Regulation of peripheral vascular tone in spinal cord-injured individuals

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Regulation of peripheral vascular tone in spinal cord-injured individuals

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Table Of Contents

Chapter 1

General introduction and outline of the thesis

The main topic of this thesis is to elucidate the regulation of peripheral vascular tone in spinal cord-injured (SCI) individuals. Since spinal cord-injured individuals have no sympathetic innervation below the level of the lesion, the regulation of the peripheral circulation at rest and during postural changes will be disturbed. The question therefore is: which factors contribute to or compensate for the lack of sympathetic innervation in the regulation of peripheral vascular tone in SCI individuals? In this introduction the focus will be on the regulation of vascular tone under baseline conditions in healthy humans and how the regulation of peripheral vascular tone is affected by deconditioning and a change in sympathetic nerve activity as a result of a spinal cord injury. The second part of the introduction deals with the regulation of peripheral vascular tone upon orthostatic challenges in healthy humans and spinal cord-injured individuals.

Spinal cord injury

Epidemiology

A spinal cord injury is a disruption of the spinal cord resulting in complete or incomplete paralysis of muscles, loss of sensation, and dysfunction of the autonomic nervous system. The severity of the consequences of a spinal cord injury is dependent on the level and completeness of the spinal lesion. Cervical lesions (tetraplegia) will result in paralysis of the arms, legs and trunk. In high thoracic lesions, trunk and leg muscles are involved, whereas lower thoracic and lumbar lesions cause paralysis of only leg muscles. The cervical cord is the most common site of injury, occurring in 38-75% of all cases.[1-6] 50-75% of the spinal lesions are traumatic of origin[4, 7] and common causes are traffic accidents, falls, violence and sports.[5, 6] Estimations of the annual incidence of spinal cord injury in the Netherlands vary between 10.4 and 27 per million population.[7-9] No reliable number is available for prevalence in the Netherlands, but extended from an overview of literature on this topic[10] it is estimated at ~12.000-15.000 of persons suffering a spinal cord injury.

Improved medical care over the past six decades increased life expectancy of people with a spinal cord injury, although there is no substantial change in either mortality or life expectancy since 1980.[11] As a consequence of the increased life expectancy SCI individuals are now more faced with secondary complications, like bladder and pulmonary infections, osteoporosis, and diseases more related to the cardiovascular system like, thromboembolism, pressure sores, and other cardiovascular diseases. In chronic SCI individuals (> 1 year post-injury) respiratory complications are nowadays the leading cause of death followed by heart disease,[12, 13] whereas in the past it was renal failure. The cardiovascular mortality is higher in SCI individuals as compared to the general population, especially in the SCI individuals aged below 55 years.[14] (see Figure 1)

Clinical consequences of a spinal cord injury

As the complications of aging accumulate in SCI individuals, the risk factors for

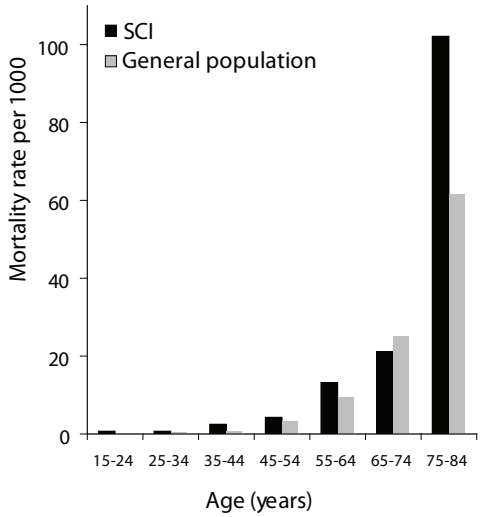

Figure 1. Mortality related to cardiovascular disease in SCI individuals and the general population.[14]

cardiovascular diseases become apparent. Risk factors that may predispose for atherosclerosis[15] include hypertension,[16] lipid abnormalities,[17-19] glucose intolerance and diabetes mellitus,[16, 20] increased weight[21] and smoking. Moreover the majority of individuals with SCI have a sedentary lifestyle and have limited physical activities, which seem to play a causal role in the onset of cardiovascular diseases, since several studies showed that regular physical exercise can decrease cardiovascular mortality.[22, 23] SCI individuals are also faced with disturbances of the autonomic nervous system, like orthostatic hypotension and autonomic dysreflexia. Orthostatic hypotension occurs in SCI individuals because their inability to regulate and maintain blood pressure to postural stress as a consequence of loss of supraspinal sympathetic control. In the acute phase these patients experience syncopal events. However, in the chronic phase they exhibit an orthostatic tolerance comparable to able-bodied subjects.[24] Autonomic dysreflexia is a potentially life-threatening episodic hypertension that develops in up to 80% of people with tetraplegia or high paraplegia (above the 6th thoracic spinal segment). This hypertension can be initiated by daily trivialities, such as bladder distension, catheterization and bowel evacuation that induce sensory input entering the spinal cord below the level of the lesion. It is characterized by exaggerated sympathetic reflexes below the level of the lesion that is unopposed by central inhibitatory pathways, and accompanied by a vagally induced bradycardia. During autonomic dysreflexia, increases in arterial pressure causes debilitating headaches, sweating, seizures, strokes and even death.

Cardiovascular adaptations

In persons with a spinal cord injury, extensive adaptations of the cardiovascular system occur as a consequence of a sedentary lifestyle and the loss of supraspinal sympathetic control below the level of the spinal lesion. Previous studies using echocardiography found a significant reduction in cardiac mass in subjects with tetraplegia,[25-29] whereas in paraplegic individuals cardiac mass was unaffected.[26, 27] Total blood volume in high spinal cord lesions is decreased, which can be explained by both deconditioning and impairment of the autonomic nervous system.[30] The basal levels of both systolic and diastolic blood pressure in subjects with high (>T4) spinal cord lesions are lower than in able bodied

subjects, whereas basal heart rate does not differ.[31, 32]

Furthermore, loss of supraspinal control of somatic efferents results in extreme deconditioning of the leg muscles which is associated with reduced oxygen demand and subsequently with profound peripheral vascular adaptations. Diameters of leg conduit arteries is approximately 20-30% lower than in able-bodied subjects.[33-36] Using echo Doppler ultrasound measurements a 30-50% decrease in common femoral artery blood flow has been reported,[33, 34, 37, 38] while other studies did not observe any difference between SCI individuals and able-bodied healthy controls in baseline femoral artery blood flow.[39, 40] Structural adaptations in the leg vascular bed of SCI individuals occur, which is reflected by a decrease in capillary density[41] resulting in a ~35% reduction of reactive hyperemic blood flow as measured by both echo Doppler[37] and venous occlusion plethysmography.[35] Baseline blood flow at the arteriolar level is ~60% lower in SCI individuals compared to able-bodied subjects as determined by thermodilution technique[32] or venous occlusion plethysmography.[42] Indeed, despite the loss of supraspinal sympathetic control, leg vascular resistance in spinal cord-injured individuals, in particular the paraplegic individuals,[32] is more than doubled in comparison with control subjects.[42]

Regulation of baseline peripheral vascular tone

Blood flow in the skeletal muscle vascular bed is governed by perfusion pressure and vascular resistance. In supine position perfusion pressure is the pressure difference between the arterial and venous ends of the circulation. Since mean arterial pressure and venous pressure are maintained within narrow limits, the control of skeletal muscle blood flow is accomplished in large part by variation of skeletal muscle vascular resistance. Skeletal muscle vascular resistance is determined by the smooth muscle tone in the arterioles. These are the major sites of control of vascular resistance in the skeletal muscle vascular bed. Compared to other tissues skeletal muscle vascular tone in supine position is high and is essentially under dual control by systemic and local factors. The systemic mechanisms are predominantly aimed at blood pressure control and cardiovascular homeostasis, whereas the local systems maintain tissue homeostasis.

Systemic factors

Neural: the sympathetic nervous system

The sympathetic nervous system plays a key role in cardiovascular homeostasis. Hemodynamic changes provoke a compensatory reflex response initiated by arterial baroreceptors and cardiopulmonary mechanoreceptors resulting in changes in sympathetic outflow and vagal-nerve activity. Afferent fibers of these receptors project to the nucleus tractus solitarii in the dorsal medulla, which in turn projects

to efferent cardiovascular neurons in the ventral medulla. From the medulla the efferent sympathetic preganglionic fibers arise from the intermediolateral and –medial cell columns in the spinal cord and join the ventral roots at each vertebral level between Thoracic 1 and Lumbar 2 and terminate in the paravertebral ganglia, which are interconnected and form the sympathetic chain. The skeletal muscle vascular bed receives postganglionic sympathetic nerve fibres. An increase in muscle sympathetic nerve activity will enhance vascular tone.

In humans, Barcroft *et al* [43] first demonstrated that the sympathetic nervous system mediates a "tonic" vasoconstriction in the skeletal muscle vascular bed; surgical sympathectomy increases skeletal muscle blood flow by ~25% and decreases vascular tone.[44, 45] Upon sympathetic stimulation noradrenaline is released from the postganglionic nerves.[46] Binding

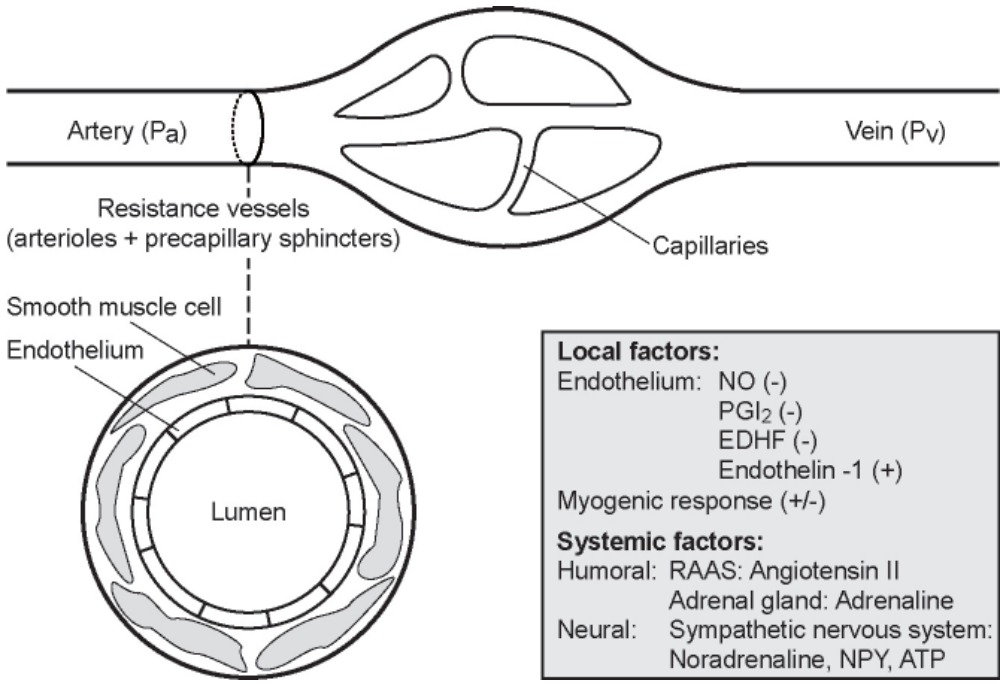

Figure 2. Schematic representation of the most important factors that increase (+) or decrease (-) vascular resistance. Angiotensin II is one of the end products of the renin-angiotensin-aldosteron-system (RAAS) and binds to the AT1-receptor on the smooth muscle cell that results in vasoconstriction. When stimulated, the adrenal gland can release adrenaline and noradrenaline into the systemic circulation. Adrenaline in low concentrations dilates muscle resistance vessels (β2-adrenergic effect) and in high concentrations produces vasoconstriction (α-adrenergic effect). Noradrenaline is the major neurotransmitter of the postganglionic sympathetic nerves. Stimulation of the α-adrenergic receptors results in vasoconstriction. The endothelium produces local factors like for example nitric oxide (NO), prostacyclin (PGI2), endothelium derived hyperpolarizing factor (EDHF), and endothelin-1 (ET-1). The myogenic response is an autoregulatory mechanism of the smooth muscle cell that contracts in response to stretch and relaxes with a reduction in vessel wall tension.

of noradrenaline to both postjunctional α_1- and α_2-adrenoceptors results in an increase in vascular tone,[47] whereas the prejunctional α_2-adrenoceptor modulates noradrenaline release via a negative feedback mechanism.[46, 48] Complete blockade of the α-adrenergic receptor, assessed by infusion of the nonselective α-adrenoceptor blocker *phentolamine*, reduces vascular tone by ~50% in the leg[49] and by ~70-80% in the forearm.[50, 51] In addition, the sympathetic nerves secrete a variety of other substances that are co-released with noradrenaline, like neuropeptide Y (NPY) and adenosine 5-triphosphate (ATP).[52, 53] NPY excites Y_1-receptors and causes vasoconstriction by direct stimulation of the receptor or by potentiation of the α-adrenergic receptor mediated vasoconstriction.[54] ATP binds to P_{2x}-purinoceptors to cause vasoconstriction.[52] Apart from transmitters co-released with noradrenaline, animal[55, 56] and human *in vivo*[57, 58] research has suggested that angiotensin II can also modulate sympathetic vasoconstriction by either increasing the release of noradrenaline through activation of presynaptic angiotensin II-receptors or by enhancing the postsynaptic noradrenaline responsiveness on vascular smooth cells.

Humoral: renin-angiotensin-aldosterone system

Angiotensin II produces arteriolar vasoconstriction and is an end product of the renin-angiotensin-aldosterone system (RAAS). The RAAS is stimulated under conditions of renal hypoperfusion, produced by hypotension or volume depletion, by reduced sodium delivery to the macula densa and by increased sympathetic activity to the kidney. Under these conditions renin is released from the juxtaglomerular cells that intitiates a sequence of steps that begins with cleavage of angiotensin I from angiotensinogen. Angiotensin I is then converted into angiotensin II. This reaction is catalyzed by angiotensin converting enzyme (ACE), which is mainly located in the lungs, but is also present at the luminal membrane of vascular endothelial cells, the kidney, adrenal gland and the brain.[59-62] Angiotensin binds to specific angiotensine II receptors: AT_1- en AT_2-receptoren.[63] Binding to AT_1-receptors results in an increase in vascular tone by a direct effect on the smooth muscle cell mediated primarily by protein kinase C generation.[64] As was explained in the previous paragraph angiotensin II can also influence vascular tone indirectly by mediating sympathetic vascular tone.[55-58] From human *in vivo* research we know that angiotensin II has a minor contribution to baseline forearm vascular tone in healthy human.[65-68] However, under conditions of RAAS activation, endogenous angiotensin II contributes to the acute local maintenance of basal peripheral vascular tone. Intrabrachial administration of losartan, a specific AT_1-receptor antagonist, in sodium depleted healthy individuals increases forearm blood flow 60%.[67] In patients with heart failure and liver cirrhosis, where plasma renin activity is increased, losartan causes local peripheral vasodilatation.[69-71]

Local factors

Endothelium derived factors

The endothelial cells control the tone of

the underlying vascular smooth muscle by releasing various relaxing factors, from which nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) are the most significant, and vasoconstrictor mediators, such as endothelin (ET), angiotensin II, and vasoconstrictor prostanoids.[72, 73]

Furchgot *et al* [74] demonstrated that the relaxation of the vascular smooth muscle cells in response to acetylcholine was dependent on the integrity of the endothelium that released a vasorelaxing factor that was later identified as NO.[75] NO is synthesized in the endothelial cell from oxygen and L-arginine by endothelial NO synthase (NOS). It diffuses to the vascular smooth muscle cell where it stimulates soluble guanylate cyclase, resulting in an increased formation of cyclic guanosine monophosphate and subsequent relaxation of the vascular smooth muscle cell.[76] The likely physiological stimulus for NO production is increased shear stress by increased blood flow. Shear stress is the frictional force of blood on the endothelium. Stimulated NO production or endothelium-dependent vasodilatation can be measured by ultrasound at the level of the conduit artery during reactive hyperemia. This flow-mediated dilatation (FMD) provides an index of conduit artery endothelium dependent NO function, whereas pharmacological stimulation of NO production by intraarterial infusion of acetylcholine is used as measure of endothelial function at arteriolar level. Apart from stimulated production of NO, NO is also released basally and is involved in the regulation of baseline arteriolar vascular tone since infusion of the L-arginine analogue and NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) into the brachial artery reduced forearm blood flow by 30-50%.[77, 78] Contribution of NO to baseline vascular tone in the leg vascular bed is unknown, and will be a topic of the present thesis. Although NO is a predominant endothelium derived vasodilator in the regulation of vascular smooth muscle tone, the contribution of the other endothelium derived vasoactive factors will also be discussed. PGI_2 is synthesized by cyclooxygenase-1 (COX-1), which is expressed in the vascular endothelium. Inhibition of COX-1 by intraarterial administration of aspirin or indomethacin in the human forearm reduced blood flow by 22-32% suggesting that prostanoids also play a role in maintaining smooth muscle vascular tone in humans.[79, 80] Debate surrounds the identity of EDHF and its mechanism of action, with the consensus being that there is no universal EDHF. Two primary mechanistic pathways are implicated: (i) myoendothelial gap junctions mediating the spread of endothelial cell hyperpolarization or small signaling molecules (or both) to the smooth muscle; and (ii) diffusible mediators released from the endothelium, including potassium ions (K^+), hydrogen peroxide (H_2O_2), epoxyeicosatrienoic acid (EET), C-type natriuretic peptide (CNP), but also PGI_2 and NO. (For review see[81-83])

In vivo studies indicate that EDHF seems to contribute little to basal blood flow and vascular tone, but makes a significant contribution to evoked blood flow. (For review see[84])

Among the vasoconstrictor factors released by the endothelium, endothelin-1 is the most potent. In humans endothelin-1 infused into the brachial artery results in a decrease in forearm blood flow.[85, 86] Endothelin-1 binding to ET_A-receptor and ET_B-receptor on the smooth muscle cell results in vasoconstriction.[87] ET_B-receptors are also located on the endothelium.[88] In contrast, activation of these ET_B-receptors mediates vasodilatation by the release of endothelium-dependent vasodilator substances such as NO and PGI_2.[89] Contribution of endothelin-1 to baseline vascular tone is not uniform in the human skeletal muscle vascular bed. Forearm blood flow increased by 35-60% during intraarterial infusion of $ET_{A/B}$-receptor antagonists,[90, 91] whereas in the leg blockade of the $ET_{A/B}$-receptor hardly influenced leg blood flow.[92]

In addition to their own distinct profile of activity, there is clear evidence that all these endothelial mediators are interacting with each other in a complex but integrated manner to maintain baseline vascular tone and the health of the vasculature.

Myogenic tone

The myogenic response was first described by Bayliss[93] in 1902 and has been proposed to participate in the establishment of basal vascular tone and autoregulation of blood flow.[94] It is characterized by an inherent ability of arterioles to contract to an increase in transmural pressure. The stimulus for the myogenic response is a pressure induced alteration of vessel wall tension[95] that produces depolarization of smooth muscle cell membranes with a subsequent depolarization-induced Ca^{2+} entry being necessary for myogenic contraction.[94] However, the exact mechanism linking the increase in transmural pressure and vascular smooth muscle constriction remains unclear.

Animal *in vitro* studies have revealed that, in particular, skeletal muscle vessels exhibit a strong myogenic tone.[96-98] Under baseline physiological levels of transmural pressure, the active myogenic tone, which is defined as the percentage of passive vessel diameter, in the rat muscle vascular bed is 50%.[97, 99] *In vivo* the muscle vascular bed is not only under the influence of transmural pressure, but is also exposed to the pulsatility of blood pressure, neurotransmitters and endothelial influences. It is known from animal research that the myogenic response is modulated by α-adrenergic stimulation by increasing the strength of the myogenic response.[100] Flow and factors released from the endothelium, like NO, can also influence the strength of the myogenic response.[101]

Regulation of baseline peripheral vascular tone in SCI individuals

In this section the focus will be on the role of the sympathetic nervous system, endothelial factors, particular NO, and the myogenic tone in the regulation of basal vascular tone in SCI individuals.

Sympathetic vascular tone

The initial phase after a spinal cord injury is characterized by "spinal shock", which is described as the sudden and transient

suppression of neural function below the level of the spinal cord lesion. During this state, a flaccid paralysis of skeletal muscle occurs and all tendon, cutaneous, and (para)sympathetic reflexes integrated in the spinal cord below the lesion are abolished or greatly reduced. As a consequence of reduced sympathetic activity, one can imagine that the contribution of the sympathetic nervous system, in maintaining vascular tone is diminished in the spinal shock phase, which will clinically result in hypotension. In humans, the spinal shock phase persists for days to weeks. Recovery is reflected by emergence of spinal (sympathetic) reflexes. The role of these reflexes in the regulation of vascular tone, and whether they contribute to the high vascular tone in the paralyzed legs, in the chronic phase after a spinal cord-injured individuals is not known. Animal research has focused on whether the deafferented spinal cord can generate significant basal sympathetic activity. In anaesthetized rats, sympathetic outflow to renal and splenogastric, but not lumbar, sympathetic activities after spinal transaction was reported to be well maintained.[102] However, in awake cervical spinal rats there was no evidence for functionally significant spinally generated sympathetic nerve activity.[103, 104] In humans, microneurographic recordings have also reported decreased activity in the cutaneous and muscle postganglionic axons situated below the level of the lesion.[105, 106] Therefore, despite the return of spinal sympathetic reflexes, there is no convincing animal or human data to demonstrate that the deafferented spinal cord can generate significant basal sympathetic activity that could contribute to baseline vascular tone.

Average sympathetic outflow can be estimated by determination of venous plasma noradrenaline.[107] Plasma noradrenaline levels depend on the level of the spinal lesion. In individuals with a cervical lesion, plasma noradrenaline is lower than in control subjects at rest.[108, 109] Individuals with high (T1-T4) thoracic lesions show low[110] to normal plasma noradrenaline levels,[111] whereas individuals with the spinal lesion below T5 have normal[108] to high[111] plasma noradrenaline levels as compared with able-bodied subjects. So, in individuals with cervical lesions, where overall sympathetic activity is reduced, circulating plasma noradrenaline has a minor contribution to baseline vascular tone, whereas in individuals with thoracic lesions circulating normal to high plasma noradrenaline levels could contribute to baseline vascular tone.

RAAS system

In the absence of adequate sympathetic innervation the renin-angiotensin-aldosteron system helps to maintain blood pressure, especially in SCI individuals with high thoracic or cervical lesions, indicated by elevated resting plasma renin levels,[31, 112-114] probably in response to low perfusion pressure of the kidneys. Whether angiotensin II levels are raised and will subsequently contribute to the high leg vascular tone in SCI individuals is unknown.

Endothelium

Deconditioning

Studies on the effect of deconditioning on

NO pathway are controversial. Previous animal studies have mimicked physical inactivity by hindlimb unloading and they report inconsistent results. Some studies demonstrate a decreased endothelial NO synthase (eNOS) expression in the endothelial cells of both conductance and resistance vessels, and an attenuated vasodilatation to acetylcholine, suggesting that the endothelial release of NO was attenuated by rat hindlimb unloading.[115-117] In contrast, another study demonstrated no alterations of eNOS expression following rat hindlimb unloading, but an upregulation of aortic inducible NOS.[118] In humans, evidence for an attenuated endothelial NO release in both conductance and resistance arteries following deconditioning is sparse. Kamiya *et al*[119] showed a decrease in plasma nitrite/nitrate concentration, an indicator of endogenous nitric oxide production, after head-down bed rest suggesting a diminished release of endothelial NO. This was not confirmed by Bonnin *et al*[120] who did not find a significant change in urinary nitrite excretion during 7 days of head-down tilt bed rest. In one study, the effect of immobilization of a forearm, by forearm casting for treatment of fractures, on NO availability was investigated by infusion of L-NMMA to block the NO production.[121] The response to L-NMMA was similar in the casted subjects and their controls. The results of this study could be explained by methodological considerations, like casting for fractures as a model for deconditioning, and the dosage of L-NMMA used, which is not likely to produce maximal NOS inhibition.

Flow-mediated dilatation (FMD) in the superficial femoral artery, which is endothelium dependent and an indicator of shear stress-induced endothelial nitric oxide production, is, surprisingly, increased after deconditioning due to unilateral lower limb suspension[122] and bed rest.[123] In these studies, endothelium-independent dilatation increased simultaneously suggesting that vascular adaptations to deconditioning occur not only in the endothelium, but also at the level of the smooth muscle. Similarly, in the brachial artery an increased FMD was reported after 7 days of bed rest deconditioning, but endothelium independent vasodilatation was not affected.

Due to hindlimb unloading circulating plasma ET-1 levels are increased.[124] In humans, deconditioning caused by microgravity, tended to increase plasma ET-1 levels.[125]

Sympathectomy

Some animal studies have shown decreased NO availability, in terms of decreased eNOS or blunted vasorelaxation to endothelial stimulation, due to surgical or chemical sympathectomy.[126-128] Data of human *in vivo* research are conflicting. Lepori *et al*[129] examined vasoconstrictor responses to systemic doses of L-NMMA in innervated calfs and denervated forearms. Sympathectomy markedly potentiated the vasoconstrictor response effect of L-NMMA infusion and they concluded that this could be related to an augmented contribution of NO to the regulation of local vascular resistance after denervation. However, local infusion of L-NMMA into the brachial artery of the sympathectomized

forearm resulted in similar vasoconstrictor responses as in non-sympathectomized limb[130] suggesting endothelial function was not changed by sympathectomy.[131]

Spinal cord-injured individuals

No studies have examined the role of baseline NO production in the increased leg vascular tone in SCI individuals. Plasma levels of the most important endothelial derived vasoconstrictor factor, ET-1, have been reported to be very high in SCI individuals,[132] which may be suggestive for an upregulation of the ET-1 pathway. Although in a recent study plasma ET-1 levels do not differ between the SCI individuals and control subjects, the contribution of ET-1 to baseline vascular tone in SCI individuals is increased.[92] As such, ET-receptor sensitivity and/or receptor signaling may explain the upregulated ET-pathway, which is rather mediated by the ET_A-receptor than the ET_B-receptor.

Superficial femoral artery FMD is increased in SCI individuals as compared with control subjects.[37, 133] If FMD is corrected for its eliciting stimulus shear rate, this difference is no longer statistically significant. However, per delta shear rate the response is still greater in SCI individuals than in controls. These results are in contrast with the posterior tibial artery FMD, which was lower in SCI individuals than in controls,[134] that could be explained by placement of the cuff proximal or distal to the measuring site. In the studies by De Groot *et al,*[37, 133] the ultrasound probe was proximal to the cuff, whereas in the study described by Stoner *et al,*[134] the site of measurement was in the ischaemic area distal to the cuff. Under these circumstances FMD is affected by vasodilators other than NO which are released in response to ischaemia, and may also be complicated by myogenic responses as a result of the pressure fall inside the artery during occlusion.[135] Endothelium independent vasodilatation of the superficial femoral artery is not altered after spinal cord injury, indicating no change in vascular smooth muscle function[37] In the non-paralyzed upper extremity, FMD does not differ between SCI individuals and control subjects.[37, 134]

Myogenic tone

Whether an increase in myogenic tone is responsible for the increase in leg vascular tone in SCI individuals has never been investigated. Results of animal research investigating effects of sympathetic stimulation or denervation, and deconditioning on myogenic tone or response are unequicoval. In the rat skeletal muscle vascular bed, it was demonstrated that α-adrenergic stimulation increases the strength of the myogenic response.[100, 136] In line with these results, one might expect that myogenic tone is attenuated in SCI individuals, who lack sympathetic activity below the level of the lesion. In contrast, chronic sympathetic denervation on resistance arteries in the rabbit ear vasculature showed that the denervated artery developed greater stretch dependent myogenic tone. It was concluded that this phenomenon may partially account for the return of vascular tone after an initial decrease in vascular tone due to sympathetic denervation.[137] After rat hindlimb

unloading, myogenic tone is attenuated in both arterioles of fast twitch fibers in the gastrocnemius muscle[138] and in mesenteric resistance arteries.[139] However, in these studies the myogenic response may be affected by chronic changes in transmural pressure rather than by deconditioning.

Regulation of peripheral vascular tone during orthostatic challenges

Regulation of peripheral vascular tone under normal physiological conditions

Assumption of the upright posture results in pooling of 500-1000 ml of blood in the lower extremities and the splanchnic circulation. This initiates an acute rapid decrease in venous return to the heart. The ensuing reduction in ventricular filling pressure results in diminished cardiac output and blood pressure. The fall in blood pressure and thoracic volume provokes the compensatory baroreflex to increase sympathetic and to reduce parasympathetic outflow. The increase in sympathetic outflow will rise peripheral vascular resistance, venous return and cardiac output. The prompt increase in peripheral vascular resistance, mediated by sympathetic α-adrenergic stimulation, is the key event in blood pressure control during postural stress.[140] Apart from baroreflex control, local sympathetic mechanisms, such as the venoarteriolar reflex, have been reported to contribute to the hemodynamic response to postural stress. The venoarteriolar reflex is triggered when venous pressure is elevated above 25 mmHg or more.[141] It has been suggested that during orthostasis as much as 45% of the increase in systemic vascular tone is due to the venoarteriolar reflex.[141-146] Several studies have been performed to unravel the mechanism of the venoarteriolar reflex.[141, 143, 147, 148] From these studies, it can be concluded that the venoarteriolar reflex concerns a local α-adrenergic sympathetic axon reflex, although a recent study makes the α-adrenergic origin of the venoarteriolar reflex uncertain.[149]

Except for α-adrenergic stimulation, non-adrenergic mechanisms, such as purinoceptor stimulation and release of neuropeptide Y, seem to contribute to the vasoconstrictor response after sympathetic stimulation.[150, 151]

In addition, the leg vasoconstrictor response to postural stress may in part be myogenic,[152] and as such not related to the sympathetic nervous system. This support from this concept comes from a human study where both arm and leg vascular resistance were measured during 40° head-up tilt, with the arm kept at heart level.[153] Despite similar increases in muscle sympathetic nerve activity in the arm and leg, the increase in vascular tone in the leg was ~2 times higher, which may be caused by an increase in leg transmural pressure evoking the myogenic response.

Postural vasoconstriction in spinal cord-injured individuals

In SCI individuals, sympathetic brainstem control of the vascular bed below the level of the spinal cord lesion is deficient. Especially in the early stages of rehabilitation, SCI

individuals are prone to orthostatic hypotension. However, chronically injured individuals show, despite the absence of central sympathetic control,[154] a remarkable orthostatic tolerance during postural stress and, in particular the paraplegic individuals, maintain blood pressure during orthostatic challenges.[155] Moreover, preservation of tilt-induced leg vasoconstriction in these individuals has been reported.[144, 156, 157]

Activation of spinal sympathetic reflexes may be one of the mechanisms contributing to the tilt-induced vasoconstriction and recovery of blood pressure upon head-up tilt.[158] These spinal reflexes function independent of brainstem control and may evoke an increase in muscle and skin vascular tone.[106] It has been suggested that spinal sympathetic reflexes contribute to the head-up tilt induced vasoconstriction in muscle[144] and subcutaneous tissue[157] in individuals with cervical spinal lesions. However, the importance of these reflexes in maintaining blood pressure upon postural change remains doubtful since the head-up tilt induced plasma noradrenaline response was almost completely absent in individuals with a cervical spinal lesion.[113]

During head-up tilt, plasma renin levels increase more in cervical spinal cord-injured individuals than in controls or in individuals with a thoracic spinal lesion.[113, 114] The renin release seem to be independent of sympathetic stimulation and is probably dependent on the fall in renal perfusion pressure upon head-up tilt.[159] The subsequent production of angiotensin II is likely to play a role in the head-up tilt induced vasoconstriction in spinal cord-injured individuals, in particular in individuals with cervical spinal lesions, which is supported by the observation that administration of captopril, which prevents angiotensin II formation, considerably enhances postural hypotension. (unpublished results by Mathias *et al* [159])

Local mechanisms, like the (sympathetic) venoarteriolar reflex and the myogenic response has been proposed to contribute to the leg vasoconstriction in SCI individuals in response to head-up tilt.[36, 144, 160, 161]

Aim and outline of the thesis

Regulation of skeletal muscle vascular tone in humans relies on systemic and local regulatory mechanisms. Systemic control consists of both sympathetic and humoral control, in particular the renin-angiotensin-aldosterone system. Myogenic regulation, which is an intrinsic mechanism of the vascular smooth muscle, and vasoactive substances derived by the endothelium, are local regulatory mechanisms. Apart from the sympathetic nervous system, which is the dominant determinant of baseline vascular tone and the increase in vascular tone upon postural stress, endothelium derived factors, like nitric oxide (NO), are important modulators of baseline vascular tone.

Individuals with a complete spinal cord injury lack sympathetic baroreflex control of the leg vascular bed. However, despite sympathetic denervation in the paralyzed legs, (1) leg vascular tone is almost twice as high as compared with able-bodied subjects, and (2) SCI individuals show head-up tilt induced increase in leg vascular tone to withstand orthostatic stress. The

aim of the present thesis is to elucidate the role of the sympathetic nervous system, and endothelial derived substances, in particular NO, in the regulation of baseline vascular tone in SCI individuals and able-bodied subjects. Furthermore, we tried to unravel the regulatory mechanisms behind the preserved head-up tilt induced vasoconstriction in SCI individuals. The studies described in this thesis are aimed to improve our understanding of the (patho) physiologic regulation of vascular tone in order to provide tools to prevent secondary complications, like pressure sores and atherosclerosis, in SCI individuals.

Chapter 2 focuses on the contribution of the sympathetic nervous system to baseline leg vascular tone in SCI individuals and controls. We hypothesized that blockade of the α-adrenergic receptor in the leg vascular bed of SCI individuals, with central loss of sympathetic control of the leg vascular bed, affects baseline vascular tone to a lesser extent than in able-bodied subjects

In *Chapter 3*, the effect of deconditioning on baseline endothelial NO production was investigated. The hypothesis was that the contribution of NO to baseline vascular tone is reduced in deconditioned skeletal muscle. Therefore, the purpose of this study was to assess the effect of extreme, long-term deconditioning, by means of a spinal cord injury, and moderate, short-term deconditioning by unilateral lower limb suspension, on the contribution of NO to baseline vascular tone in human leg skeletal muscle vascular bed.

Flow-mediated dilatation (FMD) is a valuable and non-invasive tool to evaluate conduit artery endothelial function in humans. Decreased endothelial function, characterized by a reduced bioavailability of nitric oxide (NO), has been proposed as an important early event in the pathogenesis of atherosclerosis, since NO is an important anti-atherogenic molecule. Inactivity is an independent risk factor for atherosclerosis, but surprisingly the FMD in the paralyzed and thus inactive legs of SCI individuals is preserved. Therefore, in *Chapter 4*, we examined whether superficial femoral artery FMD in healthy subjects and in SCI individuals is NO-mediated.

The applicability and reproducibility of venous occlusion plethysmography for blood flow measurements in the calf during head-up tilt at different tilt angles was assessed in *Chapter 5*.

In *Chapter 6*, the role of α-adrenergic receptor stimulation in the leg vasoconstrictor response to orthostatic stress was elucidated in able-bodied subjects, with intact sympathetic baroreflex control of the leg vascular bed, and in spinal cord-injured individuals, who lack baroreflex control of the leg vascular bed. Since α-adrenergic stimulation is the most important determinant of leg vascular tone during head-up tilt in able bodied subjects, we hypothesized that blockade of the α-adrenergic receptor in the leg vascular bed will result in a substantial blunting of the leg vasoconstrictor response to head-up tilt. This response will be larger in subjects with an intact baroreflex than in subjects who fail baroreflex control over the leg vascular bed, and therefore depend on local mechanisms of vasoconstriction.

The different local vasoconstriction mechanisms in SCI individuals were aimed to be unraveled in *Chapter 7*. In SCI individuals

and able-bodied subjects, changes in blood flow were measured in response to both limb dependency and cuff inflation above (forearm) and below (calf) the level of the spinal lesion, without interference of the baroreflex in SCI individuals. We hypothesized that these local mechanisms contribute more to vasoregulation in SCI individuals than in controls.

Methods applied in this thesis

Perfused upper leg model

The technique of leg blood flow measurements in combination with drug infusions into the femoral artery is developed collectively by the department of Physiology and Pharmacology-Toxicology. A cannula (Angiocath 16 gauge, Becton Dickinson, Sandy, Utah, USA) is introduced into the femoral artery of the leg using a modified Seldinger technique. The intra-arterial cannula is used for drug administration and for blood pressure measurement. Local anesthesia (4 ml lidocaine 20 $mg \cdot ml^{-1}$) is used.

Bilateral upper leg blood flow is measured by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges placed approximately 10 cm proximal to the patella. The thigh cuffs are simultaneously inflated to 50 mmHg using a rapid cuff inflator (Hokanson E-20, D.E. Hokanson, Bellevue, Washington, USA).[162] Cuffs below the knee were inflated to suprasystolic levels (> 200 mmHg) in order to occlude the calf circulation. This way the use of high doses of drugs was abolished reducing subsequent systemic effects. To prevent discomfort, infusions were interrupted every ten minutes and the calf circulation restored for 5 minutes.

