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Endothelial mechanotransduction proteins and vascular function are altered by dietary sucrose supplementation in healthy young male subjects

Lasse Gliemann[1], Nicolai Rytter[1], Mads Lindskrog[1], Martina H. Lundberg Slingsby[1], Thorbjörn Åkerström[1,3], Lykke Sylow[2], Erik A. Richter[2] and Ylva Hellsten[1]

[1] *Section for Integrative Physiology, University of Copenhagen, Copenhagen, Denmark*
[2] *Section Molecular Physiology, Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark*
[3] *Insulin Pharmacology Department, Novo Nordisk A/S, Maaloev, Denmark*

Key points

- Mechanotransduction in endothelial cells is a central mechanism in the regulation of vascular tone and vascular remodelling
- Mechanotransduction and vascular function may be affected by high sugar levels in plasma because of a resulting increase in oxidative stress and increased levels of advanced glycation end-products (AGE).
- In healthy young subjects, 2 weeks of daily supplementation with 3 × 75 g of sucrose was found to reduce blood flow in response to passive lower leg movement and in response to 12 W of knee extensor exercise.
- This vascular impairment was paralleled by up-regulation of platelet endothelial cell adhesion molecule (PECAM)-1, endothelial nitric oxide synthase, NADPH oxidase and Rho family GTPase Rac1 protein expression, an increased basal phosphorylation status of vascular endothelial growth factor receptor 2 and a reduced phosphorylation status of PECAM-1. There were no measurable changes in AGE levels.
- The findings of the present study demonstrate that daily high sucrose intake markedly affects mechanotransduction proteins and has a detrimental effect on vascular function.

Abstract Endothelial mechanotransduction is important for vascular function but alterations and activation of vascular mechanosensory proteins have not been investigated in humans. In endothelial cell culture, simple sugars effectively impair mechanosensor proteins. To study mechanosensor- and vascular function in humans, 12 young healthy male subjects supplemented their diet with 3 × 75 g sucrose day^{-1} for 14 days in a randomized cross-over design. Before and after the intervention period, the hyperaemic response to passive lower leg movement and active knee extensor exercise was determined by ultrasound doppler. A muscle biopsy was obtained from the thigh muscle before and after acute passive leg movement to allow assessment of protein amounts and the phosphorylation status of mechanosensory proteins and NADPH oxidase. The sucrose intervention led to a reduced flow response to passive movement (by 17 ± 2%) and to 12 W of active exercise (by 9 ± 1%), indicating impaired vascular function. A reduced flow response to passive and active exercise was paralleled by a significant up-regulation of platelet endothelial cell adhesion molecule (PECAM-1), endothelial nitric oxide synthase, NADPH oxidase and the Rho family GTPase Rac1 protein expression in the muscle tissue, as well as an increased basal phosphorylation status of vascular endothelial growth factor receptor 2 and a reduced phosphorylation status of PECAM-1. The phosphorylation status was not acutely altered with passive leg movement. These findings indicate that a regular intake of high levels of sucrose can impair vascular mechanotransduction and increase the oxidative stress potential, and suggest that dietary excessive sugar intake may contribute to the development of vascular disease.

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Corresponding author L. Gliemann: Department of Nutrition, Exercise and Sports, Section for Integrative Physiology, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark. Email: gliemann@nexs.ku.dk

Abbreviations AGE, advanced glycation end-products; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; NOX, NADPH oxidase; NOx, nitrite and nitrate; PECAM-1, platelet endothelial cell adhesion molecule; PGI_2, prostacyclin; Rac1, Rho family GTPase Rac1; ROS, reactive oxygen species; sRAGE, soluble receptor of advanced glycation end products; VEGF-R2, vascular endothelial growth factor receptor 2; VE-cadherin, vascular endothelial cadheri.

Introduction

The vascular system is precisely controlled, allowing for a tight coupling between oxygen delivery and oxygen demand in most organs throughout the body (Andersen & Saltin, 1985; Joyner & Casey, 2015). In conditions with a mismatch between oxygen delivery and oxygen demand, an impaired regulation of vascular tone (i.e. vascular dysfunction) is often the underlying cause. Vascular dysfunction commonly develops with a sedentary lifestyle, obesity and ageing (McVeigh *et al.* 1992; Taddei *et al.* 2001; Seals *et al.* 2011) and is good predictor of cardiovascular disease (Widlansky *et al.* 2003; Deanfield *et al.* 2007; Green *et al.* 2011).

The regulation of vascular tone involves a complex integration of stimuli from sympathetic nerve activity, locally produced or circulating compounds, as well as mechanical forces, including shear stress (Lamontagne *et al.* 1992). Shear stress is the frictional force of flowing blood applied to the endothelial layer lining the blood vessels. Studies on endothelial cells in culture have demonstrated that shear stress is sensed by a complex of mechanosensors consisting of vascular endothelial growth factor receptor 2 (VEGF-R2), vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule 1 (PECAM-1) (Osawa *et al.* 2002; Shay-Salit *et al.* 2002; Tzima *et al.* 2005). Mechanical deformation of the mechanosensor complex leads to phosphorylation of all three proteins (Tzima *et al.* 2005) and subsequent downstream activation of intracellular signal cascades (Fleming & Busse, 1999). Ultimately, activation of the cascade leads to increased formation of nitric oxide (NO) via phosphorylation and activation of endothelial nitric oxide synthase (eNOS), in particular serine residue 1177 (Jin *et al.* 2003; Fleming *et al.* 2005; Tzima *et al.* 2005). NO in turn causes a cGMP-dependent decrease in calcium levels and sensitivity in adjacent smooth muscle cells. This leads to shear stress induced vasodilatation.

It is well established that impaired vascular function in hypertension, diabetes and ageing is related to a dysfunctional NO system (Taddei *et al.* 2006). This dysfunction does not appear to be related to a reduced expression of eNOS (Donato *et al.* 2009; Nyberg *et al.* 2012) but rather to a reduced mechanical or chemical activation of eNOS or to an excessive removal of NO via reaction with reactive oxygen species (ROS) (Gryglewski *et al.* 1986; Guzik *et al.* 2002; Fleming & Busse, 2003). Shear stress induced activation of eNOS in the microcirculation and how it may be altered in disease has been poorly explored *in vivo* and the available data in humans are limited.

Hyperglycaemia acutely impairs shear stress induced vascular regulation in healthy humans (Kawano *et al.* 1999; Zhu *et al.* 2007) and incubation of bovine endothelial cells with high glucose suppresses both ACh- and shear stress induced activation of eNOS and NO formation (Connell *et al.* 2007). Moreover, diabetic subjects with chronic hyperglycaemia present an impaired macrovascular shear stress response (Naylor *et al.* 2011). One of the mechanisms underlying this effect in diabetic patients could be glycation of the mechanosensor proteins. Long-term high blood glucose levels have been shown to induce elevated levels of advanced glycation end-products (AGE) (Fiorentino *et al.* 2013). Glycation of the mechanosensitive proteins caused by the presence of AGE leads to decreased mechanosensor sensitivity (Otero *et al.* 2001; Liu *et al.* 2012; Naser *et al.* 2013). This link has been established in hypertension, diabetes and ageing (Goh & Cooper, 2008; Seals *et al.* 2011; Liu *et al.* 2016) and may in turn lead to impaired eNOS activation and NO production (Soro-Paavonen *et al.* 2010; Naser *et al.* 2013). In addition, high glucose levels are associated with an increased level of ROS in the vascular system, which also may affect the bioavailability of NO (Cosentino *et al.* 1997; Inoguchi *et al.* 2000; Cosentino *et al.* 2003). A period of experimental manipulation of blood glucose via intake of simple sugars would therefore be a relevant approach for manipulating endothelial mechanotransduction and vasodilatation in humans to allow study of the underlying mechanisms of vascular function.

