

Thromboxane A₂ Contributes to the Mediation of Flow-Induced Responses of Skeletal Muscle Venules: Role of Cyclooxygenases 1 and 2

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Key Words

Venule · Shear stress · Nitric oxide · Prostaglandins · Cyclooxygenases 1 and 2 · Thromboxane A₂ synthase

Abstract

Background: It has been shown that increases in intraluminal flow elicit dilation in venules, but the mediation of response is not yet clarified. We hypothesized that – in addition to nitric oxide (NO) and dilator prostaglandins (PGI₂/PGE₂) – thromboxane A₂ (TxA₂) contributes to the mediation of flow-induced responses of venules. **Methods and Results:** Isolated rat gracilis muscle venules (259 ± 11 μm at 10 mm Hg) dilated as a function of intraluminal flow, which was augmented in the presence of the TxA₂ receptor antagonist SQ 29,548 or the TxA₂ synthase inhibitor ozagrel. In the presence of SQ 29,548, indomethacin or N ω -nitro-L-arginine methyl-ester decreased flow-induced dilations, whereas in their simultaneous presence dilations were abolished. The selective cyclooxygenase (COX) 1 inhibitor SC 560 reduced, whereas the selective COX-2 inhibitor NS 398 enhanced flow-induced dilations. Immunohistochemistry showed that both COX-1 and COX-2 are present in the wall of venules. **Conclusion:** In skeletal muscle venules, increases in intraluminal flow elicit production of constrictor TxA₂, in addition to the dilator NO and PGI₂/PGE₂, with an overall effect of lim-

ited dilation. These mediators are likely to have important roles in the multiple feedback regulation of wall shear stress in venules during changes in blood flow velocity and/or viscosity.

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Introduction

Small veins and venules have an important role in determining the amount of blood flow returning to the heart and also capillary functions [1, 2]. Interestingly, less is known regarding the nature of mechanisms regulating the vasomotor tone of venules. Previously, Kuo et al. [3] and we [4] have shown that increases in flow elicit endothelium-dependent dilations in isolated coronary venules and skeletal muscle venules. These and other studies have established that flow-dependent changes in venular diameter contribute to the regulation of venular resistance, similarly to those of small arteries and arterioles [5]. Flow-dependent responses of venules can have important roles in determining venular resistance, capillary pressure and the magnitude of venous return during rest and exercise, when venules are exposed to various flow conditions.

Interestingly, the nature and the mediation of flow-induced responses seem to differ among vascular beds, and

the nature and mediators of flow-induced responses in venules are not well characterized. For example, in isolated rings of veins from rabbit ears, intraluminal injection of saline resulted in contractions, which was dependent on the presence of extracellular calcium. Thus, it was suggested that flow induces constriction by mechanically activating the vascular smooth muscle cells, inducing calcium entry into the cells [6]. In contrast, in isolated rat skeletal muscle venules increases in intraluminal flow resulted in dilations, which were mediated by nitric oxide (NO), dilator prostaglandins (PGI₂/PGE₂) and a constrictor factor [4], the nature of which remained obscure. Interestingly, in our later studies, in isolated lymphatic vessels – known to be exposed to low intraluminal pressures similar to those in venules – we have found a substantial role for the constrictor thromboxane A₂ (TxA₂) [7].

Thus, we hypothesized that, in addition to NO and PGI₂/PGE₂, flow-induced responses of venules are mediated by TxA₂ and that cyclooxygenases (COX) have different roles in producing dilator and constrictor prostaglandins.

Materials and Methods

Male Wistar rats (n = 43, approx. 350 g, purchased from Charles River Co., Budapest, Hungary) were housed separately and had free access to water and standard rat chow. All of the protocols were approved by the Institutional Animal Care and Use Committee. The animals were anesthetized with pentobarbital sodium (50 mg/kg), and small venules (inside diameter 259 ± 11 μm) from the gracilis muscle were isolated as described previously [8, 9] and transferred into an organ chamber containing standard Krebs solution (in mmol/l: NaCl 110, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.0, KH₂PO₄ 1.0, glucose 5.5 and NaHCO₃ 24.0; equilibrated with 10% O₂, 5% CO₂, 85% N₂, at pH 7.4). Then, vessels were cannulated on both sides and were continuously superfused with Krebs solution. The temperature was set at 37°C by a temperature controller (Grant Instruments), and the vessels were equilibrated at constant intravascular pressure (10 mm Hg), allowing them to develop spontaneous tone. In contrast to previous studies, we did not use norepinephrine or other vasoactive agents to precontract venules, because it may have influenced the responses to flow [7]. Instead, we allowed the venules to develop a spontaneous myogenic tone in response to the presence of 10 mm Hg intraluminal pressure. A substantial myogenic tone developed within approximately 1.5 h. The inner diameter of venules was measured by videomicroscopy [9].

