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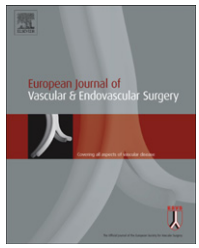

Exercise Linked to Transient Increase in Expression and Activity of Cation Channels in Newly Formed Hind-limb Collaterals

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Abstract *Objective:* This study aimed to compare arteriogenesis after femoral artery occlusion as influenced by exercise or arteriovenous shunt and follow changes in collateral transient receptor potential cation channel, subfamily V, member 4 (Trpv4).
Design: A prospective, controlled study wherein rats were subjected to femoral artery ligation (FAL), or FAL + arteriovenous shunt. Collateral Trpv4 was determined 0.5 and 6 h post exercise.
Methods: Rats were subjected to exercise for 15 min, twice daily. The number and diameter of collaterals were assessed after 7 days. Collateral Trpv4 expression was quantified by reverse transcription-polymerase chain reaction.
Results: Collateral number and diameter per limb were significantly higher in the shunt group (number: 16.0 ± 2.4 and diameter: 216.0 ± 34 μm) compared to the ligature (number: 9.4 ± 2 and diameter: 144 ± 21 μm) and exercise groups (number: 9.9 ± 2.5 and diameter: 151 ± 15 μm). Trpv4 expression in collaterals harvested 0.5 h post exercise was not significantly different from expression in shunted rats. It was significantly lower in collaterals harvested 6 h post exercise (comparable to that in ligated rats).
Conclusion: Collateral formation was greater in the shunt group than in the exercise group. Exercise-induced Trpv4 up-regulation, not significantly different from that achieved with shunt, returned to control values when evaluated 6 h post exercise. More frequent exercise to chronically increase fluid shear stress, as with a shunt model, may be required for sufficient arteriogenesis to compensate for peripheral occlusion.

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Introduction

Occlusive vascular disease remains the most important cause of worldwide death and morbidity. Treatment aims towards limb salvage, improvement of the patient's quality of life and mortality reduction and includes re-vascularisation (open or endovascular), drug therapy and exercise training.[1] In some cases, re-vascularisation is not feasible or fails, and other palliative measures, including limb amputation, may be required. Although collateral vessels bypassing an occlusion spontaneously develop in patients with ischaemic vascular disease,[2] this collateral growth insufficiently compensates for the deteriorated blood flow. Great efforts have been made in the last years in developing therapeutic approaches to stimulate pre-existing compensatory blood vessels, a process called arteriogenesis.[3] The most important triggers are physical forces, such as altered shear force, caused by the large pressure difference in the pre-existing arterioles connecting upstream with downstream branches (relative to the occlusion point) as the result of arterial occlusion.[4–6]

Redirecting the blood flow — after leaving the collateral network — directly to the venous system by creating an arteriovenous (AV) anastomosis between the distal stump of the occluded femoral artery and the accompanying vein increases flow in the collateral network with a subsequent increase in fluid shear stress (FSS).[7] In a pig and rabbit hind-limb arterial occlusion model where the collateral blood flow was directly diverted into the venous system, leading to chronically elevated FSS in the collateral system, collateral growth was markedly stimulated.[8] This new shear stress model was transferred to the rat in which genome-wide analysis is possible, whereas in the rabbit it is not.

Transient receptor potential cation channel, subfamily V, member 4 (Trpv4) has been identified as a 'shear stress regulated' channel, the activation of which triggers collateral growth and remodelling. mRNA abundance of Trpv4 is constantly up-regulated in FSS-stimulated collaterals and an elevated protein expression during collateral growth has been localised to the FSS-sensing endothelium.[9]

Exercise clearly plays a role in the treatment of patients with claudication, improving symptoms and walking ability.[10] The capability of exercise to alter FSS with the subsequent effect on Trpv4, an initial mediator of arteriogenesis, is of particular interest. This study aimed to investigate collateral development and gene expression of the FSS-regulated channel Trpv4 after exercise training or AV shunt in rats.

Materials and methods

This study was performed according to Section 8 of the German Law for the Protection of Animals, which conforms to the US National Institutes of Health (NIH) guidelines.

