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Differential Expression of VEGFA, TIE2, and ANG2 but not ADAMTS1 in Rat Mesenteric Microvascular Arteries and Veins

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Summary

Microvessels respond to metabolic stimuli (e.g. pO_2) and hemodynamic forces (e.g. shear stress and wall stress) with structural adaptations including angiogenesis, remodeling and pruning. These responses could be mediated by differential gene expression in endothelial and smooth muscle cells. Therefore, rat mesenteric arteries and veins were excised by microsurgery, and mRNA expression of four angioadaptation-related genes was quantified by real time duplex RT-PCR in equal amounts of total RNA, correlated to two different house keeping genes (ß-actin, GAPDH). The results show higher expression of VEGFA, TIE2, and ANG2 in arteries than in veins, but equal expression of ADAMTS1. Higher availability of VEGFA mRNA in endothelial cells of arteries shown here could contribute to the maintenance of mechanically stressed blood vessels and counteract pressure-induced vasoconstriction.

Key words

Microcirculation • Endothelial cells • Vascular Endothelial Growth Factor A • Receptor TIE2 • Angiopoietin-2

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Introduction

In order to understand the mechanisms underlying angioadaptive processes (Zakrzewicz *et al.* 2002) including angiogenesis, vascular remodeling, and pruning several functional parameters, e.g. shear stress, oxygen saturation, red blood cell velocity, vessel diameter or capillary density have been measured in individual microvessels by intravital microscopy (IVM) *in vivo* (Hudlicka *et al.* 1986, Andresen *et al.* 1994, Pries *et al.* 1995a, 1998, 2005, Skalak and Price 1996, Fath *et al.* 1998, Tuttle *et al.* 2002a,b, Bakker *et al.* 2003, Skalak 2005). To understand the observed vascular reactions, information on gene expression is important. Recent studies on endothelial gene expression demonstrated shear stress-dependent induction of the endothelial receptor tyrosine kinase TIE2 and its phosphorylation (Chen *et al.* 2001, Lee and Koh 2003, Chlench *et al.* 2007). Furthermore, ADAMTS1 (a disintegrin-like and metalloprotease with thrombospondin motifs-1) (Bongrazio *et al.* 2000) and VEGFA (vascular endothelial growth factor) have been reported to be shear stress-inducible in some experimental models (Da Silva-Azevedo *et al.* 2002, Rivilis *et al.* 2002, Baum *et al.* 2004, Williams *et al.* 2006), while ANG2 (angiopoietin-2) was suppressed by shear stress in endothelial cells *in vitro* (Chlench *et al.* 2007, Goettsch *et al.* 2008). Since the mean shear stress in arteries is an order of a magnitude higher than that in veins (Pries *et al.* 1995b, Pries *et al*. 2005), these results would predict higher expression of TIE2, ADAMTS1 and VEGFA in arteries compared to veins, while ANG2 should be suppressed. This would be most important for smaller arteries, which can be involved either in precapillary tone regulation or in angiogenesis.

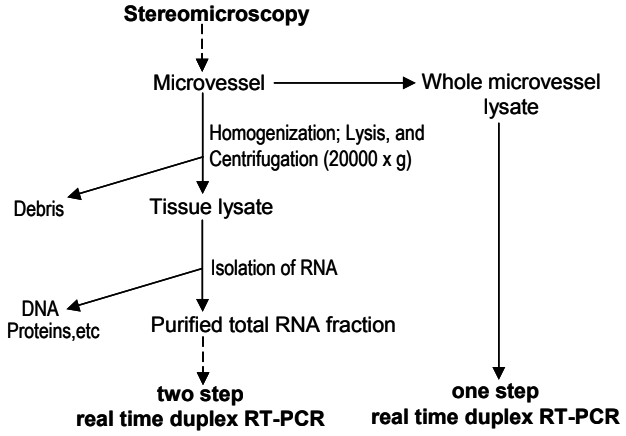

Fig. 1. Schematic outline of the two alternative procedures used to obtain RNA from single mesenteric microvessels for amplification of specific mRNA by real time duplex-RT-PCR. For details see the text.