Experimental Protocols of Flow-Induced Responses

After the equilibration period, changes in diameter of venules were assessed in response to step increases in intraluminal flow. Diameter changes were measured at the plateau phase of responses. Flow was established at a constant intravascular pressure

(10 mm Hg) by changing the inflow and outflow pressure to an equal degree, but in opposite directions. Dimensions (length and diameter) of both proximal and distal pipettes were carefully matched to provide for equal resistances. In a previous experiment, we changed the pressure difference (P_{diff}) from 0 to 16 mm Hg [4]. This study showed that above 8 mm Hg of P_{diff} the diameter did not further change; thus, we used 8 mm Hg as the maximum P_{diff}. Between 0–4 and 4–8 mm Hg P_{diff}, 1- and 2-mm Hg step changes were used. The relationship between P_{diff} and flow had been established previously and the range of perfusate flow was between 0 and 0.2 ml/min, measured by ball flow meter [4]. To elucidate the role of NO as well as dilator and constrictor prostaglandins in the mediation of flow-induced responses, we used the NO synthase inhibitor N^ω-nitro-L-arginine methyl-ester (L-NAME, 10⁻⁴ mol/l for 30 min), the nonselective COX inhibitor indomethacin (2.8 × 10⁻⁵ mol/l for 30 min) and the TxA₂ receptor (TP) antagonist SQ 29,548 (10⁻⁶ mol/l for 20 min).

To investigate the enzymatic source of prostaglandins, we used the selective COX-1 inhibitor SC 560 (10⁻⁶ mol/l for 30 min), the selective COX-2 inhibitor NS 398 (10⁻⁵ mol/l for 30 min) and the specific TxA₂ synthase inhibitor ozagrel (10⁻⁵ mol/l for 30 min) [10–12]. All inhibitors were administered extraluminally. At the end of each experiment, the maximal passive diameter of venules was obtained after incubation of venules in a Ca²⁺-free solution, which contained ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, 1 mmol/l for 20 min).

Immunohistochemistry

In order to assess the presence of COX-1 and COX-2 in the wall of gracilis muscle venules, we used immunohistochemistry to visualize them, as described previously [11]. Briefly, gracilis muscles of Wistar rats were embedded and frozen in OCT compound. Acetone-fixed consecutive sections (approx. 10 μm thick) were immunolabeled with a polyclonal anti-COX-1 and anti-COX-2 primary antibody (dilution 1:100, respectively). Immunostainings were visualized using the avidin-biotin-horseradish-peroxidase visualization systems (Vectastain kit), stained with diaminobenzidine. For nonspecific binding, the primary antibody was omitted. Images of the sections were collected with a digital camera (CFW 1310C; Scion Corp.) connected to a Nikon Eclipse 80i microscope.

Materials

OCT compound was obtained from Tissue Tek, Electron Microscopy Sciences, the polyclonal anti-COX-1 and anti-COX-2 primary antibody from Cayman Chemicals, the avidin-biotin-horseradish-peroxidase visualization systems from Vector Laboratories, the smooth muscle α-actin from Novocastra, the FITC- and Texas-red-labeled secondary antibodies from Vector Laboratories and Jackson Immuno Research. All other salts and chemicals were obtained from Sigma-Aldrich Co., and solutions were prepared on the day of the experiment. The vehicle did not have vasoactive effects. One vessel was used from each animal, and in the various protocols, 6–10 venules were investigated. Each protocol was conducted in each venule.

Data Analysis

Changes in venular diameter are expressed as a percentage of the maximal dilation of vessels, defined as the difference of the passive diameter (at 10 mm Hg intraluminal pressure, in a Ca²⁺-

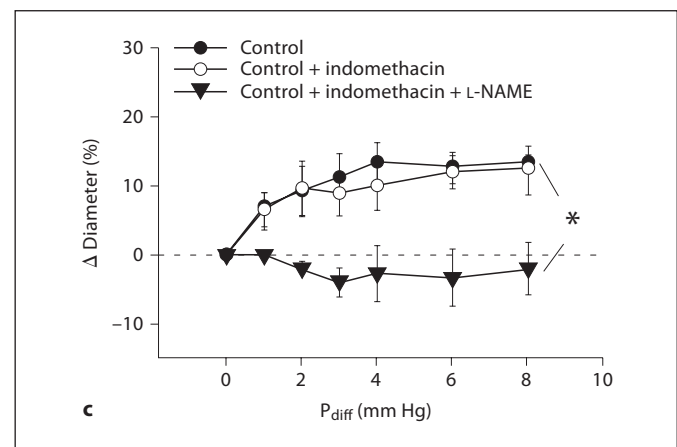
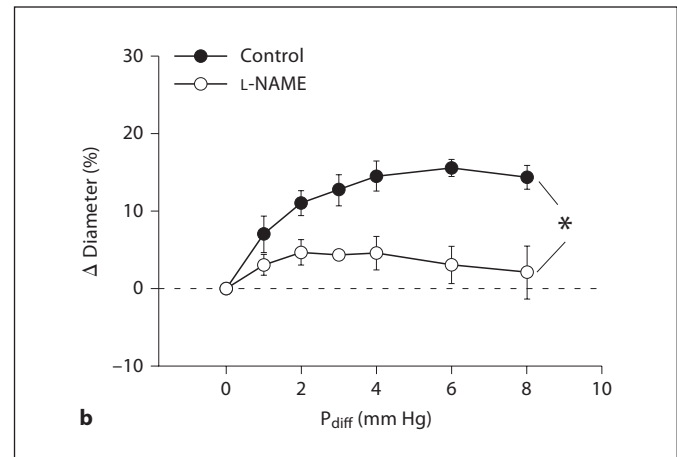
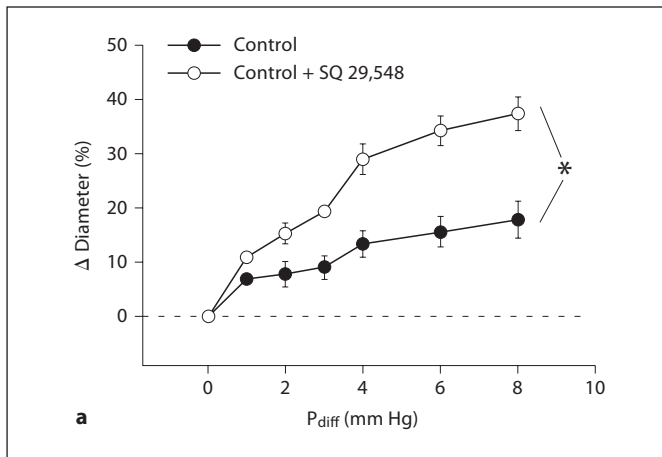


