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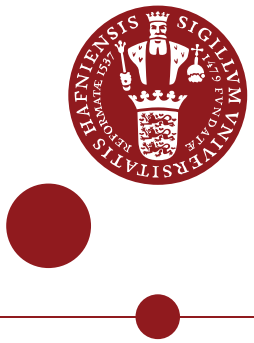

Does high sugar intake leads to an impaired vascular response to passive movement?

Medfører et højt sukkerindtag forringet vaskulær funktion som reaktion på passiv bevægelse?

Bodil Lanng Pallisgaard (RSB534)
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Supervisors: Professor Ylva Hellsten & Post.Doc. Lasse Gliemann

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Contents

Abstract

Dysfunctional endothelial cells, which show a reduced formation of nitric oxide (NO), are known to be involved in the development of cardiovascular disease in response to hyperglycaemia. An important stimulus for NO formation is passive movement of the lower leg, which activates endothelial NO synthase (eNOS) [1]. During passive movement, the blood vessels are exposed to shear stress and passive stretch [2]. Shear stress signalling is sensed on the endothelial cell surface by a mechanosensor complex consisting of platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial cadherin (VE cadherin) and vascular endothelial growth factor receptor 2 (VEGFR2) [3], which in response to an increase in shear stress activate eNOS. How this shear stress signalling pathway is affected by hyperglycaemia *in vivo* is not yet described. The formation of advanced glycation end products (AGE) is suspected to increase in response to hyperglycaemia and might adversely affect the shear stress signalling pathway.

This thesis demonstrates that the response to shear stress and passive stretch is adversely affected in young healthy male subjects by a highly elevated sugar intake (75 g x 3 per day for two weeks). Their vasodilatory response to passive movement of the leg is significantly decreased after the two weeks of high sugar intake. However, the mechanisms behind this decrease in NO formation are still unclear. Both the level and the activation of eNOS and the mechanosensor complex is found to be unaffected by the high sugar intake and so is the level of AGE. These results demonstrate that an elevated sugar intake for two weeks is able to significantly decrease the vasodilatory response to passive movement but how the shear stress and passive stretch mechanisms are inhibited by a high sugar intake is still not resolved.

Dansk resumé

Det er kendt at dysfunktionelle endothelceller, som viser reduceret dannelse af nitrogenmonooxid (NO), er involveret i udviklingen af kardiovaskulære sygdomme i respons til hyperglykæmi. Et vigtigt stimulus for NO-dannelse er passiv bevægelse af underbenet, som aktiverer endothelial NO synthase (eNOS) [1]. Under dette passive arbejde bliver blodkarrene udsat for shear stress og passivt stræk [2]. Shear stress signalering registreres på overfladen af endothelcellerne af et mekanosensorkompleks, der består af platelet endothelial cell adhesion molecule 1 (PECAM-1), vascular endothelial cadherin (VE cadherin) og vascular endothelial growth factor receptor 2 (VEGFR2) [3], som i respons til øget shear stress aktiverer eNOS. Hvordan denne shear stress signalvej bliver påvirket af hyperglykæmi *in vivo* er endnu ikke beskrevet. Det formodes at dannelsen af advanced glycation end-products (AGE) øges i respons til hyperglykæmi og kan indvirke skadeligt på shear stress signalvejen.

Denne opgave viser at responset på shear stress og passivt stræk bliver negativt påvirket i unge raske mandlige forsøgspersoner af et kraftigt forhøjet sukkerindtag (75 g x 3 per dag i to uger). Deres vasodilatoriske respons til passiv bevægelse af deres ben, er signifikant formindsket efter de to uger med højt sukkerindtag. Mekanismerne bag dette fald i NO-dannelse er dog stadig ikke afklaret. Både niveau og aktivering af eNOS og mekanosensorkomplekset er upåvirkede af det høje sukkerindtag, og det er niveauet af AGE også. Disse resultater viser at et forhøjet sukkerindtag i to uger mindsker det vasodilatoriske respons til passiv bevægelse signifikant, men hvordan mekanismerne bag shear stress og passivt stræk bliver inhiberet af et højt sukkerindtag er stadig ikke afklaret.

Introduction

It is known that hyperglycaemia leads to an increased risk of cardiovascular disease. Both chronic hyperglycaemia as seen in insulin resistance and diabetes [4] and repeated transient hyperglycaemia in response to food consumption [5, 6] has been shown to increase the risk of cardiovascular disease. Endothelial dysfunction is involved in the development of cardiovascular disease [7, 8]. One of the central mechanisms in endothelial dysfunction is reduced formation of the vasodilatory compound NO by eNOS in response to shear stress [7, 9]. This thesis examines the effect of a period of hyperglycaemia on NO formation activated by shear stress and passive stretch, with a focus on the shear stress signalling mechanisms.

Background for the project

Based on existing literature [10-19] it is relevant to investigate how an elevated sugar intake for a prolonged period of time, affect the ability to adjust the vasomotor tone in response to both passive movement and exercise. In order to examine this, 12 subjects were subjected to a high sugar intake for two weeks and their vascular function were examined both after this intervention and after a control period. Furthermore the expression of a range of different proteins, both in the plasma and muscles were measured, in order to determine possible mechanisms for changes in the ability to adjust vasomotor tone.

The circulatory system

The circulatory system is a closed loop, consisting of the heart and the blood vessels. It is divided into two circulatory systems: The pulmonary circulation, where the blood is oxygenated by the air in the lungs, and the systemic circulation where the blood delivers oxygen to all the organs in the body [20]. The blood also transports a great variety of different substances like hormones, nutrients, metabolic waste etc. between the different organs.

There are several different kinds of blood vessels in the circulatory system [20]. When the blood enters the systemic circulation, it is transported through aorta, which branches into arteries, small arteries and then arterioles. These vessels are classified as distribution and resistance vessels, since their main purpose is to distribute the required amount of blood to all organs. This is achieved by contraction or dilation of the different vessels, primarily the small arteries and arterioles, in order to allow more or less blood to pass through to different tissues. This is where the major component of the resistance in the circulatory system occurs and this markedly reduces the velocity of the blood [20]. The diameter of a given distribution vessel is regulated by contraction or relaxation of a layer of smooth muscle surrounding the vessel. The tone of the smooth muscle cells is precisely adjusted by an array of different vasoconstricting and –dilating substances released both locally and systemically.

In the organs, the arterioles branch to capillaries. These are also denoted as exchange vessels since this is where the major exchange of gasses, nutrients etc. between tissue and blood takes place [20]. The capillaries are the smallest, but also by far the most numerous vessels of the circulation. This leads to a large total cross-sectional area and hence a low mean velocity of the blood in these vessels [20]. The walls of capillaries consists of a single endothelial layer, connected by gab junctions, and a basal lamina, which enables a fast exchange across the membrane [20].

The last major class of blood vessels is the capacitance vessels, which include venules and veins. These vessels have a large compliance and low resistance and contain most of the blood [20].

All blood vessels are lined by a single layer of endothelial cells. They form the barrier between the blood and the surrounding tissue and facilitate the exchange of substances like nutrients, gasses, metabolites, hormones etc., either through the cells or via gap junctions [20]. They are also the main mediators of local vasodilatory signals [16].

Vasomotor tone

The large network of blood vessels throughout the body ensures an adequate supply of blood to all the different organs in the body, by constantly adjusting the vasomotor tone of the small arteries and arterioles [20].

The overall vasomotor tone is regulated by the balance between vasoconstrictor and vasodilator signals [16]. The autonomous nervous system is responsible for vasoconstriction and thereby resistance in the arterioles [20]. In working muscles, vasodilator systems are able to override the constriction by sympathetic activity and cause local vasodilation [16]. Most of the vasodilatory compounds are formed by the endothelial cells and include NO, ATP and prostacyclin [16]. In addition, some locally formed compounds, such as ATP, can directly influence the effect of sympathetic activity by interference by the adrenergic receptor signalling. The ability to override the systemic vasoconstricting signal by sympathetic activity and cause local vasodilation is termed functional symphatolysis [21].

Shear stress

One of the signals leading to NO formation and thus vasodilation is shear stress signalling. Shear stress is the tangential frictional force the blood flow exerts on the endothelial cells lining the blood vessels [22]. When the blood flow to a tissue increases, e.g. in muscle tissue when exercise is initiated, the shear stress in the blood vessels increases. The magnitude of the shear stress are able to increase markedly in response to these changes in local blood flow [23]. The endothelial cells are continuously exposed to shear stress due to the flow of blood but when the shear stress increases, it initiates a

signalling cascade leading to NO formation and vasodilation, decreasing the shear stress to normal levels [23].