Ultrasound measurements

Baseline red blood cell velocity and diameter of the superficial femoral artery were measured using an echo Doppler device (Megas, ESAOTE Firenze, Italy) with a 5-7.5 MHz broadband linear array transducer.[33, 37] Increased shear stress in the femoral artery, as induced by reactive hyperemia elicits an endothelium-dependent dilatation (flow-mediated dilatation, FMD) of the superficial femoral artery. For this purpose, a cuff is placed around the upper thigh distal to the imaging site of the superficial femoral artery. The cuff is inflated to a supra-systolic pressure of 220 for 5 minutes to produce local ischemia. After cuff deflation, hyperemic flow velocity in the superficial femoral artery is recorded on videotape for the first 25 seconds, followed by a continuous registration of the vessel diameter for 5 minutes to determine FMD. The reproducibility for the resting measurements in the superficial femoral artery was reported as 1.5% for diameter, and 14% for blood flow. The reproducibility for the relative FMD change was 15%.[37]

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Abstract

Background – Supraspinal sympathetic control of leg vascular tone is lost in spinal cord-injured individuals but this does not result in a reduced leg vascular tone: leg vascular resistance is even increased. The aim of this study is to assess the α-adrenergic contribution to the increased vascular tone in the lower extremity in patients without central sympathetic control of leg circulation.

Methods – Upper leg vascular resistance responses to local infusion of incremental doses of phentolamine (a competitive antagonist of the α-adrenoceptor) into the femoral artery were determined in 10 spinal cord-injured individuals (SCI) and 8 healthy age-matched controls during local β-adrenergic receptor blockade with propranolol.

Results – Basal leg vascular resistance was higher in SCI than in controls (41 ± 6 AU versus 24 ± 4 AU; P=0.034). The same accounts for minimal leg vascular resistance, assessed during reactive hyperemia, which was higher in SCI compared with controls (6.9 ± 1.0 AU versus 2.5 ± 0.2 AU; P<0.01). The maximal phentolamine-induced reduction in leg vascular resistance normalized to each individual's minimal resistance did not differ between the groups (68 ± 17% and 51 ± 4% for SCI and controls, respectively; P>0.1). A decline in mean arterial pressure was observed in both groups with increasing dosage of phentolamine. In response, baroreceptor-mediated vasoconstriction was observed in the non-infused leg of the controls, whereas in SCI individuals this reaction was absent.

Conclusion – These results indicate that the α-adrenoceptor-mediated vascular tone in the leg is preserved in spinal cord-injured individuals without sympathetic supraspinal control.

Chapter 2

Preserved α-adrenergic tone in the leg vascular bed of spinal cord-injured individuals

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Circulation 2003;108(19):2361-2367

Introduction

In healthy humans, the sympathetic nervous system contributes importantly to basal vascular tone as assessed by pharmacological blockade of the α-adrenoceptor[1-3] or acute denervation in sympathectomized patients.[4] In spinal cord-injured individuals the supraspinal sympathetic control of leg vascular tone is lost. One would expect a low leg vascular tone and orthostatic intolerance in these individuals. However, the opposite holds true: leg vascular resistance is increased in spinal cord-injured individuals[5, 6] and orthostatic tolerance seems not to be affected.[7]

In both animal[8-10] and human research,[4] sympathectomy results in a dramatic increase in blood flow to the denervated part of the body. However, this effect is short lasting and presympathectomy levels of blood flow are reached within days in animals[11] to a few months in humans.[12-14] The underlying mechanism for this biphasic response has not yet been clarified. Apart from partial denervation or reinnervation of sympathetic nerves,[12] circulating catecholamines, other systems regulating vascular resistance such as endothelial factors,[12, 13, 15, 16] the renin angiotensin system,[17, 18] or denervation-induced upregulation of α-adrenoceptors[19, 20] may substitute for the reduced sympathetic input to vascular tone. In spinal cord-injured individuals, the situation is even more complex since spinal sympathetic reflexes may still be intact. Furthermore, loss of supraspinal control of somatic efferents results in extreme deconditioning of the leg muscles which is associated with reduced oxygen demand and subsequently with profound functional and structural vascular adaptations.[5, 6] Indeed, leg vascular resistance in spinal cord-injured individuals is even increased in comparison with control subjects, despite the loss of supraspinal sympathetic control.[21]

The purpose of this study was to assess the contribution of α-adrenoceptor-mediated vasoconstriction to the vascular tone in the lower extremity of individuals with central loss of sympathetic control of the leg circulation due to a spinal cord injury. We hypothesize that the sympathetic contribution to basal vascular tone in spinal cord-injured individuals is reduced in comparison to control subjects.

Methods

Subjects

Eight healthy controls (C), seven men and one woman, and ten spinal cord-injured individuals (SCI), eight men and two women, participated in the study. Baseline characteristics of the SCI and controls are shown in Table 1 and Table 2. The SCI continued their medication throughout the study. The control group was similar with respect to age, gender and smoking habits. The spinal cord-injured individuals had complete motor and sensor spinal cord lesions of traumatic origin varying from Thoracic 4 – Thoracic 12 (American Spinal Injury Association ASIA A). The level of the spinal lesion was assessed by clinical examination. After the completion of the study, eight SCI (six men and two women) were willing to return to the laboratory to

assess the completeness of the sympathetic lesion by use of a cold pressor test of the hand. For interpretation of the cold pressor test a separate control group was assembled with similar age and gender profile (n=9; age: 39 ± 2 years; gender profile: seven men and two women).

The study was approved by the Hospital Ethics Committee. All subjects gave their written informed consent prior to the study.

Experimental procedures and protocol

All subjects refrained from caffeine, alcohol and nicotine for at least twelve hours and did not eat three hours prior to testing. All subjects had emptied their bladder in the hour before the test to minimize the influence of any reflex sympathetic activation on peripheral vascular tone.

All tests were performed in the afternoon with the subjects in supine position in a quiet temperature-controlled room (22°C-24°C). Each subject was studied on two different occasions, separated by at least three days. On the first day maximal upper leg blood flow was determined after a 12-minute arterial occlusion period[22] (non-invasive study day). On the second day phentolamine, a non-selective competitive antagonist of α-adrenergic receptors, was infused into the femoral artery (invasive study day). On both study days bilateral upper leg blood flow was measured by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D.E. Hokanson, Washington D.C., USA). Strain gauges were placed 10 cm above the patella. The thigh collecting cuffs (12 cm width) were simultaneously inflated using a rapid cuff inflator (Hokanson E-20) to a pressure of 50 mmHg during 8 heart cycles, with a 10-heart cycles interval between the venous occlusions. The lower legs were supported approximately 10 cm above

Table 1. Specific characteristics of spinal cord-injured individuals.

Subject	Age (yr)	Sex	Level of spinal lesion	Time since injury (years)	Sweating under lesion	Smoking	Medication
1	38	M	T12	16	+	-	-
2	34	M	T4	5	-	-	-
3	34	M	T4	5	-	-	tolterodine bid 2 mg baclofen tid 10 mg
4 (excluded)	31	F	T10	8	-	-	-
5	33	M	T6	10	-	-	-
6	49	M	T6	17	-	+	nitrofurantoïne
7	40	M	T4	18	-	-	-
8	45	M	T5	13	+	-	levothyroxine
9	41	M	T5	23	+	-	-
10	41	F	T12	9	+	+	baclofen tid 40 mg

heart level, to facilitate venous outflow between the venous occlusions.[23] At least one minute before upper leg blood flow measurements were performed, the calf circulation was occluded by inflating cuffs below the knee to suprasystolic values (> 200 mmHg). Calf circulation was excluded from the experimental preparation by a suprasystolic cuff in order to avoid the use of high dosages with subsequent systemic effects of these drugs (see below). In nine controls, a pilot experiment was performed using an identical experimental set-up except for the intra-arterial cannulation. Upper leg vascular resistance appeared stable over time and was not affected by the repeated arterial occlusions (data not shown).

Non-invasive study days

Reactive Hyperemia

Maximal upper leg blood flow using venous occlusion plethysmography was determined after a 12-minute arterial occlusion period.[22] A cuff around the left thigh was inflated to > 200 mmHg. Flow measurements were immediately commenced after cuff pressure release. In accordance with previous studies[24, 25] the highest value of upper leg blood flow was obtained within 30 seconds after cuff release (by the first or second measurement). Simultaneously, arterial blood pressure measurements were performed continuously, at the left third finger using Finapress (Finapress, Ohmeda 2300, BP Monitor BOC Health Care), in order to calculate minimal vascular resistance.

Cold pressor test

In order to determine the completeness of the sympathetic lesion, the hand was immersed into water of 4°C during three minutes. Calf blood flow was measured using venous occlusion plethysmography by placing a strain gauge around the thickest part of the calf at baseline and during the three minutes cold pressor test. Arterial pressure was continuously monitored using Finapress. Calf vascular resistance was calculated by dividing mean arterial pressure by calf blood flow. The mean of the three highest values of calf vascular resistance during the cold pressor test was used to calculate the percent change in vascular resistance compared with the mean vascular resistance of the baseline.

Invasive study day

Using a modified Seldinger technique, an intra-arterial cannula (Angiocath 16 gauge, Becton Dickinson, Sandy, Utah, USA) was introduced into the femoral artery of the left leg at the level of the inguinal ligament for blood pressure measurement (Hewlett Packard monitor type 78353B, Hewlett Packard GmbH, Böblingen, Germany), and for intra-arterial administration of drugs by an automatic syringe infusion pump (Type STC-521, Terumo Corporation, Tokyo, Japan). In SCI individuals, the femoral artery was cannulated without anesthesia because of the lack of sensibility under the level of the spinal cord injury. In two SCI subjects, we failed to cannulate the left femoral artery so we had to use the right femoral artery. In controls, the left femoral artery was cannulated after local anesthesia (0.4 ml lidocaine 20 mg·ml^{-1}). Heart rate

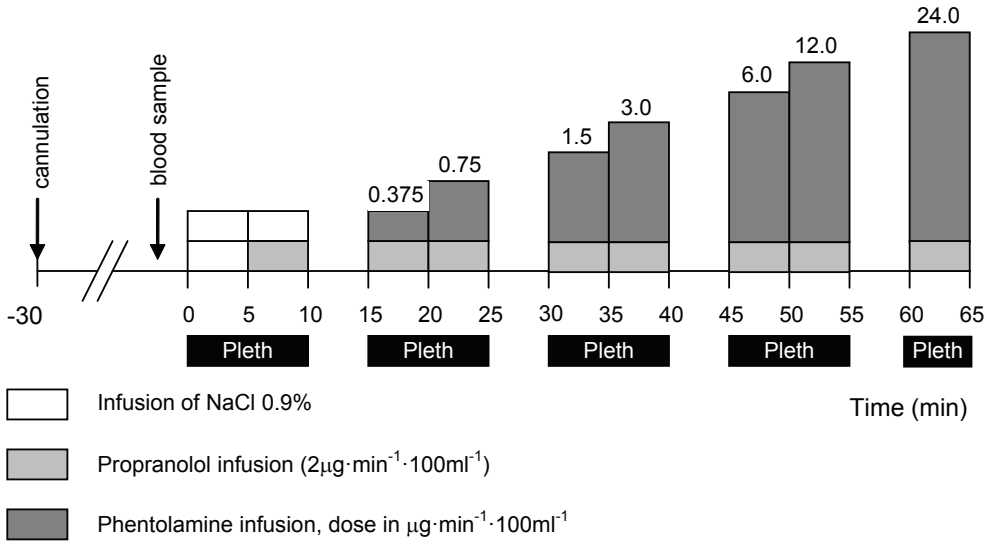

Figure 1. Schematic representation of the experimental protocol. Knee cuffs for arterial occlusion were only inflated during venous occlusion plethysmography (Pleth).

was recorded from the electrocardiogram. The schedule of the protocol is shown in Figure 1. After complete instrumentation and at least 30 minutes after cannulation of the femoral artery an arterial blood sample was taken to determine norepinephrine and epinephrine concentration. First, baseline leg blood flow was measured, during a 5-minute NaCl 0.9% infusion period, followed by infusion of propranolol during 5 minutes in a dose of 2 μg·min^{-1} per 100ml of upper leg volume. Upper leg volume was determined by anthropometry as described and validated by Jones et al.[26] Subsequently, phentolamine was infused at incremental doses of 0.375–0.75–1.5–3.0–6.0–12.0–24.0 μg·min^{-1}·100ml^{-1} of upper leg volume. Previous studies showed complete α-adrenergic blockade in the leg of healthy volunteers at a dose of 12 μg·min^{-1}·100ml^{-1}.[2] Each dose was given for 5 minutes. During the entire phentolamine infusion, propranolol was coinfused at a dose of 2 μg·min^{-1}·100ml^{-1} to prevent unopposed β-adrenergic vasodilatation during α-adrenergic blockade. Every ten minutes infusions were interrupted and the circulation to the calf was restored by deflating the arterial occlusion cuffs for five minutes to prevent discomfort. At the end of the highest dose of phentolamine and propranolol, isoproterenol (15 ng·min^{-1}·100ml^{-1}) was co-infused, in the controls only, to verify β-adrenoceptor blockade. The vasodilator action of this dose of isoproterenol was confirmed in one experiment with a healthy volunteer. In addition, in this pilot study the used propranolol dose appeared to abolish the vasodilator action of isoproterenol, confirming adequate β-adrenoceptor blockade. During the whole protocol,

infusion rate was kept constant at a rate of 20 µl·100ml^{-1}·min^{-1}.

Drugs and solutions

Phentolamine (Regitine®, 10 mg·ml^{-1}, Novartis Pharma BV, Arnhem, The Netherlands), propranolol (Inderal®, 1 mg·ml^{-1}, Zeneca Farma BV, Ridderkerk, The Netherlands), and isoproterenol (Isoprenaline sulfaat, 1 mg·ml^{-1}, Fresenius Kabi Nederland BV, The Netherlands) were dissolved in NaCl 0.9% at the beginning of each experiment.

Data analysis

Upper leg blood flow in ml·min^{-1}·100ml^{-1} was calculated as the slope of the volume change curve. Because of a cuff inflation artifact during the first second, the slope from 2 – 6 seconds after cuff inflation was used. Upper leg blood flow values of the last 2 minutes of each infusion were averaged to calculate upper leg blood flow.
Blood pressure significantly changed during the course of the intra-arterial phentolamine infusions. Therefore, upper leg vascular resistance was calculated as mean arterial pressure in mmHg divided by upper leg blood flow in ml·min^{-1}·100ml^{-1} and expressed in arbitrary units (AU=mmHg·min·100ml·ml^{-1}). For these calculations, we assumed that central venous pressure was low and remained constant throughout the protocol.
Since structural differences in the vascular bed of SCI individuals could non-specifically affect the vasodilator response to phentolamine, the individual responses in vascular resistance to infusion of phentolamine were normalized to each subject's minimal vascular resistance as assessed during reactive hyperemia. The maximal response (E_{max}) for each individual was determined using the maximal observed response to infusion of phentolamine.

Statistics

Results are expressed as mean ± SEM. Differences in baseline characteristics and in the maximal effect of phentolamine (E_{max}) between the groups were tested using the Mann-Whitney-U-test. The effect of propranolol and differences between the last two doses of phentolamine were tested with the Wilcoxon-test. Between group differences in response to infusion of phentolamine were analyzed using two factor repeated measures ANOVA with the phentolamine dose as within subject factor and the presence of a spinal cord lesion as between subject factor (Statistical Package for Social Sciences (SPSS) version 10.0). The null hypothesis was formulated, as there were no differences between the groups in response to intra-arterial infusion of phentolamine in upper leg vascular resistance.
A two-sided p-value of less than 0.05 was considered to be statistically significant.

Results

Confirmation of the lack of supraspinal sympathetic control

Individual responses in calf vascular resistance of 8 spinal cord-injured individuals (SCI) and 9 controls are shown in Figure 2. The increase in calf vascular resistance was significantly less in SCI than in controls.

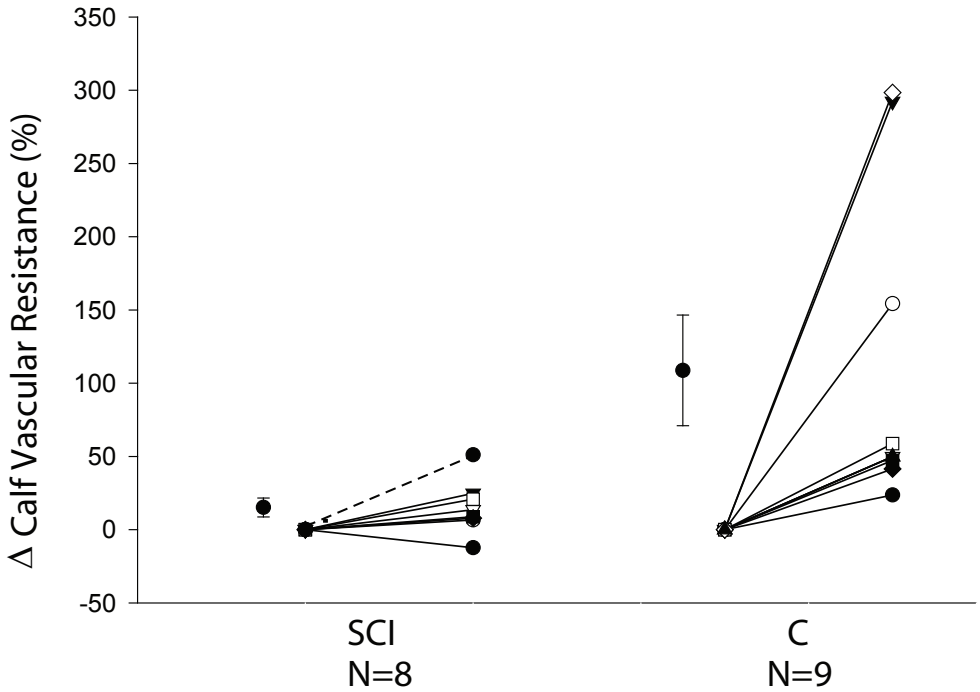

Figure 2. Individual responses of relative calf vascular resistance to the cold pressor test of the hand. Subject 8 and subject 9 did not participate in this part of the experiment. The dotted line represents subject 4, who is excluded from the analysis.

In one spinal cord-injured individual (subject 4, T10 spinal lesion), an obvious increase in vascular resistance was observed during the cold pressor test. For this reason, we excluded this individual from the analysis. Exclusion of this subject did not have any effect on overall results.

Two spinal cord-injured individuals (subject 8 and subject 9) could not participate in the cold pressor test. However, they both had complete spinal lesions at level Thoracic 5, which makes an intact sympathetic innervation of the legs unlikely.[27]

Baseline characteristics

The two groups did not differ with respect to age, systolic blood pressure, diastolic blood pressure, mean arterial pressure, heart rate, norepinephrine and epinephrine serum concentration. Body weight and thigh volume were significantly lower in SCI than in controls.

Baseline upper leg blood flow was significantly lower and leg vascular resistance was significantly higher in SCI as compared with controls.

Maximal leg blood flow was lower and minimal leg vascular resistance were higher in SCI than in controls (Table 2).

Table 2. Baseline characteristics. (Mean ± SEM)

	SCI (N=9)	C (N=8)	p-value
Age (yr)	39 ± 2	36 ± 3	0.36
Body mass (kg)	71 ± 4	81 ± 4	0.13
Thigh volume (L)	4.9 ± 0.2	7.6 ± 0.4	<0.001
Systolic blood pressure (mmHg)	125 ± 5	132 ± 5	0.18
Diastolic blood pressure (mmHg)	73 ± 4	68 ± 2	0.50
Mean arterial pressure (mmHg)	92 ± 3	90 ± 2	0.63
Heart rate (min^{-1})	67 ± 4	60 ± 4	0.44
Upper leg blood flow ($ml \cdot min^{-1} \cdot 100ml^{-1}$)	2.7 ± 0.5	4.3 ± 0.6	0.027
Upper leg vascular resistance (AU)	41 ± 6	24 ± 4	0.034
Maximal upper leg flow ($ml \cdot min^{-1} \cdot 100ml^{-1}$)	14 ± 2	32 ± 3	0.001
Minimal upper leg vascular resistance (AU)	6.9 ± 1.0	2.5 ± 0.2	0.001
Epinephrine (nmol/l)	0.10 ± 0.01	0.29 ± 0.09	0.17
Norepinephrine (nmol/l)	1.51 ± 0.26	1.34 ± 0.20	0.91

Response to drug infusions in the infused leg

Propranolol infusion alone did not affect any of the hemodynamic parameters (Table 3). Isoproterenol, co-infused with phentolamine and propranolol at the end of the protocol in the control group, did not further change upper leg vascular resistance (15 ± 4 AU to 15 ± 5).

Upper leg blood flow increased significantly and upper leg vascular resistance decreased significantly in both groups in response to phentolamine infusion. No difference in upper leg vascular resistance was observed between the doses 12 and 24 $\mu g \cdot min^{-1} \cdot 100ml^{-1}$ of phentolamine in both groups, indicating that in both groups a maximal effect of phentolamine was observed (P>0.1 for both groups; see Table 3).

The increase in upper leg blood flow in response to infusion of phentolamine was significantly less in SCI compared with controls (P<0.001 for the effect of dose in both groups, P<0.05 for the between group effect; see Table 3). However, the relative decrease in vascular resistance did not significantly differ in response to phentolamine infusion between the SCI and controls in the infused leg (P>0.1 for the effect of group and the dose by group interaction; see Figure 3a). The maximal responses in vascular resistance, expressed as percent change of baseline, normalized to each individual's minimal resistance (E_{max}) did not significantly differ between the two groups (68 ± 17% and 51 ± 4% for the SCI group and controls, respectively; P>0.1; see Figure 4).

A significant, and dose-dependent, decline

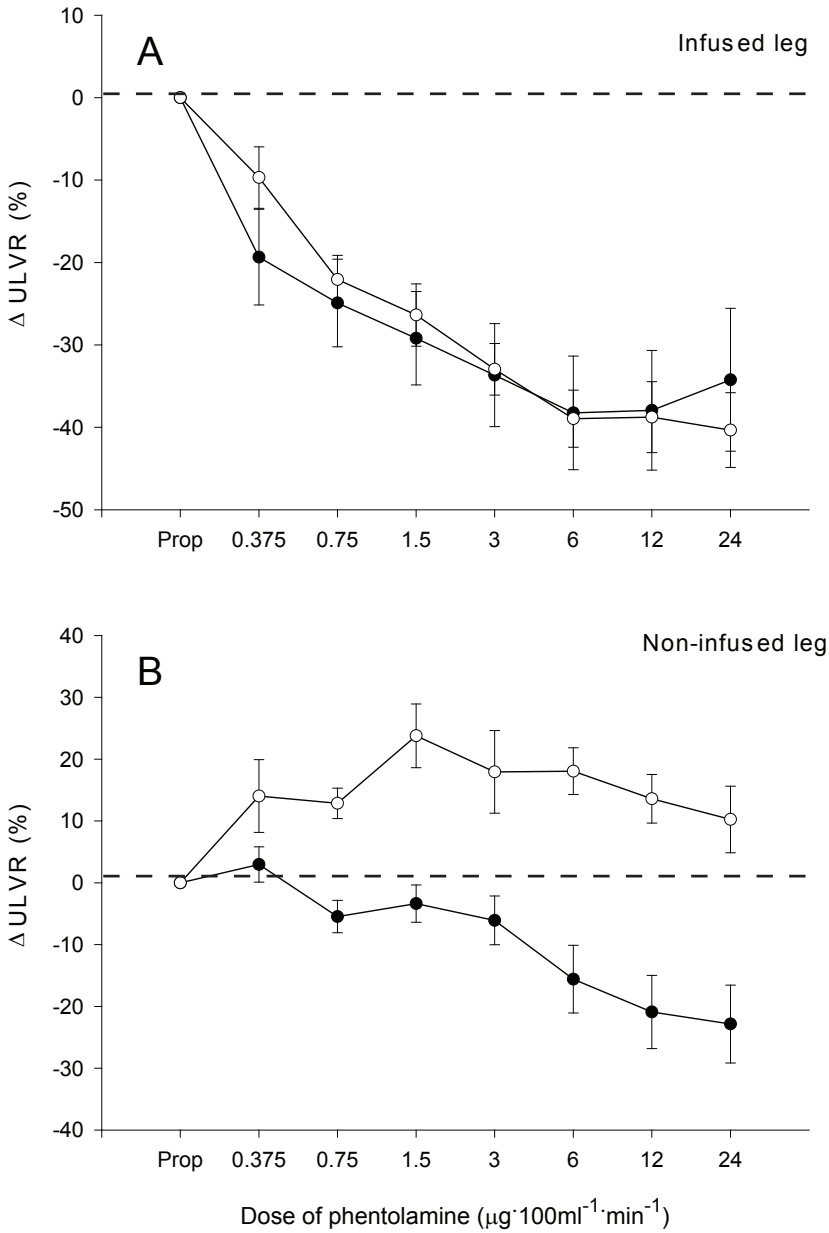

Figure 3. Mean percent change (± SEM) in upper leg vascular resistance (ULVR) ± SEM in response to infusion of incremental doses of phentolamine during local β-adrenergic blockade in the infused leg (A) and the non-infused leg (B). No difference in percent change between the groups was observed in the infused leg. ULVR of the non-infused leg in the controls increased during the first doses, which was different from the spinal cord-injured individuals, where ULVR decreased throughout the whole protocol ($P<0.01$ for the effect of group). Prop = propranolol infusion; ● = Spinal cord-injured individuals; ○ = Controls

Table 3. Effect of increasing dose of phentolamine on blood pressure, heart rate, and vascular tone of the infused leg in SCI and controls.

	SCI subjects (N=9)				Controls (N=8)			
Drug	**ULBF**	**MAP**	**ULVR**	**HR**	**ULBF**	**MAP**	**ULVR**	**HR**
NaCl 0.9%	2.7 ± 0.5	92 ± 3	41 ± 6	66 ± 4	4.3 ± 0.6	90 ± 2	24 ± 4	60 ± 4
Prop	2.8 ± 0.5	90 ± 3	40 ± 6	67 ± 4	4.2 ± 0.6	93 ± 3	24 ± 3	61 ± 4
Phe 0.375	3.5 ± 0.6	90 ± 3	31 ± 4	64 ± 4	4.7 ± 0.6	91 ± 3	21 ± 3	63 ± 5
Phe 0.75	3.7 ± 0.6	89 ± 3	29 ± 4	65 ± 4	5.4 ± 0.6	91 ± 2	19 ± 2	61 ± 4
Phe 1.5	4.0 ± 0.6	90 ± 3	27 ± 4	63 ± 3	5.6 ± 0.6	90 ± 2	18 ± 3	58 ± 4
Phe 3.0	4.2 ± 0.6	87 ± 3	25 ± 3	65 ± 3	6.1 ± 0.7	89 ± 2	16 ± 2	59 ± 4
Phe 6.0	4.4 ± 0.6	87 ± 3	23 ± 3	65 ± 3	6.7 ± 0.8	88 ± 2	15 ± 2	60 ± 4
Phe 12.0	4.3 ± 0.6	85 ± 3	23 ± 4	66 ± 3	6.8 ± 0.9	88 ± 2	15 ± 2	61 ± 4
Phe 24.0	4.2 ± 0.6	85 ± 3	24 ± 3	66 ± 3	6.8 ± 0.8	87 ± 2	14 ± 2	60 ± 4
	P<0.05	P<0.05	P<0.05	NS	P<0.05	P<0.05	P<0.05	NS

ULBF: upper leg blood flow (ml·min^{-1}·100ml^{-1}); MAP: mean arterial pressure (mmHg); ULVR: upper leg vascular resistance (AU); HR: heart rate (beats/min); Prop: propranolol in a dose of 2 µg·min^{-1}·100ml^{-1}; Phe: phentolamine (dose in µg·min^{-1}·100ml^{-1}). P-values indicate the level of significance for the effect of phentolamine dose on baseline values during propranolol infusion (repeated measures ANOVA)

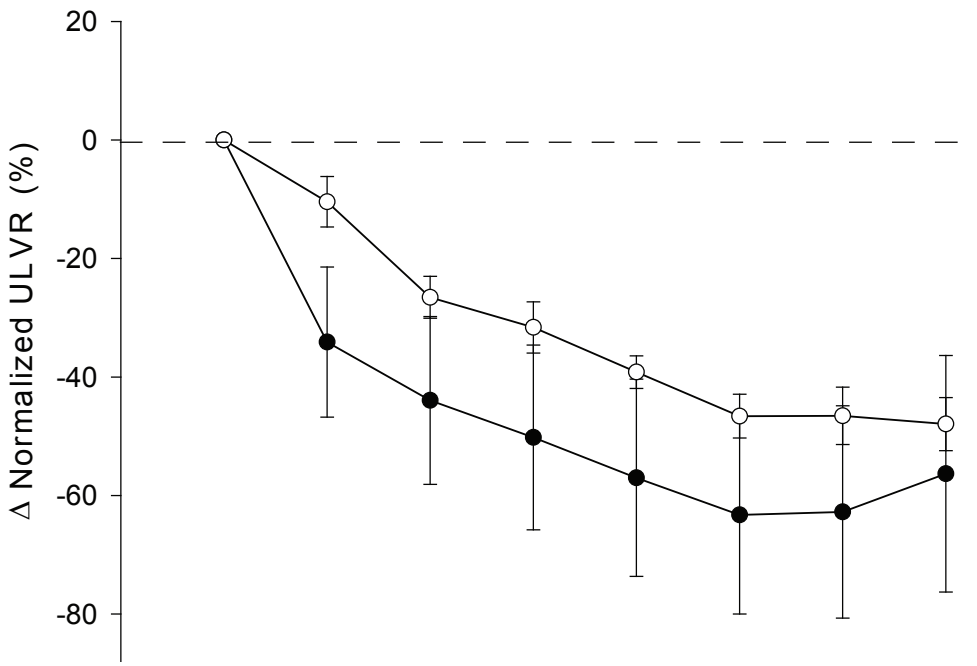

Figure 4. Mean percent change (± SEM) of upper leg vascular resistance (ULVR) ± SEM in the infused leg, normalized to each individual's minimal vascular resistance. Prop = propranolol infusion; ● = Spinal cord-injured individuals; ○ = Controls

in mean arterial pressure (MAP) was observed in both groups ($P<0.01$ for the effect of phentolamine dose; see Table 3). Heart rate (HR) did not change in either group during the protocol.

Response to drug infusions in the non-infused leg

In the non-infused leg, the controls showed a maximal increase in upper leg vascular resistance of 23.8 ± 5.1% at a dose of 1.5 μg·min^{-1}·100ml^{-1}, which decreased slightly to 10.2 ± 5.4% at a dose of 24 μg·100ml^{-1}·min^{-1}. In contrast, in the SCI group upper leg vascular resistance decreased with 22.8 ± 6.2 % throughout the incremental doses of phentolamine. ($P<0.01$ for the effect of group; see Figure 3b).

Discussion

The major observation of the present study is that α-adrenoceptor blockade in the legs of spinal cord-injured individuals decreases the vascular resistance by 68% when normalized to each individual's maximal vasodilator capacity. The decrease in vascular resistance is of the same magnitude as in controls. To our knowledge, this is the first observation that α-adrenergic tone of the leg skeletal muscle vascular bed is preserved in individuals without supraspinal sympathetic control.

Upper leg blood flow is lower and upper leg vascular resistance is higher in SCI than in controls. This is in accordance with previous observations in an independent sample of SCI and controls, using a different technique to measure leg blood flow.[21] As previously discussed,[21] this is most likely due to structural (a decrease in number of arterioles and capillaries and/or a decrease in diameter of the resistance vessels) as well as functional changes. Indeed, previous research,[6, 25] and the present observation that maximal vasodilatation during post-ischemic hyperemia is reduced in SCI, supports the concept that structural changes in SCI contribute to the reduced baseline leg blood flow.

Phentolamine-induced upper leg vasodilatation in SCI, regardless the method of expressing the results (relative or normalized), indicates preserved α-adrenergic vascular tone in these individuals. The absolute upper leg blood flow in response to infusion of phentolamine was less in SCI as compared with controls. This is due to structural changes in the leg vascular bed of SCI since this apparent difference disappeared when results were normalized to the individual maximal vasodilator capacity of the upper leg vascular bed. In controls, the vasodilator response to phentolamine was very similar to the response observed by others in a similar group of volunteers.[2] Therefore, methodological problems are not likely to explain the lack of difference between the groups in vasodilator response to phentolamine in the present study.

Since upper leg blood flow was lower in SCI than in controls, the same dose of phentolamine resulted in higher local plasma concentration of phentolamine in the infused leg in SCI compared with controls. However, in both groups, a maximal vasodilator response to phentolamine was achieved. This is supported by the observed

plateau in upper leg vascular resistance in response to infusion of the two highest doses of phentolamine. Therefore, possible differences in plasma phentolamine concentration (these concentrations were not measured) cannot explain the similarity between the two groups in their maximal response in vascular resistance to phentolamine infusion.

In addition, in both groups, blood pressure decreased during the course of the phentolamine infusions to a similar extent. This reflects a systemic effect of local vasodilatation in a large vascular bed (infused leg). During the course of the experiment, a spill of phentolamine into the systemic circulation must have occurred, as reflected by a decrease in upper leg vascular resistance in the non-infused leg in SCI. The drop in blood pressure will engage the baroreflex, resulting in an increase in sympathetic tone to the heart and peripheral circulation. In theory, the baroreflex-mediated increase in sympathetic tone could have limited the vasodilator response to phentolamine in the infused leg of the controls. However, this is very unlikely, since a possible reflex vasoconstriction in the infused leg was prevented by the local infusion of phentolamine.

The lack of a baroreflex-mediated increase in heart rate in both groups of volunteers is explained by spill of propranolol into the systemic circulation. Pilot studies clearly showed an increase in heart rate during infusion of phentolamine without simultaneous infusion of propranolol (data not shown). In the present study, subsequent blockade of cardiac β-adrenergic receptors by propranolol prevented the baroreflex-mediated increase in heart rate.

The results observed in this study reject our hypothesis that α-adrenergic tone is reduced in SCI as compared to controls. However, the elevated upper leg vascular resistance in spinal cord-injured individuals cannot be explained completely by the preserved α-adrenergic tone since in both SCI as well as controls the contribution of α-adrenergic stimulation to the leg vascular tone is of the same magnitude. In theory, several mechanisms may be responsible for the preserved α-adrenergic contribution in basal vascular tone in SCI. First, in the studied subjects, the spinal cord injury may have been incomplete or supraspinal control of sympathetic outflow to the leg may have been restored. This explanation is excluded for several reasons. In five of the SCI, sweating was disturbed under the level of the lesion, indicating loss of autonomic control of the skin. Furthermore, the lack of a baroreflex-mediated vasoconstriction in the non-infused leg in SCI as opposed to the controls, and the lack of vasoconstriction in the calf during the cold pressor test, indicates a lack of supraspinal control of sympathetic outflow to the leg vasculature in SCI. A second reason for the preserved α-adrenergic tone in SCI may be related to the spinal sympathetic reflex and the local venoarteriolar reflex. A previous study reported that both spinal sympathetic reflex activity and the venoarteriolar reflex may contribute to leg muscle vasoconstriction during tilt in tetraplegic subjects,[28] but very low sympathetic activity in cutaneous and muscle postganglionic axons situated below the level of the spinal cord injury was measured in basal supine position.[29, 30] The venoarteriolar reflex is elicited by

an increase in venous transmural pressure of more than 25 mmHg[31] and may be mediated by α-adrenoceptors,[32] which has not been confirmed by others.[33] Thus, although spinal reflexes and venoarteriolar reflex do not likely explain the preserved α-adrenergic tone, their role cannot completely be excluded.

Finally, α-adrenergic receptor hypersensitivity to circulating catecholamines, the serum concentration of which was similar in both groups, could have preserved the α-adrenergic influence on basal vascular tone. Reduced local release of norepinephrine may have induced a compensatory increase in either the sensitivity or number of α-adrenergic receptors or the efficacy of post-receptor signaling.[19, 20, 34] From the presenting data, it is not possible to assess α-adrenergic sensitivity since an α-adrenergic antagonist was used, which affinity to the α-receptor is different from the affinity of α-adrenoceptor agonists, like norepinephrine. Further research should address the question of altered α-adrenoreceptor sensitivity in SCI.

A limitation of this study is that we only used α-adrenergic blockade to assess the contribution of the sympathetic nervous system in the control of basal vascular tone below the level of spinal cord injury. Other neurotransmitters, like adenosine triphosphate and neuropeptide Y, are released simultaneously with norepinephrine by the sympathetic nerve endings and cause vasoconstriction by activating P_{2x} receptors and Y_1 receptors, respectively.[35] Due to this limitation, we may have underestimated the contribution of the sympathetic nervous system in the regulation of basal vascular tone in controls and in individuals with a spinal cord lesion.

