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Arterial stiffness is associated with adipokine dysregulation in non-hypertensive obese mice

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ABSTRACT

The aim of this study was to characterize alterations in vascular structure and mechanics in murine mesenteric arteries from obese non-hypertensive mice, as well as their relationship with adipokines. Four-week old C57BL/6J male mice were assigned either to a control (C, 10% kcal from fat) or a high-fat diet (HFD, 45% kcal from fat) for 32 weeks. HFD animals weighed 30% more than controls ($p < 0.001$), exhibited similar blood pressure, increased leptin, insulin and superoxide anion ($O_2^{\bullet-}$) levels, and reduced adiponectin levels and nitric oxide (NO) bioavailability. Arterial structure showed an outward remodeling with an increase in total number of both adventitial and smooth muscle cells in HFD. Moreover, HFD mice exhibited an increased arterial stiffness assessed by β-values ($C = 2.4 \pm 0.5$ vs $HFD = 5.3 \pm 0.8$; $p < 0.05$) and aortic pulse wave velocity (PWV, $C = 3.4 \pm 0.1$ vs $HFD = 3.9 \pm 0.1$; $p < 0.05$). β-Values and PWV positively correlated with leptin, insulin or $O_2^{\bullet-}$ levels, whereas they negatively correlated with adiponectin levels and NO bioavailability ($p < 0.01$). A reduction in fenestrae number together with an increase in type-I collagen amount ($p < 0.05$) were observed in HFD. These data demonstrate that HFD accounts for the development of vascular remodeling and arterial stiffness associated with adipokine dysregulation and oxidative stress, independently of hypertension development.

1. Introduction

Obesity is associated with progressive vascular dysfunction leading to elevated morbidity and mortality due to early cardiovascular events [1]. Mechanisms of vascular dysfunction include vascular remodeling and arterial stiffness, both actively contributing to the development of cardiovascular disease [2–4].

Obesity has emerged as a potential risk factor for arterial remodeling in both humans and rats [2–5]. In this direction, severe human obesity has been associated with profound structural alterations of subcutaneous small resistance arteries [6]. Likewise, studies performed in obese Sprague Dawley (SD) [7], obese Zucker [8] and diabetic rats [3] show vascular remodeling of middle cerebral and/or mesenteric arteries. Nevertheless, in most cases, the concomitant presence of diabetes and hypertension [9], both linked to vascular remodeling, makes difficult to discriminate the role of obesity per se in the development of the observed structural abnormalities.

Chronic alterations in vascular structure may lead to significant changes in mechanical properties, such as compliance and distensibility [3], thus accounting for arterial stiffness, an independent risk factor for cardiovascular disease [10]. Obesity is associated with an increase in aortic pulse wave velocity (PWV) and/or intrinsic stiffness (assessed by the stress–strain relationship) in human subcutaneous small resistance arteries [6,11–13], as well as in aorta of high-fat/high-sucrose SD [7] or in genetic models of obesity, i.e., *ob*/*ob* mice [14] and insulin-resistant Zucker fa/fa rats [8]. Recent evidence suggests that arterial stiffness associated to obesity might appear in the absence or prior to the development of hypertension in patients with metabolic syndrome [13]. In obese children, arterial stiffness seems to be influenced by body mass index and pulse pressure independently of systolic and diastolic blood pressure values [15]. Conversely, weight loss in overweight and obese individuals is associated with a reduction in arterial stiffness [7].

Abbreviations: AIx, augmentation index; C, control; CSA, cross-sectional area; DAF-2DA, 4,5-diaminofluorescein diacetate; DHE, dihidroethidium; DIO, diet-induced obesity; EEL, external elastic laminae; eNOS, endothelial nitric oxide synthase; HFD, high-fat diet; IEL, internal elastic laminae; KH, Krebs–Henseleit solution; MA, mesenteric artery; NO, nitric oxide; NOX, NADPH oxidase activity; $O_2^{\bullet-}$, superoxide anion; PFA, paraformaldehyde; PWV, pulse wave velocity; SMC, smooth muscle cells

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In this context, some studies suggest a possible link between adipokine levels and the development of arterial stiffness in patients with abdominal obesity [16] or increased adiposity [17].

Passive arterial mechanical properties are mainly conferred by collagen, elastin content and elastin organization [18,19]. Enhanced vascular stiffness of resistance arteries has been attributed to increases in collagen content [20,21], non-fibrous extracellular matrix proteins, and adhesion molecules [for review, see Ref. [22]], as well as to alterations in elastic fiber organization in the internal elastic lamina [23]. According to this, several studies have shown a link between arterial stiffness and abnormal increase in the collagen/elastin ratio in hypertension [18,24–26]. However, very few studies [11,12] have been performed in the context of obesity, and the role of obesity per se in the development of mechanical abnormalities remains to be elucidated.

In this context, the aim of this study was to prove the hypothesis of a direct link between obesity-derived adipokine dysregulation [27,28], vascular remodeling and arterial stiffness in obesity, without the influence of hypertension as confounding factor. Therefore, we sought to characterize structural and mechanical changes in a mouse model of long-term diet-induced obesity (DIO), which exhibits endothelial dysfunction together with an increase of oxidative stress, but does not develop hypertension [29]. We have analyzed in mesenteric arteries: i) vascular structure, ii) mechanical properties, iii) elastin content and organization, iv) types I and III collagen contents, iv) the correlation between adipokine dysregulation and arterial stiffness and v) the correlation between oxidative stress and arterial stiffness.

2. Material and methods

2.1. Animals and dietary treatment

Four-week old male C57BL/6J mice (Harlan, Spain) weighing 16–18 g were housed under controlled light (12-hour light/dark cycles from 8:00 am to 8:00 pm) and temperature (22–24 °C) conditions with standard food and water ad libitum. After one week, animals with similar average body weight, were divided into two groups and housed 8–10 per cage and assigned either to a control (C) or to a high-fat diet (HFD). Control (D12450B, 10 kcal% fat, 70 kcal% carbohydrates and 20 kcal% protein; 3.85 kcal/g) and high-fat (D12451, 45 kcal% fat, 35 kcal% carbohydrates, 20 kcal% protein; 4.73 kcal/g) diets were supplied by Test Diet Limited BCM IPS Ltd. (London, UK). HFD and their respective control mice had free access to food during 32 ± 1 weeks. The investigation conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and it was approved by the ethics committee of the University CEU-San Pablo (SAF 2009-09714, SAF2011-25303).

