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Chronic iron overload induces functional and structural vascular changes in small resistance arteries via NADPH oxidase-dependent $O_2\cdot^-$ production

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ABSTRACT

Iron overload leads to excessive free radical formation and induces cardiovascular dysfunction. Thus, our aim was to investigate the structural and endothelial modulation of vascular tone induced by chronic iron overload in mesenteric arteries. Rats were divided into two groups: the control (vehicle) group and the group treated with iron dextran for 28 days (100 mg/kg, 5 days a week). Chronic iron overload altered the following morpho-physiological parameters of third-order mesenteric resistance arteries: decreased lumen and external diameters; increased wall/lumen ratio and wall thickness; decreased distensibility and increased stiffness; and increased pulse wave velocity. Additionally, iron overload increased the vasoconstrictor response in mesenteric arterial rings *in vitro* but did not affect the relaxation induced by acetylcholine and sodium nitroprusside. It is suggested that iron overload reduces nitric oxide bioavailability by increasing free radicals, because L-NAME did not shift the concentration-response curve to phenylephrine, but L-NAME plus superoxide dismutase shifted the curve to the left. In vitro assays with DAF-2 and DHE indicated reduced NO production and increased superoxide anion ($O_2\cdot^-$) generation in the iron-overloaded group. Furthermore, tiron, catalase, apocynin and losartan induced reduced reactivity only in iron-overloaded rats. Moreover, increased ACE activity was observed in the mesenteric resistance arteries of iron-overloaded rats accompanied by an increase in gp91phox, catalase, ERK1/2 and eNOS protein expression. In conclusion, these findings show that chronic iron overload induces structural and functional changes in resistance arteries, most likely due to a decrease in NO bioavailability resulting from an increase in $O_2\cdot^-$ production by NADPH oxidase.

1. Introduction

Iron is a transition metal and is essential for many physiological processes due to its ability to donate and receive electrons. However, body iron levels must be precisely balanced because it catalyses the formation of reactive oxygen species (ROS) via Fenton and Haber-Weiss reactions (Lum and Roebuck, 2001; Marques et al., 2015). In addition, iron and ROS are recognised as important mediators of cell death in physiological and pathological situations, and acute or chronic iron loading leads to damage in different organs and systems (Dunn et al., 2007; Rossi et al., 2016), including the cardiovascular system (Marques et al., 2015; Avila et al., 2016).

Chronic iron overload occurs in patients with hereditary haemochromatosis, a recessive genetic disorder characterised by moderate iron overload. Moreover, iron overload can be secondary to a variety of pathological conditions, such as ineffective erythropoiesis, sideroblastic anaemia, primary liver disease and frequent blood transfusions or parenteral iron infusions (Bulaj et al., 1996; Siddique and Kowdley, 2012; Gattermann, 2009).

Elevated iron levels in the body are associated with increased risk of coronary artery disease and myocardial infarction (Salonen et al., 1992; Magnusson et al., 1994; Tuomainen et al., 1997). Because iron is capable of accelerating atherosclerosis and arterial thrombosis, this has been postulated as a potential mechanism by which iron overload can enhance the risk of ischaemic events (Araujo et al., 1995; Day et al., 2003). Notwithstanding, it is well known that increases in iron-mediated ROS formation also predisposes individuals to cardiovascular events by atherosclerosis-independent mechanisms (Sullivan, 1999).

Although acute ischaemic events have been directly associated with atherosclerosis and thrombosis, they can also result from a decrease in lumen diameter that could originate from structural, mechanical and/or functional alterations in small resistance arteries. Thus, we

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hypothesized that iron overload could promote vascular dysfunction and structural changes in resistance arteries since excess iron may locally catalyse ROS formation.

In the present study, our aim was to test whether, in addition to the mechanisms related to atherosclerosis, alterations in the structure and reactivity of resistance arteries might contribute to increased cardiovascular risk in iron-overloaded rats. We investigated the arterial structure, changes in pulse wave velocity, and the role of oxidative stress on the endothelial modulation of vascular tone in small mesenteric arteries. To the best of our knowledge, this is the first investigation of these parameters in resistance arteries with this model of chronic iron overload.

2. Materials and methods

Male Wistar rats (two months old) were obtained from the Animal Facility of the Federal University of Espirito Santo. During treatment, rats were kept in cages with free access to tap water and standard chow under conditions of controlled temperature and humidity and a 12-h light-dark cycle. The animals were divided into two groups: the control group (vehicle-saline solution i.p.) or the group treated with iron dextran for four weeks (100 mg/kg, 5 days a week). In a previous paper (Marques et al., 2015), this injection regimen promoted chronic iron overload with similar characteristics as that observed in humans; those characteristics are either attributed to heredity or secondary origins (Cook et al., 1992) (Beutler et al., 2003; Crownover and Covey, 2013; Salgia and Brown, 2015; Siddique and Kowdley, 2012). The phenotype included elevation of serum iron > 300 mg/dL and transferrin saturation > 50%; significant hepatic iron deposition; and typically, bronze-coloured skin.

Experiments were conducted in accordance with the American Physiological Society guidelines for animal use, the Guiding Principles in the Use of Animals in Toxicology and with the current Brazilian laws. The experimental protocol was approved by the Institutional Ethics Committee on Animal Use (007/2013 CEUA-UFES).

2.1. Intraventricular pressure

After four weeks, animals were anaesthetised with urethane (1.2 g/kg *i.p.*). The right carotid artery was carefully dissected to avoid damage to any surrounding nerves. A micromanometer (MikroTip™ SPR-320, Millar Instruments, Houston, TX) was inserted into the left ventricle through the carotid artery and left ventricular (LV) systolic and end-diastolic pressure, the rate of change in LV pressure and heart rate were acquired and analysed using a computer (Acknowledge software, Biopac Systems, Santa Barbara, USA).

