ARTICLE

High fat diet-induced metabolically obese and normal weight rabbit model shows early vascular dysfunction: mechanisms involved

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Abstract

Background Obesity contributes significantly to the development and evolution of cardiovascular disease (CVD) which is believed to be mediated by oxidative stress, inflammation and endothelial dysfunction. However, the vascular health of metabolically obese and normal weight (MONW) individuals is not completely comprehended.

Objectives The purpose of our study was to evaluate vascular function on the basis of a high fat diet (HFD)-MONW rabbit model.

Subjects Twenty four male rabbits were randomly assigned to receive either a regular diet (CD, $n = 12$) or a high-fat diet (18% extra fat on the regular diet, HFD, $n = 12$) for 6 weeks.

Results Body weight, TBARS and gluthathione serum levels were similar between the groups; fasting glucose, triglycerides, C reactive protein (CRP), visceral adipose tissue (VAT), triglyceride-glucose index (TyG index) were higher in the HFD group. Compared to CD, the HFD rabbits had glucose intolerance and lower HDL-cholesterol and plasma nitrites levels. Thoracic aortic rings from HFD rabbits exhibited: (a) a reduced acetylcholine-induced vasorelaxation; (b) a greater contractile response to norepinephrine and KCl; (c) an improved angiotensin II-sensibility. The HFD-effect on acetylcholine-response was reversed by the cyclooxygenase-2 (COX-2) inhibitor (NS398) and the cyclooxygenase-1 inhibitor (SC560), and the HFD-effect on angiotensin II was reversed by NS398 and the TP receptor blocker (SQ29538). Immunohistochemistry and western blot studies showed COX-2 expression only in arteries from HFD rabbits.

Conclusions Our study shows a positive pro-inflammatory status of HFD-induced MONW characterized by raised COX-2 expression, increase of the CRP levels, reduction of NO release and oxidative stress-controlled conditions in an early stage of metabolic alterations characteristic of metabolic syndrome. Endothelial dysfunction and increased vascular reactivity in MONW individuals may be biomarkers of early vascular injury. Therefore, the metabolic changes induced by HFD even in normal weight individuals may be associated to functional alterations of blood vessels.

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Introduction

High fat diets (HFDs) contribute to excess adipose tissue (obesity) which has proved to be harmful to multiple body organ systems through different mechanisms [1, 2]. The body mass index (BMI) was the most commonly used simple measure of obesity. However, the main limitation of BMI is that it is not a direct measure of adiposity. It measures excess weight, rather than excess fat, which is what determines whether someone is obese or not. At present, epidemiologic studies have demonstrated that central fat distribution measured by waist circumference, waist-to-hip ratio and weight-to-height ratio, is an important measure of adiposity-related risk [3]. In spite of the demonstrated association between BMI-defined obesity and mortality [4] several studies worldwide have shown that overweight

individuals have similar or even better outcomes for survival and cardiovascular events compared to people with normal body weight standards (BW) [5, 6]. Overall population comprises individuals whose BMI is considered normal but have impaired insulin sensitivity and high levels of visceral adipose tissue (VAT). This normal weight individual subgroup forms the metabolically obese but normal weight individuals (MONW) [7, 8] which are predominant in the general population (19.98% of people with normal weight) [9]. Romero Corral et al. [10] show that MONW is significantly associated to cardio-metabolic deregulation and a high prevalence of metabolic syndrome (MS) which is in fact similar to the prevalence of MS described in overweight subjects.

The causes of MS in societal terms may include several factors, but changes in nutrition habits and physical activities are the main causes of this problem. Cheap food and sedentary jobs are mainly driven by technology. Therefore, MS may be the result of technological advance and represents a major challenge to our present technological society [11]. Chronic inflammation could be a triggering factor at the root of MS: stimuli such as over-nutrition, physical inactivity, and ageing would result in cytokine hyper secretion and eventually lead to insulin resistance and diabetes in genetically or metabolically predisposed individuals [12]. The pro-inflammatory condition that accompanies the MS associates to both insulin resistance and endothelial dysfunction, connecting inflammation and metabolic processes, which are highly deleterious for vascular functions [13]. The endothelium regulation of vascular tone is affected in MS. Increase in reactive oxygen species (ROS) production and reduction in NO bioavailability have been reported [14–17]. Mitochondria plays a central role in energy metabolism, therefore its alterations may contribute to the development of metabolic disorders. Mitochondrial function is intimately associated to inflammation; it can both promote inflammation and be affected by it. It has been recently found that mitochondrial biogenesis and function are enhanced by NO. This finding indicates that changes in the production or bioavailability of NO and MS may be associated to the mitochondria [18]. Several studies suggest that cyclooxygenase (COX) derivatives might also be involved in the MS induced- vascular impairment [19, 20]. Xiang et al. [21, 22] suggest that insulin resistance and the resulting increase ROS impair functional dilation in obese Zucker rats by increasing thromboxane prostanoid (TP) receptor mediated vasoconstriction. These studies propose that inducible isoform of COX (COX-2) might be involved in the increase of thromboxane A2 (TXA$_2$) production.