In this study we induce shear stress in *m. quadriceps femoris* by passive movement of the lower leg. It has previously been shown that this model gives rise to a ~3 times increase in blood flow in young persons and that the increase is primarily mediated via NO signalling [2]. The mechanisms underlying the effect of passive movement on flow are not yet fully understood. A possible explanation could be that when the leg is passively moved, *m. quadriceps femoris* is alternately compressed and extended, which gives rise to alternately compression and opening of the capillaries. During the compression, most of the blood is presumably squeezed from the vessels and will then rush back in during the open period, due to hydrostatic forces. This rush of blood through the capillaries will then apply a periodic shear stress to the endothelial cells and lead to NO formation and consequently vasodilation. This passive movement also apply a passive stretch to the muscles and blood vessels and it is not known how much of the vasodilatory response can be contributed to the two different mechanisms [2]. In this thesis, the focus is on the shear stress signalling pathway which is known to induce NO formation [24, 25] and thus it is assumed to play an important part in the response to passive movement [2].

Muscle proteins: The mechanosensor complex and the effector protein

How the endothelial cells lining the blood vessels sense an increase in shear stress is not yet fully understood. However, a mechanosensor complex consisting of three different transmembrane proteins is likely to be involved in the signal transduction over the membrane [3]. These proteins are PECAM-1 [26], VE Cadherin [27] and VEGFR2 [28] and their structure and suggested functions will be reviewed below. They are also activated by an array of different agonist and have several signalling pathways, leading to different cell responses, including regulation of the permeability of the blood vessels and angiogenesis, but in this thesis, the focus will be on their role in vasodilation. The effector protein is eNOS, which produces NO that leads to relaxation of the smooth muscle surrounding the blood vessels causing vasodilation [1]. This protein and the function of it will also be described below.

The overall shear stress signalling pathway (Figure 1) is assumed to be initiated when PECAM-1, which form a complex with VE Cadherin, sense shear stress and is phosphorylated [29]. This leads to VEGFR2 being recruited by VE cadherin, and then cause an activation of Akt intracellularly, which in turn phosphorylates eNOS [29, 30]. Furthermore the activation of PECAM-1 might lead to a dissociation of eNOS from PECAM-1, possibly exposing the phosphorylation sites, allowing this phosphorylation by Akt [1, 31]. This leads to a maintained activation of eNOS and formation of NO, causing vasodilation [32].

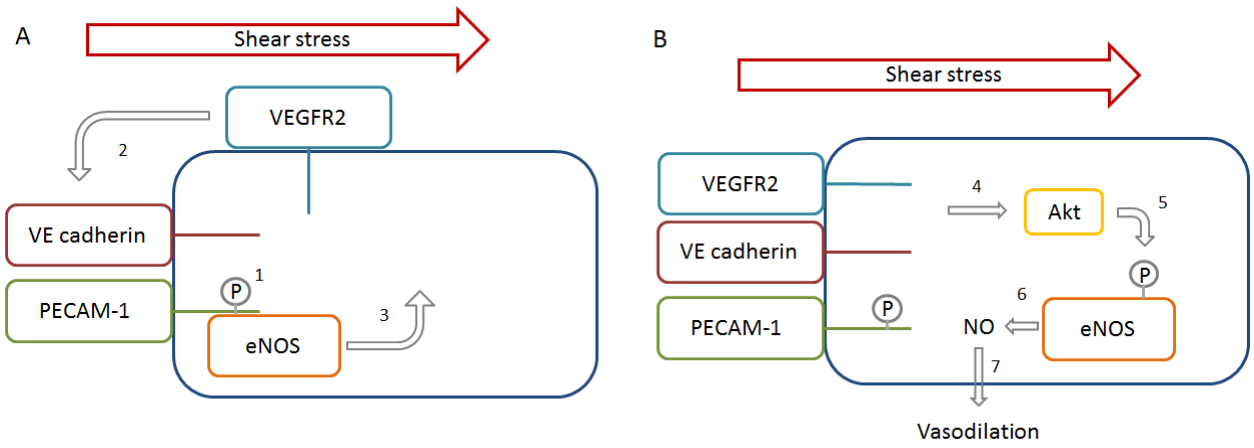

Figure 1: The mechanism behind shear stress induced vasodilation. A: When shear stress is applied to an endothelial cell it is sensed by PECAM-1, which is then phosphorylated (1). This phosphorylation leads to VEGFR2 being recruited to the PECAM-1-VE cadherin complex (2) and to dissociation of eNOS from the complex (3). B: The recruitment of VEGFR2 to the mechanosensor complex leads to activation of Akt (4) which phosphorylates eNOS (5). This activates eNOS which then produces NO (6) that leads to vasodilation (7).

PECAM-1

PECAM-1 is a transmembrane glycoprotein consisting of six extracellular Ig-like loops, a transmembrane domain and a short cytoplasmic domain [26]. There are six different known human isoforms, and the differences are all localised in the cytoplasmic tail [31]. These different isoforms have different phosphorylation sites and are assumed to be involved in different signalling pathways in the endothelial cell [31]. PECAM-1 is primarily located in the cell-cell junctions and contributes to the connection between neighbouring endothelial cells by homodimerizing to PECAM-1 proteins from adjacent cells.

Bagi *et al.* [33] have demonstrated that PECAM-1 is important in shear stress signalling by showing that the vasodilatory response of arteries from PECAM-1 knockout mice is markedly reduced compared to wildtype mice. Moreover, Tzima *et al.* [3] demonstrated that PECAM-1 is a direct transducer of mechanical force, by applying force on magnetic beads, bound to PECAM-1. The cytoplasmic domain of PECAM-1 is able to bind to β- and γ-cathenines and in that way interact with the actin filaments of the cytoskeleton [26]. The cytoskeleton may then transmit the force applied to the apical surface by shear stress, to other regions of the endothelial cell [29].

Another more recent explanation of how the transmission of shear stress through the cell occurs is presented by Conway & Schwartz [34]: According to their studies, there is an intrinsic tension the entire cell and cytoskeleton and when the endothelial cell is exposed to shear stress, an overall relaxation of the cell and of VE Cadherin, which is a part of a larger complex together with PECAM-1,

occurs. Simultaneously, the tension on PECAM-1 increases rapidly in response to flow [34]. In either way the shear stress causes a mechanical deformation of PECAM-1, leading to PECAM-1 being phosphorylated at the cytoplasmic tail at tyrosine663 (Tyr663) and tyrosine686 (Tyr686), perhaps because the deformation exposes the phosphorylation site [26]. Earlier studies have also described that Tyr713 is phosphorylated and is likely to constitute a binding site for the tyrosine kinase SH-PTP2, leading to a major increase in the phosphatase activity [35].

Upon application of shear stress, the first response, according to Hahn & Schwartz [29], is that PECAM-1 activates Src. PECAM-1 might also interact with eNOS at the cell-cell junctions [1, 31]. This means that a possible outcome of the phosphorylation of PECAM-1 in response to shear stress is that it causes a dissociation of eNOS from PECAM-1, exposing the phosphorylation sites of eNOS [31].

VEGFR2

The structure of the tyrosine kinase VEGFR2 is quite similar to PECAM-1: It is a transmembrane glycoprotein, consisting of 7 Ig-like extracellular loops, a transmembrane domain and a cytoplamic tail which contains several phosphorylation sites [28].

When PECAM-1 is activated, this activates Src while VE cadherin recruits VEGFR2 to the cell-cell adhesion site, forming a VEGFR2-VE cadherin complex [29, 36]. VEGFR2 is then phosphorylated and becomes active which leads to an activation of phosphatidylinositol-3-OH kinase (PI3K), which in turn leads to an activation of Akt [37]. This ultimately leads to an activation of eNOS, formation of NO and vasodilation.

There are two major phosphorylation sites on VEGFR2, tyrosine 1175 (Tyr1175) and tyrosine 1214 (Tyr1214), and their phosphorylation is dependent on vascular endothelial growth factor (VEGF) [38]. VEGF is ligand to VEGFR2 and induces lateral dimerization, leading to autophosphorylation [38]. Endothelial VEGF is required in endothelial cell to cause this autophosphorylation of Tyr1175 and Tyr1214 in the absence of extracellular VEGF [39]. Dela Paz *et al.* [40] hypothesize that the onset of shear stress activates some mechanisms in the endothelial cells which releases VEGF, since Tyr1175 and Tyr1214 are phosphorylated as early as 15 seconds after onset of hear stress, leading to activation of VEGFR2. When the binding of VEGF to VEGFR2 is blocked, it leads to a significant reduction in the activation of VEGFR2 and it also leads to a marked decrease in the subsequent activation of eNOS, indicating that ligand activation of VEGFR2 is important in the shear stress signalling pathway [40].

VE cadherin

VE cadherin is cell-cell adhesion molecule and the most important one in determining the endothelial cell contact integrity and the permeability of the blood vessels [27]. It consists of five extracellular domains, a transmembrane domain and a cytoplasmic tail [27].

