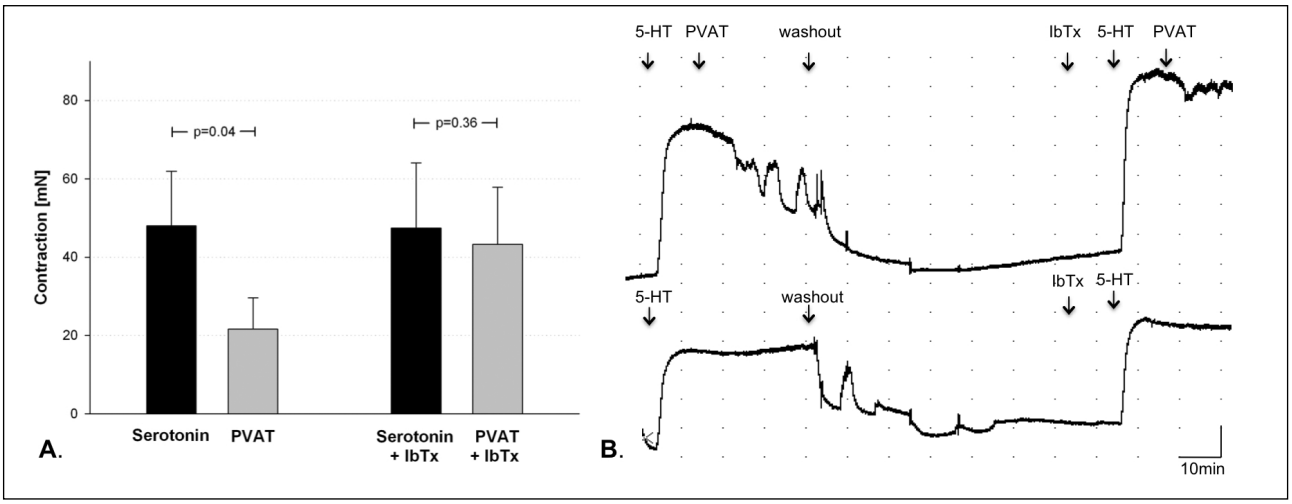

Fig. 4. (*A*) The influence of inhibition of large conductance Ca^{+2} dependent K^+ channels with iberiotoxin (100 nM) on the PVAT dependent relaxation of internal thoracic artery. The contraction response is presented as mean ±S.E.M. p value from paired t-test. (*B*) The example of original recording of the experiment. PVAT – perivascular adipose tissue, IbTx – iberiotoxin.

