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Brain-derived neurotrophic factor is a full endothelium-derived factor in rats

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Abbreviations:

BDNF, brain-derived neurotrophic factor; eNOS, endothelial nitric oxide synthase; EX, exercise; FL- TrkB, Full length - tropomyosin related kinase B; FSS, fluid shear stress; HPRT, hypoxanthine phosphoribosyl-transferase; NO, nitric oxide; p-eNOS^{Ser1177}, phosphorylated endothelial nitric oxide synthase at serine 1177; p-TrkB^{Tyr816}, phosphorylated-tropomyosin related kinase B at tyrosine 816; SED, sedentary; VSMC, vascular smooth muscle cells; vWF, von Willebrand factor.

Abstract

Most of what is known on vascular brain-derived neurotrophic factor (BDNF) derived from experiments on cultured endothelial cells. Therefore, the present study compared BDNF levels/localization in artery (aorta) vs vein (vena cava) from a same territory in rats either sedentary (SED) or exposed to treadmill exercise (EX) as a mean to stimulate endogenous endothelial nitric oxide (NO) production. In SED rats, for both artery and vein, BDNF was strongly expressed by endothelial cells, while only a faint and scattered expression was observed throughout the media. Endothelial and muscular BDNF staining as vascular BDNF protein levels were however higher in artery than in vein, while BDNF mRNA levels did not differ between vessels. Irrespective of the vessels, EX resulted in an increase (+50%) in BDNF protein levels with no change in BDNF mRNA levels, a selective endothelial BDNF overexpression (x4) and an increase in vascular levels of tropomyosin related kinase B receptors (TrkB) phosphorylated at tyrosine 816 (p-TrkB^{Tyr816}). Endothelial expressions of BDNF and p-TrkB^{Tyr816} were positively associated when SED and EX rats were simultaneously examined. The results incite to consider endothelial BDNF as a full and NO-dependent endothelium-derived factor that exerts autocrine effects.

Key words: BDNF, endothelium, artery, vein, exercise

1. Introduction

The neurotrophin brain-derived neurotrophic factor (BDNF) was discovered in the brain where activity-dependent release of neuronal BDNF plays a key role in neuroplasticity, learning, memory and cognition through phosphorylation at tyrosine 816 of neuronal full-length (FL) tropomyosin related kinase receptor (p-TrkB^{Tyr816})¹. However, cultured endothelial cells, either cerebral or peripheral, express and secrete BDNF under a bioactive form²⁻⁴. It was even reported that the rate of constitutive BDNF secretion by cultured endothelial cells was 50 times higher than that of cultured neurons⁵. More intriguingly, the rat adult cardiovascular system (aorta and heart) contains as much BDNF as the brain⁶ supporting the notion that endothelial-derived BDNF contributes to the regulation of cardiovascular function. Consistently, endothelial cells express FL-TrkB receptors and exposure to exogenous BDNF of cultured endothelial cells or isolated vessels was reported to induce angiogenesis⁷⁻⁹, inhibit leukocytes adhesion¹⁰, increase resistance to pro-apoptotic stimuli¹¹ and induce relaxation^{6,12,13}. *In vivo*, TrkB activation has also been suspected to protect against atherosclerosis and coronary artery disease¹⁴ while decreased endothelial BDNF expression results in a greater propensity to arterial thrombosis¹⁵.

Exercise (EX) is one of the most efficient strategies to improve cardiovascular health, likely as a result of fluid shear stress (FSS)-dependent increase in endothelial nitric oxide (NO) production. Indeed, NO is an endothelium-derived relaxant factor that also protects against vascular inflammation and atherogenesis¹⁶. Our laboratory revealed that EX resulted in endothelial BDNF overexpression not only in the cerebral microvasculature¹⁷ but also in peripheral conduit artery⁶. Furthermore, we showed that spontaneously hypertensive rats exhibited low aortic BDNF levels as compared to normotensive rats and that EX alleviated the decrease⁶. Given that NO and BDNF share common vascular effects, it is tempting to suggest that some effects of NO are BDNF-dependent and by extension that endothelial BDNF synthesis is controlled *in vivo* by endothelium-derived NO as suspected from *in vitro* experiments¹⁸⁻²⁰. However, *in vitro* experiments do not fully replicate *in vivo* conditions. Moreover, while BDNF was detected in cultured vascular smooth muscle cells (VSMC)^{21,22}, endothelial and muscular BDNF expressions have never been simultaneously investigated. Furthermore, studies designed to compare vascular BDNF in artery *vs* vein are lacking, while both the arterial and venous components of blood circulation benefit from EX.

The objective of the present study was to investigate vascular BDNF levels (protein and mRNA), localization and response to EX used as a strategy to modulate endothelial NO

production. For this purpose, artery and vein of a similar territory (abdominal aorta and vena cava) were collected in rats either sedentary (SED) or subjected to daily (30 min) treadmill activity for 7 consecutive days. To assess the potential autocrine effect of endothelial BDNF and the role of NO in the control of BDNF synthesis, we also investigated the relationship between endothelial BDNF and p-TrkB^{Tyr816} expressions or endothelial NO synthase phosphorylated at serine 1177 (p-eNOS^{Ser1177}) expression.

2. Material and methods

2.1 Animals

Experiments were carried out on 8 week-old male Wistar rats (n = 72) and 11 week-old male CD-1 mice (n = 2) that were purchased from Janvier (Le Genest Saint Isle, France) and the local zootechnical platform, respectively. CD-1 mice were chosen according to similar features with Wistar rats, i.e. large genetic diversity (non-inbred strain) and albinism.

Experiments were conducted according to the french Department of Agriculture guidelines (approval number R-21UB/EPHE-F1-11) and approved by the local ethic committee (agreement 105). All experimental procedures were performed in compliance with ARRIVE guidelines and EU Directive 2010/63/EU. The animals were housed five per cage, kept under a 12/12 light/dark cycle with an ad libitum access to food and water.

2.2 Induction of exercise

After an adaptation period (five days) of all rats to experimenters and treadmill apparatus as previously described in details ¹⁷, animals were then randomized in SED and EX groups (n = 36 each). Rats of EX group were trained 30 min/day in the morning at 18 m/min (~75% of maximal aerobic speed) ²³ for 7 consecutive days. SED rats were kept in their own cage at the vicinity of the treadmill apparatus.

2.3 Collection and preparation of vessel samples

After anaesthesia with chloral hydrate (400 mg/kg, i.p., Sigma-Aldrich) and transcardiac perfusion with saline to remove blood from the vasculature, the abdominal aorta and vena cava were carefully isolated in each rat. Vessels were collected either 2 h (for RT-qPCR analysis) or 24 h (for Western blotting and immunofluorescence analyses) after the last session of treadmill exercise. For RT-qPCR, isolated vessels were successively stored at +4°C

(24 h), -20°C (24 h) in RNA stabilization solution (ref. 76104, Qiagen). Then, the stabilization solution was removed before storage of vessels at -80°C. Total RNA were extracted from vessels using the RNeasy Fibrous Tissue Mini kit (ref. 74704, Qiagen) and RNA concentration was determined using NanoDrop technology. For Western blotting analysis, once isolated, vessels were immediately kept at -80°C until homogenization in 5 volumes of ice-cold lysis buffer [100 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L Ethylene Glycol Tetraacetic Acid, 1% triton X-100, 1% protease inhibitor cocktail (P8340, Sigma-Aldrich), and 1% phosphatase inhibitor cocktail (1066-8304, Fischer Scientific)] using ultra Turrax OMNI TH (1 min) and sonication (15 s). Homogenates were then centrifuged (20 min at 12,000 x g, 4°C) and protein concentration was determined in supernatant using the Lowry method. For immunofluorescence analysis, once isolated, vessels were immediately embedded in OCT (CryoMatrix, 6769006, ThermoShandon) and kept at -80°C until preparation of slices (10 µm-thickness, cryostat MM550 OPV MicromMicrotech, Francheville, France). Slices were collected on SuperFrost plus slides and stored at -80°C. Only thoracic aorta was collected in chloral-anaesthetized mice and prepared for immunohistochemical analysis in the same condition as previously described.