The importance of VE cadherin in response to shear stress is demonstrated by Tzima *et al.* [3] who showed that VE cadherin -/- cells does not show subsequent activation of PI3K in response to shear stress. Upon phosphorylation of PECAM-1 in response to the applied shear stress, VE cadherin recruits VEGFR2 to the mechanosensor complex [32]. The recruitment of VEGFR2 then leads to activation of VEGFR2 by Src [29]. VE cadherin cell-cell adhesion is not needed for the shear stress induced response [3], indicating that VE cadherin's response to shear stress is independent of the other important functions of the protein.

eNOS

eNOS is the effector protein which produces NO in response to shear stress, causing vasodilation. It is part of a larger protein complex, and eNOS can be activated by binding to a Ca^{2+}/calmodulin (CaM), a process that is activated when intracellular calcium is increased [41]. In addition, eNOS can be activated through phosphorylation at different sites.

The eNOS phosphorylation site serine 1177 (Ser1177) is rapidly phosphorylated in response to shear stress by protein kinase B (PKB), leading to activation of eNOS [30]. Threonine 495 (Thr495) on the other hand is constitutively phosphorylated and disrupts the binding of CaM to eNOS, inhibiting the production of NO [41]. Shear stress activates Src [42] and proline-rich tyrosine kinase (PYK2) [43], both of which dephosphorylate Thr495. When Thr495 is dephosporylated, more CaM is able to bind to eNOS [44]. Combined with the phosphorylation of Ser1177, this enables the resting level of Ca^{2+} in the cell to activate eNOS, causing the production of NO to increase, leading to vasodilation [1].

The activation of eNOS in response to shear stress leads to a 2-4 times increase in NO production compared to the basal level of NO, and the activation of eNOS is sustained as long as the shear stress stimulus is sustained [1]. The NO is produced from the amino acid L-arginine and under normal physiological conditions this is not a limiting factor [1].

Plasma AGE and sRAGE

AGE products are formed when reducing sugars reacts with proteins, lipids or nucleic acids in non-enzymatic reactions [45]. These sugars induce a complex series of reactions and rearrangements with the substrates and the final products are multiple, reactive species which collectively are termed AGE products [45, 46]. AGE is capable of accumulating on vascular wall collagen and basement membranes

[14] but some AGE products are soluble and are transported with the blood [47]. AGE can potentially cause damage by either reacting directly with different proteins, especially matrix-proteins, or by binding to the receptor for advanced glycation end products (RAGE) [45]. Activation of RAGE, either by AGE or other RAGE substrates, leads to formation of reactive oxygen species (ROS) and activation of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) [48].

Xu *et al.* [18] have investigated how AGE affects the endothelial-dependent vasodilation both in human vascular endothelial cells (HUVECs) and in rabbit blood vessels: When untreated rabbit blood vessels are exposed to an abrupt increase in flow, they respond by a fast increase in perfusion pressure, followed by a gradual decline, which is assumed to be caused by shear stress-dependent vasodilation. Administration of AGE inhibits this gradual decline, demonstrating that AGE inhibits the shear stress-dependent vasodilation. When HUVECs are treated with AGE, serine phosphorylation of eNOS is attenuated, inhibiting the activation of eNOS and hence inhibiting the NO-dependent vasodilation. This inhibited phosphorylation and activation of eNOS in response to AGE, provides a logical explanation for the lack of shear stress response in AGE-treated rabbit vessels.

The formation of AGE is accelerated during hyperglycaemia [14, 46] and in tissues exposed to high glucose concentrations, proteins are more prone to react with reducing sugars and form AGE [49]. This makes it relevant to investigate if two weeks of elevated sugar intake induces hyperglycaemia in healthy, moderately active subjects and in that way increases the level of AGE, inhibiting the shear stress-dependent vasodilation.

The soluble receptor for AGE (sRAGE) binds AGE and thus inhibits it [48]. An increase in the concentration of sRAGE is able to counteract potential harmful effects caused by an increase in AGE [50]. This is supported by both negative and positive results: Low concentrations of sRAGE have been found to correlate with increased possibility to suffer from high blood pressure, insulin resistance, type 2 diabetes and systemic inflammation [50] and sRAGE levels have been found to correlate inversely with fasting plasma glucose [51]. And when recombinant sRAGE is administered to arteriosclerosis-prone mice, it suppresses the acceleration of atherosclerosis [52]. These results suggest that it is relevant to determine the levels of sRAGE in response to an elevated sugar intake, since sRAGE possibly counteracts potential damaging effects caused by an elevated sugar intake, but might be decreased by the intervention.

Aim

The aim of this thesis is to investigate if the ability to vasodilate in response to both passive movement and exercise is affected by two weeks with elevated sugar intake.

To find out if this is the case, the blood flow both during rest, during passive movement of the lower leg and during exercise is measured. I will then investigate if there is a correlation between the levels or the phosphorylation status of the proteins in the mechanosensor complex, PECAM-1, VE cadherin and VEGFR2, and the ability to vasodilate. This will be done because a decrease in level or phosphorylation status of one or more of these proteins could possibly impair the NO release from the endothelial cell which is activated by the passive movement [2]. Also the level and the phosphorylation status of eNOS will be examined, to test if the activation of eNOS is impaired, since this is the effector protein which is activated in response to passive movement [1]. Furthermore, I will test if there is any link between the detected levels of AGE or sRAGE in the blood stream, the fasting blood glucose, and the possible change in the vasodilatory capacity.

Method

Experimental protocol

Subjects

12 young (mean 22.6 years, range 20-30 years), healthy, moderately active (training 1-3 times/week), male subjects with a low daily sugar intake were recruited. Before the experiment was started, their food intake was registered for a week and their maximal oxygen uptake was tested (mean 4.1 l/min, range 3.1-4.8 l/min). Of these 12, two were excluded from the data analysis. One due to illness prior to the second test and one because it became evident he was strength training 6-7 times/week and thus did not match the rest of the subjects.

Design

The experiment was designed as a cross over intervention (Figure 2). In the first test period 6 subjects underwent a period of 14 days with an elevated sugar intake of 75 g glucose drink three times a day. The remaining 6 subjects acted as control group. Both groups were then subjected to a wash out period of one month.

In the second test period, the 6 subjects formerly acting as control group were then subjected to the same elevated sugar intake and the remaining 6 subjects then constituted the control group. The subjects were tested two times during the experiment: After the first and second test period. The subjects were randomly assigned to either group 1 or group 2.

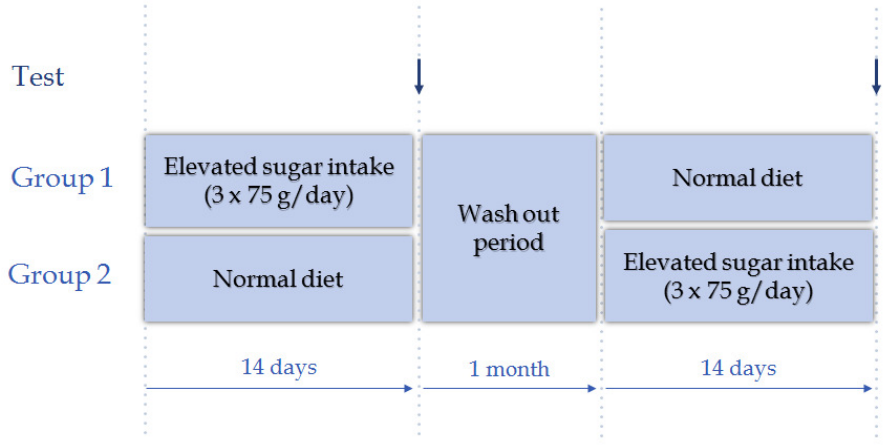

Figure 2: The experimental design. The study was designed as a cross-over experiment where the two intervention periods each lasted 14 days with a wash out period of minimum one month. The subjects were tested after both intervention periods.

Protocol, VO_2-max test

Maximal oxygen uptake was assessed on an ergometer bike using an Oxycon system (Oxycon Pro, Viasys Healthcare, Hoechberg, Germany). Starting at 100 w, the resistance was increased by 25 w every minute until exhaustion (<75 RPM). Moderate positive verbal encouragement was given during the test. The criteria for achieved VO_2-max were a respiratory exchange rate (RER) >1.15, plateau in VO_2 and visible exhaustion.

Protocol, experimental day

On the experimental day, the subject had been fasting for a minimum of 8 hours and had not ingested caffeine for the last 24 hours. A graphic overview of the experimental day is given in Figure 3.