However, gene expression studies of single vessel segments are rare. In the present study, we obtained histometrically characterized microvascular arteries and their accompanying veins by microsurgery and analyzed the expression of aforementioned genes by real time RT-PCR techniques.

Materials and Methods

Animals and excision of vessel segments

After obtaining approval from the university and state authorities for animal welfare, male Sprague Dawley rats (250-350 g) were anesthetized with urethane (1.5 g/kg body weight) and ketamine (0.5 ml/kg body weight). The mesentery was rolled out of the abdominal cavity, and placed on a stage under a stereomicroscope. Single microvascular mesenteric arteries and veins were freed from the adventitia and then excised with a length of about 10 mm under stereomicroscopic control. The vessels were collected in separate 0.2 ml Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at –80 °C until use. Alternatively, vessels were taken together with surrounding tissue for histometric analysis as described below. Animals were sacrificed by KCl injection.

Isolation of RNA from microvessels

Total RNA was isolated from the flow-through tissue lysates using a commercially available RNA isolation and purification kit (RNeasy Micro Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 350 µl of lysis buffer (RLT including 2-mercaptoethanol; RNeasy Micro Kit, Qiagen, Hilden, Germany) and RNasin at a final concentration of 1 U/µl (Promega, WI, USA) were added to flash frozen microvascular blood vessel segments. Thorough homogenization of the samples was achieved using a teflon pestle (Schütt, Göttingen, Germany) in 1.5 ml Eppendorf tubes followed by centrifugation (2 min at 20,000×g) through a spin column (QIAshredder, Qiagen, Hilden, Germany), and flow-through was used for extraction of RNA. RNA concentrations were determined using the RiboGreen RNA quantitation Kit (Invitrogen, CA, USA) for low concentrations according to the manufacturer's protocol. Isolated RNA was then analyzed in a two steps approach as described in the text below (Fig. 1).

Quantitative real time RT-PCR

Design of primers and probes

Gene-specific primers were generated for six genes: VEGFA, ANG2, TIE2, ADAMTS1, and the housekeeping genes ß-actin and GAPDH. Exon-boundaries spanning primers (18-25 nucleotides; Table 1), which do not bind to genomic DNA, and with melting temperatures (Tm) between 58 °C and 62 °C were designed for amplification specific target genes using a publicly available genome data base (NCBI, MD, USA) and the Primer3 software (Whitehead Institute for Biomedical Research, MA, USA). The primers were manufactured by MWG (Ebersberg, Germany). PCR-probes (Table 1) were designed to exhibit a Tm 8-10 °C higher than that of the corresponding primers using the gene data base and software mentioned above, manufactured by Operon Biotechnologies (Köln, Germany), and labeled at 5'end with the fluorophores FAM, JOE, TET or HEX and with the quencher BHQ at 3'end.

Real time RT-PCR

a) Amplification of mRNA from microvessel segments: one-step approach

mRNA was amplified directly from shock-frozen vessel segments without prior extraction of RNA using the respective forward inner primer for reverse transcription (Table 1). Accordingly, a single vessel segment was flash frozen in liquid nitrogen and taken directly as a template for real time duplex RT-PCR, carried out in a total reaction volume of 25 µl as described below (Fig. 1).

Table 1. Sequences of primers and probes.

