ETUDE N°1

Effet de la consommation de boissons sucrées sur la fonction vasculaire micro- et macrocirculatoire, chez des populations saines.

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Effects of sugar-sweetened beverage consumption on micro- and macrovascular function in a healthy population

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RESUME D'ARTICLE

Contexte Scientifique

Récemment, de larges études s'intéressant aux modifications du régime alimentaire ont montré chez l'enfant et l'adulte un lien fort entre la consommation de boissons sucrées, qui engendre des épisodes d'hyperglycémie (HG) aigue, et le risque de développer un diabète de type 2 (Malik et al., 2010 ; O'Connor et al., 2015 Fagherazzi et al., 2013). De plus, il est également démontré qu'une consommation excessive de ces boissons cause une augmentation de l'incidence des maladies cardiovasculaires (Xi et al., 2015 Tominaga et al., 1999). Puisque la dysfonction vasculaire est un marqueur précoce du risque cardiovasculaire, nous avons conduit récemment une méta-analyse qui a démontré qu'une HG provoquée par voie orale causait une dysfonction endothéliale transitoire chez les patients atteints de pathologies cardiométabolique, mais aussi chez des sujets sains (Loader et al., 2015). Néanmoins, les mécanismes sous-jacents impliqués restent peu décrits, même si le stress oxydant semble jouer un rôle primordial dans ces dysfonctions. (Skalicky et al., 2008 ; Demircan et al., 2008). De plus, selon les lits vasculaires considérés, les résultats disponibles à ce jour sont contradictoires ou peu robustes, notamment au niveau des adaptations microcirculatoires, réseau difficilement accessible en condition physiologique.

Ainsi, l'objectif de cette étude a été d'évaluer les effets d'une boisson sucrée sur la fonction vasculaire macro- et microcirculatoire chez des sujets sains, par une approche translationnelle allant de la clinique humaine à un modèle expérimental de rongeur. Nous avons également utilisé l'exercice physique comme une stratégie préventive des effets délétères causés par une HG aigue sur la fonction endothéliale.

Méthodologie

L'évaluation de la fonction vasculaire en condition d'HG aigue a été réalisée sur 12 hommes sains, sans contre-indications cardiovasculaires. L'HG a été induite par la consommation d'une boisson sucrée (600ml) de type soda. L'impact de cette HG a été mesuré sur la fonction endothéliale et sur la fonction du muscle lisse, au niveau de la microcirculation par Laser Speckle Contrast Imaging couplé à l'iontophorèse de drogues vasoactives (acétylcholine et nitroprussiate de sodium), et de la macrocirculation par une stimulation hyperémique (Flow mediated dilation) et un donneur exogène de monoxyde d'azote (Nitrate mediated dilation). Des mesures de pression artérielle, de glycémie et de fréquence cardiaque

ont également été effectuées tout au long du protocole pour évaluer l'impact de l'HG sur ces paramètres.

Afin de mieux comprendre les mécanismes sous-jacents des effets délétères de l'hyperglycémie aigue sur la fonction vasculaire, deux groupes de rats ont été conduits : un groupe de rats sédentaires (SED) et un groupe de rats entraînés (EX) (5sem, 5j/sem, 45mn/j, 70%VMA). Sur ces animaux, l'évaluation de la fonction vasculaire microcirculatoire a été effectuée grâce à un Laser Doppler couplé à l'iontophorèse de drogues vasoactives (acétylcholine, et nitroprussiate de sodium). Les animaux des deux groupes ont été testés en normo- et en hyperglycémie (NG et HG) (2g/kg de glucose, i.p.).. Enfin, dans le but de mettre en évidence l'influence du stress oxydant et du découplage de la eNOS, les animaux du groupe SED ont été traité avec un agent antioxydant non spécifique le N-acétylcystéine (NAC, 50mg/kg, i.p.), ou bien avec le co-facteur essentiel de la eNOS le tétrahydrobioptérine (BH_4, 10mg/kg, i.p.). Au terme des expérimentations in vivo l'aorte des animaux a été prélevée afin de tester la capacité de relaxation endothéliale en réponses à différentes concentrations de glucose. Ces artères ont également étaient incubées avec du NAC (20mM) afin d'évaluer le rôle du stress oxydant dans de potentiels dysfonctions liées à l'hyperglycémie. La production totale d'espèces réactives de l'oxygène a été évaluée par méthode de résonnance paramagnétique électronique.

Résultats majeurs

Cette étude est la première a démontré l'effet délétère causée par une HG aigue, induite par la consommation d'une boisson sucrée commerciale, sur la fonction endothéliale de sujets sains. Ce résultat est d'autant plus intéressant, qu'il a été démontré au niveau des territoires macro- et microcirculatoires. Nos explorations sur un modèle animal nous ont permis de confirmer l'altération de la fonction endothéliale en réponse à un stress HG aigue au niveau microcirculatoire, mais également lors de manipulations sur des anneaux d'aortes isolées (Krebs hyperglycémique, [Glc] : 100 et 200 mM). De plus, le stress oxydant et le mécanisme de découplage de la eNOS semblent être fortement impliqués dans la baisse de la fonction endothéliale en situation hyperglycémique. L'exercice physique permet de maintenir une capacité de relaxation endothélium-dépendante comparable à celle observée en normoglycémie.

Ces résultats démontrent qu'une consommation de boisson sucrée de type soda altère significativement la fonction endothéliale de sujets sains, quel que soit le lit vasculaire considéré. Cette dysfonction endothéliale semble fortement liée à une augmentation du stress oxydant vasculaire, qui altère la voie du NO, via un potentiel découplage de la eNOS. Enfin, une stratégie thérapeutique comme l'exercice physique semble efficace pour limiter les effets délétères d'une HG aigue sur la fonction vasculaire.

Effects of sugar-sweetened beverage consumption on micro- and macrovascular function in a healthy population

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ABSTRACT

Objective To assess vascular function during acute hyperglycemia induced by commercial sugar-sweetened beverage (SSB) consumption and its effect on the underlying mechanisms of the nitric oxide pathway.

Approach and results In a randomized, single-blind, crossover trial, twelve healthy male participants consumed 600 mL of a water beverage or a commercial SSB across two visits. Endothelial and vascular smooth muscle (VSM) function were assessed in the microcirculation using laser speckle contrast imaging incorporating transdermal iontophoresis and in the macrocirculation using brachial artery ultrasound with flow- and nitrate-mediated dilation. Compared to water, SSB consumption impaired micro- and macrovascular endothelial function as indicated by a decreased vascular response to acetylcholine iontophoresis (208.3 ± 24.3 vs. 144.2 ± 15.7 %, $P<0.01$) and reduced flow-mediated dilation (0.019 ± 0.002 vs. 0.014 ± 0.002 $\%/s^{-1}$, $P<0.01$), respectively. Systemic VSM remained preserved. Similar decreases in endothelial function were observed during acute hyperglycemia in an *in vivo* rat model. However, function was fully restored by treatment with the antioxidant, N-acetylcysteine. Additionally, *ex vivo* experiments revealed that reactive oxygen species production was elevated during acute hyperglycemia, while endothelial nitric oxide synthase activation by phosphorylation at serine 1177 was decreased.

Conclusion To our knowledge, this is the first study to assess the vascular effects of acute hyperglycemia induced by commercial SSB consumption alone. These findings suggest that SSB-mediated endothelial dysfunction is partly due to increased oxidative stress that disrupts nitric oxide synthesis.

ABBREVIATIONS

SSB	sugar-sweetened beverages
CVD	cardiovascular disease
VSM	vascular smooth muscle
NO	nitric oxide
eNOS	endothelial nitric oxide synthase

INTRODUCTION

Commercial sugar-sweetened beverages (SSB) are one of the most frequently consumed beverages worldwide.[1, 2] As such they represent a major source of added sugar in the modern diet. Habitual consumption of SSB induces frequent episodes of acute hyperglycemia and is linked to an increased risk of developing obesity, metabolic syndrome, and type 2 diabetes.[2-4] Furthermore, excessive SSB consumption is also associated with a higher incidence of advanced forms of cardiovascular disease (CVD),[2] which remains the single leading cause of death representing 31 % of the global mortality rate.[5] Impaired vascular function is considered to be a main precursor to the pathogenesis of CVD and may be present long before atherosclerotic vascular changes occur.[6]

Normal vascular function involves a continuous interaction between the endothelium and vascular smooth muscle (VSM) that is regulated by numerous vasodilators and vasoconstrictors such as the highly characterized, nitric oxide (NO).[7, 8] In a recent systematic review and meta-analysis, our research group provided evidence that acute hyperglycemia induced by an oral sugar load transiently impairs endothelial function in not only patients with cardiometabolic disease, but also in those considered to be healthy.[9] It has been suggested that such endothelial dysfunction may be attributed to increased oxidative stress mediated by acute hyperglycemia.[10, 11] Indeed, NO bioavailability in the vascular wall is highly sensitive to redox modulation of the cellular environment.[12, 13] However, due to a limited availability of microcirculatory data and discrepant reporting of macrovascular data, the impact of acute hyperglycemia on vascular function remains inconclusive and the underlying mechanisms not fully understood.

In this context and considering the surge in commercial SSB consumption, as well as its potential link to a sustained epidemic of CVD, this present study aimed to provide a comprehensive assessment of the effect of SSB-mediated acute hyperglycemia on micro- and macrovascular function in a healthy population. Additionally, considering that experimental exploration of healthy vascular tissue in humans is difficult to perform, due to ethical barriers, this study also aimed to further investigate the underlying mechanisms of the interaction between acute hyperglycemia and endothelial function via an *in vivo* and *ex vivo* rat model.

MATERIALS AND METHODS

This study was comprised of two separate protocols in order to comprehensively investigate the effect of acute hyperglycemia induced by sugar-sweetened beverage (SSB) consumption on vascular function. Protocol one was a clinical study conducted at the Australian Catholic University, Melbourne, to assess the effect of SSB consumption on micro- and macrovascular function in healthy humans. Protocol two was conducted at Avignon University, France, to provide deeper exploration into the effect of acute hyperglycemia on the underlying mechanisms of vascular function through an *in vivo* and *ex vivo* experimental rat model.

Protocol One - Clinical Study

Participants and screening

Healthy non-smoking, sedentary (<2 hours/week of exercise) males (n = 12) aged18-55 years were recruited. Participants were excluded if they had any history of cardiovascular disease; a 5 % weight gain or loss in the 6 months prior to beginning the protocol; or if they were currently using or being treated with any vasoactive medications. All participants provided informed written consent. The Human Research Ethics Committee at the Australian Catholic University, Australia, approved this protocol. This study was registered with the Australian New Zealand Clinical Trial Registry: #ACTRN12614000614695.

