Mediation of Flow Dependent Responses of Venules and its Impairement in Hyperhomocysteinemia. Role of COX-1 and COX-2 Derived Thromboxane A_2

Ph.D. Thesis

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INTRODUCTION

Small veins and venules have important role in determining the amount of blood flow returning to the heart and also the capillary functions in part by changing their diameter. However, less is known regarding the nature of mechanisms regulating the vasomotor tone and thus diameter of venules. Previously, it was shown that increases in flow, elicit endothelium-dependent dilations in isolated coronary venules and skeletal muscle venules, which could contribute to the regulation of venular resistance. Flow-dependent responses of venules can have important role in determining venular postcapillary resistance, thus capillary pressure and the magnitude of venous return during rest and exercise, when venules are exposed to various flow conditions. In isolated rat skeletal muscle venules the flow-induced dilations were mediated by nitric oxide (NO), dilator prostaglandins (PGI_2/PGE_2) and a constrictor factor, the nature of which remained obscure. In this study norepinephrine (NE) was used to preconstrict venules. However, the presence of NE has been shown to influence the nature and magnitude of the vasomotor responses of vessels. In our later studies, in isolated lymphatic vessels - known to be exposed to similar low intraluminal pressures as venules - we have found a substantial role for constrictor thromboxane A_2 (TxA_2).

We suggested that these functions of venules are impaired in different venular diseases, (such as varicosity, thrombophlebitis, orthostatic intolerance, deep vein thrombosis), the mechanisms of which we do not have enough knowledge. However, in many developed countries the incidence of venous diseases exceeds arterial diseases. These conditions emphasize the importance of a better knowledge of the causes and mechanisms of pathological changes in veins and venules in venular diseases. Recent clinical observations have clearly showed a significant relationship between hyperhomocysteinemia (HHcy) and diseases of venous circulation (venous thrombosis or venular occlusive diseases). These observations suggest that in addition to changes in coagulation-anticoagulation systems, HHcy impairs the function of endothelium to release various mediators interfering with coagulation and platelet aggregation and altering the regulation of venous tone. Thus, it seemed to be important to study the pathological changes and their mechanisms in HHcy venules, which may probably favor the development of thrombus formation and altered venular function leading to occlusive diseases of small and large venous vessels. Knowledge gained by these studies could help us to develop new therapeutic modalities to prevent and cure venular diseases.

SPECIFIC AIMS OF THE STUDY

To reassess

1) the function of **isolated venules**, which technique allows us to examine the responses of vessels intrinsic to the vascular wall, without any neural, metabolic, humoral effects,
2) **without previous preconstriction** with NE, which may mask the real nature of flow-induced responses and the mediating factors,
3) **the mechanisms** eliciting the flow-induced diameter responses.

To characterize

1. the **function of venules** isolated from rats with **HHcy**,
2. the **pathological mechanisms** responsible for the altered vasomotor function of HHcy venules.

MATERIALS AND METHODS

We investigated the diameter changes of canulated skeletal muscle venules (inside diameter 259±11 μm) isolated from control rats and rats with HHcy (induced by ~5 weeks methionine diet) to the effect of step increasing the intraluminal flow. Flow was established at a constant intravascular pressure (10 mmHg) by changing the inflow and outflow pressure to an equal degree but in opposite directions from 0 mmHg pressure difference (ΔP) up to 8 mmHg ΔP in 1 and 2 mmHg steps.

In the presence of different enzyme and receptor inhibitors and reactive oxygen species (ROS) scavengers, we reinvestigated the flow-induced responses to test the mechanism of diameter changes of control and HHcy venules. The NO synthase inhibitor, N^{ω}-nitro-L-arginine-methyl-esther (L-NAME), the non-selective cyclooxygenase (COX) inhibitor, indomethacin (INDO), the TxA_2-receptor (TP) antagonist, SQ 29,548, superoxide dismutase plus catalase (SOD/CAT) were used.

To investigate the enzymatic source of prostaglandins, we used the selective COX-1 inhibitor, SC 560, the selective COX-2 inhibitor, NS 398 and in control conditions the specific TxA_2 synthase inhibitor, Ozagrel.

To assess the presence of COX-1 and COX-2 in the wall of gracilis muscle venules, we used immunohistochemistry

The presence of ROS in the venular wall were tested by double staining of dihydroethydine (DHE) and DAPI.