2.4 Investigation of the link between vascular BDNF levels and endothelial NO production

The comparison between vascular BDNF levels in abdominal aorta vs vena cava in baseline conditions (vessels with different internal diameter and exposed to the same blood flow and consequently to different FSS) as well as the comparison of the effect of EX on arterial and venous BDNF levels (each exercise bout results in a similar increase in blood flow in abdominal aorta and vena cava) offered us the opportunity to investigate whether vascular BDNF levels mirror endothelial NO production. Indeed, endothelial NO production is mainly dependent on FSS since FSS induces a magnitude-dependent induction of eNOS phosphorylation at serine 1177²⁴. Importantly, in phosphorylated form, eNOS produces 10 more times NO than the unphosphorylated form²⁵.

2.5 Reverse Transcriptase Quantitative Real-Time Polymerase Chain Reaction

After quantification, 300 ng of RNA were reversely transcribed (5 min at 25°C, 1 h at 42°C, 5 min at 85°C, 4°C) using iScript reverse transcription supermix (BIO-RAD Laboratories, Ivry sur Seine, France). As described in Table 1, sequences of total *bdnf* (all exons of *bdnf* gene were amplified with primer), *enos* (endothelial NO synthase) and housekeeping *hprt*²⁰

(hypoxanthine phosphoribosyl-transferase) genes were obtained from the National Center for Biotechnology Information (NCBI) database while the primer sequences (Eurogentec, Angers) were designed using Primer-Basic Local Alignment Search Tool (Primer-BLAST). Real-Time PCR and quantification of gene expressions were performed as previously described²⁰. The relative transcription level of the gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Gene	Primer sequence	T _m (°C)	Product size (bp)
<i>bdnf</i>	FWD: 5' – ATGGTTATTTTCATACTTCGGTTGCA – 3' RVS: 5' – AGCTGGGTAGGCCAAGTTG – 3'	59.35 59.62	87
<i>enos</i>	FDW: 5' - CACGAGGACATTTTCGGACT – 3' RVS: 5' - ACCTAATGAAGCGACGCAGT – 3'	57.92 59.75	201
<i>hprt</i>	FWD: 5' - GCGAAAGTGGAAAAGCCAAGT -3' RVS : 5' – GCCACATCAACAGGACTCTTGAG – 3'	59.93 61.39	76

Table 1. Primer sequences for real-time RT-PCR analysis

Abbreviations: *bdnf*: brain-derived neurotrophic factor; *enos*: endothelial nitric oxide synthase; *hprt*: hypoxanthine phosphoribosyl transferase; FWD: forward; RVS: reverse; T_m: melting temperature.

2.6 Western blotting

Equal protein amounts were resolved on 8 to 15% SDS-PAGE and transferred using Turbo Transblot technology (ref. 1704150, BIO-RAD Laboratories, Ivry sur Seine, France) to polyvinylidene fluoride (PVDF) membranes. First, PVDF membranes were incubated overnight at 4°C, in Tris Buffer Saline - Tween 20 (0.1%) (TBS-T) containing 7.5% of BSA for eNOS, p-eNOS^{Ser1177} and p-TrkB^{Tyr816}, or 5% of non-fat dry milk for BDNF and β-actin proteins, in order to blockade of non-specific binding sites. Membranes were then incubated in solution containing primary antibody directed against BDNF (1:3000 in TBS-T + 5% of non-fat dry milk, rabbit monoclonal antibody, ab108319, Abcam), p-trkB^{Tyr816} (1:1000 in TBS-T + 10% of BSA, rabbit polyclonal antibody, ABN1381, Millipore), eNOS (1:2500 in TBS-T, mouse monoclonal antibody, 610297, BD Biosciences), p-eNOS^{Ser1177} (1:1000 in TBS-T + 10% of BSA, mouse monoclonal antibody, 612393, BD Biosciences) or β-actin (1:10000 in TBS-T, A5441, Sigma-Aldrich) for 3 h at room temperature (RT). Then, PVDF

membranes were incubated (RT) in TBS-T containing secondary antibodies conjugated with horseradish peroxidase anti-IgGs (1:30000 to 1:50000 according to the protein, anti- rabbit, 111-035-144, Jackson ImmunoResearch Labs and anti-mouse, 115-035-166, Jackson ImmunoResearch Labs) for 1 h 30. Protein-antibody complexes were visualized using the enhanced chemiluminescence western blotting detection system (ECL 2, 1151-7371, Fisher Scientific) and revealed on chemiluminescent film (Amersham Hyperfilm ECL, 28906837, GE Healthcare) in developer (P7042-5GA, Sigma Aldrich) and fixer (P7167-5GA, Sigma Aldrich) solution baths. The molecular weight scale used for the experiment was the Protein ladder Plus prestained (#06P-0211, Euromedex). The band density (Optical density values) was determined using a GS-800 apparatus (BIO-RAD Laboratories, Ivry sur Seine, France) and analyzed with ImageLab software. Artery and vein from SED rats were simultaneously analyzed on the same gel in order to compare protein expression between these vessels in baseline conditions. Vessels (either artery or vein) of SED and EX rats were also simultaneously analysed on the same gel in order to investigate the effect of exercise on protein expression. Importantly, the amount of protein required to detect the protein of interest results in a signal that far exceeds the linear dynamic range when detecting β -actin, thereby eliminating the usefulness of β -actin for the normalization of western blots. Thus, β -actin was used here as an internal standard only, to verify that proteins were indeed loaded on the gel. The appropriate amounts of total proteins to be analyzed were previously determined from concentration (increasing amounts of proteins)/response (optical density of the band) curves from two rats both belonging to a particular group (on the same gel) or from the two vessels belonging to a same rat. All gels were run in triplicate and data were calculated from a representative gel rather than averaged data, thus allowing not only to display corresponding immunoblots but also to perform an appropriate statistical analysis (6 samples/group for analysis of a representative gel instead and 18 samples/group for analysis of averaged data, whilst number of rats/group is 6).

2.7 Immunofluorescence

Slices were fixed with acetone before blockade of non-specific binding sites with TBS (20 mmol/L Tris/HCl, 137 mmol/L NaCl, pH 7.4) containing 5% Goat Serum (GS), 0.1% Triton X100, 0.2% Tween 20. Then, they were incubated with TBS containing 2% GS, 0.01% Triton X100, 0.02% Tween 20 and antibody against BDNF (1:200, rabbit monoclonal, ab108319, Abcam) or p-TrkB^{Tyr816} (1:100, rabbit polyclonal, ABN1381, Millipore) in the presence of

antibody directed against von Willebrand Factor (vWF), a specific endothelial cell marker (1:100 mouse monoclonal, 142828, US biological). They were then exposed to fluorescent secondary antibodies [ALEXA Fluor 488 (1:800, anti-mouse, A-11029, Thermo Fisher Scientific) and 568 (1:800, anti-rabbit, A-11036, Thermo Fisher Scientific)] diluted in TBS containing 2% GS. Finally, samples were mounted with a fluorescent mounting solution containing 1.5 µg/mL of DAPI (Vectashield, FPDT094A, Interchim) used as a nuclear marker and analysed using an epifluorescent microscope (Eclipse E600, Nikon France S.A.S., Champigny-sur-Marne, France). Intensity of staining was quantified using an automated method with Image J software. The calculated fluorescence intensity corresponded to the mean of intensity obtained by plotting a line through adjacent endothelial or vascular smooth muscle cells. The analysis was performed by two experimenters blinded from experimental conditions.