Study design

Following overnight fasting (≥10 hours), participants presented to the cardiovascular laboratory on two occasions to perform this randomized, single-blind, crossover trial (Figure 1). To standardize responses between trials, participants were instructed to maintain their current level of physical activity and to abstain from strenuous exercise for 48 hours, alcohol consumption for 24 hours, and caffeine consumption in the 12 hours prior to each trial. Clinical measurements were performed at the beginning of the first trial to collect anthropometric and hemodynamic data; and assess fasting blood glucose concentrations. Participants were then administered one of two 600 mL test beverages from an opaque container. Visit A assessed vascular function in response to consumption of 600 mL of water and visit B measured the vascular response to 600 mL of a commercial SSB. Visits A and B were randomized for each participant using an online sequence generator by a researcher not

involved in data collection or analyses (JL).[1] Participants were allowed five minutes to consume each beverage, of which the nutritional content is presented in Table 1. Blood glucose concentrations, blood pressure and heart rate were assessed throughout each visit. A 600 mL volume of each beverage was administered, as it represents the common volume of SSB commercially available for purchase by the public.

Clinical measurements

Height and mass were measured using a stadiometer and a calibrated scale, respectively. Body mass index was calculated as mass (kg) divided by height² (m). Blood glucose concentrations were assessed with a handheld blood glucose monitoring system (Freestyle Optium, Abbott Diabetes Care Ltd, UK). Systolic and diastolic blood pressure, and heart rate were measured using a digital sphygmomanometer (Dinamap, GE Medical Systems, Milwaukee, USA). Blood pressure variables were expressed as mean arterial pressure (mmHg) calculated by [(2 X diastolic blood pressure) + systolic blood pressure]/3.

Vascular function

Micro- and macrovascular measurements were conducted in a temperature-controlled room maintained at 22-24°C and proceeded 15 minutes following the consumption of the 600 mL of water beverage or the 600 mL commercial SSB, during the measured time period of acute hyperglycemia. Each participant remained in a supine position from the moment they finished consuming the 600 mL test beverage until measurements of vascular function were complete. Microvascular function was assessed on the ventral surface of the right forearm using transdermal iontophoresis in conjunction with laser speckle contrast imaging. Assessment of macrovascular function commenced next on the upper right arm using brachial artery ultrasound coupled with flow-mediated dilation and nitrate-mediated dilation. Blood glucose concentrations and hemodynamic changes were monitored throughout.

Assessment of cutaneous microvascular function

Transdermal iontophoresis delivers a pharmacologically charged solution to the skin allowing for non-invasive assessment of cutaneous microvascular endothelium and vascular smooth muscle (VSM) function.[2] The vascular response to transdermal iontophoresis was quantified by laser speckle contrast imaging using a 70mW system (PeriCam PSI System®, Perimed, Järfälla, Sweden) with a laser wavelength of 785 nm and laser head working distance set at 15

cm. The laser speckle contrast imaging head unit emits and detects light scattered in the tissue that is partially backscattered by moving blood cells, causing a change in frequency.[3] Microvascular blood flux is then calculated using the Doppler principle.[3] Laser Doppler measurements were recorded continuously at a frequency of 18 Hz using an interfaced computer with data acquisition software (PimSoft 1.2.2.0®, Perimed, Järfälla, Sweden). Two adhesive drug delivery electrodes (LI 611, Perimed, Järfälla, Sweden) were installed on the ventral surface of right forearm, avoiding any hair, broken skin, areas of increased skin pigmentation, and visible veins. A dispersive electrode (PF 384, Perimed, Järfälla, Sweden) was positioned approximately 15 cm from each drug delivery electrode to complete the electrical current circuit. The arm was then immobilized with a vacuum cushion prior to commencing the protocol to ensure the participant was positioned as recommended. Following two minutes baseline measurement of basal blood flux, transdermal iontophoresis of acetylcholine 2 % dissolved in sodium chloride 0.9 % and sodium nitroprusside 1 % dissolved in sodium chloride 0.9 % were performed to assess cutaneous microvascular endothelial and VSM function, respectively. An anodal current of 0.02 mA for acetylcholine and a cathodal current of 0.02 mA for sodium nitroprusside were administered simultaneously for 200 and 400 seconds, respectively, using a Micropharmacology system (PF 751 PeriIont USB Power Supply, Perimed, Järfälla, Sweden), avoiding nonspecific vasodilation observed with higher cathodal electrical charges.[4] Laser speckle contrast imaging was performed throughout. The manufacture's software was used to set regions of interest at 30 mm^2 that were adjusted retrospectively to find the area of max blood flux during transdermal iontophoresis. Data were exported to Microsoft Excel and analysed off-line. Cutaneous blood flux values were averaged for the 30 seconds immediately prior to the beginning of transdermal iontophoresis for baseline and for the five seconds at the maximal blood flux plateau. Data were reported as blood flux at rest, peak blood flux in response to transdermal iontophoresis, and the relative percentage increase in cutaneous blood flux from baseline measurements; and expressed as perfusion units and cutaneous vascular conductance, which is the flux in perfusion units divided by the mean arterial pressure (mmHg), to account for differences in blood pressure between tests.[5]

Assessment of macrovascular function

All macrovascular measurements were performed as previously described using high-resolution vascular ultrasonography (Vivid I, GE Medical Systems, Milwaukee, USA), with a

10-MHz multi-frequency probe.[6] Briefly, B-mode images and Doppler signals were simultaneously recorded with ECG data. Arterial diameter was measured on B-mode images in the region of the artery running perpendicular to the ultrasound beam. The operator searched for the largest diameter, strong wall signals, and the longitudinal section of the artery in each image. Time-averaged mean velocity ($cm.s^{-1}$) was recorded, at the same level, by pulsed wave Doppler with a 45-60° insonation angle. Measurements were corrected for the insonation angle, and the pulsed Doppler sample volume was adjusted to cover the entire width of the vessel. The high-pass Doppler frequency filter was kept at the lower value ensuring rejection of arterial wall motion artefacts, with a cut-off value usually below 100 Hz. The same, well-trained operator (GW) performed all measurements. To assess macrovascular endothelial function, brachial flow-mediated dilation was performed according to the International Brachial Reactivity Task Force Guidelines.[7] A pneumatic cuff was placed around the right forearm distal to the elbow and the ultrasound probe was positioned approximately midway between the antecubital and axillary regions before baseline measurements of basal brachial artery diameter were performed. The cuff was then inflated to 250 mmHg for five minutes before sudden cuff deflation induced reactive hyperemia and measurements of brachial artery diameter were performed again. Fifteen minutes later, baseline measurements of basal brachial artery diameter were repeated before nitrate-mediated dilation was performed to assess macro-VSM function. As described previously, this procedure involves sublingual delivery of 0.4 mg of glyceryl trinitrate (Nitrolingual® Pumpspray, G. Pohl-Boskamp GmbH & Co, KG, Germany).[8, 9] Data were analysed off-line as the mean of five consecutive measurements using dedicated software (EchoPac 6.0, GE Healthcare, Horten, Norway). Volume blood flow ($mL.min^{-1}$) was measured as the mean of the five cardiac cycles with the highest systolic velocity following cuff release and reported for the basal state and during peak reactive hyperemia; and expressed as the percentage change from basal measurements. Shear rate (s^{-1}) was calculated as (4 x mean systolic velocity)/mean diameter to estimate the shear stress induced by hyperemia; and was expressed as the change from basal measurements (Peak shear rate minus basal shear rate).[10] Flow-mediated dilation and nitrate-mediated dilation in response to post-occlusive reactive hyperemia and nitrate administration, respectively, were expressed as the percentage change in brachial artery diameter from baseline measurements, with and without respect to the change in shear rate.

Protocol Two - Experimental exploration

Animals

Twenty-three male Wistar rats (12 weeks old, 300 ± 50 g; Laboratoire Janvier, France) were housed in controlled conditions at 23-24°C with a normal 12 hours light/dark cycle and free access to water and a commercial standard diet conforming to current French legislation. The local Research Ethics Committee at Avignon University approved this protocol (84004).

Study design

Each rat was randomized into one of three treatment groups using an online sequence generator prior to the *in vivo* assessment of cutaneous microvascular function (Suppl Material Figure I).[1] Rats designated as normoglycemic controls received an intra-peritoneal injection of a 1.5 mL bolus of sodium chloride 0.9 % one hour prior to vascular assessment; while rats designated to perform a glucose tolerance test were treated with either a intra-peritoneal injection of a 1.5 mL bolus of sodium chloride 0.9% one hour prior to vascular assessment or a 1.5 mL bolus of the antioxidant, N-acetylcysteine, at 50 $mg.kg^{-1}$ body weight 48 hours and one hour prior to vascular assessment. These treatments prior to the glucose tolerance test allowed for assessment of the vascular effect of acute hyperglycemia alone, as well as the interaction between oxidative stress and microvascular function during acute hyperglycemia. Following overnight fasting (12-16 hours) and the designated treatment preparations, each rat was inspected to ensure that the skin on the back was hairless and the hind legs were intact. Experiments were performed in a temperature-controlled enclosure set at 34°C to maintain the skin temperature of each rat at 36-37°C. The rats were anesthetized with an intra-peritoneal injection of sodium pentobarbital at 60 $mg.kg^{-1}$ and were maintained in a prone position for the duration of the experiment.

Glucose tolerance test

Rats designated to receive the glucose tolerance test were administered an intra-peritoneal injection of a 1.5 mL bolus of 40 % glucose solution at $2g.kg^{-1}$ body weight. Rats designated as normoglycemic controls received a second intra-peritoneal injection of sodium chloride in lieu of the glucose tolerance test. Blood samples were collected from the tail for measurement of blood glucose concentrations at 0, 10, 15, 20, 30, 60, 120 minutes following the induction of acute hyperglycemia or administration of the control (sodium chloride) solution.

