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

Ph.D. Dissertation

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Budapest
2009

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Abbreviations

ACE	angiotensin converting enzyme
ADP, ATP	adenosine diphosphate, adenosine triphosphate
ANP	atrial natriuretic peptide
Ach	acetylcholine
ATIII	antithrombin III
BHMT	betaine-homocysteine *S*-methyl-transferase
cAMP	cyclic adenosine monophosphate
CAT	catalase
CBS	cystathion *ß*-synthase
COX	cyclooxygenase
CGRP	calcitonin gene-related peptide
5-CH_3-THF	5-methyltetrahydrofolate
CRVO	central retinal vein occlusion
ΔD	diameter changes
DAPI	4',6-diamidino-2-phenylindole
DHE	dihydroethydine
DPI	diphenyl-iodonium
EDRF	endothelium-derived relaxing factor
EDTA	ethylene glycol-bis(β-aminoethyl ether)-*N, N, N', N'*-tetraacetic acid
ER	endoplasmatic reticulum
ET	endothelin
GPIIb-IIIa	glycoprotein IIb/IIIa
5-HT	serotonin
Hcy	homocysteine
tHcy	total homocysteine
HHcy	hyperhomocysteinemia
H_2O_2	hydrogen-peroxide
HUVEC	human umbilical cord vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
INDO	indomethacin
K_{Ca} channels	calcium-dependent kalium chanel
L-NAME	N^{ω}-nitro-L-arginine-methyl-esther
L-NMMA	NG-monomethyl L-arginine
MS	methionine synthase
NE	norepinephrine
NF-kappaB	nuclear factor(NF)-kappaB
NO	nitric oxide
eNOS/iNOS	endothelial/inducible nitric oxide synthase
NS 398	selective COX-2 inhibitor
$O^{2.-}$	superoxide
Ozagrel	specific TxA_2 synthase inhibitor
tPA	tissue plasminogen activator
P_{diff}	pressure difference
PG	prostaglandin
PGI_2	prostacyclin
PMNLs	polymorphonuclear leukocytes

PON	peroxynitrite
ROS	reactive oxygen species
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
SC 560	selective COX-1 inhibitor
SOD	superoxide dismutase
SP	substance P
TIA	transient ischemic attack
TNF-α	tumor necrosis factor alpha
TP-R	TxA-receptor
TxA_2	thromboxane A_2
VCAM-1	vascular cell adhesion molecule
vWF	von Willebrand factor
XO	xanthine oxidase

1. 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.[1] [2] However, less is known regarding the nature of mechanisms regulating the vasomotor tone and thus diameter of venules. Previously, Kuo et al. and we have showed that increases in flow, elicit endothelium-dependent dilations in isolated coronary venules[3] and skeletal muscle venules with previous preconstriction.[4] 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 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. After the afore-mentioned observations it seems to be important to better know the mechanisms of flow-induced responses of venules**.**

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 in spite of the fact that 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. Similarly to the arterial system - in which it was showed that beside the “classic” cardiovascular risk factors, the high level of homocysteine plays a role in the development of cardiovascular diseases - recent clinical observations have clearly showed a significant relationship between hyperhomocysteinemia (HHcy) and diseases of venous circulation as well, such as venous thrombosis or venular occlusive diseases (e.g. central retinal vein occlusion), which suggest the importance of the better knowledge of the mechanisms of the development of venular diseases in HHcy.

1.1. Characteristics of Venous Vessels

1.1.1. Physiological Functions of Veins and Venules

Venous vessels have many substantial roles in the maintenance of normal cardiovascular functions, tissue circulation and also in adaptive responses of vascular system to different conditions. Among these many functions, only those are described in the following sections, which are closely related to the regulation of venous circulation.

a. Conduit (Collectors) Function

One of the main functions of veins is to forward blood from tissues to the heart and by this way to provide appropriate venous return, which is one of the main factors determining cardiac output. It is well known that skeletal muscle contractions are essential to promote venous blood flow and that venular valves play key role in maintenance of continuous perfusion.[6 7 8] However, venous vessels do not passively deliver blood from periphery,[9] but have active mechanisms to propel blood induced by local and systemic stimuli.[10 11]

The high prevalence of varicosity, which is - in part - the consequence of valvular dysfunction in veins, highlights the importance of venular valves in the regulation of normal blood flow.[12]

b. Capacitance Function

In normal, resting condition, the venular system, including large and small veins and venules, stores 60-80% of the circulating blood.[13 14] These blood storages have substantial role in various physiological and pathophysiological conditions, for example in case of increased blood demand (e.g. in physical exercise, or in blood loss).[15 16] In case of hemodynamic shock, when a large fluid loss occurs (e.g. bleeding, anaphylaxy, burning), large amount of blood loss can be compensated by mobilization of blood from reservoirs, such as large abdominal veins, liver, spleen, subcutaneous venous plexus, heart and lungs.[17] The blood reservoir and mobilization function is essential in the maintenance of normal perfusion pressure to provide suitable blood supply to tissues. In the opposite side, veins and venules can also accumulate blood in case of elevated

central venous pressure.[18] These functions are well regulated partly by sympathoexcitation,[19] and arterial baroreceptors.[20]

c. Maintenance of Filling Pressure of the Heart

Cardiac output is determined, in part, by contractile force of cardiac muscle, which depends on the preload, the volume of blood returning to the heart. Therefore, it is evident that active change in venous capacity in different conditions is essential in providing suitable filling pressure, thus cardiac output and therefore suitable tissue perfusion.[16 19 21]

d. Increasing the Orthostatic Tolerance

Distensibility of venular vessels is known to be high in low pressure range, which means that even small increases in intraluminal pressure result in substantial increase in diameter of veins. In case of changes of body position, a substantial orthostatic pressure load develops in lower limbs resulting in dilation of venous vessels. This dilation would cause a substantial systemic hypotension and thus syncope without the presence of, or in failure of several mechanisms increasing the tolerance of orthostatic pressure load.[22 23] These mechanisms include - in part - the activation of myogenic response of small veins and venules - tone, and intact venous valves, functioning neuronal activity, including arterial and cardiopulmonary baroreceptors, normal peripheral neural pathways, and central neural integration, appropriate neurohormonal secretion, etc.[22 23] Functioning skeletal muscle pump is also important in adequate venous return. For example, exogenous stimulation of the calf muscle pump in adult women, who experienced substantial pooling in their calves when their lower limbs were maintained in a dependent position, reversed fluid pooling.[24] Contrary, others suggested that respiratory muscle pressure production is the predominant factor, which modulates venous return from the locomotor limb both at rest and during calf contraction.[25]

e. Postcapillary Resistance

Changing in diameter of postcapillary venules has important role in determining the capillary hydrostatic pressure in different conditions. For example, in case of elevated demand of blood flow, when dilation of precapillary arterioles occurs to the effect of different systemic and local factors, the parallel venular dilation to flow increase is

essential to prevent the enhancement of hydrostatic pressure in the capillaries and thus the development of tissue edema. Also, this flow-induced active venular dilation reduces blood reflow to the heart, which in turn decreases the strech of cardiac muscle thus the energy need of contraction.[2 26 27 28]

f. Selective Barrier Function

Postcapillary venules participate in transmural filtration of molecules into the tissue and also in resorption of fluid which is forwarded into larger veins.[29 30] The filtration function of venous vessels is different in different region. Moreover, in pathological conditions, such as in inflammation, venules and veins have important function in diffusion of inflammatory mediators, leukocytes and other cellular elements.[31 32]

g. Angiogenesis

Among others, angiogenesis is a key adaptive mechanism, when demand of blood flow increases in different physiological conditions (e.g. physical exercise).[33 34] Venous vessels take also part, beside arteries – in this adaptational process, the mechanisms of which are still unknown.[35 36 37]

h. Others

Last, but not least veins and venules have immunological and antithrombotic role. They represent the site of the adherence of polymorphonuclear leukocytes (PMNLs) to the endothelium (margination) under physiological conditions, which is considered as a specific dynamic leukocyte pool and is regulated, in part, by endothelium and by local hemodynamic factors.[38 39] Also, venules participate in mediation of immunological responses regulating leukocyte–endothelium interactions[40 41] and in preventing platelet aggregation,[42] thus thrombus formation as well. These processes are likely mediated by the endothelium, which is an important source of biologically active substances, such as nitric oxide, prostaglandins and endothelium-derived relaxing factor (EDRF).[42 43 44] These factors can be released to changes in hemodynamic forces, environemental factors and neural and humoral substances.[45]

1.1.2. Local Regulation of Venous Tone

a. Metabolic Factors

Oxygen Tension (pO_2), Potassium, Adenosine, or ATP

Metabolic factors in regulation of the tone of arterioles is already well established,[46] contrary to postcapillary venules, in which it is much less studied. However, it is known that venules have substantial role in regulating capillary pressure, thus tissue perfusion and therefore to set the metabolic demand of tissue to its blood supply. There are studies showing that venular vessels also respond with changes in their diameter in response to vascular occlusion or physical exercise decreasing total peripheral resistance.[47] However, in large veins, such as superficial veins in the hands, physical exercise induced reduction of venous compliance (measured immediately after the exercise).[48] To the effect of vascular occlusion or physical exercise, metabolic environment (e.g. tissue oxygen tension, pH, ATP level etc.) largly changes, which suggests that metabolic factors have important role in the diameter responses of venous vessels.

For example, recent studies showed that both arteriolar and venular endothelium are sensitive to oxygen reduction in vivo or in vitro, and respond with increasing NO and prostaglandin production.[49] [257] During reduced oxygenation, arteriolar and venular diameters increased, which was inhibited by NG-nitro-L-arginine methyl ester.[49] Contrary, in pulmonary circulation, similarly to arterioles, venules respond with vasoconstriction to hypoxia and anoxia.[50] [51]

Extracellular potassium content may also play significant effect on venular functions in these conditions, influencing the permeability of the endothelium via inducing an increase of Ca^{2+} influx into endothelial cells of venules.[52] In pulmonary venular smooth muscle cell membrane the different K-channels play role in determining venular resting tone, which may modulate venous return physiologically and in disease states e.g. in pulmonary edema.[53]

An other important mechanism of adaptation of microcirculation to metabolic demand is the feedback regulation of arteriolar diameter by vasoactive agents released from the venular endothelium. The close venular-arteriolar pairing allows for diffusion of vasoactive substances from the venular blood to the arterioles. For example, ATP

released from red blood cells may stimulate the venular endothelium to release vasoactive metabolites of arachidonic acid, which dilate arterioles.[54] ATP and its derivates, mainly adenosine are the major vasorelaxant metabolite in arterial vessels,[55] but there are few data about the effect of adenosine and its derivates to the diameter of veins and venules. However, it is evident that adenosine A(1), A(2A), and A(2B) receptors are expressed in both arterioles and venules.[56] In an in vitro animal study retinal superfusion with adenosine caused dilation of retinal venules in a dose-dependent manner by activating smooth muscle K_{ATP} channels.[57] Human, superficial hand veins responded with dilation to the administration adenosine.[58, 59]

b. Humoral Factors

Effects of humoral factors on venous vessels have been investigated mainly in vitro animal models, in human umbilical vein cells or in human hand veins in vivo. Here, only those substances are mentioned, which have been investigated the most.

Constrictor Agents (Angiotensin I and II, Vasopressin, Agonists of Alpha-Adrenergic Receptors, Bradykinin)

Locally infused angiotensin I, angiotensin II, and phenylephrin produces a dose-dependent constriction of hand veins.[60] [61] [62] The presence of angiotensin converting enzyme (ACE) in human peripheral veins is supported indeed by the observation that local infusion of an ACE inhibitor into superficial human hand veins attenuated the constrictor effect of angiotensin I, but not that of angiotensin II.[60] Enalaprilat infusion into human dorsal hand vein caused dilation in norepinephrine preconstricted vessels.[62]

Vasopressin, which is also a potent vasoconstrictor factor, elicited concentration-dependent, endothelium-independent contractions in human saphenous vein by V1-receptor stimulation, but in the presence of V1-receptor blockade it caused dilation mediated by the release of endothelium-derived relaxant prostaglandins.[63]

The alpha-adrenergic receptors stimulation by noradrenalin, phenylephrin, or clonidine resulted also in venoconstriction in a dose-dependent manner in human dorsal hand veins.[62] [64] [65] [66]

Last, but not least, bradykinin has also constrictor effect in rabbit saphenous vein by increasing the Ca^{2+} influx from the extracellular space, Ca^{2+} release from intracellular storage and increasing the Ca^{2+} sensitivity of the contractile apparatus.[67 68]

Dilator Agents (Atrial Natriuretic Peptide, Stimulation of Beta-Adrenergic Receptors, Neurotransmitters and Modulators)

Local infusion of ANP increased forearm vascular volume in a dose-dependent manner, whereas ANP-receptor antagonist increased venous tone by inhibiting the venodilator effect of basal ANP.[69] However, an other human study suggests that in human forearm ANP has predominantly arterial effects, whereas its venous actions become manifest only in the presence of vasoconstriction and thus the venous effect of ANP may have only importance in disease states with elevated levels of vasoconstrictors, such as congestive heart failure.[70]

Stimulation of beta-adrenergic receptors (by isoproterenol) had also dilator effect for example in human hand veins, which was mediated in part by cyclic guanosine monophosphate-dependent mechanisms.[71 72] Dilator effect of beta-adrenerg stimulation is supported by the observation that in human umbilical vein cells the beta(2)-adrenoceptor agonist, isoprenaline increased eNOS activity, associated with an increased phosphorylation level of serine residue of eNOS.[73] Also, isolated canine saphenous vein strips responded with relaxation to isoprenaline in a dose-dependent manner.[74]