Fig. 1. Changes in diameter of skeletal muscle venules as a function of intraluminal flow elicited by increasing the P_{diff} in the absence or presence of the TP receptor blocker SQ 29,548 (**a**), the NO synthase inhibitor L-NAME (**b**) or the nonselective COX inhibitor indomethacin and indomethacin + L-NAME (**c**). * $p < 0.05$: significant difference ($n = 6$ in each group).

free physiological salt solution containing 1 mmol/l EGTA) and the initial diameter of the venules (at 0 flow condition, at 10 mm Hg). Statistical analyses were performed by 2-way ANOVA for repeated measures followed by the Tukey post hoc test, as appropriate. A value of $p < 0.05$ was considered statistically significant. Data are expressed as means \pm SEM.

Results

The mean diameter of rat gracilis muscle was $259 \pm 11 \mu\text{m}$ in the presence of intraluminal pressure of 10 mm Hg, without the use of vasoactive agents, whereas in Ca^{2+} -free solution the passive diameter of venules was $412 \pm 11 \mu\text{m}$. Thus, venules can develop an appreciable spontaneous myogenic tone allowing studying vasomotor responses without pharmacological precontraction. The various inhibitors used, such as L-NAME, indomethacin, SQ 29,548, ozagrel, SC 560 and NS 398, had no significant effect on baseline diameter.

Mediation of Flow-Induced Responses of Venules by $\text{PGH}_2/\text{TxA}_2$, NO and Prostaglandins

In control conditions, increases in intraluminal flow elicited dilations of isolated venules ($18 \pm 3\%$ at P_{diff} 8 mm Hg), which were, however, significantly increased in the presence of the TP receptor antagonist SQ 29,548 ($37 \pm 3\%$ at max.; fig. 1a) and also in the presence of the TxA_2 synthase inhibitor ozagrel ($21 \pm 1\%$ at max.; fig. 3b).

Compared to controls the NO synthase inhibitor L-NAME significantly reduced flow-induced dilations (from max. ΔD of 14 ± 2 to $2 \pm 3\%$; fig. 1b). In contrast, compared to controls the nonselective COX inhibitor indomethacin did not affect flow-induced dilations, whereas additional incubation of vessels with L-NAME abolished dilations to increases in flow (from max. ΔD of 13 ± 1 to $-2 \pm 4\%$; fig. 1c).

Next, we aimed to elucidate the contribution of NO to the mediation of the dilation in the presence of SQ 29,548;

