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

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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). For this purpose, seven SCI were compared with seven matched controls. Additionally, seven 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. Baseline leg vascular resistance tended to be higher in SCI compared with controls (37 ± 4 *versus* 31 ± 2 arbitrary units (AU), $P = 0.06$). Deconditioning altered neither the vasoconstrictor response to L-NMMA (increase in resistance in SCI *versus* controls: $102 \pm 33\%$ *versus* $69 \pm 9\%$; pre- *versus* post-ULLS: $95 \pm 18\%$ *versus* $119 \pm 15\%$), nor the vascular responsiveness to NO. In conclusion, two human *in vivo* models of deconditioning show a preserved baseline NO availability in the leg skeletal muscle vascular bed.

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 (Widlansky *et al.* 2003). Various risk factors for cardiovascular disease, such as arterial hypertension, diabetes, smoking and hypercholesterolaemia, are associated with defects in the NO pathway (Widlansky *et al.* 2003).

Reduced baseline NO production has been demonstrated in patients with hypertension and their offspring (McAllister *et al.* 1999), in smokers (McVeigh *et al.* 1996), and in chronic heart failure patients (Yoshida *et al.* 1998). Exercise training enhances baseline NO production in healthy subjects (Kingwell *et al.* 1997), in patients with hypercholesterolaemia (Lewis *et al.* 1999),

and in chronic heart failure patients (Hambrecht *et al.* 1998). 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 (Blair *et al.* 1995). Baseline blood flow to the deconditioned skeletal muscles is reduced (Takenaka *et al.* 1994; Kamiya *et al.* 2000; De Groot *et al.* 2003). In response to a reduction in blood flow, baseline NO synthesis may be attenuated, which triggers vascular remodelling and atherosclerosis (Rudic *et al.* 1998, 2000). Accordingly, Kamiya *et al.* (2000) 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

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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})	1.3 ± 0.3‡	1.4 ± 0.3	1.2 ± 0.2
Cholesterol (mmol l^{-1})	4.6 ± 0.4‡	4.5 ± 0.3	4.4 ± 0.2
Glucose (mmol l^{-1})	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 ± S.E.M. *$P = 0.047$ *versus* SCI. †$P = 0.006$ *versus* SCI. ‡Data are from 6 subjects.

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 (De Groot *et al.* 2003; Kooijman *et al.* 2003). 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 (Berg *et al.* 1991) 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 (Berg *et al.* 1991; Dudley *et al.* 1992; Berg & Tesch, 1996; Schulze *et al.* 2002).

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, seven male SCI, with extreme, long-term deconditioning, were compared with seven controls, matched for gender and age. In a second study, seven healthy subjects (three males, four 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 to 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 h per week. Exercise in the SCI (4.9 ± 0.8 h per week) consisted of voluntary arm exercise. This type of upper body exercise is limited by the amount of active muscle mass (Hopman *et al.* 1998) and does not affect the leg vasculature (Huonker *et al.* 1998). 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 h. 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 a 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 previously described (Kooijman *et al.* 2003). 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 lignocaine (lidocaine) 20 mg ml^{-1}) 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 (Thijssen *et al.* 2005a). The thigh cuffs were simultaneously inflated to 50 mmHg using a rapid cuff inflator (Hokanson E-20, D.E. Hokanson, Bellevue, Washington, USA) (Groothuis *et al.* 2003). 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 10 min and the calf circulation was restored for 5 min.

Drug infusion protocol. The drug infusion protocol is represented at the bottom of Fig. 1. The measurements started at least 30 min after cannulation of the femoral artery. Each drug dose was administered for 5 min. 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^{-1} (dl of upper leg volume)$^{-1}$. In a pilot study with doses up to 0.8 mg min^{-1} dl^{-1} maximal vasoconstriction was already achieved at 0.2 and 0.4 mg min^{-1} dl^{-1}. 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^{-1} dl^{-1}). SNP 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^{-1} dl^{-1}) served as a control vaso-constrictor 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^{-1} dl^{-1}.

Drugs and solutions. L-NMMA and angiotensin II (both from Clinalfa, Läuflingen, Switzerland) were dissolved in saline at the beginning of each experiment. SNP (department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre) was dissolved in 5% glucose and protected against light.

Upper leg volume measurement. Upper leg volume was determined by anthropometry as described by Jones & Pearson (1969).

