Long-term high-fat consumption leads to downregulation of Akt phosphorylation of eNOS at Ser1177 and upregulation of Sirtuin-1 expression in rat cavernous tissue

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Abstract Long-term consumption of high-fat diets negatively interferes with metabolic status and promotes endothelial dysfunction and inflammation. In the cavernous tissue, these outcomes become conspicuous in the elderly and strongly affect penile erection, a vascular process highly dependent on local nitric oxide bioavailability. Although epidemiological data links erectile dysfunction to nutritional patterns, the underlying molecular mechanisms remain unclear. Therefore, we investigated the effects of long-term high-fat diet on endothelial nitric oxide synthase (eNOS)–Sirtuin-1 axis and Akt/eNOS phosphorylation in the cavernous tissue of Sprague–Dawley rats, and compared with energy-restricted animals. We demonstrated that high-fat diet intake led to a noteworthy decrease in eNOS phosphorylation at Ser1177 residue through the Akt pathway, which seems to be compensated by upregulation of phosphorylation at Ser615, but without

an increment in nitric oxide production. These results are accompanied by an increase of systemic inflammatory markers and upregulation of the inducible NOS and of the deacetylase Sirtuin-1 in the cavernous tissue to levels apparently detrimental to cells and to metabolic homeostasis. Conversely, in long-term energy-restricted animals, the rate of phosphorylation of eNOS at Ser1177 diminished, but the activation of the enzyme increased through phosphorylation of eNOS at Ser615, resulting in an enhancement in nitric oxide bioavailability. Taken together, our results demonstrate that long-term nutritional conditions override the influence of age on the eNOS expression and activation in rat cavernous tissue.

Keywords Aging · Cavernous tissue · Nitric oxide synthase · Energy restriction · High-fat diet · Sirtuin-1

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Introduction

Aging is associated with structural and functional changes in the vascular system, constituting an independent risk factor for cardiovascular disease (CVD) (Csizsar et al. 2002). In addition, the incidence of metabolic syndrome (MetS), a cluster of cardiometabolic risk factors, such as insulin resistance/diabetes, hypertension, dyslipidemia, and abdominal obesity, increases dramatically along aging (Alberti et al. 2009; Lakka et al. 2002; Mottilo et al. 2010). All of these conditions are strictly related to endothelial dysfunction, an entity characterized by impaired nitric oxide (NO) bioavailability recently

recognized as an underlying component of MetS (Seals et al. 2011).

Erectile dysfunction (ED), defined as the consistent or recurrent inability to achieve and/or maintain a penile erection sufficient for satisfactory sexual performance (NIH 1993), is a common age-related complaint (Ghalayini et al. 2010). Since ED is currently considered an early manifestation of endothelial dysfunction (Montorsi et al. 2006), it is not surprising that it is highly prevalent in MetS male patients (Jacobs et al. 2011; Tomada et al. 2011). Indeed, ED constitutes a surrogate symptom of occult vascular disease (Montorsi et al. 2003) and a predictive marker for CVD (Aversa et al. 2010; Billups et al. 2008; Chew et al. 2010; Shin et al. 2011). The high susceptibility of cavernous tissue to conditions that affect hemodynamics makes it a good model to study age- and metabolism-related vascular changes, even before they become symptomatic.

Vascular health and erectile function are highly dependent on the endothelial secretion of vasoactive signaling molecules, the most important of which is the gaseous transmitter NO, predominantly produced by endothelial NO synthase (eNOS) (Ignarro et al. 1987). NO diffuses easily through the cell membrane of the endothelial cell, inducing relaxation of perivascular smooth muscle cell (VSMC) (Soucy et al. 2006), a mechanism crucial to penile erection onset and maintenance. Therefore, the endothelial dysfunction-linked ED can be explained either by the increased breakdown of NO due to augmented production of reactive oxygen species (Valko et al. 2007) or, otherwise, by changes in expression and/or activity of eNOS (Wadley et al. 2013). Several factors are critically involved in the regulation of NO synthesis by eNOS. The main pathway of eNOS activation is the phosphoinositide-3-kinase (PI3K)/Akt-dependent phosphorylation on the Ser-1177 residue (Boo et al. 2002; Butt et al. 2000; Gratzke et al. 2010; Schleicher et al. 2009). Under basal conditions, eNOS is constitutively expressed in the endothelium; nevertheless, there are conflicting reports concerning its expression and activity along aging (Cernadas et al. 1998; Challah et al. 1997; Csiszar et al. 2002; Smith et al. 2006).