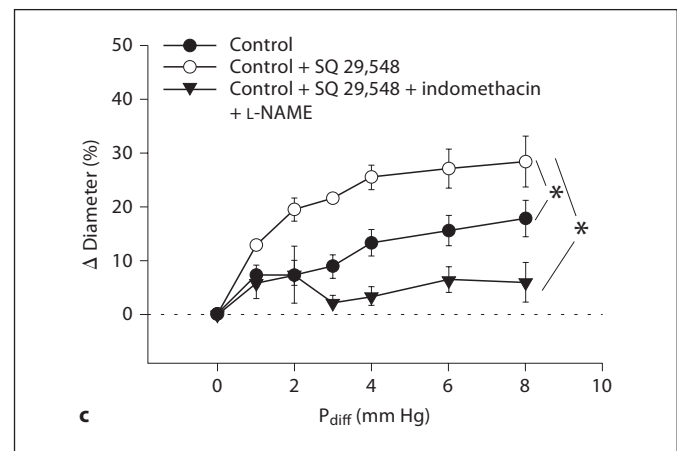
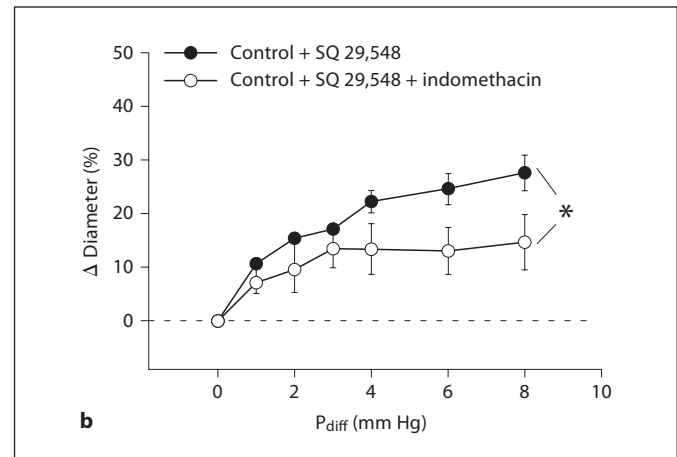
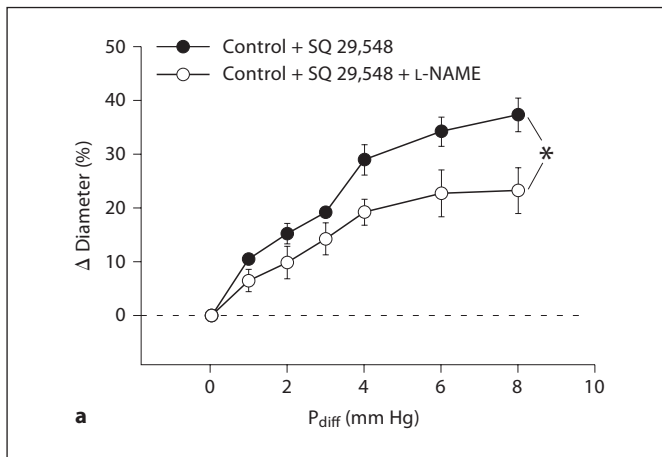


Fig. 2. Summary data show changes in diameter of skeletal muscle venules as a function of intraluminal flow elicited by increasing the P_{diff} in the presence of the TP receptor blocker SQ 29,548 or SQ 29,548 + the NO synthase inhibitor L-NAME (a), SQ 29,548 + the nonselective COX inhibitor indomethacin (b) or SQ 29,548 and both L-NAME + indomethacin (c). * $p < 0.05$: significant difference ($n = 7$ in each group).

thus, after incubation of venules with SQ 29,548, L-NAME was used. We found that in the presence of the TP receptor antagonist the augmented flow-induced dilations were significantly reduced by addition of L-NAME (from max. ΔD of 37 ± 3 to $23 \pm 4\%$; fig. 2a). Similarly, in the presence of SQ 29,548, the nonselective COX inhibitor indomethacin significantly reduced flow-induced dilations of isolated venules (fig. 2b), whereas in the presence of SQ 29,548 simultaneous addition of L-NAME and indomethacin abolished essentially flow-induced responses (fig. 2c).

Role of COX-1 and COX-2

Next, we aimed to reveal the specific roles of COX-1 and COX-2 in producing prostaglandin mediators of flow-induced dilation of venules. We found that the selective COX-2 inhibitor NS 398 did not affect flow-induced dilations in the presence of SQ 29,548 (fig. 3a). In contrast, incubation of venules with the selective COX-1

inhibitor SC 560 significantly reduced the flow-dependent dilations of venules in the presence of SQ 29,548 and NS 398 (fig. 3a).

We found similar changes in the responses using the TxA_2 synthase inhibitor ozagrel. That is, compared to controls, flow-induced dilations were significantly increased in the presence of the TxA_2 synthase inhibitor ozagrel, which were not affected by an additional COX-2 inhibitor, NS 398, but were significantly reduced by the COX-1 inhibitor SC 560 (fig. 3b).

In control conditions (in the absence of SQ 29,548 or ozagrel), addition of the selective COX-1 inhibitor SC 560 significantly decreased (fig. 4a), whereas the COX-2 inhibitor NS 398 increased flow-induced dilations of venules (fig. 4b).

Presence of COX-1 and COX-2 in the Venular Wall

Immunostaining for COX-1 and COX-2 shows that, as compared to control sections without immunostain-