In previous work we developed a HFD-induced MS and normal weight (MSNW) rabbit model characterized by central obesity, pre-diabetes, and dyslipidemia with low high-density cholesterol lipoprotein (HDL-C) and an increase of triglycerides levels. [23] This model is valuable to study the physiopathology of metabolic disorders imitating situations in MONW individuals when reproducing the main clinical manifestations of the MS in human beings.

Therefore, our main purpose is to evaluate endothelial function and vascular reactivity in this diet-induced experimental model of MONW in rabbits.

Materials and methods

Animals

The experimental protocols for this study were approved by the Animal Care and Use Committee Institution of Tucuman University. All animal care and use programs were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Publication 86 to 23, revised 1985). Male hybrid Flanders rabbits from a slaughterhouse initially weighing 850–1000 g were housed in single cages in a humidity and temperature-controlled room with a 12-h light cycle. Housing rabbits singly in cages means space restriction which does not allow normal movement, mimicking modern sedentary lifestyle. They were fed 100 g/d of standard rabbit chow. After 1-week acclimatization period, they were randomly divided into two groups: one designated to remain lean (CD, $n = 12$) and the other to become HFD ($n = 12$). The lean group continued with the same dietary regime, which is an appropriate maintenance diet for a normal adult rabbit. The HFD group was given, ad libitum, a standard rabbit chow supplemented with 18% fat. The excess fat in the diet consisted of corn oil (10%) and lard (8%). Unsaturated to saturated ratio was 2.2 ± 0.02. Composition of the diet was previously reported [23]. Experiments were performed after the rabbits had been on their respective diets for 6 weeks. Only male rabbits were used to avoid secondary variability related to sex differences in this experimental model.

Intraperitoneal glucose tolerance test (GTT)

An intraperitoneal glucose tolerance test (GTT) was performed in accordance with the Georgiev et al. [24] method. Plasma glucose levels were measured by using colorimetric reactions with commercial kits (Wiener, Rosario, Argentina).

Mean blood pressure, heart rate, and lipid determinations

Once the 6-week dietary intervention was over, food was withdrawn for 12 h, and the rabbits were weighed and then anesthetized with ketamine (20 mg/Kg) and diazepam (0.5

mg/Kg). Mean arterial blood pressure (MAP) and heart rate (HR) were measured directly in the carotid artery through a catheter connected to a pressure transducer (Gould-Statham P23, California, USA) and recorded using a data acquisition system (Biopac MP100, Aero Camino Goleta, USA). After MAP measurement, blood samples were collected in pre-chilled glass tubes containing EDTA 10^{-7} M through the catheter inserted in the carotid artery. Afterwards, a midline incision was made in the rabbit and the adipose tissues from the abdominal areas (visceral and retroperitoneal) were collected and weighed. The VAT was expressed as a percentage of the total BW: (fat weight/ animal weight) x 100.

Plasma cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TG) and glucose were measured using colorimetric reactions with commercial kits (Wiener, Rosario, Argentina). C-reactive protein (CRP) was determined by a quantitative turbidimetric test (Wiener, Rosario, Argentina), according to the manufacturer's instructions.

Lee et al. [25] show that MONW individuals could be identified based on the TyG index which is the product of fasting blood glucose and TG levels. The TyG index is a simple marker that has a high correlation with the degree of insulin resistance measured by hyperinsulinemic-euglycemic clamp studies [26]. The TyG index was calculated as ln (fasting triglycerides (mg dl^{-1}) × fasting glucose (mg dl^{-1})/2).

Nitrite levels

Nitrites were measured according to Moshage et al. [27] by using the Griess reaction. The results were expressed as nmoles/l.

Determination of lipid peroxidation and GSH/GSSH ratio in serum

Lipid peroxidation was evaluated by measuring the thiobarbituric acid reactive substances (TBARS) and GSH/GSSH was calculated from total glutathione and reduced glutathione (GSH) levels as was previously described [28].

The results were accordingly expressed as µmol of TBARS/ml/mg of protein and µg of GSH/GSSH/mg proteins determined by the Lowry's method.