Gene	Type		Primer and Probe (5' to 3' Sequences)	Product Size (bp)
ß-actin	Forward,	outer	ATATCGCTGCGCTCGTC	
	Reverse, outer		TTTCCCTCTCAGCTGTGGT	612
	Forward,	inner	**CCAGAGCAAGAGAGGCAT**	
	Reverse,	inner	AAACATGATCTGGGTCATCTTT	199
	Probe, FAM		AGATTTGGCACCACACTTTC	
VEGFA *Exon 1-3 (all splice variants)*	Forward,	outer	TTACTGCTGTACCTCCACCA	
	Reverse, outer		CATTTACACGTCTGCGGATC	518
	Forward,	inner	ATGCCAAGTGGTCCCAG	
	Reverse,	inner	CAATAGCTGCGCTGGTAG	94
	Probe, JOE		CAGAAAGCCCATGAAGTGGTGAAG	
TIE2	Forward,	outer	CGTGCTGCTGAACAACTTAC	
	Reverse, outer		TGTTTAGGGCCAGAGTTCCT	608
	Forward,	inner	TTAGTGACATTCTCCCTCCTCA	
	Reverse,	inner	CTTCATTTTTGCCCTGAACCTT	144
	Probe, TET		ACGGCTATTCGATTTCTTCCATCATC	
ANG2	Forward,	outer	TGCTGGAGAACATTCTAGAGAAC	
	Reverse, outer		CACAGTCTCTGAAGGTGGTTT	609
	Forward,	inner	CATGAAGAAGGAGATGGTGG	
	Reverse,	inner	TTTAGTACTTGGGCTTCCACA	141
	Probe, HEX		AACCAGTTTGCTCAACCAGACG	
ADAMTS1	Forward,	outer	CCTGTAACATTGAGGACTGTC	
	Reverse, outer		CTGCGGATTCTTTCCAATGC	619
	Forward,	inner	TCACTAGTACAAGACCTGGGTA	
	Reverse,	inner	AGAGTGGACAGAGTGAAGTTTC	172
	Probe, TET		TGGCAGCTTTCTGGCTATTAGAGCT	
GAPDH	Forward,	outer	TGACAACTTTGGCATCGTGG	
	Reverse, outer		TACTCCTTGGAGGCCATGT	513
	Forward,	inner	CCACTCAGAAGACTGTGGA	
	Reverse,	inner	ATCATACTTGGCAGGTTTCTC	221
	Dye, SYBR Green I			

b) Amplification of mRNA from microvessel segments: two steps approach

4 ng of total-RNA were used for reverse transcription of mRNA with the sensiscript reverse transcription kit (Qiagen, Hilden, Germany) using oligo-dT (Promega, WI, USA) as primer. Reaction mixtures were incubated at 37 °C for 1 hour. Single real-time PCR was carried out in a total volume of 50 µl containing 0.4 µM of each inner primer (sense and antisense; Table 1), 0.2 µM of the respective dual labeled probe, 10 µl template cDNA (corresponding to 2 ng of total RNA) and 25 µl multiplex mix (QuantiTect® Multiplex PCR Kit, Qiagen, Hilden, Germany). For duplex RT-PCR two primer sets were simultaneously used. PCR was run with an initial activation step at 95 °C for 15 min, followed by 45 cycles with a denaturation step at 94 °C for 60 s and a combined annealing and elongation step at 60 °C for 60 s according to the manufacturer's instructions (QuantiTect®, Qiagen, Hilden, Germany) using a real time cycler (Rotor Gene 2000, LTF, Wasserburg, Germany). Non-RT and non-template controls were run for all amplification reactions. All PCR products were controlled by melting curves and product sizes confirmed by agarose gel electrophoresis.

Separate amplification reactions for GAPDH were performed in a final volume of 20 µl, containing cDNA samples corresponding to 2 ng of total RNA as a template, primers for GAPDH at a final concentration of 0.3 µM, and 10 µl Master mix SYBR-Green (QuantiTect SYBR Green I PCR Kit, Qiagen, Hilden, Germany). PCR was performed using a Rotor Gene 2000 cycler (LTF, Wasserburg, Germany) at 94 °C for 900 s, followed by 45 cycles at 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 75 s. Amplification products were controlled by melting curves. Non-RT and non-template controls were run for all reactions.