In conclusion, α-adrenergic contribution to basal vascular tone is preserved below the level of the lesion in spinal cord-injured individuals, and is of the same magnitude as in controls. As such, the sympathetic nervous system is still a target for pharmacological intervention to improve the perfusion of the leg in SCI. However, our results do not completely explain the elevated leg vascular resistance in SCI since the contribution of the sympathetic nervous system to leg vascular tone in SCI is of the same magnitude as in controls. Therefore, other factors, like for example impaired endothelium-mediated vasodilatation, could be responsible for the observed elevation of leg vascular tone in SCI and should be subject of investigation to further unravel the mechanisms behind the observed increase in leg vascular resistance in SCI.

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Abstract

Background – Deconditioning is a risk factor for cardiovascular disease. Exercise reduces this risk, possibly by improving the vascular endothelial nitric oxide (NO) pathway. The effect of deconditioning on the NO pathway is largely unknown. This study was designed to assess baseline NO availability in the leg vascular bed after extreme, long-term deconditioning (spinal cord-injured individuals, SCI) as well as after moderate, short-term deconditioning (4 weeks of unilateral lower limb suspension, ULLS).

Methods – For this purpose, seven SCI were compared with 7 matched controls. Additionally, 7 healthy subjects were studied Pre- and Post-ULLS. Leg blood flow was measured by venous occlusion plethysmography at baseline and during infusion of 5 incremental dosages of N^G-monomethyl-L-arginine (L-NMMA) into the femoral artery. Sodium nitroprusside (SNP) was infused to test vascular responsiveness to NO.

Results – Baseline leg vascular resistance tended to be higher in SCI compared with controls (37 ± 4 versus 31 ± 2 AU, P=0.06). Deconditioning did neither alter the vasoconstrictor response to L-NMMA (increase in resistance in SCI versus controls: 102 ± 33% versus 69 ± 9%; Pre- versus Post-ULLS: 95 ± 18% versus 119 ± 15%), nor the vascular responsiveness to NO.

Conclusion – Two human *in vivo* models of deconditioning show a preserved baseline NO availability in the leg skeletal muscle vascular bed.

Chapter 3

Preserved contribution of nitric oxide to baseline vascular tone in deconditioned human skeletal muscle

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Introduction

The endothelium plays a crucial role in the regulation of vascular function and structure. Among the various mediators released by the endothelium, nitric oxide (NO) may be considered the most important vasodilator substance. The importance of the NO pathway is demonstrated by the strong link between endothelial dysfunction and cardiovascular disease.[1] Various risk factors for cardiovascular disease, such as arterial hypertension, diabetes, smoking and hypercholesterolemia, are associated with defects in the NO pathway.[1]

Reduced baseline NO production has been demonstrated in patients with hypertension and their offspring,[2] in smokers,[3] and in chronic heart failure patients.[4] Exercise training enhances baseline NO production in healthy subjects,[5] in patients with hypercholesterolemia,[6] and in chronic heart failure patients.[7] As such, the positive effect of exercise on these conditions may be partly explained by augmentation of baseline NO availability.

A sedentary lifestyle is an independent risk factor for atherosclerosis and cardiovascular disease.[8] Baseline blood flow to the deconditioned skeletal muscles is reduced.[9-11] In response to a reduction in blood flow, baseline NO synthesis may be attenuated, which triggers vascular remodelling and atherosclerosis.[12, 13] Accordingly, Kamiya *et al* [9] showed a decrease in plasma nitrite/nitrate concentration, an indicator of endogenous NO production, after head-down bed rest, suggesting a diminished release of endothelial NO. Therefore, we hypothesize that the contribution of NO to baseline vascular tone is reduced in deconditioned skeletal muscle. We tested this hypothesis in two human *in vivo* models of inactivity. The first model concerns spinal cord-injured individuals (SCI). SCI offer a unique model of nature to assess peripheral vascular adaptations to inactivity. In these individuals, the part of the body below the level of the lesion is subject to extreme and long-term deconditioning. Extensive vascular adaptations, including a reduction in baseline blood flow, occur in the leg vascular bed in SCI.[11, 14] However, the legs of SCI are not only subject to extreme deconditioning, but also to denervation. Therefore, a second model was used: unilateral lower limb suspension (ULLS). This model of deconditioning is less extreme and limited in duration, but is not confounded by denervation. The ULLS model[15] is based on the avoidance of all weight bearing activities of one leg, while the subject uses crutches for locomotion. The ULLS model induces muscle atrophy and a decrease in muscle strength.[15-18]

The purpose of this study was to assess the effect of extreme, long-term (SCI) and moderate short-term (ULLS) deconditioning on the contribution of NO to baseline vascular tone in the human leg skeletal muscle vascular bed. To address this issue increasing dosages of N^G-monomethyl-L-arginine (L-NMMA, a blocker of NO-synthase) and sodium nitroprusside (SNP, a NO donor) were infused into the femoral artery in spinal cord-injured individuals and matched controls, and in subjects before and after 4 weeks of ULLS.

Methods

Subjects

In total, 19 subjects participated and underwent the same tests to assess the contribution of NO to baseline vascular tone. In a first study, 7 male SCI, with extreme, long-term deconditioning, were compared with 7 controls, matched for gender and age. In a second study, 7 healthy subjects (3 males, 4 females) were measured twice, once before and once 4 weeks after short-term deconditioning by ULLS. Two of these subjects also served as controls for the SCI.

SCI suffered from a complete motor and sensor spinal cord lesion of traumatic origin varying from Cervical 5 – Thoracic 12 (American Spinal Injury Association ASIA A). The level of the spinal lesion was assessed by clinical examination. One of the SCI used baclofen (10 mg daily) throughout the study. Three females in the ULLS study used oral contraceptives. During their Pre-ULLS and Post-ULLS measurements, all females were in the same phase of their menstrual or contraceptive pill cycle.

All subjects met the inclusion criteria: age 18-50 years; non-smokers; diastolic blood pressure below 90 mmHg; normal fasting glucose, cholesterol, and triglyceride values. Thus, individuals with the most important risk factors that could affect endothelial function, in particular baseline NO production, were excluded from the study. Baseline characteristics are shown in Table 1. In the ULLS study, subjects exercised 2.1 ± 0.7 hours per week. Exercise in the SCI (4.9 ± 0.8 hours per week) consisted of voluntary arm exercise. This type of upper body exercise is limited by the amount of active muscle mass[19] and does not affect the leg vasculature.[20] The Hospital Ethics Committee approved the study. All subjects gave their written informed consent prior to the study. The study conforms with the principles outlined in the Declaration of Helsinki.

Experimental procedures

Leg blood flow measurement

All subjects fasted overnight, and refrained from caffeine and alcohol for 24 hours. All subjects emptied their bladder before testing to minimize the influence of reflex sympathetic activation on vascular tone. All tests were performed in the morning with the subjects in supine position in a quiet temperature-controlled room (23-24°C).

The technique of leg blood flow measurements in combination with drug infusions into the femoral artery has been described previously.[14] A cannula (Angiocath 16 gauge, Becton Dickinson, Sandy, Utah, USA) was introduced into the femoral artery of the leg using a modified Seldinger technique. The intra-arterial cannula was used for drug administration and for blood pressure measurement. Heart rate was derived from the electrocardiogram. In control and ULLS subjects, local anaesthesia (4 ml lidocaine 20 mg/ml) was applied. Because of the lack of sensibility no anaesthesia was used in SCI.

Bilateral upper leg blood flow was measured by electrocardiography-triggered venous occlusion plethysmography, using mercury-in-silastic strain gauges placed approximately

10 cm proximal to the patella. We have previously shown that the reproducibility of upper leg blood flow measurements is good.[21] The thigh cuffs were simultaneously inflated to 50 mmHg using a rapid cuff inflator (Hokanson E-20, D.E. Hokanson, Bellevue, Washington, USA).[22] Cuffs below the knee were inflated to suprasystolic levels (> 200 mmHg) in order to occlude the calf circulation. This way, the use of high doses of drugs, with subsequent systemic effects, could be minimized. To prevent discomfort, infusions were interrupted every ten minutes and the calf circulation was restored for 5 minutes.

Drug infusion protocol

The drug infusion protocol is represented at the bottom of Figure 1. The measurements started at least 30 minutes after cannulation of the femoral artery. Each drug dose was administered for 5 minutes. First, baseline leg blood flow was measured during saline (NaCl 0.9%) infusion. Subsequently, L-NMMA was infused into the femoral artery at incremental doses of 0.025–0.05–0.1–0.2–0.4 mg/min per decilitre (dl) of upper leg volume. In a pilot study with doses up to 0.8 mg/min/dl maximal vasoconstriction was already achieved at 0.2 and 0.4 mg/min/dl. Subsequently, glucose 5% was infused followed by infusion of increasing dosages of the NO donor sodium nitroprusside (SNP, 0.06–0.2–0.6 μg/min/dl). Sodium nitroprusside was infused to explore differences in smooth muscle sensitivity to exogenous NO. A one-hour washout period was scheduled before angiotensin II infusion. Angiotensin II (0.25–0.5–2.0 ng/min/dl) served as a control vasoconstrictor to detect differences in vasoconstrictor capacity due to structural vascular changes induced by deconditioning. During the whole protocol, infusion rate was kept constant at a volume rate of 10 μl/min/dl.

Drugs and solutions

N^G-monomethyl-L-arginineandangiotensin II (both from Clinalfa, Läuflingen, Switzerland) were dissolved in saline at the beginning of each experiment. Sodium nitroprusside (department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre) was dissolved in glucose 5% and protected against light.

Upper leg volume measurement

Upper leg volume was determined by anthropometry as described by Jones *et al.*[23]

ULLS protocol

In seven subjects, the right leg was exposed to deconditioning induced by 4 weeks of ULLS. We used a ULLS model very similar to the original description by Berg.[15] The right leg was suspended by attachment of a sling to a non-rigid ankle brace and to a harness on the upper body and unloaded from all weight bearing. Sole elevation of the contra-lateral foot was not used, because it produced instability of the leg. The harness was used during all locomotor activity, and the subjects used crutches for walking. Instructions were provided to minimize muscle activity of the suspended leg. Compliance was monitored with a diary, weekly interviews, and leg skin temperature. Skin temperature was consistently 1-2ºC

lower in the unloaded thigh and calf versus the control leg.

Strength measurement

In order to test the effectiveness of ULLS to induce deconditioning, the strength of the quadriceps muscle was quantified. Maximum voluntary contraction (Newton) of the quadriceps muscle of both legs was assessed with an isometric quadriceps dynamometer[24] before and after 4 weeks of ULLS. The hips and knees were positioned at 90º and 60º of flexion, respectively. The highest obtained result of three consecutive measurements represented the maximum voluntary contraction. This method has an acceptable reproducibility with a coefficient of variance of 10.2%.

Data analysis

Upper leg blood flow in ml/min/dl was calculated as the slope of the plethysmographic volume curve, as described previously.[14] Leg blood flow values of the final 2 minutes of each 5-minute infusion period were averaged.

Blood pressure significantly changed during the course of the experiment. Therefore, upper leg vascular resistance (LVR) was calculated as mean arterial pressure (MAP, in mmHg) divided by leg blood flow in ml/min/dl and expressed in arbitrary units (AU=mmHg·min/ml/dl). For these calculations, we assumed that central venous pressure was low and remained constant throughout the protocol.

We used the first baseline measurement (during saline infusion) to calculate the percentage change in outcome parameters during infusion of L-NMMA and SNP. Glucose 5% and SNP were infused immediately after infusion of L-NMMA. The NO donor SNP directly affects the vascular smooth muscle cell and overrules the effect of L-NMMA, so the effect of L-NMMA during infusion of SNP can be ignored. To calculate the percentage change during angiotensin II infusion, the baseline measurement directly prior to angiotensin II infusion was used. In order to control for changes in vasoconstrictor capacity by deconditioning, the vasoconstrictor response to L-NMMA was normalized to the average vasoconstrictor response to angiotensin II.

Statistics

Results represent means ± SEM. Differences in baseline characteristics between SCI and controls were tested using the Mann-Whitney-U-test. Changes in strength, leg volume, and body mass after ULLS were tested with Wilcoxon signed rank test. For SCI and matched controls, differences in the response to infusion of drugs were analyzed using two-way repeated measures ANOVA with the drug dose as within subject factor and the presence of a spinal cord lesion as between group factor. For ULLS, a two factor repeated measures ANOVA was used with the drug dose and Pre- or Post-ULLS as within subject factors. Differences in response between drug doses were tested with the least significant difference post-hoc test (Statistical Package for Social Sciences (SPSS) 11.0). Differences were considered to be statistically significant at a two-sided P-value of less than 0.05.

Results

Effects of extreme, long-term deconditioning (SCI) on baseline parameters

SCI and matched controls did not differ with respect to age and blood pressure. Related to their spinal cord lesion, thigh volume and body mass were lower in SCI compared with controls (Table 1). Baseline leg blood flow tended to be lower (2.8 ± 0.5 versus 3.3 ± 0.2 ml/min/dl, P=0.064) and LVR tended to be higher (37 ± 4 versus 31 ± 2 AU, P=0.064) in SCI compared with controls.

Effects of moderate, short-term deconditioning (ULLS) on baseline parameters

Body mass and thigh volume did not change after ULLS (Pre versus Post: 71.8 ± 4.6 versus 72.1 ± 4.6 kg, and 6.7 ± 0.5 versus 6.7 ± 0.5 litre, respectively). After 4 weeks of ULLS, strength of the quadriceps muscle decreased by 22.2 ± 8.3% (P<0.05). Strength of the control leg quadriceps muscle did not change (-1.8 ± 14.0%). ULLS did neither alter baseline leg blood flow, nor LVR (Pre versus Post: 3.9 ± 0.7 versus 4.5 ± 0.9 ml/min/dl and 26 ± 3 versus 23 ± 3 AU, respectively).

Response to L-NMMA

Leg vascular resistance (LVR) increased significantly in all groups during L-NMMA infusion (P<0.01). The responses of LVR to L-NMMA, expressed as percentage of baseline LVR, were not different between SCI (extreme, long-term deconditioning) and matched controls (Figure 1A). The response of LVR to L-NMMA in SCI was also compared to the data of all 12 controls (including 5 additional control subjects from the Pre-ULLS study),

Table 1. Baseline characteristics.

	Extreme deconditioning		Moderate deconditioning
	SCI (n=7)	Controls (n=7)	ULLS-subjects (n=7)
Age (years)	38 ± 2	32 ± 5	24 ± 2
Body mass (kg)	71.1 ± 5.4	81.4 ± 3.6*	71.8 ± 4.6
Upper leg volume (L)	5.0 ± 0.3	7.1 ± 0.4†	6.7 ± 0.5
Systolic blood pressure (mmHg)	123 ± 6	124 ± 4	114 ± 2
Diastolic blood pressure (mmHg)	76 ± 2	83 ± 3	77 ± 3
Triglycerides (mmol /l)	1.3 ± 0.3‡	1.4 ± 0.3	1.2 ± 0.2
Cholesterol (mmol /l)	4.6 ± 0.4‡	4.5 ± 0.3	4.4 ± 0.2
Glucose (mmol /l)	4.5 ± 0.3	4.7 ± 0.1	4.9 ± 0.1
Exercise (hours per week)	4.9 ± 0.8	3.9 ± 0.8	2.1 ± 0.7

Values represent means ± SEM. * P = 0.047 versus SCI. † P = 0.006 versus SCI. ‡ Data are from 6 subjects.

Table 2. Effect of L-NMMA, SNP and angiotensin II on blood pressure, heart rate, and vascular tone in SCI and controls.

Drug dose	SCI (n=7)					Controls (n=7)				
(dose/ min/dl)	MAP	HR	LBF	LVR		MAP	HR	LBF	LVR	
				Infusion	Control				Infusion	Control
L-NMMA										
Saline	91 ± 4	57 ± 4	2.8 ± 0.5	37 ± 4	34 ± 5	98 ± 4	57 ± 3	3.3 ± 0.2	31 ± 2	33 ± 2
0.025 mg	91 ± 4	58 ± 5	2.4 ± 0.4	43 ± 5	36 ± 4	100 ± 5	56 ± 3	2.6 ± 0.2	40 ± 3	35 ± 2
0.05 mg	94 ± 4	56 ± 5	2.2 ± 0.4	47 ± 5	33 ± 4	99 ± 4	58 ± 3	2.6 ± 0.1	40 ± 3	35 ± 2
0.1 mg	95 ± 5	55 ± 6	1.9 ± 0.3	56 ± 7	36 ± 5	100 ± 4	57 ± 3	2.3 ± 0.2	47 ± 4	33 ± 3
0.2 mg	96 ± 4	53 ± 4	1.8 ± 0.3	71 ± 16	37 ± 6	102 ± 4	55 ± 3	2.2 ± 0.1	49 ± 3	33 ± 3
0.4 mg	101 ± 4	54 ± 4	1.6 ± 0.2	71 ± 11	39 ± 5	105 ± 4	57 ± 3	2.1 ± 0.1	52 ± 3	30 ± 2
	*	*	*	*	†	*	*	*	*	‡
SNP										
Glucose	104 ± 4	53 ± 4	2.0 ± 0.2	54 ± 5	40 ± 7	109 ± 3	55 ± 5	2.9 ± 0.2	39 ± 2	30 ± 3
0.06 µg	105 ± 4	52 ± 5	3.1 ± 0.5	37 ± 4	42 ± 5	106 ± 4	58 ± 4	4.9 ± 0.6	25 ± 4	34 ± 3
0.2 µg	92 ± 4	56 ± 5	4.4 ± 0.8	25 ± 3	35 ± 5	101 ± 5	58 ± 4	5.3 ± 0.6	21 ± 3	39 ± 6
0.6 µg	83 ± 4	64 ± 5	5.5 ± 1.0	18 ± 2	33 ± 7	96 ± 5	67 ± 5	7.7 ± 0.9	15 ± 3	35 ± 6
	*	*	*	*		*	*	*	*	
ANG II										
Saline	103 ± 4	56 ± 5	2.2 ± 0.4	56 ± 12	43 ± 7	105 ± 4	60 ± 5	3.1 ± 0.4	37 ± 5	34 ± 3
0.25 ng	106 ± 3	56 ± 5	1.7 ± 0.3	80 ± 18	46 ± 9	109 ± 5	61 ± 6	2.4 ± 0.3	51 ± 6	34 ± 3
0.5 ng	106 ± 4	55 ± 5	1.5 ± 0.3	105 ± 32	55 ± 20	109 ± 5	63 ± 6	2.3 ± 0.2	53 ± 6	33 ± 3
2.0 ng	112 ± 5	57 ± 6	1.4 ± 0.3	124 ± 36	55 ± 17	112 ± 5	62 ± 5	1.8 ± 0.2	73 ± 12	32 ± 4
	*		*	*		*		*	*	

Values represent means ± SEM. MAP, mean arterial pressure (mmHg); HR, heart rate (beats/min); LBF, leg blood flow (ml/min/dl); LVR, leg vascular resistance (AU); Infusion, the infused leg; Control, the non-infused leg. Ang II, angiotensin II. significant dose effect for both groups (P<0.05, two-way-ANOVA). † trend to significant interaction for dose x group (P=0.069). ‡ significant dose effect (P<0.05, one-way-ANOVA).

but again no difference in response was detected. Likewise, moderate short-term deconditioning (ULLS) did not affect the response of LVR to infusion of L-NMMA (Figure 1B). All these observations were similar when the absolute instead of the relative (%) changes in the LVR were analyzed. After normalizing the response to L-NMMA to the individual mean response to angiotensin II (for data of angiotensin II response see next paragraph), again no differences were observed between SCI and controls, nor between Pre- and Post-ULLS.

Table 3. Effect of L-NMMA, SNP and angiotensin II on blood pressure, heart rate, and vascular tone Pre- and Post-ULLS

Drug dose	Pre-ULLS (n=7)					Post-ULLS (n=7)				
(dose/ min/dl)	MAP	HR	LBF	LVR		MAP	HR	LBF	LVR	
				Infusion	Control				Infusion	Control
L-NMMA										
Saline	90 ± 2	62 ± 3	3.9 ± 0.7	26 ± 3	29 ± 4	90 ± 2	67 ± 4	4.5 ± 0.9	23 ± 3	26 ± 3
0.025 mg	92 ± 2	62 ± 3	3.3 ± 0.6	32 ± 4	30 ± 4	91 ± 2	67 ± 4	4.2 ± 0.9	27 ± 4	28 ± 4
0.05 mg	92 ± 2	60 ± 3	2.8 ± 0.4	37 ± 4	30 ± 4	93 ± 2	65 ± 3	3.3 ± 0.7	34 ± 5	30 ± 5
0.1 mg	94 ± 2	63 ± 3	2.5 ± 0.3	41 ± 4	28 ± 4	95 ± 3	66 ± 4	2.8 ± 0.5	40 ± 6	29 ± 4
0.2 mg	97 ± 2	60 ± 3	2.2 ± 0.2	46 ± 4	30 ± 4	96 ± 2	63 ± 4	2.6 ± 0.4	43 ± 6	30 ± 5
0.4 mg	99 ± 2	60 ± 3	2.1 ± 0.2	49 ± 4	28 ± 3	99 ± 3	64 ± 3	2.3 ± 0.3	51 ± 7	29 ± 5
	*	*	*	*		*	*	*	*	
SNP										
Glucose	101 ± 2	61 ± 4	3.0 ± 0.3	36 ± 3	27 ± 4	98 ± 2	59 ± 3	2.9 ± 0.4	37 ± 4	28 ± 4
0.06 µg	100 ± 1	62 ± 4	5.7 ± 0.9	21 ± 3	28 ± 4	98 ± 2	65 ± 4	5.3 ± 0.9	22 ± 3	29 ± 4
0.2 µg	95 ± 2	62 ± 3	6.3 ± 1.4	19 ± 3	30 ± 3	93 ± 2	66 ± 4	6.4 ± 1.5	18 ± 3	31 ± 4
0.6 µg	90 ± 1	72 ± 4	8.0 ± 1.5	14 ± 3	34 ± 5	90 ± 3	72 ± 3	8.3 ± 1.7	13 ± 2	35 ± 6
	*	*	*	*	*	*	*	*	*	*
ANG II										
Saline	97 ± 2	65 ± 4	3.5 ± 0.6	32 ± 4	31 ± 4	97 ± 2	66 ± 5	4.4 ± 1.1	31 ± 6	32 ± 7
0.25 ng	101 ± 2	67 ± 3	2.9 ± 0.5	41 ± 5	28 ± 5	101 ± 3	71 ± 4	3.5 ± 0.8	40 ± 8	29 ± 6
0.5 ng	100 ± 2	66 ± 4	2.6 ± 0.4	44 ± 5	26 ± 4	99 ± 2	69 ± 4	2.9 ± 0.8	46 ± 8	29 ± 6
2.0 ng	106 ± 3	68 ± 4	2.1 ± 0.2	55 ± 5	27 ± 5	105 ± 2	72 ± 5	2.0 ± 0.4	63 ± 10	28 ± 6
	*		*	*	*	*		*	*	*

Values represent means ± SEM. MAP, mean arterial pressure (mmHg); HR, heart rate (beats/min); LBF, leg blood flow (ml/min/dl); LVR, leg vascular resistance (AU); Infusion, the infused leg; Control, the non-infused leg. Ang II, angiotensin II.

* significant dose effect for both groups (P<0.05, two-way-ANOVA).

In SCI, the matched controls and in the Pre-ULLS-tests, the maximal vasoconstrictor response to L-NMMA was already achieved at the dose of 0.2 mg/min/dl. As a consequence, doubling of the dose to 0.4 mg/min/dl did not induce a further vasoconstrictor response, indicating that maximal NO inhibition was achieved. In the Post-ULLS-tests, LVR increased further at the dose of 0.4 mg/min/dl (P=0.031). However, pilot studies in healthy volunteers showed that increasing the L-NMMA-dose to 0.8 mg/min/dl did not further increase LVR (LVR 46 ± 4, 45 ± 5, and 48 ± 4 AU during 0.2, 0.4 and 0.8 mg L-NMMA/min/dl, respectively, n=3).

L-NMMA induced a significant and dose-dependent increase in MAP (P<0.001), which did not significantly differ between SCI and controls, and between Pre- and Post-ULLS tests (Table 2 and Table 3). SCI and their controls and the ULLS-subjects showed a decrease in HR during L-NMMA infusion (Table 2 and 3).

Response to SNP

Neither the absolute nor the relative (%) response of LVR to SNP differed between SCI and controls (Table 2, Figure 1A). This also holds for the Pre- versus Post-ULLS response to SNP (Table 3, Figure 1B). MAP decreased in all groups during the higher dosages of SNP (P<0.001, Table 2 and 3).

Response to angiotensin II

The absolute and relative (%) responses of LVR to angiotensin II (Table 2, Figure 1) were not different in SCI compared with controls. The absolute responses of LVR to angiotensin II (Table 3) was not different in Pre- versus Post-ULLS. In all groups, MAP increased significantly during the higher dosages of angiotensin II (P<0.001, Table 2 and 3). This increase in MAP was not different in SCI versus controls nor in Pre-ULLS versus Post-ULLS. HR did not change significantly in either group.

Adverse effect of ULLS

Originally 8 subjects participated in the ULLS-protocol. One subject developed a deep venous thrombosis of the suspended leg during ULLS and was excluded from the study. We have reported separately on this serious adverse effect of ULLS and have proposed precautionary measures.[25]

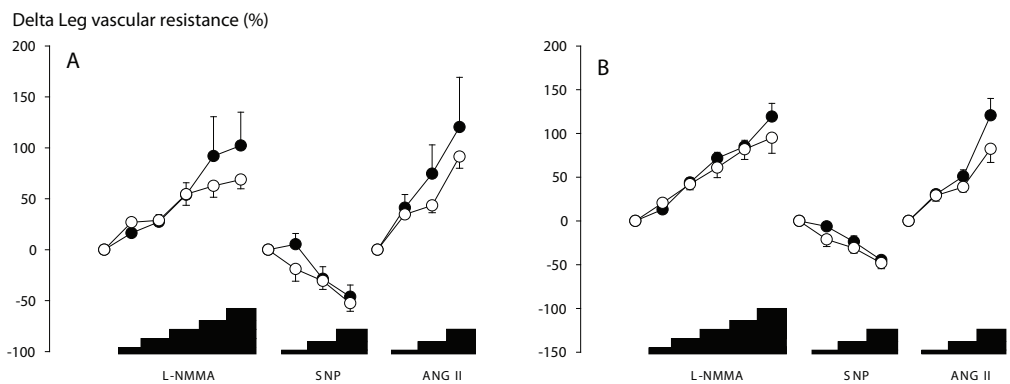

Figure 1. Change in leg vascular resistance in response to L-NMMA (0.025-0.05-0.1–0.2–0.4 mg/min/dl of leg tissue), SNP (0.06–0.2–0.6 µg/min/dl), and angiotensin II (Ang II 0.25–0.5–2.0 ng/min/dl). **A** Spinal cord-injured individuals versus matched controls (closed and open circles, respectively). **B** Healthy subjects before and after ULLS (open and closed circles, respectively). Data represent means and SEM.

Discussion

The present study showed that, in contrast to our hypothesis, the contribution of NO to baseline vascular tone is not affected by deconditioning of human skeletal muscle. This is remarkable since exercise increases the contribution of NO to baseline vascular tone.[5] Therefore, effects of deconditioning are not the inverse of effects of exercise training. Preserved contribution of NO to baseline vascular tone was confirmed in both extreme, long-term (SCI) and moderate, short-term (ULLS) deconditioning. Infusion of L-NMMA into the femoral artery increased basal LVR to a similar extent in SCI versus matched controls, as well as in Pre- versus Post-ULLS. Second, the response to the NO donor sodium nitroprusside was similar in SCI versus controls, and in Pre- versus Post-ULLS, indicating equal smooth muscle responsiveness to NO. Third, the vasoconstrictor response to angiotensin II did not differ between SCI and controls or between Pre- and Post-ULLS, indicating that there were no non-specific changes in vasoconstrictive capacity in both models. The latter observation is further strengthened by the fact that the response to L-NMMA normalized to the individual mean angiotensin II response did not differ in SCI versus controls, nor in Pre- versus Post-ULLS.

Deconditioning below the spinal cord lesion is an important consequence in SCI. In the SCI subjects in this study, leg volume was lower, indicating muscle atrophy, leg blood flow tended to be lower and LVR tended to be higher compared with controls. This is in accordance with previous observations of a significantly higher leg vascular resistance in SCI as compared with controls.[26] While in our study, ULLS did not lower baseline leg blood flow, as assessed by plethysmography, we are confident that deconditioning did occur. Using echo Doppler ultrasound, we demonstrated that the diameter of the common and superficial femoral artery significantly decreased after ULLS in the same group of subjects, while overall blood flow in these arteries was unchanged.[27] This corresponds with ultrasound data on diameter and blood flow in SCI.[28] Changes in diameter without alterations in baseline blood flow have also been shown in training studies, where an increase in femoral artery diameter occurred without changes in baseline blood flow.[29] Finally, since there was a significant decrease of 22% in strength of the quadriceps muscle, the ULLS model evidently caused deconditioning of the leg. This decrease in maximum voluntary contraction closely agrees with previous reports of a 13-21% reduction after 10 days to 6 weeks of unloading, which was invariably accompanied by muscular atrophy as assessed by MRI or CT measurements.[16-18, 30, 31]

We assessed the contribution of NO to baseline vascular tone by quantifying the vasoconstrictor response to L-NMMA. Fundamental to this technique is achievement of maximal inhibition of NO-synthase. In our SCI, their matched controls, and in the Pre-ULLS-tests, LVR did not further increase during the final L-NMMA dose, which indicates that a maximal vasoconstrictor effect was achieved. This maximal vasoconstrictor

response to L-NMMA (L-NMMA caused a 35-40% decrease in leg blood flow in the controls of the SCI-study and before ULLS) is very similar to a 31% decrease in forearm blood flow when L-NMMA is infused into the brachial artery of healthy subjects.[32] So, despite the dependent position of the leg to the heart during standing, the contribution of NO to baseline vascular tone seems comparable in the vascular beds of the leg and arm. The maximal dose of L-NMMA used in previous studies was comparable to our 0.1 mg/min/dl dose.[5, 33] If, in analogy with these studies, we limited the analysis to the lower three doses, our results and conclusion did not change. After ULLS, the vasoconstrictor response to the final L-NMMA dose (0.4 mg/min/dl) was higher than with the previous dose (0.2 mg/min/dl). Although this may indicate that maximal vasoconstriction was not achieved, data from a pilot study showed that the higher dose of 0.8 mg/min/dl of L-NMMA did not cause further vasoconstriction in the leg of healthy volunteers. Finally, if the vasoconstrictor response to L-NMMA was not maximal in the Post-ULLS test, then this would point towards augmented NO-mediated effects by moderate deconditoning. Augmented NO-mediated effects by deconditioning would be a strong argument against our hypothesis.

The results of the present study indicate that short-term and long-term deconditioning of skeletal muscle does not reduce the contribution of NO to baseline vascular tone in humans. Therefore, the observed increase in vascular resistance after deconditioning[9, 10, 26] cannot be explained by a reduced role of NO in baseline vascular tone. Training, i.e. the opposite of deconditioning, caused an increased basal NO production in the forearm vascular bed of both healthy individuals[5] and hypercholesterolemic patients,[6, 34] and an increase in nitrite-nitrate level after 8 weeks of cycle training.[35] Since these studies suggest that exercise can improve baseline NO availability in humans, the results of the present study were unexpected. Data on the effects of long-term training (months-years) on endothelial function are limited. However, baseline NO production was similar in endurance trained athletes and controls.[36] It has been proposed that changes in endothelial function represent short-term adaptations to training and are eventually replaced by structural or other adaptations.[37] This is illustrated by the observation that the endothelial function in forearm vessels of long-term tennis players was similar in the dominant and non-dominant arm.[38] One should take notice that these subjects played tennis for 13 hours per week and that this may have resulted in concomitant training of the non-dominant arm.

Data on the effects of deconditioning on the NO pathway are scarce. In agreement with our results, deconditioning induced by casting for arm fractures, does not change the vasoconstrictor response to infusion of L-NMMA in the forearm in a cross-sectional study after cast removal.[33] Our data provide additional information, since they are derived from both a cross-sectional and a longitudinal intervention study, and are not biased by the effects of trauma and fracture healing on

baseline blood flow. Plasma nitrite-nitrate concentration decreased in one bed rest study,[9] suggesting an impaired endothelial NO production, while in another bed rest study, no changes in urinary nitrite-nitrate excretion occurred.[39] As compared with the nitrite-nitrate method, which reflects total body NO metabolism, our approach more specifically quantifies the role of NO in baseline vascular tone after deconditioning. Previous animal studies have mimicked physical inactivity by hindlimb unloading and report conflicting results. Some studies demonstrate a decreased eNOS expression in the endothelial cells of both conductance and resistance vessels, and an attenuated maximal vasodilatation to acetylcholine suggesting a decrease of endothelial NO release by hindlimb unloading.[40-42] In contrast, another study demonstrated no alterations of endothelial NOS expression, but an increase in aortic inducible NOS content following hindlimb unloading.[43]
After reduction of blood flow in animals by partial ligation of conduit arteries, baseline NO synthesis is reduced, which may trigger arterial remodelling and atherosclerosis.[12, 13] However, the present study suggests that a reduction in blood flow or increase in vascular tone in humans is not explained by a diminished endogenous NO release under baseline conditions. Although, in the present study baseline NO synthesis is not affected, the NO release upon different stimuli may still play a role in arterial remodelling and in the vulnerability to atherosclerosis. Nevertheless, application of a stimulus is an artificial setting, since it is highly unlikely that the leg vascular bed of SCI is exposed to triggers that increase NO release. In this regard, it is interesting to realize that flow-mediated dilatation of the superficial femoral artery, based on endothelial NO release in response to a shear stress stimulus, is preserved and seems to be enhanced in the chronically inactive legs of SCI[28] and after 4 weeks of ULLS.[27] However, these data are derived from a conduit artery in response to a shear stress stimulus and cannot predict the response to acetylcholine or comparable drugs on the arteriolar level.[44] Moreover, in animals the short-term and long-term effects of exercise training on the NO pathway differ between conduit and resistance arteries.[45]

In the SCI and the ULLS model, the physical inactivity is most prominent in the part of the body below the spinal cord lesion and in the suspended leg, respectively. One might argue that increased activity of the upper body and of the non-suspended leg could have systemic effects on endothelial function, masking the effect of inactivity on endothelial NO release. However, in the ULLS subjects the physical activity score tended to be lower after ULLS arguing against a training effect of walking with crutches. It has been debated whether the effects of training on endothelial function are localized or systemic in nature. Leg training causes changes in baseline NO production[5] and improves endothelial function[46] in the arms. However, evidence exists that changes in endothelial function occur predominantly locally in the exercised limbs.[47-49] In SCI, neither systemic cardiovascular effects[50] nor local vascular effects of deconditioning[20] can be normalized with upper body training.

Recently, Thijssen *et al* [51] demonstrated that the adaptation to leg exercise is a local phenomenon and only occurs in the stimulated leg muscles. Collectively these data provide evidence that upper arm exercise and even exercise of adjacent muscle by electrical stimulation does not affect the vasculature in the non-exercised leg muscles in SCI. Based on the previous arguments we believe that our results are not confounded by systemic effects of increased upper body physical activity.

In the present study, we used spinal cord injury as a unique model of nature to investigate adaptations in the peripheral circulation in response to extreme deconditioning. As valuable as information is from this patient population, one should be cautious to extrapolate this information to the general population, since other conditions unique to spinal cord injury can influence the results, such as impaired supraspinal sympathetic control. However, recent human studies have shown that endothelial function does not change after chronic sympathectomy.[52] In addition, adaptations in the circulatory system in SCI are reversible by functional electrostimulation training,[26, 53] providing more evidence that these adaptations primarily result from deconditioning. We used ULLS as a second model of deconditioning. Since healthy subjects participated in this part of the study, loss of supraspinal control was not a confounder. The observations on the NO pathway in the ULLS-studies are in close agreement with the observations in the SCI-study.

In conclusion, the results of the present study demonstrate a preserved contribution of NO to baseline vascular tone in deconditioned leg skeletal muscle in man.

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Chapter 4

Flow-mediated dilatation in the superficial femoral artery is nitric oxide mediated in humans

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Abstract

Background – Flow-mediated dilatation (FMD) of the brachial and radial arteries is an important research tool for assessment of endothelial function *in vivo*, and is nitric oxide (NO)-dependent. The leg skeletal muscle vascular bed is an important territory for studies in exercise physiology. However, the role of endothelial NO in the FMD response of lower limb arteries has never been investigated. The purpose of this study was to examine the contribution of NO to FMD in the superficial femoral artery in healthy subjects. Since physical inactivity may affect endothelial function, and therefore NO availability, spinal cord-injured (SCI) individuals were included as a model of extreme deconditioning.

Methods – In 8 healthy men (34 ± 13 years) and 6 SCI individuals (37 ± 10 years), the 5-minute FMD response in the superficial femoral artery was assessed by echo-Doppler, both during infusion of saline and during infusion of the NO-synthase-blocker N^G-monomethyl-L-arginine (L-NMMA). In a subset of the controls (n=6), the 10-minute FMD response was also examined using the same procedure.