2.8 Statistical analysis

SigmaPlot 11.0 software was used for all graphs and statistical analysis. Data were expressed as means \pm standard deviations (SD). Comparison between artery and vein from the same rat was performed using a parametric or non-parametric paired-test, depending on the normality and equal variance tests. Comparison between SED and EX groups was performed using a parametric t test or non-parametric Mann–Whitney test, depending on the normality and equal variance tests. Spearman correlation was used to measure the strength of the relationship between paired data. A p-value of < 0.05 was considered statistically significant.

3. RESULTS

3.1 Arterial vs venous BDNF in sedentary rats

The results (Fig. 1) showed that artery and vein contained BDNF protein and mRNA and that BDNF protein levels were higher in artery than in vein in SED rats (Fig. 1A) while no significant difference in BDNF mRNA levels was observed between these two vessels (Fig. 1B). Immunofluorescence analysis revealed a strong BDNF staining by cells positive for the endothelial cell marker vWF (Fig. 1C, insert). This staining was uniform throughout the endothelium and higher for artery than vein ($p = 0.011$). Notably, in conditions at which endothelial expression was easily detectable, it was impossible to detect BDNF in the media. In fact, detection of BDNF in vascular smooth muscle cells (VSMC, cells positive for alpha

actin) required longer exposure time of the slices for the same field of view. In these specific experimental conditions, a faint and scattered BDNF staining was observed throughout the media. However, while specifically focusing on this area (media), we noticed that arterial VSMC exhibited higher intensity of BDNF staining than venous VSMC (3.67 ± 0.56 vs 1.68 ± 0.53 arbitrary units, $p < 0.001$, Figure 1A in Supplemental data). Collectively, these data indicated that the higher BDNF content of the arterial wall as compared to the venous wall related to a higher BDNF expression by both endothelial cells and VSMC of artery especially since the media is thicker for artery than vein from a similar territory.

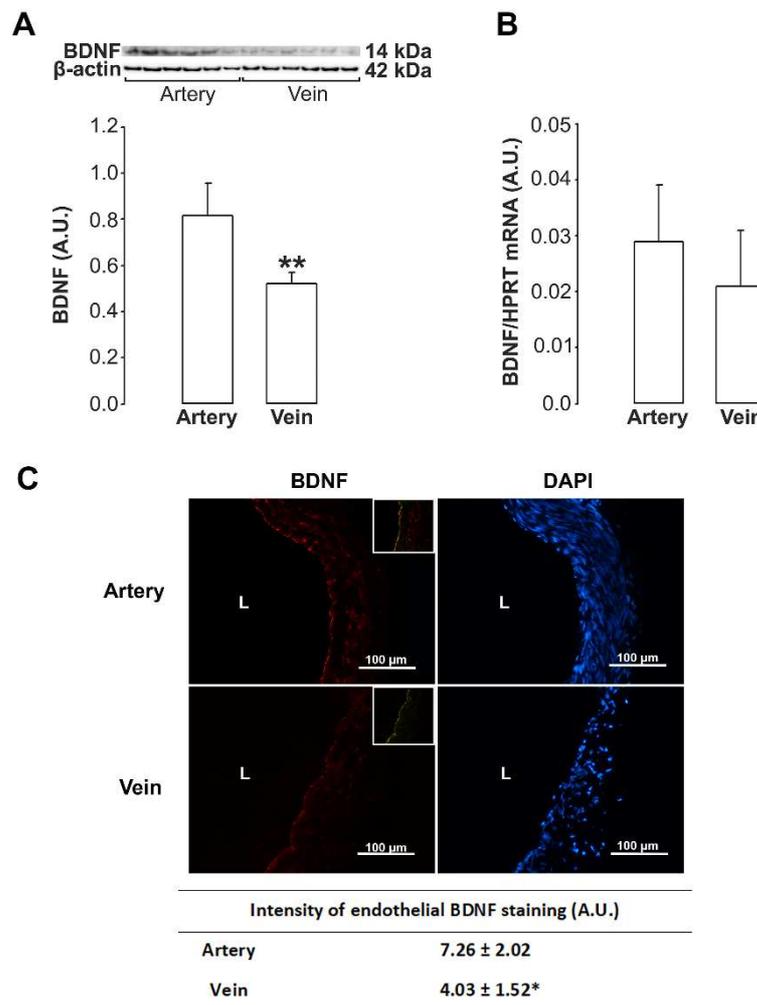


Fig. 1. Arterial vs venous BDNF levels and localization in baseline conditions.

Levels of BDNF protein (A), mRNA (B) as well as BDNF localization (C) were determined in abdominal aorta and corresponding vena cava of each sedentary rat. Immunoblots of BDNF (14 kDa) and β -actin (42 kDa) are shown above bar charts. In representative photographs of BDNF staining (red), DAPI (blue) and vWF (green) were used as nuclear and endothelial markers, respectively. Overlay (insert with vWF/BDNF) shows BDNF staining in endothelial cells in artery and vein. L = vascular lumen. Values (arbitrary units) are expressed as means \pm SD from 6 rats. * $p < 0.05$, ** $p < 0.01$ different from artery.

3.2 Effect of exercise on arterial and venous BDNF production

The effects of exercise (EX) on BDNF production are summarized in Fig. 2. As regards the effect on artery (Fig. 2A, 2B and 2C), EX rats exhibited significant higher levels of BDNF protein than SED rats (+50%, Fig. 2A) without associated significant changes in BDNF mRNA levels (Fig. 2B). Increased BDNF protein levels in response to exercise coincided with increased BDNF staining (~ x4) in endothelial cells only (Fig. 2C). Indeed, BDNF expression in arterial VSMC was not different between SED and EX rats (not shown). A similar effect of exercise was observed on vein (Fig. 2D, 2E and 2F). Thus, EX resulted in increased BDNF protein levels (+50%, Fig. 2D), no significant change in BDNF mRNA levels (Fig. 2E) and endothelial overexpression (x4) of BDNF (Fig. 2F). Again, no difference in venous VSMC BDNF expression obtained from immunofluorescence analysis, was observed between SED and EX rats (Figure 1B, C in Supplemental data).