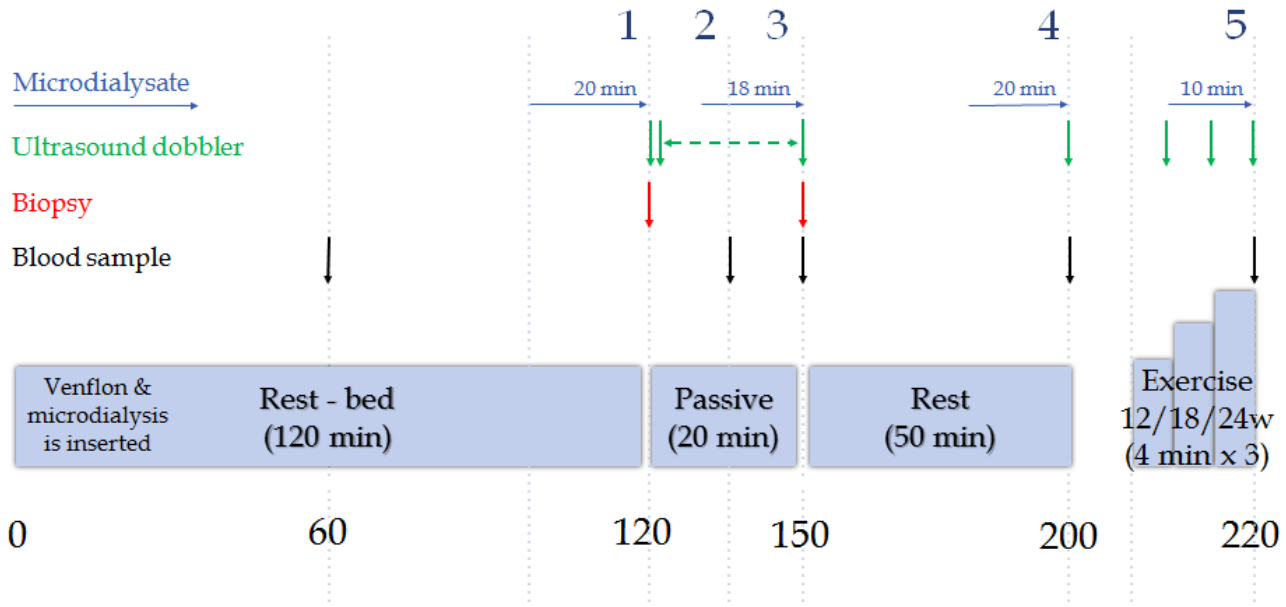

Figure 3: Overview of the experimental day. Microdialysate was collected during both periods of rest, passive work and exercise. During passive movement ultrasound Doppler was performed every 15 sec during the first three minutes, every minute the next two minutes and every 2.5 min for the last 15 minutes. Two muscle biopsies were taken, one after the first period of rest and the second immediately after the passive movement. Blood samples were collected during resting periods, passive movement and exercise.

Upon arrival, the subject was placed in bed to rest. A venflon was inserted in the left arm, in order to easily collect blood samples during the experiments. After local anaesthesia (lidocaine, 20 mg/ml), two micro dialysis probes were inserted in the right thigh in *m. vastus lateralis*, which allowed for collection of microdialysate from the muscle during the experiments [53]. After one hour of rest, blood samples were taken for analysis.

After 100 minutes of supine rest, micro dialysate was collected for 20 minutes, in order to obtain a baseline measurement. After two hours of rest a muscle biopsy was taken from *m. vastus lateralis* in the right thigh (time point 1 in Figure 3) [54].

The subject was then, without using the right leg, moved to a one-leg knee-extensor ergometer. Here resting leg blood flow in the right femoral artery was measured using ultrasound Doppler (Logic E9, GE Healthcare, Pittsburgh, PA, USA) equipped with a linear probe operating at an imaging frequency of 9 MHz and Doppler frequency of 4.2–5.0 MHz [55]. Three consecutive measurements were made, and at all time points where this was the case, the mean of the three measurements are used for statistical analysis.

The right leg was then passively moved at 60 rpm for 20 min and during this, leg blood flow was measured every 15 sec during the first three minutes, every minute the next two minutes and every 2.5 min for the last 15 minutes. Micro dialysate was collected the last 18 minutes and blood samples were collected halfway through and towards the end (time points 2 and 3 in Figure 3).

Immediately after the passive movement was terminated, a second muscle biopsy was taken from *m. vastus lateralis* (time point 3 in Figure 3). The subject then rested in supine position for 50 minutes, before exercise was begun. Micro dialysate was collected during the last 20 minutes of rest and a blood sample was collected before the exercise period was initiated (time point 4 in Figure 3).

The exercise period lasted 12 minutes, starting at a resistance of 12 watt and then increased by 6 watt every 4th minute. The leg blood flow was measured three times during the last 45 seconds at all three workloads and micro dialysate was collected during the last 10 minutes. Just before the work was terminated, blood samples were collected (time point 5 in Figure 3).

Fasting blood sample analysis

One blood sample taken after one hour of rest was transported to Rigshospitalet and analysed. The fasting blood glucose was determined from this analysis. Since one blood sample from one of the subjects was not analysed, he was excluded from this analysis and the results on blood glucose was based on nine subjects.

Protein analyses

Western blots were used to determine both the levels and the phosphorylation status of the selected proteins, PECAM-1, VE Cadherin, VEGFR2 and eNOS. All Western blot analyses were performed on two muscle biopsies taken on the experimental day, after the initial resting period and after the passive work respectively. The muscle biopsies were dissected free of blood and connective tissue and

homogenized in lysis buffer. Proteins were separated on a Tris-Tricline gel and transferred to a membrane, which was subsequently blocked by BSA. Standard samples were loaded on each gel as well, to use as reference for protein content in the samples. The membranes were incubated with primary antibodies to PECAM-1, VE Cadherin, VEGFR2 and eNOS and secondary antibody horseradish-peroxidase-conjugated goat anti-rabbit 1:5000 were used for detection of the proteins (for more details see [56]). The protein content of the samples was normalized relative to the reference proteins and expressed in arbitrary units. Due to unfinished data analysis, the level of the protein VE Cadherin was based on only four subjects.

AGE and sRAGE analysis

ELISA (enzyme-linked immunosorbent assay) was used to determine the levels of AGE protein adducts and sRAGE in plasma. Blood samples collected during the experiment were centrifuged for 15 min at 1000 g at 5° C, the plasma was collected and the samples stored at -80° C. These plasma samples were analysed according to the manufacturer's protocol (OxiSelectTM Advanced Glycation End Product (AGE) Competitive ELISA Kit; Cell Biolabs, inc., San Diego, CA, US) and the content was expressed in arbitrary units.

Statistical analysis

In order to test the effect of the period of high sugar intake, paired t-tests were performed. Nine subjects were included in the calculation of blood glucose levels, due to one missing blood sample analysis and four subjects were included in the calculation of VE cadherin levels, due to unfinished data analysis. The significance level was set to $p<0.05$.

Results

All results were obtained after both test periods, where one was a control period of two weeks and one was two weeks of elevated sugar intake (75 g x 3 per day).

Leg blood flow

Leg blood flow was measured during rest, passive movement and exercise. At rest there was no significant difference in the mean leg blood flow of the 10 subjects between the two different periods (Figure 4).

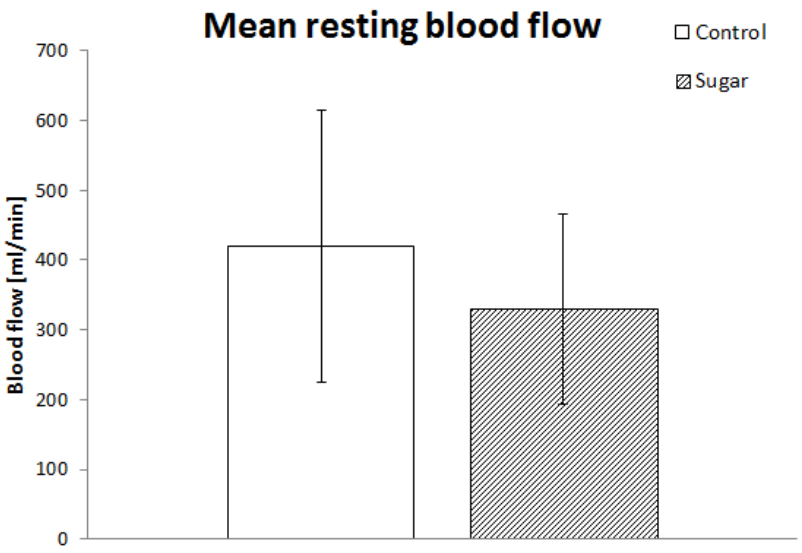

Figure 4: No significant difference in the mean resting leg blood flow of the 10 subjects after the control period (white bar) and after the period of high sugar intake (grey bar). Data are presented as mean ± SD.

During passive movement leg blood flow increased markedly during the first 30 seconds, and then decreased to a steady state after both periods (Figure 5, top). However, after the two weeks of high sugar intake, both peak value (Figure 5, top) and mean leg blood flow from 15 sec to 20 min (Figure 5, bottom) were lower than after the control period ($p<0.05$).