Results – The 5-minute FMD response in controls (4.2 ± 0.3%) was significantly diminished during L-NMMA infusion (1.0 ± 0.2%, P<0.001). In SCI, L-NMMA also significantly decreased the FMD response (from 8.2 ± 0.4% during saline to 2.4 ± 0.5% during L-NMMA). The hyperaemic flow response during the first 45 seconds after cuff deflation was lower in both groups during infusion of L-NMMA, but the effect of L-NMMA on FMD persisted in both groups after correction for the shear stress stimulus. The 10-minute FMD was not affected by L-NMMA (saline: 5.4 ± 1.6 %, L-NMMA: 5.6 ± 1.5 %).

Conclusion – Superficial femoral artery FMD in response to distal arterial occlusion for a period of 5 minutes is predominantly mediated by NO in healthy men and in the extremely deconditioned legs of SCI individuals.

Introduction

Flow-mediated dilatation (FMD) refers to the vasodilatation observed in a conduit artery in response to elevations in shear stress during reactive hyperaemia. FMD is a valuable non-invasive tool to evaluate endothelial function in humans.[1] It is reduced in patients with risk factors for cardiovascular diseases such as hypercholesterolemia, hypertension, diabetes, and smoking,[2-4] as well as in the elderly.[5, 6] Decreased endothelial function, characterized by a reduced bioactivity of nitric oxide (NO), has been proposed as an important early event in the pathogenesis of atherosclerosis,[7] since NO is an important anti-atherogenic molecule. Indeed, decreased FMD is an independent predictor for cardiovascular morbidity and mortality.[8-11]

It has been shown that the FMD response in the brachial and radial arteries, under specific conditions, is mediated by endothelial NO, since selective blockade of NO production with N^G-monomethyl-L-arginine (L-NMMA) largely abolishes the response.[12-14] NO dependency of FMD in other arteries has not been examined.

The effect of regular aerobic exercise on FMD in healthy men[15-17] and in subjects with cardiovascular disease[18, 19] has been examined primarily in the brachial artery. Since exercise-induced vascular adaptations mainly occur in the trained areas,[20] assessment of lower limb FMD may provide more direct and sensitive information about local vascular endothelial function changes. This is particularly relevant since we know that leg and arm vascular beds respond differently to endothelium-dependent and -independent vasodilator agents.[21] In parallel to this, heterogeneity in the conduit vessels in the upper and lower limbs is present regarding vessel dilatation for a given change in shear rate.[22, 23] Consequently, FMD responses observed in the brachial artery cannot necessarily be extrapolated to the leg vascular bed. Therefore, the aim of this study was to examine whether the FMD observed in the superficial femoral artery in healthy subjects is NO-mediated.

Physical inactivity is a common risk factor for cardiovascular diseases and for atherosclerosis. It has recently been demonstrated that conduit artery function in the extremely inactive legs deteriorates at a greater rate than in the arms of spinal cord-injured (SCI) individuals.[24] To examine the applicability of superficial femoral artery FMD as a marker of NO-mediated vasodilatation in a muscle vascular bed subject to extreme inactivity, SCI individuals were included. Endothelial NO production during reactive hyperaemia was blocked by infusion of L-NMMA into the common femoral artery. We hypothesize that the superficial femoral artery FMD in normal (healthy subjects) as well as in abnormal conditions (SCI individuals) is mediated through endothelial NO production.

Methods

Subjects

Eight healthy male controls (C, 34 ± 4 years, 185 ± 2 cm, 82 ± 7 kg) and 6 male spinal cord-injured individuals (SCI, 37 ± 4 years, 179 ± 1 cm, 72 ± 3 kg) participated in this study.

Subjects with a history of cardiovascular diseases, diabetes, hypercholesterolemia, or hypertension were excluded from the study. Control subjects were non-smokers who used no medication. The SCI subjects continued their medication, which did not interfere with the cardiovascular system, throughout the study. Two spinal cord-injured individuals smoked, but refrained from smoking 3 days prior to testing. The spinal cord-injured individuals had complete motor and sensory spinal cord lesions of traumatic origin varying from Thoracic 1 to 12 (American Spinal Injury Association A). The level of the spinal lesion was assessed by clinical examination. The Hospital Ethics Committee approved the study. All subjects gave their written informed consent prior to the study. The study conformed to the principles outlined in the Declaration of Helsinki.

Protocol

Measurements were carried out in the morning after an overnight fast. Subjects were asked to empty their bladder before examination and they refrained from alcohol, caffeine, vitamin C, and exercise at least 18 hours prior to the test. Room temperature was controlled at 23-24°C.

FMD with NO blockade (5-minute)

A cannula (Angiocath 16 gauge, Becton Dickinson, Sandy, Utah, USA) was introduced into the femoral artery under local anaesthesia (4 ml lidocaine 20 mg/ml) using a modified Seldinger technique. The intra-arterial cannula was used for drug administration and for continuous blood pressure monitoring. Heart rate was also

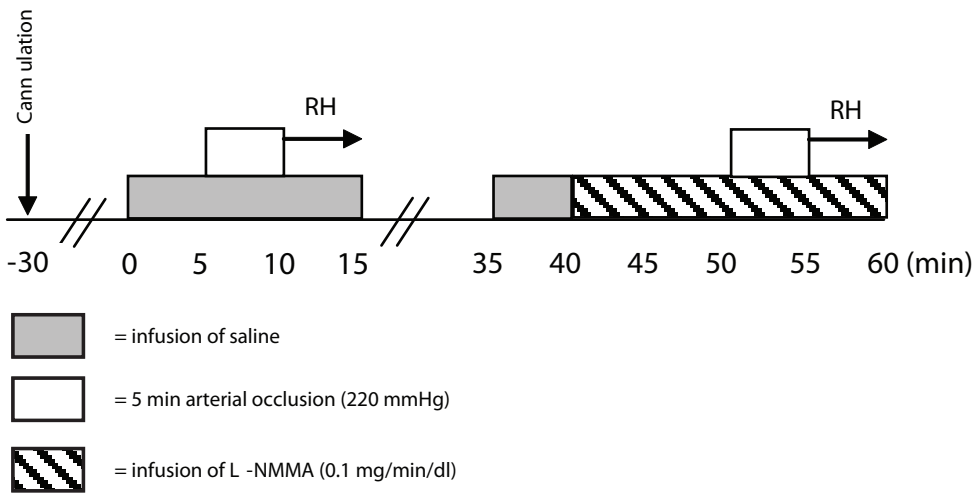

Figure 1. Schematic overview of the invasive study protocol (RH: reactive hyperaemia).

continuously monitored using lead II from an electrocardiogram. The measurements started at least 30 minutes after cannulation of the femoral artery (Figure 1). First, measurements of vessel diameter and red blood cell velocity of the superficial femoral artery (SFA) were performed during saline (NaCl 0.9%) infusion. FMD was assessed after a cuff was inflated around the upper thigh for 5 minutes to suprasystolic pressure of 220 mmHg. The 5-minute occlusion period was based on the current knowledge of NO dependency of the brachial artery FMD,[13] and on the frequent use of this protocol in previous studies examining the lower leg conduit artery FMD.[23-25] The cuff placement was distal to the arterial cannulation site, with the artery insonated between the site of the cannulation and the placement of the cuff. After cuff deflation, hyperaemic blood flow velocity in the SFA was recorded on videotape for the first 45 seconds, followed by registration of the vessel diameter for 5 minutes.[26, 27]

Twenty minutes after cuff release, baseline measurements were repeated during infusion of saline in order to verify that haemodynamic parameters had returned to pre-occlusion levels. Subsequently, L-NMMA was infused into the femoral artery at a dose of 0.1 mg/min per decilitre (dl) of leg volume. Using a similar dose in the forearm, Mullen[13] abolished FMD of the brachial artery. In addition, a previous study that infused this dose into the femoral artery showed minimal systemic effects.[28] Resting echo Doppler measurements of SFA were performed between 8 and 10 minutes during the 10-minute pre-infusion period of L-NMMA. Infusion of L-NMMA was continued during the 5-minute arterial occlusion and 5 minutes after cuff release. During the whole protocol, infusion rate was kept constant at a volume rate of 10 μl/min/dl. In a subgroup of the healthy controls (n=3, 34 ± 9 years), no effect of the infusate was found on blood flow measurement or blood flow of the superficial femoral artery (without infusate: 64 ± 15, with infusate 61 ± 12 ml/min).

FMD with NO blockade (10-minute)

To examine whether the period of arterial occlusion influences the nature of the FMD response in the superficial femoral artery, we repeated the above stated experiments after an arterial occlusion period of 10 minutes. The results of the 5-minute occlusion were compared with the 10-minute arterial occlusion.

Measurements and analyses

Red blood cell velocities and vessel diameter of superficial femoral artery were measured with an echo Doppler device (Megas Esaote, Firenze, Italy) with a 5-7.5 MHz broadband linear array transducer. The sample volume was placed in the centre of the artery and images were made approximately 3 cm distal to the bifurcation into the deep and the superficial femoral artery. The angle of insonation during velocity assessments was consistently below 60 degrees. Arterial diameter measures were assessed from arterial B-mode images where the vessel is parallel to the transducer. For baseline diameter measurements, two consecutive

images in the longitudinal view were frozen at the peak systolic (Ds) and end-diastolic phase (Dd). Off line, 3 measurements were performed per diameter image, and the mean diameter (D) was calculated by using the formula: 1/3 · systolic diameter + 2/3 · diastolic diameter. For baseline velocity measurements, four images with a total of 10-12 velocity profiles were obtained and manually traced afterwards by a single investigator. The average of these 10-12 Doppler spectra waveforms was used to calculate mean velocity (Vmean). Subsequently, mean blood flow in ml/min was calculated as $\pi(\text{radius})^2 \cdot \text{Vmean(cm/s)} \cdot 60$ and mean wall shear rate (MWSR) was calculated as $4 \cdot \text{Vmean/D}(s^{-1})$.

Hyperaemic velocity was recorded on videotape for the first 45 seconds after cuff release. After 45 seconds, superficial femoral artery diameter was recorded continuously until 5 minutes after cuff release for assessment of the FMD. Reactive hyperaemic blood velocity was calculated from the flow velocity integral (FVI) every 5 seconds, from 15 to 45 seconds after cuff release, which was manually traced by a single investigator. Subsequently, mean blood flow in ml/min was calculated as $\pi(\text{radius})^2 \cdot \text{Vmean(cm/s)} \cdot 60$ and mean wall shear rate (MWSR) was calculated as $4 \cdot \text{Vmean/D}\ (s^{-1})$.

Vessel diameters of the SFA after reactive hyperaemia were measured off line from videotape at 50, 60, 90, 120, 240, and 300 seconds after cuff release to measure FMD. All diameters were measured at the end-diastolic phase of the cardiac cycle, corresponding to the R wave of a simultaneous ECG signal. FMD in the SFA was expressed as both the maximal absolute and relative diameter change in end-diastolic baseline diameter. MWSR area-under-the-curve (MWSR-AUC) was calculated from 15-45 seconds after cuff release.[29] To correct for the shear stress stimulus, the ratio between the relative FMD response (%FMD) and the primary stimulus for vessel dilatation (MWSR-AUC) was calculated. Reproducibility of the measurements in the superficial femoral artery in our lab are reported to be 1.5% for baseline diameter, 14% for blood flow and 15% for FMD.[30]

Statistical analysis

The primary end point of this study was the superficial femoral artery FMD. We decided that with an estimated SD of 35% of the baseline value, a mean relevant effect of the L-NMMA infusion should be at least 50% (for the control and SCI comparison) and calculated that with an alpha of 0.05, 6 subjects per group would be needed to achieve a power of 90%. Statistical analyses were performed using SPSS 12.0 computer software (SPSS Inc., Chicago, Illinois, USA). Kolmogorov-Smirnov test indicated a normal (Gaussian) distribution of data. Results are expressed as mean ± SEM. Differences during the whole period of reactive hyperemia in blood flow, mean wall shear rate and diameter between the two experimental conditions (saline versus L-NMMA) were examined using a two-way repeated measures ANOVA. To assess differences in FMD response during saline and L-NMMA infusion in controls and SCI individuals (for the 5-minute as well as 10-minute ischaemic stimulus),

a Student's t-test for paired groups was used. To examine differences between groups, an unpaired Student's t-test was used. Differences were considered to be statistically significant at a two-sided probability value of ≤0.05.

Results

Baseline vascular characteristics

Resting superficial femoral artery diameters were significantly lower in spinal cord injured (SCI) individuals compared with controls (0.55 ± 0.04 and 0.78 ± 0.02 cm, respectively), while mean wall shear rate (MWSR) was higher in SCI than in controls (50 ± 4 and 13 ± 1 s, respectively). Resting blood flow through the superficial femoral artery (SFA) did not differ between controls and SCI (75 ± 12 and 98 ± 18 ml/min, respectively). Controls showed a significantly larger leg volume than SCI (11.1 ± 0.7 and 7.9 ± 0.3 L, respectively). After correction for leg volume, SCI individuals demonstrated a higher blood flow compared with controls (13 ± 2 and 7 ± 1 ml/min/L, respectively).

Before administration of L-NMMA, the superficial femoral artery blood flow and diameter had returned to baseline values in both groups (Table 1). Superficial femoral artery blood flow decreased significantly in both groups during L-NMMA infusion ($P<0.05$). Infusion of L-NMMA did not have any effect on baseline diameter of the superficial femoral artery nor did it affect mean arterial blood pressure or heart rate in either group (Table 1).

Table 1.

	Baseline 1 (Saline)	Baseline 2 (Saline)	L-NMMA
Diameter (cm)			
Controls	0.78 ± 0.02	0.79 ± 0.02	0.77 ± 0.02
SCI	0.55 ± 0.02	0.55 ± 0.02	0.55 ± 0.02
Superficial femoral artery blood flow (ml/min)			
Controls	75 ± 12	82 ± 12	46 ± 7 *
SCI	98 ± 18	85 ± 14	59 ± 14 *
Heart rate (bpm)			
Controls (n=7)	55 ± 2	57 ± 3	55 ± 3
SCI	58 ± 4	57 ± 3	58 ± 3
Mean arterial pressure (mmHg)			
Controls (n=7)	90 ± 3	93 ± 3	95 ± 3
SCI	98 ± 3	101 ± 3	103 ± 3

* $P<0.05$: Significantly different from baseline 2. Due to technical problems, no registration is present of the heart rate and mean arterial pressure of 1 control subject.

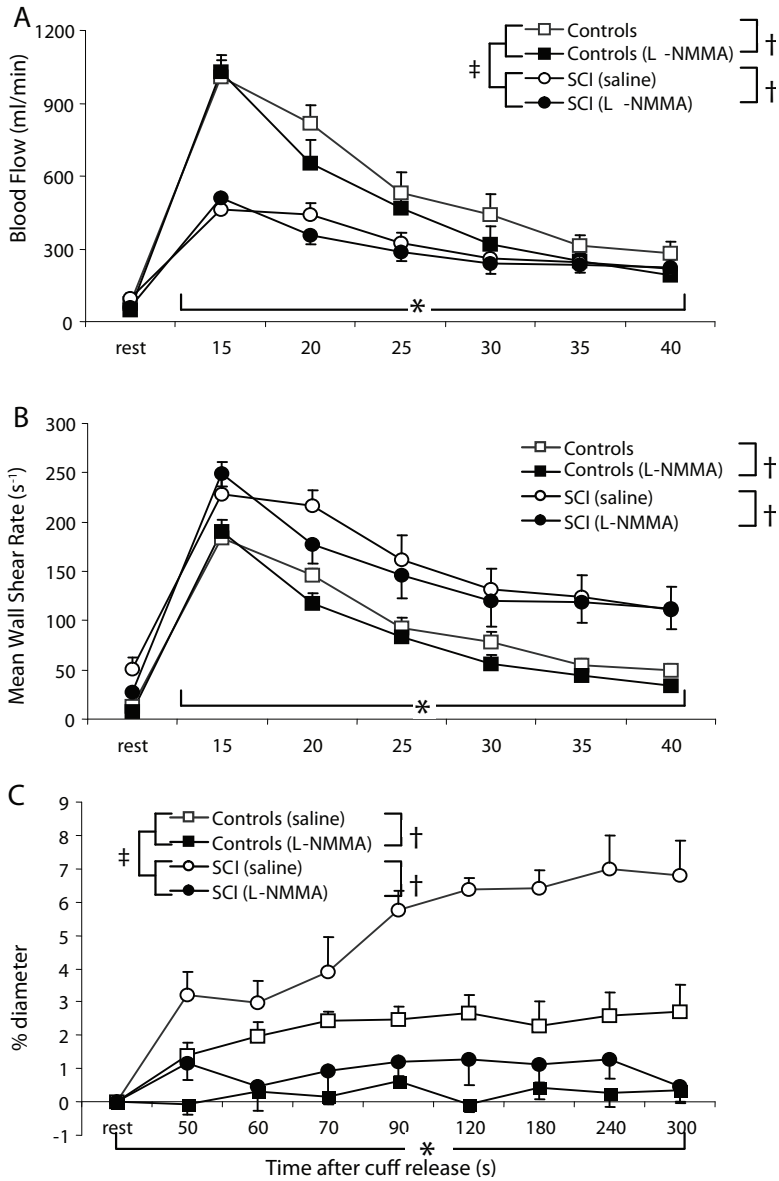

Figure 2. Average baseline and post-ischaemic blood flow (A) and mean wall shear rate (B) during the first 45 seconds of reactive hyperaemia, and (C) percentage change in superficial femoral artery diameter during the post-ischaemic period from minute 1 to minute 5 in controls (squares) and SCI individuals (circles) during infusion of saline ('open') or L-NMMA ('filled'). Data are presented as mean ± SEM. The brackets represent the ANOVA results. * significant time effect for both groups ($P<0.05$, two-way-ANOVA). † significant interaction for condition (saline or L-NMMA) x time for both group separately. ‡ significant between group effect ($P<0.05$, two-way-ANOVA). During infusion of L-NMMA, no time effect in superficial femoral artery diameter was observed in both groups (one-way-ANOVA).

FMD and hyperaemic responses

Controls After the 5-minute period of distal leg ischaemia, peak blood flow was maximal within 15 seconds with a subsequent decay in blood flow thereafter (Figure 2A). At 45 seconds post-cuff deflation, blood flow had decreased but not fully returned to baseline (Saline: 281 ± 133 versus 75 ± 34 mL/min, L-NMMA: 190 ± 74 versus 46 ± 21 mL/min). Post-ischaemic flow and MWSR were lower during L-NMMA infusion than during saline infusion (Figure 2A and Figure 2B, P=0.023 for post-ischaemic blood flow, and P=0.024 for MWSR). During reactive hyperaemia, the area-under-the-curve for MWSR during saline infusion (488 ± 33) was larger than during L-NMMA infusion (413 ± 40, P=0.02).

After cuff release, FMD in the SFA of controls was 4.2 ± 0.3% during saline infusion (Figure 3). Infusion of L-NMMA almost completely abolished the FMD response (1.0 ± 0.2%, P<0.001) (Figure 3). Analysing the overall course of the FMD, the change in diameter during reactive hyperaemia was completely abolished during L-NMMA infusion (Figure 2C). After correction for its stimulus (area-under-the-curve for MWSR), a marked difference in FMD response between saline (0.0089 ± 0.0009 AU) and L-NMMA infusion (0.0026 ± 0.0006 AU, P<0.001) was still observed (Figure 3).

SCI individuals Post-ischaemic flow and MWSR were lower during L-NMMA infusion than during saline infusion (Figure 2, P=0.006 for post-ischaemic blood flow, and P=0.003 for MWSR). At 45 seconds post-cuff deflation, blood flow had decreased but was not yet returned to baseline (Saline: 216 ± 91 versus 98 ± 43 mL/min, L-NMMA: 223 ± 91 versus 59 ± 33 mL/min). During reactive hyperaemia, the area-under-the-curve for MWSR during saline infusion (803 ± 100) was larger compared with the area-under-the-curve

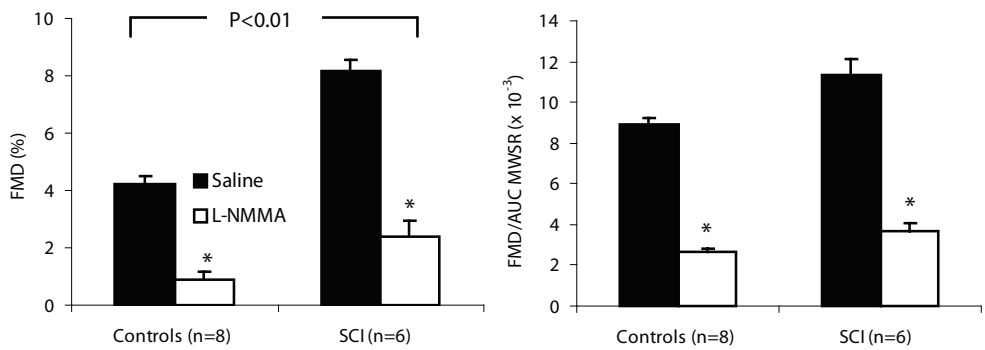

Figure 3. FMD presented as the (A) maximal relative change in superficial femoral artery diameter, and (B) the corrected FMD for the area-under-the-curve of the post-ischemic mean wall shear rate (AUC-MWSR) in controls and SCI individuals during infusion of saline (black bars) or L-NMMA (white bars). Results are presented as mean ± SEM. * Significantly different between saline and L-NMMA at P<0.05 (Student's t-test).

for MWSR during L-NMMA infusion (744 ± 99, P=0.047). FMD response in the SFA in SCI was 8.2 ± 0.4% under saline infusion, while infusion of L-NMMA significantly reduced the FMD response (2.4 ± 0.5%, P<0.001, Figure 3). By correcting the FMD response for its stimulus (area-under-the-curve for MWSR), a reduced FMD was still observed (saline: 0.0113 ± 0.0020 AU, L-NMMA: 0.0037 ± 0.0009 AU).

FMD response in SCI was significantly larger than in controls during saline (SCI: 8.2 ± 0.4%; controls: 4.2 ± 0.3%; P<0.001) as well as during infusion of L-NMMA (SCI: 2.4 ± 0.5%, controls: 1.0 ± 0.2, P=0.03). However, after correction for the eliciting stimulus (area-under-the-curve for MWSR), no difference was observed between SCI and controls, either during saline (SCI: 0.0113 ± 0.0020 AU; controls: 0.0089 ± 0.0009 AU; P=0.35) or during L-NMMA infusion (SCI: 0.0037 ± 0.0009 AU; controls 0.0026 ± 0.0006 AU, P=0.31) (Figure 3).

FMD with NO blockade (10-minute)

These experiments were performed in a subset of the original group of healthy controls (n=6). The subpopulation of healthy controls is representative for the original population regarding age (40 ± 4 versus 34 ± 4 years, respectively), body weight (86 ± 6 versus 82 ± 7 kg, respectively), systolic blood pressure (124 ± 3 versus 128 ± 3 mmHg, respectively) and diastolic blood pressure (70 ± 4 versus

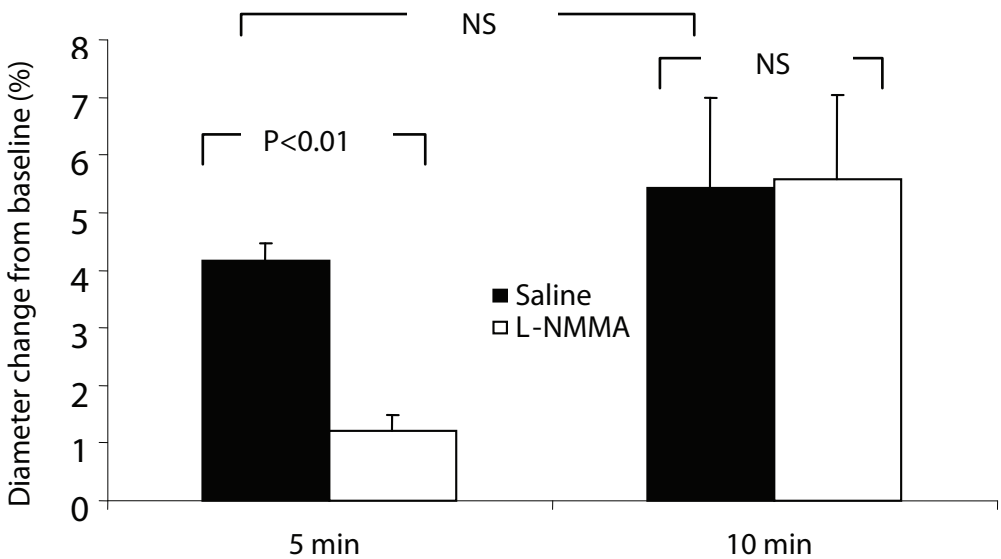

Figure 4. Relative change in the superficial femoral artery during a 5- and 10-minute FMD procedure, under infusion of saline (black bars) or L-NMMA (white bars) in a subpopulation of the original group of healthy able-bodied control subjects (n=6). A paired t-test is used to compare the diameter change to the situation with saline and L-NMMA infusion, but also between the 5- and 10-minute FMD. Error bars represent SEM.

76 ± 4 mmHg, respectively). Baseline femoral artery did not differ between both experiments (5-minute: 7.9 ± 0.2, 10-minute: 7.9 ± 0.2 mm). The 5-minute as well as the 10-minute FMD procedure resulted in a dilatation of the superficial femoral artery (Figure 4). After 5-minute occlusion, the superficial femoral artery dilatation was significantly reduced during infusion of L-NMMA, whereas no difference in artery dilatation is observed during the 10-minute FMD procedure between saline and L-NMMA (Figure 4). During reactive hyperaemia after the 10-minute occlusion, the area-under-the-curve for MWSR during saline infusion (1270 ± 345) was not different from L-NMMA infusion (1447 ± 318, P=0.42). Also correcting the superficial femoral artery dilatation after 10-min ischemia for the area-under-the-curve for MWSR, no difference was observed between saline (0.0071 ± 0.0028 AU) and L-NMMA infusion (0.0047 ± 0.0009 AU; P=0.34).

Discussion

In the present study, we demonstrate that the FMD response of the superficial femoral artery in healthy men and in spinal cord-injured (SCI) individuals predominantly reflects NO-mediated endothelium-dependent vasodilatation. FMD of the superficial femoral artery in response to a 5-minute period of ischaemia induced by a cuff placed distal to the site of artery insonation therefore represents a suitable method to examine NO bioavailability and endothelial function in the lower limbs.

Contribution of NO to FMD in controls

Previous reports utilising L-NMMA as a competitive antagonist have demonstrated a crucial role for NO in radial[13, 14] and brachial artery[12] dilatation in response to brief periods of reactive hyperaemia. In the present study, the increase in superficial femoral artery diameter in healthy men, after a brief episode of reactive hyperaemia, was almost completely abolished during infusion of L-NMMA. Thus, in accordance with data in the upper limbs, our data demonstrate that the FMD response of the superficial femoral artery in healthy men reflects NO-mediated endothelium-dependent vasodilatation. One may argue that the impact of L-NMMA on arterial diameter may be due, in part, to a diminished flow stimulus. Indeed, blood flow, MWSR and area-under-the-curve for MWSR during reactive hyperaemia were slightly lower during infusion of L-NMMA than during saline infusion. Nevertheless, when the FMD response was corrected for its stimulus (area-under-the-curve for MWSR), a marked difference between saline and L-NMMA infusion was still observed.

The FMD of the superficial femoral artery after blockade of NO production was ~1% in the controls in the present study. Lieberman *et al* [31] observed a 7% FMD of the brachial artery during administration of L-NMMA. The discrepancy with the present results can be explained by placement of the occlusion cuff relative to the ultrasound probe. In the present study, the ultrasound probe was proximal to the cuff, whereas Lieberman *et al* [31] placed the

cuff on the upper arm with the site of measurement in the ischaemic area distal to the cuff. Under these circumstances FMD is affected by vasodilators other than NO which are released in response to ischaemia, and may also be complicated by myogenic responses as a result of the pressure fall inside the artery during occlusion.[12] In other studies where the arterial measuring site was proximal to the cuff, L-NMMA reduced radial artery vasodilatation to 0.7 – 2%[13] or converted radial dilatation to vasoconstriction,[14] whilst brachial artery FMD was completely abolished.[12] In all studies, the FMD response was investigated after a 5-minute occlusion period causing a brief shear stress stimulus. The nature of the shear stress stimulus is critical to the NO-dependency, since the FMD response to more sustained stimuli (caused by release of 15 minutes ischaemia, skin warming, or distal infusion of acetylcholine) is unaffected by L-NMMA.[13] Parallel to this, we could demonstrated in a subset of the control subjects that a 10-minute period of ischaemia results in a dilatation of the superficial femoral artery that is unlikely the result of an endothelium-dependent NO-mediated mechanism only. This suggests that, similar to the brachial artery, the duration of the ischaemic period is critical for the NO-dependency of the superficial femoral artery.

During a 5-minute occlusion, FMD may be partially dependent upon other vasodilator agents such as EDHF or prostacyclin. In addition, an increase in shear stress can also stimulate endothelin production; blockade of endothelin-A receptors improves FMD in chronic heart failure patients.[32] Although these NO-independent mechanisms could explain the residual FMD during NO-blockade in the present study, we argue they play a relatively minor role in the superficial femoral artery FMD, since L-NMMA blunted the dilatation up to about 70% and the increase in diameter observed during infusion of saline was inhibited throughout L-NMMA infusion (Figure 2C). Nonetheless, our finding of NO dependency of FMD in the lower limb is limited to the superficial femoral artery in response to a 5-minute distal occlusion.

Contribution of NO to FMD in SCI

In keeping with recent reports after prolonged (spinal cord injury[30]) and short-term (unilateral lower limb suspension[33] deconditioning or deconditioning by bed rest[34, 35]), our findings demonstrate an enhanced FMD response in SCI. Recently, Stoner *et al* [24] demonstrated an attenuated posterior tibial artery FMD response in SCI individuals, measuring the artery diameter in the ischaemic area distal to the cuff. As discussed previously, one may speculate whether this response is NO-mediated or influenced by other metabolic vasoactive substances. Since deconditioning leads to marked changes in vascular function,[36-38] we considered the possibility that other mechanisms than NO might mediate FMD under these conditions. Indeed, during blockade of NO production by L-NMMA, the FMD response was attenuated but not abolished in SCI and was more preserved than in controls (~2.4% versus ~1%, respectively). However, it has been proposed that the FMD response should

be corrected for the eliciting stimulus by taking the post-occlusive area-under-the-curve of the mean wall shear rate.[29] After such correction was applied, no differences in FMD response between SCI and controls were evident. Although the contribution of other vasodilating agents cannot be ruled out in explaining the remaining FMD response during L-NMMA infusion in SCI, the FMD of the superficial femoral artery following a 5-minute ischaemic stimulus in the deconditioned legs of SCI depends predominantly on NO.

Administration of L-NMMA decreased superficial femoral artery blood flow but did not affect basal superficial femoral artery diameter, and no systemic effects were observed since mean arterial blood pressure and heart rate were not changed during infusion of L-NMMA in both groups. The decrease in blood flow is consistent with the existence of basal release of NO in the leg resistance vessels, which is in agreement with previous observations.[28] The apparent lack of effect of L-NMMA on basal superficial femoral artery diameter suggests that, consistent with the observations in the radial and brachial artery,[12-14] NO does not contribute markedly to basal vascular tone in large conduit arteries.

Limitations

A recent study demonstrated that the shear stimulus area-under-the-curve, but not the peak shear stimulus, is the critical determinant of the peak flow-mediated dilatation.[29] Although we examined the AUC during a large time window, a limitation in this study is that we could not examine the superficial femoral artery blood flow beyond 45 s after cuff release. After 45 s of cuff release, we found that superficial femoral artery blood flow was markedly reduced, yet did not return to baseline values. In a group of healthy men (n=6, 33 ± 7 years), we performed additional experiments and examined the flow response for 5 minutes after cuff release (unpublished data). We found that blood flow returned to baseline values between 90-120 s post deflation, while blood flow was 2-fold higher than baseline values at 45 s after cuff release. The latter finding was also present in the data set of controls and SCI subjects presented in this paper. In addition, based on previously reported limb differences in vascular control[21] and conduit vessel responsiveness to a certain change in shear,[22] one may argue whether correction methods for the brachial artery are equally applicable to superficial femoral artery FMD. Indeed, markedly different time-courses of post-occlusive brachial artery dilator response[29] (a peak within 50-80 s) exist compared with the superficial femoral artery change after cuff release as described in this study (peak between 1-4 minutes). Further studies will be required to fully ascertain the appropriate correction for stimulus for lower limb arteries.

Clinical relevance

The non-invasive measurement of the FMD in the brachial artery is a widely accepted and frequently used method to examine endothelial function. Based on the results of this study, we advocate the use of superficial femoral artery FMD in disease states that specifically affect the lower extremities (such as peripheral artery

disease) or to examine the effects of exercise training programs in the legs. Moreover, changes in leg conduit arteries are clinically relevant since the peripheral atherosclerotic process occurs more often in arteries of the lower limbs than in the upper limbs.[39] Taken together, we believe that the assessment of the superficial femoral artery FMD provides a relatively unexplored and interesting field of cardiovascular physiology in health and (cardiovascular) disease.

In conclusion, we have demonstrated that superficial femoral artery dilatation to a transient period of increased flow after 5 minutes of arterial occlusion of the leg represents predominantly NO-dependent dilatation in healthy men and in spinal cord-injured individuals. Consequently, superficial femoral artery FMD can be used as a valid and novel explorative tool to examine endothelial function in the lower limbs in humans.

Acknowledgements

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Abstract

Background – We tested whether venous occlusion plethysmography (VOP) is an appropriate method to measure calf blood flow (CBF) during head-up tilt (HUT).

Methods – CBF measured with VOP was compared with superficial femoral artery blood flow as measured by Doppler ultrasound during incremental tilt angles.

Results – Measurements of both methods correlated well (r=0.86). Reproducibility of VOP was fair in supine position and 30° HUT (CV: 11% to 15%).

Conclusion – This indicates that VOP is an applicable tool to measure leg blood flow during HUT, especially up to 30° HUT.

Chapter 5

Leg blood flow measurements using venous occlusion plethysmography during head-up tilt

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Paul Smits
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Clin Auton Res 2007;17(2):106-111

Introduction

The main mechanism responsible for maintaining blood pressure during orthostatic stress is arteriolar vasoconstriction.[1] In order to quantify the response in vascular resistance to postural stress, in particular in the leg, it is necessary to measure leg blood flow accurately.

Venous occlusion plethysmography (VOP) is a well-established method to measure calf blood flow, which has been used in a variety of conditions, i.e. exercise[2] and reactive hyperaemia.[3] However, its use for measuring leg blood flow in standing or head-up tilt (HUT) position remains controversial, since an empty venous system has been suggested to be requisite for this method.[4-6] Since in the upright posture veins are already distended, due to an increase in hydrostatic pressure, further collection of blood may be defined by venous compliance rather than arterial inflow. This may question the validity of measuring blood flow using VOP in dependent limbs or in the upright posture. Although VOP has been used during HUT, accuracy or reproducibility of this method has not been reported.[7-9]

Therefore, the purpose of this study was to assess the applicability and reproducibility of VOP for blood flow measurements in the calf (CBF) during HUT at different tilt angles (0°, 30°, 45°, and 70°). In a subgroup, CBF measurements by VOP were compared with blood flow measurements using Doppler ultrasound. To assess reproducibility of VOP, measurements were performed twice.

Methods

Subjects

In total eighteen healthy, normotensive subjects aged 21 to 30 years volunteered to participate in this study. In eight subjects, blood flow measurements using VOP were compared with blood flow measurements using Doppler ultrasound (DU). In ten other subjects, the VOP measurements were repeated within two weeks to assess reproducibility.

Baseline subject characteristics are illustrated in Table 1. None of the subjects used cardiovascular medication or suffered from cardiovascular disease. All were non-smokers and had no history of syncope. All volunteers refrained from caffeine and alcohol for at least eighteen hours and from food intake for three hours prior to testing. The Hospital Ethics Committee approved the study. All subjects gave their written informed consent.

Measurements

The subjects lay in supine position on a manually driven tilt table and were supported

Table 1. Subject characteristics.