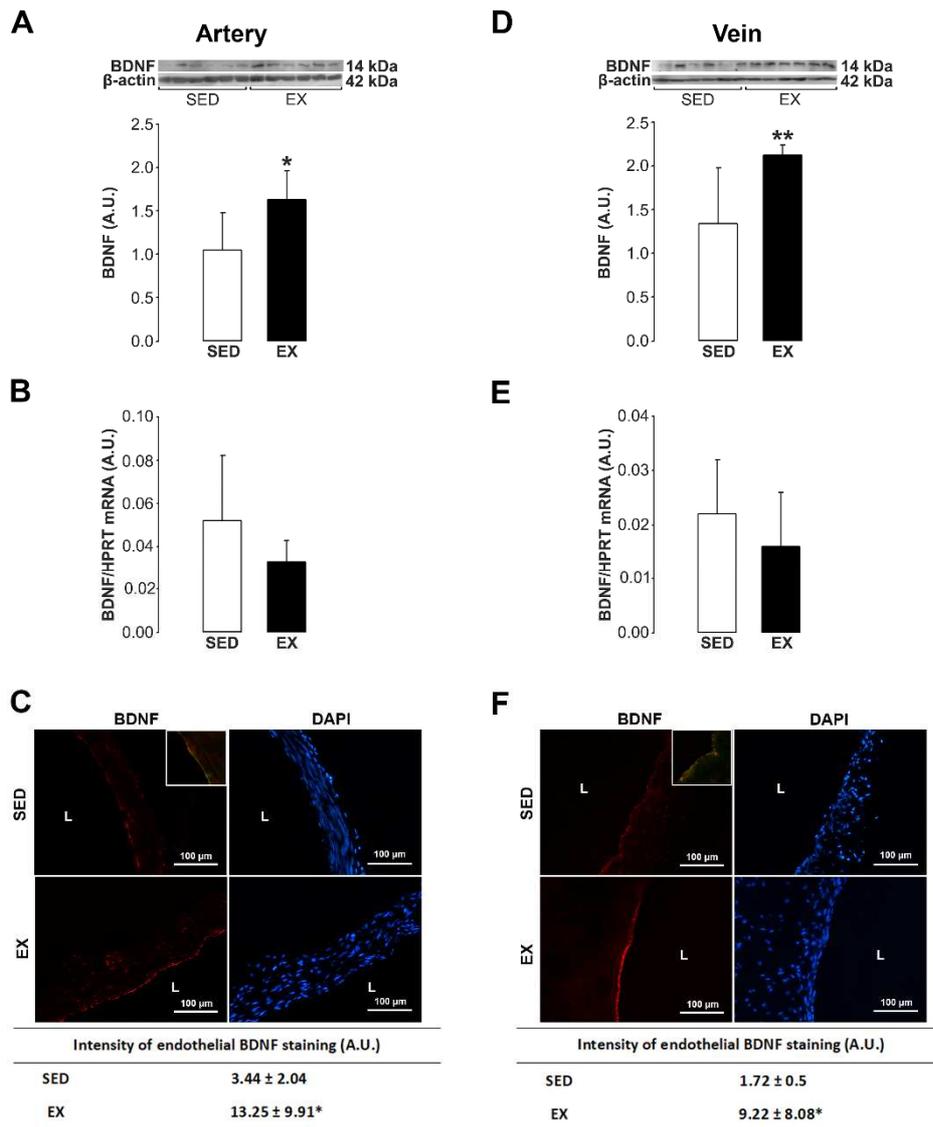


Fig. 2. Effect of physical exercise on arterial and venous BDNF production.

Levels of BDNF protein, mRNA as well as BDNF localization were investigated in abdominal aorta (A, B, C) and vena cava (D, E, F) of sedentary (SED, n = 6) and exercised (EX, n = 6) rats. Immunoblots of BDNF (14 kDa) and β-actin (42 kDa) are shown above bar charts. In representative photographs of BDNF staining (red), DAPI (blue) and vWF (green) were used as nuclear and endothelial markers, respectively. Overlay (insert with vWF/BDNF) shows BDNF staining in endothelial cells. L = vascular lumen. Values (arbitrary units) are expressed as means ± SD. * p < 0.05, ** p < 0.01 different from SED rats, n = number of rats.

3.3 Association between endothelial BDNF expression and TrkB activation

Full length TrkB receptors (FL-TrkB) and truncated TrkB receptors (t-TrkB), which are devoid of tyrosine activity, are both present in vessels^{13,22}. However, in terms of cellular localization, endothelial cells and VSMC prominently express FL- and t-TrkB receptors, respectively. Therefore, BDNF secretion by endothelial cells was assessed from staining

intensity of endothelial p-TrkB^{Tyr816}, an indicator of TrkB activation by endogenous BDNF. As shown in Fig. 3, EX resulted in a comparable augmentation (x3) of activated TrkB expression in artery (Fig. 3A) and vein (Fig. 3B). When endothelial BDNF expression in both SED and EX rats was plotted against endothelial p-TrkB^{Tyr816} expression, a positive association was observed for aorta (Fig. 3C) and vena cava (Fig. 3D). The association was not observed when SED and EX rats were examined separately (not shown). Of note, results from western blot analysis of vascular p-TrkB^{Tyr816} corroborated immunohistochemical data. Indeed, EX induced a significant augmentation (x2) of p-TrkB^{Tyr816} protein levels either in artery or in vein (Fig. 3E and 3F).

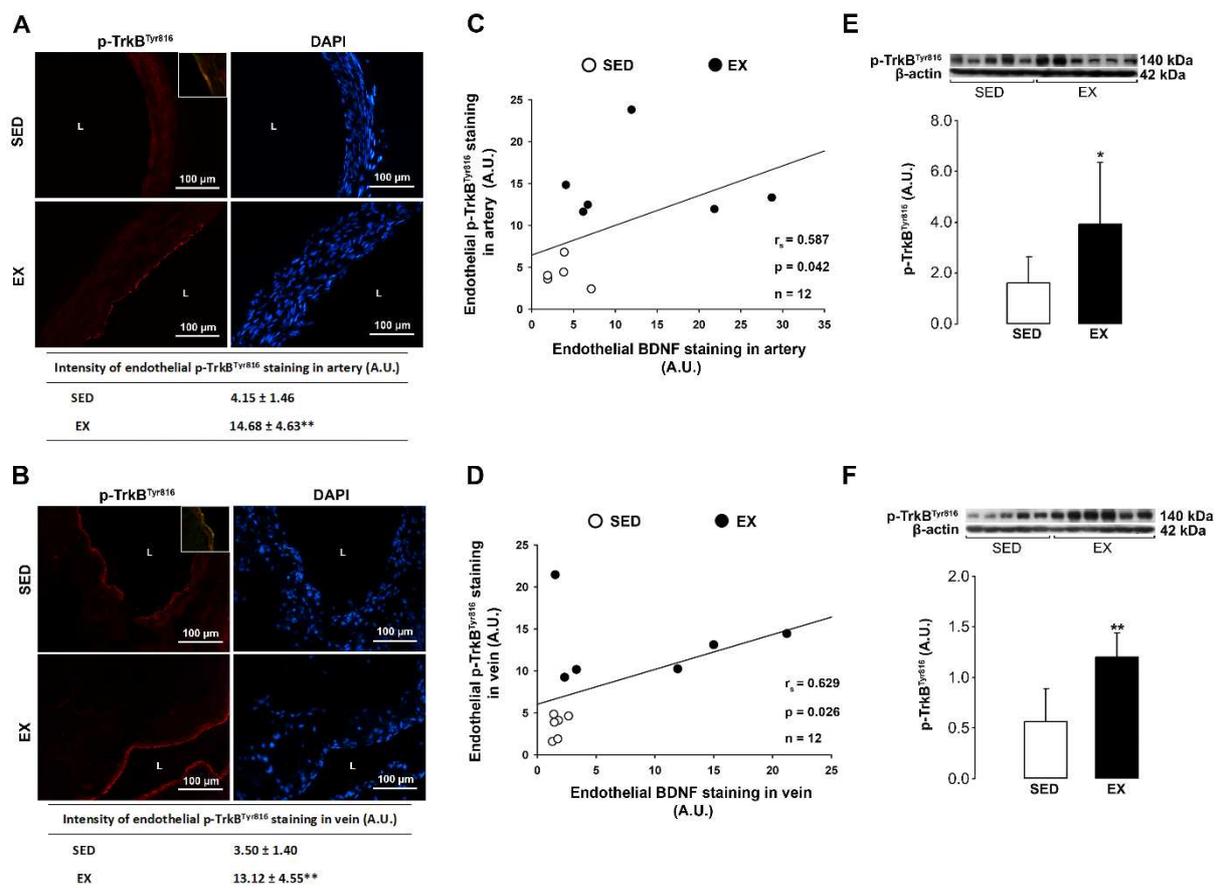


Fig. 3. Effect of exercise on endothelial TrkB receptor activation.