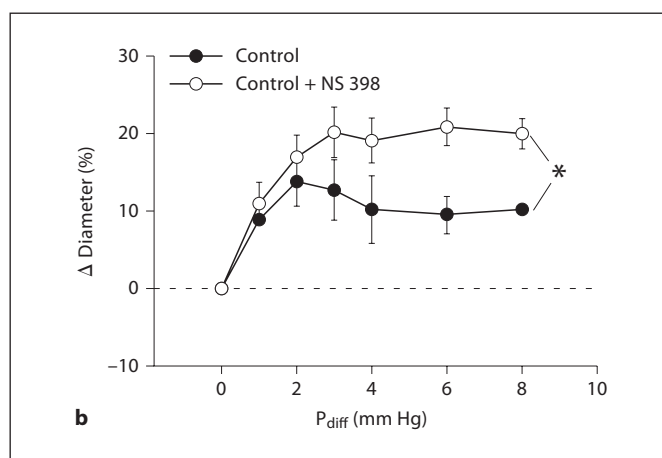
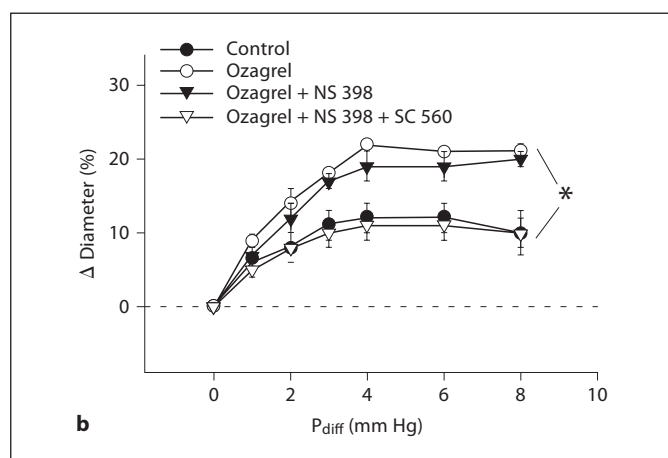
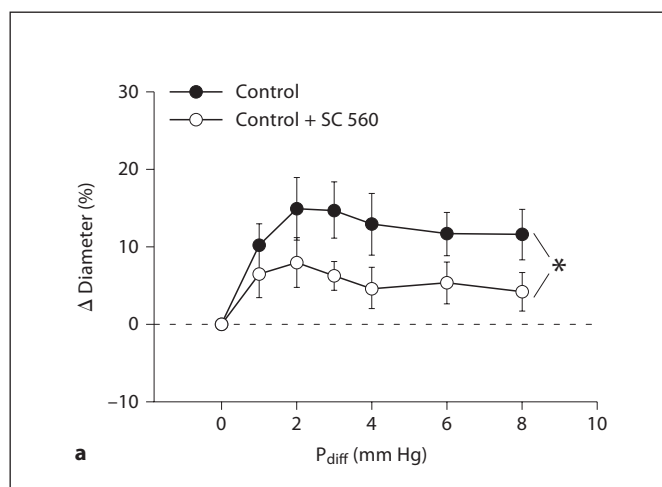
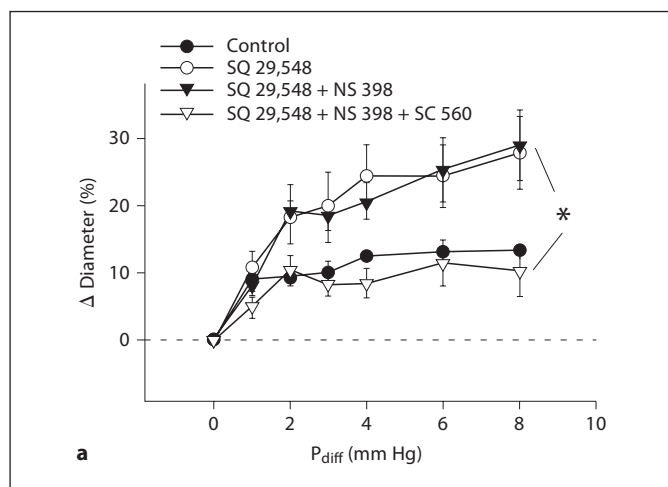


Fig. 3. Summary data show changes in diameter of skeletal muscle venules as a function of intraluminal flow elicited by increasing the P_{diff} in the presence or absence of the TP receptor blocker SQ 29,548 or SQ 29,548 + the COX-2 inhibitor NS 398 or SQ 29,548 + NS 398 + the COX-1 inhibitor SC 560 (a), and in the presence or absence of the TxA_2 synthase inhibitor ozagrel, ozagrel + NS 398 or ozagrel + NS 398 + SC 560 (b). * $p < 0.05$: significant difference ($n = 6$ in each group).

Fig. 4. Summary data show changes in diameter of skeletal muscle venules as a function of intraluminal flow elicited by increasing the P_{diff} in the presence or absence of the COX-1 inhibitor SC 560 (a) and the COX-2 inhibitor NS 398 (b). * $p < 0.05$: significant difference ($n = 7$ in each group).

ing (fig. 5a, background), both COX-1 (fig. 5b) and COX-2 (fig. 5c) labelings were present in the venular wall, primarily localized to the endothelium and subendothelial layer.