External standards

Isolation of rat liver RNA was performed using a commercially available Kit (RNeasy Mini Kit, Qiagen, Hilden, Germany). Two µg rat liver RNA was reverse transcribed (70 °C for 8 min, and 42 °C for 1 h, in a total volume of 25 µl) with oligo(dT) primer and 200 U M-MLV reverse transcriptase (Promega, WI, USA). The resulting cDNA was used for amplification of specific sequences (500-700 bp) which included the shorter sequences of target genes described above using appropriate pairs of outer primers (Table 1) with 1 U Taq DNA polymerase (Promega, WI, USA). The amplicons were purified (MinElute purification Kit; Qiagen, Hilden, Germany) and used as external standard in real time simplex and duplex RT-PCR, with the same conditions stated above.

Excluding amplification of genomic DNA

DNA (DNeasy kit, Qiagen) and RNA (RNeasy kit, Qiagen) were isolated from liver tissue, according to the protocol of the manufacturer, photometrically quantified (GeneQuant, Amersham pharmacia biotech, Cambridge, UK), and the PCR performed as described above. To exclude the amplification of genomic DNA, which is especially important during the one-step approach, all intron-exon-spanning primers were tested using genomic DNA (200 ng) as a PCR template and compared with the same amount of RNA (200 ng). No PCR products were generated from genomic DNA using the primers shown in Table 1 (data not shown).

Histology

Segmental mesenteric arteries and veins were excised, embedded in Tissue-Tek (O.T.C. compound, Sakura, USA), and kept at -20 °C until cryosection was performed. 30 µm cross-sections were placed on slides and transferred to 4 % formalin in PBS at room temperature. Vessels were stained with 0.5 % eosin for 30 s, washed gently with distilled water, incubated for 90 s with methylene blue, dehydrated with 70 %, 96 % and 100 % ethanol for 1 min each, embedded in Euparal (Carl Roth, Germany), and covered with coverslips. The histological slides were observed under a transillumination microscope (Ortholux, Leitz; Germany; objective 25/0.60). The inner circumference and the thickness of endothelial and smooth muscle cell layers of arteries and veins were measured from the images observed under the transillumination microscope, taken by a digital camera and using an image analysis system (Pries *et al.* 1997), calibrated to a standard scale taken under the same optical conditions.

Statistics

Data are presented as mean ± S.E.M. if not otherwise stated. Comparison of data sets was performed by Student´s t-test for unpaired samples if applicable. Statistical significance was assessed at $p \leq 0.05$. Measurements were done in duplicate and "n" refers to the number of blood vessels in each group.

Table 2. Morphological parameters of analyzed arteries and veins in the mesentery (n=4, mean ± S.D.).

	arteries	veins
Vessel size *[µm]*		
Inner circumference	623 ± 11.1	1044 ± 112.4
Diameter	246 ± 3.2	348 ± 35.8
Vessel wall thickness *[µm]*		
Total	24.0 ± 2.7	8.1 ± 0.4
Endothelium	2.0 ± 0.3	2.1 ± 0.1
Smooth muscle	22.0 ± 2.4	6.0 ± 0.3
Vessel wall cross-sectional area *[μm^2 . 10^3]*		
Total	16.7 ± 2.2	8.6 ± 1.0
Endothelium	1.3 ± 0.2	2.1 ± 0.3
Smooth muscle	15.5 ± 2.1	6.5 ± 0.8

Results

Morphological characteristics of mesenteric arteries and their accompanying veins

Table 2 represents morphological characteristics of the investigated vessels. The thickness of the endothelium in arteries and veins was equal. As expected, arteries exhibited a 4-fold thicker smooth muscle cell layer as compared to their accompanying veins. With respect to the total cross-sectional area of the whole vessel wall, the relative contribution of endothelial cells is approximately 0.24 in veins and 0.07 in arteries, respectively.

Gene expression in arteries and veins

For the investigated genes a high correlation between Ct-values and copy numbers was observed for both simplex and duplex PCR (Fig. 2A). The same standardization was performed for all genes analyzed in the present study exhibiting r^2 values above 0.95 in all cases.

In order to reduce any loss of RNA during isolation and purification, a one-step approach similar to that in single cell PCR was chosen as described in the Methods (Fig. 1). However, using this procedure, measurements showed extremely high variations (Fig. 2B). This was greatly reduced by a two-step approach including purification and quantification of isolated total RNA (Fig. 2B).