Localization of TrkB phosphorylated at tyrosine 816 (p-TrkB^{Tyr816}), an indicator of TrkB activation by endogenous BDNF, was investigated in abdominal aorta (A) and vena cava (B) removed from sedentary (SED) and exercised (EX) rats. In representative photographs of p-TrkB^{Tyr816} (red), DAPI (blue) and vWF (green) were used as nuclear and endothelial markers, respectively. Overlay (insert with vWF/ p-TrkB^{Tyr816}) reveals p-TrkB^{Tyr816} staining in endothelial cells. L = vascular lumen, n = 6 rats/group. Scatter plot between individual endothelial p-TrkB^{Tyr816} and BDNF stainings in artery (C) and vein (D). Levels of p-TrkB^{Tyr816} protein were investigated in abdominal aorta (E) and vena cava (F) from SED (n = 5) and EX (n = 6) rats. Immunoblots of p-TrkB^{Tyr816} (140 kDa) and β -actin (42 kDa) are

shown above bar charts. Values (arbitrary units) are expressed as means \pm SD. r_s = spearman correlation coefficient. * $p < 0.05$, ** $p < 0.01$ different from SED rats, n = number of rats.

3.4 In mouse, endothelium expresses BDNF and p-TrkB^{Tyr816}

The positive association observed between endothelial BDNF and p-TrkB^{Tyr816} expressions (Fig. 3A, 3C) supports the notion that activation of endothelial TrkB receptors involved BDNF secreted by endothelial cells. However, the possibility that circulating BDNF accounted for endothelial TrkB activation cannot be excluded even though whether BDNF is present in rat plasma *in vivo* remains an open question²⁶. In fact, the detection of BDNF in plasma sample might be due to lysis of platelets, which contain high BDNF levels, during preparation of blood sample²⁶. To further explore the source (endothelium or blood) of BDNF involved in endothelial TrkB activation in rats, we performed immunohistochemical detection of BDNF and p-TrkB^{Tyr816} in aorta collected in a mouse. Indeed, neither platelets, serum nor plasma contain BDNF in mice²⁶. As shown in Fig. 4, despite the lack of blood BDNF in mice the activated form of TrkB receptors was detected in vWF positive cells. Importantly, these cells also expressed BDNF. Collectively, these data supported the idea that endothelial TrkB receptors in rats were activated by endothelium-derived BDNF.

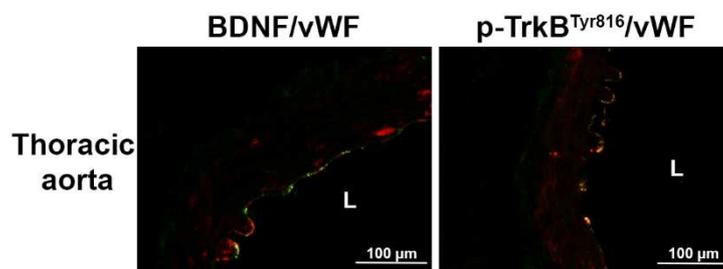


Fig. 4. Endothelial BDNF and p-TrkB^{Tyr816} expressions in aortic endothelium of mice. Overlays of BDNF/vWF and p-TrkB^{Tyr816}/vWF reveal that BDNF and p-TrkB^{Tyr816} stainings in aortic endothelium of mice. L = Lumen

3.5 Endothelial BDNF expression reflects endothelial NO production

In order to investigate the link between endothelial BDNF expression and NO production, vascular p-eNOS^{Ser1177}, eNOS protein and eNOS mRNA levels were compared in vein *vs* artery of SED rats and in SED *vs* EX rats (artery and vein). The results are summarized in Table 2. In SED rats (up part of Table 2), p-eNOS^{Ser1177} and eNOS protein levels did not differ between artery and vein. On the contrary, eNOS mRNA was lower in aorta than in vena cava ($p < 0.05$). As the proportion of endothelial tissue of the vascular wall is largely higher

for the vein than the artery, similar levels of p-eNOS^{Ser1177} and eNOS proteins in artery and vein have to be interpreted as higher endothelial NO production by arterial than by venous endothelium. Supporting a control of endothelium BDNF synthesis by NO, endothelial BDNF expression in SED rats was higher in artery than in vein (Fig. 1C). As long-term EX was reported to not change vascular tetrahydrobiopterin levels²⁷, eNOS are not uncoupled in exercised beings. In these conditions, changes in eNOS are very likely to reflect NO production as previously shown²⁸. Here, EX stimulated NO production in both artery and vein. In artery, EX increased p-eNOS^{Ser1177} and eNOS proteins by a factor 2.78 and 2.27, respectively and eNOS mRNA by a factor 2.2. The corresponding factors for the effect of EX in vein were 1.75, 2 and 1.73. Difference between artery and vein did not reach significance (down part of Table 2). These data combined with those on BDNF indicated that EX-induced changes in endothelial NO production coincided with similar variations in endothelial BDNF expression induced by EX. Of note, consistent with a non-selective localization of vascular BDNF in endothelial cells, vascular p-eNOS^{Ser1177} or eNOS (protein) levels were not correlated with vascular BDNF levels (data not shown).

Table 2. Comparative endothelial NO production in artery vs vein

Expressions in SED rats (arbitrary units)			
	p-eNOS ^{Ser1177} protein	eNOS protein	eNOS mRNA
Artery	1.09 ± 0.29	0.89 ± 0.32	0.10 ± 0.03
Vein	1.07 ± 0.32	0.85 ± 0.16	0.17 ± 0.06
] NS] p < 0.05
Effect of EX (% of SED values)			
	p-eNOS ^{Ser1177} protein	eNOS protein	eNOS mRNA
Artery	+ 278.6 % ± 52.9*	+ 227 % ± 93.7*	+ 220 % ± 67.8**
Vein	+ 175% ± 75*	+ 202.09 % ± 77.76**	+ 173.7 % ± 47.5**
] NS] NS

Levels of eNOS phosphorylated at serine 1177 (p-eNOS^{Ser1177} protein) and eNOS (protein, mRNA) were measured in rats either sedentary (SED) or subjected to exercise (EX) in artery (abdominal aorta) and vein (vena cava). Values (arbitrary units) are expressed as means ± SD. No significant difference was observed between artery and vein except for eNOS mRNA levels in baseline conditions. * p < 0.05, ** p < 0.01 different from SED values.

4. Discussion

The main results provided by the present study are that i) in baseline physiological conditions, BDNF expression in VSMC is negligible as compared to endothelial BDNF expression either in abdominal aorta or vena cava, ii) EX increases BDNF expression in arterial and venous endothelial cells with a comparable magnitude, while it does not change BDNF expression in VSMC, iii) EX-induced changes in endothelial BDNF expression and NO production are in the same way, iv) endothelium BDNF expression correlates with endothelial expression of p-TrkB^{Ty816} for both artery and vein when sedentary and exercised rats are simultaneously analyzed.