Neurotransmitters and modulators, such as histamine, calcitonin gene-related peptide (CGRP), bradykinin, neuropeptide Y, prostaglandins (PGE_1 and PGF_2 alpha), vasoactive intestinal polypeptide and substance P (SP) resulted in dilation in human pial veins.[75] The effect of CGRP was mediated in part by inhibition of the influx of extracellular Ca^{2+} and also by attenuating the release of intracellular Ca^{2+} from the sarcoplasmatic reticulum,[76] whereas histamine effected on by both an H1-receptor mediated endothelium-dependent pathway, and an H2-receptor mediated cAMP dependent pathway.[77] The SP-induced vasodilation was mediated by the activation of ATP-dependent potassium channels on vascular smooth muscle in hand veins,[78] whereas via NO and also via endothelium-dependent hyperpolarization in human omental veins.[79]

An important pharmacological agent, the exact mechanism of which is not fully known, but decreases portal pressure is somatostatin. In case of elevated portal venous pressure the pressure in the collateral veins and small veins increases, this could result in venular bleeding, most frequently in the oesophageal varices. Somatostatin has a well known and common pharmacological agent in the therapy of variceal bleeding by decreasing portal pressure. [80] Administration of somatostatin has less side-effect than administration of vasopressin with nitroglycerin. [81] Somatostatin also prevents the post-endoscopic increase in hepatic venous pressure gradient, which was shown to be increased after emergency endoscopic injection sclerotherapy and endoscopic band ligation. [82] By the way, it was shown to be as effective as injection therapy in the control of acute variceal bleeding and incidence of recurrent bleeding in the first 5 days after initiation of therapy. [83]

Factors with both Dilator and Constrictor Effects (Serotonin)

Serotonin induces both dose-dependent constriction and dose-dependent dilation of arterioles of rat skeletal muscle depending on different levels of the arteriolar microcirculation but did not alter the diameter of first-order venules.[84] However, in in vivo human investigations showed the presence of a serotonin induced constriction of human superficial veins,[85] both by 5-HT1 and 5-HT2 receptor dependent manner.[86]
In animal studies of isolated porcine pial veins, in the presence of muscle tone, veins exhibited rhythmic contractions that were inhibited by serotonin (5-HT) in a concentration-dependent manner, which was in part mediated by 5-HT_7 receptors located on the venous smooth muscle.[87]

c. Endothelial Factors

Constrictor Factors (Endothelin, Metabolites of Arachidonic Acid)

Similarly to arteries and arterioles, the most potent vasoconstrictor factor produced by the endothelium of veins and venules is endothelin (ET),[88] [89] which is also involved – in addition to dilator factors - in the control of vascular tone. ET mediates its effect by mobilization of Ca^{2+} from internal stores[90] and/or by closure of ATP-sensitive potassium channels.[91]

Arachidonic acid can also cause endothelium-dependent contraction after being converted into constrictor prostanoids, which depends upon the entry of extracellular calcium.[92] The hypoxia induced pulmonary vasoconstriction developed in venous vessels also by an endothelium-dependent manner,[93] via increased release of Ca^{2+} from the endoplasmatic reticulum.[94]

Dilator Factors (Endothelium Derived Relaxing Factor (EDRF))

It is well known that acetylcholine induces an endothelium-dependent vasodilation in arteries[95] and arterioles mediated by the so called EDRF, which later was identified as primarily nitric oxide. In most veins both in human and animal models, acetylcholine (Ach) induced endothelium-dependent relaxation[96] and nitric oxide also produced relaxation on both human arterial and venular vessels.[97]

In superficial hand veins in vivo, Collier and Vallance showed that after removal of the venular endothelium by perfusion with distilled water (which impairs endothelium), or by inhibition of nitric oxide synthase by L-NMMA (NG-monomethyl L-arginine), acetylcholine did not induce dilation, instead a vasoconstriction was observed, whereas the dilator action of glyceryl trinitrate was not impaired.[98 99]

In contrast to the above mentioned finding Arner et al. showed that in hand veins, acetylcholine induced a small relaxation, which was unaffected by endothelial removal and also suggested that the modulator role of endothelium-derived relaxing factor(s) is small in hand veins in response to constrictor factors, since contractile responses to noradrenalin, serotonin and prostaglandin F_2 alpha did not differ between vein segments with and without endothelium.[100] The role of nitric oxide in venous function of human veins is also supported by the experiment that local infusion of L-arginine into preconstricted hand veins resulted in dilation.[101] Recently, Blackman et al. showed that human forearm capacitance veins exhibit both stimulated and basal NO activity, which indicates that NO contributes not only to the regulation of venous tone, but also to the resting venous tone in healthy human subjects.[102] Moreover, in animal studies, both the Ach and flow-induced vasodilation, which was inhibited by L-NNA suggests that in isolated preconstricted venules of rats venous endothelium may produce nitric oxide both to the effect of Ach and flow.[4]

Factors with both Dilator and Constrictor Effects (Eicosanoids)

Venular vessels produce both dilator and constrictor prostaglandins. Among other constrictor factors in veins, prostaglandin $F_{2\alpha}$ resulted in a dose-dependent venoconstriction in vivo in superficial hand veins[62 65] and in vitro in isolated vein rings as well.[103]

The production of endothelial dilator prostaglandins in human hand vein has already been shown in a recent in vivo experiment, since Callow et al. have shown that in the presence of indomethacin (a non-specific COX inhibitor) venoconstriction to $alpha_1$- and $alpha_2$-stimulation increased. These findings suggest that vasodilator prostaglandins are released from the endothelium during adrenergic stimulation.[66]

The effect of vasodilator prostaglandins was inhibited by oral administration of a single dose of aspirin to healthy volunteers, and venoconstriction to norepinephrine increased.[104] Contrary to the above mentioned studies prostaglandin F_2 alpha, prostaglandin E_2 and the thromboxane A_2 analogue U46619 elicited constriction in ring segments of human hand veins, whereas prostaglandin E_1 had only dilator effect on veins suggesting at least two types of prostaglandin receptor in the venular wall.[103]

In *in vitro* experiments on human hand veins both prostaglandin (PG) F_2 alpha, PGE_1, PGE_2, and prostacyclin (PGI_2) elicited concentration-dependent relaxation, and when endothelium was removed, relaxation induced by PGF_2 alpha and PGE_2 was reduced, whereas the one, which elicited by PGE_1 and PGI_2 was not changed. This result suggested that prostaglandin mediation effects on receptors both of endothelial and smooth muscle cells.[105]

Similarly to human investigations, in isolated preconstricted rat skeletal muscle venules dilator prostaglandins (PGE_2, PGI_2) were produced in response to increasing the intraluminal flow.[4]

d. Role of Hemodynamic Forces in Regulation of Tone of Venous Vessel

There are only a few studies focused on the responses of venular system (mainly that of small veins and venules) to hemodynamic forces, such as pressure and flow.

I. Pressure-Induced Venular Tone in Veins and Venules

Myogenic tone and responses of veins and venules the pressure increase are very important in the maintenance of normal systemic blood pressure in changing pressure conditions. The pressure-induced increased myogenic tone has crucial role in preventing orthostatic hypotension and edema formation occurred in case of elevated pressure load in the lower veins and venules of the body. For example, in standing position, when gravitational force elicits a significant increase in intraluminal pressure in femoral veins, the increase of the active smooth muscle tension reduces the passive distension of these vessels. This phenomenon is essential to enhance the orthostatic tolerance of the organism.[11]

Nevertheless, there are few studies to fully understand the mechanisms of myogenic response in venules, which is probably due to the fact that venular wall is extremely sensitive, making it difficult to examine the function of its smooth muscle without any damage.[11] However, there are some experiments showing that the pressure-induced mechanism exists in several venous sites in several animal species and human, as well. For example, in rat saphenous vein, a continuous long-term elevation of mean venous pressure within physiological range (by 45 degree head-up tilt) enhanced significantly the acute pressure-induced myogenic response.[106] Also, in human studies, a substantial active tone developed in the side branches of human saphenous vein to the effect of transmural pressure, which was independent of the presence of intact endothelium.[107] Also, in isolated porcine subendocardial and rat skeletal muscle venules a substantial pressure-related myogenic tone was found, which was not influenced by removal of the endothelium.[3, 108]

Thus, pathological alterations of pressure-induced myogenic tone can result in hemodynamic changes favoring the development and maintenance of cardiovascular diseases, such as hypertension in case of increased myogenic venous tone,[106] and on the

other hand, orthostatic hypotension or peripheral edema in case of decreased myogenic tone.[11] Mechanisms mediating myogenic response of veins and venules have not yet been characterized. Endothelium seems not to be involved in the generation of myogenic response, since in isolated skeletal muscle venules, removal of the endothelium did not affect the characteristics of the pressure-diameter curves.[108] However, pressure-induced venular tone can be modulated by a continuous release of endothelium-derived vasodilator factors.[108] In certain veins (e.g. human saphenous vein) Ca^{2+}-activated and voltage-gated K^+ channels participate in myogenic response, which was showed by Szentiványi et al..[109] They have found that in isolated, cannulated human saphenous veins, administration of specific potassium ion channel blockers (iberiotoxin and 4-aminopyridine) counterregulated myogenic response, since diameter of veins at all pressure steps was decreased compared to control condition without K-channel blockers.[109] Also, K_{Ca} channels were detected by patch-clamp method in human saphenous vein smooth muscle cells, which contributed to the maintenance of the membrane potential and sustained a significant portion of the total voltage-activated, outward current. K_{Ca} channels also appeared to play a significant role in the regulation of human saphenous vein smooth muscle contractile activity.[110]

II. Flow-Induced Venular Diameter Response

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 nitric oxide.[5 111 112 113] Meanwhile, there is much less knowledge regarding the flow-induced responses of veins and venules. [3 4 114 115] *In vitro* studies showed that isolated, preconstricted large veins,[115] coronary venules[3] and isolated, preconstricted skeletal muscle venules[4] responded with dilations in response to stepwise increases in flow. This flow-induced dilation may have a very important role in vivo in the regulation of tissue blood supply in different conditions and may help to emptying capillary beds by forwarding blood toward the larger venous vessels. For example, during functional hyperemia in the arteriolar side of the microcirculation a significant vasodilation occurs induced by the increased flow and other mechanisms. This phenomenon in the arteriolar side of the circulation serves to fit the tissue pO_2 and blood supply to the elevated metabolic demand of tissues. However,

dilations of arterioles elicit an increase of capillary pressure, which would provoke an augmented transcapillary perfusion resulted in tissue edema. The simultaneous dilation of postcapillary venules and small veins is necessary to prevent the enhancement of capillary pressure and filtration, thus in physiological conditions the tissue perfusion (functional hyperemia) is suitably regulated.

III. Mediators of Flow- and Agonist-Induced Venular Responses: Role of Endothelium, Nitric Oxide (NO) and Prostaglandins (PGs)

In isolated precontracted (with $PGF_{2\alpha}$) rings of large conduit veins (canine jugular vein) flow and Ach induced dilations of the vessels, which were completely inhibited by endothelium denudation or by administration of L-NAME. Meanwhile, the non-specific inhibitor of COXs, aspirin did not influence the flow- and Ach-induced relaxation in these vessels.[115] It seems that flow- and Ach-induced dilations in canine large veins are mediated by endothelium-derived NO, whereas prostaglandins do not seem to participate in this response.[115]

However, the mechanisms of flow-induced dilation in small veins and venules have not yet established, since there have been few if any studies up to now, which have aimed to reveal this issue. In isolated small ear rabbit vein, infusion of saline resulted in contractions independently on the direction of flow and the presence of endothelium, but dependently on the extracellular calcium suggesting that flow-induced diameter changes were independent of the endothelium. It was suggested that flow mechanically activated the vascular smooth muscle cells, inducing a calcium entry into the cells[116] resulting in constrictions.

In an *in vitro* study, Davis et al. found that the frequency and amplitude of the spontaneous, permanent contractions of bat wing venules were reduced by the presence of flow, which reduction was eliminated by endothelial denudation, but – in contrast to the results of others[115] - not by the nitric oxide synthase (NOS) inhibitor NG-monomethyl-L-arginine (L-NMMA). Neither the prostaglandin synthase inhibitor indomethacin, nor the reactive oxygen species scavenger, superoxide dismutase had inhibitory effect on the flow-mediated responses. Based on these results, it was suggested that flow modulates the diameter of venules by a substance other than NO or PGs, or O^{2-} released from the endothelium.[114]

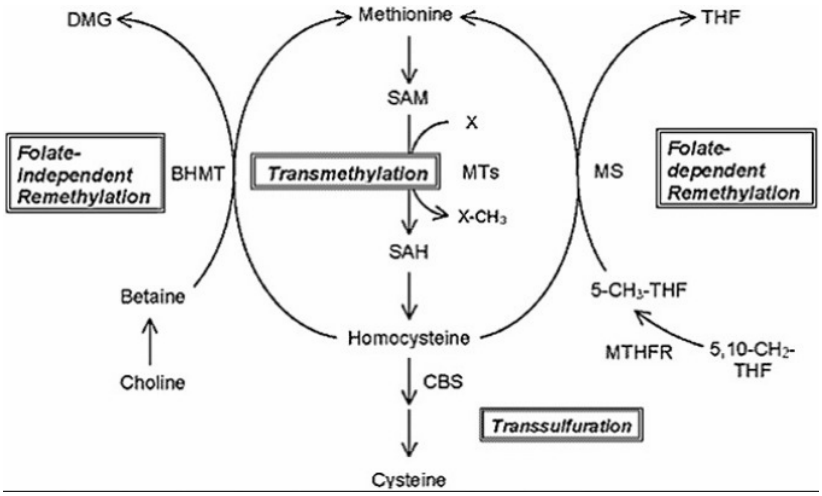
Contrary, flow-induced dilations of isolated porcine coronary venules were abolished by endothelium denudation and by L-NMMA, suggesting that flow-induced dilations are endothelium-dependent and mediated by the release of a nitrovasodilator.[3]

In isolated preconstricted skeletal muscle venules flow-induced dilations were inhibited completely only in the presence of both L-NAME and indomethacin.[4] Moreover, in the presence of both inhibitors, flow induced constrictions in these venules. This study suggests that in preconstricted skeletal muscle venules, flow induces the production of dilator PGs and NO and at the same time the production of constrictor factors as well, the nature of which has not yet been discovered, but it might be a p450 metabolite, endothelin, reactive oxygen species (ROS) or other factors.