Isometric tension measurement

After blood samples were collected, the descending thoracic aorta was exposed through a midline incision and quickly removed, cleaned of adherent connective tissues and cut into rings. Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (Grass Technologies, West Warwick, USA) to measure tension in the vessels. The rings were placed in a 10 ml organ chamber containing Krebs solution gassed with 95%O_2/5%CO_2, and maintained at 37 °C. The composition of Krebs solution was as follows (mM): 128 NaCl, 4.7 KCl, 14.4 NaHCO$_3$, 1.2 NaH$_2$PO$_4$, 0.1 Na$_2$-EDTA, 2.5 CaCl$_2$, and 11.1 glucose, at pH 7.2. The rings reached a basal tension of 2.0 g, which proved to be the optimal tension for KCl-induced contraction (96 mM) and then they were balanced for 120 min by changing the bath fluid every 15–20 min. In some of the rings, the endothelium remained intact (E+) while in the other group endothelium was removed mechanically by gently rolling the lumen off the vessel on a thin wire (E−). Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (Ach, 10^{-6} M) in the presence of contractile tone induced by phenylephrine 5 × 10^{-6} M.

Experimental protocols

Endothelial function was checked by building a concentration-response curve (CRC) to Ach (10^{-8}–10^{-5} M) in E+ aortic rings. In some phenylephrine-precontracted vessels, the vasorelaxant responses to 5 × 10^{-6} M sodium nitroprusside (SNP, a NO donor) were also examined to observe whether the vascular smooth muscle function was affected in MSNW rabbits.

The possible roles of COX products or ROS in the reduced vasorelaxant response to Ach were analyzed by incubation either with NS 398 10^{-7} M (COX-2 inhibitor) or SC 560 10^{-6} M (COX-1 inhibitor), or TEMPOL 10^{-7} M (a stable spin trap for O^{-2}) or diphenyleneiodonium 10^{-6} M (DPI, NADPH oxidase inhibitor), 30 min before phenylephrine stimulation. Relaxation was expressed as a percent change of preexisting tone (before addition of vasorelaxant).

Vascular reactivity was evaluated by measuring response to pharmacological contractile agonists. CRCs to norepinephrine (10^{-8}–10^{-3} M), angiotensin II (Ang II, 10^{-10}–10^{-6} M) or stimulation with 96 mM KCl, were assessed both in E+ and E− aortic rings from both diet groups.

To check the roles of arachidonic acid-metabolites or ROS in the altered Ang II-contractile response, some E+ vessel rings were incubated during 30 min with indomethacin 10^{-5} M (nonselective COX-inhibitor), NS 398 10^{-7} M, SC 560 10^{-6} M, SQ29548 10^{-6} M (TP receptor antagonist), CAY 10434 10^{-6} M (omega-hydroxylase inhibitor), TEMPOL 10^{-7} M or DFI 10^{-6} M, and then CRCs to Ang II were obtained. Results were expressed as mg of isometric contraction.

Immunohistochemistry

Arterial preparations (5 mm wide) of thoracic aorta were used for immunohistochemical studies. COX-1 and COX-2 were detected by using rabbit anti-COX-1 (code No. 160109) and anti-COX-2 (code No. 160106) polyclonal antibodies (Cayman Chemical, Ann Arbor, USA) diluted at 1:50 and 1:100, respectively. Negative controls were performed in absence of primary antibody. The staining was quantified using Image J software, and the results were expressed as a percentage.

Western blotting

Thoracic aortas were snap frozen in liquid nitrogen and stored at $-80\,°C$ until use. The tissue was homogenized in modified buffer for protein extraction and the homogenate was then centrifuged and the supernatant was collected. COX-1 and COX-2 proteins were analyzed by Western blot by using COX-1 polyclonal antibody, COX-2 polyclonal antibody (both raised in rabbits, 2.0 mg/mL, Cayman Chemicals, Ann Arbor, USA). The bands were visualized with biotinylated anti-rabbit immunoglobulins (Sigma Chemicals, St Louis, USA); peroxidase-labeled streptavidin complex (Sigma Chemicals, St Louis, USA); and, finally, 3,3′-diaminobenzidine tetra-hydrochloride (Sigma Chemicals, St Louis, USA and intensity was quantified by using an image system (Image J). The protein expression for COX-1 and COX-2 was normalized to alpha-actin (monoclonal antibody raised in mouse, Sigma Chemical, St Louis, USA).

Statistical analyses

Agonist CRCs were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA). Agonist potencies were calculated as pEC_{50} (negative logarithm of the molar concentration of agonist producing 50% of the maximum response), and maximum response was expressed as R_{max} (maximum effect elicited by the agonist). Investigators were blinded to treatment until data analysis. The results are reported as mean $\pm$ standard error of the mean (s.e.m). Shapiro and Wilks goodness-of-fit test was used to test for normal distribution. Statistically significant differences were calculated by one or two-way analysis of variance (followed by Duncan's post-test) or unpaired Student's t-test; $p < 0.05$ was considered statistically significant.