RESULTS

Isolated venules developed spontaneous active tone (control: 259±11 μm; HHcy: 250±30 μm) in the presence of intraluminal pressure of 10 mmHg, without the use of vasoactive agents. The passive diameter of venules was 412±11 μm and 401±11μm in Control and HHcy venules, respectively.

A.)

In control conditions increases in intraluminal flow elicited dilations of isolated venules (18±3 % at ΔP 8 mmHg), which were significantly increased in the presence of the TP receptor antagonist, SQ 29,548 (37±3 % at max.) and the TxA_2 synthase inhibitor, Ozagrel (21±1 % at max.).

The NO synthase inhibitor, L-NAME reduced significantly flow-induced dilations (from max. ΔD: 14±2 % to 2±3 %). In contrast, the non-selective cyclooxygenase inhibitor, indomethacin did not affect flow-induced dilations, whereas additional L-NAME incubation abolished dilations to increases in flow (from max. ΔD: 13±1 % to -2±4 %).

In the presence of the TP receptor antagonist, SQ 29,548 the augmented flow-induced dilations were significantly reduced by addition of L-NAME (from max. ΔD: 37±3 % to 23±4 %) or the non-selective COX inhibitor, indomethacin (from max. ΔD: 28±3 % to 15±5 %), whereas, simultaneous addition of L-NAME and INDO abolished essentially flow-induced responses (from max. ΔD: 28±5 % to 6±3 %).

The selective COX-2 inhibitor, NS 398 did not affect flow-induced dilations of Control venules in the presence of SQ 29,548 (from max. ΔD: 28±5 % to 27±8 %), but the selective COX-1 inhibitor, SC 560 reduced significantly the flow-dependent dilations of venules in the presence of SQ 29,548 and NS 398 (from max. ΔD: 27±8 % to 12±6 %).

Compared to control conditions (in the absence of any inhibitors), flow-induced dilations were significantly increased in the presence of the TxA_2-synthase inhibitor, Ozagrel (from max. ΔD: 10±3 % to 21±1 %), which were not affected by an additional COX-2 inhibitor, NS 398 (from max. ΔD: 21±1 % to 20±1 %), but were significantly reduced by the COX-1 inhibitor, SC 560 (from max. ΔD: 21±1 % to 10±2 %). Also, in control conditions (in the absence of SQ 29,548, or Ozagrel), addition of the selective COX-1 inhibitor, SC 560 significantly decreased (from max. ΔD: 12±4 % to 4±2 %), whereas the COX-2 inhibitor, NS 398 increased flow-induced dilations of Control venules (from max. ΔD: 10±1 % to 20±2 %).

Immunostaining for COX-1 and COX-2 showed, that both COX-1 and COX-2 staining were present in the wall of venules isolated from Control rats, primarily localized to the endothelium and sub-endothelial layer.

B

Stepwise increases in flow induced significant constrictions in HHcy venules (max. ΔD: -25±4 %). The TP-receptor antagonist, SQ 29,548, converted constrictions to dilations (from max. ΔD: -25±4 % to 17±4 %), which were essentially abolished by the simultaneous administration of L-NAME and INDO (from max. ΔD: 21±2 % to 5±2 %).

The specific COX-2 inhibitor, NS 398 converted flow-induced constrictions to dilations (from max. ΔD: -22±2 % to 9±3 %), which were then significantly reduced by the specific COX-1 inhibitor, SC 560 (from max. ΔD: 9±3 % to 3±3 %). Incubated the venules first with SC 560 abolished flow-induced constrictions (from max. ΔD: -17±2 % to 0±4 %), which were converted to dilations in the presence of NS 398 (from max. ΔD: 0±4 % to 12±2 %).

An enhanced HHcy-induced superoxide production was showed by ethidium bromide fluorescence method in saphenous vein of HHcy rats compared to Control vessels. The presence of SOD plus CAT reduced the fluorescence to the similar level of Control vein. Correspondingly, the presence of SOD plus CAT diminished flow-induced constrictions in venules of HHcy rats (from max. ΔD: -33±3 % to 0±2 %).

DISCUSSION

One of the important novel findings of our studies is that skeletal muscle venules developed a significant spontaneous myogenic tone (63 % of passive diameter) in response to 10 mmHg intraluminal pressure in the absence of vasoactive drugs.

A.

In isolated skeletal muscle venules thromboxane A_2, dilator prostaglandins and nitric oxide mediate diameter responses to increases in intraluminal flow. Dilator prostaglandins are produced by the COX-1, constrictor prostaglandins by the COX-2 pathway. It is likely that colocalization of enzymes on 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. It is likely that the role of the 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 extraction. In contrast, in pathological conditions, such as diabetes mellitus, elevated COX-2 expression is associated with increased production of constrictor prostaglandins in skeletal muscle arterioles. These findings could

have clinical importance during treatments with various COXs inhibitors of different selectivity.