1.2. Hyperhomocysteinemia (HHcy)

1.2.1. Homocystein (Hcy), Metabolism of Hcy, Hyperhomocysteinemia (HHcy)

Homocystein (Hcy) is a thiol-containing amino-acid that is formed from the essential amino-acid methionine. Normal fasting Hcy plasma levels are between 5,0 and 15,9 μmol/l. Methionine, an essential amino-acid, building element in animal and plant proteins, is metabolized to Hcy by methionine-adenosyl-transferase via the transmethylation pathway. In the reaction *S*-adenosyl-methionine (SAM) and then *S*-adenosyl-homocysteine (SAH) - in a methyl-transferase reaction - is formed. The transmethylation pathway is present in most mammalian tissues. SAH is converted to homocysteine by SAH hydrolase. Homocysteine may be remethylated to methionine by either folate-dependent or folate-independent mechanisms. For folate-dependent remethylation, the B_{12}-dependent enzyme methionine synthase (MS) utilizes a methyl group from 5-methyltetrahydrofolate (5-CH_3-THF). Betaine-homocysteine *S*-methyl-transferase (BHMT) catalyzes the folate-independent remethylation of homocysteine using betaine, a methyl group donor derived from choline oxidation. Alternatively, homocysteine can be catabolized through the transsulfuration pathway to cysteine, beginning with the irreversible conversion to cystathion by cystathion *ß*-synthase (CBS). Cysteine can be further metabolized into other important biological compounds such as glutathione. Whereas SAM-dependent transmethylation occurs in nearly all tissues, the transsulfuration pathway and the remethylation of homocysteine by BHMT are tissue specific, existing primarily in the liver and kidney.[117]



Due to its high reactivity to proteins, Hcy is almost always bound to the cysteine and lysine containing proteins by its thiol group and can permanently affect protein function. In blood, it is found bound to albumin and to hemoglobin. It affects enzymes with cysteine-containing active sites; for example, it inhibits lysyl oxidase, a key enzyme in the production of collagen and elastin, which are two main structural proteins in artery, bone and skin.

The Hcy level > 16 μmol/L is defined as hyperhomocysteinemia (HHcy), when the risk for atherothrombotic diseases increases independently from other risk factors.[118] [119] [120] [121] Mild HHcy is between 16-30 μmol/L, mean 31-100 μmol/L, and severe HHcy >100 μmol/L.

1.2.2. Factors Involved in the Development of HHcy

Plasma Hcy concentration may be increased by genetic defects in the enzymes involved in homocysteine metabolism (most frequently cystathion β-synthetase, methylene-tetrahydrofolate reductase[122] [123] or nutritional deficiencies in vitamin cofactors, such as folic acid, vitamin B12, and B6 (pyridoxal phosphate).[124] [125] Also, HHcy is frequent in smoking,[121] in alcoholism.[126] Some medications can block the metabolism of Hcy either by affecting on the synthesis of folate (e.g. phenytoin, carbamasepin, methotrexat) or vitamin B6 (e.g. theophyllin).[118] Hyperhomocysteinemia is also attributed to suppressed homocysteine clearance, as indicated by kinetic studies in type 2 diabetics with nephropathy,[127] in kidney insufficiency, psoriasis, and some malignancies.[118]

1.2.3. Vascular Effects of HHcy

a. Effects of HHcy on Arterial Vessels

Epidemiological and experimental evidence suggest that increased plasma concentration of homocysteine is associated with the development of atherothrombotic diseases.[119] [128] [129] Even mild elevation of plasma homocysteine level (> 16 μmol/L) increases the risk for the development of stroke, transient ischemic attack (TIA), myocardial infarction, peripheral vascular disease and atherosclerosis, independently

from other risk factors.[120 121 130 131] Every 1 μmol/L increase in total homocysteine (tHcy) increased the risk of stroke and myocardial infarction by 6% to 7%.[132] In a meta-analysis it was reported, that 25% (i.e. about 3 μmol/L) increase in plasma Hcy levels was associated with 11% and 19% risk for ischemic heart disease and stroke, respectively, after correction for other cardiovascular risk factors.[133] Also an other meta-analyses of 72 genetic studies, in which the prevalence of a mutation in the MTHFR gene (which increases homocysteine level) was determined, serum homocysteine and disease risk showed a significant causal association between Hcy and ischemic heart disease, deep vein thrombosis, and stroke. On the basis of this relation the authors suggested that lowering homocysteine concentrations by 3 μmol/l from current levels (by increasing folic acid intake) would reduce the risk of ischemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24%.[134] Odds ratio for stroke was 1.26 for TT genotype versus CC homozygotes. Mean difference in Hcy concentration between TT and CC homozygotes was 1.93 micromol/L. Thus a 1.93 μmol/L increase in Hcy was associated with a 1.26 -fold increase in stroke in case of TT homozygotes.[135]
In cognitive impairment, and Alzheimer's disease level of Hcy was elevated eliciting a cerebral microvascular rarefaction.[136] Modest correlation between Hcy and higher diastolic and systolic blood pressures was also suggested.[137]
In patients affected by retinal vascular occlusive disease either the arterial or venular site, mean tHcy levels were significantly higher than in healthy man.[138] In recent animal studies HHcy impaired coronary resistance vessel reactivity to acetylcholine (Ach), increased vascular tone in the presence of nitric oxide synthase inhibition, increased carotid arterial permeability, and initiated arterial stiffening in rats with hyperhomocysteinemia,[139] and also in mice.[139] Taken together, evidence from case-control, prospective, and animal studies supports an association between elevated plasma Hcy levels and increased cardiovascular risk.[140]
The mechanism(s) by which elevated Hcy promotes atherothrombotic vascular diseases is still not clearly elucidated and is likely to be multifactorial. HHcy stimulates smooth muscle proliferation,[141] platelet activation,[142] and endothelial injury,[143] promotes LDL oxidation,[144] and activation of the coagulation system.[118 143 145 146 147 148]
On the basis of the aformentioned, it is logical to suggest that lowering Hcy levels by administration of folate and vitamins B6 and B12 would be associated with decrease in

vascular events in populations at risk. Indeed, an analysis indicated a 21% benefit of vitamin B12 treatment on major cardiovascular events, ischemic stroke, coronary disease, or death.[149] Combined treatment of folic acid, vitamin B6 and vitamin B12 was shown to reduce significantly the rate of restenosis and the need for revascularisation after coronary angioplasty.[150] However, there are controversial data regarding the beneficial effects of vitamin therapy in this respect. One of them showed that high dose vitamin B6, B12 and folate therapy did not lower the risk of recurrent cardiovascular disease after acute myocardial infarction.[151]

b. Effects of HHcy on Isolated Arterioles

Previous studies in isolated arterioles have shown that acetylcholine- (Ach) and histamine-induced NO-mediated dilations, which are known to be dependent on endothelium-mediated mechanisms, are reduced in HHcy vessels.[152] Also, in an endothelium-dependent way, bradykinin elicited a greater constriction of arterioles of HHcy rats than in controls. The difference between the responses of control and HHcy arterioles was eliminated by the inhibitor of cyclooxygenases (COXs), indomethacin, and the thromboxane A_2 (TxA_2) receptor (TP) antagonist, SQ 29,548, suggesting that HHcy enhances TxA_2 synthesis in the arteriolar endothelium. The ADP or collagen-induced enhanced platelet aggregation was also decreased by these inhibitors.[145] These studies made our laboratory suggest that the important function of endothelium, which regulates peripheral resistance and tissue blood supply in normal healthy conditions, namely the flow-dependent arteriolar dilation, might be also injured. Indeed, isolated arterioles responded with constriction in hyperhomocysteinemia instead of dilation to the effect of increasing the flow, which was due to a decreased bioavailability of NO and an enhanced thromboxane A_2 mediation.[153] This adverse vascular effect of HHcy was prevented by the administration of antioxidant enzymes,[154] and also by chronic vitamin C treatment[155] indicating the role of an enhanced production of superoxide ($O^{2\cdot}$), thus oxidative stress, in the vascular dysfunction. Enhanced level of superoxide scavenges NO released from the endothelium to flow, thus in part reduces the bioavaibility of the dilator NO, and forms peroxynitrite, which promotes the release of TxA_2, resulting in arteriolar constriction.[154] Vascular sources of superoxide ($O^{2\cdot-}$) might be NAD(P)H oxidase, xanthine oxidase (XO), cyclooxygenase, NO synthase (NOS). In

skeletal muscle arterioles of HHcy rats, inhibition of XO or COX abolished pathological vasoconstriction,[154] whereas in HHcy coronary arteries flow-induced dilation was restored by the NAD(P)H oxidase inhibitor diphenyl-iodonium (DPI),[156] thus in skeletal muscle arterioles $O^{2.-}$ are produced mainly by XO, and COX, whereas in coronary arterioles by NAD(P)H oxidase. In coronary arteries, HHcy increased TNF-α expression, which enhanced the upregulation of NAD(P)H oxidase and inducible nitric oxide synthase (iNOS), producing ROS.[157] These studies suggest that the mechanisms of altered vascular function in HHcy vary among vascular beds and disease conditions.

c. Effects of HHcy on Venular Vessels

Clinical observations show that diseases of venous circulation are also frequent in HHcy. Case-control and cross-sectional studies indicated that mild-to-moderate HHcy is associated with high risk venous thromboembolism[128 129] and a 2 to 3-fold elevated relative risk for deep-vein thrombosis and pulmonary embolism.[158] A recent meta-analysis on 24 retrospective studies demonstrated that 5 μmol/L increase of total plasma Hcy was associated with a ~ 60% increase in risk of venous thrombosis. These and other studies suggest an increased risk for venous thrombosis in HHcy, due to the coexistence of disturbed coagulant and anticoagulant systems.[52 129 159 160 161 162] An important role for elevated levels of Hcy were also found in cerebral,[163 164 165] portal, or splenic venous thrombosis.[166 167 168 169] Moreover, a close relationship has been revealed in clinical studies between hyperhomocysteinemia and venular occlusive diseases, such as central retinal vein occlusion (CRVO).[138 170 171 172 173 174] 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 that thromboxane A_2 (TxA_2) plays a significant role in these pathological events.[145] However, these same mediators have an important role in the regulation of venular tone as well, significantly affecting blood supply of tissues and organs. However, there are few, if any studies conducted to reveal the effect of HHcy on the function of veins and venules.

d. Effects of HHcy on Human Venular Endothelial Cells (HUVEC)

Human umbilical cord vein endothelial cell (HUVEC) is a model, which provides possibilities to examine in vitro several cellular functions in different conditions. In HUVEC Hcy has pro-inflammatory[175 176] and pro-apoptotic effect,[177] inducing elevated expression of inflammatory molecules and increasing oxidative stress. Among others the expression of vascular cell adhesion molecule (VCAM-1) at the protein and mRNA levels via nuclear factor(NF)-kappaB (NF-kappaB) activation and the production of intracellular reactive oxygen species (ROS) by NAD(P)H oxidase activation increases, as shown by the membrane translocation of its p47(phox) subunit. Dietary polyphenolic antioxidants, such as trans-resveratrol and hydroxytyrosol, but not vitamin B6 or folate reduced Hcy-induced VCAM-1 expression and monocytoid cell adhesion to the endothelium.[175]

After others, Hcy alone had no effect on the expression of VCAM-1, but sensitizes HUVEC to the effect of inflammatory mediators (such as thrombin and lypopolisacharides), at least in part through VCAM-1 expression and function.[176]

Also, Hcy increased the intracellular production of ROS in HUVEC, enhanced NF-kappaB activation, and stimulated intercellular adhesion molecule-1 (ICAM-1) RNA transcription and cell surface expression leading to increase in monocyte adhesion to HUVECs, which suggested that in HHcy ROS induce a proinflammatory situation in the vessel wall that may initiates and promotes atherosclerotic lesion development.[178]

The pro-apoptotic effect of Hcy was reduced by the antioxidant vitamin C and vitamin E suggesting the role of oxidative stress in the cytotoxic effect of Hcy in venous endothelial cells.[177]