Shear stress induced vasodilatation can be evaluated in humans by the flow response to passive movement of the lower leg (Hellsten *et al.* 2008; Mortensen *et al.* 2012). Passive movement of the lower leg causes a rapid biphasic increase in femoral arterial blood flow and a passive stretch of the muscle in the absence of muscle activity or metabolic disturbances (Hellsten *et al.* 2008; Mortensen *et al.* 2012). Both shear stress (Pohl *et al.* 1986) and stretch probably contribute to the flow response, although the stretch effect is probably short lasting (Venturelli *et al.* 2017). The response to passive movement is highly NO-dependent, as indicated by an almost completely abolished flow response

in the presence of an NO synthase blocker (Mortensen *et al.* 2012). Thus, passive leg movement induced hyperaemia is a valid method for studying the impact of mechanotransduction in the vascular system *in vivo*.

The two hypotheses of the present study are that, in healthy young men, the increase in blood flow during passive leg movement would be associated with phosphorylation of endothelial mechanotransductor proteins and eNOS and that 14 days of high sucrose intake would impair mechanosensing during passive leg movement with a consequent reduction in mechanosensor phosphorylation and blood flow.

Methods

Ethical approval

The present study was approved by the Ethics Committee of Copenhagen and Frederiksberg communities (H-6-2014-085) and was conducted in accordance with the latest guidelines of the *Declaration of Helsinki*. Written informed consent was obtained from all subjects before enrollment in the study.

Subjects

Twelve young healthy men (aged 20–25 years) were recruited. All subjects were of normal weight (body mass index < 28 kg m^{-2}), non-smokers and without known chronic diseases.

Intervention

The study was of a randomized, cross-over design. Each subject completed two 14 day periods of either a control or a high sucrose diet, with a 30 day washout period between the end of the first and the start of the second period. Before the first experimental day, subjects were randomly allocated to start with either the control or the sucrose diet in a balanced order. During the control treatment, subjects maintained their normal diet and during the sucrose treatment subjects supplemented their normal diet with three daily doses of 75 g of sucrose dissolved in 200 ml of water. The sucrose diet introduced a daily increase in energy intake of 886 kcal. With normal diet and energy expenditure maintained during the 2 week intervention, this corresponds to 1.58 kg of body fat. Each daily dose of sucrose was ingested after a meal, morning, midday and afternoon. Subjects registered the time of sucrose ingestion and any discomfort experienced. A 7 day diet diary was completed before the start of the intervention and normal diets of the subjects were maintained from enrollment to end of the intervention. All analyses were blinded to the investigators.

Pre-testing

Before the first experimental day, subjects visited the laboratory where pulmonary maximal oxygen uptake ($\dot{V}_{O_2max}$) was determined (Oxycon Pro; Viasys Healhtcare, Hoechberg, Germany) with an incremental exercise test on a mechanically braked cycle ergometer (Monark Ergomedic 839E; Monark, Vansbro, Sweden). Subjects completed a 5 min warm-up at 125 W, after which the workload was increased by 25 W min^{-1} until exhaustion. $\dot{V}_{O_2max}$ was calculated as the average of the three highest consecutive 15 s values. For recognition of true $\dot{V}_{O_2max}$, three of the following five criteria had to be met: individual perception of exhaustion, respiratory exchange rate > 1.15, $\dot{V}_{O_2}$ curve plateau, heart rate approaching age-predicted maximum, and inability to maintain pedalling frequency above 80 rpm. Verbal encouragement was given throughout the test. To confirm normal glucose metabolism and liver function, blood samples were drawn from the antecubital vein and analysed for glucose, HbA_{1c}, insulin and hepatic enzymes at the clinical biochemical unit at the main hospital in Copenhagen (Rigshospitalet) (Table 1).

Experimental days

On experimental days, subjects arrived at the laboratory after an overnight fast. Subjects rested in the supine position and a catheter (DB Venflon Pro Safety, 18 GA; Becton Dickinson Infusion Therapy AB, Stockholm, Sweden) was inserted into the antecubital vein for blood sampling. Blood was drawn at rest, after 20 min of passive leg movement (Mortensen *et al.* 2012), and before and after active knee extensor exercise. Two microdialysis probes (CMA63 with 30 mm membrane length and 20 kDa cut-off; M Dialysis AB, Stockholm, Sweden) were inserted into the musculus vastus lateralis under local anaesthesia (xylocaine, ∼3 ml, 20 mg ml^{-1}; AstraZeneca, Gothenburg, Sweden). After insertion of the probes, subjects rested for 120 min to allow the muscle to attain a resting state and to recover from insertion trauma. The probes were perfused with Ringer acetate buffer (Fresenius Kabi AB, Oslo, Norway) at a rate of 5 ml min^{-1} and, to determine the relative exchange over the membrane, a small amount (2.7 nM) of [2-^{3}H] labelled adenosine was added to the perfusate to allow for calculation of probe recovery. After the 120 min of initial rest, blood pressure was measured three consecutive times with an automatic sphygmomanometer (M7; OMRON, Vernon Hills, IL, USA) on the left and right upper arm and microdialysate was collected for 20 min of subsequent rest. Microdialysate was then collected during 20 min of passive leg movement, excluding the first 2 min to account for delay in the probe perfusate. Immediately after collection, samples were weighed and triplicates of 5 ml of dialysate

Table 1. Subject characteristics and blood values

Age (years)	22.8 ± 0.9	–	
Height (cm)	186.1 ± 1.6	–	
$\dot{V}_{O_2max}$ (ml O_2 min^{-1})	4130 ± 141	–	
$\dot{V}_{O_2max}$ (ml O_2 min^{-1} kg^{-1})	50.4 ± 1.9	–	
	Control	After sucrose	*P*
Body weight (kg)	82.2 ± 3.3	83.5 ± 3.5	0.21
Systolic blood pressure (mmHg)	116.4 ± 2.8	118.3 ± 2.3	0.43
Diastolic blood pressure (mmHg)	66.6 ± 2.0	69.9 ± 1.6	0.09
Rest heart rate (beats min^{-1})	55.0 ± 1.9	56.6 ± 2.6	0.32
Erythrocytes (10^{12} l^{-1})	4.7 ± 0.1	4.8 ± 0.1	0.52
Hemoglobin (mmol l^{-1})	8.5 ± 0.1	8.7 ± 0.1	0.38
Plasma endothelin-1 (pg ml^{-1})	2.6 ± 0.1	2.3 ± 0.1	0.21
Fasting blood glucose (mmol l^{-1})	5.1 ± 0.1	4.9 ± 0.1	0.07
HbA1c (mmol l^{-1})	5.4 ± 0.1	5.3 ± 0.1	0.09
Insulin (pmol l^{-1})	38.0 ± 4.4	45.5 ± 5.5	0.18
QUICKI	0.44 ± 0.01	0.43 ± 0.01	0.34
Total cholesterol (mmol l^{-1})	3.7 ± 0.2	3.8 ± 0.2	0.25
HDL cholesterol (mmol l^{-1})	1.3 ± 0.1	1.3 ± 0.1	0.72
LDL cholesterol (mmol l^{-1})	2.2 ± 0.2	2.4 ± 0.2	0.06
Triglycerides (mmol l^{-1})	0.7 ± 0.1	0.7 ± 0.1	0.64