Discussion

The salient findings of the present study are that in isolated skeletal muscle venules (1) a substantial spontaneous myogenic tone developed in response to the pres-

ence of intraluminal pressure, (2) increases in intraluminal flow elicited dilations, (3) which were significantly augmented in the presence of the TP receptor antagonist or the TxA_2 synthase inhibitor, (4) dilations were significantly reduced by inhibition of the NO synthase, but (5) were not affected by the nonselective inhibition of COXs, (6) inhibition of COX-1 reduced, whereas inhibition of COX-2 enhanced flow-induced dilations, and (7) both COX-1 and COX-2 are expressed in the venular wall. Collectively, these findings suggest that in skeletal muscle venules, TxA_2 , dilator prostaglandins and NO

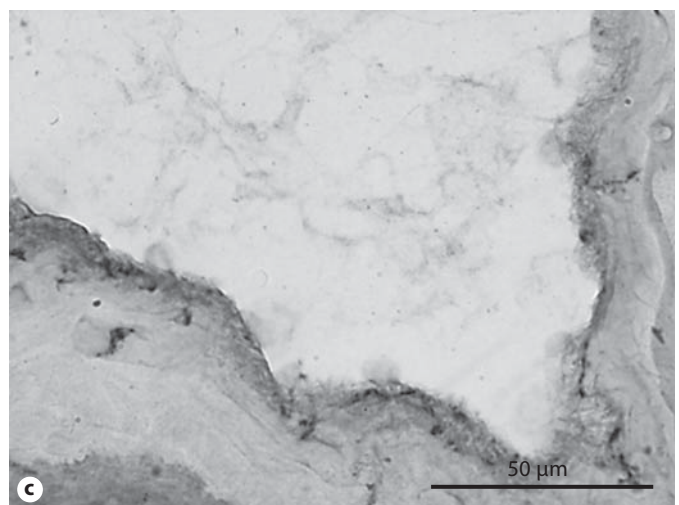
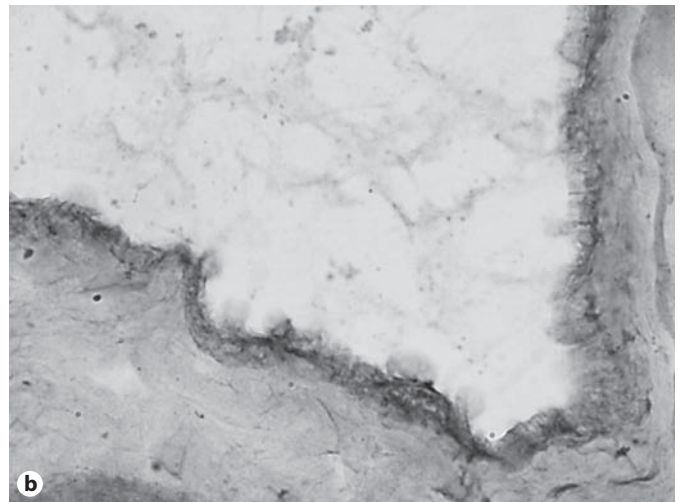
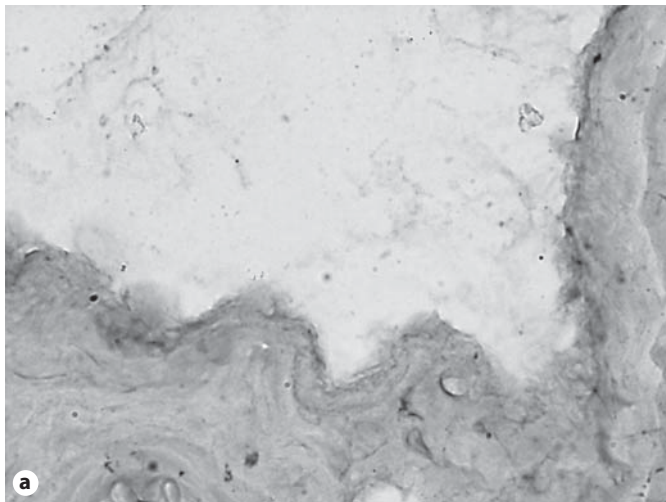


Fig. 5. Representative pictures of immunohistochemical staining of COX-1 and COX-2 in gracilis muscle venules. **a** Background without immunolabeling. **b** COX-1 immunostaining (dark gray; brown in the online version). **c** COX-2 staining (dark gray; brown in the online version). Methyl green was used to stain the nuclei (light gray; green in the online version). Scale bar = 50 μ m.

mediate diameter responses to increases in intraluminal flow.

It has been established that flow-induced responses of arteries and arterioles play an important role in the modulation of vascular resistance and tissue blood flow and that the primary mediators are dilator prostaglandins and NO [5, 13–15]. Much less is known regarding the nature of flow-induced responses and their mediation of venules [3, 4, 16, 17]. Thus, we aimed to investigate the diameter changes of isolated venules in response to increases in intraluminal flow and to elucidate the mediators of the response.

In most previous studies, constrictor agents were used to induce tone for venous vessels, and then flow-induced responses were investigated. These studies

showed that isolated, precontracted large veins [17], coronary venules [3] and skeletal muscle venules [4] responded with dilations to stepwise increases in flow, but the effect of flow on isolated skeletal muscle venules without previous precontraction has not been clarified yet.