But not only the elevated production of ROS has been shown in HHcy. Hcy caused oxidative stress in HUVEC also by decreasing the expression of the antioxidant enzymes - glutathione peroxidase and natural killer-enhancing factor B – which in turn could potentially enhance the cytotoxic effect of agents or conditions known to cause oxidative stress. In addition, in these cells Hcy did not induce the gene expression of heat shock proteins (as normally to the effect of inflammation and cell damaging effects), which are responsible for the prevention of cell damage and also Hcy inhibited H_2O_2-mediated HSP70 gene induction.[179] Also, Hcy impairs ER by altering its redox

potential and thus prevents cell-surface expression of thrombomodulin and causes aberrant protein processing.[179] Changing the redox state of ER alters the expression of genes sensitive to ER stress among them genes, which are known to mediate cell growth and differentiation leading to changes in gene expression in human vascular endothelial cells.[179]

e. Effects of HHcy on Coagulation System

As mentioned above, HHcy results in endothelial injury,[143] enhances the activity of platelets,[142] and coagulation system.[118 143 145 146 147 148] Hcy activates tissue factor in endothelial cells,[180] and monocytes,[181] which promotes the adhesion of thrombocytes to the endothelium via GPIIb-IIIa/fibrinogen binding.[182] Other coagulation factors are also activated in HHcy,[146 147] such as factor V,[183] factor VIII,[184] von Willebrand factor (vWF).[184] Others also showed that Hcy elicits the binding of the thrombogenic lipoprotein(a) to fibrin mediating atherothrombotic events.[185]

Homocystein induces thrombotic processes not only by activating prothrombotic factors, but also alters the antithrombotic effect of the endothelium,[186] and other antithrombotic factors. In the presence of Hcy endothelial cells can not inhibit the aggregation of thrombocytes, probably due to the decrease of the bioavailability of NO.[187] Moreover, hyperhomocysteinemia inhibits thrombomodulin surface expression, and the activity of antithrombin III (AT III),[188] and protein C,[189] in part by altering the structure rich in disulfide bindings inside the molecules, or in case of the thrombomodulin expression, by alteration of intracellular protein transport.[190] In addition, homocysteinilation of protein S results in the inhibition of the inactivation of activated factor V (Va).[191]

Last, but not least, fibrinolysis is also altered, for example adhesion and activation of tissue plasminogen activator (tPA) - which is a proteolytic enzyme catalyzing the formation of plasmin - impaires.[192]

1.2.4. Mechanisms of the Development of Vascular Disorders in HHcy

a. Oxidative Stress

In all of the above mentioned disorders, oxidative stress may be a key process leading to endothelial dysfunction and thrombus formation in HHcy,[142] [193] in part by enhancement of membrane lipid and lipoprotein peroxidation,[194] or by inactivation of endothelium-derived NO via peroxynitrite (PON) formation.[154] [195] In the presence of trace metal ions and oxygen, the sulfhydryl group (-SH) of Hcy undergoes autooxidation forming disulfide (RSSR, 2 RSH + O_2 → RSSR + [O^{2-}] → H_2O_2) leading to the formation of reactive oxygen species (H_2O_2, O^{2-}, hydroxyl radicals).[184] [196] Reactive oxygen species (ROS) result in lipid peroxidation in the cell membrane[196] elevating the permeability of membranes, and cell dysfunctions via receptor injuries. Also, ROS have direct oxidative effect on circulating LDL,[196] participating in atherosclerotic processes.

HHcy results in oxidative stress not only extracellulary, but intracellulary as well.[197] For example, in HHcy the nitric oxide (NO)-mediated relaxation in aortic rings was injured, which was inhibited by the intracellulary effective superoxide scavenger, Tiron. Also, in endothel cell culture, an elevated intracellular O^{2-} -production was detected by lucigenin-chemiluminescence method.[197] Intracellular oxidative stress changed the expression of several genes altering the growth of vascular cells and other important functions.[198] Thus, enhanced Hcy provides an oxidative environment, since via its autooxidation the level of ROS enhances intra- and extracellulary, increases the level of reduced metal ions participating in Fenton reactions, and changes the redox state of intra- and extracellular thiol-containing groups, and intracellular redox potential.[199] Changes in intracellular redox potential result in an endoplasmatic reticulum stress leading to reduced production of different proteins and enzymes, such as antioxidant enzymes (glutathione peroxidase).[179] Thus, the balance of ROS production and elimination by antioxidant enzymes also gets injured in HHcy by enhanced ROS production and decreased antioxidant capacity leading to vascular dysfunctions, altered endothelial functions, and atherothrombosis. The HHcy induced endothelial dysfunction and decreased NO effect is not developed via decreased NO production,[200] but via inactivation of NO.[154] [201]

b. Homocysteinilation

An other mechanism leading to prothrombotic processes is the hypersulfation of different proteins and extracellular matrix proteoglycanes injuring basal membranes and connective tissue structures.[184] All of these changes can be induced by Hcy itself and its oxidative derivates (homocysteinic acid, homocystein sulfinic acid, homocysteine thiolactone, which is the main by-product of alternative Hcy metabolism), the sulfur atom of which sulfates extracellular matrix proteoglycanes impairing basal membrane of the vascular wall and contributes to LDL sequestration,[202] [203] eliciting atherothrombotic plaques formation. Modified proteins by linking to Hcy and its derivates by lysine residues leads to protein damage and cell death, and induces immune responses via autoantibodies that recognize the Hcy-N-Lys-epitope on Hcy-thiolactone-modified proteins. These pathogenetic disorders are likely to contribute to the development of human diseases.[204]

c. Hypomethylation

Elevated level of Hcy elicits the failure of intracellular methylation processes leading to the formation of several injured proteins, which may participate in vascular dysfunctions. In vitro enhanced level of Hcy inhibits the regeneration of endothelium and activates smooth muscle cells by decreasing the carboximethylation of p21-ras gene.[205] Chronic elevation of Hcy decreases the methylation of DNS, RNS, proteins, phospholipids, since the elevated S-adenosyl-homocysteine (SAH) inhibits the S-adenosyl-methionine (SAM)-dependent transmethylation reactions.[206]

2. Hypotheses and Aims (Own Experiments)

2.1. Hypotheses A

The nature and the mediation of flow-induced responses are not well characterized in venules but seem to differ among vascular beds. For example, in isolated rings of veins from rabbit ear, intraluminal injection of saline resulted in contractions by mechanically activating the vascular smooth muscle cells, inducing a calcium entry into the cells.[116] In contrast, in isolated rat skeletal muscle venules increases in intraluminal flow resulted in dilations, which were mediated by nitric oxide (NO), dilator prostaglandins (PGI_2/PGE_2) and a constrictor factor,[4] 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.[207] Interestingly, 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).[207]

Thus, on the basis of previous findings, we hypothesized that

1) increases in intraluminal **flow elicit dilation** in isolated, **non-preconstricted venules**,
2) these flow-induced dilations of venules are mediated by:
    a. **nitric oxide** (NO),
    b. **dilator prostaglandins** (PGI_2/PGE_2),
    c. and **thromboxane A_2** (TxA_2),
3) **cyclooxygenases** have different roles in producing dilator and constrictor prostaglandins.

2.2. Aims A

Thus first, we found important to reassess

1) the diameter changes of **isolated venules** in response to increases in intraluminal **flow**, 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 underlying intracellular **mechanisms** eliciting the flow-induced diameter responses.

2.3. Hypotheses B

After the above mentioned studies it seems that HHcy impairs the function of endothelium to release various mediators interfering with coagulation and platelet aggregation and changing the regulation of venous tone. Up to now there are no studies about 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.

Thus secondly, we hypothesized that

1) elevated level of plasma **homocysteine impairs the regulation of vasomotor tone of venules**,
2) due to the **alterations** in the **arachidonic acid pathway**,
3) and due to an enhanced production of **reactive oxygen species** (ROS).

2.4. Aims B

Thus secondly, we found important

1) to characterize the potential changes in **flow-dependent regulation** of vasomotor tone in **venules** isolated from rats with **HHcy**,
2) the **pathological mechanisms** responsible for the altered vasomotor function of HHcy venules.

3. Methods

Male Wistar rats (n= 43, ~350 g, purchased from Charles River Co, Budapest, Hungary) were housed separately and had free access to water and standard rat chow. In case of control rats, animals were fed by standard diet, whereas in studies of HHcy, moderate HHcy was induced by administration of L-methionine (1 g/kg body wt/day, ~ 5 weeks) in the drinking water, which increased plasma homocysteine concentration (~ 46 μmol/L in HHcy vs ~ 6 μmol/L in control group), as shown in our previous studies.[145] The doses of L-methionine administered were based on average daily fluid intake.

All of the protocols were approved by the Institutional Animal Care and Use Committee. Animals were anesthetized with pentobarbital sodium (50 mg/kg) and small venules (inside diameter 259±11 μm) from gracilis muscle were isolated as described previously[208] [209] and transferred into an organ chamber containing standard Krebs solution (in mmol/L: NaCl 110, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0, KH_2PO_4 1.0, glucose 5.5, and $NaHCO_3$ 24.0; equilibrated with 10% O_2, 5% CO_2, 85% N_2, 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 mmHg) allowing them to develop spontaneous tone. In contrast to previous studies, we did not use norepinephrine or other vasoactive agent to preconstrict venules, because it may have influenced the responses to flow.[207] Instead, we allowed the venules to develop a spontaneous myogenic tone in response to the presence of 10 mmHg intraluminal pressure. A substantial myogenic tone developed within ~1.5 hour. The inner diameter of venules was measured by videomicroscopy.[209]

3.1. 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 the responses. 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. 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 mmHg.[4] This study showed that above 8 mmHg of P_{diff} the diameter did not further change, thus we used 8 mmHg as the maximum pressure difference. Between 0-4 and 4-8 mmHg P_{diff}, 1- and 2 mmHg step changes were used. The relationship between P_{diff} and flow was established previously and the range of perfusate flow was between 0-0.2 ml/min, measured by ball-flow meter.[4] In a recent in vivo study of rat spinotrapezius preparation the blood flow in postcapillary venules of 8,6±1,9 μm in diameter was in the range of 3.7×10^{-4} to 6.5×10^{-2} nl/s with a mean flow of 0.011 ± 0.014 nl/s. [210] On the basis of these measurements and that flow is proportional with the fourth power of the vessel radius after the Hagen-Poisseuille equation, we can assume that flow in vessels of our studies is in the normal range to their dimensions. On the basis of in vivo studies of Lipowksy et al. in venules, it seems that the range of wall shear stress is around 10-40 dynes/cm^2 in resting conditions, [211] whereas the calculated WSS in these size of isolated venules is around 10-20 dynes/cm^2.

To elucidate the role of NO, as well as dilator and constrictor prostaglandins in mediation of flow-induced responses, we have used the NO synthase inhibitor, N^{ω}-nitro-L-arginine-methyl-esther (L-NAME, 10^{-4} mol/L for 30 min.), the non-selective cyclooxygenase (COX) inhibitor, indomethacin (INDO, 2.8×10^{-5} mol/L for 30 min.) and the TxA_2-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 in control conditions the specific TxA_2 synthase inhibitor, Ozagrel (10^{-5} mol/L for 30 min.).[212] [213] [214] 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 Ca^{2+}-free solution, which contained ethylene glycol-bis(β-aminoethyl ether)-*N, N, N', N'*-tetraacetic acid (1 mmol/L for 20 min.).

Also, flow-induced responses were measured in the presence and absence of superoxide dismutase plus catalase (SOD/CAT, incubation for 30 minutes) to elucidate the potential role of reactive oxygen species in mediation of response.

3.2. 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.[213] 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.

3.3. Ethidium Bromide Fluorescence

In previous studies of isolated arterioles[154 156] an elevated level of ROS were detected in HHcy, which were responsible for the impaired vascular functions. To investigate whether in venules isolated from HHcy rats the level of ROS in the vascular wall are also elevated compared to control venules double staining of dihydroethydine (DHE) and DAPI was used.[157] In the presence of superoxide, DHE is oxidized to fluorescent ethidium bromide, which is a cell permeable product, intercalated into the DNA of the nuclei. DAPI, a fluorescent dye, was used for staining the nuclei. Simultaneous enhancement of the fluorescence intensity of the two dyes indicated the presence of elevated level of superoxide in the DNA. Accordingly, saphenous veins were removed from rats, cleared of connective tissue, immersed in normal PSS in the absence or presence of SOD (120 U/L) plus CAT (80 U/L) at 37°C. DHE (5×10^{-6} M; Molecular Probes) was then added to the PSS for 10 minutes followed by washing in normal PSS. Frozen sections of veins were overlaid with DAPI, and then visualized and photographed by a digital camera (Olympus DP12) attAched to a fluorescence microscope (Olympus BX51). Intensity of fluorescence of the venular wall was measured and quantified by ImageJ software. Relative fluorescence intensity was counted by extracting the intensity of the background from a standard size of the

arteriolar wall. Measurement was repeated five times, and the mean intensity and SE were calculated.

3.4. 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.

3.5. Data Analysis

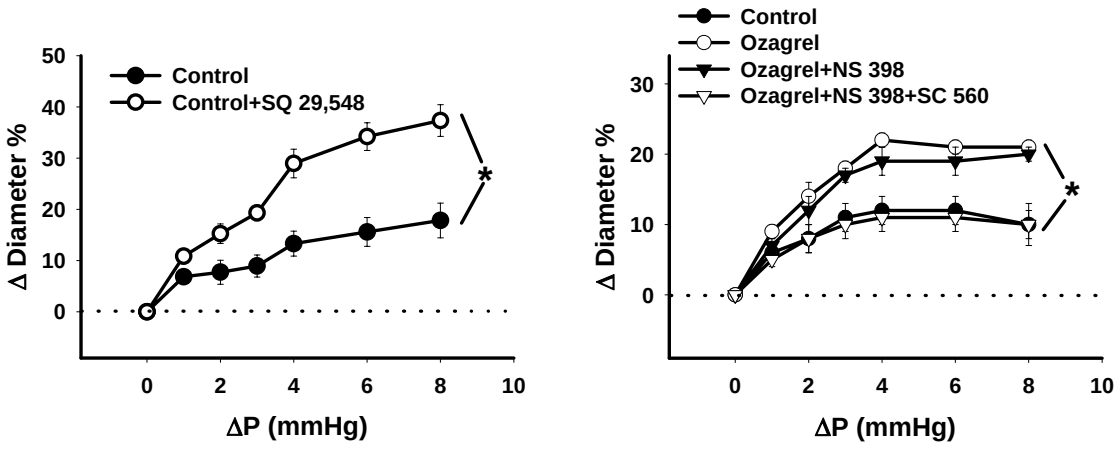
Changes in venular diameter are expressed as a percentage of the maximal dilation of vessels, defined as the difference of the passive diameter (at 10 mm Hg intraluminal pressure, in a Ca^{2+} -free physiological salt solution containing 1 mmol/l EGTA) and the initial diameter of the venules (at 0 flow condition, at 10 mmHg). 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 ± SEM.