$\dot{V}_{O_2max}$, maximal oxygen optake; QUICKI, quantitative insulin sensitivity check index (Katz *et al.* 2000). HDL, high-density lipoprotein; LDL, low-density lipoprotein. Data are presented as the mean ± SD.

were allocated into 3 ml of Ultimate Gold scintillation liquid (Perkin Elmer, Waltham, MA, USA). The remaining dialysate was frozen at −80°C. Probe recovery (PR) was calculated as [PR = ($dpm_{infusate}$–$dpm_{dialysate}$/$dpm_{infusate}$)], where dpm denotes disintegrations per minute (Scheller & Kolb, 1991; Jansson *et al.* 1994). The probe recovery was used to obtain an estimate of probe recovery at rest and during passive movement. The main purpose for this estimation was to take into account changes in recovery in going from rest to movement. Accordingly, the resulting values are presented as estimated interstitial concentrations. The [2-^{3}H] adenosine activity of the dialysate was measured on a liquid scintillation counter (Tri-Carb 2910 TR; Perkin Elmer).

Femoral arterial blood flow was measured at rest, during passive leg movement and during active knee extensor exercise (at 12, 18 and 24 W) with ultrasound Doppler (GE Vivid E9; GE Healthcare, Pittsburgh, PA, USA) equipped with a linear probe (L5) operating at an imaging frequency of 9 MHz and a Doppler frequency of 4.2 MHz. The site of blood velocity measurements in the common femoral artery was distal to the inguinal ligament but above the bifurcation into the superficial and profound femoral branch to avoid turbulence from the bifurcation. All recordings were obtained at the lowest possible insonation angle and always below 60°. The sample volume was maximized by choosing the widest section of the vessel and the measurements were made without interference of the vessel walls. A low-velocity filter (velocities < 1.8 m s^{-1}) rejected noises caused by turbulence at the vascular wall. Doppler tracings and B-mode images were recorded continuously and Doppler tracings were averaged over 45 s. Resting femoral arterial blood flow was measured before passive leg movement and before active knee extensor exercise. During the 20 min passive leg movement, femoral arterial blood flow was measured at 15 s intervals during the initial 3 min, after 4 and 5 min, and at 2.5 min intervals from 7.5 to 20 min. After 30 min of rest, active knee extensor exercise was performed for 3 min at each load, allowing for steady-state blood flow. Femoral arterial blood flow was measured during the final minute of each exercise bout.

Immediately before and immediately after the 20 min passive leg movement bout, a muscle biopsy was obtained from the musculus vastus lateralis under local anaesthesia (xylocaine, ∼5 ml, 20 mg ml^{-1}; AstraZeneca) using the percutaneous needle biopsy technique with suction (Bergström, 1975). The biopsies were immediately frozen in liquid nitrogen. Frozen samples were stored at 80°C until further analysis.

Quantification of protein expression

Biopsies were freeze-dried and dissected free from fat, blood and connective tissue. Approximately 5 mg dry weight of muscle tissue was homogenized in a fresh batch buffer (10% glycerol, 20 mM sodium-pyrophosphate, 150 mM NaCl, 50 mM Hepes, 1% Nonidet P-40, 20 mM β-glycerophosphate and proteolytic inhibitors) two times for 30 s (Qiagen Tissuelyser II; Retsch, Haan, Germany). After rotation end-over-end for 1 h, the samples were centrifuged for 30 min at 17 500 *g* at 4°C and the lysates

were collected as the supernatant. Protein concentrations were determined in the lysates using BSA standards (Pierce Reagents, Rockford, Il, USA). The lysates were diluted to appropriate protein concentrations in a concentrated sample buffer (0.5 M Tris-base, dithiothreitol, SDS, glycerol and bromphenol blue) and equal amounts of total protein were loaded for each sample in different wells on precasted Tris-HCL gels (Bio-Rad, Hercules, CA, USA). For comparisons, samples from the same subject were always loaded on the same gel. After gel electrophoresis, the proteins were transferred (semidry) to a polyvinylidene difluoride membrane (Immobilon Transfer Membrane; Millipore, Billerica, MA, USA), which was incubated with ∼10 ml of primary antibody overnight and then washed three times for 5 min in Tris-buffered saline-Tween before incubation with secondary antibody for 1 h. The membranes were incubated with the primary antibodies: VEGF-R2 (sc-19530; Santa Cruz Biotechnology, Santa Cruz, CA, USA); VEGF-R2 Tyr1175 (2478, Cell Signaling Technology, Danvers, MA, USA); eNOS (BD 610287, BD Biosciences, San Jose, CA, USA-9; eNOS Ser1177 (Calbiochem 482737; Merck Milipore, Darmstadt, Germany); PECAM-1 (AF806; R&D Systems Inc., Minneapolis, MN, USA); PECAM-1 Tyr 713 (BS4666; Bioworld Technology, St Louis Park, MN, USA); VE-cadherin (ab33168; Abcam, Cambridge, UK); VE-cadherin Tyr731 (NBP1-51393; Novus Biologicals, Littleton, CO, USA); NADPH oxidase (NOX) p67 (610912; BD Biosciences, San Jose, CA, USA); S-nitrosocysteine (ab94930; Abcam); and Rho family GTPase Rac1 (Rac1) (610651; BD Biosciences). Secondary antibodies used were goat anti-rabbit or rabbit anti-goat HRP-conjugated antibodies (P-0448 and P-0449; DakoCytomation, Glostrup, Denmark; dilution 1:5000). Following detection and quantification (ChemiDoc MP system; Bio-Rad), the protein content was expressed in arbitrary units. To control for loading differences, the blots were also analysed for glyceraldehyde 3-phosphate dehydrogenase (ab9484; Abcam), actin (A2066; Sigma-Aldrich, St Louis, MO, USA) or α-tubulin (ab4076, Abcam).

To confirm that the mechanosensor proteins PECAM-1, VE-cadherin and VEGF-R2 were not present in skeletal muscle cells, pure human primary skeletal muscle cell cultures (human skeletal muscle cells; PromoCell, Heidelberg, Germany) were run on a gel together with whole human muscle homogenate (both samples loaded with the same protein concentration). Blots are shown in Fig. 3*C*.