Results

Clinical characteristics

The BW increased in the two dietary groups throughout the treatment without significant differences between

Table 1 Biometrical, biochemical, and hemodynamic characteristics from rabbits fed a control diet (CD), and a CD supplemented with 18% fat (HFD)

	CD	HFD
Body weight (g)	2138 ± 82	2043 ± 46
Visceral abdominal fat (g)	0.92 ± 0.06	2.31 ± 0.14^a
Fasting glucose (mg/dl)	113.2 ± 2.7	126.1 ± 5.8^a
Total cholesterol (mg/dl)	61.3 ± 6.0	77.7 ± 4.8
LDL-cholesterol (mg/dl)	23.8 ± 3.1	46.8 ± 7.2
HDL-cholesterol (mg/dl)	53.5 ± 4.1	24.2 ± 2.8^a
Triglycerides (mg/dl)	104.9 ± 14	191.8 ± 22.0^a
Blood pressure (MAP) (mmHg)	57.2 ± 2.7	56.7 ± 5.3
Heart rate	277.6 ± 29	281.8 ± 27

Data are expressed as mean $\pm$ SEM of 12 rabbits $^a p < 0.05$ indicates statistically significant differences between rabbits fed a CD and rabbits fed a HFD (unpaired Student's t test)

them. When the experiment ended, animal BW did not differ significantly between the two diet groups (Table 1). However, rabbits fed on HFD displayed a significant increase of VAT (Table 1) compared to the CD group.

GTT showed abnormal blood glucose levels at 60 min (CD: 167 ± 10 mg/dl vs HFD: 200 ± 7 mg/dl) and 120 min (CD: 138 ± 10 mg/dl vs HFD: 163 ± 4 mg/dl; $p < 0.05$, one way ANOVA). In addition, feeding the HFD significantly increased fasting glucose (Table 1).

MAP and HR of rabbits fed on HFD were similar to CD group (Table 1).

Biochemical assays

After 6 weeks of dietary treatment, we found an increase of TG and a decrease HDL-C in rabbits fed on HFD compared to CD. TC and LDL-C did not show differences between MSNW and control rabbits (Table 1).

CRP levels and the TyG index were significantly higher in rabbits fed on HFD. The values obtained were: CRP (mg/dl): CD: 5.1 ± 0.9 vs HFD: 22.0 ± 3.3; $n = 12$, $p < 0.05$, unpaired Student's t test. TyG index: CD: 8.27 ± 0.22 vs HFD: 9.28 ± 0.11, $n = 12$; $p < 0.001$, unpaired Student's t test.

Nitrites in plasma were reduced in rabbits fed on HFD (CD: 1752 ± 784 nmoles/l vs HFD: 324 ± 109 nmoles/l; $p < 0.05$, unpaired Student's t test).

There were no differences between TBARS levels (CD: 53.3 ± 7 μmoles/mg protein vs HFD: 36 ± 6 μmol/mg protein) and GSH/GSSH ratio (CD: 0.2 ± 0.04 μg/mg proteins vs HFD: 0.12 ± 0.1 μg/mg proteins) in serum from rabbits fed on CD with respect to rabbits fed on HFD.
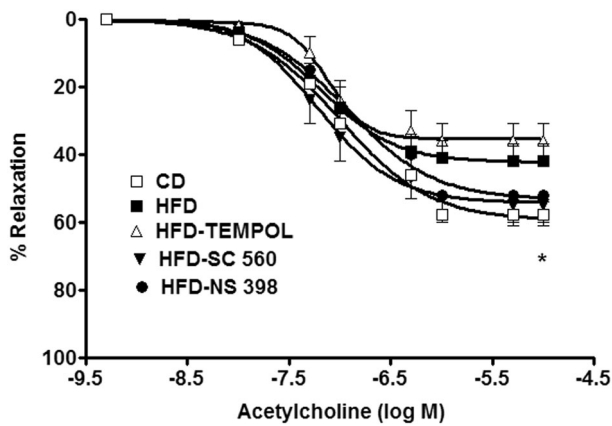
Fig. 1 Concentration response curves to acetylcholine. Arteries from rabbits fed on control diet (CD) and rabbits fed on high fat diet (HFD) were incubated with vehicle (control, $n = 8$) or NS 398 10^{-7} M (cyclooxygenase-2 inhibitor, $n = 8$), SC 560 10^{-6} M (cyclooxygenase-1 inhibitor, $n = 8$) or TEMPOL 10^{-7} M (antioxidant, $n = 8$). *$p < 0.05$ indicates statistically significant differences in maximal relaxation between control arteries and incubated with NS 398 or SC 560 arteries (one way ANOVA)