2.2. Pulse wave velocity and arterial wave reflection index determination

On the last day, both carotid and femoral arteries were catheterized under anesthesia (80 $mg \cdot kg^{-1}$ ketamine hydrochloride and 12 $mg \cdot kg^{-1}$ xylazine hydrochloride, ip) and blood pressure waves were recorded in a PowerLab system (ADInstruments). Pulse wave velocity (PWV) represents the pressure waveform that travels along the aorta and large arteries during each cardiac cycle and it was calculated with the following formulae: D (meters) / Δt (seconds), where the time delay (Δt) was measured by using the two pressure waves (carotid and femoral) and D was the distance between the two arteries. Arterial wave reflection was determined by using arterial pressure waveforms from the right carotid artery and the augmentation index (AIx, magnitude of wave reflection) was calculated as previously described [30]. After pulse wave determination, anesthetized animals were euthanized by decapitation. The mesenteric bed was immediately dissected, blood was collected in chilled EDTA-coated polypropylene tubes and plasma samples were frozen at −80 °C for further analysis.

2.3. Plasma measurements

Plasma leptin and adiponectin concentrations were analyzed by specific RIA for murine leptin (Linco Research) and adiponectin (Linco Research). Insulin was determined by means of a specific EIA kit for mouse insulin (Mercodia).

2.4. Structural and mechanical properties in mesenteric arteries

Mesenteric bed was removed and placed in Krebs–Henseleit solution (KH, in mM: 115 NaCl, 4.6 KCl, 2.5 $CaCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 0.01 EDTA and 11.1 glucose). A first-order branch of mesenteric artery (MA) was isolated from the mesenteric bed and was carefully cleaned of surrounding adipose tissue under a dissecting microscope. MAs structural and mechanical properties were studied with a pressure myograph (Model P100, Danish Myo-Tech), as previously described [23,31]. Briefly, vessels were placed on two glass cannulas, secured with surgical nylon suture and vessel length was adjusted so that the vessel walls were parallel without stretch. To equilibrate MA segments, intraluminal pressure was set at 70 mm Hg for 60 min at 37 °C in calcium-free KH ($0Ca^{2+}$; omitting calcium and adding 10 mM EGTA), bubbled with carbogen (95% O_2/5% CO_2). Thereafter, intraluminal pressure was increased at 20 mm Hg intervals (3, 20, 40, 60, 80, 100, 120, and 140 mm Hg), and external and internal diameters (D_{i0Ca}, D_{e0Ca}) were recorded at each pressure level with a video camera coupled to Myoview software. After maximal relaxation in $0Ca^{2+}$, MA segments were pressure-fixed at 70 mm Hg with 4% paraformaldehyde (PFA, in 0.2 mol/l phosphate buffer, pH 7.2–7.4) at 37 °C for 45 min and stored at 4 °C for confocal microscopy studies.

From the D_{e0Ca} and D_{i0Ca} values we calculated structural [wall thickness, cross-sectional area (CSA), and wall-to lumen ratio] and mechanical parameters [incremental distensibility, circumferential wall strain, circumferential wall stress, and β-values obtained from stress–strain relationship] as described [23].

2.5. Confocal microscopy

2.5.1. Confocal microscopy study of nuclei distribution

Pressure-fixed intact MA arteries were stained with the nuclear dye DAPI (1:500, Molecular Probes) for 15 min at room temperature (RT) in the darkness. After washing, the arteries were mounted on a slide provided with a small well of spacers to avoid artery deformation, filled with Citifluor (glycerol-antifade agent; Sigma Aldrich) mounting medium, and visualized with a Leica TCS SP5 confocal system (Leica Microsystems) and the cell nuclei in the adventitia, media, and endothelium were visualized at excitation 405 nm/emission 410–475 nm. For each artery, a single image was captured in the midpoint of the artery with a ×20 objective. In addition, three randomly selected regions were visualized with a ×63 objective zoom 4. In each of the regions, stacks of 1-μm-thick serial optical sections were taken from the first visible adventitial cell nuclei to the first visible endothelial cell nuclei. An additional group of images focusing of the endothelial monolayer were captured along the entire segment length. Metamorph images analysis software (Universal Imaging) was used for quantification. To allow comparison of C and HFD animals, the following calculations were performed on the basic of 1-mm-long segments: artery volume (in mm^3) (volume = wall CSA (mm^2) × 1 mm); total number of adventitial and smooth muscle cells (cell n = n of nuclei per stack × n of stack per artery volume). Endothelial cells were quantified in several single images obtained along the arterial length and calculated per area, since endothelium is a monolayer. Data are expressed as endothelial cell number per luminal surface area of each vessel, which was calculated from the internal diameter measured from images captured with the ×20 objective. CSA (μm^2) was calculated on the basis of the wall and lumen measurements.

2.5.2. Confocal microscopy study of elastin content and organization

The content and organization of elastin in the external (EEL) and internal elastic lamina (IEL) was assessed in intact pressure-fixed MA by fluorescent laser scanning confocal microscopy (Leica TCS SP5) as previously described [23,32]. Arterial segments were mounted as described above and they were visualized at a wavelength of the 488/515 nm. Serial optical sections (stacks of images) from the adventitia to the lumen (z step = 0.5 μm) and the assessment of fenestrated elastic lamella were captured with a ×63 objective, zoom 4. Three randomly selected regions were studied for quantitative analysis. A minimum of two stacks of images of different regions were captured in each arterial segment. All the images were taken under identical conditions of laser intensity, brightness and contrast.

Quantitative analysis was performed with Metamorph images analysis software as described [23]. Briefly, from each stack of serial images, individual projections of IEL were reconstructed and total fenestra number and fenestra area were measured. In addition, elastin density was estimated from fluorescence intensity values [33]. The EEL was also reconstructed and the content of elastin fibers in the image was also quantified (% of fibers versus background).

2.5.3. Confocal microscopy study of determination of collagen fiber content

Types I and III collagen contents were assessed in MA fixed in PFA 4% by immunofluorescence. Briefly, arteries were incubated with anti-type I or type III collagen antibodies (1:200, Abcam) for 60 min at RT. Segments were washed and incubated with Alexa Fluor 647® anti-rabbit IgG (1:200 dilution, 1 h, RT, Molecular Probes). Finally, nuclei were stained with DAPI. After rinsing, MA segments were mounted as described above and visualized with a confocal microscope (SP5 Leica Microsystems) by using ×40 objective zoom 2. A minimum of three regions, were randomly selected. To avoid biased selection, the regions were chosen in the DAPI wavelength, as described above. Once selected the images were acquired at identical conditions of brightness, contrast, and laser power at 633 nm excitation/−640–650 nm emission wavelength (secondary antibody-Alexa 647) to detect collagen, either type I or type III. Quantitative analysis of collagen content in vascular wall was performed with Metamorph images analysis software as follows. An extended focus image was reconstructed from the serial images. Thereafter, total and background fluorescence intensity values were measured in the reconstructed image. Collagen content was then estimated by subtracting the background from the total intensity fluorescence values.