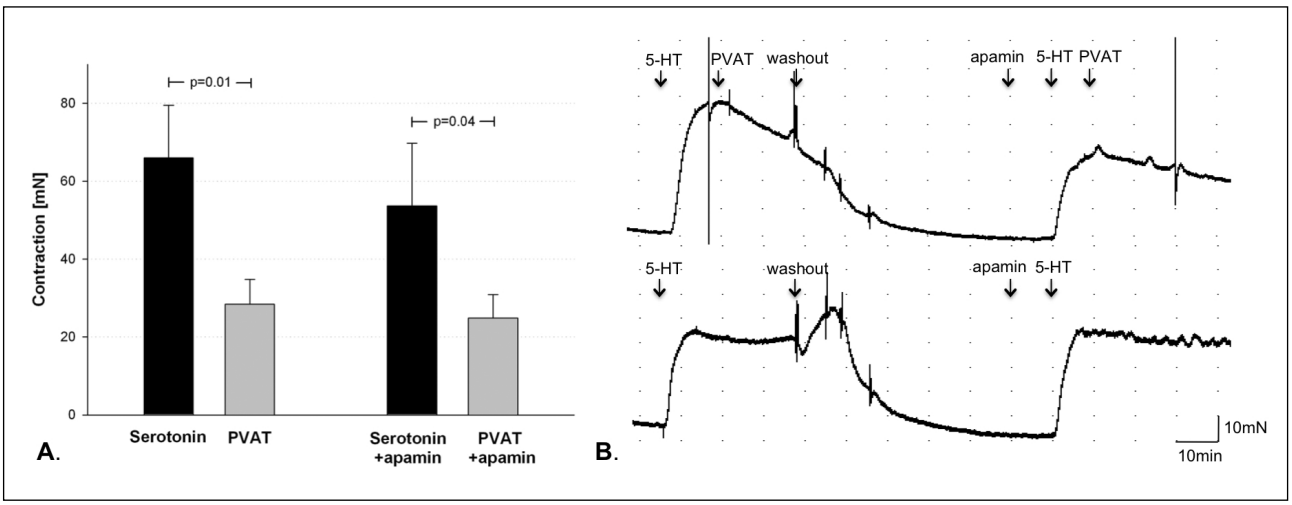

Fig. 5. (*A*) The influence of inhibition of small conductance Ca^{+2} dependent K^+ channels with apamin (1 μM) on the PVAT dependent relaxation of internal thoracic artery. The contraction response is presented as mean ± S.E.M.; p value from paired t-test. (*B*) The example of original recording of the experiment. PVAT – perivascular adipose tissue.

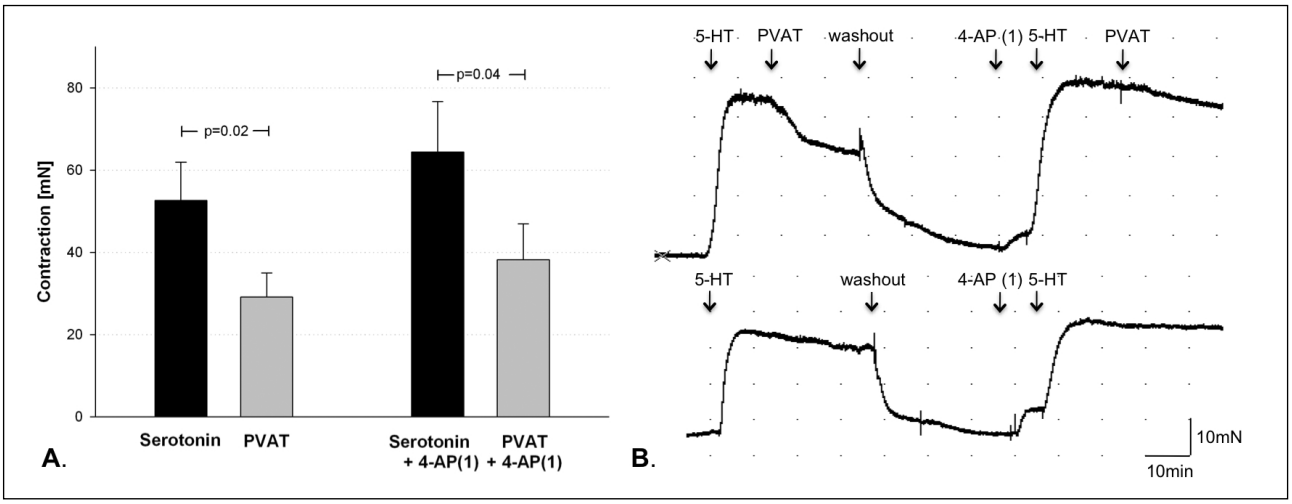

Fig. 6. (*A*) The influence of inhibition of voltage dependent K^+ channels with 4-aminopyridine (1 mM) on the PVAT dependent relaxation of internal thoracic artery. The contraction response is presented as mean ± S.E.M.; p value from paired t-test. (*B*) The example of original recording of the experiment. PVAT – perivascular adipose tissue, 4-AP – 4-aminopyridine.