4. Results

Venules isolated from gracilis muscle of Control and HHcy rats developed similar 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. Whereas, in Ca^{2+}-free solution the passive diameter of venules was 412±11 μm and 401±11μm in Control and HHcy venules, respectively. Thus, venules can develop an appreciable spontaneous myogenic tone allowing studying vasomotor responses without pharmacological preconstriction. The various inhibitors used, such as L-NAME, indomethacin, SQ 29,548, Ozagrel, SC 560 and NS 398, and the ROS scavengers SOD and CAT had no significant effect on baseline diameter.

4.1. Flow-Induced Responses of Control and HHcy Venules: Role of TxA_2, NO and Prostaglandins

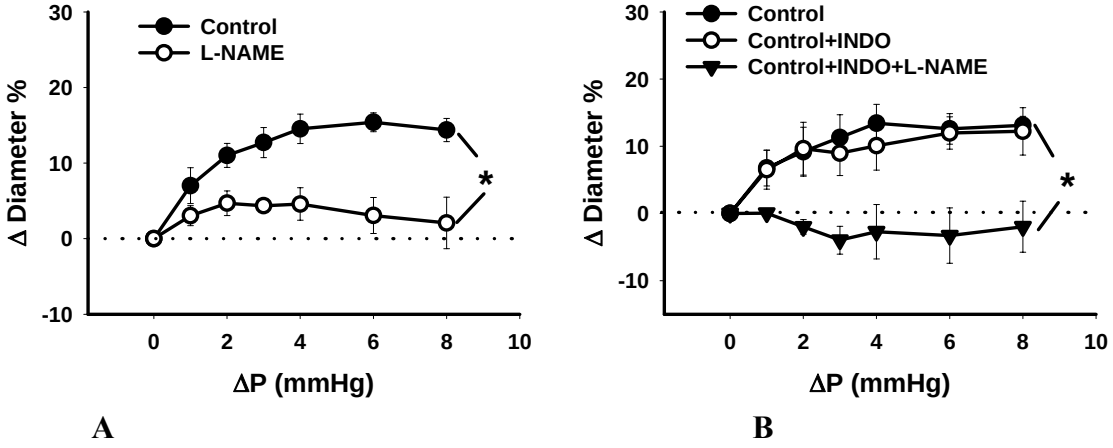
A. In control conditions, increases in intraluminal flow elicited dilations of isolated venules (18±3 % at P_{diff} 8 mmHg), which were significantly increased in the presence of the TP receptor antagonist, SQ 29,548 (37±3 % at max.) (Figure 1A), and also in the presence of the TxA_2 synthase inhibitor,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



A **B**

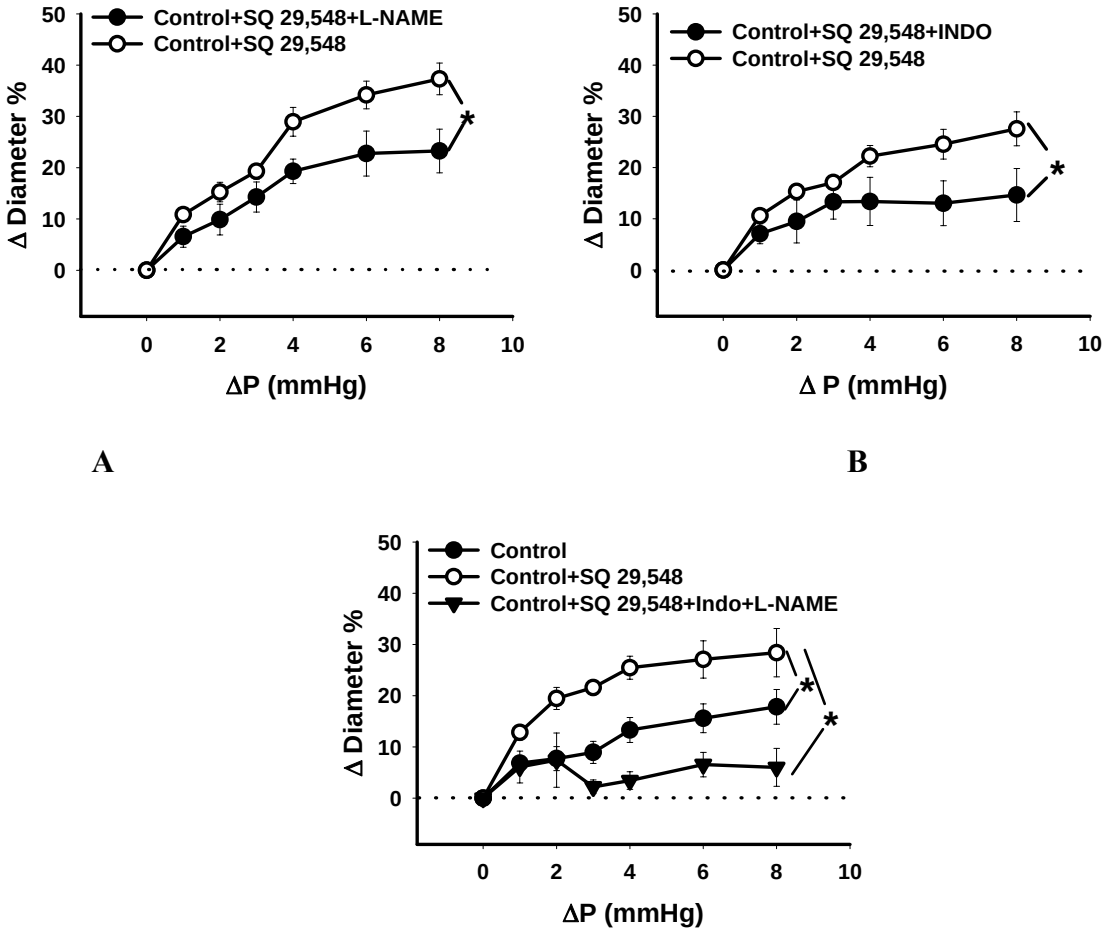
1. Changes in diameter to increases in intraluminal flow (ΔP) in Control venules in the presence and absence of the (A) PGH_2/TxA_2 receptor antagonist, SQ 29,548 and the (B) TxA_2-synthase inhibitor, Ozagrel.

Also, in control conditions the NO synthase inhibitor, L-NAME reduced significantly flow-induced dilations, (from max. ΔD: 14±2 % to 2±3 %) (Figure 2A). In contrast, the non-selective cyclooxygenase 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: 13±1 % to -2±4 %) (Figure 2B).



2. Changes in diameter to increases in intraluminal flow (ΔP) in Control venules in the presence and absence of the (A) NO synthase inhibitor, L-NAME and the (B) non-specific cyclooxygenase inhibitor INDO and INDO+L-NAME.

Next, we aimed to elucidate the contribution of NO to the mediation of the dilation in the presence of SQ 29,548, thus after incubation of Control 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: 37±3 % to 23±4 %) in Control venules (Figure 3A).

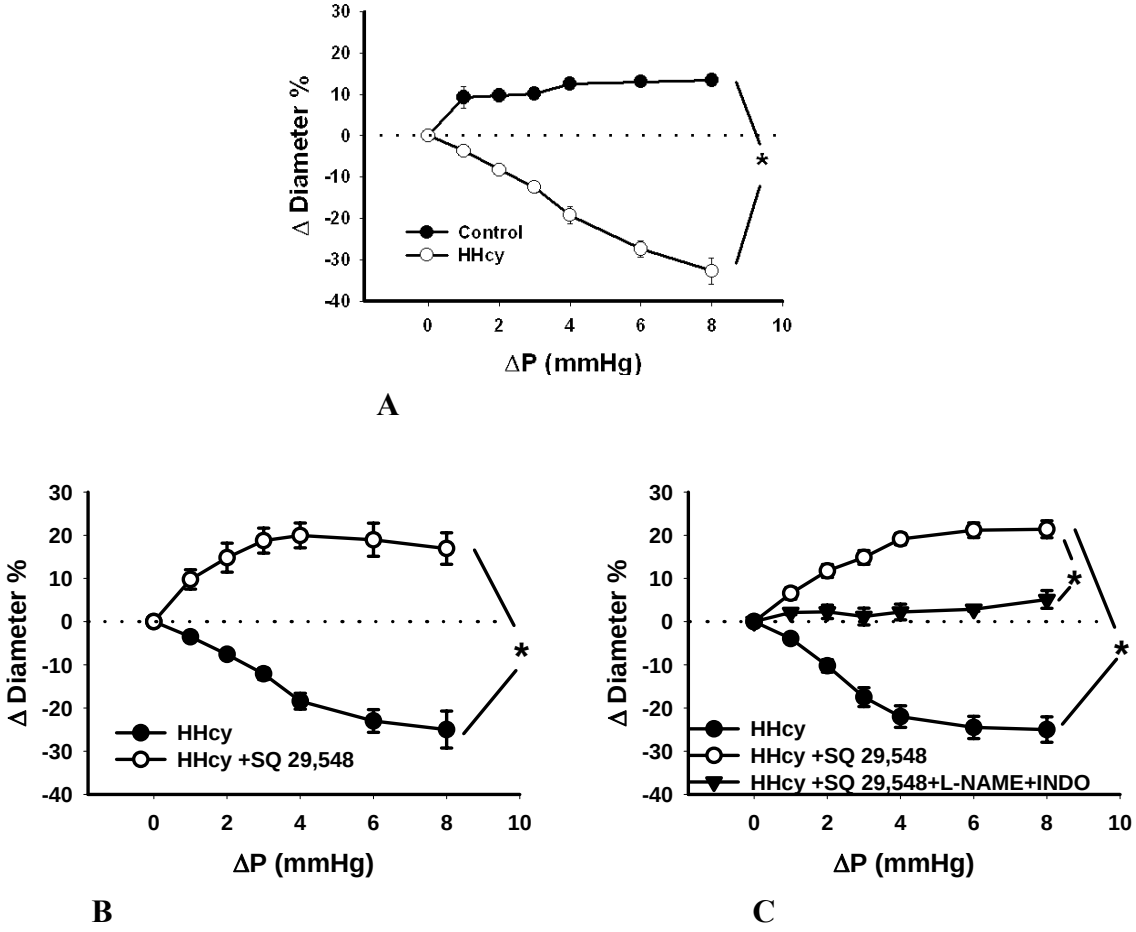


3. Changes in diameter to increases in intraluminal flow (ΔP) in Control venules in the presence of the (A) PGH_2/TxA_2 receptor antagonist, SQ 29,548 and SQ 29,548+the NO synthase inhibitor, L-NAME, (B) SQ 29,548 and SQ 29,548+the non-specific cyclooxygenase inhibitor INDO, and (C) SQ 29,548 and SQ 29,548+INDO+L-NAME.

Similarly, in the presence of SQ 29,548, the non-selective COX inhibitor, indomethacin reduced significantly the flow-induced dilations of isolated Control venules (from max. ΔD: 28±3 % to 15±5 %) (Figure 3B), whereas, in the presence of SQ 29,548 simultaneous addition of L-NAME and INDO abolished essentially flow-induced responses (from max. ΔD: 28±5 % to 6±3 %) (Figure 3C).

B. Whereas stepwise increases in flow induced dilation in control venules it resulted in significant constrictions in HHcy venules (Figure 4A). The TP-receptor antagonist,

SQ 29,548, converted constrictions to dilations in HHcy venules (from max. ΔD: -25±4 % to 17±4 %) (Figure 4B). Flow-induced dilations of HHcy venules in the presence of SQ 29,548 were essentially abolished by the simultaneous administration of L-NAME and INDO (from max. ΔD: 21±2 % to 5±2 %) (Figure 4C).

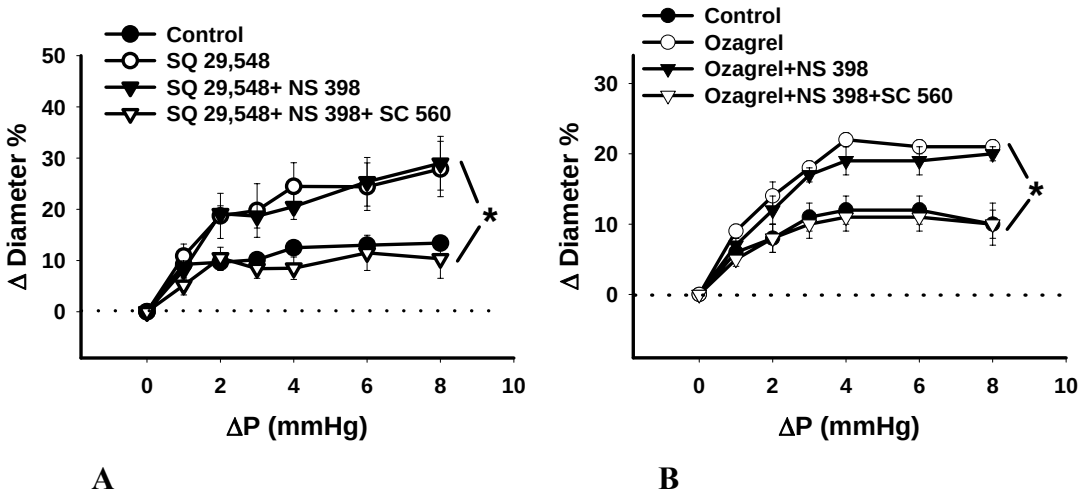


4. Changes in diameter to increases in intraluminal flow (ΔP) in control and HHcy venules (A). Changes in diameter to increases in intraluminal flow (ΔP) in HHcy venules in the presence and absence of the (B) PGH_2/TxA_2 receptor antagonist, SQ 29,548, and (C) SQ 29,548 and SQ 29,548+the NO synthase inhibitor, L-NAME+the non-specific cyclooxygenase inhibitor INDO.