The gene expression levels of four genes were compared between arteries and veins (Fig. 3). Values are given as quotients of the copy number of the gene of interest relative to that of β-actin as house keeping gene measured by duplex RT-PCR (Fig. 3A). In addition, all measured values were normalized to a second house keeping gene GAPDH (Fig. 3B). The expression of VEGFA, TIE2, and ANG2 was higher in arteries compared to veins, while ADAMTS1 was equally expressed in both vessel types.

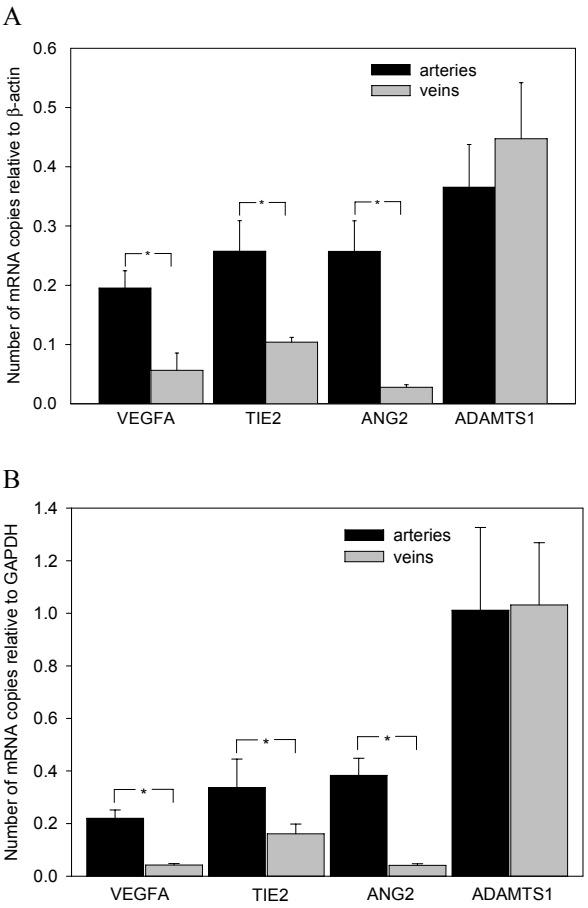

Fig. 3. Expression profiles of four genes in arteries and veins. The copy numbers of VEGFA-, Tie2-, ANG2- or ADAMTS1 mRNA are given relative to that of ß-actin (**A**, upper panel) and GAPDH (**B**, lower panel) as a house keeping gene. Data are given as mean ± S.E.M., n=11. * p≤0.05.

Discussion

In this study, the expression of four genes related to angioadaptation and vascular remodeling has been quantified for paired microvascular arteries and veins using β-actin or GAPDH as house keeping genes. The results show higher expression of VEGFA, TIE2, and ANG2 in arteries as compared to veins, but equal expression of ADAMTS1.