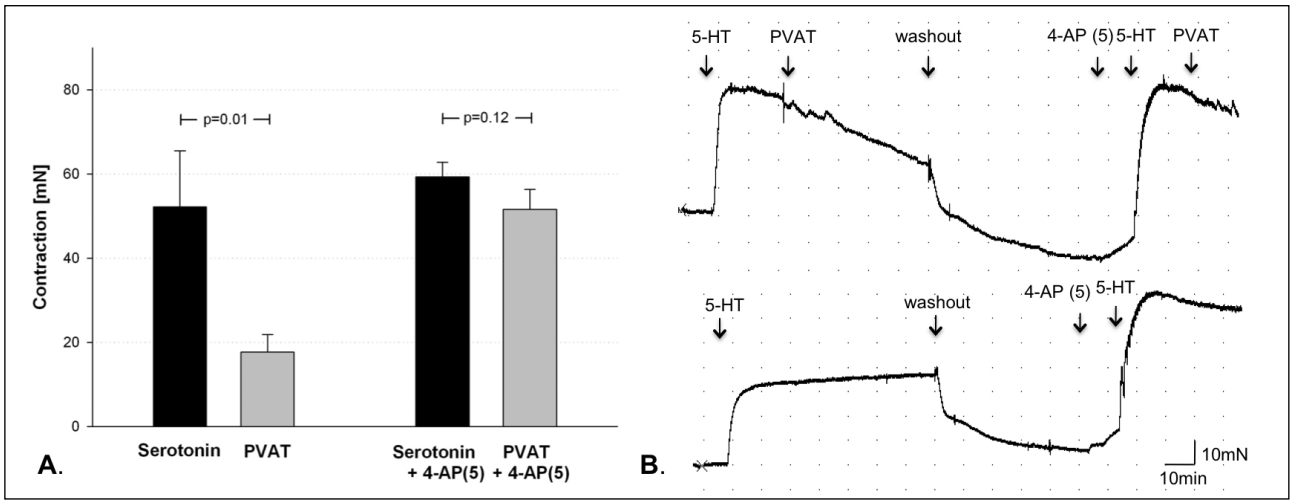

Fig. 7. (*A*) The influence of inhibition of Ca^{+2} dependent potassium channels K^+ channels with 4-aminopyridine (5 mM) on the PVAT dependent relaxation of internal thoracic artery. The contraction response is presented as mean ± S.E.M.; p value from paired t-test. (*B*) The example of original recording of the experiment. PVAT – perivascular adipose tissue, 4-AP – 4-aminopyridine.

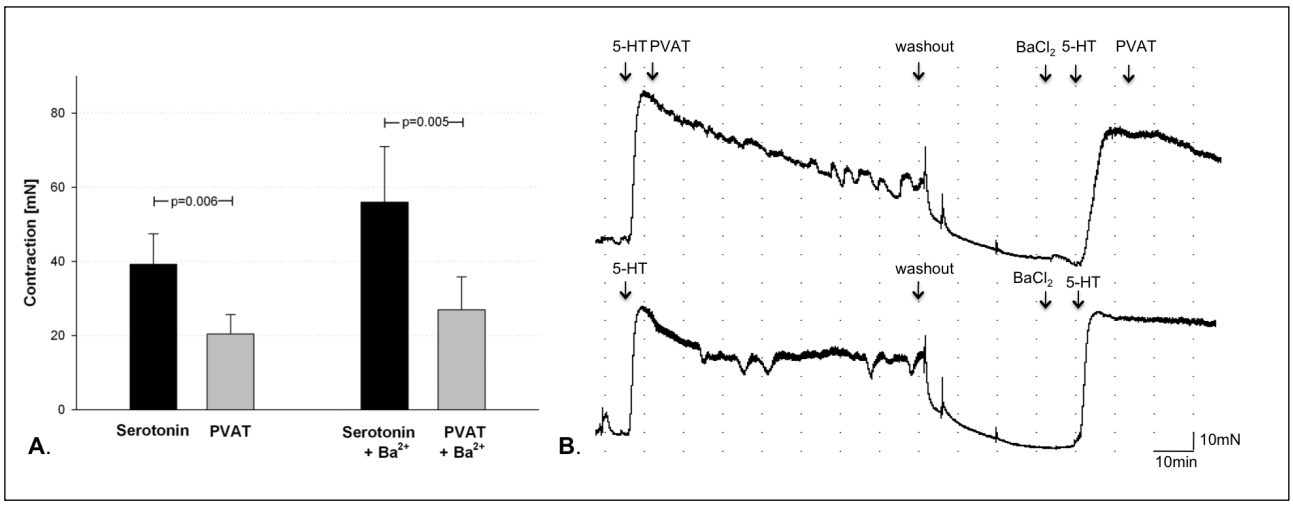

Fig. 8. (*A*) The influence of inhibition of inward-rectifier K^+ channels with $BaCl_2$ (100 μM) on the PVAT dependent relaxation of internal thoracic artery. The contraction response is presented as mean ± S.E.M.; p value from paired t-test. (*B*) The example of original recording of the experiment. PVAT – perivascular adipose tissue.