***In vivo* vascular exploration**

Assessment of cutaneous microvascular function was assessed 10 minutes following the induction of acute hyperglycemia or administration of the control (sodium chloride) treatment using a laser Doppler flowmetry system (Periflux PF5000; Perimed, Järfälla, Sweden) and a thermostatic laser Doppler flowmetry probe (PF 481; Perimed) with an effective surface area of 0.95 cm^2; in conjunction with transdermal iontophoresis. Two adhesive drug delivery electrodes containing acetylcholine 2 % dissolved in sodium chloride 0.9 % and sodium nitroprusside 2 % dissolved in sodium chloride 0.9 % were positioned on the inferior-posterior surface of the right and left leg of each rat. Each adhesive drug delivery electrode was inserted with a laser Doppler flowmetry probe. Dispersive electrodes were placed on front paws to complete the electrical current circuit and were connected to a battery powered iontophoresis device (Perilont, Perimed, Järfälla, Sweden). For 60 seconds prior to the commencement of transdermal iontophoresis, baseline cutaneous blood flux of the leg was measured using laser Doppler flowmetry. A single pulse of a 0.1 mA anodal and cathodal current was administered for 20 seconds for acetylcholine and sodium nitroprusside, respectively,[11] followed by a further 20 minutes of laser Doppler flowmetry measurements. Data was exported to Microsoft Excel and analysed off-line. Cutaneous blood flux values were averaged for the 10 seconds immediately prior to the beginning of transdermal iontophoresis for baseline measurements and for the period at the maximal blood flux plateau. Data were reported as perfusion units and expressed as the percentage increase in cutaneous blood flux relative to baseline measurements.

***Ex vivo* vascular exploration**

Following *in vivo* assessment, rats were anesthetized using an intra-peritoneal injection of sodium pentobarbital at 120 $mg.kg^{-1}$ and the intact thoracic aorta was quickly removed and placed in cold Krebs-Henseleit bicarbonate buffer (composition in mM: NaCl 118; $NaHCO_3$ 25; Glucose 11; KCl 4.8; KH_2PO_4 1.2; $MgSO_47H_2O$ 1.1; $CaCl_2$ 1.25). Following removal of adherent tissue, the thoracic aorta was cut into 2-3 mm rings and suspended between two wire hooks. The suspended rings were then mounted in an organ chamber with 5 mL Kreb's solution with a pH level of 7.4 at 37°C and continuously gassed with 95 % oxygen and 5 % carbon dioxide, under a resting tension of 2 g. The rings were connected to an isometric force transducer (EMKA technologies, EMKA Paris, France) and linked to an amplifier (EMKA technologies, EMKA Paris, France), as well as a computer acquisition system to record

changes in isometric force. The rings were then allowed to equilibrate for 45 minutes. During this period the tension was verified every 15 minutes and washed with Kreb's solution. Following the equilibration period, the rings were pre-contracted with phenylephrine (10^{-6} M), and relaxed with acetylcholine (10^{-5} M) to functionally confirm the muscular and endothelium integrity. Each ring was then pre-contracted with phenylephrine (10^{-6} M). When a plateau in the effect of pre-contraction was reached, endothelium function was examined by challenging the aortic rings with two cumulative concentration-response curves to administration of acetylcholine (10^{-10} to 10^{-5} M), firstly in the normoglycemic state and then during acute hyperglycemia. Acute hyperglycemia was induced by two hours of hyperglycemic Kreb's solution incubation at glucose concentrations of 30 mM, 100 mM and 200 mM. The interaction between acute microvascular function and oxidative stress was further examined during acute hyperglycemia induced by hyperglycemic Kreb's solution incubation (100 mM) in aortic rings that were pre-treated with N-acetylcysteine (20 mM) for 30 minutes. Data acquisition was performed using IOX (EMKA technologies, EMKA Paris, France). The relaxation response was expressed as a percentage of the pre-contraction induced by phenylephrine.

Western blotting analysis

Proteins from aorta homogenates were separated on polyacrylamide-sodium dodecyl sulphate gels and transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight with primary antibodies, anti-endothelial nitric oxide synthase (eNOS) III and anti-eNOS-$P^{Ser1177}$ (1:1000 and 1:500, respectively; BD Transduction Laboratory), at 4°C in 10 % milk with Tris-buffered saline containing 0.05 % Tween 20. The eNOS and eNOS-$P^{Ser1177}$ protein contents were expressed relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content (anti-GAPDH antibody 1: 5000; incubated in 3 % bovine serum albumin, Santa Cruz Biotechnology). Immunodetection was performed using an ECL or ECL Plus System (Supersignal West Pico Chemiluminescence Substrate, Thermo Scientific); and the membranes were exposed to X-ray films for visualization.

Measurement of reactive oxygen species

Production of reactive oxygen species in both normoglycemic and hyperglycemic aortic ring preparations were evaluated by electron paramagnetic resonance in fresh frozen aortic homogenates as previously described.[12, 13] Aortic ring preparations were then treated with 1

mM CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine solution) (1:1 v/v), put in the electron paramagnetic resonance glass capillary tube (Noxygen Science Transfer and Diagnostics, Germany) and placed inside the e-scan spectrometer (Bruker, Germany) for data acquisition. Production of reactive oxygen species was normalized to the protein content of each sample and then expressed in µmol/mn/mg.

Statistical analyses

Sample size was calculated using the results of a similar intervention, which demonstrated that flow-mediated dilation decreased from 6.96 ± 1.56 % to 4.0 ± 2.6 % during acute hyperglycemia when compared to the normoglycemic state.[14] Considering this, it was estimated that a total of six participants would be needed to detect a difference between trials, with a one-tailed α of 0.05 and a 1-β of 0.80. All clinical microvascular and macrovascular data were exported and coded by a researcher (JL) not involved in data collection and analyses in order to blind the investigator. Following checks of distribution, clinical data was then analysed using repeated measures analysis of variance (ANOVA) with a post-hoc Tukey's multiple comparison test to assess differences over time in vascular, hemodynamic, and blood glucose responses to water or SSB consumption. A one-way ANOVA with Fisher post hoc tests was performed to determine differences in the cutaneous microvascular blood flow response to the induction of acute hyperglycemia in rats designated with normoglycemic, acute hyperglycemic only, or acute hyperglycemic-N-acetylcysteine treatments. Differences in *ex vivo* vascular reactivity between the hyperglycemic and normoglycemic conditions were determined using a two-way ANOVA with Bonferroni post hoc tests. All statistical analyses were performed using MedCalc software (bvba, Mariakerke, Belgium) and significance was accepted at $P<0.05$. All values are reported as mean ± SEM.

RESULTS

Twelve healthy male participants were recruited and completed this randomized, single-blind, crossover trial between June 9th and July 4th, 2014 (Figure 1). To examine the acute vascular effects of commercial SSB consumption in humans, variables of blood glucose concentration, heart rate, arterial pressure and vascular function were assessed after the ingestion of a commercial SSB and compared to values measured following water consumption (Figure 2). Nutritional information for each beverage is presented in Table 1.

Participant characteristics

Participants included in the study were aged 31 ± 1.9 years with a body mass index of 24.68 ± 0.71 kg/m^2. At each visit, prior to the consumption of the test beverage, baseline measurements of fasting blood glucose (4.95 ± 0.15 vs. 4.78 ± 0.16 mmol/L, $P>0.05$), heart rate (64.08 ± 3.1 vs. 62.25 ± 3.2 $beats.min^{-1}$, $P>0.05$), and mean arterial pressure (80.50 ± 1.66 vs. 79.86 ± 1.48 mmHg, $P>0.05$) were found to be similar (Figure 3).

Sugar-sweetened beverage consumption increased blood glucose concentration with no major effect on heart rate or blood pressure.

Ingestion of the SSB significantly elevated blood glucose concentrations 20 minutes (109 ± 6 %) following the beginning of consumption, with peak hyperglycemia (127 ± 3 %) recorded at 40 minutes (Figure 3A). A progressive decrease in blood glucose concentrations was observed between 40 and 75 minutes thereafter. However, blood glucose concentrations at 75 minutes were still significantly greater than baseline blood glucose values (91 ± 7 %) and all blood glucose values measured over time following water consumption. Blood glucose concentrations did not deviate from baseline measurements following water consumption. The SSB-mediated acute hyperglycemia had no major effect on mean arterial pressure. However, a slight but significant reduction in mean arterial pressure was observed during peak hyperglycemia when compared to that at the baseline measurement ($P<0.05$) (Figure 3B). No variations in heart rate were observed during acute hyperglycemia (Figure 3C).

Sugar-sweetened beverage consumption decreased micro- and macrovascular endothelial function

All assessments of micro- and macrovascular function were completed between 20 and 75 minutes following the beginning of SSB consumption, during acute hyperglycemia. Vascular function was assessed in the same time period following water consumption to allow for comparison between the two test beverages.

Assessment of cutaneous microvascular function

Cutaneous microvascular endothelial and VSM function were assessed using laser speckle contrast imaging in conjunction with transdermal iontophoresis of acetylcholine and sodium nitroprusside, respectively. Prior to the beginning of transdermal iontophoresis, there were no differences in baseline measurements of basal cutaneous blood flux between each visit (23.6 ± 1.8 vs. 26.4 ± 1.6 perfusion units, P=0.23) (Table 2). An increase in cutaneous blood flux was observed following transdermal iontophoresis of acetylcholine and sodium nitroprusside (Figure 4A). However, the relative percentage increase in cutaneous blood flux in response to acetylcholine iontophoresis was significantly lower during SSB-mediated acute hyperglycemia when compared to that during normoglycemia following water consumption (129.76 ± 11.18 vs. 196.78 ± 20.61 %, respectively, P<0.01) (Figure 4B). Even after accounting for differences in blood pressure between visits by converting cutaneous blood flux from perfusion units to cutaneous vascular conductance, the relative increase in cutaneous blood flux mediated by acetylcholine iontophoresis was still lower following ingestion of the SSB than that measured after water consumption (144.2 ± 15.7 vs. 208.3 ± 24.3 %, respectively, P<0.01). In contrast, the vascular responses to transdermal iontophoresis of sodium nitroprusside were similar following the consumption of each beverage.