Response to acetylcholine and sodium nitroprusside

The endothelium-dependent relaxation induced by Ach was significantly reduced in arteries from rabbits fed on HFD (CD-R_{max}: $66.2 \pm 5.2\%$ vs HFD-$R_{max} = 43 \pm 5.5\%$, $n = 10$, $p < 0.05$, unpaired Student's t test). The Ach-induced vasorelaxation was improved in HFD rabbits by the pre-incubation with SC560 and NS 398, and the difference between CD and HFD groups of rabbits disappeared (Fig.1). Incubation with TEMPOL did not change the Ach-response but surprisingly the pretreatment with the NADPH inhibitor (DFI) decreased Ach-evoked vasorelaxation in aortic rings obtained from rabbits fed on HFD (HFD-R_{max}: $43 \pm 5.7\%$ vs HFD-DFI-R_{max}: $14 \pm 4\%$; $n = 8$, $p < 0.05$, unpaired Student's t test).

The relaxation responses to SNP 5×10^{-6} M, a direct vasodilator of vascular smooth muscle, were the same between CD and HFD rabbits (E+: CD: $94 \pm 5\%$ vs HFD: $88 \pm 2\%$; E−: CD: $89 \pm 2\%$ vs HFD: $91 \pm 7.4\%$).

Response to norepinephrine, angiotensin II, and KCl

The norepinephrine-induced vasoconstriction was increased by E+ aortic rings in rabbits fed on CD compared to those in E− aortic rings. Fat addition to diet significantly improved both R_{max} and pEC$_{50}$ to norepinephrine in E− arteries. Thus, the difference between E+ and E− in both groups disappeared (Fig. 2).

The Ang II-induced R_{max} was similar in E+ aortic rings from both diet groups. When endothelium was removed Ang II-induced R_{max} was significantly reduced in HFD rabbit arteries compared to CD rabbits (Fig. 3). Upward
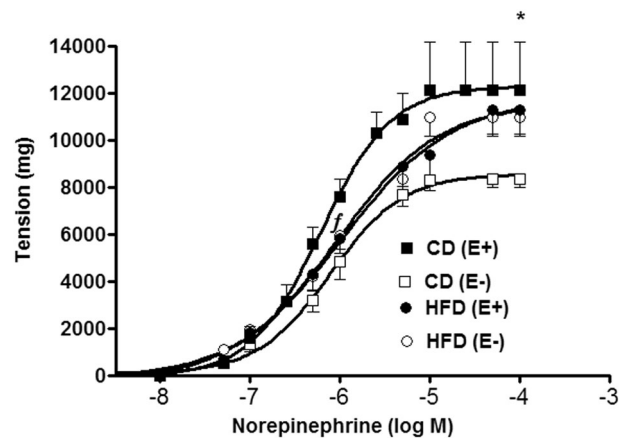

Fig. 2 Concentration response curves to norepinephrine in endothelium intact (E+, $n = 8$) and endothelium removed (E−, $n = 8$) arteries from rabbits fed on control diet (CD) or on high fat diet (HFD). *$p < 0.05$ indicates statistically significant differences between R_{max} of E+ and E− arteries from rabbits fed a CD. f $p < 0.05$ indicates statistically significant differences between R_{max} of E− arteries from rabbits fed on CD and rabbits fed on HFD (two way ANOVA and Duncan's post test)
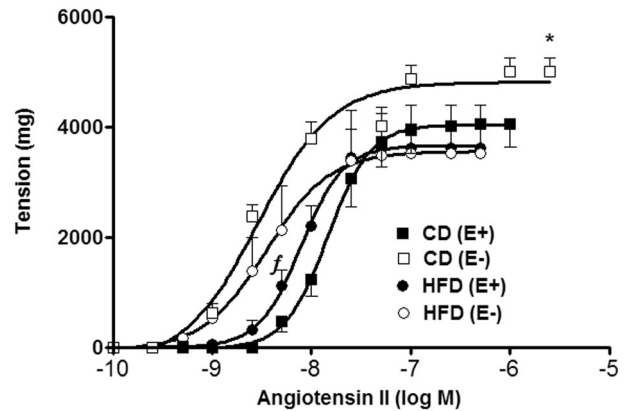

Fig. 3 Concentration response curves to angiotensin II in endothelium intact (E+, $n = 8$) and endothelium removed (E−, $n = 8$) arteries from rabbits fed on control diet (CD) or on high fat diet (HFD). *$p < 0.05$ indicates statistically significant differences between R_{max} of E+ and E − arteries from rabbits fed a CD (two way ANOVA and Duncan's post test). f $p < 0.05$ indicates statistically significant differences between pEC$_{50}$ of E+ arteries from rabbits fed on CD and rabbits fed on HFD (two way ANOVA and Duncan's post test)

shift of the CRC for Ang II was found in E− arteries from control rabbits (pEC$_{50}$ E−: 8.46 ± 0.07 vs E+: 7.82 ± 0.08, respectively; $n = 8$, $p < 0.01$, unpaired Student's t test). This difference in both groups disappeared in HFD rabbit arteries (pEC$_{50}$ E−: 8.04 ± 0.08 vs E+: 8.15 ± 0.06; $n = 8$, n.s). When both diet groups were compared, results showed that Ang II-contractile responses were sensitized in E+ and desensitized in E− arteries in HFD rabbits ($p < 0.05$, two way ANOVA and Duncan's post test; Fig.3).