2.5.4. Confocal microscopy study of determination of superoxide anion ($O_2^{\bullet-}$) availability and nitric oxide (NO) bioavailability

Basal $O_2^{\bullet-}$ availability and NO bioavailability were determined with dihidroethidium (DHE, 3 μM) and 4,5-diaminofluorescein diacetate (DAF-2DA, 10 μM), respectively in MA segments fixed in PFA 4%. Both $O_2^{\bullet-}$ and NO levels in MA were determined by quantification DHE and DAF-2DA fluorescence intensity, respectively in MA as previously described [29,34].

2.6. Statistical analyses

Results are expressed as mean ± SEM and n denotes the number of animals used in each experiment. Statistical analyses were performed with Stat View software (SAS Institute, EEUU). One-way ANOVA followed by Newman–Keuls post hoc test were used as appropriate. Differences were considered statistically significant at $p < 0.05$.

2.7. Chemical compounds

DHE and DAF-2DA were obtained from Sigma Aldrich, DHE was dissolved in dimethylsulfoxide (DMSO) and kept in dark conditions under argon.

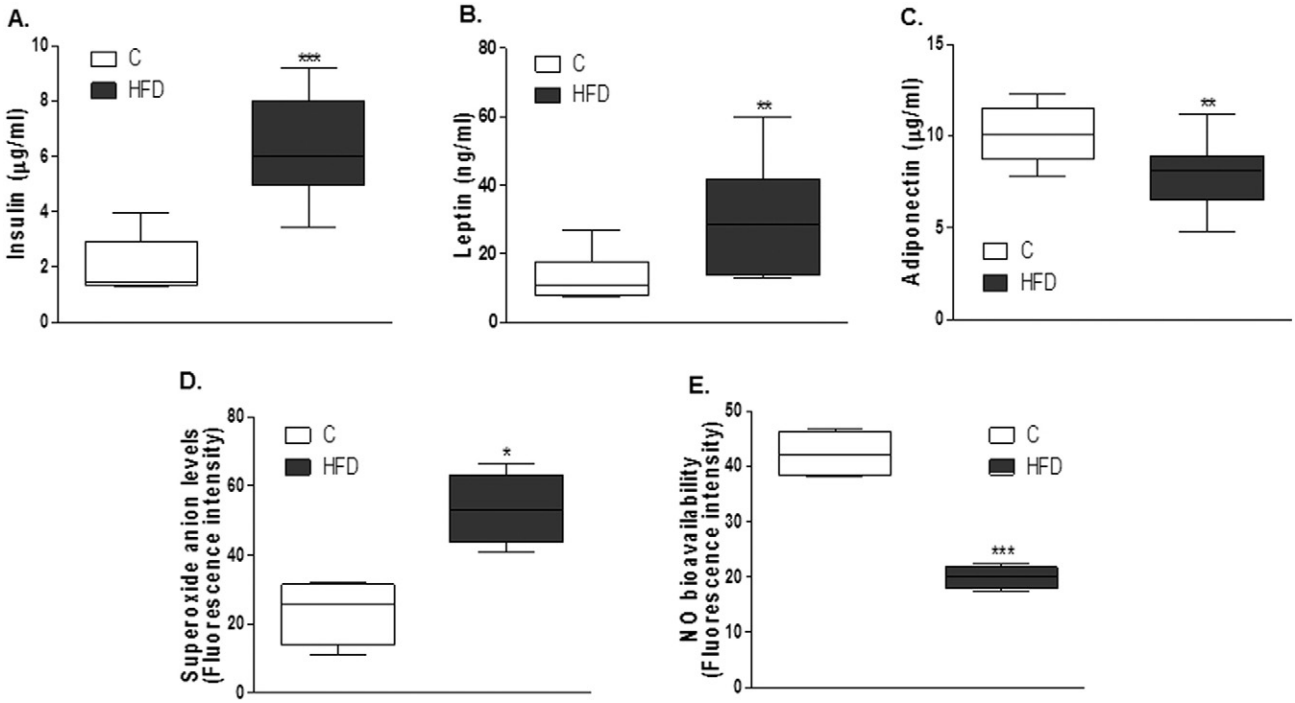

Fig. 1. Effect of HFD on plasmatic parameters. Plasmatic (A) insulin, (B) leptin and (C) adiponectin levels and (D) superoxide anion ($O_2^{\bullet-}$) levels and (E) NO bioavailability in mesenteric artery (MA) segments of C and HFD animals. $O_2^{\bullet-}$ levels were determined by quantification DHE fluorescence intensity and NO bioavailability was determined by quantification DAF-2DA fluorescence intensity. Data are expressed as mean ± S.E.M. of seven determination by group. Statistical analysis was performed by 1-way ANOVA. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, HFD compared with C group. Newman–Keuls test.

3. Results

3.1. Physiological variables and plasma parameters after 32-week HFD

As previously shown [29,35], HFD animals exhibited an increase in body and adipose tissue weight as well as in glucose levels, together with an impairment of insulin sensitivity (results not shown). No changes were detected in both systolic (C = 85.7 ± 6.3 mm Hg, HFD = 90.1 ± 5.5 mm Hg; p = 0.639) or diastolic blood pressure levels (C = 63.8 ± 5.1 mm Hg, HFD = 60.6 ± 2.4 mm Hg; p = 0.644).

32-wk of HFD induced a 3-fold increase in plasma insulin (Fig. 1A; $p < 0.001$) and a 2-fold increase in plasma leptin concentrations (Fig. 1B; $p < 0.01$). However, plasma adiponectin levels were significantly lower (Fig. 1C; $p < 0.01$) in HFD compared to controls. In MA from HFD, basal $O_2^{\cdot-}$ levels (Fig. 1D; $p < 0.05$) were significantly higher, whereas endothelial NO availability (Fig. 1E; $p < 0.001$) was significantly lower.

3.2. 32-week HFD induced a hypertrophic outward remodeling in mesenteric arteries

Fig. 2 shows structural parameters of MA from HFD and control animals mounted on a pressure myograph and measured under fully relaxed conditions. Internal and external MA diameters were significantly higher after 32-wk of HFD compared with control diet at all intraluminal pressure levels (3–140 mm Hg) tested (Fig. 2A and B; $p < 0.05$). As a result, wall-to-lumen ratio was significantly decreased in HFD compared to control animals (Fig. 2C; $p < 0.05$). Wall thickness was similar in both groups (Fig. 2D).