Adult male Sprague-Dawley rats were subjected to femoral artery ligation (FAL). FAL was followed either by AV shunt formation between the distal femoral artery stump and accompanying vein (Fig. 1) or by exercise training. Trpv4 was determined 0.5 h and 6 h post exercise, after 7 days of training. Rats with FAL, with no shunt or exercise training, were termed the ligature group. In six rats, an AV shunt was re-occluded 1 day prior to tissue harvesting and used for quantification of gene expression (shunt re-occlusion group). The number of values for each group for collateral number and size were ligature-14, shunt-10, and exercise-12. The number of values for each group for Trpv4 expression were shunt-11, exercise early (harvested 0.5 h post exercise)-6, exercise late (harvested 6 h post exercise)-6, shunt re-occlusion-6 and ligature-7.

Femoral artery ligature

Anaesthesia was induced by administering ketamine hydrochloride (100 mg per kg of body weight) and xylazine

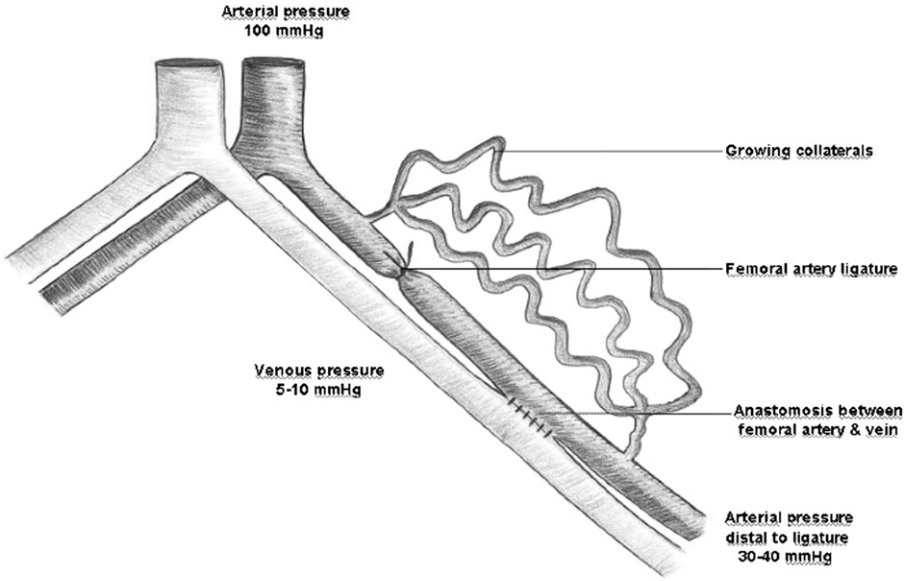

Figure 1 Shunt model. An anastomosis is made between the femoral artery and vein and the femoral artery is ligated proximal to the anastomosis.

(4 mg kg^{-1}) intra-peritoneally. The femoral arteries were exposed and ligated below the inguinal ligament with a 3/0 silk ligature. Carprofen (Rimadyl 5 mg kg^{-1}) was given subcutaneously to prevent pain immediately post-operatively and on the first postoperative day.

Shunt model

Anaesthesia was performed as described above. The femoral artery and vein were exposed and a side-to-side anastomosis with 10/0 ethylon under ×10 magnification was constructed. The femoral artery was ligated proximal to the anastomosis. As a result, blood distal to the occlusion is directed to the venous side. The persistently low pressure leads to a high-pressure gradient across the occlusion site. AV shunt function was confirmed by Doppler examination, identifying the typical holosystolic—diastolic flow signal.

Exercise training

Before operation, the rats were familiarised with a treadmill (self-constructed belt type) by running for 15 min 1 day prior to ligation. Exercise was started on the first day after FAL with a 15° ascending slope belt speed of 15 m min^{-1} for 15 min twice daily. Starting from the second day, each rat ran at a belt speed of 25 m min^{-1} for 15 min twice daily or until the fifth dropout.

On the seventh day, collaterals were assessed in all groups by post-mortem angiogram. Collaterals were harvested from the rats' hind-limbs, mRNA was isolated and gene expression analysis of Trpv4 was performed.

Post-mortem angiograms and tissue harvesting

To quantify visible collaterals, post-mortem angiograms were performed on the seventh postoperative day. Homogenous contrast medium (CM) composed of 80 g gelatin, 200 g barium sulphate and 200 ml distilled water was prepared and kept at 4 °C until use. The anaesthetised animals were euthanised under anaesthesia by bleeding, using an approved protocol. The aorta was exposed, cannulated and pre-warmed CM (37 °C) was injected at 80 mmHg pressure. The inferior vena cava was opened, and the animals were placed in a water bath at 38 °C to prevent hardening of CM and vasoconstriction of the vessels. After the leg arteries were filled with CM, the animals were placed in crushed ice for 10 min to harden the CM and angiograms were prepared. Visible collaterals were counted and their diameters measured (Fig. 2). According to the Longland classification,[11] only collaterals >0.1 mm diameter were counted with visible stem, middle and re-entry zones.