	Mean ± SD
Age, yrs	25 ± 4
Length, cm	187 ± 9
Body mass, kg	80 ± 11
Calf circumference, cm	38 ± 2
Systolic blood pressure, mmHg	125 ± 12
Diastolic blood pressure, mmHg	74 ± 5
Heart rate, bpm	64 ± 10

by a saddle. The venous occlusion cuff was placed around the right thigh and connected to a rapid cuff inflator (Hokanson Stopler E-20, Bellevue, WA 98005, USA). The mercury-in-silastic strain gauge was placed around the thickest part of the calf and was connected to the plethysmograph. Red blood cell velocities and systolic and diastolic vessel diameter of the right superficial femoral artery were measured with a pulsed-colour Doppler device, which is described in detail elsewhere.[10] Reproducibility of DU in the superficial femoral artery was 1.5% for diameter, 14% for blood flow.[11]

Protocol

Supine blood flow measurements using DU and VOP were performed after subjects were 30 minutes quietly in supine position. When the subject was 2 minutes into 30° HUT, DU measurements started until 3.5 minutes where after CBF continued for another 3.5 minutes. The venous occlusion pressure was adjusted to the hydrostatic pressure column, which is derived from the vertical distance heart level – thigh level and was calculated as the sinus of the tilt angle * actual distance heart – thigh, and was 75 mmHg during 30° HUT. The same

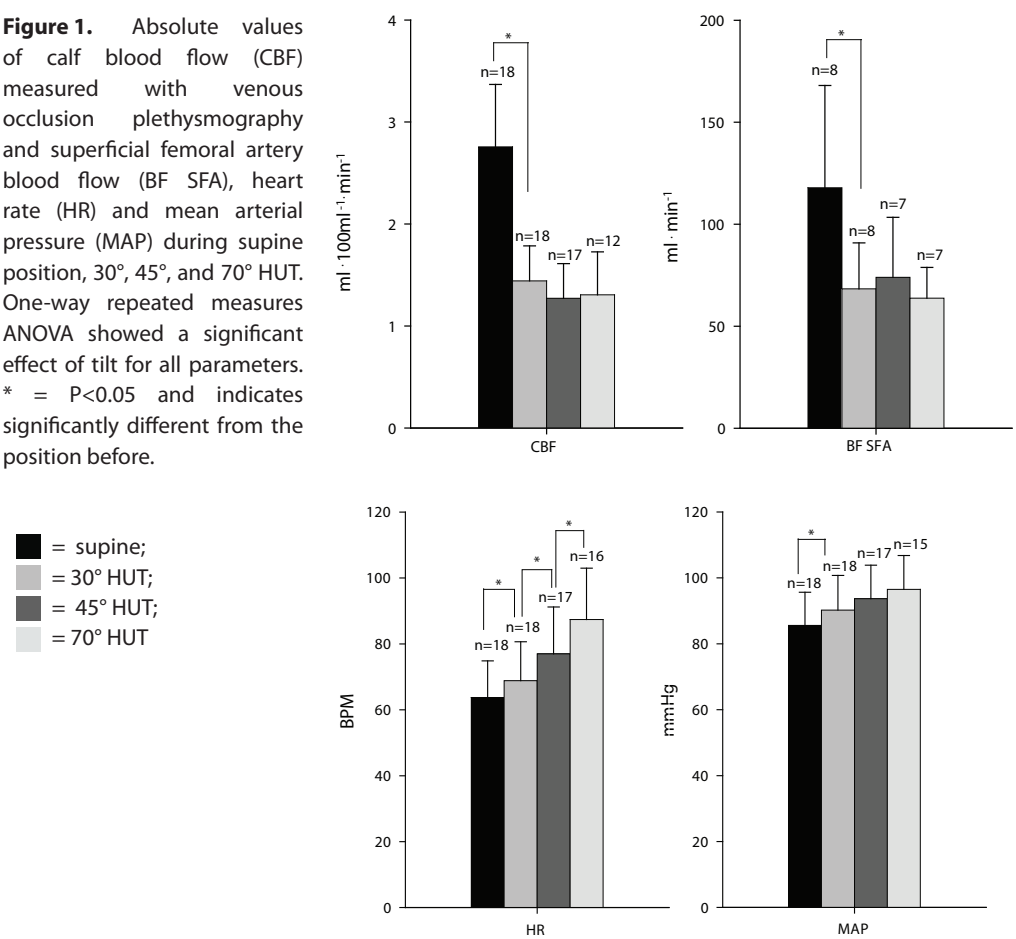

Figure 1. Absolute values of calf blood flow (CBF) measured with venous occlusion plethysmography and superficial femoral artery blood flow (BF SFA), heart rate (HR) and mean arterial pressure (MAP) during supine position, 30°, 45°, and 70° HUT. One-way repeated measures ANOVA showed a significant effect of tilt for all parameters. * = P<0.05 and indicates significantly different from the position before.

procedure was repeated for 45°, and 70° HUT using a venous occlusion pressure of 87, and 105 mmHg, respectively.

Data analysis

CBF in ml·100ml^{-1}·min^{-1} was calculated as described previously[12] and values from minute 3.5 until 7 were averaged to calculate CBF for each HUT position. Doppler ultrasound was analyzed as described before[10] to obtain superficial femoral artery blood flow. To compare CBF measurements with superficial femoral artery blood flow, CBF measurements in ml·100ml^{-1}·min^{-1} were multiplied by lower leg volume (ml) as measured by water displacement.

Statistics

Data are expressed as mean ± standard deviation (SD).

The results of each method were correlated and agreement evaluated according to the method described by Bland and Altman.[13] The limits of agreement are defined as the mean of the relative differences between the two methods ± 2 SD. Student's t-test was used to test for systemic differences between the two methods.

Reproducibility of the CBF was assessed by calculating the coefficient of variance (CV) from two measurements.[12]

To determine whether hemodynamic responses were dependent on the angle of tilt, one-way repeated measures ANOVA's were applied. If significant effects of tilt were observed, post-hoc paired t-tests with Bonferroni correction for multiple testing were used. A P-value of <0.05 was considered to indicate significance.

Results

In 5 volunteers, CBF could not be measured during 70° head-up tilt (HUT), due to a poor plethysmography signal or near fainting of the subject. One subject was not available for the second VOP measurements. Therefore, reproducibility data were analysed over nine subjects.

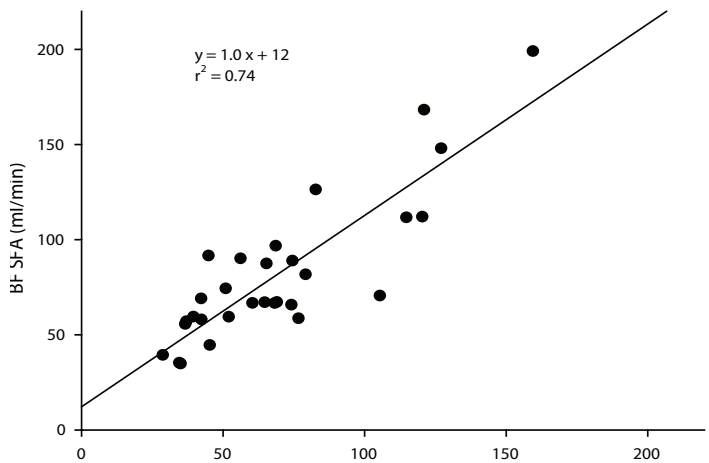

Figure 2. Blood flow in the superficial femoral artery (BF SFA) measured by Doppler ultrasound during different angles of head-up tilt versus calf blood flow (CBF) measured by venous occlusion plethysmography corrected for lower leg volume. Pearson correlation coefficient is 0.86.

Hemodynamic responses to HUT (Figure 1)

CBF and superficial femoral artery blood flow (BF SFA) decreased significantly from supine to 30° with no further decrease with increasing tilt angle (Figure 1). The relative decrease in CBF (46% ± 11%) from supine to 30° was significantly larger than the decrease in BF SFA (40% ± 12%) (P<0.001).

Agreement VOP and DU

The Pearson correlation between the two methods was 0.86 (P<0.001) for all data points (Figure 2).

The agreement between the two methods was evaluated by plotting the relative difference in each measurement against the mean for all data points and separated

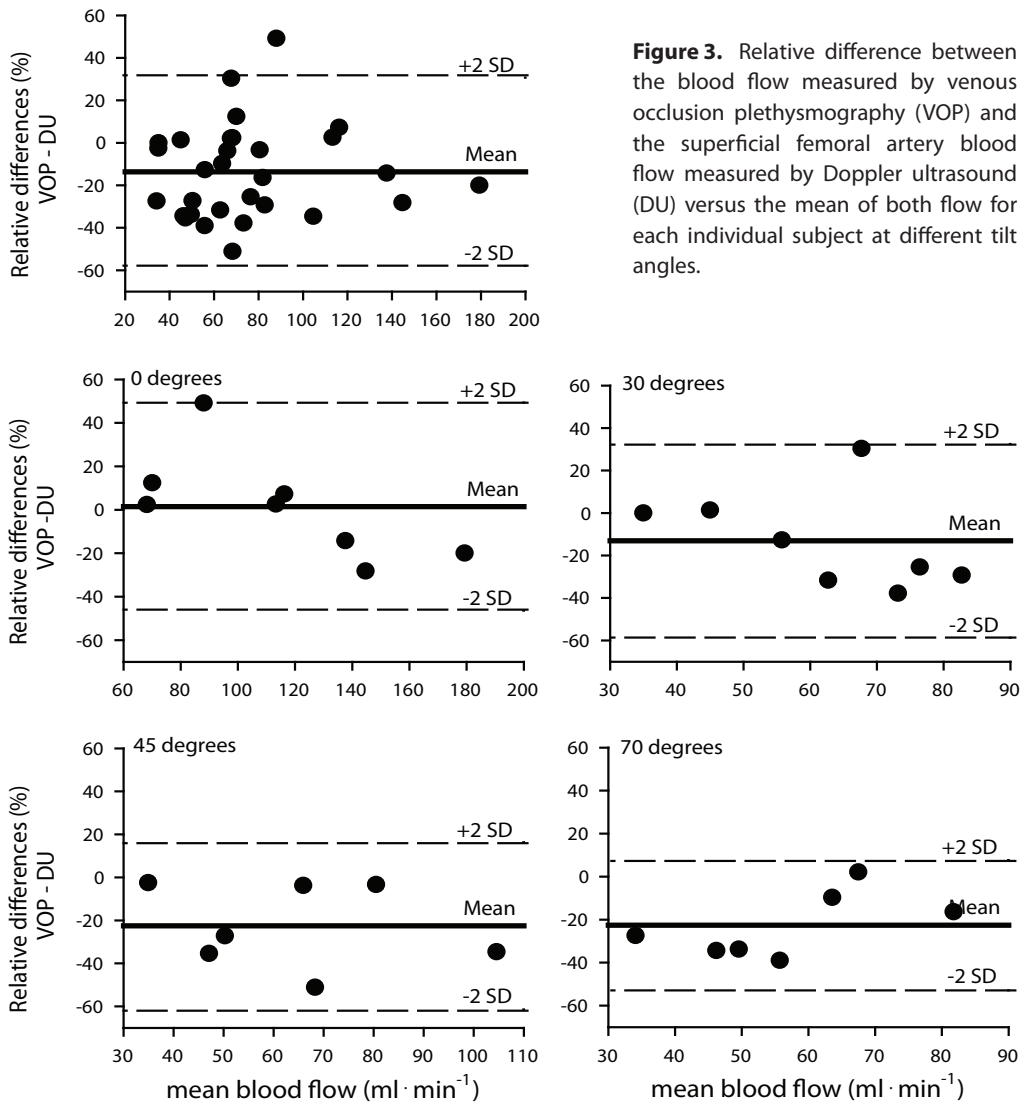

Figure 3. Relative difference between the blood flow measured by venous occlusion plethysmography (VOP) and the superficial femoral artery blood flow measured by Doppler ultrasound (DU) versus the mean of both flow for each individual subject at different tilt angles.

Table 2. Values of calf blood flow (n=9).

	Supine		30° HUT		45° HUT		70° HUT (n=4)	
Subject	test 1	test 2	test 1	test 2	test 1	test 2	test 1	test 2
Mean ± SD	2.6±0.6	2.6±0.8	1.3±0.3	1.3±0.3	1.1±0.3	1.1±0.2	1.1±0.2	1.2±0.2
%change± SD			-45±11	-49±12	-54±11	-57±13	-44±17	-44±14
CV	15.0%(CI 10.1 – 29.1)		11.0%(CI 7.4 – 21.3)		14.9%(CI 10.0 – 28.9)		8.7%(CI 4.9 – 33.2)	

Values of calf blood flow in $ml \cdot 100ml^{-1} \cdot min^{-1}$ and mean absolute and relative data ± SD in supine position, 30°, 45°, and 70° head-up tilt (HUT) for the first and second test. Coefficients of Variation (CV). Missing data in 70° HUT are due to near fainting or a poor plethysmography signal.

for the different tilt angles (Figure 3). The relative mean difference ((VOP – DU)/ DU) was –14% ± 22 for all data points in supine position and HUT indicating that overall CBF (VOP) is lower than BF SFA (DU); for supine position the relative mean difference between VOP and DU was 1.5% ± 24; for 30°: -13% ± 23; for 45°: -23% ± 19; for 70°: -23% ± 15. Limits of agreement for all data points in supine position and during HUT were -58% to 31% and became smaller during HUT. The limits of agreement are reasonable, although CBF during HUT is lower than BF SFA.

Reproducibility of VOP during head-up tilt

The coefficient of variation (CV) of CBF ranged between 8.7 and 15.0% (Table 2). In 70° HUT, the CV for both parameters was calculated over 4 subjects only.

Discussion

Calf blood flow (CBF) measured with VOP correlates well with superficial femoral artery blood flow (BF SFA) measured with DU, and can be measured reproducibly during HUT. Since the most profound changes in blood flow with both techniques were already measured in 30° HUT, and the increase in hydrostatic and venous pressure, and concomitant technical difficulties are smallest from supine to 30° HUT, we recommend to use VOP for leg blood flow measurements during HUT up to 30°.

The decrease in leg blood flow assessed with VOP and DU is comparable to tilt-induced blood flow changes in other studies using DU (33%-59%).[14-17] The strong relationship between VOP and DU blood flow measurements in the present study is in line with previous studies reporting correlation coefficients varying from 0.57 to 0.99 at rest and during exercise.[18-20] Head-up tilt affects muscle blood flow more than skin blood flow.[21, 22] Since skin blood flow contributes more to superficial femoral blood flow than to calf blood flow, this may explain the observed discrepancy between the decrease in CBF (-48%) versus the decrease in superficial femoral artery blood flow (~40%) in response to HUT.
Reproducibility of baseline CBF (15.0%)

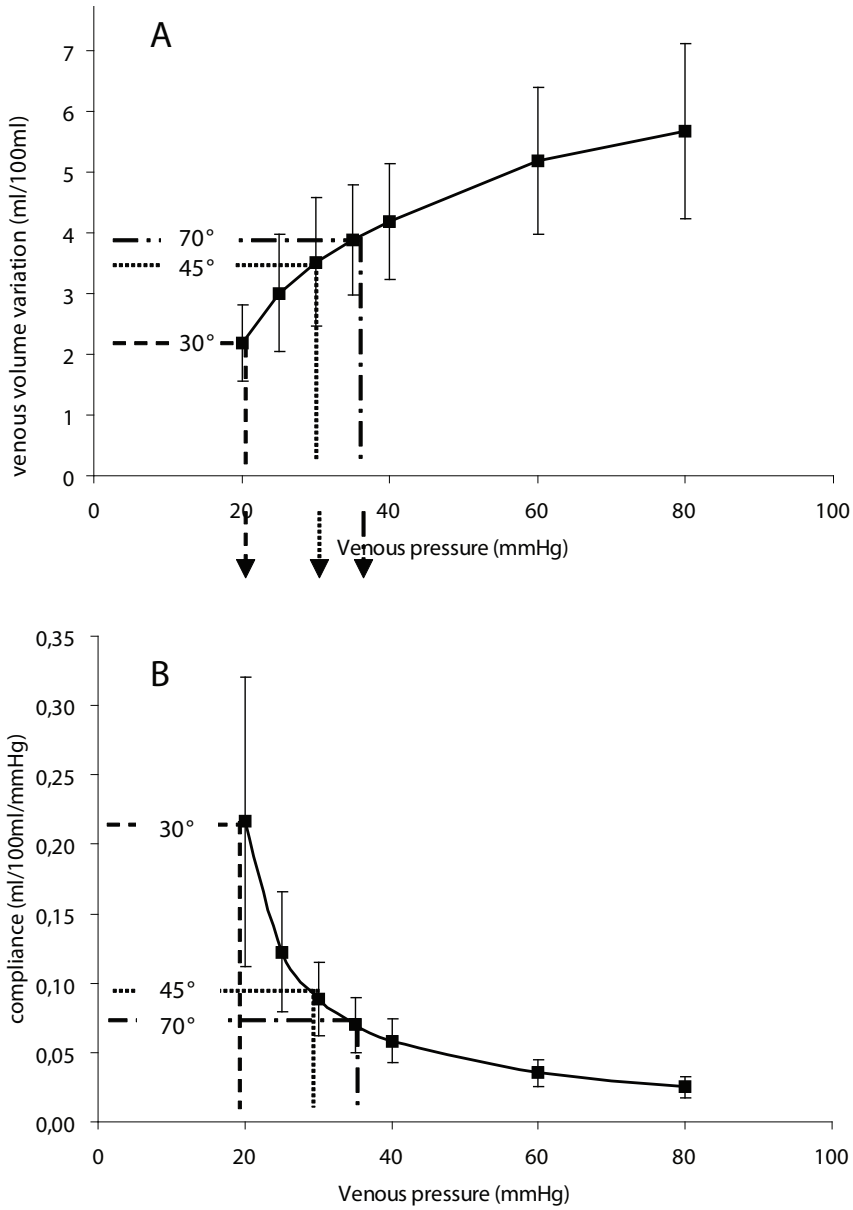

Figure 4. (A) Pressure-Volume curve and (B) Pressure-Compliance curve based on data of a similar group of volunteers measured by venous occlusion plethysmography at different cuff occlusion pressures (for method and protocol see[25]). The increases in calf volume in response to 30°, 45°, and 70° HUT are marked by the dotted lines in the pressure volume curve (A). The different tilting angles correspond with different venous pressures (X-axis). Transferring these venous pressures into the pressure-compliance curve (B) clearly demonstrate that during 30° HUT venous compliance is still on the steep linear part of the curve, whereas during 45°, and 70° HUT the venous compliance is compromised.

is in range with other studies using similar techniques to measure leg blood flow.[12, 23, 24] The coefficient of variation of CBF during HUT was even better (11.0-17.9%), which indicates that VOP is a reproducible tool to measure tilt-induced vasoconstriction repetitively. The low coefficients of variation of CBF during 70° (8.7-8.9%) are not representative since these coefficients of variation were calculated over no more than 4 subjects. Not all subjects were able to abstain from moving their legs in 70° HUT position, and some subjects fainted in this position. Moreover, the quality of the plethysmographic tracing became worse at 70° HUT whereas at the lower tilt angles the plethysmography signal is of good quality indicated by the volume pulsations in the plethysmographic tracing for the period of venous occlusion.

Our data and previous studies[26-29] show that at 30° HUT peripheral vascular responses are accomplished to a large extent. The increase in hydrostatic pressure and concomitant increase in venous pressure is low at 30°. From Figure 4B it can be concluded that at 30° HUT venous compliance is still at the steep portion of the venous compliance curve whereas during 45°, and 70° HUT the venous compliance has shifted to the non-linear part. At 30° HUT increase of the plethysmography

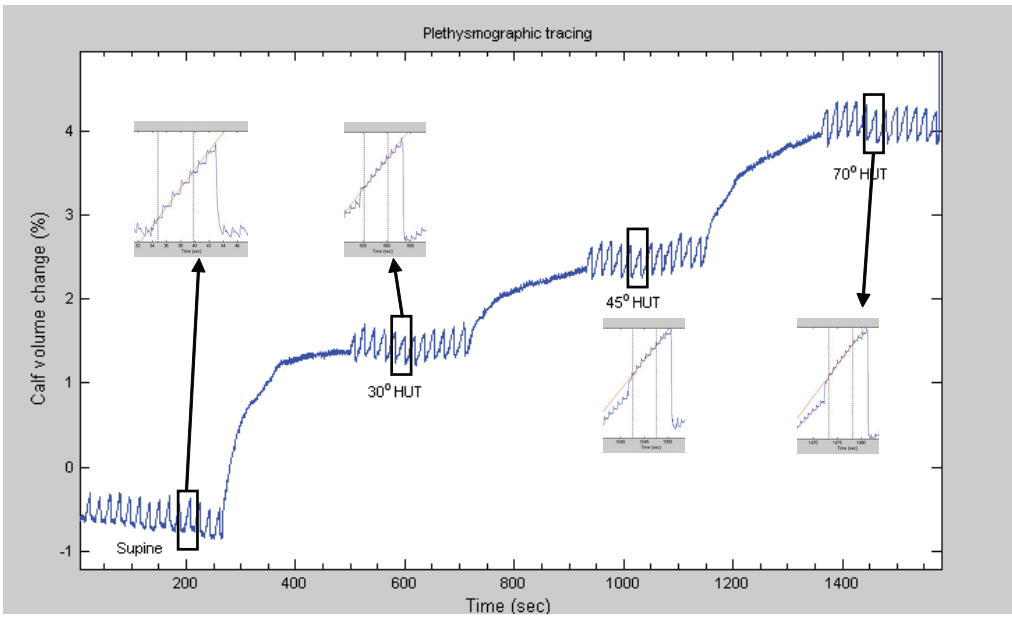

Figure 5. Typical plethysmographic tracing of one individual subject during a complete experiment. Blood flow measurements at 30° HUT start when the plethysmography signal does not change anymore, meaning that venous volume reached a steady state situation. Besides, looking at a typical VOP tracing at 30° HUT, the increase in venous volume is linear during the first 5 seconds of cuff inflation, indicating that blood flow measurements using VOP during 30° HUT are not compromised by a decrease in venous compliance.

signal during venous occlusion is linear while at 45° and 70° HUT, however, venous distensibility is reduced and consequently results in a non-linear increase in leg volume during inflation of the venous occlusion cuffs, which is illustrated in Figure 5. We therefore recommend using VOP at 30° HUT. For studies focussing on syncope at the endpoint of HUT, which requires larger tilt angles, other techniques to measure leg blood flow should be used.

Limitation

Using VOP, blood flow is defined as limb volume changes over time. During HUT, when the leg is below heart level, volume changes can still be measured using VOP, however, the physiological determinants of these volume changes are complex and it is no longer possible to say with reasonable certainty that a change in volume over time, which most likely reflects flow, is determined by resistance vessel tone. For example, limb blood flow measured using VOP in HUT position can decrease due to an increase in venous pressure, as a result of venous congestion and the associated fall in arterio-venous pressure gradient, without any increase in resistance at the arteriolar level.

In conclusion, this study demonstrates that CBF measured by VOP during HUT is suitable and reproducible. The method is easy applicable and recommended in tilt angles equal to 30° to avoid high hydrostatic and leg venous pressures.

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Abstract

Background - The prompt increase in peripheral vascular resistance, mediated by sympathetic α-adrenergic stimulation, is believed to be the key event in blood pressure control during postural stress. However, despite the absence of central sympathetic control of the leg vasculature, postural leg vasoconstriction is preserved in spinal cord-injured individuals (SCI). This study aimed to assess the contribution of both central and local sympathetically induced α-adrenergic leg vasoconstriction to head-up tilt (HUT) by including healthy individuals and SCI, who lack central sympathetic baroreflex control over the leg vascular bed.

Methods - In 10 controls and 9 SCI the femoral artery was cannulated for drug infusion. Upper leg blood flow (LBF) was measured bilaterally using venous occlusion strain gauge plethysmography before and during 30° HUT throughout intra-arterial infusion of saline or the non-selective α-adrenergic receptor antagonist *phentolamine*, respectively. Additionally, in six controls the leg vascular response to the cold pressor test was assessed during continued infusion of phentolamine, in order to confirm complete α-adrenergic blockade by phentolamine.

Results - During infusion of phentolamine HUT still caused vasoconstriction in both groups: leg vascular resistance (mean arterial pressure/LBF) increased by 10 ± 2 AU (compared to 12 ± 2 AU during saline infusion), and 13 ± 3 AU (compared to 7 ± 3 AU during saline infusion) in controls and SCI, respectively.

Conclusion - Effective α-adrenergic blockade did not reduce HUT-induced vasoconstriction, regardless intact baroreflex control of the leg vasculature. Apparently, redundant mechanisms compensate for the absence of sympathetic α-adrenoceptor leg vasoconstriction in response to postural stress.

Chapter 6

The role of the α-adrenergic receptor in the leg vasoconstrictor response to orthostatic stress

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Acta Physiologica, in revision

Introduction

The prompt increase in peripheral vascular resistance, mediated by sympathetic α-adrenergic stimulation, is believed to be the key event in blood pressure control during postural stress[1]. In individuals who fail arterial and cardiopulmonary baroreflex-mediated control over the legs, for instance individuals with a complete spinal cord injury (SCI), leg vasoconstriction upon postural change is preserved.[2-7] In SCI individuals preservation of postural vasoconstriction may, apart from structural vascular adaptations, such as vascular atrophy, explain their remarkable orthostatic tolerance and suggests the importance of local mechanisms in the regulation of vascular tone during postural challenges. So, apart from *central* sympathetic control, *local* sympathetic mechanisms, such as the venoarteriolar reflex, contribute to the hemodynamic response to postural stress. The venoarteriolar reflex is triggered when venous pressure is elevated above 25 mmHg or more.[8] During orthostasis as much as 45% of the increase in systemic vascular tone is due to the venoarteriolar reflex.[9] Several studies have been performed to unravel the mechanism of the venoarteriolar reflex.[4, 8-10] From these studies, it can be concluded that the venoarteriolar reflex concerns a *local* α-adrenergic sympathetic axon reflex.

Apart from α-adrenergic stimulation, non-adrenergic mechanisms, such as purinoceptor stimulation and release of neuropeptide Y, seem to contribute to the vasoconstrictor response after sympathetic stimulation.[11, 12] In addition, the leg vasoconstrictor response to postural stress may in part be myogenic,[13] and as such not related to the sympathetic nervous system. Nevertheless, the importance of the sympathetic nervous system during orthostasis is supported by the fact that patients who fail an adequate increase in sympathetic activity during postural stress, such as patients with autonomic failure, are not able to maintain blood pressure. In spite of this, in humans the sympathetic contribution to leg vascular tone in response to orthostasis has not been quantified. Therefore, the present study aimed to quantify the involvement of sympathetic α-adrenergic vasoconstriction to head-up tilt-induced increase in leg vascular tone by including healthy individuals and individuals who lack baroreflex control over the leg vascular bed, due to a spinal cord injury.

We hypothesize that blockade of the α-adrenergic receptor in the leg vascular bed will result in a substantial blunting of the leg vasoconstrictor response to head-up tilt (HUT), which will be larger in subjects with an intact baroreflex than in subjects who fail baroreflex control over the leg vascular bed and therefore depend on local vasoconstriction mechanisms. To address this hypothesis, the vasoconstrictor response to HUT was quantified, both during infusion of saline and during infusion of phentolamine (an α-adrenergic blocking agent) into the femoral artery in controls and spinal cord-injured individuals. Appropriate control experiments were performed for relevant confounding mechanisms.

Methods

Subjects

Nine male spinal cord-injured individuals (SCI) and ten healthy male controls participated in the study (Table 1). All subjects were normotensive (auscultatory blood pressure measurement) and the controls used no medication. The SCI continued their medication throughout the study. The control group was similar with respect to smoking habits (one subject in each group smoked). All subjects had no history of syncope. The two groups did not differ with respect to age, systolic and diastolic blood pressure, and heart rate. Body weight and thigh volume were significantly lower in SCI than in controls. The SCI had complete spinal cord lesions of traumatic origin varying from Cervical 5 – Thoracic 12 (American Spinal Injury Association ASIA A[14]). The motor and sensor level of the spinal lesion was assessed by clinical examination. The completeness of the sympathetic lesion was confirmed by the cold pressor test by immersing the hand into ice water and assessing the effect on leg vascular tone, except for one individual suffering from a cervical 5 spinal lesion

Table 1. Subject characteristics.

Subject	Level of spinal lesion	Time since injury (years)	Smoking	Medication	Age (yr)	Body weight (kg)	Upper leg volume (L)	Syst/Diast blood pressure (mmHg)	Heart rate (bpm)
1	C5	22	+	Baclofen 10 mg tid, Bisacodyl, Brindley stimulator	43	60	5.6	110/74	48
2	T6	11	-		34	71	5.7	128/68	47
3	T4	20	-		42	74	5.9	115/75	59
4	T5	6	-		35	68	5.0	102/64	63
5	T5	20	-		49	67	5.1	110/70	64
6	T12	12	-		38	73	4.3	150/90	56
7	T6	3	-	Detrusitol	23	74	6.5	122/78	41
8	T8	10	-		45	77	4.9	110/72	70
9	T8	7	-	Intrathecal Baclofen	35	85	5.6	134/84	62
SCI Mean±SD					38±8	72±7	5.4±0.6	120±15/75±8	57±9
C Mean±SD					36±9	88±12	8.2±1.2	127±9/80±5	58±6

SD - standard deviation

who lacked sensibility of the hand[15]. The study was approved by the Hospital Ethics Committee. All subjects gave their written informed consent prior to the study.

Experimental procedures and protocol

Instrumentation and measurements

All subjects refrained from caffeine and alcohol for at least twelve hours, from nicotine for at least 48 hours, and from food for three hours prior to testing. All subjects had emptied their bladder in the hour before the test to minimize the influence of any reflex sympathetic activation on peripheral vascular tone. All tests were performed in the afternoon with the subjects in supine position in a quiet temperature-controlled room (22°C - 24°C). The subjects lay in supine position on a manually driven tilt table and were supported by a saddle to prevent gliding down during HUT.

Using a modified Seldinger technique, an intra-arterial cannula (Angiocath 16 gauge, Becton Dickinson, Sandy, Utah, USA) was introduced into the femoral artery of the right leg at the level of the inguinal ligament for intra-arterial administration of drugs by an automatic syringe infusion pump. The right femoral artery was cannulated after local anesthesia (0.4 ml lidocaine 20 mg/ml). Heart rate was recorded from the electrocardiogram. Blood pressure measurements were performed continuously using a portable blood pressure device (Portapres, TNO, The Netherlands). This device corrects for hydrostatic pressure changes. A finger cuff was attached to the middle phalanx of the left third finger. Data were collected beat to beat during the experiment at a sampling rate of 200 Hz. Finger arterial blood pressure measurements during HUT accurately reflect intra-arterial blood pressure changes in young healthy volunteers.[16]

Bilateral upper leg blood flow was measured by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, D.E. Hokanson, Washington D.C., USA), which is a valid method to measure upper leg blood flow.[17] In supine position the thigh collecting cuffs (12 cm width) were simultaneously inflated using a rapid cuff inflator (Hokanson E-20) to a pressure of 50 mmHg[18] during 8 heart cycles, with a 10-heart cycles interval between the venous occlusions. For further description of this method see.[15] During the 30° HUT cuff pressure was adjusted to the hydrostatic pressure, i.e. to 75 mmHg.[19]

A 30° HUT was chosen since this position results in significant cardiovascular effects [20-22] with a significant increase in peroneal muscle sympathetic nerve activity,[23] without fainting of the subjects. In HUT position, calf blood flow measured with venous occlusion plethysmography correlates well with superficial femoral artery blood flow measured with Doppler ultrasound, and can be measured reproducibly during HUT up to 30°.[19] Besides, using 30° HUT the hydrostatic pressure column, that represents venous pressure, is ~25 mmHg (vertical distance right atrium to strain gauge at the upper leg). At this venous pressure the venous system is still at its steep compliant part of the venous pressure-compliance curve, which indicates that venous occlusion plethysmography represents arterial inflow and is not affected by venous compliance.

HUT-protocol

For a schematic presentation and time-table of the protocol see . Measurements started after complete instrumentation and at least 30 minutes after cannulation. During the experiment leg blood flow measurements were performed during three subsequent HUT-sessions. Each session consisted of 5 minutes of supine blood flow measurements. Measurements were continued during the subsequent 5 minutes with the subject in 30° HUT position.

During the first session NaCl 0.9% was infused continuously to measure the effect of HUT on leg blood flow under baseline conditions. The HUT-session was repeated during complete blockade of the α-adrenergic receptor by continuous infusion of phentolamine in a dose of 12 μg/min/dl upper leg volume. Propranolol was coinfused with phentolamine at a dose of 2 μg/min/dl to prevent unopposed β-adrenergic vasodilatation during α-adrenergic blockade. At this dose propranolol does not affect the haemodynamic parameters.[15, 24] Phentolamine will increase baseline blood flow and this in itself may affect the leg vasoconstrictor response to HUT, since there is a large vasomotor reserve available for vasoconstriction. This has been referred to as the vasoconstrictor reserve.[25, 26] Therefore, an additional HUT was performed to control for this by increasing supine blood flow values during infusion of sodium nitroprusside (SNP) in a dose of 0.2 μg/min/dl. So, SNP was infused in order to serve as a vasodilator control for phentolamine. Moreover, it was used to distinguish between the effect of specific α-adrenergic receptor blockade by phentolamine versus the non-specific effect of an altered baseline blood flow by vasodilatation.

During the experiment NaCl 0.9% was infused during the first HUT session. The second HUT was always performed in the presence of SNP. The third and final HUT was performed during infusion of phentolamine-propranolol. This fixed

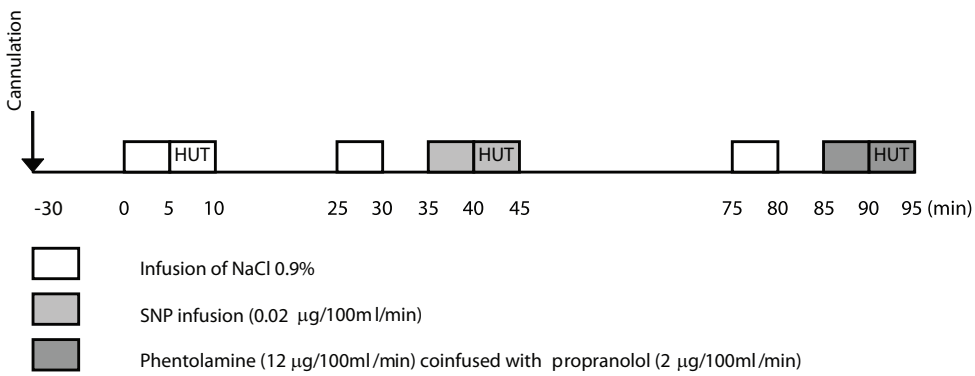

Figure 1. Schematic representation of the experimental protocol. HUT: 30° head-up tilt

sequence was chosen to prevent carry over effects because the time to wash out for SNP is shorter than for phentolamine-propranolol. To ensure return of blood flow to baseline values each HUT-session was preceded by measurements of supine leg blood flow measurements with infusion of NaCl 0.9% during 5 minutes. During the whole protocol, infusion rate was kept at 20 μl/min/dl.

Cold pressor test

Ten minutes after completing the last HUT a cold pressor test was performed in six of the control individuals to confirm that phentolamine was able to block the α-adrenergic receptors completely. Phentolamine in a dose of 12 μg/min/dl was coinfused in the femoral artery with propranolol (2 μg/min/dl) during 5 minutes to perform baseline measurements. After that the hand was immersed into water of 4°C for 3 minutes Bilateral leg blood flow measurements continued and heart rate and intra-arterial blood pressure were recorded continuously.

Time-control experiment

In another group of six healthy normotensive individuals (age: 29 ± 6 years; 4 men, 2 women) a time-control experiment was performed. The same *protocol* as described above (three subsequent HUT-sessions), without intra-arterial cannulation and drug infusion, was used to examine the effect of time and three repetitive HUT-sessions.

Drugs and solutions

Phentolamine (Regitine®, 10 mg/ml, Novartis Pharma BV, Arnhem, The Netherlands), and propranolol (Inderal®, 1 mg/ml, Zeneca Farma BV, Ridderkerk, The Netherlands) were dissolved in NaCl 0.9% at the beginning of each experiment. Sodium nitroprusside (department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre) was dissolved in glucose 5% and protected against light.

Data analysis

Upper leg blood flow (LBF) in ml/min/dl was calculated as the slope of the volume change curve. Because of a cuff inflation artifact during the first second, the slope from 2 – 6 seconds after cuff inflation was used. LBF values obtained during the last 2 minutes in supine position and during the last 2 minutes of HUT were averaged to represent LBF at baseline and during HUT, respectively. To calculate steady state heart rate and mean arterial pressure in response to HUT, values obtained during the last 2 minutes of HUT were averaged.

Upper leg vascular resistance (LVR) was calculated as mean arterial pressure (MAP) in mmHg divided by LBF in ml/min/dl and expressed in arbitrary units (AU). For these measurements we used mean arterial blood pressure measurements of the portapres device, because these were adjusted to hydrostatic pressure during HUT, assuming no net change in perfusion pressure when the leg is in dependent position[27, 28].

During the cold pressor test the mean of the three highest values of LVR in the infused and non-infused leg was used to calculate the percent change in LVR from baseline.

Statistics

Results are expressed as the mean ± SEM. Baseline leg blood flow values were not normally distributed across the SCI group. Therefore, possible differences in baseline measurements of leg blood flow (LBF) and leg vascular resistance (LVR) between SCI and controls were assessed by means of a Mann-Whitney U-test.

Because HUT-induced vasoconstriction, defined as the absolute increase in LVR, were normally distributed for both groups, parametric tests were used to test for differences in vasoconstrictor responses. Possible differences between the groups were identified by an unpaired t-test. A paired Student's t-test was used to test for different HUT-induced vasoconstriction within the groups between SNP, saline and phentolamine infusion. Hemodynamic effects in response to HUT for each HUT-session and each group, separately, were tested using a paired t-test.