Analysis of skeletal muscle mRNA content: RNA isolation, reverse transcription and PCR

Total RNA was isolated from the muscle biopsies using TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA by SuperScript^{TM} II Reverse Transcriptase (Invitrogen) as described previously (Pilegaard *et al.* 2000). The mRNA content of VEGF-R2, eNOS and PECAM-1, was determined by real-time RT-PCR (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). The cDNAs were amplified using TaqMan Gene expression assays from Applied Biosystems. For each sample, the amount of target gene mRNA was normalized to β-actin mRNA content. The effect of the experimental condition on the level of β-actin mRNA was determined statistically and no significant effect was found with acute exercise.

Analysis of NOx, ET-1, AGE and RAGE in plasma

The stable metabolites of NO in plasma, nitrite and nitrate (NOx), were measured using a fluorometric EIA kit (Cayman Chemical Co., Ann Harbor, MI, USA). ET-1 and soluble receptor of advanced glycation end products (sRAGE) in plasma were measured with a Quantikine ELISA kit (R&D Systems). AGE in plasma was measured with ELISA kits (Oxyselect Advanced Glycation End Products Competitive ELISA kit; Cell Biolabs, San Diego, CA, USA).

Analysis of NOx and prostacyclin (PGI_2) in microdialysate

NOx and the stable metabolites of of PGI_2, 6-keto prostaglandin $F_1\alpha$, in microdialysate were measured using fluorometric kits (Cayman Chemical Co.). Interstitial concentrations were estimated based on the relative recovery of [2-^{3}H] adenosine in the microdialysis probe. Based on previous findings, it was assumed that the relative recovery of [2-^{3}H] adenosine is similar to the recovery of other compounds (Höffner *et al.* 2003).

Statistical analysis

Subject number is based on power calculations of selected primary outcome measures. These include average differences and variability in femoral arterial blood flow in response to passive leg movement and protein expression and phosphorylation of eNOS in muscle homogenates from musculus vastus lateralis (Altman, 1980). The area under the curve for the analysis of total blood flow to the leg during the passive leg movement was calculated using the trapezoidal rule. Significance level for all tests was set at an α-level of $P < 0.05$ at a power level of 0.8. Data are reported as the mean ± SD.

A linear mixed-model approach (RStudio, version 0.99.903; RStudio, Boston, MA, USA) was used to investigate the effects of (i) the sucrose intervention and (ii) passive leg movement and active exercise. Fixed factors were 'intervention' (control or sucrose) and 'time'

(rest, passive leg movement and active exercise). Subjects were specified as a repeated factor and identifier of random variation. The homogeneity of variance and normal distribution was confirmed through residual and Q–Q plots. Pairwise differences were identified using the Tukey's honestly significant difference *post hoc* procedure.

Results

Compliance with the intervention

Based on self-reports, the subjects exhibited very high compliance (100%) with the intervention, resulting in no deviations from the sucrose ingestion protocol or their normal diet during the intervention period.

Body composition, blood values and blood pressure

Under control conditions, subjects had a body mass index of $23.7 \pm 0.7\ \mathrm{kg\,m^{-2}}$, in addition to normal blood pressure, blood glucose, insulin and lipid profiles (Table 1). The sucrose intervention did not affect body weight, body composition, blood pressure, plasma endothelin-1, fasting blood glucose, insulin, calculated QUICKI index or lipid profile (Table 1). Hepatic enzymes were not affected by the sucrose intervention (data not shown).

Blood flow response to passive leg movement

Femoral arterial blood flow was increased at 15 s of passive leg movement and remained elevated throughout the 20 min period ($P = 0.029$) (Fig. 1). After the sucrose intervention, the blood flow response was significantly lower compared to the control condition ($P = 0.033$). The area under the curve comprising total blood flow during the 20 min period was 17% lower under the sucrose condition compared to the control condition (10.4 ± 1 *vs.* 12.5 ± 1 l; $P = 0.018$).

Blood flow response to active exercise

Femoral arterial blood flow at rest was no different between the two conditions (Fig. 2). During active exercise, blood flow increased at 12, 24 and 36 W under both conditions ($P < 0.0001$). Flow was lower at 12 W after the sucrose intervention compared to the control condition ($P = 0.033$) (Fig. 2).

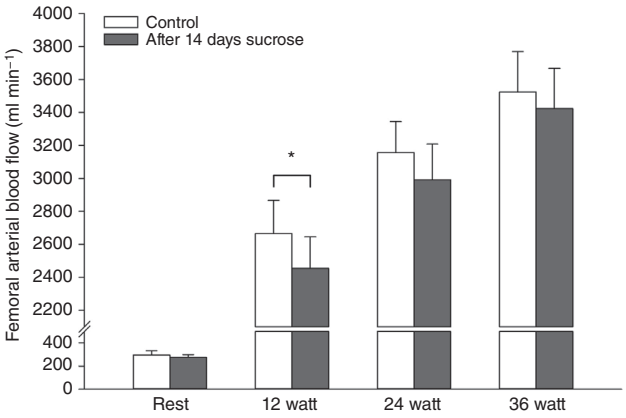

Figure 2. Femoral arterial blood flow during active one leg knee extensor exercise
Blood flow response to 12, 24 and 36 W exercise under control conditions (open bars) and after 2 weeks of high sucrose intake (grey bars). Data are presented as the mean ± SD ($n = 12$). *Significantly different compared to the control condition.

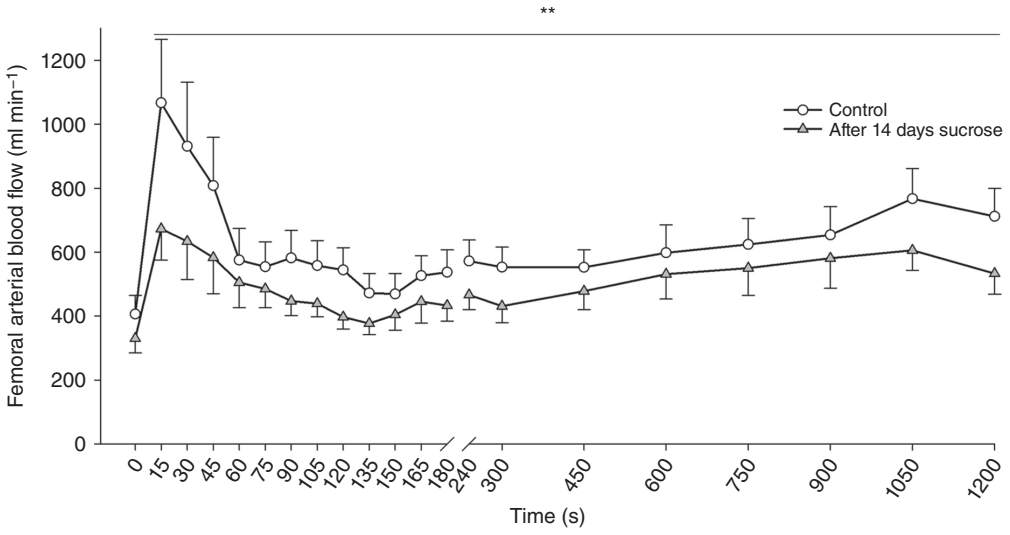

Figure 1. Femoral arterial blood flow during rest and passive leg movement
Blood flow response to 20 min of passive leg movement under control conditions (open circles) and after 2 weeks of high sucrose intake (grey triangles). Data are presented as the mean ± SD ($n = 12$). **Overall significant effect of the sucrose intervention compared to the control condition.