Most of what is known on vascular BDNF originates from studies on cultured endothelial cells and VSMC from different origins. However, experiments on cultured cells cannot fully replicate the *in vivo* environment. In addition, endothelial and muscular phenotypes differ between artery and vein^{29,30}. Despite this observation, studies aiming to compare endothelial and muscular BDNF productions by cells derived from arterial *vs* venous vessels are lacking. Our results revealed that BDNF in baseline conditions is very weakly expressed by the media of both abdominal aorta and vena cava as compared to endothelial expression and that muscular as well as endothelial BDNF expressions were higher in artery than in vein. Another major difference between endothelial and muscular BDNF is that EX exclusively changed endothelial BDNF expression (x4) irrespective of the vessel. These data emphasize a role of endothelial but not muscular BDNF in vascular physiology and support the idea that FSS is a main regulator of vascular BDNF synthesis. Indeed, FSS is higher in artery than in vein from a similar territory but EX increases FSS to a same extent in these two vessels.

A well-documented effect of FSS on endothelial cells is an intensity-dependent increase in endothelial NO production²⁴. Evidence that FSS resulted in BDNF overproduction by HUVEC (Human umbilical vein endothelial cells) cells^{6,9} and that incubation of vessels or exposure of cultured endothelial cells with a slow releasing NO donor increased and with a NOS inhibitor decreased BDNF levels^{6,12,13,18} strongly suggests that NO may be the intermediate between FSS and endothelial BDNF synthesis *in vivo*. Consistently, our results showed that changes in endothelial NO production translate into changes in endothelial BDNF production in the same way. Indeed, endothelial BDNF expression as NO production were higher in artery than in vein (Fig. 1 and Table 2). In addition, EX that stimulated NO production to a same extent in artery and vein increases endothelial BDNF expression by a factor 4 in both vessels (Fig. 2 and Table 2). These data resonate with the demonstration that the angiogenic effect of exercise involves the activation of the BDNF/TrkB axis in an NO-

dependent manner⁹. Intriguingly, EX-induced elevation of vascular BDNF levels in either artery or vein occurred without an associated trend in elevation of vascular BDNF mRNA levels (Fig. 2). Such mismatch between protein and mRNA BDNF levels in response to EX suggests the involvement of NO in the maturation of BDNF from its precursor pro-BDNF. Of note, FSS was reported to increase activity and synthesis of pro- protein convertases including furin, responsible for the processing of pro-BDNF into BDNF^{31,32}. However, it cannot be excluded that exercise does well induce *bdnf* gene transcription but that vascular BDNF mRNA processing occurs more rapidly than mRNA synthesis as previously reported for skeleton muscle³³. Indeed, in the present study BDNF mRNA and protein levels were assessed 2h and 24h after the last session of exercise. More speculative is that the mismatch between changes in BDNF mRNA and protein levels might relate to the fusion of BDNF-containing exosomes with endothelial cells.

As already stated in the Introduction section, exposure to exogenous BDNF of cultured endothelial cells and isolated vessels was reported to induce relaxation, angiogenesis and protect against cytotoxicity and inflammation^{6,7,9-13} indicating that full-length TrkB receptors are expressed by endothelial cells. Given that cultured endothelial cells secrete BDNF according to a constitutive and regulated manner² and that both arterial and venous endothelium expressed simultaneously BDNF and activated TrkB receptors, it is tempting to hypothesize that endothelium-derived BDNF acts as an autocrine factor *in vivo*. In accordance with this hypothesis, the present study showed that endothelial BDNF expression correlated with endothelial TrkB activation in rats (Fig. 3A and 3C) and that endothelial cells expressed activated TrkB receptors in mouse (Fig. 4) whose blood does not contain BDNF²⁶. These data are in line with a recent study in which FSS-related angiogenic response of EX was reported to be attenuated by TrkB inhibition⁹. Importantly, an activation of endothelial TrkB receptors by BDNF secreted by VSMC is unlikely. Indeed, a widespread extracellular diffusion of BDNF is prevented by its high molecular weight (28 kDa for the mBDNF homodimer). Moreover, the basement membrane (negative charge) that surrounds each VSMC may rather serve to a reservoir of BDNF (positive charge at physiological pH³⁴), thereby hampering an action of BDNF on endothelial cells. Nevertheless, plasma BDNF might contribute to endothelial TrkB receptors activation observed in exercised rats providing that exercise increase BDNF in plasma.

As a study limitation, we are aware that both shear stress-dependent and -independent mechanisms may account for the cardiovascular benefits of exercise³⁵ even though shear stress is an important physiological stimulus for the control of endothelial function by

exercise. Therefore, the present study cannot exclude that mechanisms unrelated to NO are involved in exercise-induced endothelial BDNF overexpression. For instance, while exercise decreases circulating TNF- α levels ³⁶, the exposure of peripheral endothelial cells to the cytokine results in BDNF downregulation ³. Moreover, exercise increases blood levels of adrenalin and irisin, factors already identified as positive modulators of BDNF synthesis ³⁷⁻³⁹.

In conclusion, our results reveal that BDNF is highly expressed by both arterial and venous endothelium as compared to muscular cells with higher expression in the former and that EX results in uniform response of BDNF and TrkB activation in artery and vein from a similar territory. These data suggest that endothelial BDNF should be included to the already long list of endothelium-derived factors and that this factor is positively controlled by EX. Further studies are required to explore the role of endothelial BDNF in exercise-induced vascular benefits.

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Disclosures

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References

1. Nagahara, A.H. & Tuszynski, M.H. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. *Nature reviews. Drug discovery* **10**, 209-219 (2011). 10.1038/nrd3366
2. Leventhal, C., Rafii, S., Rafii, D., Shahar, A. & Goldman, S.A. Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. *Molecular and cellular neurosciences* **13**, 450-464 (1999). 10.1006/mcne.1999.0762
3. Bayas, A., Hummel, V., Kallmann, B.A., Karch, C., Toyka, K.V. & Rieckmann, P. Human cerebral endothelial cells are a potential source for bioactive BDNF. *Cytokine* **19**, 55-58 (2002)