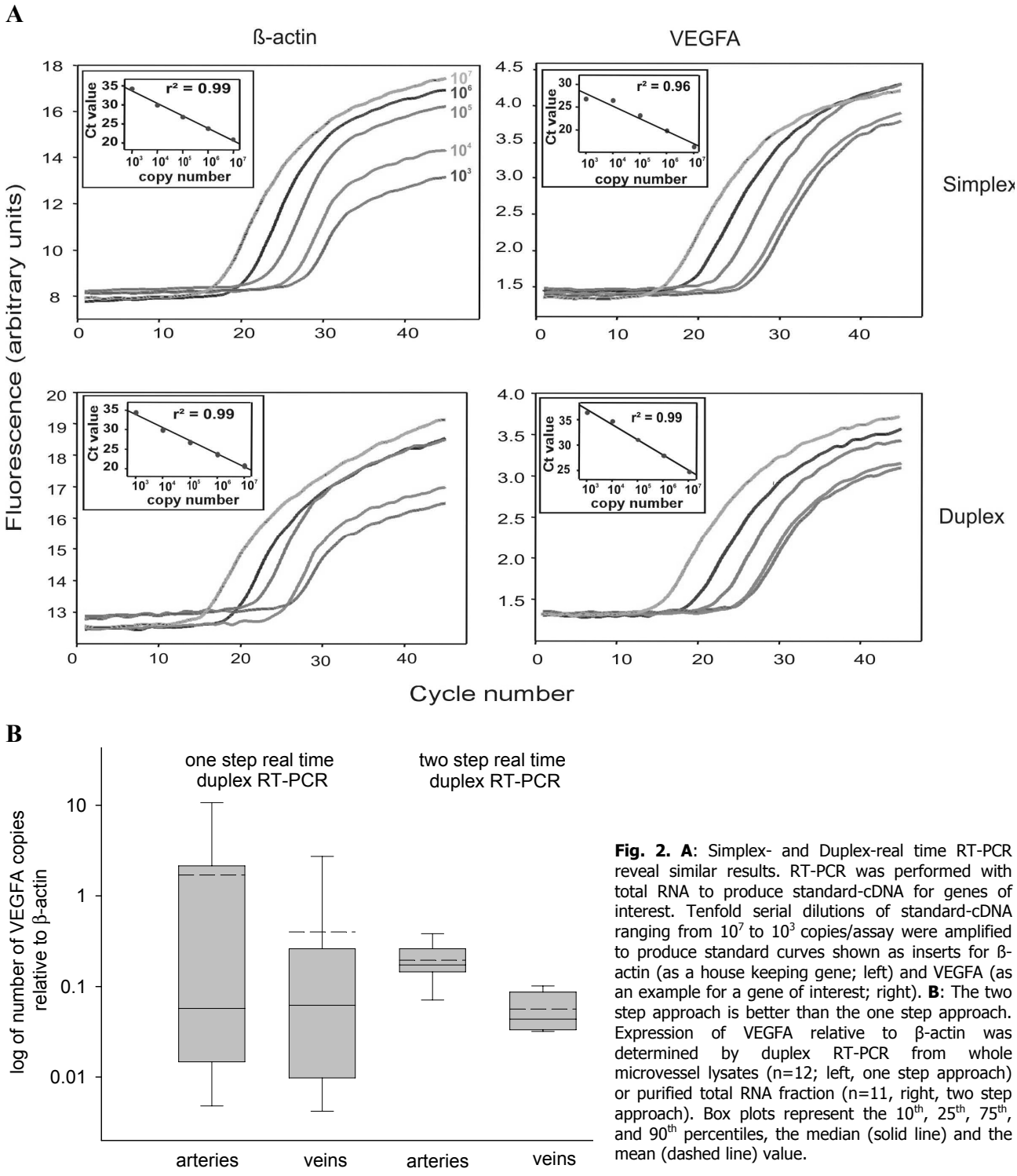

Fig. 2. A: Simplex- and Duplex-real time RT-PCR reveal similar results. RT-PCR was performed with total RNA to produce standard-cDNA for genes of interest. Tenfold serial dilutions of standard-cDNA ranging from 10^7 to 10^3 copies/assay were amplified to produce standard curves shown as inserts for ß-actin (as a house keeping gene; left) and VEGFA (as an example for a gene of interest; right). **B**: The two step approach is better than the one step approach. Expression of VEGFA relative to β-actin was determined by duplex RT-PCR from whole microvessel lysates (n=12; left, one step approach) or purified total RNA fraction (n=11, right, two step approach). Box plots represent the 10^{th}, 25^{th}, 75^{th}, and 90^{th} percentiles, the median (solid line) and the mean (dashed line) value.