4.2. Role of COX-1 and COX-2 in the Flow-Induced Responses of Venules

Next, we aimed to reveal the specific roles of COX-1 and COX-2 in producing prostaglandin mediators of flow-induced dilation of venules.

A. We found that 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 %) (Figure 5A). In contrast, incubation of Control venules with 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 %) (Figure 5A).

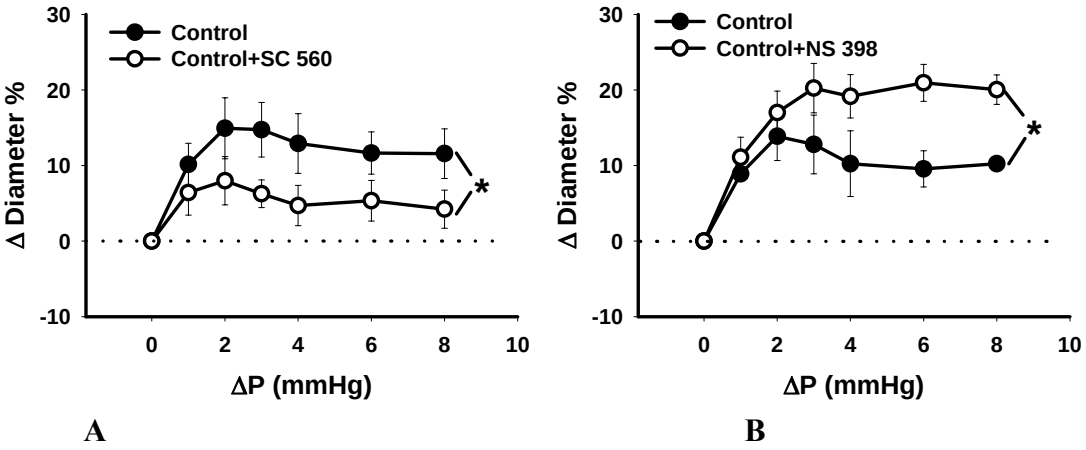


5. Changes in diameter to increases in intraluminal flow (ΔP) in Control venules in the presence and absence of the (A) PGH_2/TxA_2 receptor antagonist, SQ 29,548, SQ 29,548 +the specific COX-2 inhibitor, NS 398 and SQ 29,548+NS 398+the specific COX-1 inhibitor, SC 560, and (B) TxA_2-syntase inhibitor, Ozagrel, Ozagrel+NS 398 and Ozagrel+NS 398+SC 560.

We found similar changes in the responses using the TxA_2 synthase inhibitor, Ozagrel. That is, 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

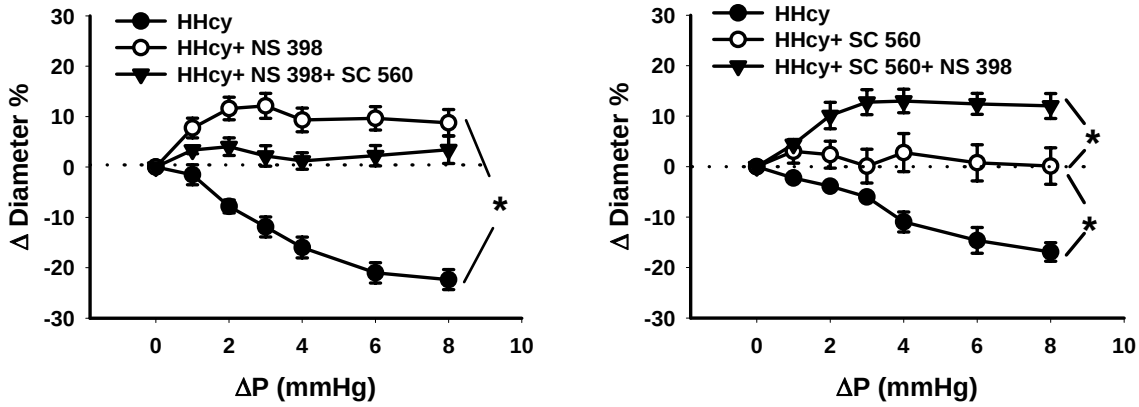
COX-2 inhibitor, NS 398 (from max. ΔD: 21±1 % to 20±1 %), but were significantly reduced by the COX-1 inhibitor, SC 560 (from max. ΔD: 21±1 % to 10±2 %) (Figure 5B).

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 %) (Figure 6A), whereas the COX-2 inhibitor, NS 398 increased flow-induced dilations of Control venules (from max. ΔD: 10±1 % to 20±2 %) (Figure 6B).



6. Changes in diameter to increases in intraluminal flow (ΔP) in Control venules in the presence and absence of (A) the specific COX-1 inhibitor, SC 560, and (B) the specific COX-2 inhibitor, NS 398.

B. In HHcy venules, the specific COX-2 inhibitor, NS 398 converted flow-induced constrictions to dilations (from max. ΔD: -22±2 % to 9±3 %) (Figure 7A), which were then significantly reduced by the specific COX-1 inhibitor, SC 560 (from max. ΔD: 9±3 % to 3±3 %) (Figure 7A). 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 %) (Figure 7B).

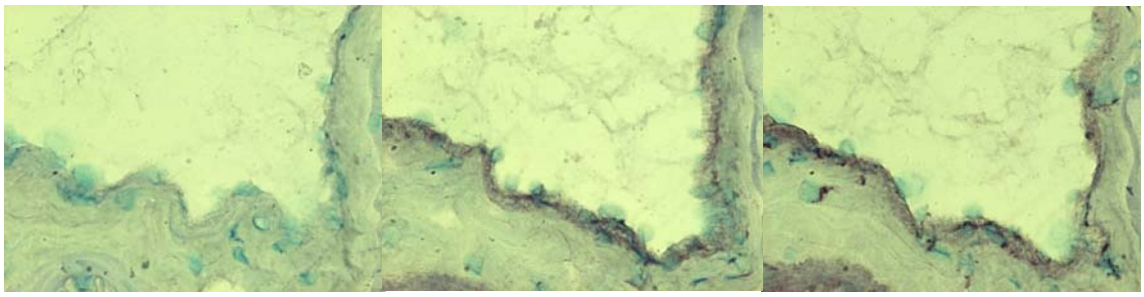


A **B**

7. Changes in diameter to increases in intraluminal flow (ΔP) in HHcy venules in the presence and absence of (A) the specific COX-2 inhibitor, NS 398 and NS 398+ the specific COX-1 inhibitor, SC 560, and (B) first SC 560 and then SC 560+NS 398.

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

Immunostaining for COX-1 and COX-2 shows, that, as compared to sections without immunostaining (Figure 8A, background), both COX-1 (Figure 8B) and COX-2 (Figure 8C) staining were present in the wall of venules isolated from Control rats, primarily localized to the endothelium and sub-endothelial layer.

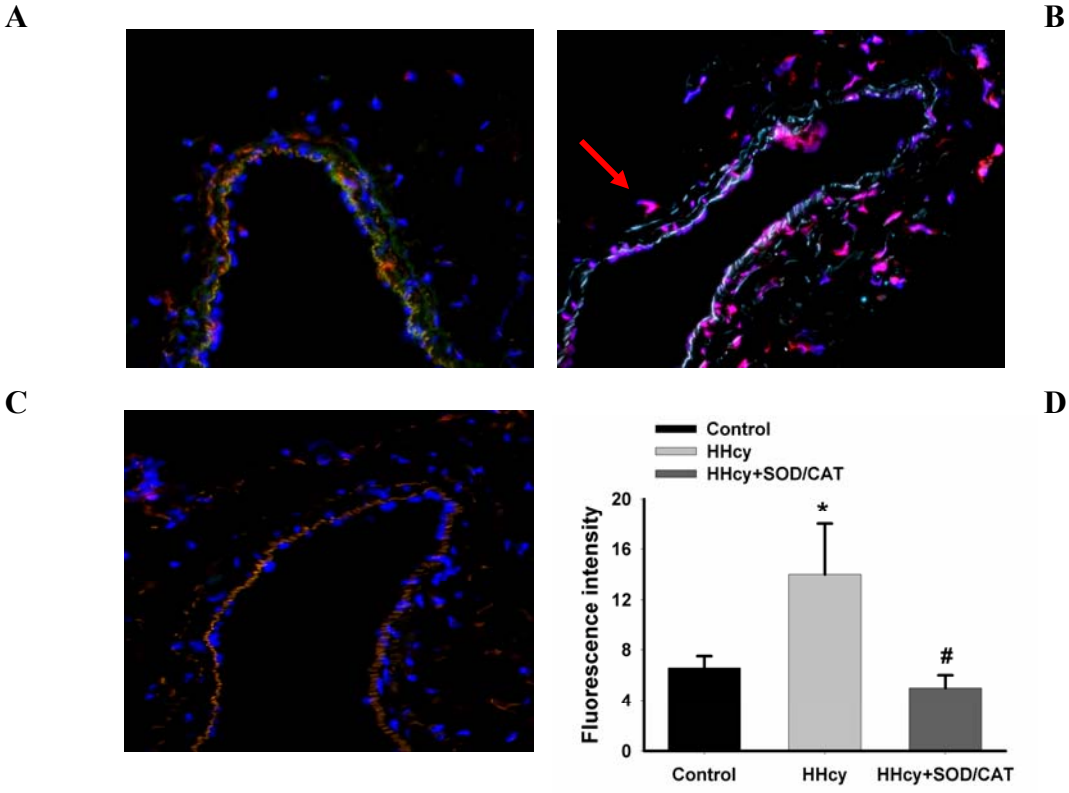


A **B** **C**

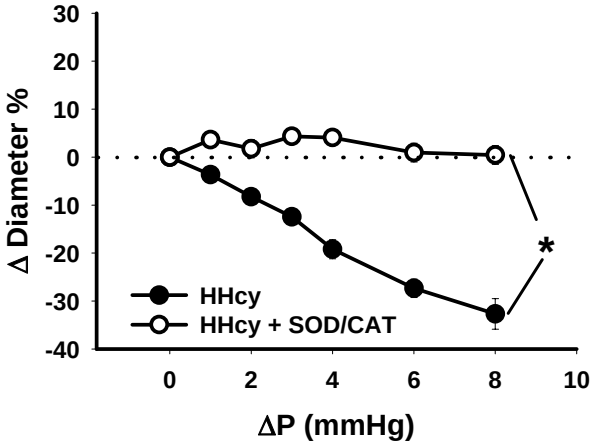
8. Representative pictures of immunohistochemical staining of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in gracilis muscle venules. (A) background, (B) COX-1, (C) COX-2

4.4. Role of Reactive Oxygen Species (ROS)

Fluorescent digital images of ethidium bromide and DAPI-stained sections showed an enhanced fluorescence in HHcy veins (Figure 9B), indicating an enhanced HHcy-induced superoxide production in saphenous vein compared to Control vessels (Figure 9A). The presence of SOD plus CAT reduced the fluorescence to the similar level of Control vein (Figure 9C). Summary data show that the intensity of fluorescence was substantially enhanced in HHcy venules compared to Control venules, which was significantly reduced in the presence of the ROS scavenger SOD plus CAT (Figure 9D). Correspondingly, the presence of SOD plus CAT diminished flow-induced constrictions in venules of HHcy rats (from max. ΔD: -33±3 % to 0±2 %) (Figure 10).



9. Isolated venules with DHE-staining overlaying with DAPI-staining (arrow) in (A) control, (B) HHcy and (C) HHcy+SOD plus CAT conditions. (D) Summary data showing fluorescence intensity in control, HHcy and HHcy plus SOD/CAT conditions.



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10. Changes in diameter of skeletal muscle venules of HHcy rats in response to increases in flow in the presence or absence of SOD/CAT.

4.5. Summary of our Results

In control conditions in isolated skeletal muscle venules 1) a substantial spontaneous myogenic tone developed in response to the presence 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 nitric oxide synthase, but 5) were not affected by the non-selective inhibition of COXs, 6) inhibition of COX-1 reduced, whereas inhibition of COX-2 enhanced flow-induced dilations, 7) both COX-1 and COX-2 are expressed in the venular wall.

Collectively, these findings suggest that in skeletal muscle venules thromboxane A_2, dilator prostaglandins and nitric oxide mediate diameter responses to increases in intraluminal flow. Dilator prostaglandins are released mostly by the COX-1 pathway, whereas constrictor prostaglandins are released mostly by the COX-2 pathway.