4. Kallmann, B.A., Wagner, S., Hummel, V., Buttman, M., Bayas, A., Tonn, J.C. & Rieckmann, P. Characteristic gene expression profile of primary human cerebral endothelial cells. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **16**, 589-591 (2002)
5. Guo, S., Kim, W.J., Lok, J., Lee, S.R., Besancon, E., Luo, B.H., Stins, M.F., Wang, X., Dedhar, S. & Lo, E.H. Neuroprotection via matrix-trophic coupling between cerebral endothelial cells and neurons. *Proc Natl Acad Sci U S A* **105**, 7582-7587 (2008). 10.1073/pnas.0801105105
6. Prigent-Tessier, A., Quirie, A., Maguin-Gate, K., Szostak, J., Mossiat, C., Nappey, M., Devaux, S., Marie, C. & Demougeot, C. Physical training and hypertension have opposite effects on endothelial brain-derived neurotrophic factor expression. *Cardiovascular research* **100**, 374-382 (2013). 10.1093/cvr/cvt219
7. Kermani, P. & Hempstead, B. Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. *Trends in cardiovascular medicine* **17**, 140-143 (2007). 10.1016/j.tcm.2007.03.002
8. Cao, L., Zhang, L., Chen, S., Yuan, Z., Liu, S., Shen, X., Zheng, X., Qi, X., Lee, K.K., Chan, J.Y. & Cai, D. BDNF-mediated migration of cardiac microvascular endothelial cells is impaired during ageing. *Journal of cellular and molecular medicine* **16**, 3105-3115 (2012). 10.1111/j.1582-4934.2012.01621.x
9. Wang, B.L., Jin, H., Han, X.Q., Xia, Y. & Liu, N.F. Involvement of brain-derived neurotrophic factor in exercise-induced cardioprotection of post-myocardial infarction rats. *International journal of molecular medicine* **42**, 2867-2880 (2018). 10.3892/ijmm.2018.3841
10. Takeda, K., Obinata, Y., Konishi, A., Kajiya, M., Matsuda, S., Mizuno, N., Sasaki, S., Fujita, T. & Kurihara, H. Brain-Derived Neurotrophic Factor Inhibits Intercellular Adhesion Molecule-1 Expression in Interleukin-1beta-Treated Endothelial Cells. *Cell Biochem Biophys* **74**, 399-406 (2016). 10.1007/s12013-016-0749-2
11. Takeda, K., Kermani, P., Anastasia, A., Obinata, Y., Hempstead, B.L. & Kurihara, H. BDNF protects human vascular endothelial cells from TNFalpha-induced apoptosis. *Biochem Cell Biol* **91**, 341-349 (2013). 10.1139/bcb-2013-0005
12. Meuchel, L.W., Thompson, M.A., Cassivi, S.D., Pabelick, C.M. & Prakash, Y.S. Neurotrophins induce nitric oxide generation in human pulmonary artery endothelial cells. *Cardiovasc Res* **91**, 668-676 (2011). 10.1093/cvr/cvr107
13. Totoson, P., Pedard, M., Marie, C. & Demougeot, C. Activation of endothelial TrkB receptors induces relaxation of resistance arteries. *Vascul Pharmacol* **106**, 46-53 (2018). 10.1016/j.vph.2018.02.005
14. Jiang, H., Huang, S., Li, X., Li, X., Huang, S., Zhang, Y. & Chen, Z.Y. Endothelial tyrosine kinase receptor B prevents VE-cadherin cleavage and protects against atherosclerotic lesion development in ApoE^{-/-} mice. *Oncotarget* **6**, 30640-30649 (2015). 10.18632/oncotarget.5855
15. Sandrini, L., Ieraci, A., Amadio, P., Veglia, F., Popoli, M., Lee, F.S., Tremoli, E. & Barbieri, S.S. Sub-Chronic Stress Exacerbates the Pro-Thrombotic Phenotype in BDNF(Val/Met) Mice: Gene-Environment Interaction in the Modulation of Arterial Thrombosis. *Int J Mol Sci* **19**(2018). 10.3390/ijms19103235
16. Sukhovshin, R.A., Yepuri, G. & Ghebremariam, Y.T. Endothelium-Derived Nitric Oxide as an Antiatherogenic Mechanism: Implications for Therapy. *Methodist DeBakey cardiovascular journal* **11**, 166-171 (2015). 10.14797/mdcj-11-3-166
17. Quirie, A., Hervieu, M., Garnier, P., Demougeot, C., Mossiat, C., Bertrand, N., Martin, A., Marie, C. & Prigent-Tessier, A. Comparative effect of treadmill exercise

- on mature BDNF production in control versus stroke rats. *PLoS One* **7**, e44218 (2012). 10.1371/journal.pone.0044218
18. Marie, C., Pedard, M., Quirie, A., Tessier, A., Garnier, P., Totoson, P. & Demougeot, C. Brain-derived neurotrophic factor secreted by the cerebral endothelium: A new actor of brain function? *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, 271678X18766772 (2018). 10.1177/0271678X18766772
 19. Pedard, M., Quirie, A., Totoson, P., Verhoeven, F., Garnier, P., Tessier, A., Demougeot, C. & Marie, C. Vascular brain-derived neurotrophic factor pathway in rats with adjuvant-induced arthritis: Effect of anti-rheumatic drugs. *Atherosclerosis* **274**, 77-85 (2018). 10.1016/j.atherosclerosis.2018.05.004
 20. Monnier, A., Prigent-Tessier, A., Quirie, A., Bertrand, N., Savary, S., Gondcaille, C., Garnier, P., Demougeot, C. & Marie, C. Brain-derived neurotrophic factor of the cerebral microvasculature: a forgotten and nitric oxide-dependent contributor of brain-derived neurotrophic factor in the brain. *Acta Physiol (Oxf)* **219**, 790-802 (2017). 10.1111/apha.12743
 21. Donovan, M.J., Miranda, R.C., Kraemer, R., McCaffrey, T.A., Tessarollo, L., Mahadeo, D., Sharif, S., Kaplan, D.R., Tsoulfas, P., Parada, L. & et al. Neurotrophin and neurotrophin receptors in vascular smooth muscle cells. Regulation of expression in response to injury. *The American journal of pathology* **147**, 309-324 (1995)
 22. Otani, K., Okada, M. & Yamawaki, H. Expression pattern and function of tyrosine receptor kinase B isoforms in rat mesenteric arterial smooth muscle cells. *Biochem Biophys Res Commun* **467**, 683-689 (2015). 10.1016/j.bbrc.2015.10.084
 23. Pedard, M., Cefis, M., Ennequin, G., Quirie, A., Garnier, P., Prigent-Tessier, A., Pernet, N. & Marie, C. Brain-derived Neurotrophic Factor Pathway after Downhill and Uphill Training in Rats. *Medicine and science in sports and exercise* (2018). 10.1249/MSS.0000000000001771
 24. Xu, S., Li, X., LaPenna, K.B., Yokota, S.D., Huke, S. & He, P. New insights into shear stress-induced endothelial signalling and barrier function: cell-free fluid versus blood flow. *Cardiovascular research* **113**, 508-518 (2017). 10.1093/cvr/cvx021
 25. Corson, M.A., James, N.L., Latta, S.E., Nerem, R.M., Berk, B.C. & Harrison, D.G. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res* **79**, 984-991 (1996)
 26. Chacon-Fernandez, P., Sauberli, K., Colzani, M., Moreau, T., Ghevaert, C. & Barde, Y.A. Brain-derived Neurotrophic Factor in Megakaryocytes. *J Biol Chem* **291**, 9872-9881 (2016). 10.1074/jbc.M116.720029
 27. Sindler, A.L., Delp, M.D., Reyes, R., Wu, G. & Muller-Delp, J.M. Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles. *J Physiol* **587**, 3885-3897 (2009). 10.1113/jphysiol.2009.172221
 28. Mota, M.M., Mesquita, T.R., Braga da Silva, T.L., Fontes, M.T., Lauton Santos, S., dos Santos Aggum Capettini, L., Guedes de Jesus, I.C., Quintans Junior, L.J., De Angelis, K., Wichy, R.B. & Santos, M.R. Endothelium adjustments to acute resistance exercise are intensity-dependent in healthy animals. *Life Sci* **142**, 86-91 (2015). 10.1016/j.lfs.2015.10.007
 29. Richardson, M.R., Lai, X., Witzmann, F.A. & Yoder, M.C. Venous and arterial endothelial proteomics: mining for markers and mechanisms of endothelial diversity. *Expert review of proteomics* **7**, 823-831 (2010). 10.1586/epr.10.92
 30. Eriksson, E.E., Karlof, E., Lundmark, K., Rotzius, P., Hedin, U. & Xie, X. Powerful inflammatory properties of large vein endothelium in vivo. *Arteriosclerosis*,