Macrovascular measurements

Endothelial and VSM function were then assessed in the macrocirculation using ultrasound of the brachial artery in conjunction with flow-mediated dilation and nitrate-mediated dilation, respectively. Following the consumption of each test beverage, there were no differences in basal brachial artery diameter or blood flow between visits (4.7 ± 0.1 vs. 4.7 ± 0.1 mm, P=0.68; and 48.0 ± 5.7 vs. 53.0 ± 6.5 $ml.min^{-1}$, P=0.67, respectively) (Table 2). Increases in brachial blood flow and the Δshear rate induced during hyperemia were also similar between each visit (408.3 ± 27.6 vs. 445.1 ± 34.5 $ml.min^{-1}$, *p*=0.19; and 484.3 ± 32.8 vs. 501.6 ± 37.1 $\%/s^{-1}$, P=0.51, respectively). However, flow-mediated dilation was significantly reduced (-23.86 ± 5.33 %) during SSB-mediated acute hyperglycemia when compared to that during

normoglycemia following water consumption; when expressed solely as the percentage change in diameter (6.53 ± 0.61 vs. 8.56 ± 0.54 %, respectively, P<0.01) (Figure 5) and as the percentage change in diameter with respect to the change in shear rate (0.014 ± 0.002 vs. 0.019 ± 0.002 %/s^{-1}, respectively, P<0.01). In contrast, there were no differences in the responses to nitrate-mediated dilation between each visit.

Endothelial dysfunction following SSB consumption may be associated with an acute hyperglycemic-mediated increase in oxidative stress that decreases NO bioavailability

Given that both flow-mediated dilation and the vascular response to acetylcholine iontophoresis were reduced during SSB-mediated acute hyperglycemia, whilst the vascular responses to nitrate administration were preserved, clinical findings clearly suggest that acute hyperglycemia impairs micro- and macrovascular function via an endothelium-dependent pathway. However, considering that further experimental exploration within vascular tissues of healthy humans is, for ethical reasons, very difficult to perform, the underlying mechanisms by which acute hyperglycemia mediates endothelial dysfunction were evaluated further, both *in vivo* and *ex vivo*, using an experimental rat model (Suppl Material Figure I).

In vivo experimental exploration

Acute hyperglycemia was induced in each rat using an intra-peritoneal injection of glucose that mediated a hyperglycemic plateau between 10 and 20 minutes following administration, with peak hyperglycemia occurring at 15 minutes (Suppl Material Figure IIA). To allow for comparisons between the acute hyperglycemic and normoglycemic conditions, a group of control rats received a placebo injection of sodium chloride. During the acute hyperglycemic plateau, iontophoresis of acetylcholine and sodium nitroprusside were performed inducing a significant increase in cutaneous microvascular blood flux (Figure 6A). However, similar to the findings obtained in human trials, the relative increase in cutaneous blood flux in response to transdermal iontophoresis of acetylcholine was decreased during acute hyperglycemia when compared to the normoglycemic controls, whilst the vascular response to sodium nitroprusside iontophoresis remained preserved (Figure 6B). Such endothelial dysfunction may be primarily caused by an acute hyperglycemic-mediated increase in oxidative stress.[14] Considering this, a third group of rats were treated with a dosage of the antioxidant, N-acetylcysteine, prior to the induction of acute hyperglycemia. Although there was no difference in the acute hyperglycemic response to the intra-peritoneal injection of glucose

between N-acetylcysteine-treated and untreated rats (Suppl Material Figure IIB), the increase in cutaneous blood flux in response to acetylcholine iontophoresis was fully restored in rats treated with the antioxidant (Figure 6B); supporting the implication of increased oxidative stress in acute hyperglycemia-mediated endothelial dysfunction.

Ex vivo experimental exploration

Given that endothelial function is highly dependent on NO bioavailability, which itself is dependent on endothelial nitric oxide synthase (eNOS) and its activation by phosphorylation at serine 1177 ($eNOS^{ser1177}$), *ex vivo* experiments were then performed on rat aortic tissue to investigate the interaction between oxidative stress and the NO pathway; and whether this interaction contributes to the endothelial dysfunction observed during acute hyperglycemia. Similar to that observed following SSB consumption in human trials and the intraperitoneal injection of glucose in *in vivo* experimental exploration, acute hyperglycemia induced by the hyperglycemic Kreb's solution decreased the relaxation response to acetylcholine administration in pre-contracted aortic rings in a dose-dependent manner (Suppl Material Figure IIIA and B). However, pre-treatment with N-acetylcysteine restored the relaxation response in aortic rings during acute hyperglycemia (Figure 6C and 6D). Western blotting revealed that acute hyperglycemia tended ($p=0.054$) to mediate a decrease in $eNOS^{ser1177}$ expression in the aortic rings (Figure 6E), while electron paramagnetic resonance measured an elevation in the concentration of reactive oxygen species when compared to the normoglycemic condition (Figure 6F).

DISCUSSION

The present study aimed to assess the effect of acute hyperglycemia induced by the ingestion of a commercial SSB on vascular function in healthy sedentary participants. It was found that SSB consumption decreased both micro- and macrovascular endothelial function, whereas VSM function remained unaffected during acute hyperglycemia. Considering these findings, *in vivo* and *ex vivo* experimental explorations were then performed in an experimental rat model to further investigate the interaction between acute hyperglycemia and vascular function; and examine the underlying mechanisms that may mediate such endothelial dysfunction. Similar to that observed in human trials, acute hyperglycemia mediated a decrease in endothelial function in both *in vivo* and *ex vivo* experimental rat models.

Interestingly, endothelial function was fully restored during acute hyperglycemia in rats and aortic rings that were pre-treated with the antioxidant, N-acetylcysteine. Further to this, it was found that acute hyperglycemia was associated with an increased production in arterial reactive oxygen species and reduced expression of eNOS phosphorylation. Collectively, these findings suggest that acute hyperglycemia induces endothelial dysfunction by mediating an increase in oxidative stress that disrupts normal processes of the eNOS/NO pathway.

It is currently unknown how acute hyperglycemia induced by commercial SSB consumption affects vascular function due to limited and discrepant data. Previous research has most commonly assessed vascular function during acute hyperglycemia induced by a typical oral glucose load using measures of macrovascular reactivity such as brachial artery ultrasound with flow-mediated dilation.[9] Given that the microcirculation represents most of the arterial vascular network and exerts dominant control over local blood flow,[15] and that emerging evidence suggests that coronary microvascular dysfunction may appear before macrovascular disease, collectively highlights the need to comprehensively assess both micro- and macrovascular function when investigating mechanisms that contribute to the pathogenesis of CVD.[16] The results of this present study are consistent with those of previous research demonstrating that commercial SSB consumption mediates a decrease in macrovascular endothelial function as indicated by reduced flow-mediated dilation during acute hyperglycemia.[17] In contrast, the microcirculatory findings contradict a separate study, which found that consumption of commercial SSB enhances microvascular endothelial function, as indicated by an increased cutaneous blood flux response to transdermal iontophoresis of acetylcholine during acute hyperglycemia.[18] Importantly, it should be noted that the former study of the macrocirculation induced acute hyperglycemia by ingestion of a commercial SSB in conjunction with consumption of a high caloric commercial candy bar. Moreover, the latter study of the microcirculation used a commercial SSB that is defined as an energy drink and contains caffeine, which has been found to enhance microvascular function in healthy subjects.[19] Considering these methods, this present study is, to our knowledge, the first to examine the effects of acute hyperglycemia induced by commercial SSB consumption alone on micro- and macrovascular function. The results of this study also contribute to clarifying the effect of acute hyperglycemia on systemic vascular endothelial function, which in a recent systematic review and meta-analysis was found to be inconclusive due to limited microvascular data and discrepant reporting of shear stress data in studies that used flow-mediated dilation to assess changes in vascular function from normoglycemic to

hyperglycemic states.[9] Given that this present study found a decrease in flow-mediated dilation during acute hyperglycemia with no reduction in shear stress, a disruption of the NO pathway may be implicated in SSB-mediated endothelial dysfunction.[20, 21]

Current evidence suggests that acute hyperglycemia induces endothelial dysfunction by mediating an abnormal elevation in oxidative stress that disturbs normal underlying mechanisms of NO synthesis.[22, 23] In the postprandial state, oxidative metabolism initiates oxidative phosphorylation of adenosine triphosphate at the electron transport chain of the mitochondria, which via the phenomenon of electron leakage causes superoxide generation of reactive oxygen species.[24] Moreover, it has been clearly reported that increased glycemia is responsible for the activation of NADPH oxidase, which also contributes to production of the superoxide anion.[10] Although these reactive oxygen species are normally readily detoxified, elevated activity within this mechanism such as that following commercial SSB consumption increases production to a rate beyond suppressive capabilities of the antioxidant systems.[25] The implication of oxidative stress in acute hyperglycemia-mediated endothelial dysfunction is further supported by *ex vivo* findings in this present study that demonstrated that acute hyperglycemia increased reactive oxygen species in rat aortic rings. Moreover, treatment with the antioxidant, N-acetylcysteine, was found to attenuate the impaired relaxation response to acetylcholine iontophoresis observed during acute hyperglycemia. Importantly, the applicability of this oxidative stress-dependent mechanism to acute hyperglycemia-mediated endothelial dysfunction in living organisms was also demonstrated in this study, which revealed for the first time in an *in vivo* experimental rat model that antioxidant treatment also fully restores cutaneous microvascular endothelial function during acute hyperglycemia. Given that a decrease in eNOS activation by reduced phosphorylation at serine 1177 was also observed, it is plausible that acute hyperglycemia mediates an increase in oxidative stress that disrupts eNOS phosphorylation, which reduces NO bioavailability and, subsequently, induces endothelial dysfunction. Such mechanisms may also in turn explain the observed protective effect of N-acetylcysteine administration on endothelial function.

Despite not being affected in this present study, previous research has demonstrated decreased VSM function mediated by VSM cell proliferation in as little as 6 hours following the induction of hyperglycemia in animal and *in vitro* studies,[26] suggesting a need to extend the typical assessment period of vascular function following SSB consumption in future research. Moreover, considering the global rate of commercial SSB consumption and its role in transient endothelial dysfunction, even in a healthy population, research must also quantify

the relative loading of SSB over time that mediates significant vascular remodelling and contribute to the pathogenesis of CVD in humans.[9] In addition to examining the underlying mechanisms of NO-mediated microvascular function, future research may also assess the effect of SSB consumption on other main vasoactive mediators such as prostaglandin I_2, endothelium derived hyperpolarizing factor, and endothelin-1, all of which have varying influence between the micro- and macrocirculation.[7, 11, 27] Finally, these studies need to be conducted across a variety of ethnicities, some of which have previously demonstrated decreased vascular function even at rest and therefore may be more severely impacted by the deleterious vascular-related effects of acute hyperglycemia than that observed in Caucasian populations.[28, 29]

Several inherent limitations must be considered when interpreting this data. Whereas the main sugar in SSB is sucrose, which is comprised of glucose and fructose, only glucose was used to induce acute hyperglycemia in the experimental rat model used to explain the effects of SSB consumption on underlying mechanisms of vascular function. However, it has previously been suggested that the deleterious cardiovascular effects of sucrose are more related to glucose rather than fructose.[11, 30] It must also be considered that ingredients other than sucrose that comprise commercial SSB were not evaluated individually in this research and, therefore, it is not known how they may contribute to the observed SSB-mediated endothelial dysfunction. Additionally, it was not possible to blind participants to the intervention by using a sugar-free placebo such as a commercial diet soda due to previous research suggesting that even artificial sweeteners may interact with taste receptors stimulating insulin secretion, which may induce a vascular response.[31] Given that changes in blood insulin concentration in response to SSB consumption were not monitored in this study, it was not possible to explore what effect SSB consumption may have on mechanisms of insulin-mediated vasodilation. Finally, vascular function was assessed in a focussed sample and, therefore, the effect of SSB consumption may vary across health groups, ethnicities and genders.