The incubation with CAY 10434 10^{-6} M, indomethacin 10^{-5} M, TEMPOL 10^{-7} M or DFI 10^{-6} M did not modify Ang II-contractile response both in rabbits fed on CD (data

not shown) and rabbits fed on HFD (Table 2). However, Ang II-induced R_{max} in rabbits fed on HFD aortic rings was significantly reduced by pretreatment with NS 398 10^{-7} M or SQ29548 10^{-6} M (Fig.4). The pretreatment with SC 560 10^{-7} M unexpectedly improved the sensitivity to Ang II-induced contraction in E+ arteries in both diet groups. This effect was higher in HFD rabbits' arteries (pEC$_{50}$-CD: 7.82 $\pm$ 0.08 vs CD-SC560: 8.12 $\pm$ 0.06 vs HFD: 8.15 $\pm$ 0.06 vs HFD-SC560: 8.48 $\pm$ 0.07, $p < 0.05$, one way ANOVA and Duncan's post test).

HFD rabbit arteries exhibited a significantly larger contractile response to KCl 96 mM compared to control rabbits

Table 2 Maximal contractile response (R_{max}) and pEC$_{50}$ to angiotensin II of endothelium intact aortic rings from rabbits fed a high fat diet (HFD)

	R_{max}	pEC$_{50}$
Control	3880 $\pm$ 542	8.15 $\pm$ 0.06
NS 398	1933 $\pm$ 304[a]	7.88 $\pm$ 0.15[a]
SQ 29548	2473 $\pm$ 239[a]	7.92 $\pm$ 0.14[a]
CAY 10434	3606 $\pm$ 873	8.13 $\pm$ 0.05
Indomethacin	3528 $\pm$ 436	8.12 $\pm$ 0.12
SC 560	2437 $\pm$ 473[a]	8.48 $\pm$ 0.07[a]
Tempol	3820 $\pm$ 643	8.00 $\pm$ 0.08
DFI	3172 $\pm$ 376	8.02 $\pm$ 0.07

Data are expressed as mean $\pm$ SEM of eight experiments

[a]$p < 0.05$ indicates statistically differences between untreated (control) and treated arteries
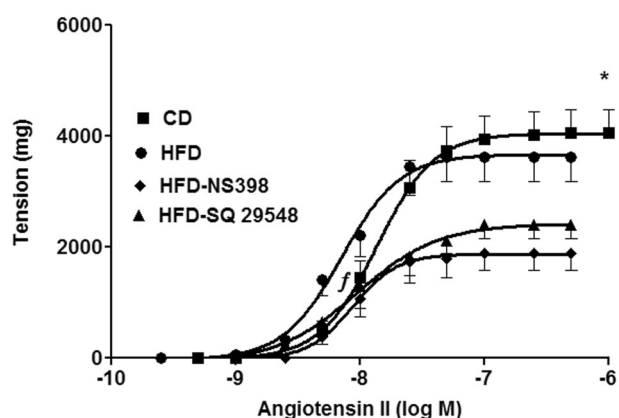

Fig. 4 Concentration response curves to angiotensin II. Endothelium intact arteries from rabbits fed on control diet (CD, $n = 8$) and rabbits fed on high fat diet (HFD) were incubated with vehicle (untreated, $n = 8$) or NS 398 10^{-7} M (cyclooxygenase-2 inhibitor, $n = 8$) or SQ 29548 10^{-6} M (TP receptor antagonist, $n = 8$). *$p < 0.05$ indicates statistically significant differences in R_{max} between untreated arteries and incubated with NS 398 or SQ 29548 arteries (one way ANOVA). f $p < 0.05$ indicates statistically significant differences in pEC$_{50}$ between untreated arteries and incubated with NS 398 or SQ 29548 arteries (one way ANOVA)

in both E+(CD: 3907 $\pm$ 270 mg vs HFD: 5993 $\pm$ 616 mg, $n = 12$, $p < 0.05$, unpaired Student's t test) and E− (CD: 2849 $\pm$ 288 mg vs HFD: 5175 $\pm$ 608 mg, $n = 12$, $p < 0.05$, unpaired Student's t test) aortic rings.