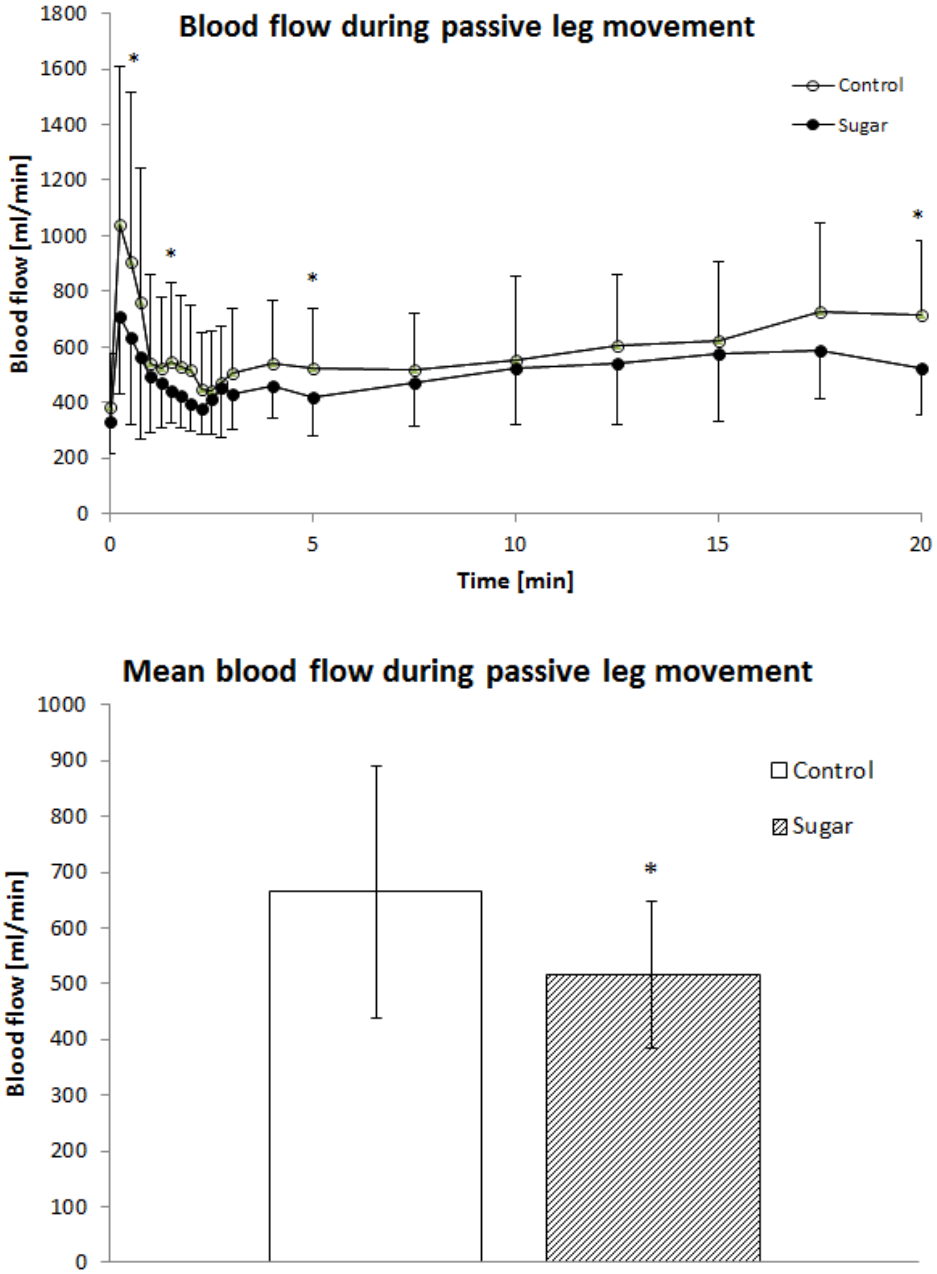

Figure 5: Leg blood flow during passive leg movement plotted as a function of time (top). There was a significant difference in the blood flow between the two groups to t=30 sec, t=45 sec, t=5 min and t=20 min ($p<0.05$). Also the mean leg blood flow of all 10 subjects from t=15 sec to 20 min during passive movement was plotted (bottom). This revealed a significantly lower blood flow after the period of high sugar intake (grey bar) compared to after the control period (white bar)($p<0.05$). Data are presented as mean ± SD.

Blood flow during exercise after the two periods were also determined and no difference were observed ($p>0.05$). Blood flow were plotted both as a function of workload (Figure 6, top) and as an average blood flow during the entire working period (Figure 6, bottom).

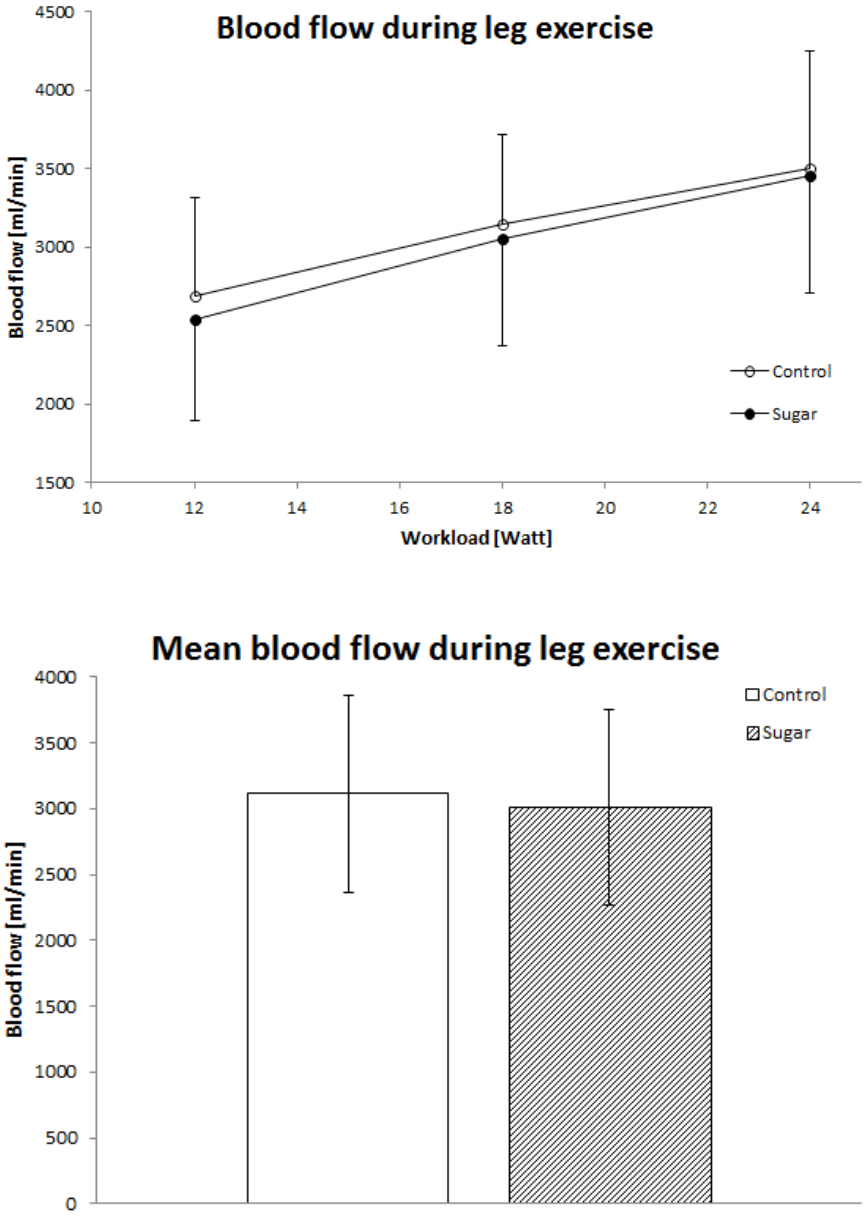

Figure 6: Leg blood flow during one-leg exercise at three workloads: 12 watts, 18 watts and 24 watts (top) and the average blood flow during the three workloads (bottom). There was no significant difference in the blood flow after the control period (white bar) and after the period of high sugar intake (grey bar). Data are presented as mean ± SD.

Blood glucose levels

No differences in fasting blood glucose levels are detected after the two test periods (Figure 7).