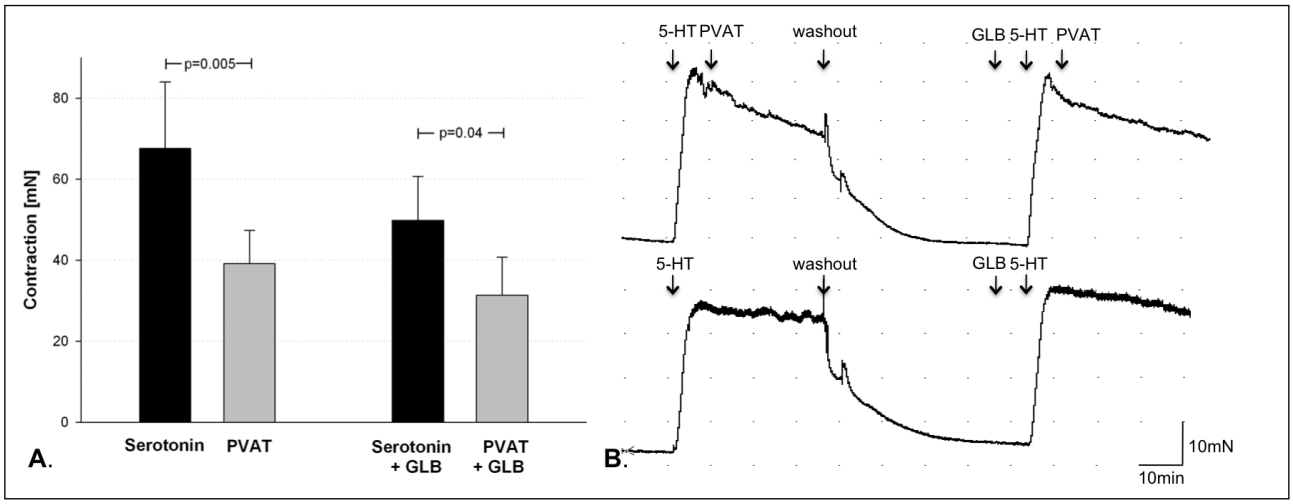

Fig. 9. (*A*) The influence of inhibition of ATP dependent K^+ channels with glibenclamide (10 μM) on the PVAT dependent relaxation of internal thoracic artery. The contraction response is presented as mean ± S.E.M.; p value from paired t-test. (*B*) The example of original recording of the experiment. PVAT – perivascular adipose tissue, GLB – glibenclamide.

solution. We previously also showed that the relaxation to PVAT is NO and PGI_2 independent (1). In this sequel work we aimed to further evaluate the mechanisms of relaxing properties of this substance. We concentrated on K^+ channels, as it was suggested that they may play a crucial role in relaxing properties of PVAT derived factor (2, 4). The initial interest in the role of K^+ channels was based on the works that showed that high extracellular concentration of K^+ ions diminished the relaxing properties of ADRF and, also that PVAT elicits hyperpolarization of vessel smooth muscle cell membrane (2, 4, 8).

In our study only iberiotoxin and 4-AP in the concentration of 5 mM diminished relaxing properties of PVAT-derived relaxing factor. Iberiotoxin is a specific inhibitor of large conductance Ca^{+2} dependent K^+ channels (BK_{Ca}). The role of BK_{Ca} in the mechanisms of action of ADRF on ITA was first observed by Gao *et al*. (2). The authors showed the ARDF was blocked by both iberiotoxin and less specific BK_{Ca} inhibitor - tetraethyloamonium (TEA). Similar to our results they found that relaxation to PVAT is not susceptible to neither 4-AP (1 mM) nor glibenclamide. The contribution of K_{Ca} channels blocked by TEA in the relaxation induced by aliquots from PVAT(+) vessels was also proved in wild-type mice aorta (7). On the contrary, in the model of isolated mice mesenteric vascular bed the inhibition of BK_{Ca} with both TEA and iberiotoxin was found ineffective (9).

The small conductance Ca^{+2} dependent potassium channel (SK_{Ca}) blocked with apamin is not a mediator of relaxing properties of PVAT in our model. However its role was presented by Gao and colleagues when PVAT from aortic rings from Wistar rats was studied (10). In the very first paper about relaxing mechanisms of ADRF Lohn *et al*. found that ADRF dependent relaxation of rat aorta was partially mediated by K_{ATP} channel modulated by glibenclamide (3 μM) and cromakalim (300 nM) (4). Nevertheless, the next works with glipizide (10 μM) did not confirm K_{ATP} channel involvement in the PVT dependent relaxation of rat aorta (10). Based on our results and similar results from Gao *et al*. K_{ATP} channel's role in this relaxation in human ITA seems unlikely.