The collateral tissue visualised by the CM was dissected from the surrounding muscle as described.[12] In brief, after contrast-agent hardening, collaterals spanning tissue from the deep femoral artery to the distal branches of the superficial femoral artery were dissected free and sampled, according to the classification of Longland, into stem, midzone (the tortuous portion) and re-entry. Only midzones were used for expression studies.

RNA isolation, amplification and quantitative real-time polymerase chain reaction

Total RNA was isolated (RNeasy Mini kit, Qiagen) from collateral wall tissue on the seventh postoperative day in all groups. In the exercise group, collateral wall tissue was harvested either early (0.5 h) after training ($n = 6$) or later (6 h) after training ($n = 6$). Gene-specific real-time polymerase chain reaction (RT-PCR) primers (Trpv4: forward: acg gtg gac tac ctg agg ctg g; reverse: tgg aag gag cca tcg acg aag; Pecam: forward: tcg aac cgc atc tcc aag gcc ag; reverse: tta gct aca ggc gca ccc gag a; eNOS: forward: agc gct gat gga aat gtc ggg cc, reverse: cgc caa gag gat acc agt ggg tc; Vegfa: forward: agt gcc aac cag atg gca gcc tg; reverse: tgc ca acct gga cgg cag ttg c; Vegfc: forward: ccg tct aca gat gtg ggg gtt gc; reverse: tgt gac tgg ttt ggg gcc ttg ag) were selected using FastPCR software (Institute of Biotechnology, University of Helsinki, Helsinki, Finland). cDNA was synthesised according to the manufacturer's protocol (Invitrogen). qRT-PCR was performed in a 25-μl reaction, 96-well format 1 μl cDNA (1:20); 200 nM each primer; 1X IQ SYBR Green Super Mix (BioRad) using a CFX96 RT-PCR system (BioRad). Samples were measured in triplicate, with a minimum of two independent experiments. The relative amount of target mRNA normalised to 18S was calculated according to the method described by Pfaffl.[13]

Shunt re-occlusion

To quantify gene expression of Trpv4 after abolishing the chronically elevated FSS in the shunt model, shunts were re-occluded in one group after 6 days. mRNA isolation was done 1 day later (seventh day from shunt creation), to demonstrate the effect of absent high FSS on the previously up-regulated gene. To observe the effect of shunt re-occlusion on vessel growth, rats were either subjected to

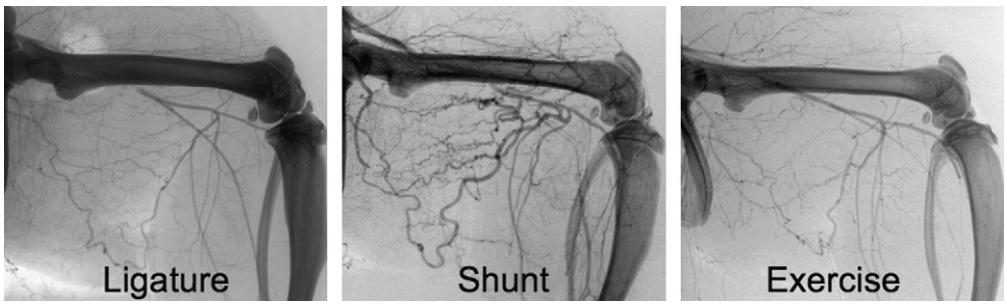

Figure 2 Post-mortem angiograms of rat hind-limbs after the indicated treatment.

14 days of FAL, 14 days shunt or 7 days shunt + 7 days re-occlusion. After 14 days the number of visible collateral vessels was determined in all groups.

Statistics

All values are expressed as mean ± standard deviation (SD). Statistics were calculated using the unpaired *t*-test with Bonferroni adjustment for multiple comparisons. *P*-value <0.05 was considered to represent statistically significant differences.

Results

No animals were lost due to surgery or postoperatively. No clinical signs of peripheral ischaemia were present in any animal. In the shunt group, only animals with a functional shunt were included in the study.