In conclusion, this present study is, to our knowledge, the first to assess vascular function during acute hyperglycemia induced by SSB consumption alone. The findings of this study demonstrate that commercial SSB consumption induces micro- and macrovascular endothelial dysfunction in a healthy population. Furthermore, data from the experimental rat model suggest that this commercial SSB-mediated endothelial dysfunction is partly due to increased oxidative stress, which reduces NO bioavailability by disrupting processes crucial

to its synthesis. Ultimately, such results inform international public health policy on the adverse effects of both commercial SSB and general excess sugar consumption; and how they contribute so acutely, even in those considered healthy, to the upregulation of mechanisms that are the primary precursors to the pathogenesis of CVD.[32]

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REFERENCES

1. Brown C, Dulloo A, Montani J. Sugary drinks in the pathogenesis of obesity and cardiovascular diseases. *International Journal of Obesity*. 2008;32:S28-S34.
2. Huang C, Huang J, Tian Y, Yang X, Gu D. Sugar sweetened beverages consumption and risk of coronary heart disease: A meta-analysis of prospective studies. *Atherosclerosis*. 2014;234:11-16.
3. Hu FB, Malik VS. Sugar-sweetened beverages and risk of obesity and type 2 diabetes: Epidemiologic evidence. *Physiol Behav*. 2010;100:47-54.
4. O'Connor L, Imamura F, Lentjes MA, Khaw KT, Wareham NJ, Forouhi NG. Prospective associations and population impact of sweet beverage intake and type 2 diabetes, and effects of substitutions with alternative beverages. *Diabetologia*. 2015;58:1474-1483.
5. World Health Organisation. Cardiovascular diseases fact sheet. http://www.who.int/mediacentre/factsheets/fs317/en/. Accessed April 1, 2016.
6. Souza EG, De Lorenzo A, Huguenin G, Oliveira GM, Tibirica E. Impairment of systemic microvascular endothelial and smooth muscle function in individuals with early-onset coronary artery disease: Studies with laser speckle contrast imaging. *Coron Artery Dis*. 2014;25:23-28.
7. Feletou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: Where are we now? *Arterioscler Thromb Vasc Biol*. 2006;26:1215-1225.
8. Maiorana A, O'Driscoll G, Taylor R, Green D. Exercise and the nitric oxide vasodilator system. *Sports Med*. 2003;33:1013-1035.
9. Loader J, Montero D, Lorenzen C, Watts R, Meziat C, Reboul C, Stewart S, Walther G. Acute hyperglycemia impairs vascular function in healthy and cardiometabolic diseased subjects: Systematic review and meta-analysis. *Arterioscler Thromb Vasc Biol*. 2015;35:2060-2072.
10. Mah E, Bruno RS. Postprandial hyperglycemia on vascular endothelial function: Mechanisms and consequences. *Nutr Res*. 2012;32:727-740.
11. Mah E, Noh SK, Ballard KD, Matos ME, Volek JS, Bruno RS. Postprandial hyperglycemia impairs vascular endothelial function in healthy men by inducing lipid peroxidation and increasing asymmetric dimethylarginine:Arginine. *J Nutr*. 2011;141:1961-1968.

12. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: From marvel to menace. *Circulation*. 2006;113:1708-1714.
13. Li PL, Zhang Y. Cross talk between ceramide and redox signaling: Implications for endothelial dysfunction and renal disease. *Handb Exp Pharmacol*. 2013:171-197.
14. Ceriello A, Taboga C, Tonutti L, Quagliaro L, Piconi L, Bais B, Da Ros R, Motz E. Evidence for an independent and cumulative effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial dysfunction and oxidative stress generation: Effects of short- and long-term simvastatin treatment. *Circulation*. 2002;106:1211-1218.
15. Roustit M, Cracowski JL. Assessment of endothelial and neurovascular function in human skin microcirculation. *Trends Pharmacol Sci*. 2013;34:373-384.
16. Jzerman RG, de Jongh RT, Beijk MA, van Weissenbruch MM, Delemarre-van de Waal HA, Serne EH, Stehouwer CD. Individuals at increased coronary heart disease risk are characterized by an impaired microvascular function in skin. *Eur J Clin Invest*. 2003;33:536-542.
17. Weiss EP, Arif H, Villareal DT, Marzetti E, Holloszy JO. Endothelial function after high-sugar-food ingestion improves with endurance exercise performed on the previous day. *Am J Clin Nutr*. 2008;88:51-57.
18. Grasser EK, Yepuri G, Dulloo AG, Montani JP. Cardio- and cerebrovascular responses to the energy drink red bull in young adults: A randomized cross-over study. *Eur J Nutr*. 2014;53:1561-1571.
19. Noguchi K, Matsuzaki T, Sakanashi M, Hamadate N, Uchida T, Kina-Tanada M, Kubota H, Nakasone J, Sakanashi M, Ueda S, Masuzaki H, Ishiuchi S, Ohya Y, Tsutsui M. Effect of caffeine contained in a cup of coffee on microvascular function in healthy subjects. *J Pharmacol Sci*. 2015;127:217-222.
20. Mitchell GF, Parise H, Vita JA, Larson MG, Warner E, Keaney JF, Jr., Keyes MJ, Levy D, Vasan RS, Benjamin EJ. Local shear stress and brachial artery flow-mediated dilation: The framingham heart study. *Hypertension*. 2004;44:134-139.
21. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME, Green DJ. Assessment of flow-mediated dilation in humans: A methodological and physiological guideline. *Am J Physiol Heart Circ Physiol*. 2011;300:H2-12.

22. Monnier L, Mas E, Ginet C, Michel F, Villon L, Cristol JP, Colette C. Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes. *Jama*. 2006;295:1681-1687.
23. Zheng F, Lu W, Jia C, Li H, Wang Z, Jia W. Relationships between glucose excursion and the activation of oxidative stress in patients with newly diagnosed type 2 diabetes or impaired glucose regulation. *Endocrine*. 2010;37:201-208.
24. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med*. 2000;29:222-230.
25. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. *Nature*. 2001;414:813-820.
26. Sun J, Xu Y, Dai Z, Sun Y. Intermittent high glucose enhances proliferation of vascular smooth muscle cells by upregulating osteopontin. *Mol Cell Endocrinol*. 2009;313:64-69.
27. Muniyappa R, Sowers JR. Role of insulin resistance in endothelial dysfunction. *Rev Endocr Metab Disord*. 2013;14:5-12.
28. Ozkor MA, Rahman AM, Murrow JR, Kavtaradze N, Lin J, Manatunga A, Hayek S, Quyyumi AA. Differences in vascular nitric oxide and endothelium-derived hyperpolarizing factor bioavailability in blacks and whites. *Arterioscler Thromb Vasc Biol*. 2014;34:1320-1327.
29. Melikian N, Wheatcroft SB, Ogah OS, Murphy C, Chowienczyk PJ, Wierzbicki AS, Sanders TA, Jiang B, Duncan ER, Shah AM, Kearney MT. Asymmetric dimethylarginine and reduced nitric oxide bioavailability in young black african men. *Hypertension*. 2007;49:873-877.
30. Grasser EK, Dulloo A, Montani JP. Cardiovascular responses to the ingestion of sugary drinks using a randomised cross-over study design: Does glucose attenuate the blood pressure-elevating effect of fructose? *Br J Nutr*. 2014;112:183-192.
31. Pepino MY. Metabolic effects of non-nutritive sweeteners. *Physiol Behav*. 2015
32. Sugar intake: Lowering the bar. *Lancet Diabetes Endocrinol*. 2015;3:305.
33. United States Department of Agriculture. National nutrient database for standard reference release 27. http://ndb.nal.usda.gov/ndb/search/list. Accessed March 11, 2015

Significance

- To our knowledge, this study is the first to examine the effects of acute hyperglycemia induced by consumption of a commercial sugar-sweetened beverage alone on vascular function.
- Consumption of commercial sugar-sweetened beverages induces acute hyperglycemia that mediates a transient endothelial dysfunction in the micro- and macrocirculation, even in those considered to be healthy.
- Findings from an *in vivo* and *ex vivo* experimental rat model suggest that acute hyperglycemia is associated with an increase in oxidative stress that decreases endothelial nitric oxide synthase activation by disrupting phosphorylation at serine 1177, thus reducing nitric oxide bioavailability that subsequently induces endothelial dysfunction.

TABLES

Table 1. Nutrient composition of each test beverage

	WATER	SSB
Volume, *mL*	600	600
Energy, *kJ*	0	1200
Protein, *g*	0	0.30
Fat		
- Total, *g*	0	0
- Saturated, *g*	0	0
Carbohydrate, *g*	0	72.4
- Sugars, *g*	0	72.4
Calcium, *mg*	18	N/A
Magnesium, *mg*	6	N/A
Sodium, *mg*	24	108
Zinc, *mg*	0.06	N/A
Ingredients	Tap water	Carbonated water, sugar, reconstituted lemon juice (5 %), food acids (330,331, natural flavor, preservatives (211, 223), natural colour (safflower extract)

Nutrient composition of water was obtained from the United States Department of Agriculture Nutrient Database Standard Reference Release 27.[33] Nutrient composition for the commercial SSB was obtained from the nutrition information label on packaging. Participants consumed 600 mL of water or commercial SSB 15 minutes prior to the beginning of vascular assessment. Participants completed trials on separate days for each test beverage in a randomized order.

Table 2. Microvascular and macrovascular function in response to water and commercial SSB consumption in healthy subjects.