Confocal microscopy in pressure-fixed segments at 70 mm Hg allowed confirming that adventitial and medial layer thickness was not different between control and HFD animals (data not shown). Total wall CSA was significantly higher after 32-wk of HFD compared to control diet (1-ANOVA, $F_{(1,15)} = 7.318$, $p < 0.05$; Fig. 3A). These differences were due to a significant increase in both the adventitial CSA (1-ANOVA, $F_{(1,16)} = 4.457$; $p < 0.05$) and the medial CSA (1-ANOVA, $F_{(1,14)} = 5.216$; $p < 0.05$). Total numbers of adventitial and smooth muscle cells (SMC) were significantly increased in HFD compared with the control group (Fig. 3B; $p < 0.05$). As result, cell density was not different between experimental groups in any layer, although SMC density showed a tendency to be reduced in HFD animals (Fig. 3C; $p = 0.081$). Endothelial cell density was higher in HFD group, but it did not reach statistical significance (Fig. 3D; $p = 0.075$), probably due to the fact that luminal surface area was also significantly increased in HFD animals (C = 0.27 ± 0.04; HFD = 0.46 ± 0.06; $p < 0.05$). These data evidenced a hypertrophic outward remodeling after 32-wk of HFD.

3.3. 32-week HFD induced an increase in arterial stiffness in mesenteric arteries

Incremental distensibility was significantly lower at low pressure (20 and 60 mm Hg) in HFD compared with control animals (Fig. 4A). Media stress (Fig. 4B) was similar in MA from both groups. However, media strain (Fig. 4C) was significantly smaller in MA from HFD animals than controls. In addition, MA from HFD animals also exhibited a decreased elasticity as shown by the leftward shift of the stress–strain relationship and the significantly larger β-value compared with the control diet (1-ANOVA, $F_{(1,7)} = 8.848$, $p < 0.05$; Fig. 4D).

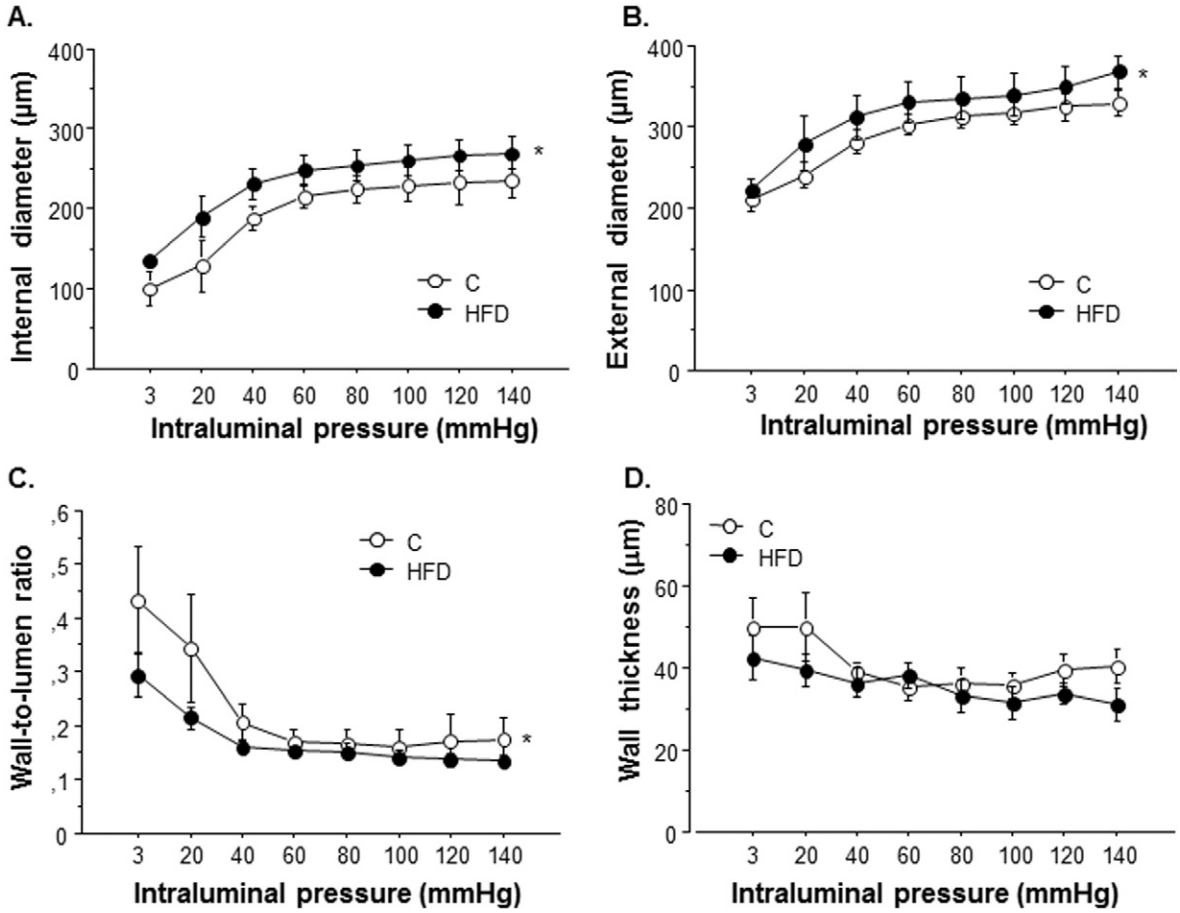

Fig. 2. Effect of HFD on structural parameters. (A) Internal diameter, (B) external diameter, (C) wall-to-lumen ratio and (D) wall thickness–pressure curves in fully relaxed MA segments of C and HFD animals, determined with pressure myography. Data are expressed as mean ± S.E.M. ($n \geq 5$). Statistical analysis was performed by 1-way ANOVA. *$p < 0.05$, HFD compared with C group. Newman–Keuls test.