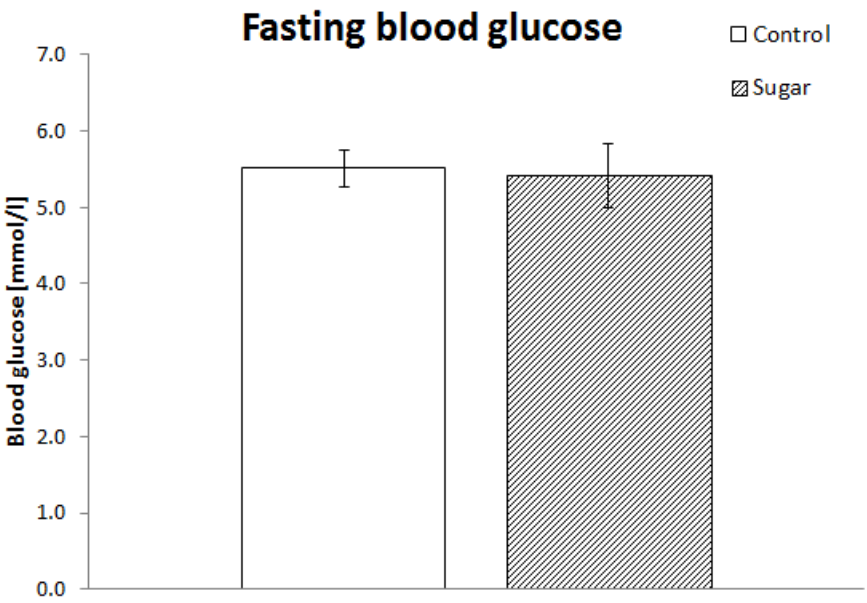

Figure 7: The fasting blood glucose levels after the control period (white bar) and after the two weeks of high sugar intake (grey bar). The levels are based on data from nine subjects, since one blood sample from one subject was not analysed. Data are presented as mean ± SD.

Muscle protein levels

Levels of mechanosensor proteins PECAM-1, VE Cadherin and VEGFR2 and of effector protein eNOS were determined by Western blot (Figure 8). All the protein levels were constant between the two periods.

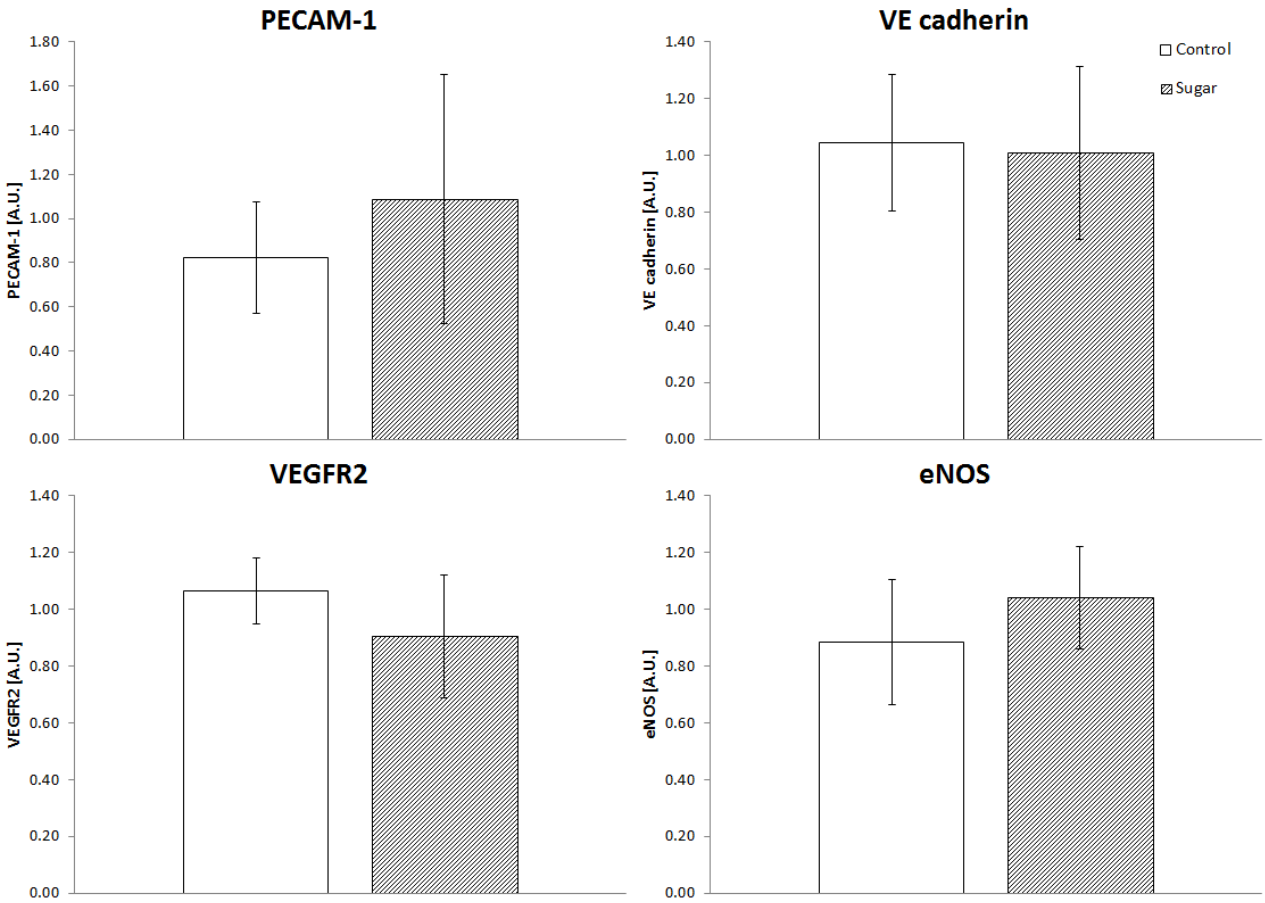

Figure 8: Average levels of the four investigated muscle proteins after the control period (white bars) and after the two weeks of high sugar intake (grey bars). VE cadherin levels are based on data from four subjects, since data analysis are not finished. Data are presented as mean ± SD. Protein levels are expressed in arbitrary units.

The phosphorylation status of the different proteins was determined by Western blot as well (Figure 9). None of the proteins displayed any differences in phosphorylation status on the investigated phosphorylation sites. The phosphorylations were compared both between rest and passive movement after each test period and after rest and passive movement respectively, between the two test periods.

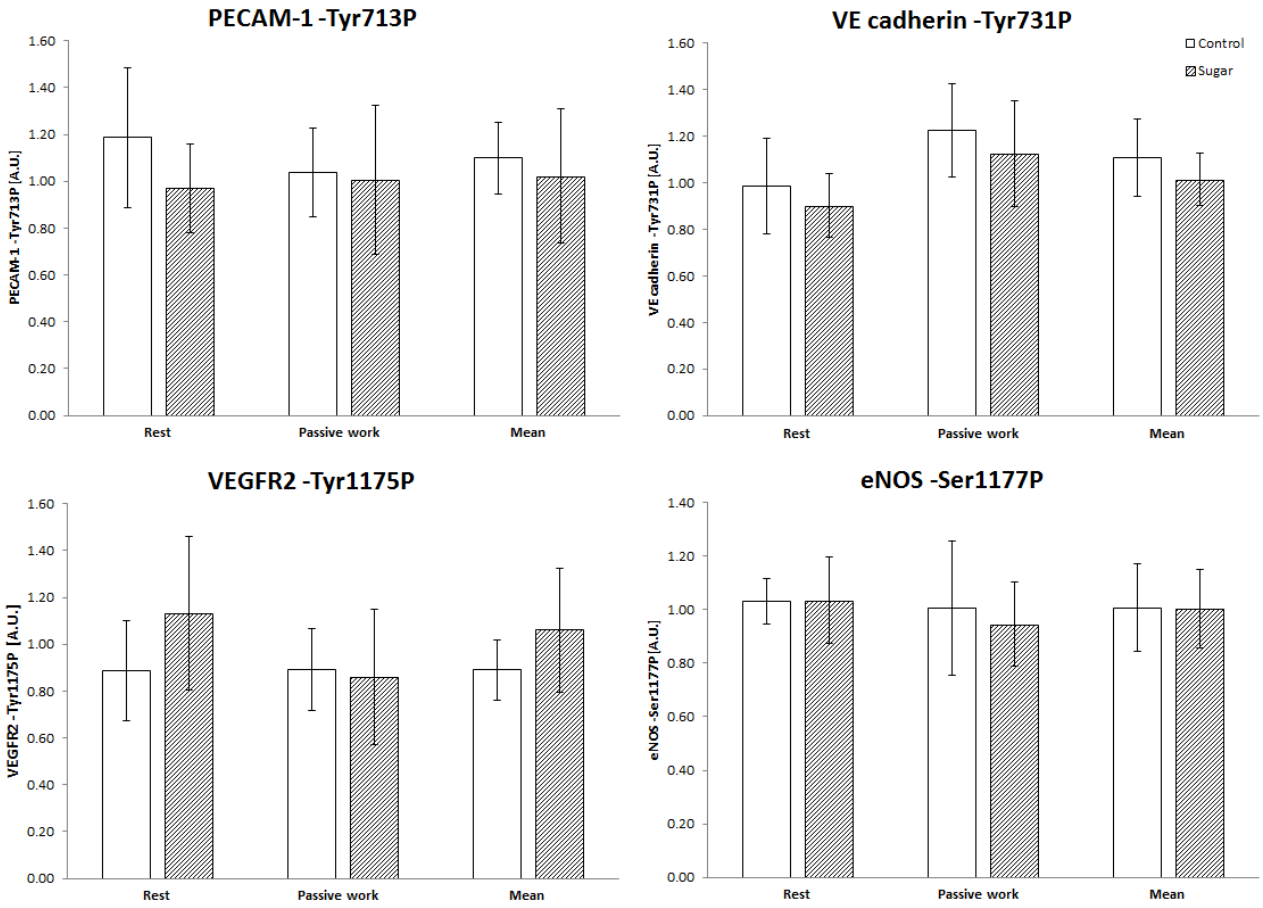

Figure 9: The phosphorylation status of the four proteins after the control period (white bars) and after the sugar intervention (grey bars). The phosphorylations of the proteins were determined both after rest and passive work and the mean of the two. There are no significant difference in phosphorylation status of any of the proteins between the control period and the sugar intervention. Data are presented as mean ± SD. Protein levels are expressed in arbitrary units.