The effect of time on hemodynamics and responses to HUT in the non-infused leg of controls and during the time-control experiment were tested using a one-way repeated measures ANOVA. Because LVR in response to the cold pressor test was not normally distributed, especially for the non-infused leg, possible change in LVR in response to the cold pressor test for the infused and non-infused leg, separately, were identified using a Wilcoxon's signed rank test. (Statistical Package for Social Sciences (SPSS) 14.0). Statistical significance level was set at 5% (two sided).

Results

Baseline characteristics

Baseline supine leg blood flow (LBF) and leg vascular resistance (LVR) of the infused leg did not differ between the groups (Table 2).

α-adrenergic contribution to HUT-induced vasoconstriction (Figure 2)

Controls

The HUT-induced vasoconstriction was 12 ± 2 AU (62 ± 13%) during infusion of saline. During infusion of the non-selective α-blocking agent, phentolamine, HUT still caused vasoconstriction (10 ± 2 AU; 66 ± 13%), and this response did not differ from HUT-induced vasoconstriction during saline infusion (Figure 1).

Because supine LVR was lower during infusion of phentolamine compared to saline a more pronounced response to HUT during infusion of phentolamine can be expected (see method section). To further analyse this confounding effect of differences in baseline LVR between saline and phentolamine infusion an increase in baseline supine blood flow was induced by sodium nitroprusside (SNP) infusion prior to HUT. Supine LVR during infusion of SNP decreased to 12 ± 2 AU, which significantly differed from the values obtained during both saline (23 ± 4 AU; P=0.002) and phentolamine infusion (16 ± 3 AU; P=0.019). The HUT-induced vasoconstriction during infusion of SNP (18 ± 2 AU; 165 ± 21%; P=0.002 and P=0.04, respectively) was significantly higher than during infusion of phentolamine and saline.

Spinal cord-injured individuals

The HUT-induced vasoconstriction during infusion of SNP or phentolamine did not differ, but tended to be significantly lower during saline infusion within SCI subjects (P=0.055 and P=0.065 for comparison saline-phentolamine, and saline-SNP, respectively) (Figure 2). Like in controls supine LVR was significantly lower during phentolamine and SNP infusion than during saline infusion. During infusion of phentolamine supine LVR did not differ between the groups. The vasoconstrictor responses upon HUT during saline, SNP and phentolamine infusion did not differ between the groups (saline: SCI: 7 ± 3 AU (n=8), controls: 12 ± 2 AU; SNP: SCI: 16 ± 2 AU, controls 18 ± 2 AU; phentolamine: SCI: 15 ± 3 AU, controls: 10 ± 2 AU)

Blood pressure and heart rate in response to HUT

In controls, MAP increased in all HUT sessions, but in SCI a HUT-induced increase in MAP was only observed during saline infusion (Table 2). In controls heart rate did not increase from supine to HUT

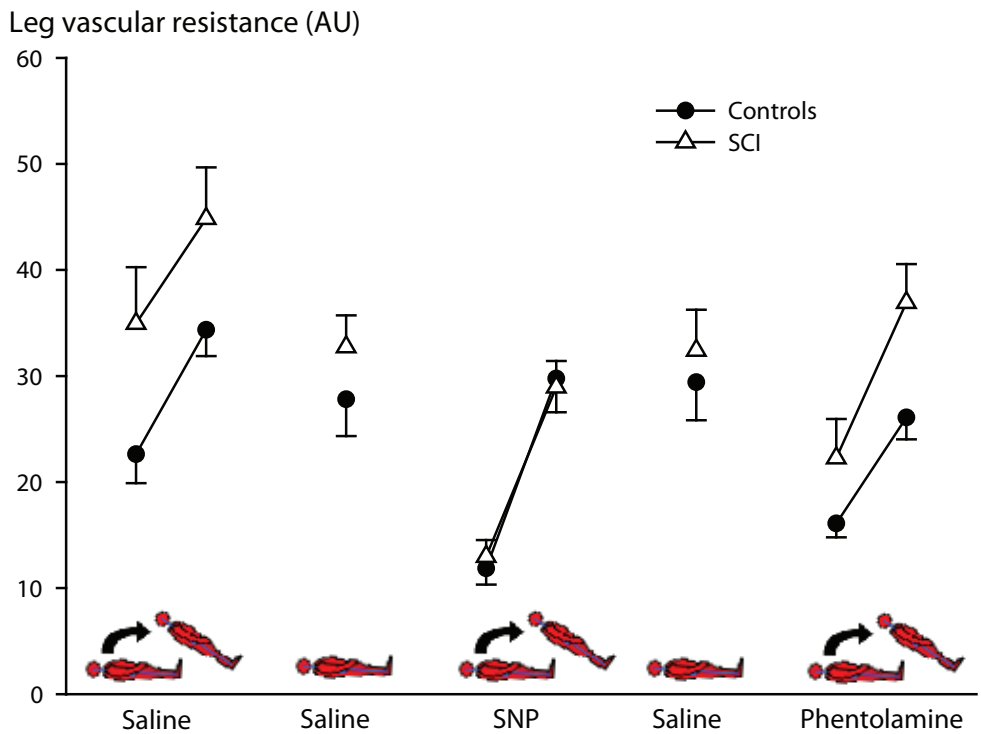

Figure 2. Leg vascular resistance in arbitrary units (AU) ± SEM in the infused leg of controls and spinal cord-injured individuals (SCI). This figure clearly demonstrates that during infusion of phentolamine head-up tilt still caused vasoconstriction, and the vasoconstriction during infusion of phentolamine was independent of intact sympathetic baroreflex control since head-up tilt-induced vasoconstriction in SCI was comparable with controls.

Table 2. Mean absolute values ± SEM for all parameters.

Controls							
		LBF		MAP	LVR		HR
Dose of drugs		Infused	Non-infused		Infused	Non-infused	
Saline	Supine	4.2 ± 0.5	4.1 ± 0.6	83 ± 4	23 ± 4	24 ± 3	58 ± 2
	HUT	2.7 ± 0.2	2.5 ± 0.3	88 ± 5	34 ± 2	39 ± 4	58 ± 2
Saline	Supine	3.8 ± 0.5	3.7 ± 0.5	91 ± 4	28 ± 3	32 ± 6	56 ± 2
SNP	Supine	8.2 ± 1.0	3.1 ± 0.5*	87 ± 3	12 ± 2	33 ± 5*	59 ± 1
	HUT	3.4 ± 0.3	2.1 ± 0.3*	93 ± 4	30 ± 3	54 ± 8*	62 ± 2
Saline	Supine	3.8 ± 0.4	3.5 ± 0.4*	98 ± 4	30 ± 4	33 ± 5*	55 ± 1
Phent	Supine	6.2 ± 0.5	3.4 ± 0.6*	94 ± 4	16 ± 3	34 ± 5*	62 ± 2
	HUT	4.1 ± 0.3	2.4 ± 0.2*	102 ± 5	26 ± 2	47 ± 6*	68 ± 2
Spinal cord-injured individuals							
		LBF		MAP	LVR		HR
Dose of drugs		Infused	Non-infused‡		Infused	Non-infused‡	
Saline	Supine	3.4 ± 0.6	3.0 ± 0.8	95 ± 5	35 ± 4	47 ± 10	57 ± 3
	HUT	2.5 ± 0.3‡	2.1 ± 0.5	101 ± 6	45 ± 5‡	60 ± 8	61 ± 3
Saline	Supine	3.4 ± 0.5	3.0 ± 0.7	102 ± 7	33 ± 3	41 ± 5	58 ± 3
SNP	Supine	7.9 ± 1.0	3.7 ± 0.9	92 ± 6	13 ± 2	30 ± 4	67 ± 4
	HUT	3.6 ± 0.5	3.4 ± 0.5	96 ± 6	29 ± 2	50 ± 7	71 ± 7
Saline	Supine	3.5 ± 0.6	3.2 ± 0.8	99 ± 4	32 ± 4	39 ± 6	56 ± 4
Phent	Supine	5.3 ± 0.9	3.7 ± 0.7	97 ± 4	22 ± 3	30 ± 4	62 ± 3
	HUT	2.9 ± 0.4	2.5 ± 0.5	98 ± 5	37 ± 4	43 ± 4	68 ± 3

* n=9 ‡ n=8

Phent = phentolamine; SNP = sodium nitroprusside; HUT = 30º head-up tilt; LBF = leg blood flow in ml/min per dl; MAP = mean arterial pressure in mmHg; LVR = leg vascular resistance; HR = heart rate in beats per minute.

during saline, but showed an increase in response to HUT during infusion of SNP and phentolamine (4.6% ± 0.9, P=0.001; and, 10.2 % ± 1.2, P<0.001, respectively). In SCI, heart rate tended to increase in response to HUT during saline and phentolamine infusion (7.8% ± 3.8, P=0.073, and 10.6% ± 6.2, P= 0.099, respectively), but did not increase during infusion of SNP.

Non-infused leg in controls and time-control experiment

Throughout the experiment **supine** LBF during saline infusion did not change. In

supine position LVR, in both the infused and non-infused leg, and mean arterial pressure increased throughout the whole experiment (Table 2). These results are consistent with the time-control experiments (data not shown).

In the time-control experiment absolute changes in LVR in response to HUT became significantly less with subsequent HUT-manoeuvres (1st: 19 ± 5 AU, 2nd: 17 ± 3 AU, 3rd: 10 ± 3 AU; P=0.014) In the non-infused leg of the control subjects absolute changes in response to HUT did not decrease throughout the experiment (LVR: saline: 16 ± 3 AU, SNP: 21 ± 5 AU, phentolamine: 14 ± 4 AU).

Confirmation of complete α-adrenergic blockade by the cold pressor test (n=6)

Complete α-adrenergic blockade was confirmed by a cold pressor test. In the phentolamine infused leg a slight, but irrelevant, increase in LVR was observed (phentolamine: 19 ± 3 AU; phentolamine and cold pressor test: 20 ± 2; P=0.046), while in the non-infused leg the cold pressor test induced an increase in LVR from 31 ± 6 AU to 53 ± 12 AU (P=0.026).

Discussion

In the present study the α-adrenergic contribution to head-up tilt induced vasoconstriction in the leg was assessed by infusing an α-adrenergic blocking agent – phentolamine – into the femoral artery in supine and head-up tilt position. The major finding is that after pharmacological blockade of the α-adrenergic receptor, leg-vasoconstrictor responses are still present upon head-up tilt (HUT) in humans, irrespective of an intact sympathetic baroreflex. Other mechanisms, such as the myogenic response, and stimulation of non-adrenergic receptors by sympathetic neurotransmitters that are co-released with norepinephrine, possibly contribute to leg vasoconstriction during postural stress. This study reveals that redundant mechanisms compensate for the absence of α-adrenergic vasoconstriction in the leg of healthy volunteers during postural stress. In spinal cord-injured individuals these redundant mechanisms do not depend on an intact baroreflex.

Phentolamine, a non-selective α-adrenoceptor blocker, was infused into the femoral artery to block the α-adrenergic receptor. Nevertheless, HUT still induced leg vasoconstriction comparable to that during saline infusion. Could incomplete α-adrenoceptor blockade explain this preserved HUT-induced leg vasoconstriction? Using a similar dose, a maximal and comparable vasodilator effect was achieved in previous studies, for both controls and spinal cord-injured individuals (SCI), indicating complete α-adrenoceptor blockade in the leg vascular bed.[15, 24] Moreover, during an endogenous sympathetic stimulus (the cold pressor test) vasoconstriction was abolished in the phentolamine infused leg as compared to the non-infused leg. This confirms that intra-arterial infusion of phentolamine achieved effective intrasynaptic drug concentrations.

A possible inhibiting effect of phentolamine on leg vasoconstriction could have been masked by phentolamine-induced vasodilatation prior to HUT. Therefore, HUT was also performed under SNP infusion. Supine vasodilatation induced by phentolamine was less than during SNP, and the HUT-induced vasoconstriction was smaller during phentolamine than during SNP. Although a small confounding effect of differences in supine leg vascular resistance cannot be ruled out, this does not explain the preserved leg vasoconstriction during HUT under phentolamine infusion. In addition, the time-control experiment demonstrated that during consecutive HUT procedures leg vascular responses subside. Therefore, the lack of difference in HUT-induced vasoconstriction during infusion of phentolamine (third) and saline (first) only strengthens our conclusion that HUT-induced vasoconstriction is to a large extent preserved during α-adrenergic blockade.

Spinal cord-injured individuals exhibited a similar HUT-induced vasoconstriction compared with controls during infusion of SNP, when supine blood flow values were comparable between the groups. Former observations where leg vasoconstriction, in either skin or muscle vasculature, in response to postural change was hardly influenced by *central* sympathetic blockade[2] (epidural[29] or proximal nerve blockade,[4] acute sympathectomy[2] or in spinal cord-injured individuals[5-7, 30]) suggested that the venoarteriolar reflex is responsible for the observed vasoconstriction. The venoarteriolar reflex involves α-adrenergic sympathetic nerves.[9,29] However, the present study shows that in SCI, where HUT-induced vasoconstriction relies on local mechanisms solely, leg vasoconstriction is not affected by local α-adrenergic blockade. This observation questions the importance of the venoarteriolar reflexes in HUT-induced vasoconstriction or, alternatively, makes the α-adrenergic origin of the venoarteriolar reflex uncertain.[31]

Besides α-adrenergic neurotransmitters, other neurotransmitters, such as ATP and neuropeptide Y, are released simultaneously with norepinephrine by the sympathetic nerve endings and can cause vasoconstriction by activating P_{2x} receptors and Y_1 receptors, respectively, as has been assessed in animal research.[32] When the sympathetic nervous system is stimulated by Lower Body Negative Pressure (LBNP) in humans, blockade of the α-adrenoceptor did not abolish, but reduced, vasoconstriction in the forearm arterioles.[11] Complete elimination of vasoconstriction with bretylium revealed the presence of a certain amount of non-adrenergic sympathetic vasoconstriction in humans. In the forearm these non-adrenergic sympathetic mechanisms could not fully compensate for the absence of α-adrenergic vasoconstriction, whereas the present study showed that in the leg the HUT-induced vasoconstrictor response was completely compensated during α-adrenoceptor blockade. This discrepancy may be related to the HUT-induced change in transmural pressure in the leg, eliciting the myogenic response, which is absent in the forearm during LBNP.

The co-release of other neurotransmitters in spinal cord-injured individuals is not

likely, since they have no central control of baroreflex activity which is confirmed by the lack of a rise in levels of plasma noradrenaline upon HUT.[33, 34] Non-adrenergic sympathetic mechanisms can, therefore, not explain the observed leg vasoconstriction during HUT in SCI. Most likely, the observed vasoconstriction during α-adrenoceptor blockade upon HUT in SCI, can be explained by myogenic vasoconstriction in arterioles in response to the increase in lower limb transmural pressure.[35] Sympathoexcitation may facilitate myogenic responses to changes in transmural pressure.[36] The present study, however, showed that even when the sympathetic α-adrenergic contribution to vascular tone is blocked, and in individuals who lack supraspinal sympathetic control, HUT-induced leg vasoconstriction is still largely preserved. This suggests that when normal sympathetic regulatory mechanisms fail, like in SCI, the myogenic response comes into play.[13]

Questions may arise about the involvement of humoral vasoconstrictor factors, like renin-angiotensin, vasopressin, aldosterone, and epinephrine. No changes of these hormones have been found within 10 minutes of HUT in healthy controls and paraplegics, whereas a slight increase was observed in quadriplegics.[21, 37, 38] Since in the present study HUT only lasted for 5 minutes and all, but one, were paraplegic individuals humoral mechanisms will not explain the observed vasoconstriction.

Conclusions could only be drawn on the vasoconstriction in the leg vascular bed and not on other vascular beds, i.e. the splanchnic area, which also plays a role in restoring blood pressure upon HUT. SCI maintained, but did not enhance blood pressure as observed in controls in response to HUT during infusion of phentolamine and SNP. This is in accordance with former observations in SCI, [7]where both blood pressure and total peripheral vascular resistance did not increase. In most of our SCI subjects (6 to 8 out of 9) the splanchnic area is not under baroreflex control. Therefore, HUT-induced increase in splanchnic vascular tone may be blunted, since it is not known whether compensatory mechanisms, like for example myogenic responses, may also play a role in the splanchnic area.

Limitations

Thirty degrees HUT provokes mild orthostatic stress and it may be considered to what extent the sympathetic outflow to the leg is increased by unloading the baroreceptors. Although sympathetic nervous activity was not measured, 30° HUT causes a significant increase in muscle sympathetic nerve activity,[23] and results in significant cardiovascular effects.[20-23] Although in controls heart rate did not show a statistically significant increase upon HUT during infusion of saline in the steady state situation (the average over the last 2 min. of each HUT session), heart rate initially increased upon HUT (from 59 ± 2 beats/min just before HUT to 63 ± 2 beats/min during the first minute into HUT), indicating autonomic changes compatible with baroreflex activation. Moreover, a more pronounced HUT-induced increase in heart rate was observed during infusion of both SNP and phentolamine supporting that the

orthostatic challenge is not reduced during subsequent head-up tilts and sufficient to alter autonomic outflow. In addition, the most profound changes in leg blood flow as measured by echo Doppler and venous occlusion plethysmography were observed from supine to 30° HUT and did not further change when HUT continued to 45° and 70°.[19]

This study shows that in humans under conditions of effective α-adrenergic blockade, leg vasoconstriction upon head-up tilt is still preserved, regardless of an intact baroreflex control of the leg vascular bed. Thus, in humans redundant mechanisms compensate for the absence of α-adrenergic vasoconstriction in the leg vascular bed that is subject to orthostatic changes. In humans with intact baroreflex control of the leg vascular bed stimulation of non-adrenergic receptors and myogenic mechanisms may come into play, whereas in spinal cord-injured individuals, who lack baroreflex control of the leg vascular bed, head-up tilt-induced vasoconstriction relies most likely on the myogenic response.

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Abstract

Background – Local vasoconstriction plays an important role in maintaining blood pressure in spinal cord-injured individuals (SCI). We aimed to unravel the mechanisms of local vasoconstriction (venoarteriolar reflex (VAR) and myogenic response) using both limb dependency and cuff inflation in SCI and compare these with control subjects.

Methods – Limb blood flow was measured in 11 male SCI (age: 24-55 year) and 9 male controls (age: 23-56 year) using venous occlusion plethysmography in forearm and calf during three levels of (1) limb dependency, and (2) cuff inflation. During limb dependency vasoconstriction relies on both the VAR and the myogenic response. During cuff inflation the decrease in blood flow is caused by the VAR and by a decrease in arterio-venous pressure difference, whereas the myogenic response does not play a role.

Results – At the highest level of leg dependency the percent increase in calf vascular resistance (mean arterial pressure/calf blood flow) was more pronounced in SCI than in controls (SCI: 186 ± 53% and controls: 51 ± 17% (P=0.032)). In contrast, during cuff inflation no differences were found between SCI and controls (SCI: 17 ± 17% and controls: 14 ± 10%). Percent changes in forearm vascular resistance in response to either forearm dependency or forearm cuff inflation were equal in both groups.

Conclusion – Local vasoconstriction during dependency of the paralyzed leg in SCI is enhanced. The contribution of the VAR to local vasoconstriction does not differ between the groups, since no differences between groups existed for cuff inflation. Therefore, the augmented local vasoconstriction in SCI during leg dependency relies, most likely, on the myogenic response.

Chapter 7

Local vasoconstriction in spinal cord-injured and able-bodied individuals

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Introduction

In individuals with a spinal cord injury (SCI), sympathetic brainstem control of the vascular bed below the level of the spinal cord lesion is deficient. Despite the absence of *central* sympathetic control,[1] these patients show a remarkable orthostatic tolerance during postural stress and, in particular the paraplegic individuals, maintain blood pressure during orthostatic challenges.[2] Moreover, preservation of tilt-induced leg vasoconstriction in these individuals has been reported.[3-5]

Local mechanisms have to play an important role in the tilt-induced increase in leg vascular tone in SCI. The myogenic response, which is triggered when transmural pressure across an arteriole is increased, seems to be partly responsible for the increase in leg vascular resistance in healthy able-bodied individuals upon head-up tilt[6] but could also explain vasoconstriction in SCI individuals. Former research suggests that the local venoarteriolar reflex (VAR),[7-10] which is elicited when venous transmural pressure exceeds 25 mmHg, contributes to the observed tilt-induced vasoconstriction in the leg muscle of SCI.[4, 5] Originally the VAR was thought to be mediated via a sympathetic α-adrenergic mechanism, but recent evidence suggests a non-adrenergic mechanism.[11]

The mechanisms of local vasoconstriction, i.e. VAR and myogenic response, have been evaluated during venous congestion by *cuff inflation*, and by lowering the limb below heart level (*limb dependency*).[12-15] During cuff inflation the reduction in blood flow may not be solely due to the VAR. A decrease in local perfusion pressure between arteries and veins may also reduce blood flow upon

Table 1. Characteristics of the spinal cord-injured individuals.

Subject	Age, Years	Level of spinal lesion	Complete-ness of lesion (ASIA score)	Time since injury, Years	Spastic paresis	Sweating below spinal lesion	Smoking	Medication
1	24	T5	A	4	+	-	-	-
2	44	T5	A	22	+	+	-	-
3	46	T10	A	6	+	-	+	Antibiotic
4	33	T11	A	6	-	-	+	-
5	46	T8	A	11	+	-	-	-
6	42	T12	A	20	-	+	-	Furadantine 50mg
7	37	T4	A	9	+	-	-	Detrusitol, Microlax
8	54	T9	A	15	+	+	-	-
9	43	T9	B	21	+	-	-	Bisoprofumaraat 20mg
10	51	C6	B	21	+	-	-	-
11	44	T10	B	3	+	-	-	Oxybutynine HCL5, Sirdalud 2mg, Baclofen

cuff inflation. It was previously found that upon lowering the hand or foot below heart level venous as well as arterial pressure rise equally in the hand and foot, respectively.[16, 17] So, assuming that limb dependency does not affect perfusion pressure, the consequent reduction in blood flow may not be solely due to the VAR via an increase in venous transmural pressure, but may also be due to the myogenic response, which is elicited when arteriolar transmural pressure increases (Figure 1). By use of limb dependency and cuff inflation of the leg, Okazaki *et al* calculated that in healthy men the VAR and the myogenic response contribute 55% and 45%, respectively, to local postural skin vasoconstriction.[12]

Because of the lack of sympathetic baroreflex control below the level of the spinal cord lesion, SCI individuals depend more on local vasoconstriction mechanisms during orthostatic challenges. Therefore, in this study we aimed to unravel the different local vasoconstriction mechanisms in SCI individuals by comparing the vasoconstriction in SCI individuals with able-bodied control subjects in response to both limb dependency and cuff inflation above (forearm) and below (calf) the level of the spinal lesion. Based on previous research,[18] demonstrating the highest initial decrease in cutaneous blood flow upon leg dependency in SCI individuals, we hypothesize that the vasoconstriction to both limb dependency and cuff inflation is more pronounced in SCI individuals than in controls because of enhancement of both myogenic and venoarteriolar vasoconstriction.

Table 2. Baseline characteristics.

	SCI (n=11)	C (n=9)	P-value
Age, y	42 ± 2	38 ± 1	0.384
Body mass, kg	82 ± 4	80 ± 3	0.712
Height, m	1.83 ± 0.02	1.83 ± 0.01	0.785
SBP, mmHg	119 ± 2	126 ± 3	0.04
DBP, mmHg	77 ± 3	82 ± 2	0.122
MAP, mmHg	91 ± 2	96 ± 2	0.06
CBF, ml·100ml^{-1}·min^{-1} (*)	3.0 ± 0.5	2.0 ± 0.2	0.086
CVR, AU (*)	38 ± 6	44 ± 6	0.446
FBF, ml·100ml^{-1}·min^{-1} (*)	4.4 ± 0.8	5.2 ± 0.8	0.467
FVR, AU (*)	30 ± 6	20 ± 3	0.138
Exercise, h/wk	1.8 ± 2.2	1.8 ± 1.6	0.964

Values are mean ± SEM. (*) Mean of two baseline values; SCI, spinal cord-injured individuals; C, controls; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; CBF, calf blood flow; CVR, calf vascular resistance; FBF, forearm blood flow; FVR, forearm vascular resistance.

Methods

Subjects

Eleven spinal cord-injured individuals (SCI) and nine able-bodied controls (all men) volunteered to participate in this study. Individual baseline characteristics of the SCI are shown in Table 1, and baseline characteristics of both groups are presented in Table 2.

The SCI continued their medication throughout the study. The study was carried out according to the principles of the Declaration of Helsinki. All subjects gave their written informed consent before participation and the study was approved by the Hospital Ethics Committee.

Measurements

Calf and forearm blood flow were measured by electrocardiography-triggered venous occlusion plethysmography using mercury-in-silastic strain gauges (E-4 Hokanson, Hokanson, Bellevue, WA.). The electrically calibrated gauges were stretched around the thickest part of the right forearm and calf and connected to the plethysmograph (Loosco, Amsterdam, The Netherlands). The "plethysmography" cuff was wrapped around the right upper leg and arm (10-cm width) and connected to an adjustable air pressure source (E-20 Hokanson Rapid Cuff Inflator and AG-101 Cuff Inflator Pressure source, Hokanson). A double occlusion cuff arrangement was used to measure limb blood flow during increases of local venous pressure. We, therefore, placed a second cuff (=congesting cuff) proximal to the "plethysmography" cuff, that was connected to a manometer to raise local venous pressure during "cuff inflation" (see Figure 1 for set-up). A finger cuff was attached to the middle phalanx of the left third finger, which was kept at heart level, in order to measure finger arterial blood pressure using Portapres (TNO, The Netherlands). Data were collected beat to beat during the experiment at a sampling rate of 200 Hz. A built in expert system, physiocal, was in operation to establish and adjust a proper volume clamp setpoint. Heart rate was recorded, beat-to-beat, from a 3-lead ECG.

Protocol

Subjects were asked to refrain from drinking coffee, tea, cola or alcohol in the 18 hours before testing, and not to eat 2 hours before testing. All individuals had emptied their bladder 1.5 hours prior to testing to minimize the possibility of any reflex sympathetic activity from bladder filling on vascular tone.

All tests were performed in the afternoon with the subjects in supine position in a quiet temperature-controlled room.

Each experiment consisted of four measurements: forearm blood flow as well as calf blood flow were measured during both cuff inflation and limb dependency. The order of the measuring site (forearm or calf) and the order of the condition (limb dependency or cuff inflation) were randomized among the subjects.

Cuff inflation (Figure 1)

Calf and forearm blood flow were measured during an increase in supine local venous pressure by inflating a cuff (=congesting

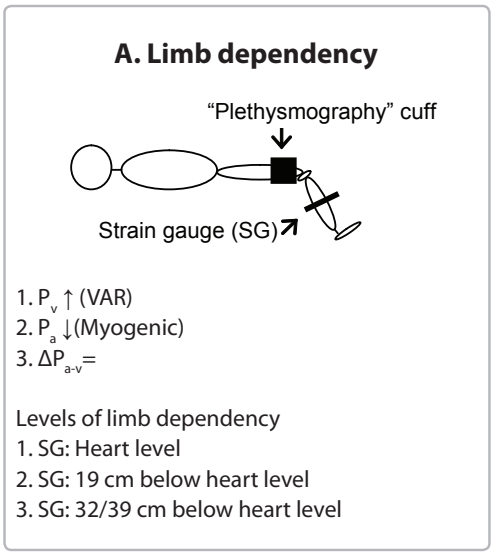

Figure 1A. During limb dependency venous (transmural) pressure (P_v) and arteriolar (transmural) pressure (P_a) rise equally due to an increase in hydrostatic pressure. Therefore, the arterio-venous pressure difference does not change. So, limb blood flow decreases by eliciting the venoarteriolar reflex (VAR) via an increase in P_v and the arteriolar myogenic response via an increase in P_a.

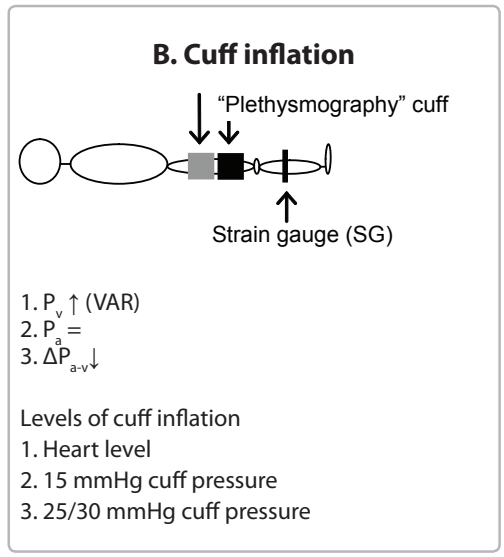

Figure 1B. Limb cuff inflation impedes venous return resulting in venous congestion. Subsequently P_v rises whereas inflation of the cuff minimally affects P_a. Therefore, arterio-venous pressure difference (ΔP_{a-v}) is decreased, resulting in a decrease in driving pressure. Since P_a is minimally affected, the arteriolar myogenic response is not likely to be activated. So, during cuff inflation limb blood flow decreases by a decrease in driving pressure (ΔP_{a-v}) and by eliciting the VAR via an increase in P_v independently of the arteriolar myogenic response.

cuff) proximal to the "plethysmography" cuff. Congesting cuff pressure was set at different levels: baseline, 15 mmHg, and 25 mmHg for the arm; and baseline, 15, and 30 mmHg for the leg. Calf or forearm blood flow were first measured in supine position without increasing local venous pressure, with the limb just above heart level, for four minutes with the "plethysmography" pressurized intermittently to 40 mmHg.[19] To increase local venous pressure, the proximal congesting cuff at the arm and thigh were inflated to 15 mmHg for four minutes, which increases local venous pressure to a similar magnitude.[20-22] Blood flow was measured with the "plethysmography" cuff pressure adjusted to local venous pressure and was set at 55 mmHg. After four minutes, local venous pressure was raised to 25 mmHg and 30 mmHg for the forearm and calf, respectively, and blood flow was measured with the "plethysmography" cuff pressurized to 65, and 70 mmHg, respectively. For all individuals, diastolic blood pressure was more than 70 mmHg, so blood flow measured by venous occlusion plethysmography was not affected.[19]

Limb dependency (Figure 1)

In order to match the increase in local venous pressure during limb dependency with cuff inflation, the limb was lowered such that the strain gauge was 0, 19, and 32 cm below heart level for the forearm and 0, 19, and 39 cm below heart level for the calf. The hydrostatic pressure column in mmHg was calculated with the formula: 0.776*hydrostatic pressure in cm blood. Calf or forearm blood flow were first measured in supine position. Thereafter the limb was lowered such that the strain gauge was 19 cm below heart level and blood flow measurements continued for four minutes. Subsequently, blood flow was measured with the forearm and calf lowered to 32 cm and 39 cm below heart level, respectively. Cuff pressure for blood flow measurements was adjusted to the hydrostatic pressure column when the "plethysmography" cuff was below heart level. Due to a hip contracture or an increase in spasm in two SCI individuals, the limb was lowered to 32 and 36 cm, respectively.

With limb dependency, transmural pressure in the arterioles increases in accordance

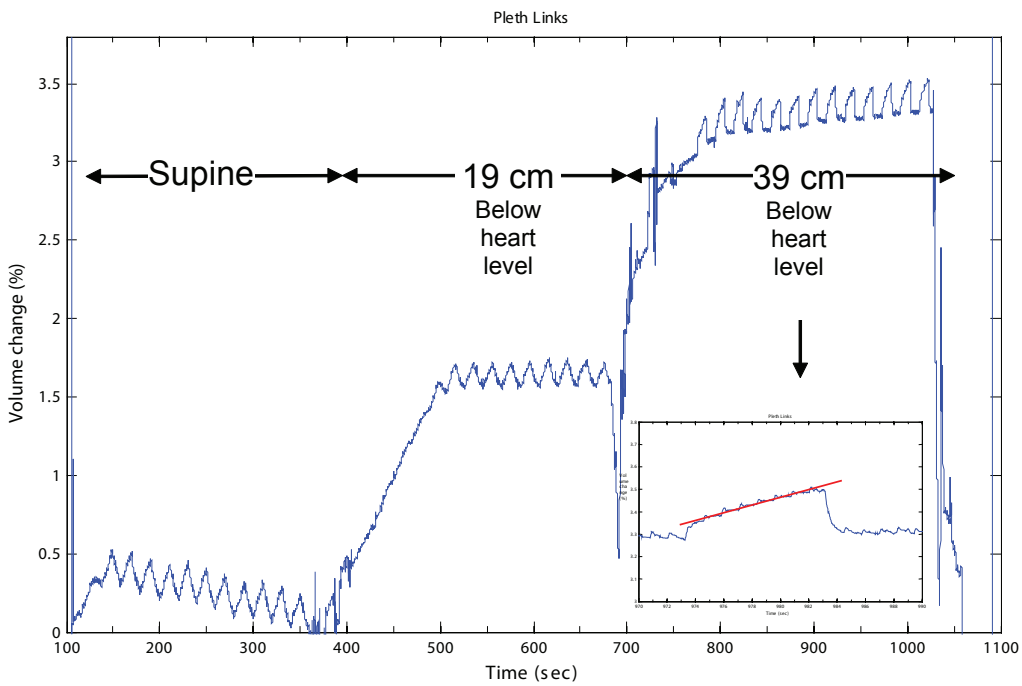

Figure 2. A typical plethysmographic tracing of the blood flow measurements during leg dependency of one control individual. The arrows indicate the levels of leg dependency. Notice that calf volume increases with increasing levels of leg dependency. Besides, looking at a typical plethysmographic tracing at 39 cm leg dependency, the increase in venous volume is linear during the first 5 seconds of cuff inflation, indicating that blood flow measurements using venous occlusion plethysmography during leg dependency are not compromised by a decrease in venous compliance.

with the hydrostatic pressure gradient between the heart and the limb.[16] Initially, the venous valves restrict the backward flow preventing limb venous pressure to increase to the same hydrostatic pressure. However, as blood continues to flow from the arteries into the dependent veins, they are filled with blood and the valves are forced open in a heart ward progression until there is an uninterrupted hydrostatic column between the central circulation and the limbs.[16] Thus, once all venous valves are open, the limb venous pressure is the sum of the dynamic pressure and the hydrostatic pressure.[16] Therefore, the premise of this study is that there is no net change in perfusion pressure during limb dependency.

When local venous pressure is 30 mmHg the venous system is still at its steep compliant part of the curve, which indicates that venous occlusion plethysmography represents arterial inflow and is not affected by venous compliance.[23] This is illustrated in Figure 2 where upon the highest level of leg dependency the increase in leg volume is linear during the first 5 seconds of cuff inflation, indicating that blood flow measurements using venous occlusion plethysmography during leg dependency are not compromised by a decrease in venous compliance.

Data analysis

Data were digitalized with a sample frequency of 100 Hz (MIDAC, Instrumentation Department, Radboud University Nijmegen Medical Centre, The Netherlands) and analyzed by a customized computer program (Matlab 6.1; Mathworks, Natick, MA, USA). Blood flow was calculated as the slope of the volume change over a 4-seconds interval. An initial steep rise, previously observed and attributed to a cuff inflation artifact,[24] was skipped. Registrations with artifacts, due to movements, were excluded. The blood flow values over the last 2 minutes in each stage of cuff inflation or limb dependency (12-17 slopes) were averaged and represent arterial inflow in $ml \cdot 100ml^{-1} \cdot min^{-1}$.

Baseline vascular resistance was calculated by dividing [(arterial pressure, Pa) – (local venous pressure, Pv)]/arterial inflow. Pa was assumed to be equal to MAP. For supine Pv 5 mmHg was used since pilot experiments and previous literature[20] demonstrates that when the limb is above heart level resting venous pressure varies between 0 and 7 mmHg. During cuff inflation vascular resistance was estimated from [(arterial pressure, Pa) – (local venous pressure, Pv)]/arterial inflow. Pa was assumed to be equal to MAP and Pv was equal to the cuff inflation pressure. Experiments in our laboratory, where venous pressure upon venous congestion was measured in the vena saphena magna at the ankle in a group of three healthy volunteers, have demonstrated that cuff pressure is a good index for local venous pressure (a cuff pressure of 30 mmHg corresponds with a venous pressure of 27 ± 2 mmHg). This is also in line with previous literature.[20-22] During limb dependency Pa and Pv were assumed to increase similarly with no change in perfusion pressure.[16, 17] In three volunteers, we verified the increase in venous pressure during limb dependency: local venous pressure during limb dependency,

Table 3. Hemodynamic responses during cuff inflation and limb dependency in the forearm and calf.