	WATER	**SSB**	*P*-value
n	12	12	
Microcirculation			
Basal CBF (PU)	23.6 ± 1.8	26.4 ± 1.6	*p*=0.231
Peak ACh CBF (PU)	70.9 ± 8.0	63.0 ± 6.4	*p*=0.346
Peak SNP CBF (PU)	101.2 ± 6.0	101.9 ± 7.4	*p=0.934*
ACh CVC increase (%)	208.3 ± 24.3	144.2 ± 15.7*	*p*=0.008
SNP CVC increase (%)	360.3 ± 26.7	355.9 ± 29.3	*p*=0.926
Skin resistance (Ω)	399.4 ± 41.3	395.6 ± 30.2	*p*=0.872
Macrocirculation			
Basal brachial artery diameter (mm)	4.7 ± 0.1	4.7 ± 0.1	*p*=0.684
Basal brachial blood flow ($ml.min^{-1}$)	48.0 ± 5.7	53.0 ± 6.5	*p*=0.673
Peak brachial blood flow ($ml.min^{-1}$)	408.3 ± 27.6	445.1 ± 34.5	*p*=0.194
Δshear rate ($\%/s^{-1}$)	484.3 ± 32.8	501.6 ± 37.1	*p*=0.506
FMD/Δshear rate ($\%/s^{-1}$)	0.019 ± 0.002	0.014 ± 0.002*	*p*<0.001
Brachial hyperemia (%)	886.4 ±	855.5 ± 107.7	*p=0.693*

Values are mean ± SEM. SSB indicates sugar-sweetened beverage; CBF, cutaneous blood flux; PU, perfusion units; ACh, acetylcholine; SNP, sodium nitroprusside; CVC, cutaneous vascular conductance; FMD, flow-mediated dilation. Δshear rate means peak brachial shear rate minus resting brachial shear rate. P-value was estimated by repeated measures ANOVA followed by post-hoc Tukey's multiple comparison tests. *P<0.05 vs. water.

Figure 1. Study design: sequence of testing. In a fasted state (≥10 h) participants rested for 20 minutes before consuming 600 mL (20 oz) of water or sugar-sweetened beverage (SSB) within a 5 minute period. Participants then rested for a further 15 mins before iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) were performed. Immediately following the conclusion of iontophoresis, flow-mediated dilation (FMD) and nitrate-mediated dilation (NMD) were also performed. Blood glucose (GL), blood pressure (BP), and heart rate (HR) were measured after the 20 min resting period, 15 minutes after consuming the test beverage, at the end of iontophoresis, at the end of FMD, and at the end of NMD. All 12 participants were administered the water beverage or commercial SSB in a randomized order.

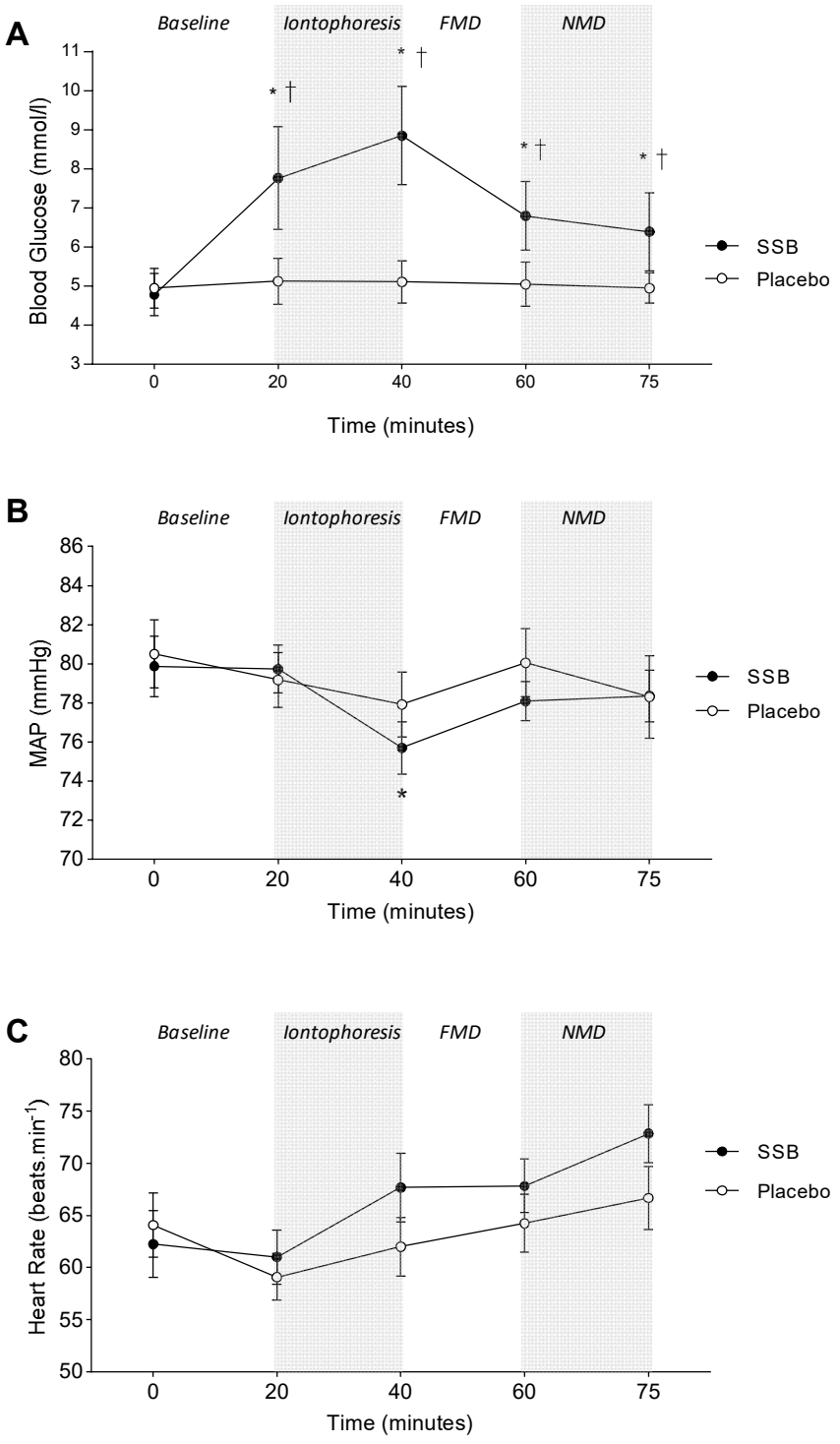


Figure 3. Changes in A) blood glucose concentrations, B) mean arterial pressure (MAP), and C) heart rate over time in response to consumption of 600mL of water or commercial sugar-sweetened beverage (SSB). *P<0.05 vs. baseline; †P<0.05 vs. water.

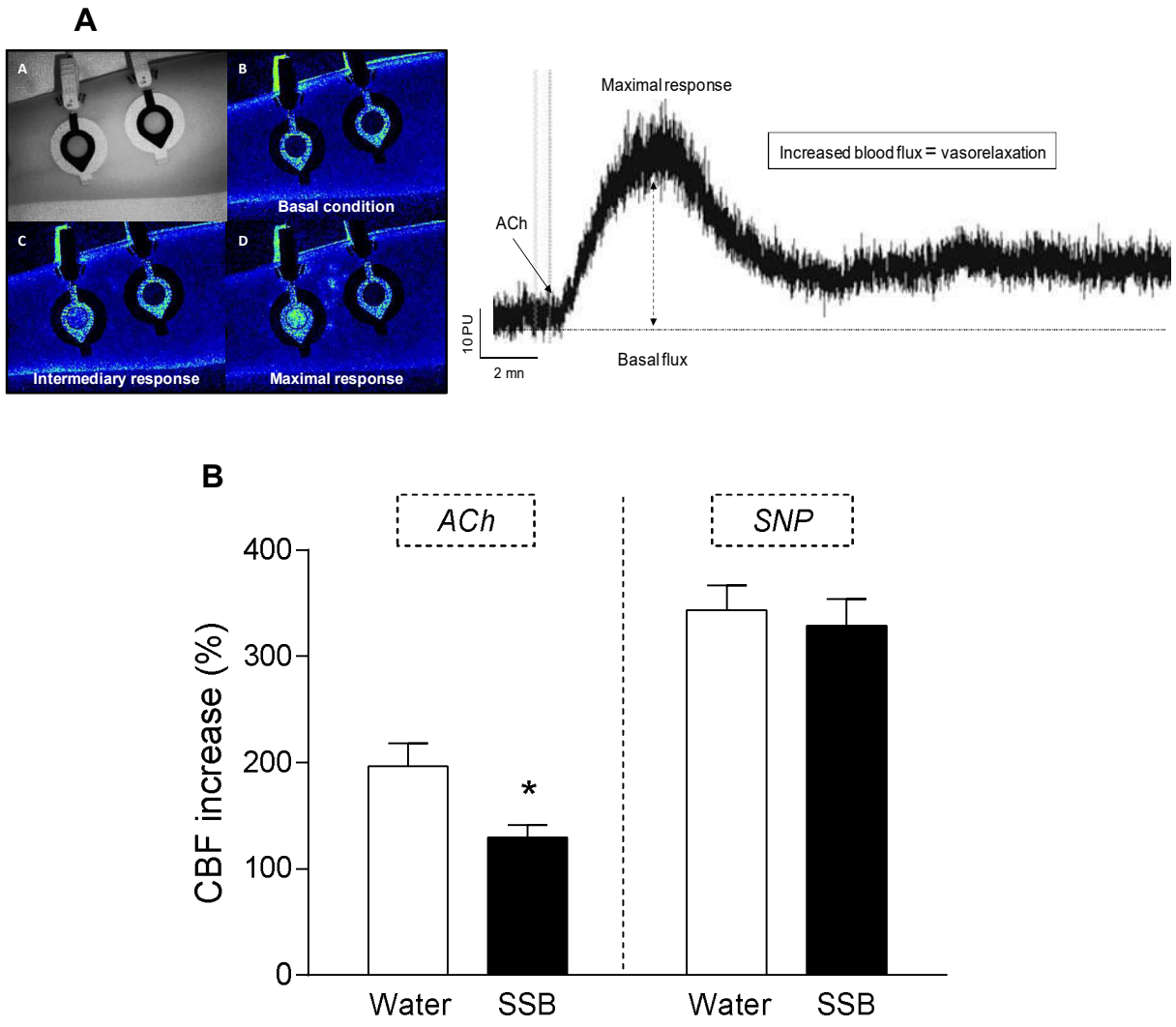


Figure 4. Microvascular function in healthy participants following consumption of 600 mL of water or commercial sugar-sweetened beverage (SSB). (A) Left: Laser speckle contrast imaging electrode position on the right forearm. Right: Representative curve of cutaneous blood flux (CBF) during acetylcholine (ACh) iontophoresis. **(B)** The percentage increase from baseline in CBF in response to transdermal iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP). *P<0.01 vs. water.