Protein expression and phosphorylation of mechanosensors

Baseline protein levels of VEGF-R2 and VE-cadherin were no different between the two conditions, whereas baseline protein PECAM-1 levels were higher overall after the sucrose intervention ($P = 0.022$) (Fig. 3*A*). There was no effect of acute passive leg movement on content of any of the proteins. The ratio of phosphorylation at tyrosine residue 1175 to total protein of VEGF-R2 was increased after the sucrose intervention overall and was higher after passive leg movement subsequent to the sucrose intervention compared to the control ($P = 0.014$) (Fig. 3*B*). The ratio of phosphorylated PECAM-1 at tyrosine residue 713 to total protein of PECAM-1 was reduced after the sucrose intervention ($P = 0.037$) (Fig. 3*B*). The phosphorylation status of VE-cadherin at tyrosine residue 731 was no different between the sucrose intervention and the control condition. Passive leg movement did not change the phosphorylation status of any of the mechanosensor proteins in the control or after the sucrose intervention (Fig. 3*B*).

Comparison of PECAM1, VE-cadherin and VEGF-R2 protein expression in whole muscle homogenates compared to purified primary skeletal muscle cells confirmed a lack of expression of these proteins in the skeletal muscle cells (Fig. 3*C*)

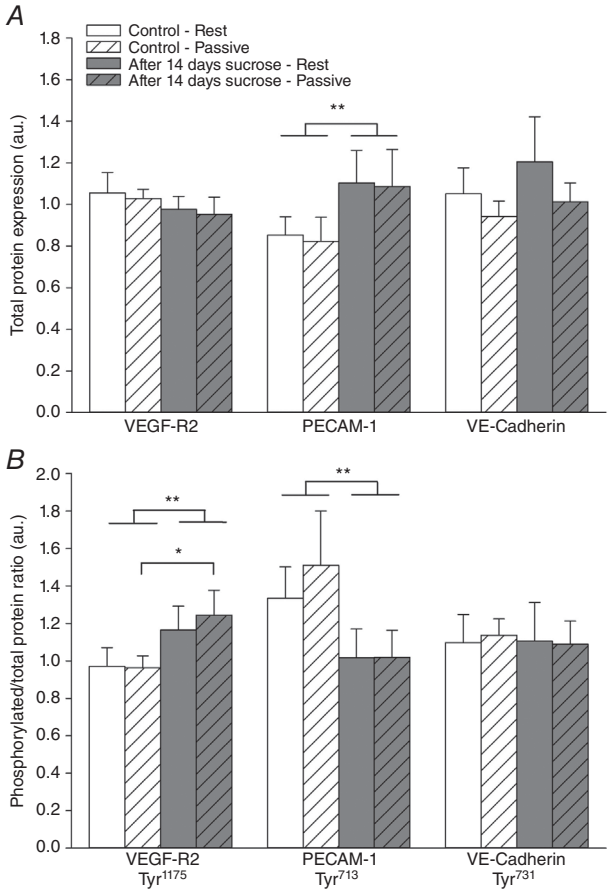

Figure 3. Protein expression and phosphorylated/total protein ratio of mechanosensor complex before and after 14 days of sucrose intervention
A, protein expression of VEGF-R2, PECAM-1 and VE-cadherin. *B*, phosphorylated VEGF-R2 (tyr^{1175}), PECAM-1 (tyr^{713}) and VE-cadherin (tyr^{731}) to total protein ratio in muscle homogenates from musculus vastus lateralis at rest and after 20 min of passive leg movement. *C*, expression of VE-CAD, VEGF-R2 and PECAM-1 in isolated primary skeletal muscle cells (SkMu Cell) and skeletal muscle tissue homogenate (SkMu Tissue). *D*, representative blots are shown from one subject in duplicates from rest (1) and after passive leg movement (2) under the control situation and from rest (3) and after passive leg movement (4) after the sucrose intervention. Data are presented as the mean ± SD ($n = 12$). *Significantly different compared to before the sucrose intervention during the condition of passive leg movement. **Overall significant effect of the sucrose intervention.

Protein expression and phosphorylation eNOS and NOX

eNOS protein content was higher after the sucrose intervention ($P = 0.015$) (Fig. 4*A*). As expected, the protein expression of eNOS was not affected by passive leg movement under either condition. Total phosphorylation of eNOS at serine residue 1177 and the ratio to eNOS