	Limb dependency							
	Spinal cord-injured individuals (n=11)				Control (n=9)			
Cm below heart level	0	19	F32/C39	max % D	0	19	F32/C39	max % D
Calf								
BF	3.0 ± 0.5	1.7 ± 0.2	1.3 ± 0.2	-44 ± 9	1.9 ± 0.2	1.7 ± 0.2	1.5 ± 0.2 (*‡)	-18 ± 8
VR	39 ± 8	67 ± 14	95 ± 22	186 ± 53	45 ± 6	58 ± 7	67 ± 9 (*)	51 ± 17 (†)
HR	64 ± 3	63 ± 3	62 ± 3		61 ± 2	61 ± 3	61 ± 3	
MAP	85 ± 3	88 ± 4	91 ± 4		84 ± 3	87 ± 3	87 ± 3 (*)	
Forearm								
BF	4.8 ± 1.0	2.3 ± 0.2	1.7 ± 0.2	-54 ± 7	5.3 ± 0.8	3.2 ± 0.4	2.5 ± 0.3 (*)	-45 ± 8
VR	30 ± 7	48 ± 8	70 ± 12	248 ± 94	19 ± 4	31 ± 4	39 ± 4 (*)	163 ± 57
HR	66 ± 3	66 ± 4	64 ± 4		61 ± 2	60 ± 2	60 ± 3	
MAP	89 ± 5	89 ± 4	93 ± 4		83 ± 3	84 ± 3	85 ± 3 (*)	
	Cuff inflation							
	Spinal cord-injured individuals (n=11)				Control (n=9)			
Inflation pressure (mmHg)	0	15	F25/C30	max % D	0	15	F25/C30	max % D
Calf								
BF	3.0 ± 0.5	2.7 ± 0.4	1.9 ± 0.2	-29 ± 8	2.1 ± 0.3	1.8 ± 0.2	1.3 ± 0.1(*)	-34 ± 4
VR	37 ± 7	34 ± 5	37 ± 6	17 ± 17	43 ± 5	43 ± 4	47 ± 4	14 ± 10
HR	63 ± 4	66 ± 3	62 ± 3		61 ± 3	60 ± 3	62 ± 3 (‡)	
MAP	85 ± 3	86 ± 3	86 ± 3		86 ± 4	86 ± 4	88 ± 4	
Forearm								
BF	4.0 ± 0.7	3.3 ± 0.6	2.6 ± 0.4	-34 ± 5	5.2 ± 0.9	4.4 ± 0.6	3.5 ± 0.4(*)	-30 ± 4
VR	28 ± 4	31 ± 5	35 ± 7	18 ± 7	20 ± 3	19 ± 3	21 ± 3	9 ± 6
HR	64 ± 3	63 ± 3	63 ± 3		61 ± 3	61 ± 3	62 ± 2	
MAP	87 ± 3	88 ± 3	89 ± 3		87 ± 4	87 ± 3	87 ± 4	

Values are mean ± SEM. BF, blood flow (ml·100ml^{-1}·min^{-1}); VR, vascular resistance (AU); HR, heart rate (min^{-1}); MAP, mean arterial pressure (mmHg); F, forearm; C, calf; max % D, maximal percent change from baseline (= 0); (*) statistically significant (P<0.05) cuff or limb dependency (=pressure) effect; (†) statistically significant (P<0.05) between the SCI and C group. (‡) statistically significant (P<0.05) group * pressure interaction.

measured invasively, with the ankle ranging from 39-54 cm below heart level, increased up to 40 mmHg, which corresponds with the calculated increase in venous pressure based on the height difference between ankle and heart.

Vasoconstriction was quantified by normalizing blood flow and vascular resistance during cuff inflation and limb dependency to baseline values and was presented as percent change from baseline.

Statistical analysis

All values are reported as means ± SEM, unless otherwise indicated. Baseline characteristics were tested using Student's t-test. Differences between the groups were analyzed by means of two way repeated measures ANOVA, with the pressure (levels of limb dependency or cuff inflation) as within subject factor and the presence of a SCI as between subject factor. The pressure factor represents the overall effect of limb dependency or cuff inflation. The pressure by group factor was used to test for differences of limb dependency or cuff inflation between the groups. Statistically significant differences were further analyzed by unpaired t-tests at the highest level of limb dependency or cuff inflation. Statistically significance level was set at $P<0.05$.

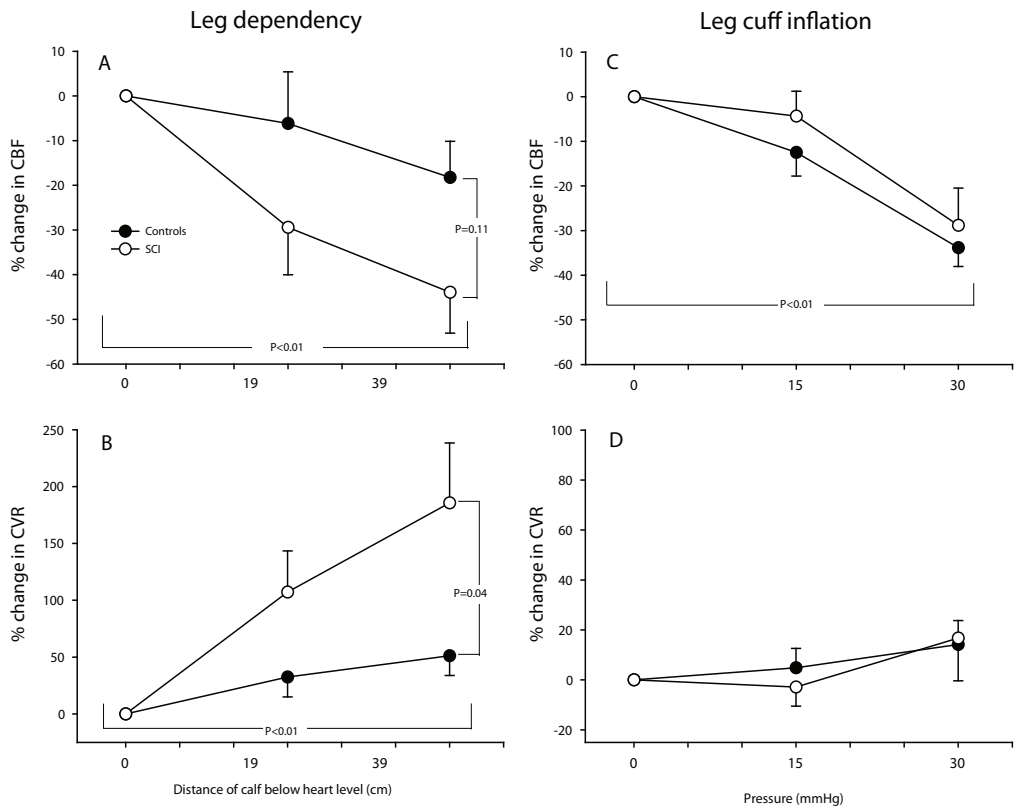

Figure 3. Percent changes in calf blood flow (CBF) (A) and calf vascular resistance (CVR) (B) during leg dependency, and percent changes of CBF (C), and CVR (D) during leg cuff inflation in spinal cord-injured individuals (SCI) and controls (C). Data are presented as means ± SEM. The brackets respresent the results of the repeated measures ANOVA (at the bottom of the figure for venous pressure and next to the figure for the group by pressure interaction).

Results

Baseline characteristics

The two groups did not differ with respect to age, body mass, height, diastolic blood pressure, mean arterial pressure (MAP), and hours exercise per week. Systolic blood pressure was significantly lower in SCI than in control subjects. Baseline calf and forearm blood flow and vascular resistance did not differ between the groups (Table 2).

Calf: SCI versus controls

Cuff inflation

During cuff inflation, calf blood flow decreased with increasing cuff pressure in both groups (Table 3; Figure 3C). However, calf vascular resistance did not change with increasing cuff pressure (Table 3; Figure 3D). The responses in both calf blood flow and calf vascular resistance during cuff inflation did not differ between the groups.

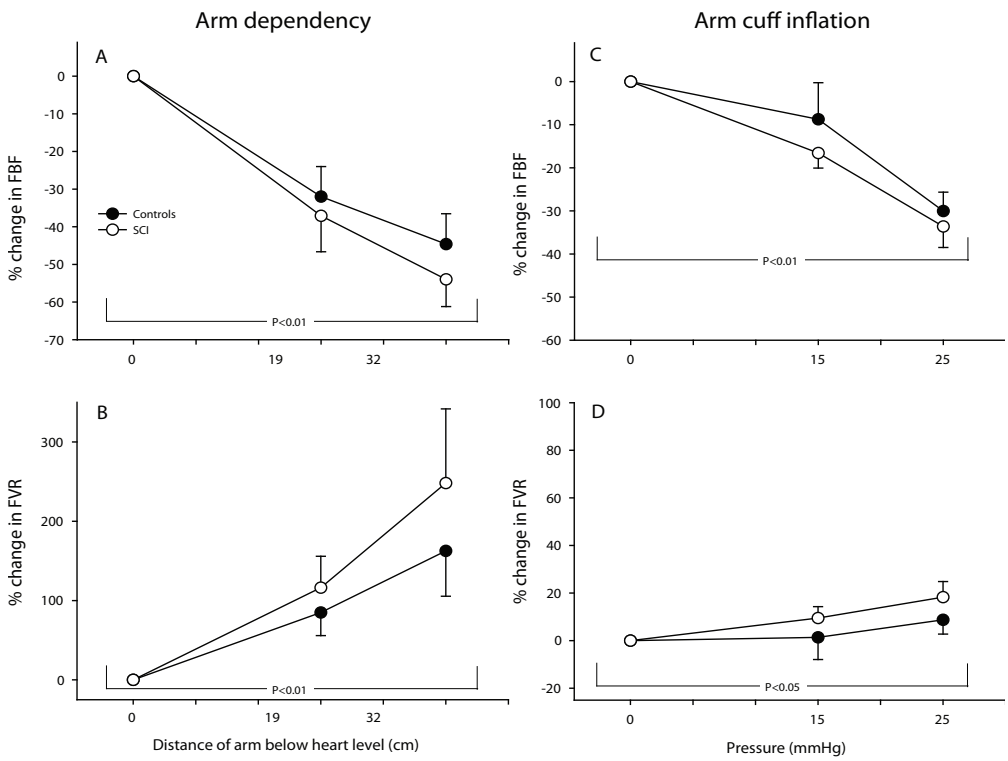

Figure 4. Percent changes in forearm blood flow (FBF) (A) and forearm vascular resistance (FVR) (B) during arm dependency, and percent changes of FBF (C), and FVR (D) during arm cuff inflation in spinal cord-injured individuals (SCI) and controls (C). Data are presented as means ± SEM. The brackets respresent the results of the repeated measures ANOVA (at the bottom of the figure for venous pressure and next to the figure for the group by pressure interaction).

Limb dependency

During leg dependency, calf blood flow decreased and calf vascular resistance increased in both groups (Table 3; Figure 3A). During the levels of leg dependency the percent decrease in blood flow tended to be higher in SCI (two-way repeated measures ANOVA; group*pressure interaction: P=0.11).

During leg dependency calf vascular resistance increased with increasing level of dependency (Table 3; Figure 3B). The relative increase in calf vascular resistance at the different levels of leg dependency was significantly higher in SCI than in controls (two-way repeated measures ANOVA; group*pressure interaction: P=0.04). With the calf 39 cm below heart level the percent increase in calf vascular resistance was 186 ± 53% in SCI, which was significantly higher than the 51 ± 17% increase in controls (Student's t-test: P=0.032).

Forearm: SCI versus controls

During arm dependency, forearm blood flow decreased significantly and forearm vascular resistance increased significantly in both groups. Changes in forearm blood flow and resistance did not differ between both groups (Table 3, Figure 4). During cuff inflation, forearm blood flow decreased and forearm vascular resistance increased statistically significant in both groups.

Heart rate and MAP

Heart rate did not change during limb dependency nor during cuff inflation. During forearm and leg cuff inflation, MAP did not change in both groups. During both arm and leg dependency MAP increased in both groups (P=0.012 for arm, and P<0.001 for leg) (Table 3).

Discussion

The results of this study show that vasoconstriction below the level of the spinal cord lesion in response to depending the leg is more pronounced in spinal cord-injured (SCI) individuals than in controls. In contrast, no differences in forearm vascular responses between the groups, i.e. above the level of the spinal cord lesion, were observed upon arm dependency. When the forearm as well as the calf were subjected to an increase in venous pressure during cuff inflation blood flow decreased equally in both groups, whereas in both groups the increase in vascular resistance was less marked.

Venous congestion by cuff inflation

Limb blood flow in response to venous congestion by cuff inflation decreased. Interestingly, the increase in limb vascular resistance upon cuff inflation was less marked; in the forearm an increase in vascular resistance was observed, whereas in the calf the increase in vascular resistance did not reach statistical significance. The consistency of this finding is underlined since it was found in both controls and SCI individuals. It was previously described that the reduction in blood flow within human skeletal muscle when subjected to venous congestion was attributed to a local vasoconstrictor mechanism, the venoarteriolar reflex (VAR), which is elicited when venous transmural pressure exceeds 25 mmHg.[9] To assess vasoconstriction upon

venous congestion, in the present and former studies[12, 15, 25] changes in perfusion pressure have been taken into account to calculate limb vascular resistance. The observations are ambiguous. In the cutaneous circulation of the human finger, vascular tone did not change upon venous congestion indicating that a venoarteriolar reflex mechanism is not operative in that specific region.[15] However, this is in contrast to an increase in vascular resistance upon venous congestion in the human forearm and calf skin.[12] There are several reasons for this striking difference. First, regulation of blood flow in the finger (apical skin) and forearm (nonapical skin) is different; sympathetic tone in the apical skin in a thermoneutral environment at rest is substantial, whereas nonapical skin at rest demonstrates little vasoconstrictor activity. So, the vasoconstrictor effect of the VAR in the preconstricted finger skin vascular bed may be marginal. Second, calculation of perfusion pressure differed between the studies. To calculate perfusion pressure Richardson *et al* [15] used capillary pressure, which is higher than venous pressure that was used in the present study and the study by Okazaki *et al*.[12] The consequent underestimation of vascular resistance may explain the lack of increase in vascular tone upon venous congestion in the study by Richardson *et al*.[15] However, calculation of vascular resistance in the present study, which is similar to that used by Okazaki *et al*,[12] can not explain the attenuated increase in vascular resistance upon venous congestion. Using venous occlusion plethysmography to measure blood flow a previous study described that a venous congestion of 40 mmHg caused an increase in calf vascular resistance of 25%.[13] Although a venous pressure of 25-30 mmHg used in the present study exceeds the threshold to elicit the VAR, it may explain the less marked increase in vascular resistance upon venous congestion. So, up to a venous congestion pressure of 30 mmHg the present study casts doubt on a leading role of an active venoarteriolar vasoconstriction mechanism in the arm or leg vascular bed. In other words, the reduction in limb blood flow upon venous congestion up to 30 mmHg seems to be mostly a passive effect caused by a reduced arterio-venous pressure difference with a minor role of additional active vasoconstriction mechanism.

Limb dependency

Upon forearm and calf dependency limb blood flow decreased in both groups. In contrast to the observations during cuff inflation, limb dependency resulted in a marked increase in limb vascular resistance. Upon limb dependency the increase in vascular tone is elicited by (i) the myogenic response via an increase in arteriolar pressure, and it was assumed that (ii) the increase in venous pressure will activate the VAR. However, taking into account the vascular responses during cuff inflation, one may question to what extent the venoarteriolar response contributes to the increase in limb vascular resistance upon depending a limb, since local venous pressure upon cuff inflation matches local venous pressure upon limb dependency and results in a local venous pressure of 25-30 mmHg. Hence, the increase in vascular resistance upon limb dependency in the present study seems to be predominantly

due to arterial myogenic vasoconstriction, which has been suggested before.[11]

Leg cuff inflation induced similar changes in calf vascular tone in SCI and controls. However, upon leg dependency, SCI showed a more pronounced increase in calf vascular resistance than controls, where no differences in forearm vascular responses were observed between the groups upon arm dependency. Since calf blood flow tended to be higher in the SCI group, a larger vasoconstrictor reserve in the SCI than in the control group could have influenced the results of the study. Therefore, results of matched supine blood flows of a subgroup of 5 SCI individuals and 5 control subjects are presented in Figure 5. Using these subgroups of subjects the trend towards an enhanced vasoconstriction during leg dependency in SCI is evident. In addition, the decrease in calf blood flow upon venous congestion in the subgroup of subjects is equal in both groups. This underlines our conclusion that local vasoconstriction upon leg dependency is enhanced in spinal cord-injured individuals. So, an exaggerated myogenic response, below the level of the spinal lesion, could explain the disparity in

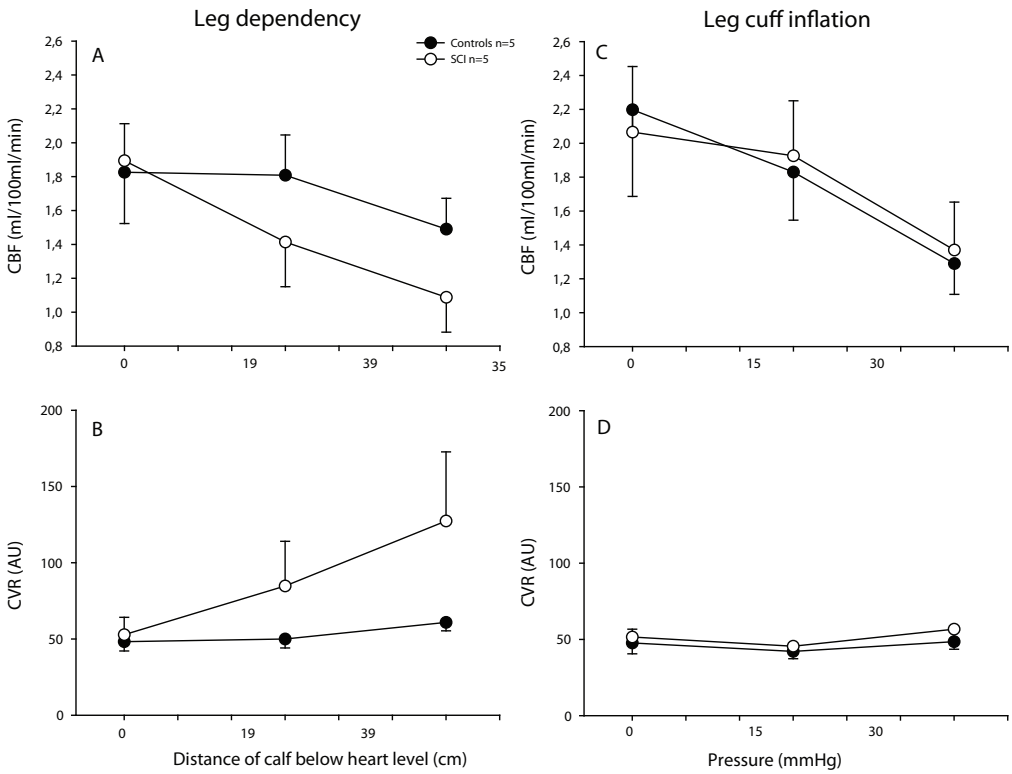

Figure 5. Absolute values of calf blood flow (CBF) and calf vascular resistance (CVR) (left panel) during leg dependency, and absolute values of CBF and CVR (right panel) during leg cuff inflation in a subgroup of five spinal cord-injured individuals (SCI) and five controls. Data are presented as means ± SEM.

vascular responses found in the calf upon leg dependency between SCI and controls.

The myogenic response is inherent to vascular smooth muscle. It is considered to be attributable to an increase in smooth muscle intracellular free calcium concentration, after depolarization of the smooth muscle cell. The myogenic response seems to be independent of metabolic, hormonal or neural influences.[26] However, recently it has been suggested that vascular sensory fibers are involved in mediating the myogenic response in rat mesenteric arteries.[27] C-fibre nerves are depolarized, via arachidonate metabolites generated by an increase in transmural pressure, and release vasoactive sensory neuropeptides. This could explain that lidocaine treatment abolished cutaneous vasoconstriction during limb dependency.[11, 12] The myogenic response is affected by chronic changes in transmural pressure. After rat hindlimb unloading, causing a decrease in transmural pressure in the hindlimbs and an increase of transmural pressure in the forelimbs, myogenic tone is attenuated in both arterioles of fast twitch fibers in the gastrocnemius muscle[28] and in mesenteric resistance arteries.[29] In contrast to hindlimb unloading, the calf muscles of the wheelchair bound SCI individuals are always dependent with a constantly high transmural pressure across the vessels due to the absence of the calf muscle pump and an invariable high hydrostatic pressure gradient. It has been suggested that elevation of transmural pressure, as in small cerebral arteries in rats subjected to hindlimb unloading[30] and in spontaneously hypertensive rats myogenic tone is enhanced.[31] So, the constantly high transmural pressure in calf muscles of SCI could increase myogenic responsiveness, which may contribute to the increase in peripheral vascular tone and maintaining blood pressure during orthostatic stress, and may explain the observations in the present study. The mechanisms whereby the myogenic response may be increased could involve alterations in components that transduces changes of transmural pressure into myogenic tone, by increasing the calcium content within the smooth muscle cell, such as for example stretch activated channels or voltage dependent Ca^{2+} channels.[32]

Clinical relevance

In SCI individuals, the importance of local vasoconstriction in withstanding orthostatic tolerance has been emphasized by former research.[3-5, 18, 33] The uniqueness of the present study is that mechanisms of local vasoconstriction were investigated without interference of baroreflex-mediated vasoconstriction by limb dependency and during venous congestion above and below the level of the spinal lesion. No differences between the groups were found in changes of calf blood flow and calf vascular resistance upon venous congestion, indicating that the reduction in blood flow upon venous congestion relies on a change in perfusion pressure and to a minor extent on the VAR, which are equal in both groups. Upon leg dependency, however, changes in calf blood flow and calf vascular resistance were more pronounced in the SCI group. This supports the idea that the enhanced local vasoconstriction in the paralyzed legs

of SCI individuals is most likely due to the myogenic mechanisms.

Limitations

The mechanisms of local vasoconstriction are investigated by both limb dependency and cuff inflation. This approach is based on several assumptions. We do not exactly know how arteriolar pressure is changed upon cuff inflation and limb dependency. Nonetheless, it may be possible for these two models to demonstrate differences that may reasonably be attributed to different mechanisms. For example, upon cuff inflation back pressure from the increased local venous pressure does exert some unknown influence on arteriolar pressure. These unknown mechanisms, however, do not differ between the control and spinal cord-injured group, and do, therefore, not influence the observations in the present study. In order to verify the assumptions made in the present study leg venous pressure under different conditions was measured invasively by cannulation of the vena saphena magna at the ankle in a group of three healthy volunteers. Like previous studies,[20, 22] where venous pressure was measured invasively, these experiments demonstrated that cuff pressure is a good index for local venous pressure (a cuff pressure of 30 mmHg corresponds with a venous pressure of 27 ± 2 mmHg).

We did not measure arterial pressure during venous congestion and assumed that arterial pressure remained similar to the period just prior to venous congestion. So, perfusion pressure is calculated as mean arterial pressure minus cuff pressure. As confirmed in three volunteers with invasive venous pressure measurements, leg dependency results in an increase in local venous pressure that corresponds with the calculated hydrostatic pressure gradient (see method section). We assumed that the hydrostatic pressure gradient causes a similar increase in the arteries[16] and that, therefore, perfusion pressure was not affected by limb dependency. This is confirmed in a study where arterial and venous pressure, both measured in the depending limb, changed linearly with the height of the column of blood.[17]

In conclusion, upon arm dependency, above the level of the spinal lesion, both groups exhibited similar vasoconstriction responses. However, upon leg dependency vasoconstriction was more pronounced in the paralyzed legs of SCI than in the legs of controls. No differences between the groups were observed upon cuff inflation, where the myogenic response is not involved. So, most likely the myogenic response is responsible for the augmented vasoconstriction upon leg dependency in SCI. Therefore, the myogenic response may play a pivotal role in the orthostatic tolerance in SCI, where sympathetic baroreflex control over the leg vasculature is absent.

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Chapter 8

General discussion and future implications

A spinal cord injury leads to numerous vascular adaptations below the level of the spinal lesion. Since supraspinal sympathetic control of the leg vascular bed is lost in spinal cord-injured (SCI) individuals, one might expect that blood flow in the legs is increased and that leg vascular resistance is decreased. However, the opposite is true; leg blood flow is decreased and leg vascular resistance is increased in SCI individuals. These vascular changes likely contribute to increased risk of secondary complications in SCI individuals, such as pressure ulcers and impaired wound healing in the paralyzed and therefore inactive lower part of the body. The mechanisms responsible for the increase in leg vascular resistance in SCI individuals are not completely understood. The studies described in this thesis aimed to elucidate the role of the α-adrenergic receptor and endothelial nitric oxide (NO) in the regulation of baseline vascular tone

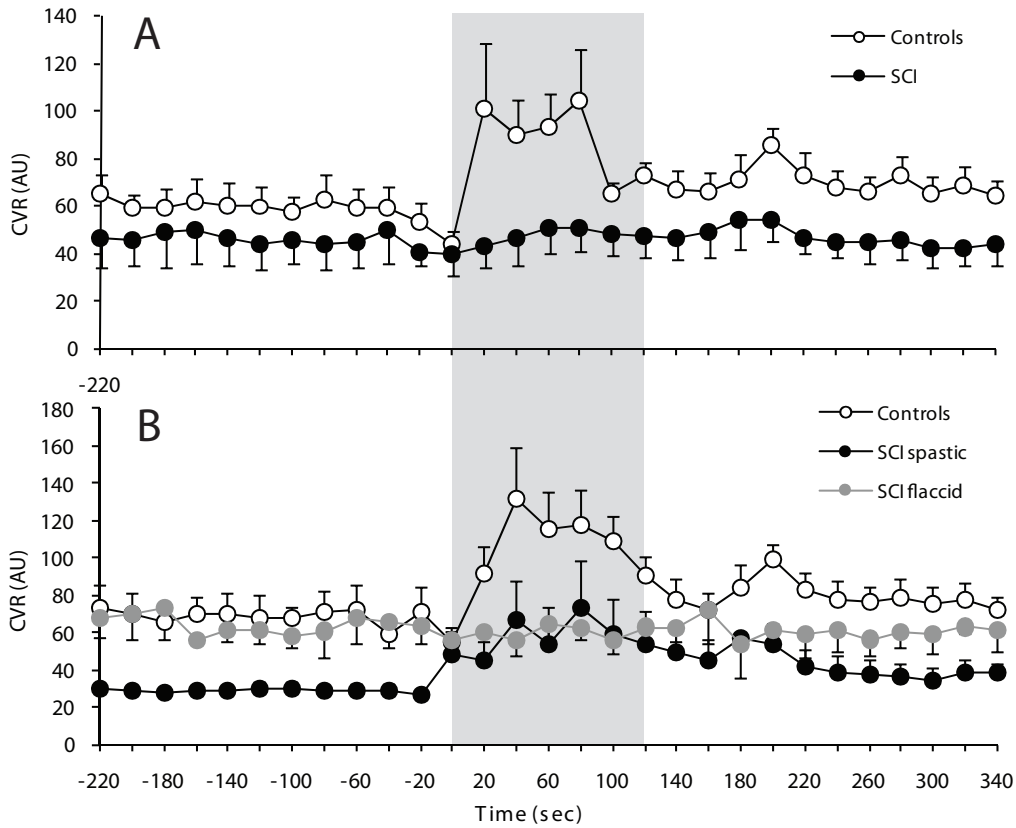

Figure 1. Calf vascular resistance (CVR) in response to a cold pressor test of the hand (A) and the foot (B) in control subjects and SCI individuals. The cold pressor test lasted for 2 minutes and is marked in grey. In response to the cold pressor test of the hand in controls, CVR increased, whereas in SCI, CVR did not change, that confirms disruption of supraspinal sympathetic control in SCI. When immersing the foot in ice water in both controls and SCI with a spastic paresis, CVR increased, whereas in SCI with a flaccid paresis and without spinal sympathetic activity, CVR did not change. (Data derived from this thesis, Chapter 2)

in SCI individuals. The results of those studies will be discussed in the first part of this chapter.

Although leg vasculature of SCI individuals is not under supraspinal baroreflex control, an orthostatic challenge will increase leg vascular resistance, a vascular response that is widely regarded to be regulated solely via the sympathetic nervous system. Accordingly, the second part of this chapter will discuss the potential mechanisms responsible for this observation.

Regulation of vascular tone in SCI individuals: resistance arteries

Preserved sympathetic α-adrenergic tone

In SCI individuals, the leg vascular bed is not under supraspinal control (Figure 1A), which is confirmed by the absence of baroreceptor related sympathetic nerve activity and very low baseline leg sympathetic nerve activity in these individuals.[1, 2] Therefore, the observations in *Chapter 2,* describing a preserved sympathetic α-adrenergic tone of the leg skeletal muscle vascular bed in SCI individuals, were surprising. In theory, several mechanisms may be responsible for the preserved α-adrenergic contribution to basal vascular tone in SCI individuals. First, the spinal cord injury may have been incomplete or supraspinal control of sympathetic outflow to the leg may have been restored. However, a cold pressor test, that drives sympathetic activity, did not result in an increase in leg vascular resistance below the spinal lesion level in SCI individuals as is shown in Figure 1A. This confirms a complete disruption of supraspinal sympathetic control. Although supraspinal sympathetic control is lost, spinal sympathetic reflex activity may be intact and could contribute to the preservation of α-adrenergic tone in SCI individuals. Figure 1B shows that in individuals with a flaccid pareses, stimulation of the sympathetic nervous system (cold pressor test of the foot) below the level of the spinal lesion does not evoke a change in calf vascular resistance. Therefore, in individuals with a flaccid pareses spinal sympathetic reflex activity is unlikely to contribute to the preserved α-adrenergic vascular tone. However, individuals with a spastic pareses demonstrate an increase in vascular resistance during a similar sympathetic stimulus. This indicates that spinal sympathetic reflexes are intact and may contribute to basal vascular tone. Although previous research[1, 2] showed very low leg sympathetic nerve activity in SCI individuals, one may hypothesize that inflation of a cuff to 50 mmHg during blood flow measurements by venous occlusion plethysmography act as a stimulus to trigger leg spinal sympathetic nerve activity. Since we did not measure muscle sympathetic nerve activity below the level of the spinal lesion, we can only speculate on this. Nonetheless, intra-arterial blood pressure can be used as derivative of sympathetic nerve activity. In all SCI individuals, regardless the level or type of the spinal lesion, blood flow measurements did hardly influence blood pressure. Therefore, the role of spinal

sympathetic reflexes in the regulation of basal vascular tone is probably not affected by our experimental procedures.

Finally, local release of noradrenaline in SCI individuals is reduced, which may induce a compensatory increase in either the sensitivity or number of α-adrenergic receptors or the efficacy of post-receptor signaling, leading to preservation of basal α-adrenergic tone.[3-5] Although there is hardly any scientific evidence, this explanation can be verified by observations in disease states in which the sympathetic control of the leg vascular bed is impacted, such as in patients with primary autonomic failure. Although leg vascular resistance is reported to be slightly decreased in autonomic failure patients,[6] suggestive for a reduced sympathetic vasoconstrictor tone, ~50% of these individuals suffer from supine hypertension.[7, 8] Interestingly, in patients with multiple system atrophy, residual sympathetic activity drives supine hypertension; both blockade of autonomic ganglia as well as administration of phentolamine reduced blood pressure not only by a decrease in cardiac output but also by influencing peripheral vascular tone.[7] Although α-adrenergic peripheral vascular tone is not quantified in autonomic failure patients, these results suggest that in autonomic failure patients supine hypertension reflects a state of increased postganglionic sympathetic neurovascular tone and, like in SCI individuals, the sympathetic nervous system still contributes to peripheral vascular tone. Since the preservation of α-adrenergic tone cannot be explained by an increase in noradrenaline release in both autonomic failure patients and SCI individuals, the sensitivity to endogenously-released noradrenaline must be increased.

In summary, in contrast to our hypothesis baseline α-adrenergic vascular tone is preserved in SCI individuals. The increased responsiveness of the α-adrenergic receptor to noradrenaline, released systemically or locally by the sympathetic nerve, seems to be most likely to explain our results. However, the role of spinal sympathetic reflexes can not be completely ruled out.

The role of the endothelium in the regulation of baseline vascular tone in SCI individuals

Nitric oxide (NO) is an important anti-atherogenic molecule. Therefore, decreased endothelial function, characterized by a reduced bioavailability of NO, has been proposed as an important early event in the pathogenesis of atherosclerosis.[9] Since physical inactivity is an important and independent risk factor for atherosclerosis, we hypothesized that the NO availability is reduced in the inactive legs of SCI individuals. Previous animal data suggested that physical inactivity can reduce vascular expression of endothelial nitric oxide synthase (eNOS) and consequently lead to a reduction of NO bio-availability.[10] By use of intra-arterial administration of N^G-monomethyl-L-arginine (L-NMMA), a blocker of eNOS, NO availability and the contribution of NO to baseline vascular tone can be assessed. In contrast to our hypothesis, the results presented in *Chapter 3* indicate that the contribution of NO to baseline vascular tone is not impaired in the inactive legs of SCI individuals. Therefore,

a reduction in NO availability can not explain the increase in leg vascular tone in chronic SCI subjects.

The likely physiological stimulus to endothelial NO production has been identified as increased flow through the vessel lumen with acute NO-mediated vasodilatation tending to normalize shear stress. Changes in shear stress, as a result of exercise training or physical inactivity, may, respectively, improve or reduce the bioavailability of NO. Four weeks of cycling exercise caused an increase in basal NO production in the forearm vascular bed.[11] In contrast, baseline NO production in endurance trained athletes did not differ from controls, which suggests that long-term training does not affect NO production in the forearm vascular bed. In mice, physical inactivity leads to an initial decrease in baseline NO production in response to a low-flow state.[12] However, after 14 days of physical inactivity, baseline NO production was partly normalized while the decrease in arterial diameter has become mostly structural. So, short-term functional endothelial adaptations to normalize shear stress, lead to arterial remodelling and structural normalization of shear stress. Subsequently, endothelial function and NO availability return towards baseline levels.[13] In SCI individuals, structural vascular adaptations are accomplished with weeks.[14] Collectively, it may be possible that chronic SCI would have demonstrated an initial reduction in NO bioavailability in the acute phase after the spinal cord injury, but that after normalization of shear the contribution of NO to baseline vascular tone is not affected as described in *Chapter 3*.

Since the increase in basal vascular resistance in chronic SCI individuals is unlikely to be explained by the NO pathway, other endothelial factors may be involved. The role of the other endothelial vasodilating substances, i.e. prostacyclin and EDHF, have never been investigated in SCI individuals. Recently it has been demonstrated that the contribution of vasoconstrictive agent endothelin-1 (ET-1) to baseline leg vascular tone in SCI individuals is increased,[15] suggesting that ET-1 could be a regulator of changes in vascular tone during deconditioning.

Vascular adaptations in conduit arteries in SCI individuals: the role of NO

Endothelial function at conduit artery level can be assessed using flow-mediated dilatation (FMD), which represents the conduit artery dilatation that occurs in response to a 5-minute distal arterial occlusion. Our findings described in *Chapter 4* demonstrate an enhanced FMD response in SCI individuals, when FMD is expressed as change of baseline diameter, which is in accordance with a study by De Groot *et al.*[16] FMD is strongly dependent on arterial size,[17-19] as is shown in Figure 2. Therefore, larger relative FMD responses can be expected in SCI subjects, who have smaller baseline superficial femoral arteries, than in able-bodied controls. This strong link is suggested to result from differences in the level of shear rate during reactive hyperaemia, which is regarded as the eliciting stimulus of the FMD. It is proposed that

smaller arteries, exposed by virtue of their size to a great shear stress stimulus, exhibit enhanced dilator responses compared to arteries of larger dimension. Therefore, it has been proposed that the FMD response should be corrected for the eliciting stimulus by taking the post-occlusive area-under-the-curve of the mean wall shear rate.[20] After correcting FMD data in this manner, FMD is similar in controls and SCI subjects, indicating that FMD seems not to be affected by deconditioning.