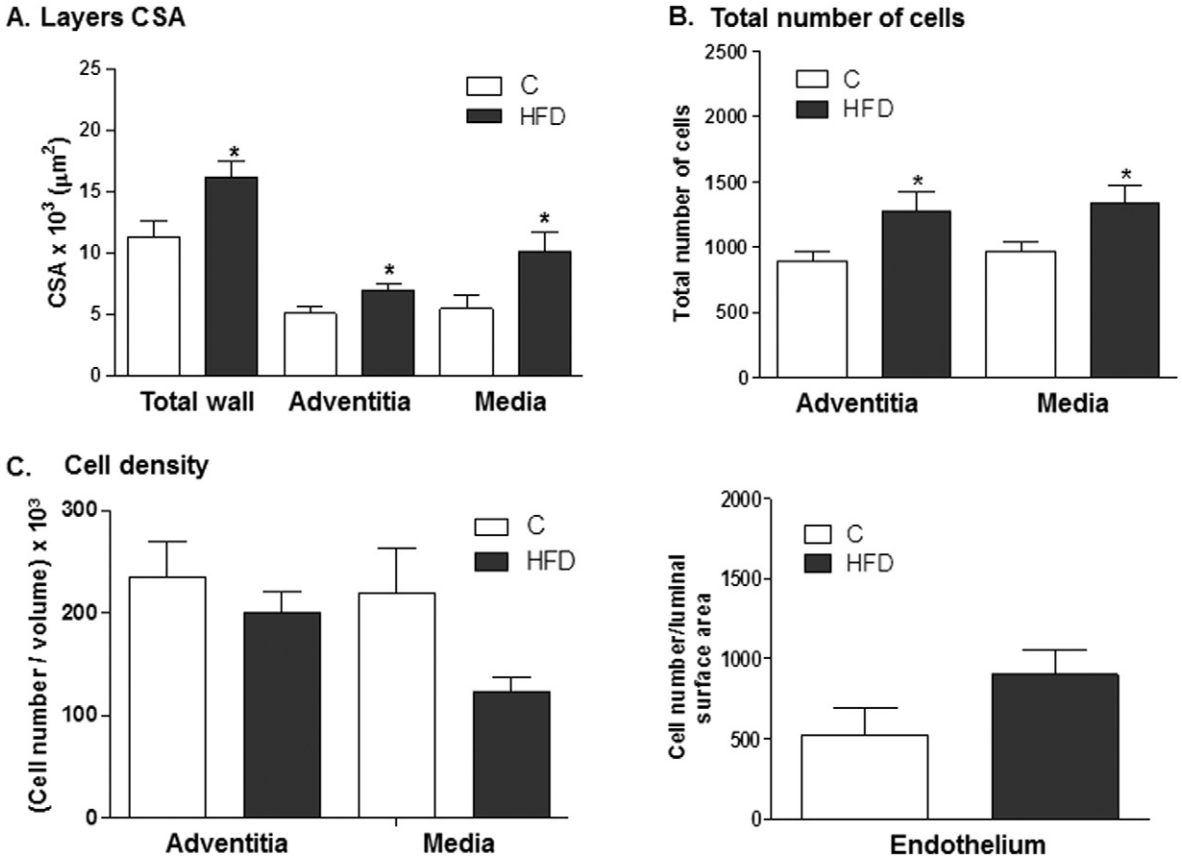

Fig. 3. Effect of HFD on wall composition. Diagram bars show (A) layers cross-sectional area (CSA), (B) total number of cells and (C) cell density in pressure-fixed MA segments of C and HFD animals at 70 mm Hg and visualized intact with a laser-scanning confocal microscope (excitation 405 nm/emission 410–475 nm). Quantification was obtained with Metamorph analysis software. All calculations were performed on the basis of 1-mm-length segment. Data are expressed as mean ± S.E.M. ($n \geq 5$). Statistical analysis was performed by 1-way ANOVA. *$p < 0.05$, HFD compared with C group. Newman–Keuls test.

A positive correlation was found between stiffness index β and plasma levels of insulin (Fig. 5A) or leptin (Fig. 5B). A negative correlation was found between MA β-value and plasma adiponectin levels (Fig. 5C). In addition, stiffness index β positively correlated with $O_2^{\bullet-}$ levels in MA (Fig. 5D), but it negatively correlated with arterial NO bioavailability (Fig. 5E).

PWV, an index of aortic stiffness, was significantly higher after 32-wk of HFD compared with the control diet (C = 3.4 ± 0.1 m/s vs HFD = 3.9 ± 0.2 m/s; 1-ANOVA, $F_{(1,7)} = 6.354$; $p < 0.05$). Similarly, data obtained from carotid blood pressure waveform revealed a significant increase in AIx in HFD compared with control animals (C = −12.88 ± 4.5% vs HFD = 13.6 ± 0.3%; $p < 0.05$; Supplementary Fig. 1A). Interestingly, a negative correlation was observed between AIx and both NO bioavailability and p-eNOS/e-NOS expression in MA (Supplementary Fig. 1B and C). We also found a positive correlation between PWV and insulin (Supplementary Fig. 2A) or leptin plasma levels (Supplementary Fig. 2B), as well as with arterial $O_2^{\bullet-}$ levels (Supplementary Fig. 2D). By the contrary, plasma adiponectin concentrations (Supplementary Fig. 2C) and NO bioavailability in MA (Supplementary Fig. 2E) negatively correlated with PWV.

3.4. 32-weeks HFD-induced alterations in elastin organization and collagen content

In order to assess the role of collagen and elastin in arterial elasticity changes, they were analyzed in MA by confocal microscopy. Elastin organization in the IEL was altered in MA from HFD animals (Fig. 6A) that showed a significant reduction ($p < 0.05$) in total number of fenestrae than the control group (Fig. 6C) without changes in fenestrae area (Fig. 6B). No differences were found neither in elastin content in the EEL (C = 65.6 ± 5.1% vs HFD = 63.06 ± 3.4%; $p = 0.68$) nor in elastin density in the IEL, estimated from autofluorescence values (C = 68.0 ± 10.4 vs HFD = 74.8 ± 9.1; $p = 0.64$).

HFD significantly increased type I collagen content in the arterial wall ($p < 0.05$, Fig. 7A and B). However, HFD did not affect type III collagen content (Fig. 7C and D). Altogether, these data demonstrate that reduced elasticity in MA from HFD animals results from both an increased type I collagen content and alterations in IEL organization.

Simple regression analysis revealed a positive correlation between type I collagen content and plasma insulin ($r = 0.676$; $p < 0.01$) and leptin ($r = 0.559$; $p < 0.05$) concentrations and aortic PWV ($r = 0.786$; $p < 0.01$) and a negative correlation with plasma adiponectin levels ($r = 0.57$; $p < 0.05$).

4. Discussion

The present study provides first evidence for the development of arterial stiffness by long-term high-fat feeding per se independently of hypertension. The observed increase in PWV is likely due to an outward remodeling together with an increase in total number of adventitial and SMC. Reduced intrinsic vascular elastic properties go along with an increase in collagen type I and an alteration in elastin organization. We propose a key contribution of adipokine dysregulation and oxidative stress in this process, since vascular stiffness correlates with altered adipokine levels and superoxide ($O_2^{\bullet-}$) anion levels.