The next K^+ channel that was tested as a potential mediator of PVAT dependent relaxation was voltage dependent K^+ channel (K_V). Its role in anticontractile effect of PVAT was discovered in rat mesenteric arteries (8). The molecular structure of these channels has not been finally described yet. However it is known that they are different in various vascular beds and their properties depend on their subunits conformation (12, 13). K_V function in PVAT dependent relaxation was analyzed based on pharmacological inhibition and measurement of intracellular membrane potential that does not allow for precise description of which K_V was involved. The concentration of 4-AP needed for K_V inhibition ranges from 0.2–1.1 mM (14). In this range the relaxation obtained with ADRF was blocked in rat (6, 8) and mouse mesenteric artery (9). Pharmacological K_V blockade was not observed to be involved in the human ITA relaxation to PVAT in the research by Gao *et al*. (2). This is concordant to our results.

The interesting finding of our research is that ARDF was inhibited by high (5 mM) concentration of 4-AP. This chemical has various inhibition properties depending on its concentration. In high concentration used by us it is a non-selective K_{Ca} channel inhibitor (15, 16). The inhibition in this concentration is agreeable to the inhibition obtained with iberiotoxin. Rogers *et al*. have discovered that 3 mM of 4-AP blocks vessels relaxation obtained with H_2O_2 (17). Similar results were observed in human ITA when the authors used the same concentration as we did (5 mM) (18). Having in mind the suggestions that H_2O_2 may mediate the ADRF dependent relaxation (10), we cannot exclude that in our model it may be the factor that is partially responsible for vasorelaxation blocked by 5 mM 4-AP. Moreover it may be the case because H_2O_2 was proved to be a vasorelaxant of vessel wall through BK_{Ca} channels inhibited also by iberiotoxin (19, 20).

The least known potassium channel in vascular wall is inward rectifier K^+ channel (K_{ir}). It is activated by the increase of extracellular concentration of K^+ and hyperpolarization of the membrane (21). Barium ions inhibit K_{ir} in arterial smooth muscle (14). We, similar to the others (9), were not able to find the involvement of K_{ir} in PVAT dependent relaxation.

As discussed above, the diversity of various K^+ channels involved in PVAT depending vessel relaxation is significant. It depends on the animal species and different vascular beds tested. The lack of universal mediator causing relaxation to PVAT may suggest that ADRF is not one substance but a group of similar vasorelaxants. This hypothesis however still needs to be verified. The nature of PVAT derived relaxing factor is not known. Recently palmitic acid methyl ester (PAME) was suggested to act as PVAT derived relaxing factor (22). Its vasorelaxation is mediated by opening K_V channels blocked by 4-AP (2 mM) and TEA (5 and 10 mM) but not iberiotoxin. PAME induced relaxation was analyzed in Wistar Kyoto rat so it stll needs to be tested in human vessels.

In conclusion, our results confirm that human internal thoracic artery releases from its perivascular adipose tissue a transferable relaxing factor. This factor reveals its relaxing properties through the involvement of large conductance Ca^{+2} dependent K^+ channels. However the precise mechanism and nature of PVAT derived relaxation in human ITA still needs to be verified. Similarly the precise role of ADRF in clinical practice and cardiovascular pathology remains unknown especially in the context of "brain - adipocyte" axis (23).

Limitations of the study

The obvious limitation of our study stems from the fact that we studied human tissue coming from the patients subjected to cardiac surgery. As a consequence, we have to accept a myriad of various factors that might influence vascular function like various disease state and therapy, consisting of varying agents in many dosages. The other obvious limitation includes the limitation of isolated organ bath set-up used. In organ bath studies all vessel surfaces are exposed to the drugs used. This may complicate instances where both intimal and adventitial surfaces may be involved in drug responses. Additionally, we cannot exclude that the behaviour of a vessel segment with PVAT removed, would be expected to be altered when compared with conditions *in vivo*, simply due to altered 'biomechanics'.

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Conflict of interest: None declared.

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