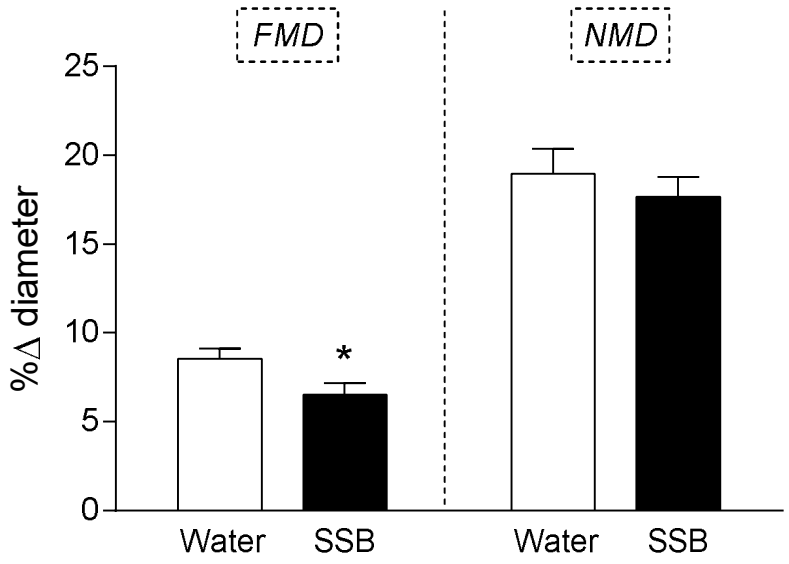


Figure 5. Macrovascular function in healthy participants following consumption of 600 mL of water or commercial sugar-sweetened beverage (SSB). The percentage change from baseline in brachial artery diameter in response to flow-mediated dilation (FMD) and nitrate-mediated dilation (NMD). *P<0.01 vs. water.

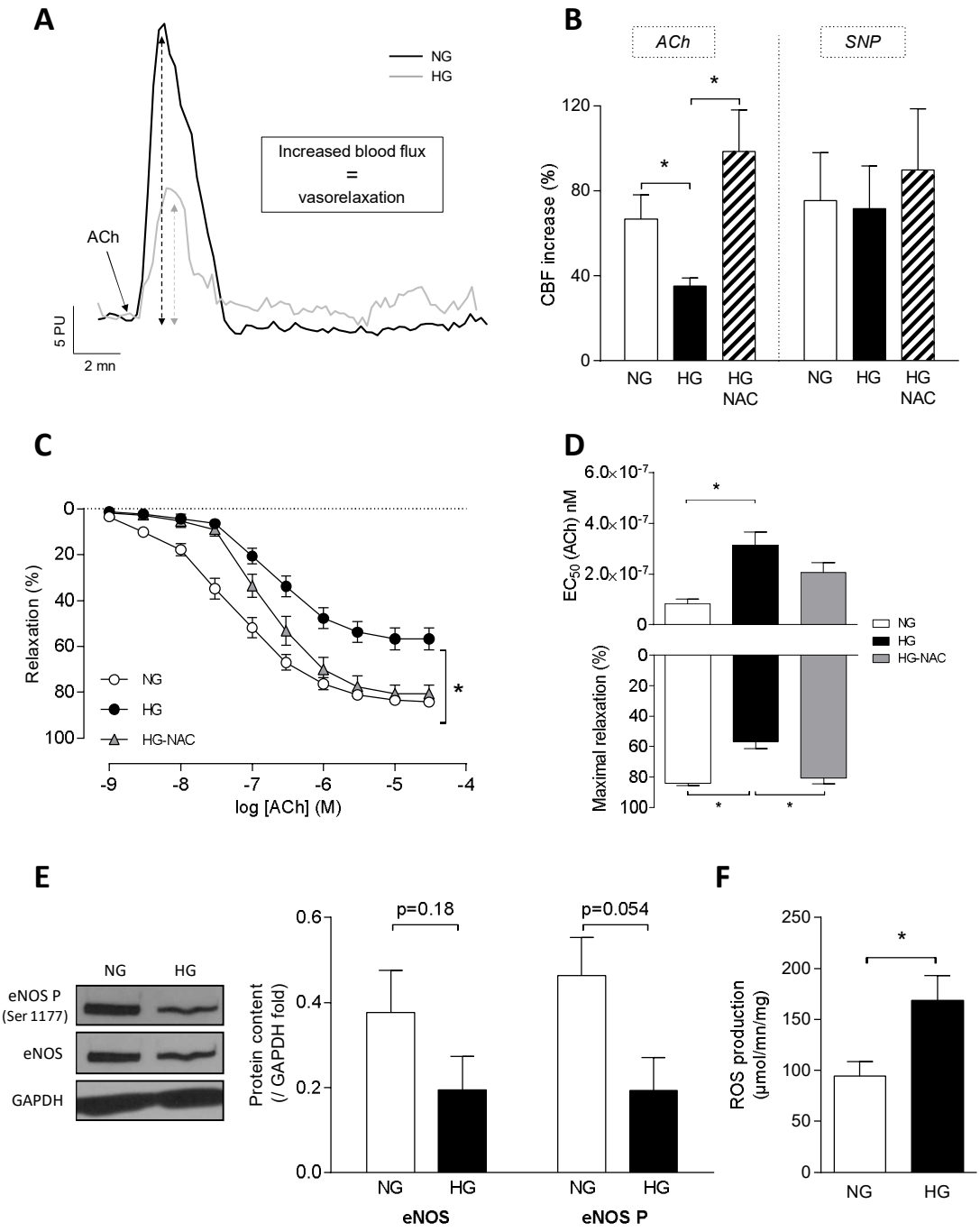
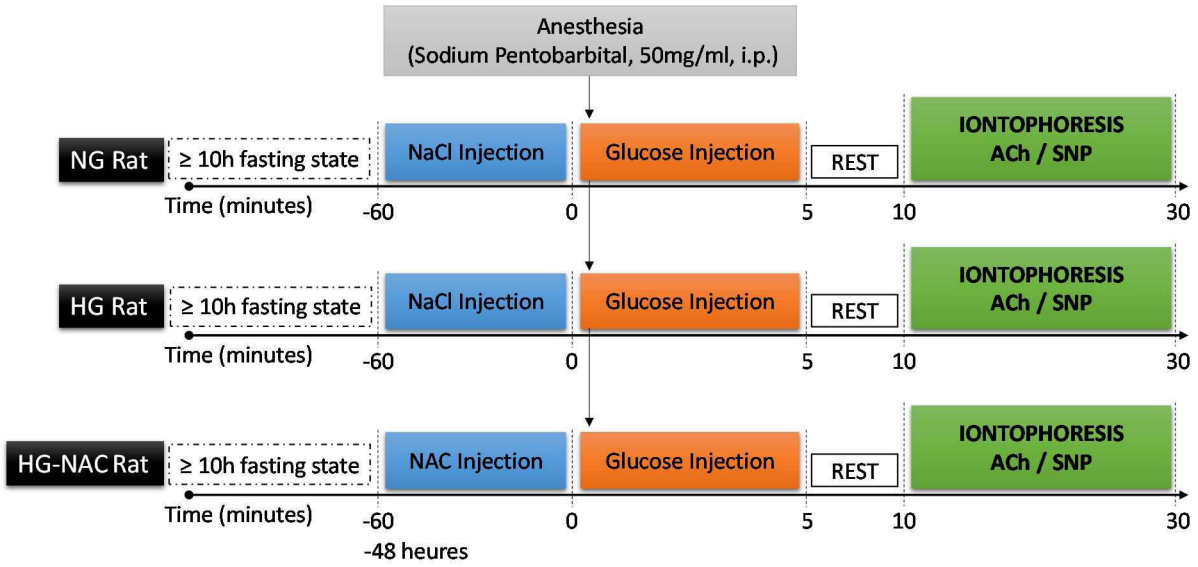
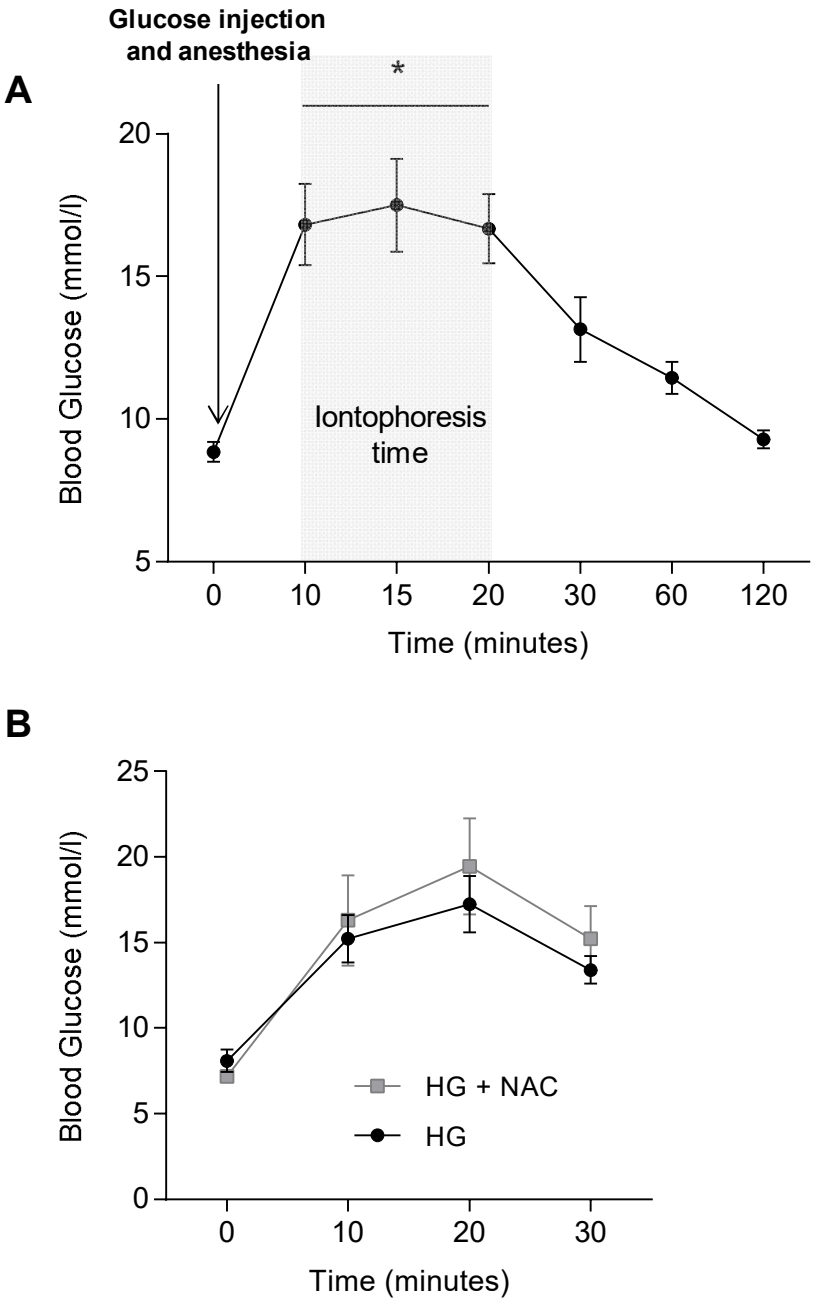