Cyclooxygenase expression

Data derived from the immuno histochemical analysis showed that the COX-1 isoform was slightly and homogeneously expressed in CD preparations. In HFD rabbits' arteries COX-1 expression was mainly localized in the endothelium and the adventitia. In contrast, the COX-2 isoform was only detected in HFD preparations and was predominantly localized in the adventitia of the vascular wall (Supplement 1).

Western blot studies evidenced COX-2 expression in HFD rabbits and no differences in COX-1 expression between both diet groups (Supplement 2).

Discussion

Our results demonstrated that the administration of HFD for a period of 6 weeks resulted in: (1) central obesity, (2) a state of pre-diabetes characterized by impaired fasting glucose and glucose intolerance, (3) alterations in the lipid profile revealed by an increase in triglycerides and a decrease of HDL-C, (4) unchanged in total cholesterol levels and BW. Thus, this model had metabolic features characterizing the human MS definition and may be named as the MSNW rabbit model. Increase in TyG Index supports the view that this model could be useful to study the early mechanisms involved in metabolic disorders induced by feeding on HFD therefore imitating situations in MONW individuals. At present, almost all animal models responding to MS are obese. Cao et al. [29] developed a rat model of abdominal obesity with normal weight using a modified high-sucrose diet. These authors reported steatosis, mitochondrial morphologic changes, and insulin resistance as metabolic disorders appeared in MONW individuals. However other features characterizing the MS (high blood pressure, increased triglycerides, reduced HDL-C) have not been analyzed in that model.

Vascular dysfunction associated to MS and obesity has been well documented [30]. However, there are no studies characterizing the early vascular changes related to HFD-induced MONW. Our findings demonstrated that endothelial dysfunction was present in the MSNW model. There are many hypotheses on the origin of endothelial dysfunction in obesity. One of them is that insulin resistance leads to endothelial dysfunction [31]. An alternative hypothesis is that both insulin resistance and endothelial dysfunction have an antecedent in common, possibly inflammatory

mediators released from adipose tissue [32]. Endothelial dysfunction may be the earliest stage in the cardiovascular disease continuum [33], therefore, it could represent a biomarker of early vascular injury. Increase in systemic blood pressure may be a later stage in the evolution of MS to cardiovascular damage. We have previously characterized an obesity and hypertension rabbit model by feeding rabbits on a HFD during 12 weeks [34]. There are some differences between obese and MSNW rabbit models. Although both models showed an increase in VAT, fasting glucose and abnormal glucose tests compared to the one with control showed that only obese rabbits reported an increase in blood pressure levels. Taking into account prospective studies showing glucose intolerance as a predictor of hypertension [35], we may hypothesize that a shorter high fat-feeding period in MSNW rabbits may be insufficient to raise blood pressure above the normal levels.

In the presence of impaired NO bioavailability, a dysfunctional endothelium becomes the source of mediators that are detrimental to the arterial wall. Accumulating evidence suggests that pro-inflammatory arachidonic acid metabolites through the three pathways, cyclooxygenase (COX), lipoxygenase, and cytochrome P450 oxygenases as well as increased oxidative stress are involved in endothelial dysfunction. Previous studies [19, 20] reported that an increase in free radical production may account for the enhancement of COX-metabolites involved in endothelial dysfunction. Therefore, the effects of NS 398 (COX-2 inhibitor), SC 560 (COX-1 inhibitor) and TEMPOL (antioxidant) on Ach-relaxation were checked. NS 398 and SC 560 reached Ach relaxation to CD levels. TEMPOL did not modify Ach-relaxation of HFD arteries compared to untreated arteries. These results imply that COX may play a part in the HFD-induced endothelial dysfunction.

Previous investigators have reported either enhanced, [36] reduced [37] contractile responses to agonists, or no change in reactivity, using a variety of different models of MS. In the present model of MSNW rabbit, endothelium-independent increase of contractile response to KCl was found. Norepinephrine contractile response in E+ aortic rings was similar in both diet groups. However, endothelium-removal increased R_{max} and pEC_{50} to norepinephrine in rabbits fed on HFD with respect to E- arteries from rabbits fed on CD. Considering these results, an increased vascular smooth muscle reactivity induced by feeding on HFD may be hypothesized. Central sympathetic over-activity plays a pivotal role in the etiology and complications of MS [38]. However, in the present study HR (marker of sympathetic activity) was found unchanged in HFD with respect to CD rabbits. That means sympathetic nervous system activation may not account for any improvement in the vascular tone. Contribution of NO in maintaining resting vasodilator tone has been widely described [39]. Taking into account that reduced nitrite levels in plasma from HFD-rabbits were found in our MSNW model, reduction of NO may be responsible for the vascular reactivity increase.