In HHcy conditions 1) increases in flow - instead of dilations as observed in controls (normal Hcy level) - resulted in constrictions of venules isolated from rats with hyperhomocysteinemia, 2) these constrictions were converted to dilations by inhibition of TP-receptors, 3) and that these dilations were abolished by L-NAME and INDO. Furthermore, 4) inhibition of COX-2 converted flow-induced constrictions to dilations, which were then significantly reduced by COX-1 inhibition, also 5) inhibition of COX-1 abolished constrictions, which were further converted to dilations by COX-2 inhibition, 6) dihydroethidine staining indicated an elevated venous superoxide production in HHcy, which was reversible by SOD plus CAT, and 7) SOD plus CAT treatment abolished flow-induced constrictions in HHcy venules.

Collectively, these findings suggest that 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.

5. Discussion

At present, still few studies have been conducted to investigate the vasomotor function of small venous vessels in response to hemodynamic forces. This could be due primarily to technical difficulties, since these type and size of vessels have very thin vulnerable wall. Also, any intervention during in vivo investigation elicits complex responses of the microcirculation preventing the clear assessement of the diameter response of venules to hemodynamic forces.

Thus in our first series of experiments we aimed to establish that isolated skeletal muscle venules develop a substantial spontaneous tone in response to intraluminal pressure. This was important, because in most previous studies, constrictor agents were used to induce tone for venous vessels, which may have posed difficulties to reveal the real nature of vasomotor function of venules. 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. This pressure is likely to be in the physiological range for this size of venules.[215] Having this spontaneous venular tone, flow-dependent responses could be investigated without the potential interference of vasoactive agents on vascular vasomotor mechanisms.

In arterial vessels, such as small arteries and arterioles, it has been established that flow-induced responses play an important role in the modulation of vascular resistance and tissue blood flow and that the primary mediators are dilator prostaglandins and nitric oxide.[5 111 113 216] Much less is known regarding the nature of flow-induced responses and their mediation of venules. [3 4 114 115] Previous studies showed that isolated, preconstricted large veins,[115] coronary venules[3] and skeletal muscle venules[4] responded with dilations to stepwise increases of flow, but the effect of flow on isolated skeletal muscle venules without previous preconstriction has not been clarified yet. Thus next we aimed to investigate the diameter changes of isolated skeletal muscle venules in response to increases in intraluminal flow and elucidate the mediators of the response.

5.1. Nature and Mediation of Flow-Induced Responses in Venules

5.1.1. Characteristics of Flow-Induced Response of Venules

We found that isolated skeletal muscle venules respond with a complex manner, resulting in moderate 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.[217 218] 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.

5.1.2. Mediators of Flow-Induced Responses of Venules

Nitric Oxide 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[115] and also in isolated porcine coronary venules,[3] whereas, in bat wing venules neither NO,[114] nor prostaglandins were involved.[114] Our findings that inhibition of NO synthase reduced significantly flow-induced dilations (in control conditions, or after indomethacin, or TP receptor antagonist), suggest that increases in flow elicit a release of NO, responsible - in part - for dilation (Figure 2A, B, Figure 3A).

We have found that indomethacin (non-selective 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 (Figure 2B, 3B) and were eliminated by further administration of L-NAME (Figure 3C).

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

Based on these findings we conclude that in isolated skeletal muscle venules in addition to constrictor TxA_2, NO, and dilator prostaglandins are simultaneously produced in response to increases in intraluminal flow. The nature of dilator prostaglandins can be assumed from previous findings, which could be both PGI_2 and/or PGE_2.[111 216 219 220 221 222]

Thromboxane A_2: 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 thromboxane A_2 synthase inhibitor, Ozagrel. We found that in the presence of the TP receptor antagonist, or the TxA_2 synthase inhibitor, flow elicited significantly greater dilations in venules, indicating a substantial contribution of constrictor TxA_2 (Figure 1A, 1B and Figure 3A, B, C). These findings suggest that in skeletal muscle venules, increases in flow elicit the release of TxA_2, responsible for a constrictor response. It is known however, that in addition to the stable metabolite TxA_2, TxB_2 also has constrictor effects, shown previously in pulmonary vasculature,[223 224] thus it may also contribute to the response.

Role of COX-1 and COX-2: It seemed to be important to elucidate the source(s) of prostaglandins released to increases to 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.[225] For example, our recent studies in arterioles proposed a role for COX-2 derived constrictor TxA_2 in microvascular function in diabetes mellitus and an enhanced COX-2 expression in the arteriolar wall.[212] 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.[213] 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.[226 227 228] However, there are few, if any studies extant regarding the presence and function of COX isoforms in venular vessels.

In the present studies, we found that in the presence of TP receptor blockade or inhibition of TxA_2 synthase, the COX-1 inhibitor, SC 560 reduced significantly flow-

induced dilations of isolated skeletal muscle venules, whereas the COX-2 inhibitor, NS 398 was without effect (Figure 5A). We 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 (Figure 6A, B and Figure 2B). These findings suggest that in venules COX-1 participates primarily in the production of dilator, whereas COX-2 participates primarily in the production of constrictor PGs 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.[229] 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 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.[228] 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.[212] These findings could have clinical importance during treatments with various COXs inhibitors of different selectivity.

5.1.3. Physiological Importance of Regulation of Wall Shear Stress in Venules

Previous in vivo studies emphasized the important 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.[215] The findings of our studies indicate that isolated venules have an appreciable pressure-induced tone and respond with dilation to increased 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.[216] According to Murray' theory, the "purpose" of the regulation of wall shear stress - especially in the arteriolar network - is to minimize the power loss in the circulation.[230] [260] This can be achieved by the

regulation of wall shear stress in a negative feed back 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,[211] thus in venules, wall shear stress is determined primarily by the viscosity of blood,[211] as opposed to arterioles, in which diameter is more important in this regard.[216]

This is the reason, we believe, why in venules both dilator and constrictor factors are released, which could limit the increase in diameter, hence increase in viscosity due to the reduction of shear rate.[230] This is achieved by the release of constrictor TxA_2 as well, 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 higher wall shear rate to lower the hematocrit-related viscosity.

5.2. Mediation of Flow-Induced Responses of Venules in Hyperhomocysteinemia

Previous studies have suggested an important relation between elevated levels of plasma homocysteine and venous diseases, such as venous thromboembolism[128] in the lungs,[129] brain,[163] portal and splenic circulation,[166 168] which can lead to venous occlusive diseases. Among others, central retinal vein occlusion is frequently observed in HHcy.[172] Several mechanisms could be proposed to be responsible for the development of occlusive diseases in the venous circulation in HHcy, such as decreased expression of thrombomodulin,[189] enhanced level of tissue factor[180] or increased platelet activation.[142]

It seems, however, that changes in mechanisms regulating coagulation of blood and platelet activation are insufficient to explain entirely the development of venous occlusive diseases in HHcy. Thus, in our studies we hypothesized that endothelial

mechanisms regulating the vasomotor function of venules are altered in HHcy, which may also favor the development of occlusive venous diseases. Thus, we investigated the flow-induced responses of isolated skeletal muscle venules, known to be mediated by factors released from the endothelium.[3 4] In addition, our recent study revealed that in control conditions an increase in flow elicited dilation of isolated venules, a response, which is mediated by the simultaneous release of NO, PGI_2/E_2 and TxA_2, as indicated by the finding that inhibition of TP receptors augmented the dilation.[231]

In contrast, our previous studies in skeletal muscle arterioles isolated from HHcy rats showed that increases in flow-induced constrictions, instead of dilations, which were due to the altered function of endothelium, including the impaired bioavailability of NO, the elevated synthesis of TxA_2 and reactive oxygen species.[154 157]

Thus, it was logical to assume that HHcy affects - not only the arterial, but also the venous side of circulation. Indeed, venous diseases are frequently observed in HHcy.[232] Thus it seemed to be important to elucidate the effects of HHcy on venular vessels known to provide a large endothelial surface area of the circulation and thus responsible for the release of numerous factors that are involved, not only in the maintenance of rheological properties of blood, but also in the regulation of resistance of venous circulation. On the basis of previous findings, we hypothesized that endothelial regulation of venular tone is impaired in HHcy.

5.2.1. Flow-induced Constrictions of Venules in HHcy

Increases in intraluminal flow resulted - instead of dilations - in substantial constrictions in venules isolated from HHcy rats (Figure 4A), which were then converted to dilations in the presence of TP receptor antagonist (Figure 4B), suggesting that increases in flow/shear stress – in addition to dilator factors - elicit a substantial release of constrictor TxA_2 in skeletal muscle venules of HHcy rats. In the presence of TP receptor blockade, a role for NO and dilator prostaglandin (likely PGI_2/E_2), could be revealed in mediating flow-induced dilations, as shown by the findings that these dilations were inhibited by L-NAME and indomethacin (Figure 4C). Thus, it seems that in HHcy the dilator effects of NO and prostaglandins are overcome by the substantial release of TxA_2.

5.2.2. Role of COX-1 and COX-2 in HHcy

To elucidate the role of COX-1 and COX-2 pathways in the development of flow-induced venular constrictions in HHcy, first we used the specific COX-2 inhibitor, NS 398 then add the COX-1 inhibitor, SC 560. We found that the specific COX-2 inhibitor, NS 398 converted the flow-induced constrictions to dilations suggesting that primarily the COX-2 pathway are responsible for the production of TxA_2. This dilation in the presence of NS 398 was reduced by the selective COX-1 inhibitor, SC 560, which suggests that primarily the COX-1 pathway produces dilator prostaglandins (Figure 7A). The finding that the presence of the COX-1 inhibitor, SC 560 abolished constriction, then additional incubation of the venules with the COX-2 inhibitor NS 398 resulted in dilation suggests that constrictor PGs are produced both by COX-1 and COX-2 pathways, whereas dilators by COX-1 pathway (Figure 7B).

Collectively, we interpret these findings to mean that in skeletal muscle venules of HHcy rats COX-1 produces primarily dilator PGs, whereas COX-2 produces primarily TxA_2. These may be due to the fact that COX-1 and COX-2 have different compartmentalization with downstream enzymes, such as prostaglandin synthase and thromboxane synthase.

There are still controversial data in the literature regarding the specific role of COX-1 and COX-2, in normal and pathological conditions. It seems that both COX-1 and COX-2 are expressed in physiological and pathological conditions, but their roles, levels of activation and affinity to arachidonic acid could be different.[233] In most tissues, COX-1 is constitutively expressed and produces dilator prostaglandins mediating basic housekeeping functions that are important in the maintenance of normal function (e.g. renal function, gastric mucosal integrity, platelet aggregation, autocrine response to circulating hormones).[233] In contrast, COX-2 is believed to be primarily an inducible enzyme, activated by proinflammatory conditions (e.g. in inflammation, during hyperalgesia, cell proliferation),[233] which produces several prostaglandins, leading to inflammatory processes, thrombogenesis or angiogenesis. However, in some tissues, such as brain and kidney, COX-2 is constitutively expressed, although its physiological role remains obscure.[233]

5.2.3. Role of Oxidative Stress in HHcy

In previous studies in arterioles isolated from HHcy rats the increased level of reactive oxygen species and their role in the adverse vascular effect of HHcy has been already demonstrated.[154] In vivo studies of humans and animals also supported a role of oxidative stress in the development of vascular dysfunction in HHcy, because oral administration of the antioxidant ascorbic acid prevented HHcy-induced endothelial dysfunction in both conduit and resistance vessels.[155 234] Interestingly, homocysteine can elicit generation of oxygen free radicals either via autooxidation of the sulfhydryl group[235] or by decreasing the antioxidant mechanisms, such as glutathione-peroxidase or SOD.[236] On the basis of these findings and the above mentioned studies we suggest that oxidative stress plays an important role in the altered flow-induced responses in HHcy.

Correspondingly, in the present study, we have found that in saphenous veins isolated from HHcy rats there was an increased oxidative stress as indicated by the elevated number of ethidium-bromide staining in the wall of vessels compared to controls (Figure 9) and scavengers of ROS (SOD/CAT) abolished flow-induced constriction in HHcy venules (Figure 10).