Figure 6. Implication of oxidative stress in endothelial dysfunction during acute hyperglycemia. (A) Representative curve of cutaneous microvascular blood flux (CBF) during normoglycemia (NG) and acute hyperglycemia (HG) in response to acetylcholine (ACh) iontophoresis in rats **(B)** The percentage increase from baseline in CBF following sodium chloride administration in NG rats; and following induction of HG in rats pre-treated with sodium chloride in order to examine the effect of HG alone, or rats pre-treated with N-acetylcysteine (NAC) to evaluate implication of oxidative stress in endothelial dysfunction. **(C)** A dose response curve to ACh during NG and HG (100 mM) in pre-contracted aortic rings and during HG (100 mM) in pre-contracted rings pre-incubated in NAC (20 mM) **(D)** Up: Concentration of ACh administered to induce 50 % of the maximal relaxation response to

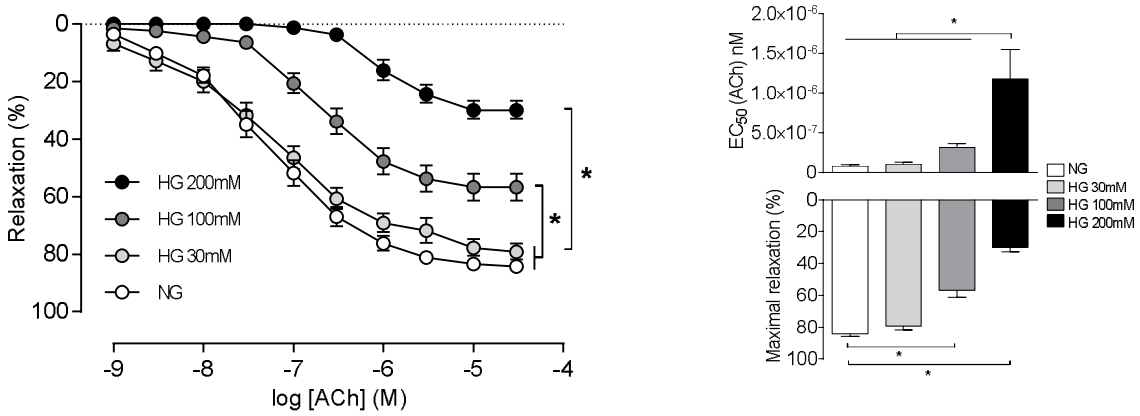
ACh in NG, HG and HG-NAC aortic ring preparations; Down: Maximal relaxation of aortic ring preparations in response to administration of ACh expressed as the percent relaxation relative to the measured pre-contraction with phenylephrine (10^{-6} mM). **(E)** Expression of endothelial nitric oxide synthase (eNOS) and its activation by phosphorylation (P) at serine 1177 analysed by western blotting in NG aortic preparations; and aortic preparations pre-incubated in HG Kreb's solution (glucose concentration: 100 mM). **(F)** Production of reactive oxygen species (ROS) evaluated by electron paramagnetic resonance in NG aortic preparations; and aortic preparations pre-incubated with HG Kreb's solution (glucose concentration: 30 mM). *P<0.05.



Supplemental Material Figure 1 : Study design of experimental exploration. In a fasted state (≥ 10 hours), following pre-treatments with sodium chloride (NaCl), N-acetylcysteine (NAC), or tetrahydrobiopterin (BH_4), rats were anesthetized using sodium pentobarbital (50mg.kg-1). Rats then received an intra-peritoneal injection of NaCl (for NG rats) or glucose (2g/kg) before acclimatizing for 10 minutes in an incubator to maintain skin temperature at 37°C. Iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) were then performed. Rats were randomized each treatment group.



Supplemental Material Figure II. (A) Time course of blood glucose concentrations following glucose injection (2 g/kg, 1.5 ml) in rats. **(B)** Time course of blood glucose concentrations following glucose injection in rats pre-treated with sodium chloride only (HG) or N-acetylcysteine (HG-NAC). *P<0.05 *vs* baseline.



Supplemental Material Figure III. **(A)** Dose response curve to acetylcholine (ACh) in normoglycemic (NG) pre-contracted aortic ring preparations; and pre-contracted aortic preparations incubated with incremental concentrations of a hyperglycemic (HG) Kreb's solution (glucose concentrations: 30, 100 and 200 mM). **(B) Up:** Concentration of ACh administered to induce 50 % of maximal relaxation response to ACh in NG aortic ring preparations; and aortic preparations pre-incubated with incremental concentrations of a HG Kreb's solution (glucose concentrations: 30, 100 and 200 mM). **Down:** Maximal relaxation of aortic ring preparations in response to administration of ACh expressed as the percent relaxation relative to the measured pre-contraction with phenylephrine (10^{-6} mM). *P<0.05.

Résultats additionnels

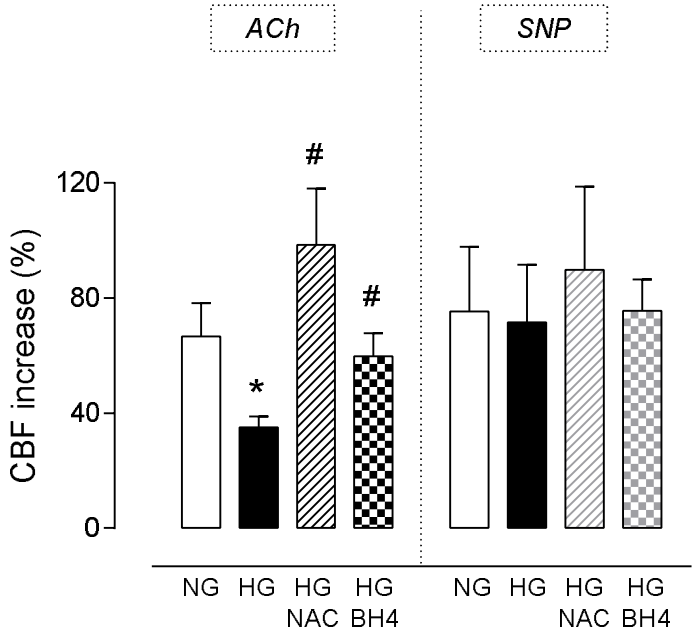


Figure 34 : Flux sanguin cutané (CBF : cutaneous blood flow) de rats sains en situation normoglycémique (NG) ou en réponse à une hyperglycémie aigue (HG).

Pourcentage d'augmentation par rapport à l'état basal du flux sanguin cutanée mesuré par Laser Doppler, en réponse à une iontophorèse d'acétylcholine (ACh) et de nitroprussiate de sodium (SNP), permettant d'évaluer respectivement la fonction de relaxation vasculaire endothélium-dépendante et - indépendante. Ces tests ont été réalisés après avoir administré du chlorure de sodium (i.p.) chez les rats NG, ou du glucose (2g/kg, i.p.) pour les rats HG, HG-BH4, et HG-NAC. Les animaux HG-NAC étaient prétraités au N-acétylcystéine afin d'évaluer l'implication du stress oxydant, et les animaux HG-BH_4 étaient prétraités à la tétrahydrobioptérine afin d'évaluer l'état de couplage de la eNOS. *: $p<0.05$ vs NG, # : $p<0.05$ vs HG. Les valeurs sont exprimées en moyenne ± SEM.

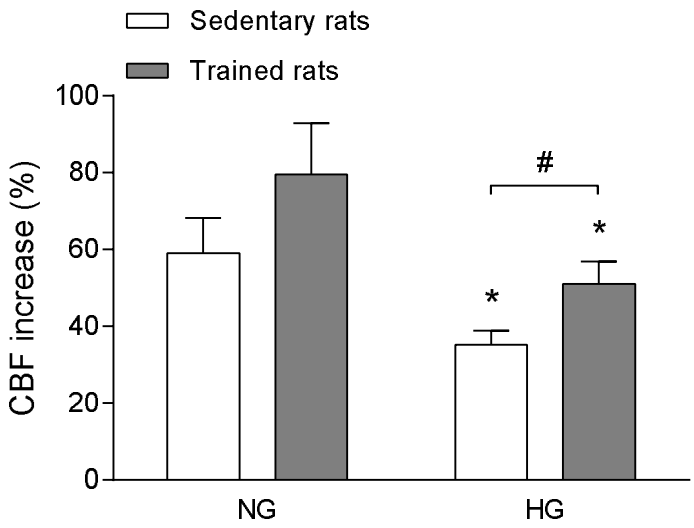


Figure 35 : Flux sanguin cutané (CBF : cutaneous blood flow) de rats sédentaires et entraînés en situation normoglycémique (NG) ou en réponse à une hyperglycémie aigue (HG). Pourcentage d'augmentation par rapport à l'état basal du flux sanguin cutanée mesuré par Laser Doppler, en réponse à une iontophorèse d'acétylcholine. Ces tests ont été réalisés après avoir administré du chlorure de sodium (i.p.) chez les rats NG, ou du glucose (2g/kg, i.p.) pour les rats HG, sur des rats sédentaires et entraînés, au terme du protocole d'entraînement. *: $p<0.05$ vs NG, # : $p<0.05$ vs HG. Les valeurs sont exprimées en moyenne ± SEM.