Post-mortem angiograms

Preliminary experiments evaluating post-mortem angiograms showed an approximate doubling of hind-limb collaterals after ligature. Therefore, ligated rats were used as the control group instead of sham-operated rats. A marked increase occurred in the number of visible collaterals per limb in the shunt group (16.0 ± 2.4) compared to the ligature group (9.4 ± 2.0, $p < 0.05$). There was no difference in the number of visible collaterals between the exercise group (9.9 ± 2.5) and the ligature group as shown in Figure 3A. The average diameter of collaterals was also markedly increased in the shunt group (216 ± 34 μm) compared to the ligature group (144 ± 21 μm, $p < 0.05$) as shown in Figure 3B. There was no significant difference between average collateral diameter in the exercise group (151 ± 15 μm) compared to the ligature group.

Gene expression: analysis of Trpv4

To further elucidate the lack of exercise-induced improvement in collateral diameter and number, we determined the mRNA profile of FSS-dependent endothelial genes from collaterals dissected 0.5 and 6 h post exercise (Fig. 4A). Neither eNOS, Pecam (CD31), Vegfa nor Vegfc showed a significant alteration in mRNA abundance when compared early and late after exercise. The only sensitive marker for FSS after exercise was Trpv4 (Fig. 4B).

mRNA quantification, as shown in Figure 4C, revealed 3.5-fold ± 0.5 up-regulated Trpv4 in the shunt group compared to the ligature group ($p < 0.05$). There was no significant difference in the up-regulation of gene expression between the shunt group and the exercise group when collaterals were harvested 0.5 h after exercise. However, a return to the expression level seen in the ligated rats was apparent 6 h after exercise.

To mimic a decline in FSS, shunts were re-occluded and mRNA isolated 1 day later. Gene expression of Trpv4 dropped significantly, showing that Trpv4 is, indeed, related to an increase in FSS, confirming that Trpv4 expression is only sustained as long as the FSS persists. The effect of re-occlusion of the shunt on vessel growth was evaluated after 14 days of FAL, 14 days of shunt and 7 days shunt + 7 days re-occlusion. As shown in Figure 4D, the number of visible collateral vessels after 14 days in the re-occluded animals (20.2 ± 2.1) was significantly reduced when compared to the shunt group (28.5 ± 4).

Discussion

The amount of exercise required of the rats in the current study caused no significant increase in the number and size of collaterals when compared to ligature. In a previous shunt model (pig) with increased FSS, marked collateral remodelling occurred with a significant increase in the number of collaterals in shunted compared to ligated limbs (13.4 ± 1.4 vs. 5.6 ± 0.6, $p < 0.001$).[13] This compares well with the current study showing a significant increase in both number and diameter of collaterals after AV shunt compared to ligation. In another study with more prolonged exercise training (25 days), collaterals in FAL-exercised rats showed a statistically significant increase in size compared to FAL-sedentary rats.[14]

At the molecular level, it has previously been shown that exercise may exert its beneficial effect on blood flow by influencing endothelial function[15] or muscle metabolism, morphology, blood rheology, development of atherosclerosis, walking economy, pain perception and cardiac adaptation.[16] Exercise's relation to FSS, the most influential factor in arteriogenesis, was not clear. With each exercise session, increased heart rate, blood pressure and myocardial contractility plus a transient increase in shear stress occurs, due to increased blood flow. Whether exercise induces similarly extensive changes in shear-dependent gene

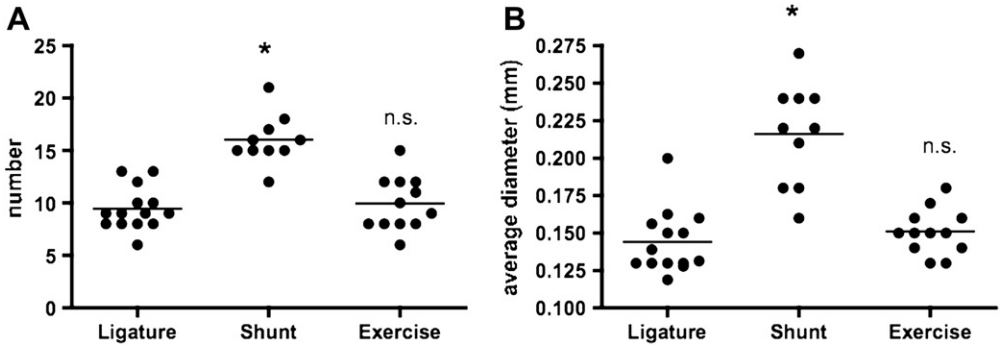

Figure 3 A. Number of visible collaterals. B. Average diameter of visible collaterals. Values are means ± SD. n.s. = not significantly different from ligature. *$p < 0.05$ with Bonferroni adjustment, compared to ligature.