Pressure-Induced Venular Tone

An important aspect of the present study is that skeletal muscle venules developed a significant spontaneous myogenic tone (63%) in response to 10 mm Hg intraluminal pressure in the absence of vasoactive drugs. This pressure is likely to be in the physiological range for this size of venules [18]. Having this spontaneous venular tone, flow-dependent responses could be investigated

without the potential interference of vasoactive agents on vascular vasomotor mechanisms.

Flow-Induced Venular Response

We have found that isolated skeletal muscle venules respond with dilations to increases in intraluminal flow as shown in figure 1. Flow-induced dilations of venules may have an important role in the regulation of tissue blood supply [3]. Dilations of postcapillary venules during increased flow conditions may play an important role to reduce venular wall shear stress, but also prevent the development of tissue edema [3] especially during exercise, which elicits substantial dilations on the arteriolar side of the microcirculation [19, 20]. A simultaneous increase in venular diameter in response to increased venular blood flow would help to empty capillary beds by forwarding blood toward the larger venous vessels.

Mediators of Flow-Induced Venular Responses

Thromboxane A₂

In our previous studies in venules, we have found that – unlike in arterioles – constrictor factor(s) is released to flow [4]. To elucidate the nature of constrictor factors mediating flow-induced venular response, we used the TP receptor antagonist SQ 29,548 and the TxA₂ synthase inhibitor ozagrel. We found that in the presence of the TP receptor antagonist or the TxA₂ synthase inhibitor, flow elicited significantly greater dilations in venules, indicating a substantial contribution of constrictor TxA₂ (fig. 1a, fig. 3a, b). These findings suggest that in skeletal muscle venules, increases in flow elicit the release of TxA₂, responsible for a constrictor response. It is known, however, that in addition to the stable metabolite TxA₂, TxB₂ also has constrictor effects, shown previously in pulmonary vasculature [21, 22]; thus, it may also contribute to the response.

NO and Dilator Prostaglandins

A role for NO in mediating flow-induced responses of venous vessels has been shown in isolated precontracted rings of dog jugular veins [17] and also in isolated porcine coronary venules [3], whereas in bat wing venules neither NO [16] nor prostaglandins were involved [16]. Our findings that inhibition of NO synthase significantly reduced flow-induced dilations (in controls, or after indomethacin or TP receptor antagonist) suggest that increases in flow elicit a release of NO, responsible – in part – for dilation (fig. 1b, c, fig. 2a).

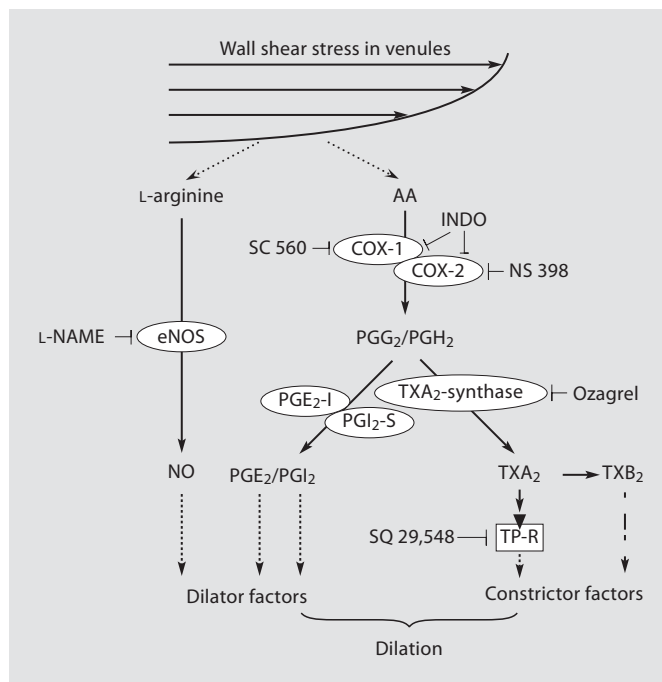


Fig. 6. Schematic drawing showing the proposed mechanisms of flow/shear-stress-induced dilation in skeletal muscle venules. Increases in flow/shear stress stimulate the production of (1) NO from L-arginine by NO synthase, (2) dilator PGI₂ and PGE₂ via the COX-1-PGI₂ synthase and PGH₂-E₂ isomerase pathway, and (3) constrictor TxA₂ via the COX-2-TxA₂ synthase pathway. The final effect of these dilator and constrictor factors is dilation. AA = Arachidonic acid; eNOS = endothelial NO synthase; INDO = indomethacin.

We have found that indomethacin (nonselective inhibitor of COXs) did not affect flow-induced dilations, but in the presence of TP receptor antagonist the augmented flow-induced dilations were significantly reduced by indomethacin (fig. 2a, b) and were eliminated by the further administration of L-NAME (fig. 2c).

These findings suggest that a nonselective inhibitor of COX-1 and COX-2 inhibits the production of both constrictor and dilator prostaglandins to increases in flow, and they are likely produced in equal amounts; thus, the overall effect (in the absence of TP receptor blockade) is no change in diameter.