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 (Berg *et al.* 1991). 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 contralateral foot was not used, because it produced instability of the leg.
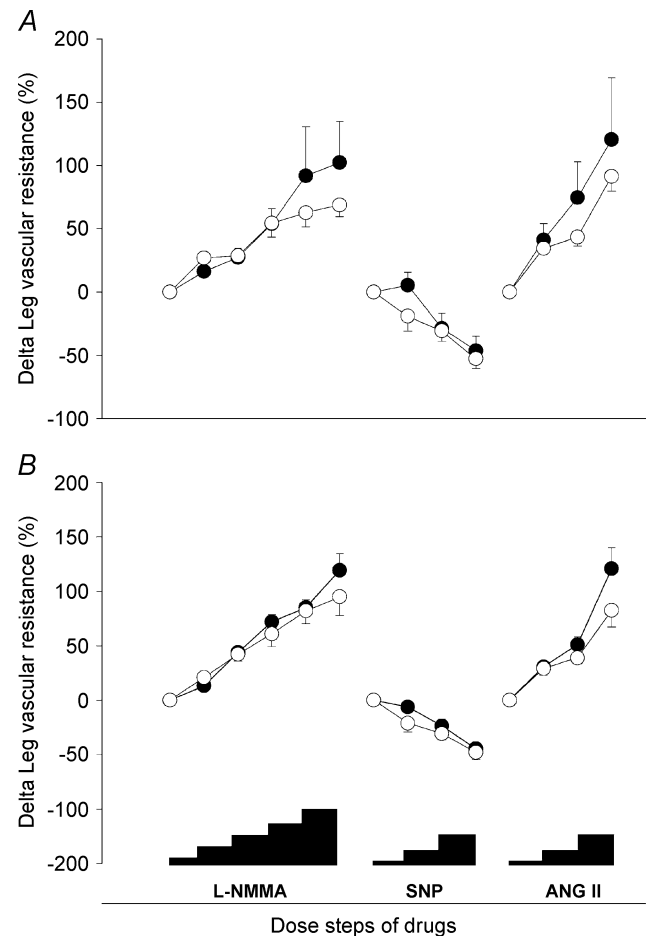

Figure 1. Change in leg vascular resistance in response to L-NMMA (0.025–0.05–0.1–0.2–0.4 mg min^{-1} dl^{-1} of leg tissue), SNP (0.06–0.2–0.6 μg min^{-1} dl^{-1}), and angiotensin II (Ang II 0.25–0.5–2.0 ng min^{-1} dl^{-1})
A, spinal cord-injured individuals *versus* matched controls (● and ○, respectively). *B*, healthy subjects before and after ULLS (○ and ●, respectively). Data represent means and s.e.m.

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 measurement of leg skin temperature. Skin temperature was consistently 1–2°C 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 (Newtons) of the quadriceps muscle of both legs was assessed with an isometric quadriceps dynamometer (Gerrits *et al.* 2001*b*) before and after 4 weeks of ULLS. The hips and knees were positioned at 90 deg and 60 deg 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^{-1} dl^{-1} was calculated as the slope of the plethysmographic volume curve, as previously described (Kooijman *et al.* 2003). Leg blood flow values of the final 2 min 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^{-1} dl^{-1} and expressed in arbitrary units (AU = mmHg·min ml^{-1} dl^{-1}). 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 ± s.e.m. 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 analysed using two-factor 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^{-1} dl^{-1}, $P = 0.064$) 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 l, 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 not alter baseline leg blood flow, or LVR (pre *versus* post: 3.9 ± 0.7 *versus* 4.5 ± 0.9 ml min^{-1} dl^{-1} and 26 ± 3 *versus* 23 ± 3 AU, respectively).

Response to L-NMMA

Leg vascular resistance 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 (Fig. 1*A*). The response of LVR to L-NMMA in SCI was also compared to the data of all 12 controls (including five additional control subjects from the pre-ULLS study), 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 (Fig. 1*B*). All these observations were similar if the absolute instead of the relative (%) changes in the LVR were analysed. After normalizing the response to L-NMMA to the individual

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

Drug dose (Dose min^{-1} dl^{-1})	SCI (*n* = 7)					Controls (*n* = 7)				
	MAP	HR	LBF	LVR Infusion	LVR Control	MAP	HR	LBF	LVR Infusion	LVR 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
	*		*	*		*		*	*	

MAP, mean arterial pressure (mmHg); HR, heart rate (beats min^{-1}); LBF, leg blood flow (ml min^{-1} dl^{-1}); 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 × group ($P = 0.069$). ‡Significant dose effect ($P < 0.05$, one-way ANOVA).

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.

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^{-1} dl^{-1}. As a consequence, doubling of the dose to 0.4 mg min^{-1} dl^{-1} 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^{-1} dl^{-1} ($P = 0.031$). However, pilot studies in healthy volunteers showed that increasing the L-NMMA dose to 0.8 mg min^{-1} dl^{-1} 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^{-1} dl^{-1}, 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 (Tables 2 and 3). SCI and their controls and the ULLS subjects showed a decrease in HR during L-NMMA infusion (Tables 2 and 3).