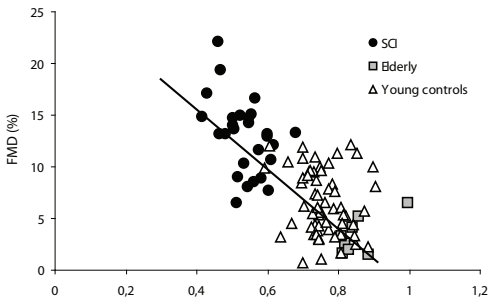

Figure 2. Relation between resting superficial artery diameter and flow-mediated dilatation in SCI individuals,[16] older men, and young control subjects. (Pearson correlation coefficient (r) = 0.696, P<0.001)

To gain better insight into the mechanisms responsible for the preserved FMD in the deconditioned legs of SCI individuals, experiments described in *Chapter 4* were performed. First, we aimed to assess whether the FMD response of the superficial femoral artery in SCI individuals is mediated exclusively by NO. The dilatation during the FMD response may be affected by vasodilators other than NO, such as prostacyclin or endothelium-derived hyperpolarizing factor, which could be co-released in response to an increase in shear stress after 5 minutes of ischaemia. Second, smooth muscle cell responsiveness to NO could be increased in response to deconditioning.[16, 21, 22]

For brachial and radial arteries it is known that, using a 5-minute ischemic period distal from the imaging site, FMD in healthy young subjects is mediated by endothelial-derived NO.[23, 24] However, the role of NO in the superficial femoral artery FMD is not known, neither in healthy subjects nor in SCI individuals. One cannot simply extrapolate the findings in upper limb conduit arteries to the superficial femoral artery, since leg and arm vasculature beds have been demonstrated to respond differently to endothelium-dependent and -independent vasodilator agents.[25] In *Chapter 4*, it was described that superficial femoral artery FMD in healthy men, after a brief episode of an increase in shear stress, was almost completely abolished during infusion of L-NMMA. In accordance with data in the upper limbs, these results demonstrate that the FMD response of the superficial femoral artery in healthy men, using 5 minutes of distal ischemia, reflects a NO-mediated endothelium-dependent vasodilatation. In SCI individuals, the FMD response during blockade of NO production by L-NMMA was similarly attenuated as in the able-bodied controls. Since NO-blockade could not completely block dilatation after cuff release, the contribution of other vasodilating agents to FMD in SCI individuals can not completely be ruled out. Nonetheless, the FMD of the superficial femoral artery in the deconditioned legs of SCI individuals

is predominantly NO-mediated.
Hindlimb unweighting in rats indicates that deconditioning can lead to a down-regulation of endothelial NO-synthase expression.[26] Accordingly, one explanation for the preserved FMD in the legs of SCI subjects relates to the possibility that smooth muscle cell sensitivity to NO is enhanced in response to deconditioning. Previous studies[16, 21, 22] concluded that deconditioning induced an increase in smooth muscle cell responsiveness of conduit arteries to NO. However, this is usually assessed using a single dose of sublingual administration of a NO donor, rather than by construction of a dose-response curve. In resistance arteries we already showed, as is described in *Chapter 3,* that deconditioning, as a result of a spinal cord injury or due to unilateral lower limb suspension, does not affect smooth muscle cell responsiveness. Recently, we published a study that demonstrates that superficial femoral artery dilatation to intra-arterial administration of three doses of SNP is not affected by deconditioning due to a spinal cord injury.[27] This suggests that NO sensitivity of the smooth muscle cells of conduit arteries in SCI individuals is not elevated. Furthermore, both resting diameter and maximal conduit artery vasodilator responses were less in SCI individuals, which demonstrates an inward remodelling of the superficial femoral artery in SCI individuals. In conclusion, conduit arteries supplying chronically deconditioned limbs seem to undergo structural changes, while vascular function of the same artery is normalized.

Baseline blood flow and vascular resistance in SCI individuals

In this thesis, different methods to measure blood flow have been used. In Table 1 pooled data from studies of blood flow measurements using venous occlusion plethysmography and Doppler ultrasound, conducted at the department of Physiology, Radboud University Nijmegen Medical Centre, are presented. Venous occlusion plethysmography primarily reflects muscle blood flow normalized for leg volume. Doppler ultrasound reflects blood flow within a specific conduit artery vessel and detects functional as well as structural vascular adaptations. Although Doppler ultrasound does not actually measure blood flow within the muscles, it may be expected that altered muscle blood flow will ultimately lead to changes in blood flow at conduit arteries level. Maximal post-occlusive reactive hyperaemia, as measured by Doppler ultrasound or venous occlusion plethysmography, has been proposed as a measure of structural changes in the microvasculature, such as capillaries.
Interestingly, using Doppler ultrasound superficial femoral artery blood flow as well as artery dimensions do not differ between SCI individuals and able-bodied subjects after correction for leg volume (Table 1).[28, 29] This finding suggests that in chronic SCI individuals, structural vascular adaptations and muscular atrophy have changed in a similar degree, and seem to have a strong functional link. From a physiological perspective, it would be interesting to examine whether vascular

Table 1. Pooled blood flow data using venous occlusion plethysmography and Doppler ultrasound.

	SCI	Controls	
Venous occlusion plethysmography			
ULBF (ml·100ml^{-1}·min^{-1})	2.7 ± 1.2 (n=26)	3.7 ± 1.4 (n=42)	↓
ULVR (AU)	41 ± 18 (n=26)	27 ± 8 (n=40)	↑
CBF (ml·100ml^{-1}·min^{-1})	2.9 ± 1.5 (n=21)	2.2 ± 0.7 (n=58)	=
CVR (AU)	42 ± 19 (n=21)	44 ± 19 (n=50)	=
Doppler Ultrasound			
CFA blood flow (ml·min^{-1})	238 ± 133 (n=56)	265 ± 106 (n=38)	=
CFA diameter (cm)	0.69 ± 0.11 (n=56)	0.97 ± 0.11 (n=38)	↓
SFA blood flow (ml·min^{-1})	109 ± 72 (n=39)	96 ± 39 (n=50)	=
SFA diameter (cm)	0.54 ± 0.06 (n=39)	0.76 ± 0.08 (n=50)	↓
After correction for total leg volume			
CFA blood flow (ml·min^{-1}·L^{-1})	34 ± 17 (n=19)	25 ± 10 (n=8)	=/↑
CFA diameter (cm·L^{-1})	0.10 ± 0.02 (n=19)	0.12 ± 0.01 (n=8)	=
SFA blood flow (ml·min^{-1}·L^{-1})	15.5 ± 8.0 (n=16)	8.5 ± 3.5 (n=16)	=/↑
SFA diameter (cm·L^{-1})	0.08 ± 0.02 (n=16)	0.08 ± 0.01 (n=16)	=

ULBF, upper leg blood flow; ULVR, upper leg vascular resistance; CBF, calf blood flow; CVR, calf vascular resistance; CFA, common femoral artery; SFA, superficial femoral artery.

structural adaptations precede changes in muscle volume, or vice versa or simultaneously, during the initial stage of deconditioning. Pooled blood flow data by venous occlusion plethysmography show discrepancies between thigh and calf blood flow in SCI individuals; thigh blood flow and vascular resistance are decreased and increased, respectively, whereas, in contrast to prior observations[30] no differences in calf blood flow and vascular resistance were found between SCI individuals and able-bodied subjects.

Based on these blood flow data, one may question whether the increase in leg vascular resistance reflects a structural (i.e. a decrease in number of arterioles and capillaries and/or a decrease in the diameter of the vessels) or functional adaptation (endothelium-derived factors, circulating humoral factors and/or sympathetic vascular regulation). As demonstrated in this thesis (*Chapter 2* and *3*), endothelium derived NO and α-adrenergic stimulation do not contribute to the increase in upper leg vascular resistance. Although the contribution of endothelin-1 to baseline vascular tone in SCI individuals is increased,[15] the increase in vascular resistance can also be explained by structural adaptations of the muscle vascular bed. The latter is supported by the fact that sympathectomy in rats results in an increase in vascular resistance, which is due to a decrease in density of the vascular network.[31] Moreover, a reduction in maximal vasodilatation during post-

ischemic hypaeremia in SCI individuals, as is shown in *Chapter 2* and *Chapter 4,* supports structural vascular changes. So, the increase in vascular resistance in SCI individuals can therefore be explained by a decrease in capillary density, and in part by functional changes at resistance artery level.

Denervation and deconditioning seem to inconsistently affect the decrease in capillary density in the legs of SCI individuals, since thigh vascular resistance is more increased than vascular resistance in the calf (Table 1). Variability of changes in muscle fibre type and associated changes in capillarisation may explain this apparent difference between areas of the lower limbs. Within a group of SCI individuals, the percentage slow, well capillarised, fibres in the vastus lateral muscle may vary between 0 and 62%.[32] Even within SCI individuals, fibre types can vary from the exclusively slow twitch fibres in the tibial anterior muscle to predominantly fast twitch fibres in the vastus lateralis.[33] Although speculative, changes in muscle fibre composition to merely fast twitch low oxidative fibres could explain the increase in thigh vascular resistance, whereas in our population of SCI individuals muscle fibre type in the calf seems to be less deteriorated. Variability in muscle fibre types could also explain the large variation found in calf as well as thigh blood flow within the group of SCI individuals.

Regulation of vascular tone in response to orthostatic challenges

The prompt increase in peripheral vascular resistance is the key event in blood pressure control during postural stress.[34] It was suggested that this response is primarily mediated by baroreflex sympathetic activation with subsequent α-adrenergic stimulation. However, in *Chapter 6*, we found that after pharmacological blockade of the α-adrenergic receptor in both healthy young men and SCI individuals, who lack baroreflex control of the leg vascular bed, leg-vasoconstrictor responses are still present in a similar magnitude upon head-up tilt in humans, irrespective of the presence of an intact sympathetic baroreflex. Apart from α-adrenoceptor stimulation, non-adrenergic mechanisms, such as purinoceptor stimulation by ATP and release of neuropeptide Y acting on Y_1 receptors, seem to contribute to the vasoconstrictor response after sympathetic stimulation.[35-37] However, this does not explain the preserved vasoconstriction upon postural change in SCI individuals,[38] where supraspinal sympathetic activation is lost. In these individuals other, presumably local, vasoconstriction mechanisms may compensate for the lack of supraspinal sympathetic control.

Local vasoconstriction mechanisms

It has been suggested that in able-bodied individuals during orthostasis as much as ~45% of the increase in systemic vascular tone is due to the venoarteriolar reflex (VAR).[39] Accordingly, the VAR potentially

contributes to the increase in vascular tone in SCI individuals during orthostatic challenge. However, from the results presented in *Chapter 7*, the contribution of the VAR to leg vasoconstriction upon postural changes seems to be minimal. It has been suggested that the VAR runs via a sympathetic axon where the receptors are presumably stretch receptors placed in the small veins and the effector site are the arterioles.[40] However, research to confirm a venoarteriolar axon by histobiochemical techniques is sparse. Only one study[41] showed that sporadic fibres from the dense plexus of sympathetic fibres around arterioles innervate the venules directly and return from the venule back to the arteriolar network. In light of the results of *Chapter 7*, where the VAR seems to play a minor role in local arteriolar vasoconstriction upon orthostatic challenges, the existence of a venoarteriolar axon should be questioned.

As was suggested in *Chapter 7*, other local vasoconstriction mechanisms such as the myogenic response could also explain vasoconstriction upon orthostatic challenges in SCI individuals.[42] In human *in vivo* research, local vasoconstriction upon orthostatic challenges is mostly examined by lowering the limb.[43-45] Apart from an increase in venous transmural pressure, this manoeuvre also increases transmural pressure on the arteriolar level. An increase in arteriolar transmural pressure causes a myogenic response. The mechanisms that sense the increase in arteriolar transmural pressure resulting in myogenic arteriolar vasoconstriction are not known. In theory, nerve endings in the adventitia, that are exposed to the influence of transmural pressure, can be the sensor element that detects the change in vessel wall tension and initiates the myogenic response. Human *in vivo* research suggested a neurally mediated response in the microcirculation of the skin since application of local anaesthetics abolished skin vasoconstriction upon lowering of the limb.[39, 40, 45-49] This is in contrast with an equal reduction in blood flow in response to limb dependency in normal innervated and denervated skin flaps.[50] Also in SCI individuals with a flaccid pareses, where nerves are completely atrophied, vasoconstriction upon lowering the leg was preserved (*Chapter 7*). Moreover, *in vitro* research showed that after elimination of a tonic nervous influence on the vessel by blocking nerve endings in the adventitia no alteration of the myogenic response was observed,[51] suggesting no neural involvement in the initiation of the myogenic response. On the other hand, neural influences, especially from adrenergic nerves, may modulate the myogenic response by increasing the strength of the myogenic response.[51] So, it is still of debate what role nerves play in the initiation or modulation of the myogenic response.

In conclusion, based on the results presented in *Chapter 7*, myogenic mechanisms are most likely responsible for the preserved vasoconstriction in SCI individuals upon postural changes. Although venoarteriolar reflexes seem not to initiate arteriolar vasoconstriction upon changes in vascular transmural pressure, the role of neurally mediated mechanisms via initiating or influencing the myogenic response can not be ruled out.

Redundancy

Blood flow upon orthostatic stress is tightly regulated and depends on many different mechanisms, that interact and can compensate when one of the regulatory mechanisms fail. Such a process is often referred to as redundancy, which reflects the versatility of blood flow regulation mechanisms. Research in the field of exercise physiology is being faced with this phenomenon; it is hard to find clear evidence for a dominant vasodilatating factor or factors that link muscle metabolism to exercise hyperaemia.[52, 53] In these studies, it is found that when one of the key pathways is blocked others can compensate and evoke a normal rise in blood flow during exercise. To our opinion, the study described in *Chapter 6* is the first that suggests redundant mechanisms in the regulation of peripheral vascular tone upon orthostatic challenges. In this perspective, it is of interest whether peripheral vasoconstriction is preserved in different groups of patients who suffer from various causes of orthostatic intolerance. Currently, most research to elucidate the mechanisms that cause orthostatic intolerance focuses on central regulatory mechanisms and baroreflex function. However, based on our findings in *Chapter 6*, peripheral vascular responses may be underexposed and play an important role in compensating baroreflex dysfunction. A better knowledge of these peripheral vascular mechanisms of vasoconstriction in response to orthostatic challenge may provide new targets for intervention to improve orthostatic tolerance of patients with baroreflex failure.

Implications of the studies and future directions

The studies presented in this thesis importantly contributed to the understanding of the regulation of leg vascular resistance in spinal cord-injured individuals. The contribution of nitric oxide to leg vascular tone is preserved in SCI individuals. Whilst, the sympathetic nervous system still contributes to basal vascular tone in SCI individuals, we demonstrated that the α-adrenergic tone is not increased in SCI individuals compared with able-bodied counterparts. Based on these results, we question whether other mechanisms contribute to the increased vascular resistance found in SCI individuals. Atrophy of skeletal muscles as a result of paralyses of the legs of SCI individuals coincide with vascular atrophy, not only of conduit arteries, but also of the muscular vascular bed. Functional vascular changes may precede the structural vascular changes. To support this view, in the chronic phase of a spinal cord injury, we found that endothelial function as well as smooth muscle cell function have returned to normal. Accordingly, we hypothesize that the increase in vascular resistance is merely caused by structural changes of the vascular bed, although the effect of endothelin-1 and angiotensin II on baseline vascular tone may not be ruled out. While recent data demonstrate a role for endothelin-1 to contribute to the increased vascular resistance in SCI subjects, the role of angiotensin II on baseline vascular tone, especially in SCI individuals with cervical or high thoracic spinal lesions where the renin-

angiotensin-aldosteron system is affected, should be topic for further research.

How can we explain these results in light of the spinal cord-injured individual complaining of cold legs or confronted with pressure sores and poor wound healing? The development of pressure sores is strongly dependent on skin tissue oxygenation, which is significantly lower in individuals with SCI than in controls.[54] An increase in arteriovenous shunting in SCI individuals[55] could explain the impaired skin tissue oxygenation. Arteriovenous shunting is hypothesized to increase susceptibility to local ischemia with prolonged pressure and subsequently increasing the risk of skin ulceration. Venous occlusion plethysmography or Doppler ultrasound are unable to examine the arteriovenous shunting since it measures total limb blood flow, which can be unaffected despite an increase in arteriovenous shunting with subsequent impairment of tissue oxygenation.
The occurrence of cold legs and impaired wound healing could also be explained by the lack of variation of leg blood flow due to inactivity. Furthermore, wound healing is negatively influenced in subjects who are frequently suffering from autonomic dysreflexia, i.e. increases sympathetic vascular tone and decreases blood flow. Finally, sitting in a wheelchair, the legs of SCI subjects are continuously in dependent position. The increase in transmural pressure while sitting will engage the myogenic response that decreases both muscle and skin blood flow. In theory, the increase in myogenic tone can result in a decrease in blood flow resulting in a decrease in tissue oxygenation of the leg skin and muscles in SCI individuals while sitting,[56] and can therefore be a primary factor in the causation and poor healing of pressure sores and wounds in SCI individuals. Taken together, even when vascular tone is not increased in SCI individuals, tissue oxygenation can be compromised by atrophy of the vascular bed resulting in an increase in vascular resistance, and/or by arteriovenous shunting.

The protective behaviours and risk factors associated with the development of pressure sores and poor wound healing in SCI individuals have been identified in several studies.[57-59] A healthy life style appears to be strongly associated with avoiding pressure sores. Moreover, previous cross-sectional and longitudinal studies of subjects with SCI showed that the prevalence of pressure ulcers increased as time since injury increased.[60, 61] Chronic denervated skin with long-term structural and physiologic (vascular) changes may play an important role in the development of pressure sores among subjects with longstanding spinal cord injury. So, future research could focus more on changes in skin blood flow (regulation) and skin tissue oxygenation in relation to the occurrence of pressure sores in recently injured SCI individuals and SCI individuals aged with a spinal cord injury. The pathophysiology behind the changes in skin blood flow can be unravelled by elucidating the role of autonomic dysregulation and other factors that could importantly influence skin vascular tone, like endothelial and myogenic

factors. Moreover, the effect of pressure and friction on skin blood flow and the recovery of skin blood flow after pressure is removed would be an interesting topic for further research. After the understanding of the changes in skin blood flow regulation and skin tissue oxygenation has improved, the effect of therapeutic interventions (i.e. functional electrical stimulation (FES), that can improve blood flow but also can influence the effect of pressure on skin blood flow by reducing muscular atrophy, or pharmacological interventions) on reducing secondary complications, like pressure sores or impaired wound healing, can be evaluated.

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Summary

Individuals with a complete spinal cord injury (SCI) lack sympathetic baroreflex control of the leg vascular bed. However, despite sympathetic denervation in the paralyzed legs, (1) leg vascular tone is almost twice as high as compared with able-bodied subjects, and (2) SCI individuals show head-up tilt induced increase in leg vascular tone to withstand orthostatic stress. The aim of the present thesis is to elucidate the role of the sympathetic nervous system and endothelial derived substances, in particular NO, in the regulation of baseline vascular tone in SCI individuals and able-bodied subjects. Furthermore, we tried to unravel the regulation mechanisms behind the preserved head-up tilt induced vasoconstriction in SCI individuals.

Chapter 2 focuses on the contribution of the sympathetic nervous system to baseline leg vascular tone in SCI individuals and controls. Upper leg vascular resistance responses to local infusion of incremental doses of phentolamine (a competitive antagonist of the α-adrenoceptor) into the femoral artery were determined in a group of SCI individuals and control subjects during local β-adrenergic receptor blockade with propranolol. In contrast to our hypothesis the phentolamine induced decrease in leg vascular resistance did not differ between SCI individuals and the control group. This indicates that the α-adrenoceptor-mediated vascular tone in the leg is preserved in SCI individuals, who lack sympathetic supraspinal control.

Decreased endothelial function, characterized by a reduced bioavailability of nitric oxide (NO), has been proposed as an important early event in the pathogenesis of atherosclerosis, since NO is an important anti-atherogenic molecule. Whether the increase in leg vascular tone due to deconditioning as a result of SCI can be explained by an attenuated release of endothelium derived NO is not known. Therefore, the study described in *Chapter 3* was designed to assess baseline NO availability in the leg vascular bed after extreme, long-term deconditioning (SCI individuals) as well as after moderate, short-term deconditioning (4 weeks of unilateral lower limb suspension, ULLS). The NO production was blocked by infusion of N^G-monomethyl-L-arginine (L-NMMA) in five incremental dosages into the femoral artery. Sodium nitroprusside (SNP) was infused to test the vascular responsiveness to NO. Deconditioning did neither alter the vasoconstrictor response to L-NMMA, nor the vascular responsiveness to NO. Therefore, it can be concluded that two human *in vivo* models of deconditioning show a preserved baseline NO availability in the leg skeletal muscle vascular bed.

Apart from the preserved baseline NO availability in SCI individuals at resistance artery level, the FMD, which is an non-invasive tool to assess endothelial function, in the paralyzed and thus inactive legs of SCI individuals is preserved as well. Therefore, in *Chapter 4*, we examined whether superficial femoral artery FMD in healthy subjects and in SCI individuals is NO-mediated. 5-minute FMD response in the superficial femoral artery both during infusion of saline and during infusion of the NO-synthase-blocker N^G-monomethyl-L-arginine (L-NMMA) was

assessed in a group of SCI individuals and control subjects. In controls, the FMD response was almost completely abolished during L-NMMA infusion. L-NMMA also significantly decreased the FMD response in SCI individuals. The effect of L-NMMA on FMD persisted in both groups after correction for the shear stress stimulus. So, superficial femoral artery FMD in response to distal arterial occlusion for a period of 5 minutes is predominantly mediated by NO in healthy men and in the extremely deconditioned legs of SCI individuals.

Venous occlusion plethysmography is a well-established method to measure limb blood flow in humans. However, its use for leg blood flow measurements during head-up tilt (HUT) is questionable, since the leg venous system is not empty and venous pressure exceeds zero. For that reason, in *Chapter 5*, the accuracy and reproducibility of venous occlusion plethysmography for measuring calf blood flow was assessed during different angles of head-up tilt. Calf blood flow measured with venous occlusion plethysmography was compared with superficial femoral artery blood flow as measured by Doppler ultrasound. Measurements of both methods correlated well and reproducibility of venous occlusion plethysmography was fair in supine position and 30° HUT. This indicates that venous occlusion plethysmography is an applicable tool to measure leg blood flow especially up to 30° HUT.

The prompt increase in peripheral vascular resistance, mediated by sympathetic α-adrenergic stimulation is the key event in blood pressure control during postural stress. Interestingly, this orthostatic leg vasoconstriction is preserved in SCI individuals, despite the fact that they lack baroreflex sympathetic control of the leg vasculature. This suggests that α-adrenergic mechanisms are not obligate in maintaining blood pressure during postural stress. So, in *Chapter 6*, the contribution of both *central* and *local* sympathetic α-adrenergic leg vasoconstriction to head-up tilt was assessed by including healthy individuals and individuals who lack baroreflex control over the leg vascular bed, due to a spinal cord injury. Upper leg blood flow was measured before and during 30° HUT throughout intra-arterial infusion of saline or the non-selective α-adrenergic receptor antagonist *phentolamine*, respectively. Interestingly, during infusion of phentolamine, HUT still caused vasoconstriction in controls as well as in the SCI group. So, effective α-adrenergic blockade, which was confirmed by a cold pressor test, did not reduce HUT-induced vasoconstriction, regardless of intact baroreflex control of the leg vasculature. Apparently, redundant mechanisms compensate for the absence of sympathetic α-adrenoceptor leg vasoconstriction in response to postural stress.

In *Chapter 7*, we aimed to unravel these redundant mechanisms, in particular the local mechanisms of vasoconstriction (venoarteriolar reflex (VAR) and myogenic response) using both limb dependency and venous stasis by cuff inflation in SCI individuals and compare responses with those in control subjects. Limb blood flow was measured in the forearm and calf during three levels of (1) limb dependency, and (2) cuff inflation. During limb dependency vasoconstriction relies on both the VAR

and the myogenic response. During cuff inflation the decrease in blood flow is caused by the VAR and by a decrease in arterio-venous pressure difference, whereas the myogenic response does not play a role. At the highest level of leg dependency, the percent increase in calf vascular resistance was more pronounced in SCI individuals than in controls. In contrast, during cuff inflation no differences were found between SCI individuals and controls. In the forearm, where the vascular bed is not influenced by the spinal cord injury, responses in vascular resistance were equal in both groups in response to both forearm dependency as well as forearm cuff inflation. Thus, local vasoconstriction during dependency of the paralyzed leg in SCI individuals is enhanced. The contribution of the VAR to local vasoconstriction does not differ between the groups, since no differences between groups existed for cuff inflation. Therefore, the augmented local vasoconstriction in SCI individuals during leg dependency relies, most likely, on the myogenic response.

In C*hapter 8*, the mechanisms responsible for the increase in basal leg vascular resistance, and the increase in leg vascular resistance upon orthostatic challenges in SCI individuals are discussed. Furthermore suggestions for future research are given.

Samenvatting

Het sympathisch zenuwstelsel is een deel van het autonome of onwillekeurige zenuwstelsel. Het speelt een grote rol in het reguleren van de doorbloeding in het menselijk lichaam. Bij een intact sympathisch zenuwstelsel resulteert toename van sympathische activiteit vanuit de hersenstam in een afname van doorbloeding. In "rust" is er een basale beïnvloeding van de bloedvaten door het sympathisch zenuwstelsel; als het sympathisch zenuwstelsel ter hoogte van de bloedvaten geblokkeerd wordt neemt, de doorbloeding toe. In rechtopgaande houding neemt de activiteit van het sympathisch zenuwstelsel toe. Dit leidt onder andere tot vermindering van doorbloeding en verhoging van de hartslag om een daling van de bloeddruk, orthostatische hypotensie genoemd, te voorkomen.

Bij mensen met een complete dwarslaesie is door het ruggenmergletsel het sympathisch zenuwstelsel onderbroken. De hersenstam kan dan via het sympathisch zenuwstelsel de bloedvaten onder het niveau van de dwarslaesie niet meer beïnvloeden. Door het niet goed meer functioneren van het sympathisch zenuwstelsel verwacht je een toename van de doorbloeding onder het niveau van het ruggenmergletsel. Echter bij mensen met een dwarslaesie blijkt het tegenovergestelde waar: de doorbloeding in de verlamde en daardoor inactieve benen is fors verminderd. De verminderde doorbloeding speelt een rol in het ontstaan van allerlei complicaties, zoals vertraagde wondgenezing en doorligwonden.

Opmerkelijk is dat mensen met een dwarslaesie, met name in de chronische fase, goed in staat zijn een rechtopgaande houding aan te nemen zonder flauw te vallen. Dit wordt orthostatische tolerantie genoemd. Ondanks dat het sympathisch zenuwstelsel niet meer onder controle van de hersenstam staat, vernauwen de bloedvaten in de benen nog wel bij mensen met een dwarslaesie die een rechtopgaande houding aannemen. Deze vaatvernauwing is mogelijk één van de mechanismen die de opvallende orthostatische tolerantie bij deze groep mensen kan verklaren.

Tot nu toe is nog niet bekend welke factoren bijdragen aan de verminderde doorbloeding in de benen in rust bij mensen met een dwarslaesie. Ook de mechanismen verantwoordelijk voor de afname van de doorbloeding in de benen tijdens rechtopgaande houding zijn nog grotendeels onbekend. Daarom is het doel van het huidig promotie-onderzoek de regulatie van de doorbloeding bij mensen met een dwarslaesie te ontrafelen, zowel in rust als tijdens een rechtopgaande houding.

In Hoofdstuk 1 wordt een globaal overzicht gegeven van de factoren die betrokken zijn bij de regulatie van de doorbloeding onder normale omstandigheden. Daarnaast komen verschillende mechanismen aan bod die mogelijk de verminderde doorbloeding bij mensen met een dwarslaesie kunnen verklaren. Niet alleen de rol van het sympathisch zenuwstelsel in de regulatie van de doorbloeding wordt belicht. Aan de hand van verschillende proefdier en humane modellen wordt ook het effect van fysieke inactiviteit op de functie van de bloedvatwand beschreven. Met name het endotheel, dat de binnenwand van het

bloedvat bekleedt, is van belang vanwege de productie van verschillende vaatverwijdende stoffen, waaronder stikstofmonoxide, en vaatvernauwende stoffen. De regulatie van de doorbloeding tijdens het recht op gaan staan en de mogelijke mechanismen die verantwoordelijk zijn voor de vernauwing van de bloedvaten in de benen van de mensen met een dwarslaesie worden eveneens besproken.

De bijdrage van het sympathisch zenuwstelsel aan de doorbloeding van de benen van zowel mensen met een dwarslaesie als controle personen is in Hoofdstuk 2 onderzocht. Fentolamine, een middel dat de sympathische zenuwoverdracht in de bloedvaten van de spieren blokkeert, werd in oplopende doseringen via de liesslagader toegediend en het effect op de doorbloeding werd gemeten. Onze hypothese was dat blokkeren van het sympathisch zenuwstelsel bij mensen met een dwarslaesie geen effect zou hebben op de doorbloeding. Echter tijdens het toedienen van fentolamine nam de doorbloeding niet alleen in de controle groep, maar ook bij de personen met een dwarslaesie toe. Heel verrassend blijkt bij mensen met een dwarslaesie het sympathisch zenuwstelsel, ondanks dat het niet meer onder controle van de hersenstam staat, nog wel bij te dragen aan de doorbloeding van de benen. Omdat de bijdrage van het sympathisch zenuwstelsel aan de doorbloeding niet verschilt tussen de beide groepen kan dit niet de verminderde doorbloeding van de benen verklaren bij mensen met een dwarslaesie.
Verminderde functie van het endotheel, dat wordt gekarakteriseerd door een verminderde stikstofmonoxide productie, speelt een belangrijke rol in de pathogenese van hart- en vaatziekten. Een belangrijke onafhankelijke risicofactor voor hart- en vaatziekten is lichamelijk inactiviteit. Of de verminderde doorbloeding in de verlamde en dus inactieve benen van mensen met een dwarslaesie verklaard wordt door een verminderde productie van stikstofmonoxide werd onderzocht in Hoofdstuk 3. De bijdrage van stikstofmonoxide aan de doorbloeding in de benen na langdurige fysieke inactiviteit als gevolg van een dwarslaesie was behouden. Dit zelfde geldt wanneer een "gezond" been gedurende korte tijd niet gebruikt wordt. Fysieke inactiviteit had geen effect op de vermindering van doorbloeding na remmen van de productie van stikstofmonoxide. Ook de vaatverwijding nadat stikstofmonoxide was toegediend was niet veranderd. Fysieke inactiviteit blijkt tevens de mogelijkheid van het bloedvat om te vernauwen niet te beïnvloeden, zoals blijkt na toedienen van een vaatvernauwend middel (angiotensine II). Concluderend kan dus, evenmin als het sympathisch zenuwstelsel, ook een verminderde productie van stikstofmonoxide de verminderde doorbloeding in de benen van mensen met een dwarslaesie niet verklaren. Naast het bepalen van de bijdrage van stikstofmonoxide aan de doorbloeding van de spieren om endotheelfunctie te bepalen, wordt FMD (Flow Mediated Dilatation) gebruikt om de endotheelfunctie van een groot bloedvat (bijvoorbeeld de liesslagader) te meten. De FMD wordt uitgedrukt als de procentuele toename van het bloedvat na een snelle toename van de

bloedstroom in het bloedvat; hoe groter de bloedvatverwijding, hoe beter de functie van het endotheel. Uit eerder onderzoek blijkt dat fysieke inactiviteit als gevolg van een dwarslaesie de endotheelfunctie van de grote bloedvaten in het been, uitgedrukt als FMD, behouden blijft. Hoewel er vanuit gegaan wordt dat de FMD veroorzaakt wordt door het vrijkomen van stikstofoxide, is dit echter in de grote beenvaten nooit onderzocht. Ook is niet bekend of de behouden FMD na inactiviteit volledig door stikstofoxide wordt veroorzaakt. Daarom is in Hoofdstuk 4 de FMD uitgevoerd zonder en met remming van de productie van stikstofmonoxide in een groep mensen met een dwarslaesie en in een controle groep. In beide groepen was na remming van de productie van stikstofmonoxide de FMD fors verminderd tot nagenoeg afwezig. Dus, zowel in controle personen als bij mensen met een dwarslaesie wordt de FMD veroorzaakt door stikstofmonoxide. De behouden FMD bij mensen met een dwarslaesie lijkt dus een behouden endotheelfunctie te weerspiegelen.

Veneuze occlusie plethysmografie is een veel gebruikte methode om doorbloeding in de armen of benen te meten. Echter, één van de voorwaarden voor het gebruik van deze methode is dat de venen (aderen) leeg zijn. Daarom was het gebruik van deze methode tijdens het staan voor het meten van de doorbloeding in de benen, wanneer de venen gevuld zijn met bloed, discutabel. In Hoofdstuk 5 is de toepasbaarheid en reproduceerbaarheid van veneuze occlusie plethysmografie om de doorbloeding van de kuit tijdens het gaan staan gemeten. Daartoe werd middels een kanteltafel verschillende posen aangenomen tussen liggen en staan (met een kantelhoek variërend van 0°-70°). Doorbloeding gemeten met veneuze occlusie plethysmografie werd vergeleken met de doorbloeding van een bovenbeenslagader gemeten met echo Doppler. De correlatie tussen beide methoden was goed en de reproduceerbaarheid van veneuze occlusie plethysmografie was redelijk tot goed in liggende positie en in 30º kanteling. Dus, veneuze occlusie plethysmografie kan toegepast worden voor het meten van de doorbloeding in het been tot een kantelhoek van 30º.

Tijdens het gaan staan is de vaatvernauwing door activatie van het sympathisch zenuwstelsel, en als gevolg daarvan de afname van doorbloeding in de benen en de buik van groot belang voor het op peil houden van de bloeddruk. Ondanks dat het sympathische zenuwstelsel bij personen met een dwarslaesie niet meer onder controle staat van de hersenstam, vernauwen de bloedvaten in de benen wel tijdens het gaan staan. Dit suggereert dat een goed werkend sympathisch zenuwstelsel niet cruciaal is voor het handhaven van de bloeddruk tijdens het gaan staan. In Hoofdstuk 6 wordt de bijdrage van het sympathisch zenuwstelsel aan de vaatvernauwing tijdens het gaan staan onder een kantelhoek van 30º bepaald in zowel personen met een intact sympathisch zenuwstelsel, als in personen met een dwarslaesie. De zenuwoverdracht van het sympathisch zenuwstelsel in het bloedvat van het been werd wederom geblokkeerd door fentolamine. Blokkade van de sympathische zenuwoverdracht had geen effect op de vaatvernauwing tijdens

het gaan staan, niet alleen bij de mensen met een dwarslaesie, maar ook niet bij de controle personen. Blijkbaar zijn andere mechanismen, bij het tekortschieten van het sympathisch zenuwstelsel, in staat de vaatvernauwing volledig te compenseren.
In Hoofdstuk 7 worden locale mechanismen in het been onderzocht die mogelijk verantwoordelijk zijn voor de vaatvernauwing tijdens het staan bij mensen met een dwarslaesie. Deze locale mechanismen zijn: 1) de venoarteriolare reflex die actief wordt wanneer de druk in de aderen toeneemt en via een sympathische zenuw een vaatvernauwing veroorzaakt; 2) de myogene respons: druktoename in het bloedvat doet gladde spiercellen samentrekken dat vaatvernauwing veroorzaakt. Bij de mensen met een dwarslaesie is de locale vaatvernauwing groter dan bij de controle personen. De bijdrage van de venoarteriolaire reflex aan de locale vaatvernauwing verschilt niet tussen de beide groepen. Dit wijst erop dat de toename van locale vaatvernauwing bij mensen met een dwarslaesie voor een groot deel is toe te schrijven aan de myogene response. Dit suggereert dat de myogene respons een rol speelt in de vaatvernauwing tijdens het gaan staan bij mensen met een dwarslaesie.

Hoofdstuk 8 bevat een algemene discussie over de regulatie van de doorbloeding bij mensen met een dwarslaesie en over de kennis die dit proefschrift daarover heeft bijgedragen.

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Lieve Tommy, het leven is met jou, en door jou, mooi!

Curriculum Vitae

Miriam Kooijman werd geboren op 11 mei 1973 in Werkhoven, gemeente Bunnik. Na het behalen van het VWO diploma aan het St Bonifatiuscollege in Utrecht begon zij aan de studie Gezondheidswetenschappen (momenteel Biomedische Wetenschappen) aan de Katholieke Universiteit Nijmegen (momenteel Radboud Universiteit Nijmegen). Zij stroomde door in het verkorte doctoraal programma van de studie Geneeskunde aan dezelfde universiteit na het behalen van haar doctoraal Gezondheidswetenschappen.

Tijdens haar studie Gezondheidswetenschappen deed zij onderzoek naar de anatomische "pitfalls" van triple osteotomie van het bekken op de afdeling Anatomie-Embryologie van de Radboud Universiteit in samenwerking met de afdeling Orthopaedie van de Sint Maartenskliniek in Nijmegen. Ook heeft zij stage gelopen op de afdeling Fysiologie, alwaar zij onderzoek deed naar het gebruik van Near Infrared Spectroscopy om doorbloeding te meten bij patiënten met "etalage"- benen. Daar werden de eerste schreden gezet naar het onderzoek dat geleid heeft tot het in dit proefschrift beschreven promotie-onderzoek.
Voor de afronding van haar medische opleiding heeft Miriam 3 maanden co-schap gelopen in Biharamulo Hospital, Tanzania. In 1999 behaalde zij het arts-examen, waarna zij eerst als poortarts in het Bernhoven Ziekenhuis in Veghel heeft gewerkt. In 2000 startte zij met de opleiding tot revalidatiearts in de Sint Maartenskliniek (opleiders: drs. HJM van Kuppevelt en prof. dr. ACH Geurts). Sindsdien heeft zij haar klinische opleiding afgewisseld met het doen van promotieonderzoek op de afdeling Fysiologie in samenwerking met de afdeling Farmacologie-Toxicologie onder begeleiding van prof. dr. MTE Hopman, prof. dr. P Smits en dr. GA Rongen. Bij de Trans European Scientific Contest, gehouden tijdens het twee-jaarlijkse congres van 'European Society of Physical and Rehabilitation Medicine' in 2004, ontving zij de tweede prijs voor haar mondelinge voordracht.

Op dit moment is Miriam bezig met de laatste fase van haar opleiding tot revalidatiearts. Na het afronden van haar opleiding gaat zij als revalidatiearts werken in revalidatiecentrum de Vogellanden in Zwolle waarbij haar interesse uitgaat naar de kinderrevalidatie.
Miriam is getrouwd met Tommy Visscher. Zij hebben twee kinderen, Isa en Suze.