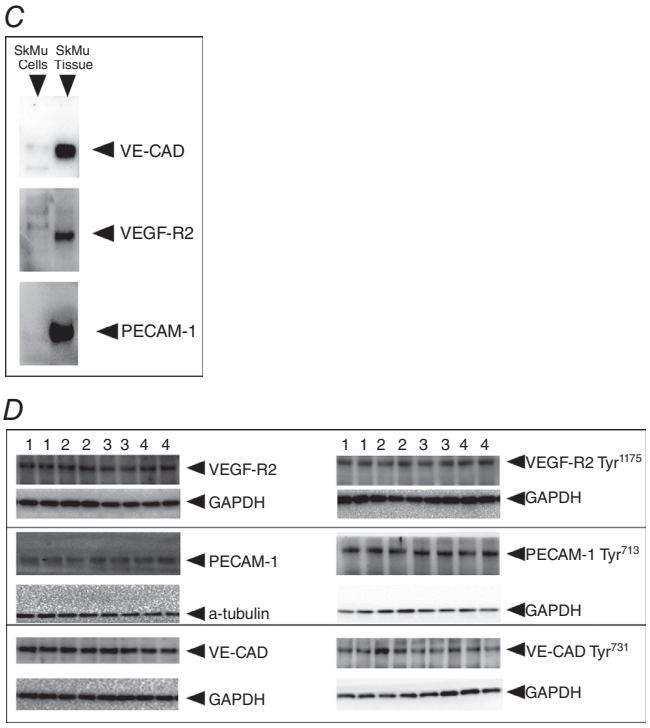

Figure 3. Continued

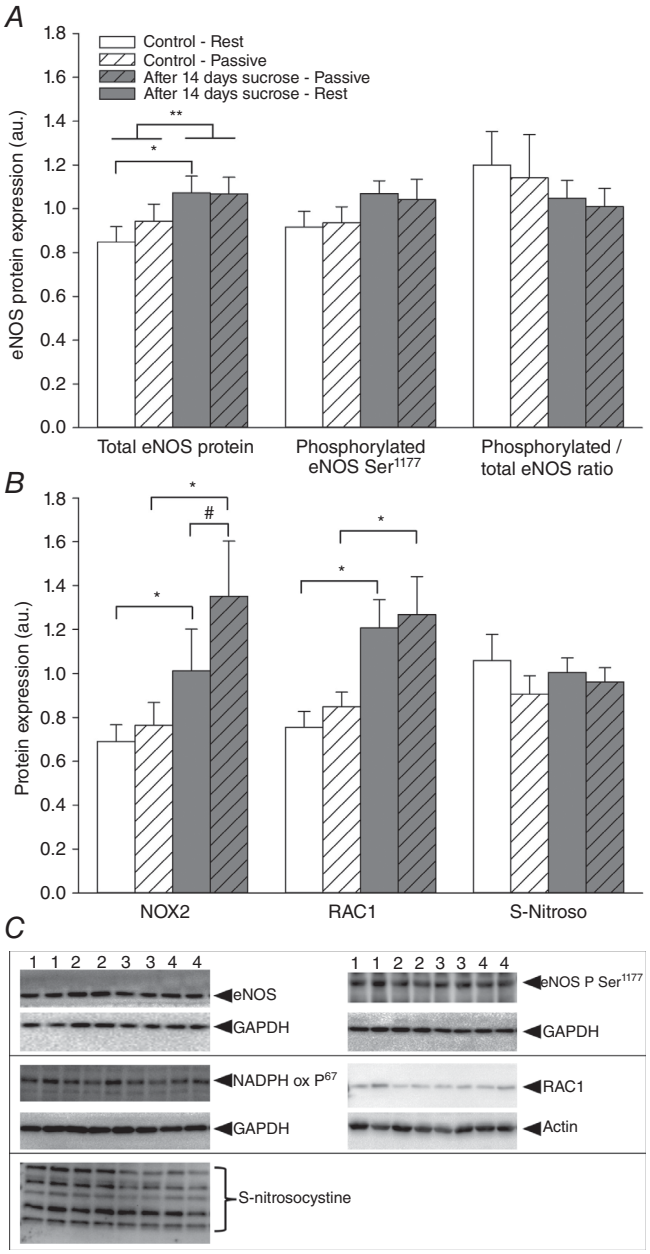

Figure 4. Protein expression and phosphorylation of eNOS, NOX, Rac1 and *S*-nitrosocystine before and after 14 days of sucrose intervention
A, protein expression of eNOS, phosphorylated eNOS (ser^{1177}) and phosphorylated *to* total eNOS ratio. *B*, NOX subunit p-67, Rac1 and *S*-nitrosocystine in muscle homogenates from musculus vastus lateralis at rest and after 20 min of passive leg movement. *C*, representative blots are shown from one subject in duplicates from rest (1) and after passive leg movement (2) under the control situation and from rest (3) and after passive leg movement (4) after the sucrose intervention. Data are presented as the mean ± SD ($n = 12$). *Significantly different compared to before the sucrose intervention under the same condition. **Overall significantly different compared to before the sucrose intervention.

protein were no different after the sucrose intervention and were not affected by passive leg movement (Fig. 4*A*).

NOX subunit p67 levels was markedly increased after the sucrose intervention ($P < 0.001$) (Fig. 4*B*) but unaffected by passive leg movement under both conditions.

Protein expression of Rac1 and S-nitrosocysteine

The protein expression of Rac1 levels was higher after the sucrose intervention ($P < 0.001$) (Fig. 4*B*) and was not changed from rest to acute passive leg movement for either of the conditions. The protein expression of *S*-nitrosocysteine was not changed by the sucrose intervention or from rest to acute passive leg movement under any condition (Fig. 4*B*).

Skeletal muscle homogenate mRNA levels

eNOS, PECAM-1 and VEGF-R2 mRNA levels were higher after the sucrose intervention ($P < 0.001$) (Fig. 5) and were not changed from rest to acute passive leg movement under any condition.

Plasma AGE and sRAGE

Plasma concentrations of AGE and sRAGE were no different between the control condition and the sucrose intervention (Fig. 6).

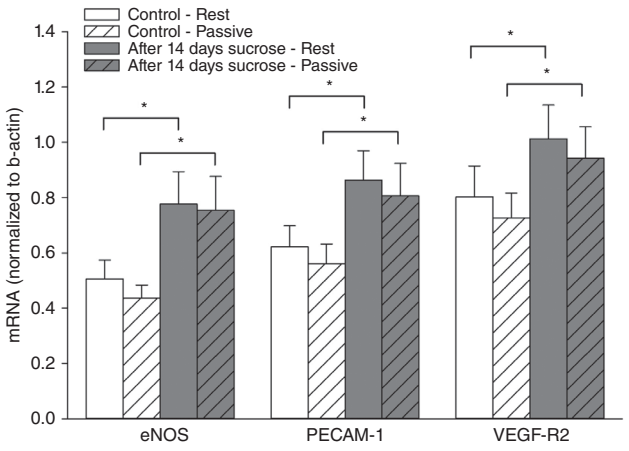

Figure 5. Skeletal muscle mRNA levels of eNOS, PECAM-1 and VEGR-R2 before and after 14 days of sucrose intervention
mRNA levels of eNOS, PECAM-1 and VEGF-R2 in muscle homogenates from musculus vastus lateralis at rest and after 20 min of passive leg movement. Data are presented as the mean ± SD ($n = 12$). *Significantly different compared to before the sucrose intervention under the same condition. There was no significant effect of passive leg movement.

Skeletal muscle interstitial and plasma NOx and PGI_2 levels

The muscle interstitial concentration of NOx was higher overall for all conditions ($P = 0.024$) and during passive movement ($P = 0.017$) after the sucrose intervention compared to the control condition (Fig. 7). Interstitial NOx was not altered acutely by passive leg movement or by active exercise compared to at rest. Plasma levels of NOx were not affected by the sucrose intervention (82.2 ± 11 and 86.6 ± 8 μM, control and sucrose, respectively).

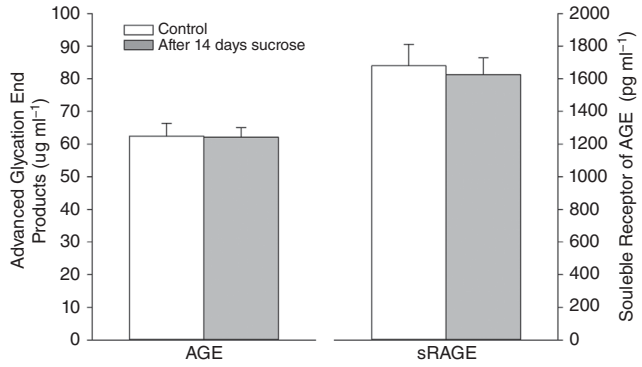

Figure 6. Plasma concentrations of AGE and sRAGE before and after 14 days of sucrose intervention
Plasma concentration of AGE and sRAGE collected at rest. Data are presented as the mean ± SD ($n = 12$). There was no significant effect of the sucrose intervention on AGE and sRAGE levels.

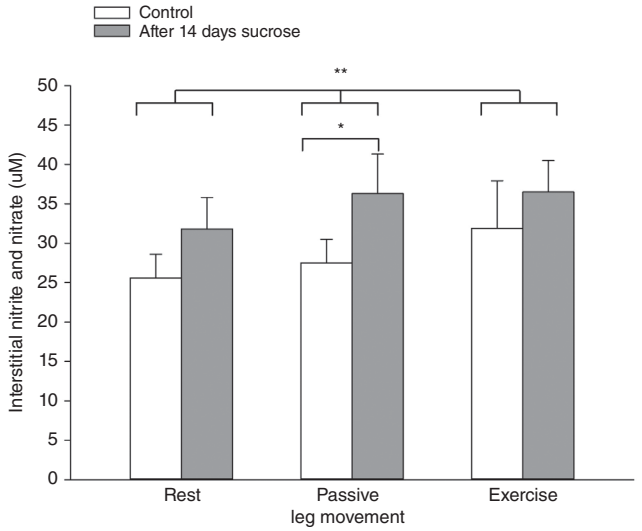

Figure 7. Skeletal muscle interstitial nitrite and nitrate before and after 14 days of sucrose intervention
Muscle interstial levels of the stable metabolites of nitric oxide, nitrite and nitrate (NOx) at rest, during 20 min of passive leg movement and active one leg knee extensor exercise. Data are presented as the mean ± SD ($n = 12$). *Significantly different compared to before the sucrose intervention during passive leg movement. **Overall significantly different compared to before the sucrose intervention.