Previous studies have shown that reactive oxygen species (ROS), such as superoxide anion and H_2O_2 elicit constrictions of isolated venules[237] primarily by activating TxA_2 receptors. Thus, it is possible 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[238] and PGH_2 synthase.[239]

5.2.4. The Pathophysiological and Clinical Importance of Vasomotor Dysfuntion of Venules in HHcy

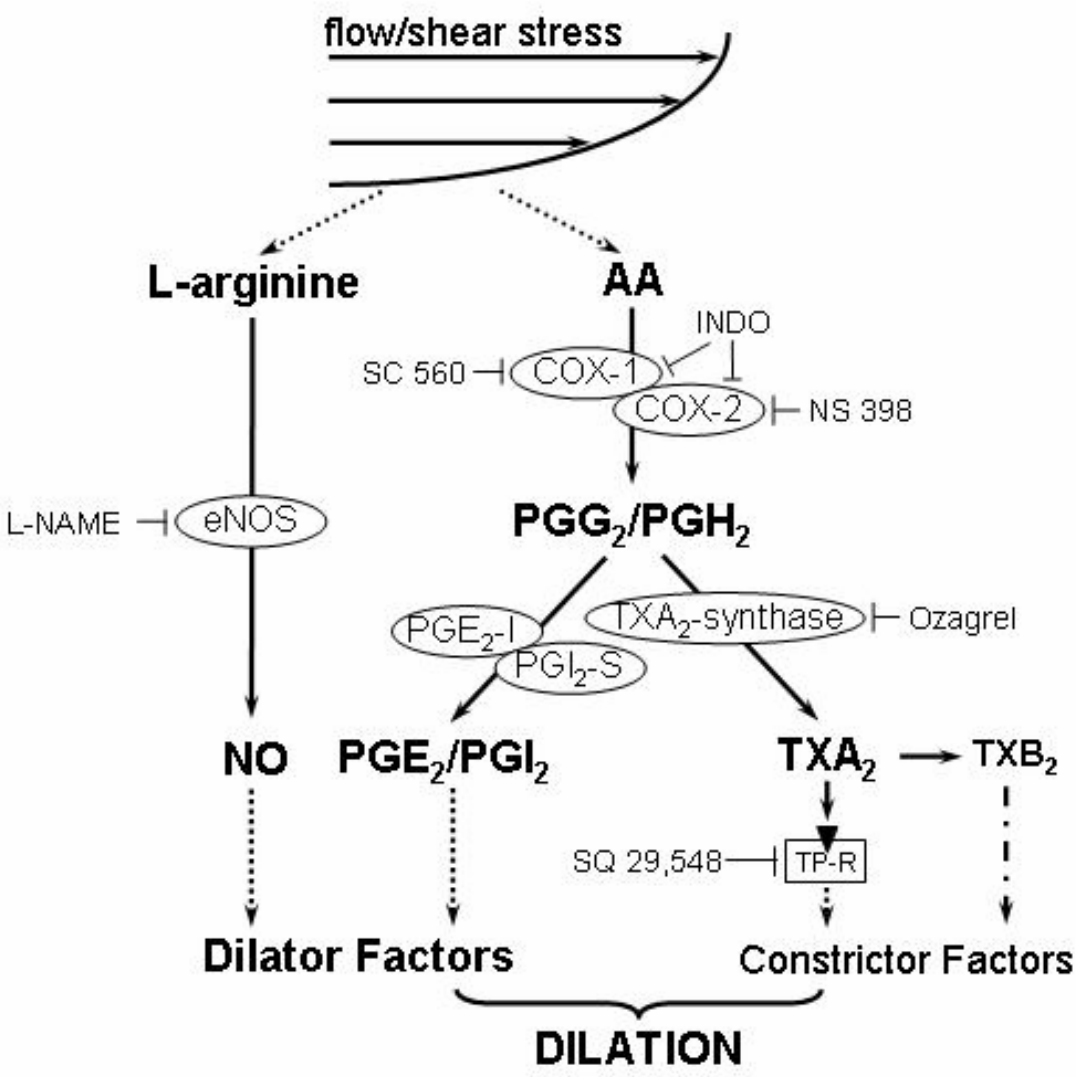
The pathophysiological and clinical importance of our findings in HHcy venules is that, in addition to changes in coagulation and anticoagulation systems,[180 189] shown previously, HHcy alters the function of venular endothelium. Intact function of endothelium is important both in the regulation of 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[4] 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.[145]

Indeed, 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,[142] which in turn produced constrictor TxA_2 as suggested by the present study, as well. Activation of arachidonic acid pathway and oxidative stress could be due to the development of proinflammatory conditions in HHcy as demonstrated by previous studies.[157 236]

6. Conclusion

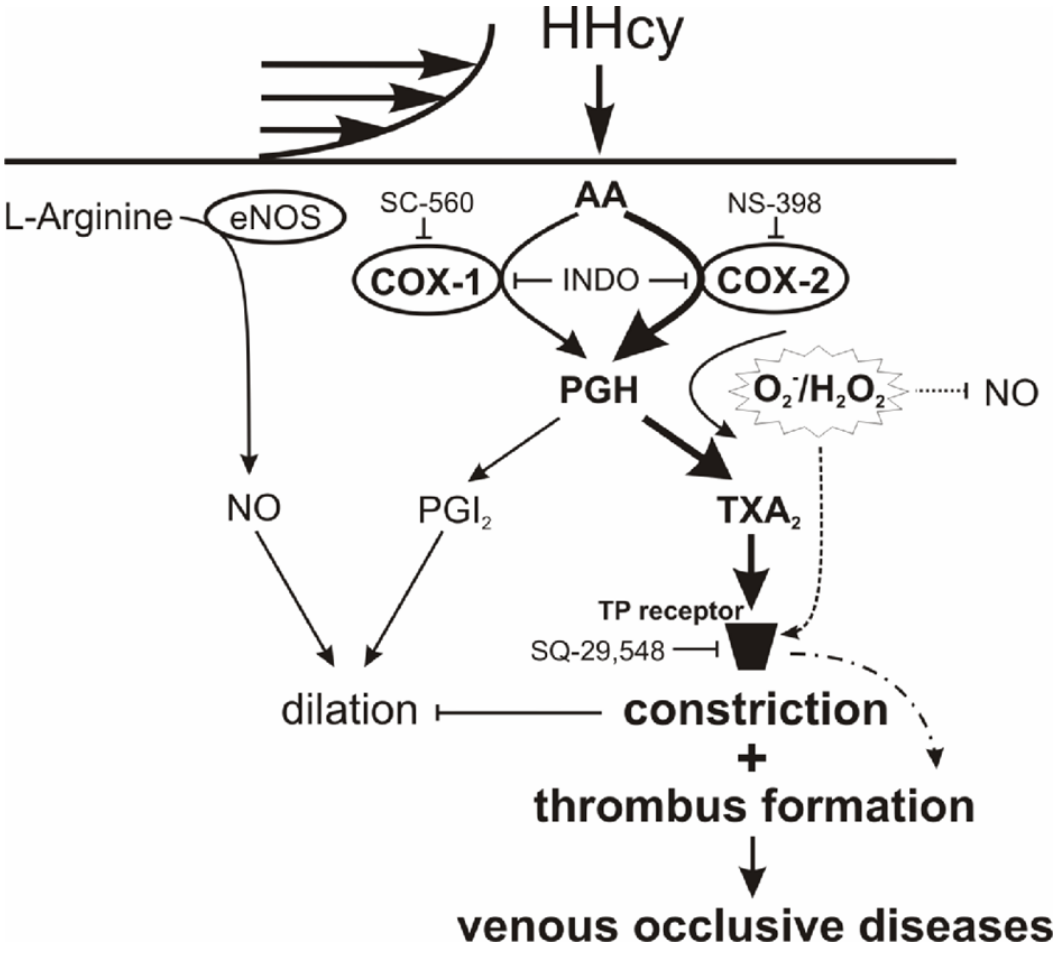
6.1. In Control Venules

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.



6.2. In HHcy Venules

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.



7. Summary

There are several studies regarding the function of large veins, whereas we do not have many knowledge about functions of small veins or venules. Nevertheless, in isolated preconstricted venules flow-dependent mechanism was showed to be present, the mediation of which is not fully clarified. In addition, preconstriction could largely influence the responses of venules. However, flow-dependent responses of venules without preconstriction and the underlying mechanisms have not been studied yet. We hypothesized that - in addition to nitric oxide (NO) and dilator prostaglandins (PGI_2/PGE_2) – thromboxane A_2 (TxA_2) contributes to the mediation of flow-induced responses of venules. This mechanism can be injured in different pathological conditions such as in venular occlusive diseases and in venous thrombosis. After clinical evidences, in these diseases plasma level of homocysteine (Hcy) is elevated, suggesting that hyperhomocysteinemia (HHcy) has pathological effects on venular functions, the mechanisms of which have not yet been studied. We hypothesized that HHcy leads to the impairment of venular functions due to alterations in the arachidonic acid pathway and due to an enhanced production of reactive oxygen species (ROS).

Thus we aimed to study the flow-induced diameter responses of venules isolated from normal and HHcy rats, because these responses indicate the function of endothelium known to be importantly involved in the regulation of venular tone. We have found that increases in intraluminal flow elicited dilations in control, whereas constrictions in HHcy venules. In controls, flow-induced responses were mediated by nitric oxide (NO), dilator and constrictor prostaglandins (PGs), with an overall effect of dilation. Dilator PGs are produced by cyclooxygenase-1 (COX-1), whereas constrictor PGs are produced by cyclooxygenase-2 (COX-2) pathway. In HHcy the flow-induced altered venular response is the result of an altered balance of dilator and constrictor factors. The mediative role of NO and dilator PGs is reduced, whereas the role of constrictor PGs became enhanced. In HHcy, dilator PGs are produced by the COX-1, whereas constrictors both by COX-1 and COX-2 pathway. Moreover, enhanced level of ROS also contributes to the altered vasomotor functions of venules. Because venules determine postcapillary resistance - thus capillary pressure and venous return to the heart - the flow-induced diameter responses of venules may have an important role in

the regulation of tissue blood supply, especially in increased flow conditions. We propose that in HHcy, the altered flow-induced venular vasomotor function contributes to the pathological changes of venous circulation and the development of thrombosis and occlusive venular diseases in HHcy.

8. Összefoglalás

A nagy vénák funkcióit illetően számos tanulmány áll rendelkezésünkre, azonban a kis vénák és venulák funkcióiról kevés ismeretünk van. Izolált, prekonstrihált venulákban kimutatták az áramlás-indukálta átmérőváltozás jelenlétét, mely hátterében álló mechanizmusok nem teljesen ismertek még. Azonban a prekonstrikció nagyban befolyásolhatja a venulák vazomotor válaszát. Mindazonáltal az áramlás-indukálta válaszokat prekonstrikció nélkül és az azt létrehozó mechanizmusokat ezideig még nem tanulmányozták. Feltételeztük, hogy a nitrogén-monoxidon (NO) és dilatátor prosztaglandinokon kívül (PGI_2/PGE_2), a tromboxán A_2 (TxA_2) szintén hozzájárul az áramlás-indukálta válaszok, kialakulásáért venulákban. Ez a mechanizmus károsodhat különböző patológiás körülmények között, mint például vénás okkluzív betegségekben, vénás trombózisban. Klinikai tanulmányok alapján ezen betegségekben a plazma homocisztein (Hcy) szint emelkedett, mely arra utal, hogy a hiperhomociszteinémiának (HHcy) káros hatása van a venulák funkciójára, melynek mechanizmusa még nem ismert. Feltételeztük, hogy a HHcy az arachidonsav kaszkád megváltoztatása és a fokozott mértékben termelődő reaktív oxigén gyökök (ROS) által károsítja a venulák funkcióját.

Tehát célul tűztük ki normál és HHcy patkányokból izolált venulák áramlás-indukálta átmérőváltozásainak tanulmányozását, mivel ezen válaszok jelzik az endothelium funkcióját, melyről jól ismert, hogy fontos szerepet játszik a venulák tónusának szabályozásában. Azt találtuk, hogy az intraluminalis áramlás emelése kontroll venulákban dilatációt, míg HHcy venulákban konstrikciót hoz létre. Kontrollban az áramlás-indukálta válaszokat a nitrogen-monoxid (NO), dilatátor és konstriktor prosztaglandinok (PG) hozzák létre, mely összességében dilatációt alakít ki. A dilatátor prosztaglandinok a ciklooxigenáz-1 (COX-1), míg a konstriktor PG a ciklooxigenáz-2 (COX-2) útvonalon termelődnek. HHcy-ban a venulák károsodott áramlás-indukálta válaszai a dilatátor és konstriktor faktorok megváltozott aránya miatt alakul ki. Az NO

és dilatátor PG-k szerepe csökken, míg a konstriktor PG-k szerepe nő. A dilatátor PG-k a COX-1, míg a konstriktor PG-k a COX-1 és COX-2 útvonalon egyaránt képződnek. Az emelkedett ROS képződés szintén hozzájárul a megváltozott vazomotor funkcióhoz HHcy venulákban. Mivel a venulák határozzák meg a posztkapilláris ellenállást, így a kapilláris nyomást és befolyásolják a szív felé történő vénás visszaáramlást, ezért az áramlás-indukálta átmérőváltozásoknak venulákban fontos szerepe lehet a szöveti vérellátás szabályozásában, különösen fokozott áramlás esetén. Feltételezzük, hogy HHcy-ban a megváltozott áramlás-indukálta vazomotor funkció hozzájárul a vénás keringés patológiás változásaihoz, trombózis és vénás okkluzív betegségek kialakulásához HHcy-ben.

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Publications related to the dissertation

- A. Racz, Z. Veresh, N. Erdei, Z. Bagi, A. Koller. Thromboxane A_2 Contributes to the Mediation of Flow-Induced Responses of Skeletal Muscle Venules: Role of Cyclooxygenases 1 and 2. Journal of Vascular Research, 2009;46:397–405.

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Other publications

- Janos Toth, Anita Racz, Pawel Kaminski, Michael Wolin, Zsolt Bagi, Akos Koller Asymmetric Dimethylarginine (ADMA) Inhibits Flow/Shear Stress Dependent Dilation of Isolated Arterioles and Increases Basal Tone via Superoxide Release. Hypertension, 2007;49;563-568.

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Acknowledgement

I am owing to render thanks first of all for my tutor Akos Koller MD, PhD who ensured me the possibility to carry out my studies in his laboratory and who provided several idea and advice during my work.

Also, I would like to thank all of my collegues, Zsolt Bagi MD, PhD, Zoltan Veresh MD, Janos Tóth MD PhD, Erika Tóth MD, Nóra Erdei MD PhD, Csongor Csekő MD PhD for participating in my work or for their advice, such as for collegues of other departments; Gabor Lotz MD PhD, Mrs Pásztor T. Enikő.

I am also especially grateful for Ágnes Cser for the irreplaceable help in my works.

Last but not least I am thankful for my family for the persistent support which I have always continuously received.

Acknowledgement for Financial Support

This work was supported by grants from: AHA Founders Aff. 0855910D, Hungarian Sci. Res. Funds/OTKA -T48376, K67984; K71591.