Arteries and veins of the rat mesentery differ in the hemodynamic and metabolic conditions to which they are exposed. The magnitude of wall shear stress in the mesentery of the rat is lower in veins ($\approx$15 dyn/cm^2) than in arteries ($\approx$90 dyn/cm^2) (Pries *et al.* 1995b, Pries *et al.* 2005). Furthermore, oxygen saturation measured during intravital microscopy has been reported to be 98 % in arteries and 76 % in veins in the mesentery of rats (Styp-Rekowska *et al.* 2007). From this, an oxygen tension can be estimated of about 100 mmHg for arteries and of about 50 mmHg for veins. These significant hemodynamic and metabolic differences could play a role in differential gene expression between arteries and veins under steady-state conditions.

Analyzing gene expression in single segments of microvessels still remains to be a challenge for two

reasons, the amount of mRNA derived from each sample is relatively low (and the protein content not sufficient for immunoblotting), and the vessel wall consists of more than one cell type. We took advantage from real time RT-PCR techniques to quantify even low copy numbers of mRNA. Most often, the amount of RNA, isolated from a single microvessel segment, was enough for one or two PCRs only.

The amplification of mRNA directly from shock-frozen microvessel segments using intron-exon-spanning primers without RNA isolation by real time RT-PCR to reduce any loss of mRNA by the isolation techniques showed comparable results, but with extremely variable values (Fig. 2, panel B, left). It is evident from these results, that further RNA isolation and purification is necessary to obtain reproducible values of gene expression (Fig. 2, panel B, right) from single segments of microvessels. The resulting protocol describes reduction of the coefficient of variation (CV) from about 200 % to about 45 %. Thus, elaborate isolation and purification steps seem to be necessary, especially for small but complex tissue samples.

VEGFA mRNA was about two- to fourfold higher in arteries than in veins. VEGFA mRNA can be produced by both endothelial as well as smooth muscle cells, but its effects are restricted to the endothelium, because VEGFA-receptors are expressed on endothelial but not smooth muscle cells. The amount of VEGFA which is probably available per endothelial cell will even be higher in arteries, since the relative contribution of endothelial cells to arterial walls is lower than to venular walls. VEGFA is a main inducer of sprouting type angiogenesis (Ferrara *et al.* 2003), and tip cell guidance (Gerhardt *et al.* 2003). In addition, maintenance of blood vessels (Lee *et al.* 2007), vascular permeability (Guidi *et al.* 1995) and dilation of blood vessels (Ku *et al.* 1993) can be enhanced by VEGFA. Consequently, the pharmacological lowering of active VEGFA concentrations is accompanied by an increase in blood pressure in some patients (Hurwitz *et al.* 2004). Since mature arteries do not sprout and they have relatively low permeability during steady-state conditions, the most relevant biological function of higher amounts of VEGFA mRNA copies in arteries, found in this study, should be vasodilation, counterbalancing the Bayliss´ effect. This may be supported by our previous finding, that eNOS-knockout mice, lacking endothelial NO as a main vasodilator, show an increased endothelial baseline VEGFA concentration (Da Silva-Azevedo *et al.* 2002, Baum *et al.* 2004).

Hypoxia is well known as a main inducer of VEGFA expression. Because oxygen tension is higher in arteries than in veins, this cannot explain the differences observed. On the other hand, endothelial VEGFA expression can be induced by shear stress *in vitro* (Chen *et al.* 2001, Fong *et al.* 2005) and with enhanced wall shear stress during prazosin treatment of mice *in vivo* (Da Silva-Azevedo *et al.* 2002), during which the VEGFA concentration in the surrounding tissue declined (Baum *et al.* 2004). So, higher shear stress could be an explanation for higher amounts of VEGFA mRNA in arteries. Since shear stress induces VEGFA in endothelial cells mainly, it should then work in an autocrine fashion. Endothelial autocrine VEGFA effects have indeed recently been shown to be necessary for blood vessel maintenance rather than for the induction of sprouting type angiogenesis (Lee *et al.* 2007). Thus our results could indicate that (for some unknown reason) arteries need stronger signals for their maintenance than veins. However, the complex interaction of different cell types in a vessel wall *in vivo* may further contribute to a differential gene expression.