Thus we found that isolated skeletal muscle venules respond with a complex manner, resulting in moderate dilations to increases in intraluminal flow. This response of postcapillary venules may have an important role in the regulation of tissue blood supply and to reduce venular wall shear stress, but also prevent the development of tissue edema especially during exercise, which elicits substantial dilations on the arteriolar side of the microcirculation. 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.

It is important that in venules wall shear stress is low, because of the low wall shear rate (low velocity and large diameter) and at low wall shear rate the viscosity of blood increases. This is the reason, we believe, why in venules constrictor factors are released beside dilator factors, which could limit the increase in diameter, hence increase in viscosity due to the reduction of shear rate. Thus venules regulate wall shear stress not only by changing their diameter (as arterioles do), but also by maintaining 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 dilation. Thus when blood flow velocity and/or viscosity change, these mediators are likely contribute importantly to the multiple feed-back regulation of wall shear stress in venules.

B.

In skeletal muscle venules HHcy mediates constrictions in responses to increasing in flow, which is mediated in part by an elevated production of TxA_2, and in part by an elevated production of ROS. TxA_2 is produced both by COX-1 and COX-2 pathways. COX-1 produces both dilator and constrictor prostaglandins, whereas COX-2 produces constrictor prostaglandins. In the presence of TP receptor blockade, a role of NO and dilator prostaglandin (likely PGI_2/E_2), could be revealed in mediating flow-induced dilations suggesting that the dilator effects of NO and prostaglandins are overcome by the substantial release of TxA_2.

After previous studies, which showed that reactive oxygen species (ROS), such as superoxide anion and H_2O_2 elicited constrictions of isolated venules primarily by activating TxA_2

receptors we supposed that in venules isolated from HHcy rats – in addition to TxA_2 - ROS also contribute to the development of flow-induced constrictions, either directly via facilitation of constrictor prostanoid production or via decreasing the bioavailability of NO. One of the potential sources of ROS can be the upregulation of arachidonic acid metabolism via cyclooxygenase, lipoxygenase and PGH_2 synthase.

The pathophysiological and clinical importance of our findings in HHcy venules is that, in addition to changes in coagulation and anticoagulation systems, HHcy alters the function of venular endothelium. Intact function of endothelium is important both in the regulation of vasomotor tone and rheological properties of blood. In HHcy, the increased release of TxA_2 and ROS may significantly alter the regulation of the resistance of small veins and venules. It is known that during physical activity, such as locomotion, venular blood flow increases substantially, which in normal conditions would increase the diameter of venular vessels allowing the increase in venular and venous blood flow. However, in HHcy the increased release of TxA_2 and ROS to flow could increase the resistance of venular circulation, hence changes venous return and may promote platelet aggregations as well.

In previous clinical studies, the level of 8-iso-$PGF_{2\alpha}$, a peroxidized product of arachidonic acid, increased in urine of HHcy patients compared with healthy subjects, which correlated with plasma levels of Hcy and with the rate of TxA_2 biosynthesis measured by the level of TxB_2 in urine. These findings suggest the presence of oxidative stress induced platelet and endothelial cell activation in HHcy patients, which in turn produced constrictor TxA_2 as suggested by the present study, as well.

In conclusions, we have found that the intrinsic regulation of the vasomotor function of venules is substantially altered in HHcy, which is due to the increased production of thromboxane A_2 produced primarily by COX-2 and elevated levels of reactive oxygen species. These factors can increase the resistance of venular blood circulation and at the same time - we propose - could contribute to increased platelet aggregation and thrombus formation, all of which favor the development of occlusive venous diseases in HHcy. The clinical importance of our finding is that appropriately interfering with the upregulated arachidonic metabolism and production and/or elimination of reactive oxygen and nitrogen species may help to prevent the development of venular and venous thrombotic and occlusive diseases in hyperhomocysteinemia.

Publications related to the thesis

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5. **A. Racz**, Z. Veresh, G. Lotz, N. Erdei, Z. Bagi, A. Koller. Cox-2 derived prostaglandin H_2/thromboxane A_2 (PGH_2/TXA_2) mediates flow dependent constrictions of gracilis muscle venules in hyperhomocysteinemia (HHcy). 25th European Conference on Microcirculation, Budapest, 2008.