Interstitial PGI_2 in musculus vastus lateralis was not changed with the sucrose intervention, but levels were increased during passive leg movement ($P = 0.032$) and active exercise ($P = 0.027$) compared to resting conditions (Fig. 8).

Discussion

The principal findings of the present study were that 14 days of supplementation of the diet with sucrose attenuated vascular function in young healthy individuals, as indicated by a lower hyperaemic response to both passive leg movement and active exercise. This impairment in vascular function was paralleled by an up-regulation of PECAM-1, eNOS, NOX and Rac1 protein expression in whole muscle tissue, as well as by an increased phosphorylation status of VEGF-R2 and a reduced phosphorylation status of PECAM-1. We propose that the impaired hyperaemic response after the sucrose intervention was the result of a reduced activation of eNOS caused by impairments in the mechanosensory complex and enhanced removal of NO because of increased superoxide formation by NOX. These findings indicate that regular intake of large amounts of sucrose, as commonly observed in unhealthy diets, is detrimental to vascular health and function and may be one of the underlying causes of cardiovascular disease.

Another novel finding was that there was no detectable increase in phosphorylation status of the mechanosensory

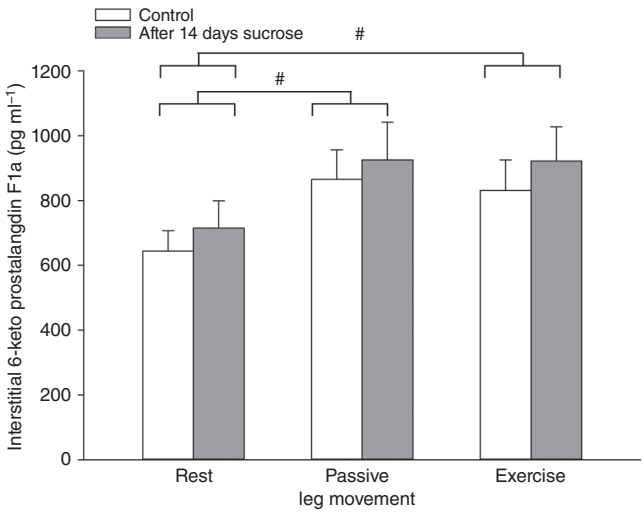

Figure 8. Skeletal muscle interstitial 6-keto prostaglandin $F_1\alpha$ before and after 14 days of sucrose intervention
Muscle interstitial levels of the stable metabolites of PGI_2, 6-keto prostaglandin $F_1\alpha$, at rest, during 20 min of passive leg movement and active one leg knee extensor exercise. Data are presented as the mean ± SD ($n = 12$). #Overall significantly different compared to resting conditions. There was no significant effect of the sucrose intervention.

complex in response to increased shear stress induced by acute passive leg movement. This finding is in contrast to cell culture findings showing increased phosphorylation with added shear stress and may also reflect the continuous shear stress influence on endothelial cells *in vivo*, rendering them less sensitive to experimental changes in flow.

Two weeks of high sucrose intake impairs vascular function

The supplementation of a normal diet with 3 × 75 g of sucrose results in a repeated transient ∼50% and 700% increase in blood glucose and insulin, respectively (Jameel *et al.* 2014). The response curve to sucrose is similar to that of a standard oral glucose tolerance test and blood glucose returns to initial levels after 2 h in the morning and after 3 h at midday and in the evening (Jarrett *et al.* 1972; Carroll & Nestel, 1973). The present intervention thus led to high blood glucose levels during at least 8 h every day and, as hypothesized, the diet had adverse effects on vascular function in young healthy subjects. This was indicated by a marked reduction in the blood flow response to passive leg movement and a reduction in blood flow during low intensity exercise. The hyperaemia in response to passive leg movement has previously been shown to be almost completely dependent on the NO system (Mortensen *et al.* 2012). Thus, the present finding of a reduced blood flow response to passive leg movement after high sucrose intake indicates that a high sucrose intake interferes with the function of the NO system, either by affecting NO formation, NO availability or NO responsiveness.

The detrimental effect of the sucrose intervention on vascular function was further confirmed by our finding that blood flow during low intensity active exercise also was decreased after the sucrose trial compared to the control condition. Interventions that reduce exercise hyperaemia are unusual because the oxygen requirement during exercise provides a strong and multifactorial impact on several interacting vasodilator systems, allowing for redundancy, including between the NO and the prostacyclin systems (Boushel *et al.* 2002; Hillig *et al.* 2003; Mortensen *et al.* 2007). However, a compensatory formation of prostacyclin during exercise was not evident after the sucrose intervention, as indicated by similar interstitial prostacyclin levels in in the control and sucrose conditions.

Chronic high blood glucose levels have been linked to increased endothelin-1 vasoconstrictor tone in humans (Diehl *et al.* 2013) and NO has been shown to inhibit the expression and release of endothelin-1; thus, reduced NO availability may increase endothelin-1 levels (Bourque *et al.* 2011). However, endothelin-1 did not appear to have influenced the impaired vascular reponse in the present study because levels were similar in the sucrose and control intervention.

It should also be noted that, considering the limited muscle mass involved in knee extensions, the observed reductions in blood flow after the sugar intervention were probably not a result of alterations in cardac function, as also confirmed by similar heart rates in the sucrose and control conditions.

Vascular mechanosensors are widely affected by high sucrose ingestion

Our functional data suggest an impairment in the NO system, which was not related to the amount of eNOS. By contrast, the enzyme was up-regulated both at the mRNA and protein level. This finding fits well with previous observations of up-regulation of eNOS in endothelial cells collected from the brachial artery of aged subjects (Seals *et al.* 2011) and may reflect a compensatory mechanism for the impaired function of the NO system. Therefore, the reduced response to passive leg movement after the sucrose intervention may have been related to limitations in the signal transduction leading to activation of eNOS (Fleming & Busse, 1999). Tyrosine phosphorylation of PECAM-1 is central to the translation of shear stress-induced activation of eNOS (Fleming *et al.* 2005). In the present study, we show that the impaired flow response to passive leg movement is paralleled by a marked reduction in basal phosphorylation status of PECAM-1 at tyrosine residue 713, suggesting that this mechanosensor was negatively affected by the sucrose intervention.