Moreover, HFD modified the contractile response to Ang II. Sensitization was found in E+ arteries with respect to arteries from rabbits fed on CD. Previous studies demonstrated that vasoconstrictor COX-omega hydroxylase metabolite-release sensitized Ang II-response during endothelial dysfunction [40]. The role of arachidonic acid metabolites on endothelium-dependent sensitization to Ang II was checked. CAY 10434 (omega hydroxylase inhibitor) and indomethacin (COX-inhibitor) had no effects on the contractile response to Ang II. However NS 398 reached the pEC_{50} to control values and even blunted the R_{max} to Ang II. Unexpectedly, SC 560 improved Ang II affinity in arteries from both diet groups. The major prostaglandin released by endothelium from rabbit aorta in basal conditions is prostacyclin (PGI_2) [41]. This study showed that only COX-1 was expressed in rabbits fed on CD. Therefore, inhibition of PGI_2-release may account for the SC560 effect. Considering that both isoenzymes COX-1 and COX-2 were expressed in arteries from rabbits fed on HFD, inhibition of COX-1 may unmask COX-2 effects in such conditions. That means COX-2 up regulation may be involved in vascular dysfunction associated to HFD. In fact, the lack of effect of indomethacin supports this view. As was stated in the introduction, Xiang et al. [21, 22] found in obese Zucker rats increased vasoconstriction TP-mediated. These studies suggest that COX-2 might be involved in TXA_2 over production. Taking this data into account, the effect of SQ 29548 was checked. SQ 29548 reversed the sensitization and blunted the contractile response to Ang II compared to untreated arteries. In addition, endothelium-removal reduced contractile response and pEC_{50} to Ang II with respect to E- arteries from rabbits fed on CD. These results support the hypothesis that COX-2 up regulation may contribute to endothelial dysfunction by TP-receptors activation induced-sensitization to Ang II in rabbits fed on HFD.

Plenty of data from the bibliography reported role of ROS on the up-regulation of COX-2. Tian et al. [20] demonstrated that endothelium dependent contractile factors derived from COX-2 were released in response to ROS stimulation in hypertensive rats. In addition, MS has been related to increased oxidative stress [14, 15]. Considering this data, the oxidative stress was checked in the present model of MSNW rabbit. TBARS level and GSH/GSSH ratio in plasma were similar in both diet groups. This result was consistent with non existing effect of TEMPOL either on the Ach-relaxation or the Ang II contractile response in aorta from rabbit fed on HFD. Thus, ROS increase may not account for the COX-2 up regulation in the present model.

While superoxide reacts with NO three to four times faster than it reacts with superoxide dismutase (SOD), this reaction only becomes a significant biological regulatory factor when superoxide levels rise or when NO levels are very high and approach the concentrations of SOD that are present. As NO levels increase, it competes with SOD for the metabolism of superoxide, forming reactive species, including peroxynitrite (ONOO), a reactive molecule that causes the modification of multiple other biological molecules and key sites on proteins potentially involved in signaling mechanisms that influence vascular function. Considering that we did not find increase of lipid peroxidation in our MSNW model, we infer that antioxidant systems of the body may be sufficient to maintain normal levels of ROS.

The Western-type dietary pattern showed a positive relation with CRP [42]. The present model of MSNW showed higher CRP levels in plasma than in metabolically healthy rabbits. Modulation of inflammatory gene expression by lipids have been described [43]. Saturated fatty acids (SFA) induce expression of COX-2 [44, 45]. Omega-6 fatty acids, and especially linoleic acid can cause endothelial cell dysfunction as well as potentiate TNF-α-mediated endothelial injury [46]. Recently, Hennig et al. [47] have demonstrated that both the extracellular signal regulated kinase (ERK1/2) and phosphoinositide-3 kinase/amino kinase terminal (PI3K/Akt) signaling pathways can contribute to the effect of linoleic acid on nuclear factor-kappa B-dependent transcription and endothelial cell activation. Previously, we demonstrated increase of omega 6 fatty acids levels (linoleic and arachidonic acids) and SFA in plasma from rabbits fed on HFD [41]. Moreover, considering that both COX-1 and COX-2 contribute to the inflammatory response and the extent to which each isoform contributes may depend on the inflammatory stimuli, post-period insult, and the relative levels of each isoform in the target tissue [48], HFD-induced COX-2 expression may have a fundamental role in the pro-inflammatory status of MONW model.

Conclusion

Our research demonstrated that a rabbit model feeding on HFD imitates some metabolic features of MONW individuals. A pro-inflammatory status characterized by COX-2 expression, increase of the CRP levels, reduction of NO release and oxidative stress-controlled conditions were found at an early stage in metabolic alterations characterizing MS. Endothelial dysfunction and increased vascular reactivity in MONW individuals may be biomarkers of early vascular injury. Therefore, our results support the view that metabolic changes induced by HFD even in normal weight individuals are associated to functional alterations of blood vessels that in turn could be responsible for cardiovascular disease continuum.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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