We observe a hypertrophic outward remodeling in MA from animals fed a long-term high-fat diet. Similarly, MA from Zucker rats, a model of obesity associated to type 2 diabetes, show a hypertrophic outward remodeling [2,36]. However, these rats show elevated blood pressure, which is a strong stimulus for vascular remodeling. In contrast, our

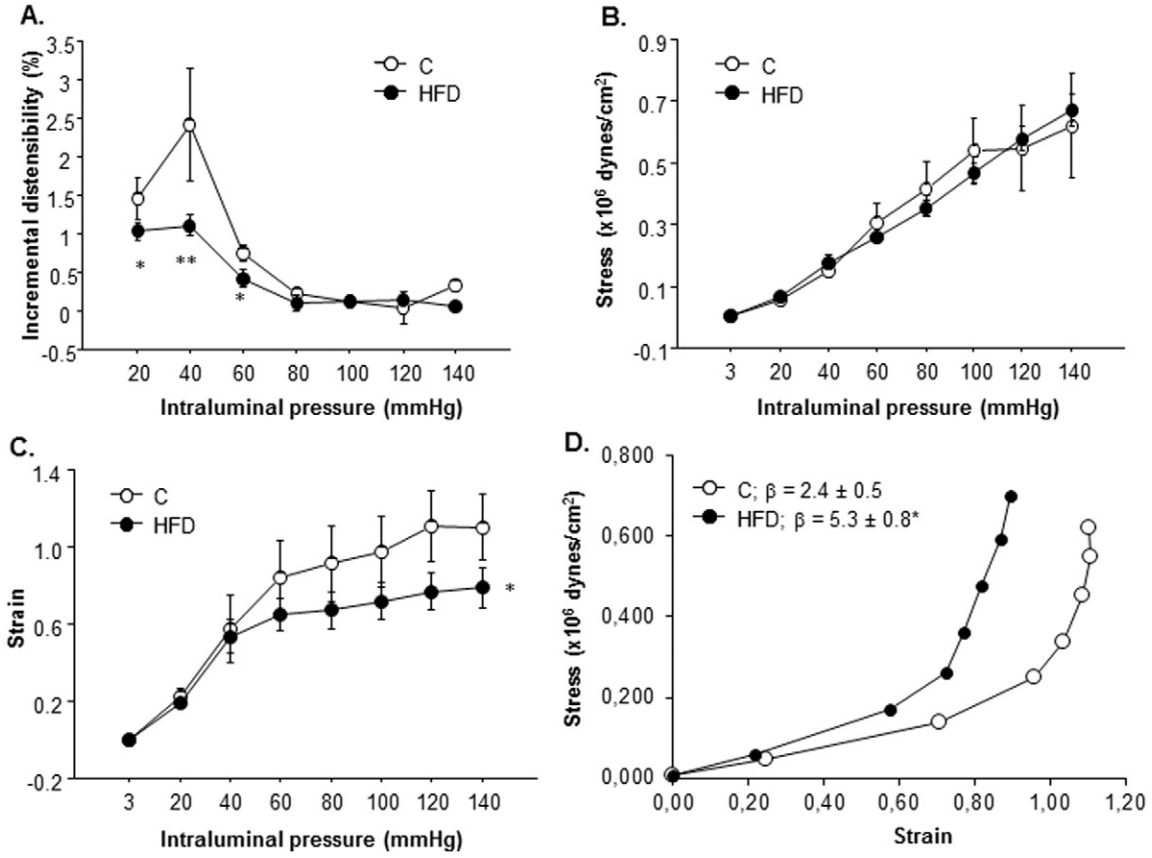

Fig. 4. Effect of HFD on mechanical parameters. (A) Incremental distensibility–pressure curve, (B) stress and (C) strain–pressure curves and (D) stress–strain relationships with β-values obtained from fully relaxed MA segments of C and HFD animals, calculated from pressure myography data. Data are expressed as mean ± S.E.M. ($n \geq 5$). Statistical analysis was performed by 1-way ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$, HFD compared with C group. Newman–Keuls test.

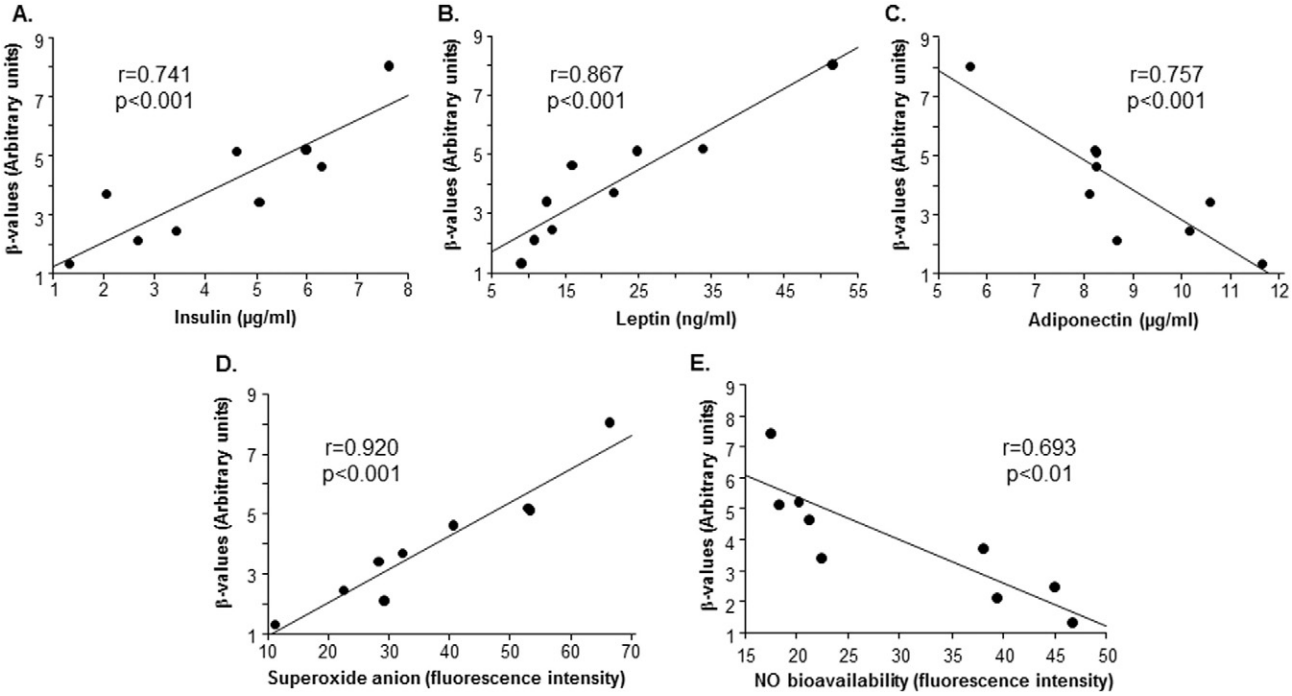

Fig. 5. Association between adipokines and plasmatic parameters, superoxide anion ($O_2^{\bullet-}$) levels and NO bioavailability in mesenteric arteries with arterial stiffness (β-values). Correlation between β-values and plasmatic insulin (A), leptin (B) and (C) adiponectin concentrations, superoxide anion ($O_2^{\bullet-}$) levels (D) and NO bioavailability (E) in MA segments of C and HFD animals.