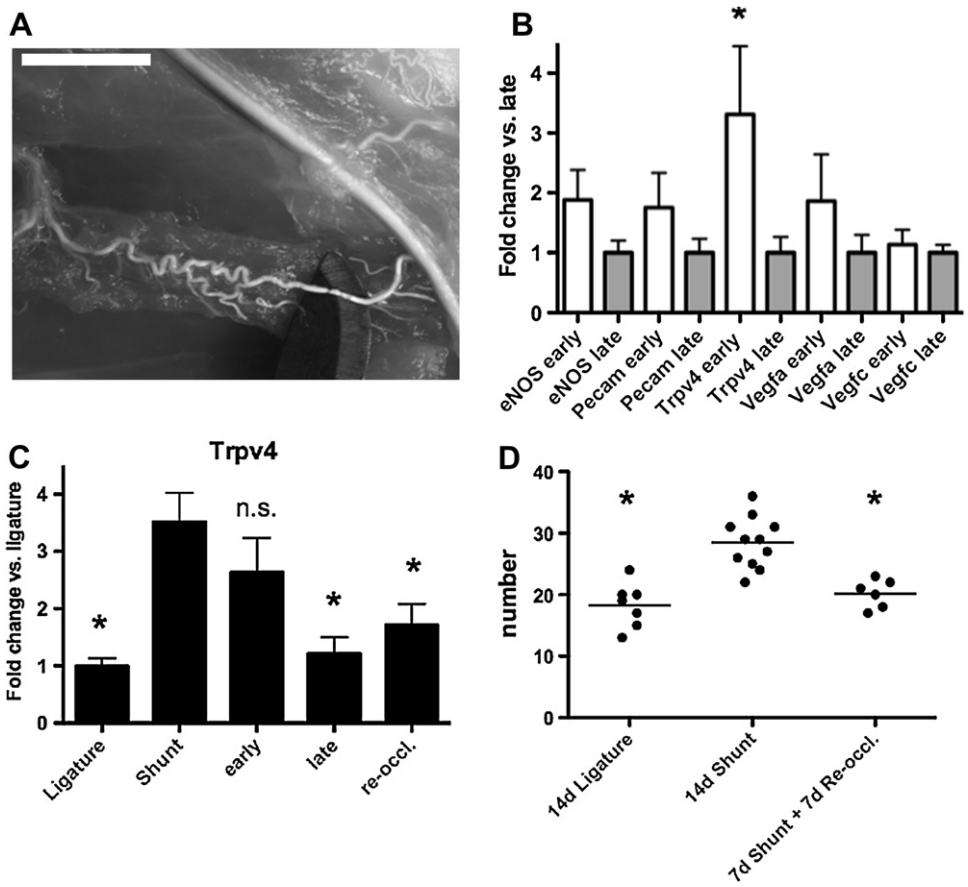

Figure 4 A. Superficial adductor muscle collateral arteries as visualised under the stereo-microscope. Collateral midzones were dissected from the surrounding muscle and subjected to RNA isolation. B. Relative mRNA abundance of selected endothelial markers in collateral arteries after exercise. C. Relative expression of Trpv4 as detected by real-time PCR (ligature group set at 1). Exercise early and late correspond to collateral harvest 0.5 and 6 h post exercise, respectively. D. Number of visible collateral arteries 14d after the indicated treatment. Values are means $\pm$ SD. n.s. = not significantly different from shunt. *$p < 0.05$ with Bonferroni adjustment, compared to shunt.

expression was unclear, since exercise not only increases shear but also intermittently changes the frequency and magnitude of all physical forces in the vascular system.[17]

Trpv4 is a calcium (Ca) channel, activated by osmotic pressure changes, temperature and FSS changes exerted on the inner lining of the vascular wall.[18,19] In a recent study, Trpv4 was identified as an initial mediator of physical stimuli to intracellular signals. Microarray data of growing collateral vessels, exposed to chronically elevated FSS in the AV shunt model, showed increased mRNA transcription of this Ca^{2+} channel. Experiments also showed that chronic stimulation of Trpv4 was followed by active proliferation of vascular cells with subsequent collateral remodelling and development. Furthermore, pharmacological activation of Trpv4 by 4α-phorbol 12,13-didecanoate causes a strong increase in collateral blood flow.[9]

Since Trpv4 is an important initial mediator for FSS-induced arteriogenesis, we studied gene expression of its mRNA in exercised FAL-rats compared to FAL-shunt rats. We aimed to determine whether alterations of FSS during exercise are sufficient to affect Trpv4 gene expression.