Based on these findings, we can assume that in isolated skeletal muscle venules in addition to constrictor TxA₂, NO and dilator prostaglandins are simultaneously produced in response to increases in intraluminal flow (fig. 6). The nature of dilator prostaglandins can be assumed from previous findings, which could be both PGI₂ and/or PGE₂ [14, 23–27].

Role of COX-1 and COX-2

It seemed to be important to elucidate the source(s) of prostaglandins released to increases in flow/shear stress. Initially, COX-1 was thought to be a constitutive isoform of the COX enzyme and expressed in physiological conditions, whereas COX-2 was believed to be a form induced by inflammatory stimuli [28]. For example, our recent studies proposed a role for the COX-2-derived constrictor TxA_2 in microvascular dysfunction in diabetes mellitus and revealed an enhanced COX-2 expression in the arteriolar wall [10]. An enhanced expression of COX-2 was also found in isolated coronary arterioles of diabetic patients, and its selective inhibition reduced bradykinin induced dilation, a response known to be mediated by dilator prostaglandins [11]. Recently, it has been shown that both COX-1 and COX-2 could be involved in the synthesis of prostaglandins in physiological conditions and expressed in the wall of arterial vessels of animals and humans [29–31]. However, there are few, if any, studies extant regarding the presence and function of COX isoforms in venular vessels.

In the present study, we found that in the presence of TP receptor blockade or inhibition of TxA_2 synthase, the COX-1 inhibitor SC 560 significantly reduced flow-induced dilations of isolated skeletal muscle venules, whereas the COX-2 inhibitor NS 398 was without effect (fig. 3a, b). We have also found that in the absence of SQ 29,548, the COX-1 inhibitor SC 560 reduced, whereas the COX-2 inhibitor NS 398 increased flow-induced dilations and that indomethacin was without effect (fig. 4a, b, fig. 1c). These findings suggest that in venules, COX-1 participates primarily in the production of dilator, whereas COX-2 participates primarily in the production of dilator prostaglandins in response to increases in flow. It is likely that colocalization of enzymes in the arachidonic cascade, such as COX-1 with PGI_2 synthase and COX-2 with TxA_2 synthase, is responsible for the specific action of COX-1 and COX-2 to release dilator and constrictor factors to increases in flow/shear stress [32]. Corresponding to these functional findings, immunohistochemical staining showed that both COX-1 and COX-2 are expressed in the wall of rat skeletal muscle venules and are localized primarily to the endothelium and subendothelial layer. It is likely that the role of various prostaglandins produced by COX-1 and COX-2 could be different in vascular beds, as previous studies showed that inhibition of COX-2 in healthy humans results in a suppressed prostacyclin synthesis and its urinary excretion [31]. In contrast, in pathological conditions, such as diabetes mellitus, elevated COX-2 expression is associated with increased produc-

tion of constrictor prostaglandins in skeletal muscle arterioles [10]. These findings could have clinical importance during treatments with various COX inhibitors of different selectivity.

Physiological Importance of Venular Responses to Increase in Flow/Wall Shear Stress

Previous *in vivo* studies emphasized the role of hemorheological factors in the regulation of postcapillary resistance, because they found little or no changes in diameter of venules to various interventions, such as changes in pressure and flow [18]. The findings of the present study indicate that isolated venules have an appreciable pressure-induced tone and respond with dilation to increases in wall shear stress elicited by increases in intraluminal flow. Interestingly, the magnitude of shear-stress-sensitive dilations of venules is less than that of arterioles [14]. In theory, the purpose of regulation of wall shear stress – especially in the arteriolar network – is to minimize the power loss in the circulation [33]. This can be achieved by the regulation of wall shear stress in a negative feedback manner, which maintains it at an appropriate level.

Because the levels of hemodynamic forces and the rheology of blood are different in venules and arterioles, one can assume that regulation of shear stress is achieved by different mechanisms. Wall shear stress is the function of wall shear rate and viscosity (dependent primarily on hematocrit), both of which are substantially different in arterioles and venules. In the arterioles, wall shear stress is high due to high wall shear rate (high velocity and narrow diameter), whereas in venules, wall shear stress is low because of the low wall shear rate (low velocity and large diameter). It is known that at low wall shear rate the viscosity of blood increases [34]; thus, in venules, wall shear stress is determined primarily by the viscosity of blood [34], as opposed to arterioles, in which diameter is more important in this regard [14].

This is the reason, we believe, why in venules both dilator and constrictor factors are released, which could limit the increase in diameter, hence the increase in viscosity due to the reduction of shear rate [33]. This is achieved by the release of constrictor TxA_2 , preventing substantial reduction in wall shear rate. That is venules regulate wall shear stress not only by changing their diameter (as arterioles do), but also by maintaining a higher wall shear rate to lower the hematocrit-related viscosity.

In summary, in isolated skeletal muscle venules, increases in intraluminal flow/shear stress elicit production of constrictor TxA_2 , in addition to NO and dilator prostaglandins, resulting in an overall effect of limited dila-

tion. Thus, when blood flow velocity and/or viscosity change, these mediators are likely to contribute importantly to the multiple feedback regulation of wall shear stress in venules.

Acknowledgments

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