Response to SNP

Neither the absolute nor the relative (%) response of LVR to SNP differed between SCI and controls (Table 2, Fig. 1*A*).

This also holds for the pre- *versus* post-ULLS response to SNP (Table 3, Fig. 1*B*). MAP decreased in all groups during the higher dosages of SNP ($P < 0.001$, Tables 2 and 3).

Response to angiotensin II

The absolute and relative (%) responses of LVR to angiotensin II (Table 2, Fig. 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 ($P < 0.001$, Tables 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 eight 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 (Bleeker *et al.* 2004).

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

Drug dose (Dose min⁻¹ dl⁻¹)	Pre-ULLS ($n = 7$)					Post-ULLS ($n = 7$)				
	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
	*		*	*	*	*		*	*	*

MAP, mean arterial pressure (mmHg); HR, heart rate (beats min⁻¹); LBF, leg blood flow (ml min⁻¹ dl⁻¹); 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).

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 (Kingwell *et al.* 1997). Therefore, the effects of deconditioning are not the inverse of the 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 either of the 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 (Hopman *et al.* 2002). 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 (Bleeker *et al.* 2005). This corresponds with ultrasound data on diameter and blood flow in SCI (De Groot *et al.* 2004). 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 (Dinenno *et al.* 2001). 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 magnetic resonance imaging (MRI) or computed tomography (CT) measurements (Dudley *et al.* 1992; Hather *et al.* 1992; Ploutz-Snyder *et al.* 1995; Berg & Tesch, 1996; Schulze *et al.* 2002).

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 (Vervoort *et al.* 1999). 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 $0.1 \text{ mg min}^{-1} \text{dl}^{-1}$ dose (Green *et al.* 1997; Kingwell *et al.* 1997). 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 ($0.4 \text{ mg min}^{-1} \text{dl}^{-1}$) was higher than with the previous dose ($0.2 \text{ mg min}^{-1} \text{dl}^{-1}$). Although this may indicate that maximal vasoconstriction was not achieved, data from a pilot study showed that the higher dose of $0.8 \text{ mg min}^{-1} \text{dl}^{-1}$ 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 (Takenaka *et al.* 1994; Kamiya *et al.* 2000; Hopman *et al.* 2002) 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 (Kingwell *et al.* 1997) and hypercholesterolaemic patients (Lewis *et al.* 1999; Walsh *et al.* 2003), and an increase in nitrite–nitrate level after 8 weeks of cycle training (Maeda *et al.* 2001). 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 (Kingwell *et al.* 1996). It has been proposed that changes in endothelial function represent short-term adaptations to training and are eventually replaced by structural or other adaptations (Green *et al.* 2004*a*). 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 (Green *et al.* 1996). One should take notice that these subjects played tennis for 13 h 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 (Green *et al.* 1997). 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 (Kamiya *et al.* 2000), suggesting an impaired endothelial NO production, while in another bed rest study no changes in urinary nitrite–nitrate excretion occurred (Bonnin *et al.* 2001). 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 endothelial nitric oxide synthase (eNOS) expression in the endothelial cells of both conductance and resistance vessels, and an attenuated maximal vasodilation to acetylcholine suggesting a decrease of endothelial NO release by hindlimb unloading (Jasperse *et al.* 1999; Delp *et al.* 2000; Schrage *et al.* 2000). In contrast, another study demonstrated no alterations of eNOS expression, but an increase in aortic inducible NOS (iNOS) content following hindlimb unloading (Vaziri *et al.* 2000).

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 (Rudic *et al.* 1998, 2000). 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 dilation 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 (de Groot *et al.* 2004) and after 4 weeks of ULLS (Bleeker *et al.* 2005). 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 (Green *et al.* 2004*b*). Moreover, in animals the short-term and long-term effects of exercise training on the NO pathway differ between conduit and resistance arteries (Laughlin *et al.* 2001).

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 (Kingwell *et al.* 1997) and improves endothelial function (Linke *et al.* 2001) in the arms. However, evidence exists that changes in endothelial function occur predominantly locally in the exercised limbs (Gokce *et al.* 2002; Allen *et al.* 2003; Kobayashi *et al.* 2003). In SCI neither systemic cardiovascular effects (Phillips *et al.* 1998) nor local vascular effects of deconditioning (Huonker *et al.* 1998) can be normalized with upper body training. Recently, Thijssen *et al.* (2005*b*) 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 (Eisenach *et al.* 2002). In addition,

adaptations in the circulatory system in SCI are reversible by functional electrostimulation training (Gerrits *et al.* 2001*a*; Hopman *et al.* 2002), 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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