Trpv4 was highly up-regulated in the shunt group when compared to the ligature group (3.5-fold), indicating increased FSS in the shunt model, and explaining the marked increase in collateral number and diameter in the shunt group. In the exercise group, however, there was a significant difference in gene expression when tissue was harvested early (0.5 h) or late (6 h) after exercise (Fig. 4C). A significant decrease was apparent compared to shunt in the collaterals harvested late but no significant difference was noted in the collaterals harvested early. This shows that exercise-induced up-regulation is transient.

When the chronically elevated FSS in the shunt model was abolished by re-occluding the shunt, and mRNA isolated 1 day later, down-regulation of Trpv4 expression occurred (Fig. 4C). This links Trpv4 expression to FSS,[9,18,19] as has previously been established. In addition, the decline of FSS results in significant deceleration of collateral growth.

Many studies have investigated vascular endothelial growth factor (VEGF) expression as a possible molecular explanation for the effect of exercise on blood vessels. They have shown that exercise training results in increased VEGF secretion in hypoxic tissue and increased VEGF receptor expression,[20–22] and induces enhancement of the peripheral uptake of VEGF.[23] However, VEGF is

involved in exercise-induced skeletal muscle angiogenesis[24]and not arteriogenesis.[25] We did not examine the effect of exercise on VEGF, as arteriogenesis is the process potentially able to fully replace an occluded artery, whereas angiogenesis cannot.[26]

In a recent review by Duncker and Bache concerning the regulation of cardiac blood flow during exercise training, it was shown that exercise training does not stimulate growth of coronary collateral vessels in the normal heart. However, if exercise produces ischaemia, which is absent or minimal during rest, there is evidence that collateral growth can be enhanced. In addition to ischaemia, the pressure gradient between vascular beds, which is a determinant of the flow-rate and, therefore, the shear stress on the collateral vessel endothelium, may also be important in stimulation of collateral vessel growth.[27] We believe that the same relationship between exercise training and growth of collateral vessels exists in hind-limb blood flow. However, it seems limited to the time during exercise and, directly thereafter, not persistent and chronic as in the shunt model. While the increase in FSS in the shunt group was responsible for the marked increase in number and size of collaterals in this group, the exercise-induced increase in FSS was transient, failing to produce a long-lasting increase in Trpv4 expression. These data support data of other investigators. Prior et al. stated that longer exercise duration is needed to induce sufficient arteriogenesis. High-flow demand through conduit arteries has been shown to remodel them with larger, thicker walls.[28] Long-term adjustments including recruitment and growth of collateral vessels in response to arterial occlusion have been shown to be time dependent, while rapid short-term adjustments result from active vasomotor activity of the collateral vessels.[27]

The beneficial effects of exercise are not restricted to arteriogenesis. Increased vascular compliance and anti-oxidative capacity may contribute to the atheroprotective effects associated with physical exercise in conduit vessels.[29] Improvement of endothelium-dependent vasodilatation in response to flow or acetylcholine infusion is well established after long-term physical activity. A shear stress- and hydrogen peroxide-related increase in endothelial nitric oxide synthase expression/activity and a reduction in vascular oxidative stress are the most important mechanisms contributing to this effect.[17] Training alters local control of resistance vessels. Thus, arterioles exhibit increased myogenic tone, likely due to a calcium-dependent protein kinase C signalling-mediated alteration in voltage-gated calcium channel activity in response to stretch.[27] This adds to the importance of the role of Ca^{2+} homeostasis after physical forces have changed within the blood vessel.

Conclusion

A time-limited effect of exercise on Trpv4 provides a relationship between exercise and FSS and subsequent arteriogenesis. In the current study, Trpv4 was transiently up-regulated by exercise, but not chronically up-regulated, as in the steady elevation of FSS in the shunt model. Future experiments with more frequent or more extensive exercise are planned to further elucidate the role of exercise therapy. Based on consistently high levels of Trpv4, as mediator for FSS-induced arteriogenesis, new therapeutic concepts may be developed to treat occlusive vascular disease. It would be of interest to determine when the effect of exercise sets in, how long it must last and what interval between exercise sessions is allowable, to more effectively increase Trpv4 levels.

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Conflict of Interest

None declared.

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