An additional explanation for the lower shear stress response after the sucrose intervention is the parallel increase in basal phosphorylation of VEGF-R2 serine residue 1175. The enhanced phosphorylation of VEGF-R2 is in agreement with observations in endothelial cell cultures, showing that the glucose induced phosphorylation occurs via the Src family kinases and is dependent on oxidative stress (Warren *et al.* 2014). Importantly, phosphorylation was shown to result in impaired trafficking of VEGF-R2 to the cell membrane with a consequent reduction in available receptors at the cell surface (Warren *et al.* 2014). Such an effect could reduce shear stress sensing and the increased VEGF-R2 phosphorylation after the sucrose intervention may partly explain the observed lower flow response during passive movement.

Regulation and activation of eNOS

The period of high sucrose intake led to an increase in eNOS expression both at the mRNA and protein level. This observation is in agreement with observations in endothelial cell cultures showing that incubation

of cells with high glucose enhances eNOS expression (Cosentino *et al.* 1997). The mechanisms behind the up-regulation of eNOS could, in the present set-up, be related to oxidized low-density lipoprotein and/or hydrogen peroxide because these have been shown to increase eNOS gene transcription and prolong the half-life of eNOS mRNA, respectively (Cosentino *et al.* 1997; Ramasamy *et al.* 1998; Drummond *et al.* 2000).

We found no changes in phosphorylation of eNOS serine residue 1177, which is known to be an important site of activation (Dimmeler *et al.* 1999; Fisslthaler *et al.* 2000), although one or several of the other mechanisms of eNOS activation could have been impaired, in particular those influenced by oxidative stress, as described below.

High sucrose intake leads to high NOX activity

NO reacts rapidly with superoxide radicals, resulting in the formation of peroxynitrite, and NO is no longer available for vasodilatation. A high concentration of superoxide can also lead to uncoupling of eNOS, which leads to reduced NO formation and increased superoxide formation by the enzyme (Fleming & Busse, 2003). The most important source of superoxide radicals in the vasculature is NOX in endothelial cells. High glucose levels are known to be associated with increased levels of oxidative stress in endothelial cell cultures in part via up-regulation (Cosentino *et al.* 1997, 2003) and increased activity (Inoguchi *et al.* 2000) of NOX. In the present study, the sucrose intervention resulted in a two-fold increase of the regulatory subunit p67 on NOX compared to the control condition. This was also paralleled by an increased protein expression of the NOX assembler Rac1 suggesting increased activity of the enzyme. NOX is known to be present both in endothelial cells and in skeletal muscle (Cocks *et al.* 2012, 2016) and the observed increases could have occurred in either or both tissues.

Thus, the level of superoxide production was probably enhanced and the bioavailability of NO was correspondingly reduced. We could, however, not detect an increase in *S*-nitrosocysteine levels in the muscle samples. An increased level of *S*-nitrosocysteine is suggested to reflect an increased formation of peroxynitrite (Hlaing & Clément, 2014; Hsieh *et al.* 2014). The data should, however, be interpreted with caution because the sensitivity of the assay is limited and sensitivity may also be low considering that the measurements were made on whole muscle homogenates of which endothelial cells make up only a small fraction.

AGE are not associated with reduced vascular function

High levels of AGE have been linked to reduced mechanotransduction (Otero *et al.* 2001; Soro-Paavonen *et al.* 2010; Liu *et al.* 2012; Naser *et al.* 2013) and, based on previous findings, a diet high in sucrose was hypothesized to increase the circulating levels of AGE (Schalkwijk *et al.* 2004). Plasma AGE levels were, however found to be unaltered after the sucrose intervention. One explanation could be that the diet intervention was too short and that a limited AGE formation plasma was removed by RAGE. The RAGE levels of the young healthy subjects in this project were relatively high compared to the general male population (Prakash *et al.* 2015). Thus, the changes observed in vascular function were not related to changes in AGE plasma concentration.

Phosphorylation of the mechanosensor complex

The present study attempted to assess the acute effect of passive movement and thereby shear stress on the activation status of the mechanosensor complex comprising PECAM-1, VEGF-R2 and VE-cadherin. Interestingly, activation of the mechanosensor complex was not detectable after 20 min of passive leg movement. This finding contrasts with findings from cell culture studies in which endothelial cells (of varying origin) have been cultured and stimulated with laminar fluid shear stress (Osawa *et al.* 1997; Orsenigo *et al.* 2012; dela Paz *et al.* 2013). The lack of effect of passive movement induced hyperaemia on phosphorylation of the mechanosensor proteins could be related to the endothelial cells *in vivo* continuously experiencing large variations in shear stress according to heart cycle, muscle activation and changes in flow, and thus the increased flow during passive leg movement provides a relatively small stimulus. By contrast, cells in culture are habituated to static no-flow conditions and, subsequently, they are exposed to shear stress that probably provides a greater stimulus than passive movement in the *in vivo* setting.

Conclusions

In the present study, we demonstrate that a diet high in sucrose impairs vascular function in young healthy subjects. The impaired vascular function was paralleled by alterations of the endothelial mechanosensory protein complex, suggesting that vascular function was compromised at the site of mechanosensing in addition to potential disruption of the NO system. Interference of eNOS was also indicated by a compensatory up-regulation of this protein. A probable mechanism behind a reduced NO availability was inactivation of eNOS and/or removal of NO due to increased superoxide formation from the up-regulation of NOX.

Another important finding was that acute passive leg movement did not lead to measureable activation of the mechanosensor complex or changes in NO formation

despite leading to increased blood flow. Prostacyclin formation, on the other hand, is increased during passive leg movement to the same extent as during low-intensity active exercise.

Perspective

A single bottle of sweetened soft drink alone can contain ∼50 g of simple sugars and, in western societies, soft drinks are often a companion throughout the day. The findings of the present study indicate that the regular intake of large amounts of sucrose impairs vascular mechanosensory proteins, increases the oxidative stress potential and disrupts the regulation of vascular tone. Impaired vascular regulation is the first step on the way to vascular disease and the findings of the present study indicate that a sucrose-rich diet can be a factor contributing to the development of vascular disease.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

All authors were resonsible for the conception and design of the study; the collection of data; and the drafting of the article or revising it critically for important intellectual content. LG, NM, ML, LS, EAR and YH were responsible for the analysis and interpretation of data. All authors approved the final version and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Translational perspectives

The present study shows that dietary sugar impairs vascular function in healthy humans. The translation of the mechanical stress that flowing blood applies to the blood vessel wall into an intracellular signal, termed 'mechanotransduction', is important for vascular function. Sugar could impair this mechanosensing; therefore, we aimed to evaluate the impact of a diet high in simple sugars on vascular function in healthy young men. We hypothesized that endothelial mechanotransductor proteins are important for vascular regulation and that a high sucrose diet impairs vascular function in part by decreasing mechanotransduction in vascular endothelial cells. Young healthy men were supplemented with three daily 75 g sucrose drinks for 2 weeks. The sucrose intervention greatly affected vascular function, which is a novel finding, emphasizing the impact of sugar intake on the risk of vascular diseases. Moreover, our molecular findings indicate that the regular intake of high amounts of sucrose disrupts the normal regulation of blood vessels by impairing vascular mechanosensor proteins. The findings of the present study suggest that a sucrose-rich diet can contribute to the development of vascular disease. Future studies are encouraged to investigate whether sugar-induced vascular dysfunction is reversible after several years of 'sugar abuse' and more work is needed to fully understand the mechanisms involved in dietary-induced vascular diseases.