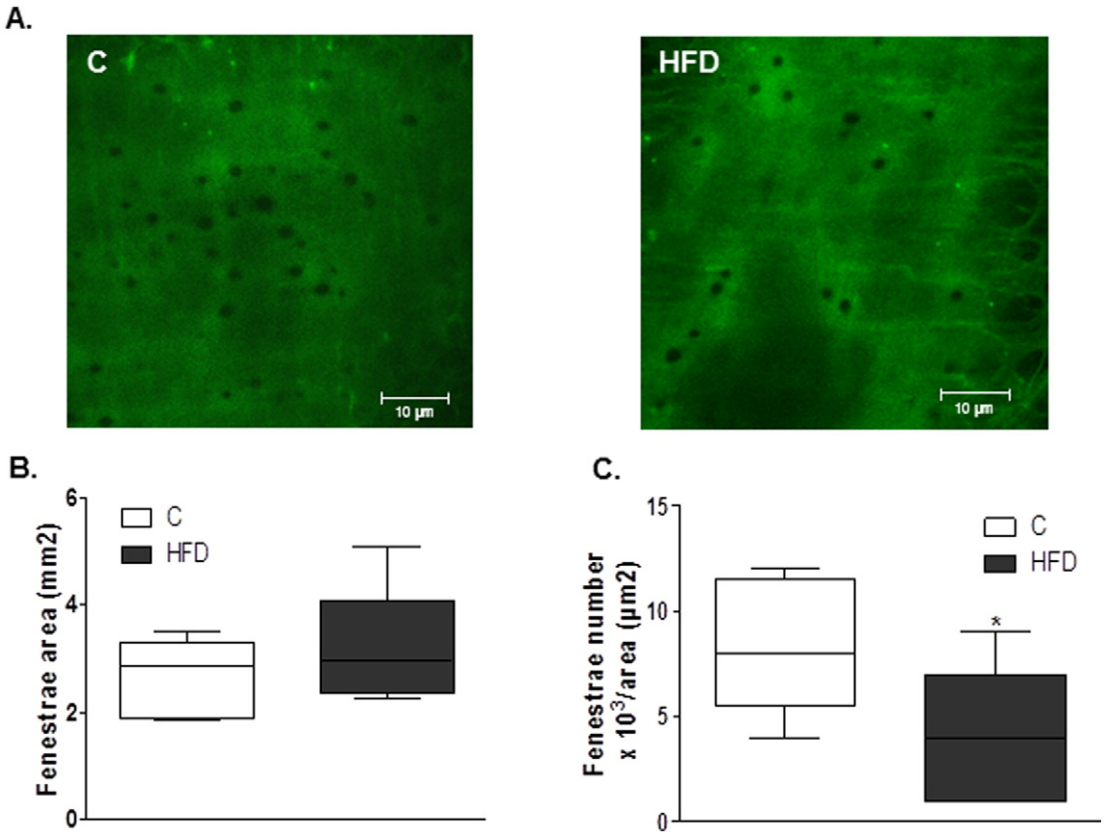

Fig. 6. Effect of HFD on elastin organization in the internal elastic lamina (IEL) in mesenteric arteries. (A) Representative confocal projections of the IEL of MA segments from C and HFD animals. Arteries were pressure-fixed at 70 mm Hg and mounted intact on a slide. Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×63 oil immersion objective, zoom ×4, scale bar = 10 μm). Diagram bars show quantification of (B) fenestrae area and (C) fenestrae number/area by Metamorph analysis software. Results are expressed as mean ± SEM of n ≥ 5. *p < 0.05, HFD compared with C group. Newman–Keuls test.

DIO model does not exhibit hypertension [29] but is characterized by a progressive development of insulin resistance during the onset of obesity [35]. In accordance, hypertrophic outward remodeling has been found in type 2 diabetic *db*/*db* mice [37] and in obese humans [6] in the absence of hypertension. Therefore, we might exclude elevated blood pressure as the initiating stimulus for remodeling.

One plausible candidate implicated in the development of obesity-derived hypertrophic outward remodeling seems to be oxidative stress. Indeed, excessive reactive oxygen species production in MA from Zucker rats might affect the remodeling process by reducing nitric oxide (NO) availability, thus accounting for peroxynitrite ($ONOO^-$) production and matrix metalloproteinase activation, both essential in the remodeling process [38–40]. Interestingly, our HFD model also exhibits a significant increase in superoxide ($O_2^{\bullet-}$) anion production together with a reduced NO bioavailability, thus leading to endothelial dysfunction [29]. Hypertrophic outward remodeling can be also attributed to chronic flow alterations, which is another stimulus able to modify vascular structural and mechanical properties [3]. This hypothesis is supported by the fact that vascular disorders associated with metabolic syndrome involve alterations in local flow supply. In this context, genetic models of obesity show a chronic elevation of blood flow in mesenteric resistance arteries [41,42], which results in a higher lumen diameter and outward remodeling [43]. In addition, chronic increases in blood flow are often accompanied by wall hypertrophy to normalize circumferential wall stress that increases during vessel expansion [43]. This is likely what occurs in our HFD mice, where circumferential stress was not different between control and obese mice.

Obesity is also associated with arterial stiffness in patients determined by PWV representing an integrated index of aortic stiffness [11,12,44,45]. Similarly, an increased arterial stiffness has been described in conduit arteries from *ob*/*ob* mice [14], Zucker fa/fa rats [8] and humans with insulin resistance [46]. In accordance, MA from our HFD animals exhibit i) higher values of both aortic PWV and AIx and ii) a significant increase in the β-values indicative of elevated intrinsic stiffness of the wall material independently of the geometry. Moreover, our results are in the same line as the results obtained by Weisbrod et al. in a model of high-fat/high-sucrose feeding, in which arterial stiffness in conduit arteries precedes the onset of hypertension [7].

Since elastin contributes to arterial biomechanical properties at low pressures [23], both reduced distensibility and the leftward shift of the stress–strain relationship at the low pressure range, suggest alterations in this protein in MA from HFD. We did not find modifications in elastin content, but a change in elastin organization, characterized by a reduction in fenestrae number in the IEL. We have previously demonstrated that a reduction in the proportion of fenestrae versus elastin, either due to smaller fenestrae or to reduced total number, affects vascular mechanical properties, thus making the vessel stiffer [19,23,32]. Moreover, IEL organization is more relevant than elastin content as contributor to arterial stiffening [23]. These results evidence the role of elastin to the observed mechanical alterations in HFD.

Moreover, alterations in collagen turnover that favor type I collagen synthesis are also related to a decreased aortic elasticity [47,48]. Fibrillar type I/III collagens are the most abundant in vascular walls [49] and they play an important contribution to the rigidity of the arterial wall of resistance arteries in hypertension [6,9,31,50]. In this study, MA from HFD show augmented content of type I collagen, which comprises 60% of the vascular wall collagens [51], without changes in type III collagen. Similar findings have been described in conduit arteries from a Wistar rat DIO model [52].