2.2. Pulse wave velocity

Pulse wave velocity (PWV) was measured as previously described (Ribeiro et al., 2016; Fitch et al., 2001; Marque et al., 2001; Cosson et al., 2007). Under anaesthesia, arterial pulses were simultaneously recorded at the thoracic and abdominal portions of the aorta by inserting two polyethylene catheters through the left common carotid and the left femoral arteries, respectively. For this, each catheter [a PE-10 (24 mm length, 0.28 mm ID, 0.61 mm OD; Clay Adams, Parsippany, NJ) fused to a PE-50 (36 mm length, 0.58 mm ID, 0.96 mm OD; Clay Adams, Parsippany, NJ)] filled with heparinized 0.9% NaCl (50 U/ml) was connected to a low-volume pressure transducer (TSD104A; Biopac Systems, Santa Barbara, USA). PWV (cm/s) was calculated as the distance between the two cannula tips (measured *in situ* following euthanasia) divided by the pulse wave transit time.

2.3. Structural, mechanical and functional properties of the resistance arteries in vitro

2.3.1. Pressure myography

The structural and mechanical properties of the third-order mesenteric resistance arteries (MRAs) were studied with a pressure myograph as previously described (Ribeiro et al., 2010; Souza-Smith et al., 2011; Briones et al., 2006). Rats were euthanized by exsanguination, and MRA segments were dissected and positioned on two glass microcannulae and tied with surgical nylon suture. The artery was mounted in a pressure myograph (Danish Myo Tech, model P110, J. P. Trading, Aarhus, Denmark) and perfused with calcium-free ($0Ca^{2+}$) krebs-henseleit buffer (in mM: 130 NaCl, 14.9 $NaHCO_3$, 3.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$-$7H_2O$, 11 glucose, 10 HEPES and 10 EGTA, pH 7.4) for 30 min. The artery length was adjusted so that the arterial walls were parallel without stretch. Intraluminal pressure was then raised to 140 mmHg, and the artery was unbuckled by adjusting the cannulae. The artery was set to a pressure of 45 mmHg and allowed to equilibrate for 30 min at 37 °C in krebs-henseleit buffer. Intraluminal pressure was then reduced to 10 mmHg, and a pressure-diameter curve was acquired by increasing intraluminal pressure in 20-mmHg steps between 10 and 140 mmHg. Internal and external diameters (D_i and D_e) were acquired under passive conditions ($0Ca^{2+}$-KHS) for 3 min at each intraluminal pressure.

From D_i and D_e, the following structural parameters were calculated: wall thickness = $(D_e - D_i)/2$; cross-sectional area (CSA) = $(\pi/4) \times (D_e^2 - D_i^2)$; and wall/lumen = $(D_e - D_i)/2D_i$. In addition, based on Baumbach and Heistad's method (Baumbach and Heistad, 1989), the following mechanical parameters were calculated: circumferential wall strain (ε) = $(D_i - D_0)/D_0$, where D_0 is the internal diameter at 10 mmHg, and D_i is the observed internal diameter for a given intravascular pressure; and circumferential wall stress (σ) = $(P \times D_i)/(2WT)$, where P is the intraluminal pressure (1 mmHg = 133.4 N/m^2), and WT is wall thickness at each intraluminal pressure. Arterial stiffness independent of geometry was determined by Young's modulus of elasticity (E = stress/strain). Because the stress-strain relationship is non-linear, a tangential or incremental elastic modulus (E_{inc}) was determined from the slope of the stress-strain curve ($E_{inc} = \delta\sigma/\delta\varepsilon$) (Dobrin, 1978). E_{inc} was obtained by fitting each stress-strain data point to an exponential curve using the equation $\sigma = \sigma_{orig}e^{\beta\varepsilon}$, where σ_{orig} is the stress at the original diameter (diameter at 10 mmHg). The derivation of that equation is as follows: $E_{inc} = \beta\sigma$, and thus, if σ is constant, then E_{inc} is directly proportional to β. As a result, an increase in β implies an increase in E_{inc}, which means an increase in stiffness.

2.3.2. Vascular reactivity

Vascular reactivity of the MRAs was analysed in a wire myograph (Model 410A; Danish Myo Tech, Aarhus, Denmark); the isometric tension was measured based on the method described by Mulvany and Halpern (Mulvany and Halpern, 1977). The third-order MRA segments (2 mm in length) were dissected and placed in a small-vessel chamber containing krebs-henseleit buffer (in mM: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$ $7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose, and 0.01 Na_2EDTA) gassed with 95% O_2 and 5% CO_2 (pH = 7.4). The arteries were stretched to their optimal lumen diameter for active tension development based on the internal circumference/wall tension curve. Their internal circumference (L_0) was set to 90% of what the vessels would exhibit if they were exposed to passive tension equivalent to the transmural pressure of 100 mmHg (L_{100}). The internal diameter (I_1) was determined according to the equation $I_1 = L_1/\pi$, using specific software for the normalization of resistance arteries (DMT Normalization Module; ADInstruments, Castle Hill, Australia). All data were acquired by hardware connected to a computer (Powerlab/800 ADInstruments, Castle Hill, Australia) and analysed by software (Labchart 8 ADInstruments, Castle Hill, Australia). After a 45-min equilibration period, MRAs were exposed to 120 mM KCl to confirm smooth muscle integrity. The presence of endothelium was estimated by the ability of