- thrombosis, and vascular biology* **25**, 723-728 (2005).
10.1161/01.ATV.0000157578.51417.6f
31. Negishi, M., Lu, D., Zhang, Y.Q., Sawada, Y., Sasaki, T., Kayo, T., Ando, J., Izumi, T., Kurabayashi, M., Kojima, I., Masuda, H. & Takeuchi, T. Upregulatory expression of furin and transforming growth factor-beta by fluid shear stress in vascular endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology* **21**, 785-790 (2001)
 32. Kang, H., Duran, C.L., Abbey, C.A., Kaunas, R.R. & Bayless, K.J. Fluid shear stress promotes proprotein convertase-dependent activation of MT1-MMP. *Biochemical and biophysical research communications* **460**, 596-602 (2015).
10.1016/j.bbrc.2015.03.075
 33. Yu, T., Chang, Y., Gao, X.L., Li, H. & Zhao, P. Dynamic Expression and the Role of BDNF in Exercise-induced Skeletal Muscle Regeneration. *Int J Sports Med* **38**, 959-966 (2017). 10.1055/s-0043-118343
 34. Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. & Barde, Y.A. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149-152 (1989). 10.1038/341149a0
 35. Nystoriak, M.A. & Bhatnagar, A. Cardiovascular Effects and Benefits of Exercise. *Front Cardiovasc Med* **5**, 135 (2018). 10.3389/fcvm.2018.00135
 36. Petersen, A.M. & Pedersen, B.K. The anti-inflammatory effect of exercise. *J Appl Physiol (1985)* **98**, 1154-1162 (2005). 10.1152/jappphysiol.00164.2004
 37. Juric, D.M., Loncar, D. & Carman-Krzan, M. Noradrenergic stimulation of BDNF synthesis in astrocytes: mediation via alpha1- and beta1/beta2-adrenergic receptors. *Neurochem Int* **52**, 297-306 (2008). 10.1016/j.neuint.2007.06.035
 38. Jodeiri Farshbaf, M., Ghaedi, K., Megraw, T.L., Curtiss, J., Shirani Faradonbeh, M., Vaziri, P. & Nasr-Esfahani, M.H. Does PGC1alpha/FNDC5/BDNF Elicit the Beneficial Effects of Exercise on Neurodegenerative Disorders? *Neuromolecular Med* **18**, 1-15 (2016). 10.1007/s12017-015-8370-x
 39. Wrann, C.D., White, J.P., Salogiannis, J., Laznik-Bogoslavski, D., Wu, J., Ma, D., Lin, J.D., Greenberg, M.E. & Spiegelman, B.M. Exercise induces hippocampal BDNF through a PGC-1alpha/FNDC5 pathway. *Cell Metab* **18**, 649-659 (2013).
10.1016/j.cmet.2013.09.008

Supplemental data:

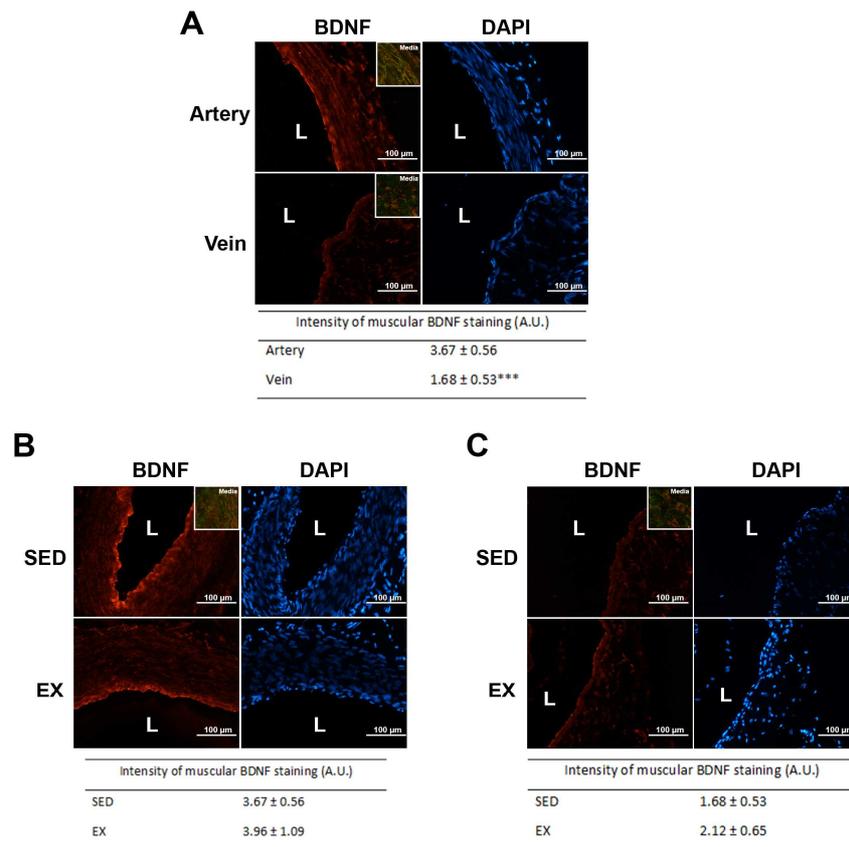
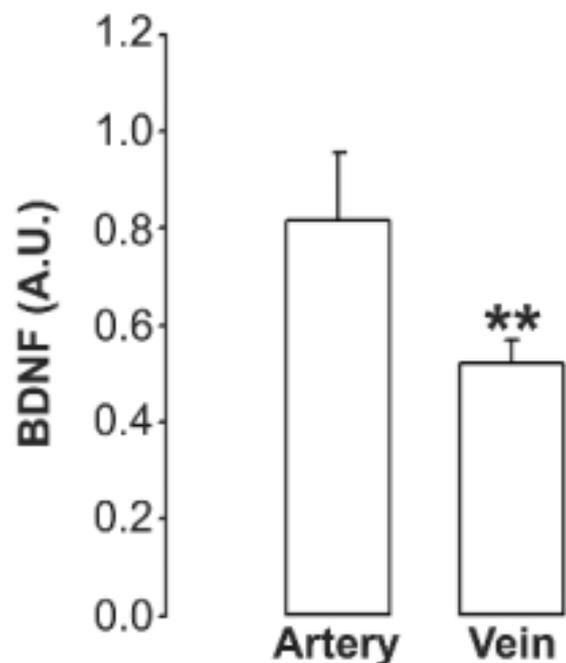
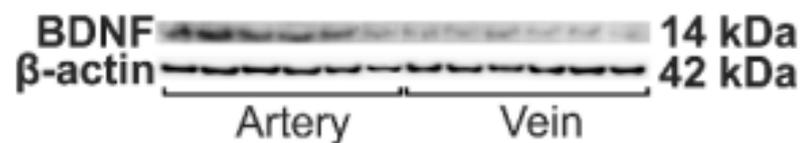


Figure 1 S.A: Muscular expression of BDNF in artery and vein in basal and stimulated conditions.

(A) In representative photographs of BDNF staining (red), DAPI (blue) and alpha-actin smooth muscle cells (green) were used as nuclear and vascular smooth muscle cells (VSMC) markers, respectively. Overlay (insert with alpha-actin/BDNF) shows BDNF staining in VSMC in artery and vein. Localization of BDNF was investigated in abdominal aorta (B) and vena cava (C) removed from sedentary (SED) and exercised (EX) rats. L = vascular lumen. Values (arbitrary units, A.U.) are expressed as means ± SD from 6 rats. *** p < 0.001 different from artery.

Sedentary rats



Endothelial BDNF labelling