Insulin resistance has been proposed as a link between obesity and vascular stiffness. In this context, studies in humans show a strong correlation between visceral adipose tissue and stiffness index β,

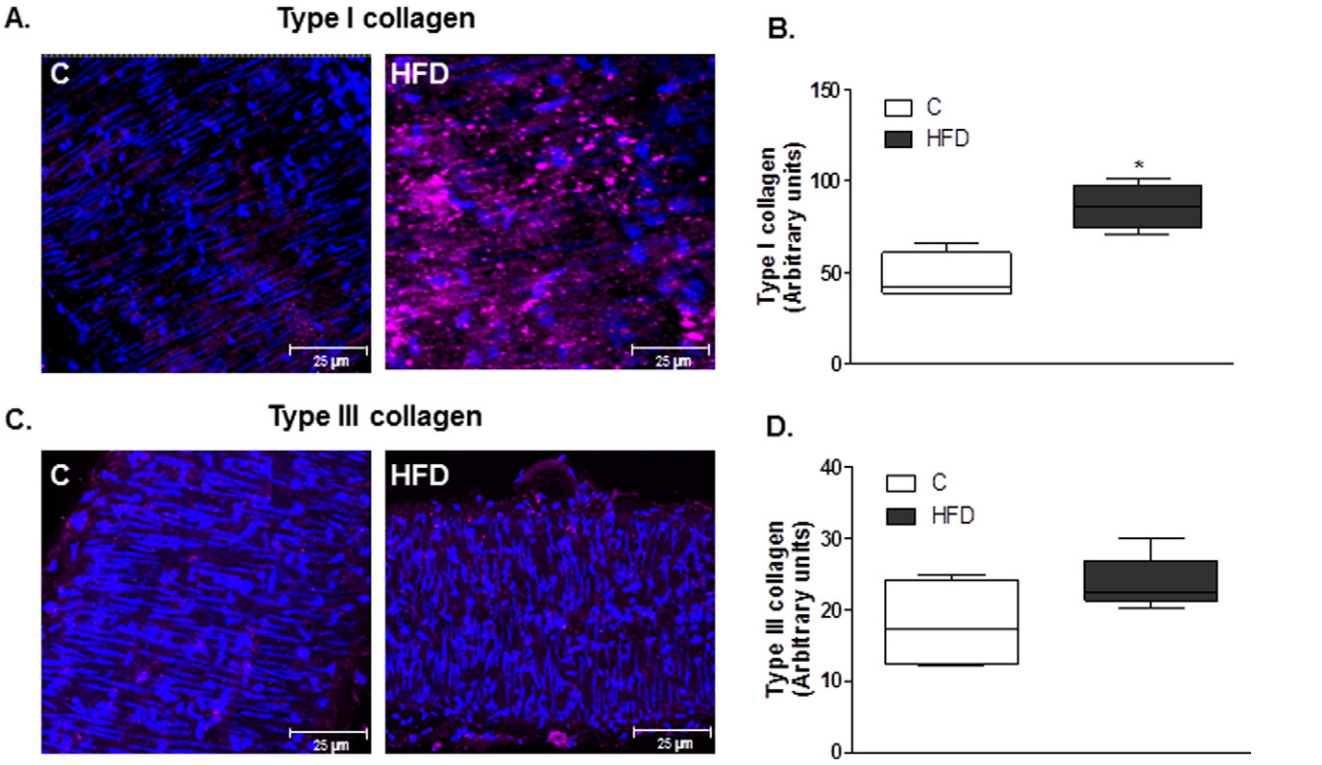

Fig. 7. Effect of HFD on types I and III collagen contents in mesenteric arteries. Laser confocal microscopic images of type I collagen (A) and type III collagen (C) in MA segments from C and HFD animals. Vessels were labeled with DAPI for nuclei (blue), anti-collagen I/III for types I and III collagens (pink). Projections were obtained from serial optical sections captured with a fluorescence confocal microscope (×40 objective, zoom ×4, scale bar = 25 μm). Diagram bars show quantification of type I collagen (B) and type III collagen (D) by Metamorph analysis software. Results are expressed as mean ± SEM of n ≥ 5. *p < 0.05, HFD compared with C group. Newman–Keuls test.

suggesting that aortic stiffness might be mediated through elements of the insulin resistance syndrome [53]. In addition, elevated circulating insulin levels stimulate proliferation and growth of SMC and the increase collagen formation [54,55]. According to this, we show an increase in adventitial and SMC number, as well as a strong correlation between insulin levels and stiffness index β in HFD animals. Therefore, our data support the theory of insulin resistance as one possible connexion between obesity and arterial stiffness.

A further question raised by our data concerns the eventual influence of hyperleptinemia and/or hypoadiponectinemia on mechanical alterations. We show a positive correlation between plasma leptin and stiffness index β or PWV, as well as collagen type I in HFD. These results are in agreement with the association between leptin levels and impaired arterial distensibility in humans [56]. A possible underlying mechanism for this association might be a leptin-induced increase of collagen type I and $O_2^{\bullet-}$ levels in vivo. Results in cultured VSMC suggest that leptin could participate in vascular remodeling and stiffness through the activation of oxidative stress-PI3K/Akt pathway and the production of the profibrotic factors TGF-β and CTGF [52]. Accordingly, the correlation between both stiffness index β or PWV and $O_2^{\bullet-}$ levels (Fig. 5 and Supplemental Fig. 2), as well as with NOX (Supplemental Fig. 3) supports the role for oxidative stress in these alterations.

Hypoadiponectinemia has been associated with a decreased arterial elasticity in both hypertensive and diabetic conditions [16,50,57]. Moreover, hypoadiponectinemia is associated with SMC hypertrophy, as well as collagen accumulation [58,59]. Accordingly, we also find a negative correlation between adiponectin levels and collagen type I in our non-hypertensive obese mice, as well as with stiffness index β or PWV. A possible mechanism for the described changes might be the decrease in NO availability since hypoadiponectinemia is closely associated with endothelial dysfunction in humans [60] and reduced p-eNOS levels [61]. The correlation between stiffness index β, PWV and AIx with p-eNOS levels (Supplemental Figs. 1 and 3) also supports this mechanism.

5. Conclusions

The present data show that diet-induced obesity per se might account for the development of vascular remodeling and arterial stiffness through mechanisms independent of hypertension. Since vascular stiffness correlates with adipokine levels, we do suggest a key contribution of adipokine dysregulation in this process. Hyperleptinemia and hypoadiponectinemia might initiate the observed alterations in vascular cells and extracellular matrix through an increase in oxidative stress. However, further work at cellular and molecular level will be required to elucidate the precise mechanisms underlying the alteration of arterial mechanical properties. Understanding these mechanisms might lead to additional options for prevention and treatment of obesity-related vascular complications.

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Conflict of interest

None declared.

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