AGE and sRAGE levels

Also the levels of both AGE and sRAGE were the same after the two test periods (Figure 10).

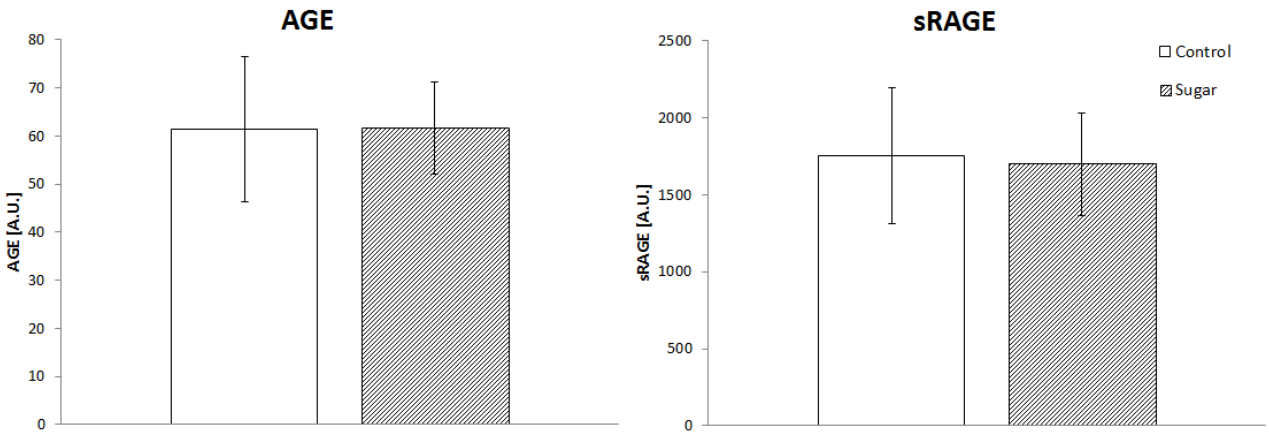

Figure 10: Average levels of AGE and sRAGE for all participants. All protein levels are shown both for the control period (white bars) and for the period of high sugar intake (grey bars). Data are presented as mean ± SD. Protein levels are expressed in arbitrary units.

Discussion

The key result of this study was that an intervention consisting of two weeks with a substantially elevated sugar intake had a negative effect on the cardiovascular system, namely a decreased ability to vasodilate in response to shear stress and passive stretch.

Leg blood flow

During passive movement, the blood flow was impaired after the high sugar intake and it has been shown that release of NO from the endothelial cells is the primary stimulus for vasodilation in this experimental setup [2]. This NO release has previously been shown to be stimulated by both shear stress and passive stretch during passive movement [2] and the shear stress response is the one primarily evaluated here.

Resting leg blood flow was not affected by the high sugar intake, which was expected. Since an adequate blood supply, constantly to all organs throughout body is vital, many different and robust systems are in place to ensure this, and a change in diet is not enough to overrule all these systems [16, 20]. Leg blood flow during exercise was also not affected after the intervention. This was not surprising either, since several different complementary pathways are involved in the adjustment of the vasomotor tone during exercise and if one is impaired, others will assure an adequate blood supply to the working muscles [57, 58].

Together these results suggest that it is primarily the NO release from the endothelial cells, activated by shear stress and passive stretch, which is affected by the high sugar intake.

Muscle protein levels and phosphorylation status

The levels of the muscle proteins investigated, PECAM-1, VE cadherin, VEGFR2 and eNOS, were not affected by the sugar intervention. This was not particularly surprising, since these proteins are all involved in an array of different functions of the endothelial cells, such as regulation of vessel permeability and the arrangement of the endothelial cells, and their expression and degradation is not only depending on the ability to sense shear stress and passive stretch [1, 26-28]. This leads to a diverse regulation of the protein levels and ensures that the levels are not affected after two weeks of high sugar intake.

The degree of phosphorylation of the muscle proteins, PECAM-1, VE cadherin, VEGFR2 and eNOS, were not significantly altered by the intervention either: The levels of phosphorylation were unchanged after the period of high sugar intake compared to the control period. Also the levels of phosphorylations during rest were compared to the phosphorylations after passive movement and did not show and changes, neither after the control period nor after the high sugar intake. This was more

surprising, since the phosphorylations that were investigated are assumed to happen in response to passive movement.

A possible explanation for this could be that the expected rises in phosphorylation levels primarily happens during the first seconds to minutes of the passive work when the blood flows increases rapidly and then returns to resting levels. In order to elucidate this, it would be relevant to take muscle biopsies to t = 30 seconds and 1 minute, where the greatest changes in blood flow take place.

It might also be that the Western blot method is too insensitive to detect smaller changes in phosphorylation and immunohistochemistry [59] might yield more precise results, when investigating the levels of phosphorylation.

It is also a possibility that the phosphorylations are unstable and become dephosphorylated during the time it takes from the passive work is terminated, the muscle biopsy is taken and until the muscle sample is frozen. Even though this is done as fast as possible, it may take 15 to 30 seconds, and it is possible that even such a short time might be enough to significantly decrease the phosphorylation status of some proteins.

This means that the lack of difference in phosphorylation status detected in this study might not prove that there is no difference. It is possible that there were some changes correlated to the high sugar intake and the following decrease in blood flow in response to passive movement and either Western blot was to imprecise or the handling time of the muscle sample too long.

There are also other muscle protein phosphorylations which could be relevant to investigate, e.g. Thr495 on eNOS and Tyr663 and Tyr686 on PECAM-1, which are all known to be phosphorylated in response to shear stress [26, 42, 43]. Even if the phosphorylation status on all of the relevant sites were determined, there is still the uncertainty regarding the dephosphorylation occurring while handling the muscle samples, making it difficult to know if we could discover any changes in the samples that are already obtained.

Blood glucose levels

In this study the subjects have been exposed to a highly elevated sugar intake (75 g x 3 per day) during two weeks. This has been done to increase the postprandial hyperglycaemia [5] markedly and subsequently evaluate the vascular response to this induced hyperglycaemia. The amount of sugar was chosen due to several considerations: It constitutes a substantial increase in sugar intake for the subjects and is thus expected to give rise to a substantial increase in the postprandial hyperglycaemia. It is within a physiologically relevant range, since this is what some persons might ingest every day

[60]. Also, to expose the subjects to a higher sugar intake per day or a longer intervention would not be ethical, since this would be likely to adversely affect their health [6].

The blood glucose levels of the subjects throughout this period have not been monitored and their fasting blood glucose levels are not affected after the period of high sugar intake. This means we do not know for how much of the time they have been hyperglycaemic. Since the subjects are young, healthy and moderately active, the increase in blood glucose in response to ingestion of 75 g sugar is expected to be moderate, from resting levels of around 5 mmol/l to a maximum of 7 mmol/l, and return to resting levels in one to three hours [61, 62]. If they engage in high-intensity exercise regularly, it is possible there is almost no change in blood glucose in response to the sugar ingestion [61].

This might be a contributing reason why we do not detect the expected changes, which has previously been connected to hyperglycaemia. To investigate if this is the case it would be relevant to test how the blood glucose levels are affected by the ingestion of a 75 g sugar dose, both at the beginning and at the end of the two week period. This will elucidate how the high sugar intake affects the blood glucose levels both acutely and in response to the test period.

AGE and sRAGE levels

The levels of both AGE and sRAGE are also unaffected after two weeks of elevated sugar intake.

The level of AGE is known to increase in response to hyperglycaemia [14, 46], but as described above, it is unclear how much the blood glucose levels are raised and for how long in response to each ingestion of 75 g of sugar in young healthy subjects. Since the levels of AGE are not measured during the two weeks of high sugar intake but only after, it is possible that the AGE levels did increase after the onset of the sugar intervention and subsequently returned to normal. It is also worth noticing that the AGE levels are determined after an overnight fast of minimum 9 hours. Depending on the clearing rate of AGE it might be that the AGE level is acutely elevated in response to each sugar dose but the surplus of AGE is cleared after nine hours of fasting.

To thoroughly investigate how the AGE levels are affected by this intervention, it would be relevant to take several blood samples during the two weeks of high sugar intake. This would allow us to test if the AGE levels changed during the two week period, or if they are unaffected throughout the entire intervention.