mRNA expression of endothelial tyrosine kinase receptor TIE2 was about twofold higher in arteries compared to veins. Since TIE2 is known to be specifically expressed in endothelial cells (Dumont *et al.* 1993), its expression could be correlated to the relative contribution of endothelial cells to different types of blood vessel walls (Table 2). Correlated to endothelial cells, the expression of TIE2 mRNA would be even seven times higher in arteries compared to veins. The observed differences between arteries and veins must be caused by differences between their endothelial cells. Endothelial cells are directly exposed to shear stress – frictional forces exerted by the flowing blood. Thus, higher expression of TIE2 mRNA in arteries might be in agreement with our previous finding, that the expression of TIE2 is induced by shear stress in endothelial cells *in vitro* (Chlench *et al.* 2007) and could contribute to the stabilization of arterial walls, since the activation of TIE2 (or the inhibition of ANG2 respectively) has been shown to stimulate the recruitment of perivascular cells (Maisonpierre *et al.* 1997) and vessel wall maturation (Jain 2003). However, the endogenous TIE2-antagonist ANG2 was also more expressed in arteries than in veins, and could thus compensate for the induction of TIE2. However, in some situations, ANG2 has been shown to be an activator of TIE2-dependent signal transduction,

especially at higher concentrations (Daly *et al.* 2006). So, higher expression of ANG2 is not necessarily linked to the antagonism of TIE2 effects. Nevertheless, higher expression of ANG2 in arteries was completely unexpected, since its expression can be suppressed by shear stress *in vitro* (Chlench *et al.* 2007). Alternatively, induction of ANG2 can be achieved by hypoxia (Mandriota and Pepper 1998), but this again would not explain its prevalence in arteries. ANG2 has been shown to be stored in Weibel-Palade bodies (Fiedler *et al.* 2004) which were originally described in arterial endothelial cells (Weibel and Palade 1964). This would probably explain or at least correlate with a higher amount of ANG2 mRNA in arteries. However, some authors reported higher numbers of Weibel-Palade bodies in veins than in arteries (Yamamoto *et al.* 1998). Most probably, higher copy numbers of ANG2 mRNA in arteries will be due to a contribution of smooth muscle cells. Since ANG2 has recently been shown to be a significant cofactor of TNFα-induced inflammatory reactions (Fiedler *et al.* 2006), it could contribute to the development of atherosclerosis. Furthermore, a higher expression of ANG2 in arteries compared to veins as shown in this study could contribute to the restriction of atherogenesis to arteries.

ADAMTS1, although induced by shear stress in endothelial cells (Bongrazio *et al.* 2000), was found to be equally expressed in arteries and veins. In cultured human endothelial cells without flow, ADAMTS1 is nearly completely suppressed. If these cells are exposed to increasing amounts of shear stress (up to 6 dyn/cm^2), its expression is induced (Zakrzewicz *et al.* 2006). However, shear stress in the microcirculation of the rat is significantly higher. Thus, from the data reported here, we have to assume, that the shear stress-induced expression of ADAMTS1 reaches a plateau somewhat below the typical wall shear stress in the veins tested, so that there is no further increase produced by the higher shear stress in arteries. By the release of anti-angiogenic peptides from thrombospondin (Lee *et al.* 2006), ADAMTS1 could inhibit vascular sprouting from well perfused, mature blood vessels of every type.

Several studies identified arterial-venous differentiation markers in endothelial cells like EphB4 for instance (Wang *et al.* 1998). However, EphB4 is not a venous specific marker in mesenteric blood vessels of the rat (Taylor *et al.* 2007). This indicates that arterial-venous differentiation can be accompanied by different marker molecules in different vascular beds. Therefore, the differential expression of endothelial genes has to be tested in single vessel segments from every vascular bed, since the results from one part of the vasculature cannot be necessarily generalized to all vascular regions.

Taken together, microvascular arteries and their accompanying veins differ significantly in the expression of important angioadaptive genes, even during steady-state-conditions. This is probably a part of the physiological adaptation mechanisms of blood vessels to different hemodynamic conditions.

Conflict of Interest

There is no conflict of interest.

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