Another aspect to take into consideration is the testing method used to determine the AGE level: According to Nowotny *et al.* [45] is ELISA an unreliable method for this and both HPLC and gas or

liquid chromatography are more precise methods which could be applied in order to obtain more precise results.

The levels of sRAGE might be negatively correlated to a high level of resting plasma glucose [51], but since we do not detect any changes in the fasting plasma glucose or in AGE levels, no changes in sRAGE was expected.

Alternative mechanisms affecting the vasodilatory response

There are also other mechanisms that could be involved in the decrease in blood flow in response to passive movement, which we have not yet investigated.

Thickness of glycocalyx

The luminal surface of the endothelial cells is covered by a layer of proteins and polysaccharides collectively named glycocalyx. The primary structure of glycocalyx is constituted by proteoglycans and glycoproteins, both of which are transmembrane proteins [63]. In addition, a lot of soluble proteins, which either originate in the endothelial cells or are transported by the blood stream, form a substantial part of the glycocalyx. The glycocalyx layer is approximately 0.5 μm in the capillaries and up 4.5 μm in the carotid arteries [63].

The thickness of the glycocalyx is dynamic and changes easily in response to e.g. disturbed flow and hyperglycaemia: Where the blood vessels are branching the flow is disturbed and this leads to a thinner glycocalyx in these areas [63]. Hyperglycaemia is also capable of degrading the glycocalyx: In healthy subjects the volume of the glycocalyx has been reported to be halved six hours after acute hyperglycaemia [15].

That the thickness of glycocalyx might be involved in the shear stress response has been demonstrated by Lopez-Quintero *et al.* [11]: When bovine aortic endothelial cells are grown in hyperglycaemic conditions, the content of some components of glycocalyx in decreased together with an inhibited activation of eNOS, compared to cells grown in normal glycaemic conditions. Also Mochizuki *et al.* [64] have demonstrated that the NO-production in response to shear stress was significantly decreased after degradation of the glycocalyx in rabbit arteries, implying an important role for glycocalyx in sensing of shear stress.

How the glycocalyx responds to a two week period of elevated sugar intake has, to my knowledge, not been investigated yet, so it would be relevant to measure the thickness of the glycocalyx to see if it is affected. This is possible to test by ELISA [15], so it can be done on the samples already obtained in this study.

Ca^{2+} signalling pathway induce vasodilation

The intracellular Ca^{2+} concentration is known to increase in response to shear stress, which leads to an increase in the CaM-dependent activation of eNOS causing vasodilation [65]. This signalling pathway does not involve the mechanosensor complex and it has not been tested if this system is affected after the sugar intervention. However, this system is assumed to respond primarily to changes in shear stress which causes transient increases in $[Ca^{2+}]$ and not give rise to a sustained response to a sustained increase in shear stress [31, 65].

$P2Y_2$ and G_q/G_{11} might act as upstream mechanosensors

Two studies have demonstrated that the G-proteins G_q/G_{11} is likely to be involved in shear stress signalling: Otte *et al.* [66] have demonstrated that G_q/G_{11} and PECAM-1 are co-localized at the cell-cell junction and that a change in shear stress leads to rapid dissociation of the two proteins. Subsequently, Melcior & Frangos [67] demonstrated that silencing the G_q/G_{11} proteins inhibits the activation of Akt in response to shear stress. According to a study by Burnstock & Ralevic [68], ATP released from endothelial cells in response to shear stress acts on the G-protein coupled receptors P2X and P2Y, increasing the NO production. Together these studies indicate that more proteins than the mechanosensor complex described by Tzima *et al.* [3] might be involved in the sensing of shear stress and release of NO.

Wang *et al.* [32] follow up on these studies by demonstrating that both the purinergic receptor $P2Y_2$ and the G-proteins G_q/G_{11} are involved in the shear stress signalling and most likely function upstream from the mechanosensor complex. They demonstrate the importance of the three proteins in the shear stress signalling pathway both in cell studies and in arteries from $P2Y_2$ and G_q/G_{11} knock-out mice respectively.

In both human vascular endothelial cells (HUVECs) and in bovine aortic endothelial cells (BAECs) the α-subunit of the two heterotrimeric G-proteins G_q and G_{11} are knocked down, inhibiting the function of the two G-proteins simultaneously. This effectively reduces the phosphorylation of PECAM-1, VEGFR2, Akt and eNOS and the NO production in response to shear stress in both cell types. When arteries from G_q/G_{11} knock-out mice are exposed to shear stress, there is no vasodilatory response. That the general ability to vasodilate is not affected is demonstrated by the fact that the NO-donor sodium nitroprusside is able to induce vasodilation. These two results support the notion that G_q/G_{11} proteins are involved in the shear stress signalling pathway leading to NO formation.

The G-protein coupled receptor $P2Y_2$ is also demonstrated to play a role in shear stress induced vasodilation: When $P2Y_2$ is knocked down in HUVECs the phosphorylations of PECAM-1, VEGFR2, Akt

and eNOS are inhibited, just as they were in the G_q/G_{11} knock-down cells, leading to a lowered NO production. This knock-down also inhibits ATP induced phosphorylation of Akt and eNOS, but not insulin induced phosphorylation. ATP is released from endothelial cells in response to shear stress [69] and acts on P2X and P2Y, leading to formation of NO, most likely through the shear stress signalling pathway [68]. Also arteries from $P2Y_2$ knock-out mice do not vasodilate in response to shear stress, but do dilate when exposed to the NO-donor sodium nitroprusside.

Together these results demonstrate that both $P2Y_2$ and G_q/G_{11} are likely to be involved in the vasodilatory response to shear stress. Thus it would be relevant to determine the levels and, if possible, the activation of these protein in our study, to elucidate if they might explain the lowered response to passive movement we have detected in response to the elevated sugar intake.

Conclusion

The vasodilatory response to passive movement of the lower leg is impaired after two weeks of elevated daily sugar intake. This is most likely due to an impairment of the NO release in response to shear stress and passive stretch.

In this study we have not been able to detect any changes in the mechanosensor complex comprised of PECAM-1, VE cadherin and VEGFR2 which senses the shear stress or in the effector protein eNOS. The results show that neither the levels of these proteins nor the phosphorylation status has changed in response to the high sugar intake. It is possible that there have been changes in the levels of phosphorylation of the proteins but that the changes have not been detected.

The levels of AGE is also unaffected by the sugar intervention. This is probably because the subjects are young, healthy and moderately active, causing them to stabilize their blood glucose fast after the ingestion of each sugar dose. Consequently, they are only exposed to short bouts of hyperglycaemia for two weeks which might not be enough to cause a chronic elevated level of AGE. Since the resting blood glucose levels are not affected, changes in sRAGE are not expected to occur either, since the expression of sRAGE has been shown to be inversely correlated to the glucose levels and this is also what we see in this study.

Perspectives

In order to elucidate which factors are impaired in the NO release in this experiment, it is necessary to continue the investigations.

Using the samples already obtained in this study, it would be relevant to investigate both levels and phosphorylation status of other proteins which are either known or expected to be involved in the shear stress signalling pathway, e.g. PI3K and Akt. It would also be interesting to use immunohistochemistry to test the phosphorylation status of especially eNOS, but also the mechanosensor complex, to investigate if this method is able to detect smaller changes in the phosphorylation status. Also, it might be worth considering checking the determined AGE levels by HPLC or chromatography [45], to test if the methods used here, are the reason we do not detect any changes.

Two proteins have been identified, which plays a critical role in the vasodilatory response to shear stress and probably do so upstream from the mechanosensor complex, namely $P2Y_2$ and G_q/G_{11} [32]. It would be interesting to examine the levels and phosphorylations of these proteins as well, either by Western blot or immunohistochemistry. Furthermore it would be interesting to test if any detectable changes occur in glycocalyx, which can be done by ELISA [15] and these tests can also be done on the samples already obtained.

A more ambitious plan to further investigate this issue would be to carry out the same experimental protocol on 12 hyperglycaemic subjects and test the same array of proteins and AGE as we have done in this experiment. It is expected that their levels of AGE would be markedly increased [70] but it has, to my knowledge, not been tested how chronic hyperglycaemia affects the ability of to release NO from the endothelium in response to passive movement. If there are no changes in the proteins, PECAM-1, VE cadherin, VEGFR2 and eNOS, and their phosphorylations in chronic hyperglycaemic subjects, this could indicate that some other mechanisms are at play.

Furthermore, this study is already now being followed by a second study: Here the vasodilatory effect in 15 elderly, inactive subjects is assessed before and after they engage in a ten week training period. The subjects are subjected to all the same tests performed in this study. Thus it is investigated if there are any connections between the levels and phosphorylation status of the muscle proteins or AGE and the impaired vasodilatory response to passive movement. Furthermore it is investigated if a training period improves the response to shear stress and passive stretch and if this can be explained by changes in the muscle proteins or AGE levels.

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