Vascular aging associates with changes in gene expression that are in part regulated by the NAD⁺-dependent protein deacetylase Sirtuin-1 (Sirt1) (Morris 2013; Zahn et al. 2007). Accumulating evidence suggests that Sirt1 acts as a longevity factor in vascular tissue, particularly due to its critical role in endothelial

homeostasis by regulating eNOS activity (Ota et al. 2010b). Sirt1 promotes endothelial-dependent vasodilatation by targeting eNOS for deacetylation, which increases NO production (Mattagajasingh et al. 2007). Likewise, eNOS has also been implicated in the regulation of the expression of Sirt1 (Nisoli et al. 2005), and NO itself could act as an inducer of Sirt1 activity in endothelium (Ota et al. 2010b).

Sirt1 is upregulated in tissues by energy restriction (ER) (Rippe et al. 2010), the reduction in energy intake without undernourishment. Apart from Sirt1 activation (Mattagajasingh et al. 2007), ER increases eNOS expression and activity, and reduces the expression of inducible NOS (iNOS) that synthesizes NO under inflammatory conditions (Park et al. 2012; Ungvari et al. 2008). In fact, numerous studies demonstrate that ER reduces atherosclerosis and improves endothelial function both in rodents (Csiszar et al. 2009; Kondo et al. 2009; Ungvari et al. 2008; Zanetti et al. 2010) and in humans (Fontana et al. 2004; Pierce et al. 2008) that results in amelioration of cardiovascular risk profile.

In opposition to ER healthy effects, the regular intake of high-fat diets (HFD) affects NO production and promotes inflammation (Martins et al. 2010). In line with that, we have previously shown that rats on a chronic HFD along aging develop MetS, noticeable by insulin resistance, hyperlipidemia, and high blood pressure, associated with an irreversible diminution of adiponectin secretion; conversely, ER prevents most of those changes and the underlying endothelial dysfunction. We have also found that HFD treatment led to the increase of connective tissue deposition in cavernous tissue, a structural modification reversed by HFD withdrawal (Tomada et al. 2013a). Thus, considering that diet pattern modulates not only metabolic status but also the structural organization of the cavernous tissue, and that the molecular mechanisms associated with age-related endothelial dysfunction remains unclear, we aimed to investigate the effects of long-term HFD versus ER on eNOS-Sirt1 axis, and Akt/eNOS phosphorylation in rat cavernous tissue.

Materials and methods

All animal procedures were undertaken according to the European Community Guidelines (86/609/EEC) and the Portuguese Act (129/92) for the use of experimental animals.

Animals Male Sprague–Dawley rats ($n=30$) (Charles River, Barcelona, Spain) were housed in single cages and maintained under controlled standard laboratory conditions (12:12 h light–dark cycle; 20–22 °C temperature; 40–60 % humidity). In brief, at 2 months old, rats were randomly divided into three experimental groups ($n=10$ per diet group): control group (C), rats with free access to a standard rat chow containing 4 % fat (mainly derived from fish oils), 76 % carbohydrate, and 20 % protein (A04, Panlab® SL, Barcelona, Spain); HFD group, rats with free access to a purified rodent diet with 45 % fat (derived from lard), 36 % carbohydrate, and 19 % protein (#58V8 TestDiet®, Purina Mills®, LLC/PMI Nutrition International®, Richmond, USA); energy-restricted group (ER), animals submitted to energy restriction, correspondent to 75 % of daily standard rodent chow individual intake of C rats. All animals were maintained in the same dietary pattern, with free access to tap water, until reach 6 or 18 months of age (young adult and old rats, respectively). The endpoint at 18 months was chosen as the maximum reasonable limit at which pain or distress, due to experimental manipulation or spontaneously occurring disease, is prevented or relieved.

Collection of samples Rats were euthanized by decapitation, after an overnight fast, when reached 6 or 18 months of age ($n=5$ for each subgroup of age). Trunk blood samples were collected for biochemical determinations. After centrifugation ($1,000\times g$ for 30 min at 4 °C), plasma was recovered and aliquots of 250 μL were stored at -80 °C until assays. The penises were removed after dissection from skin and surrounding fat, and divided in two fragments: one was immediately stored at -80 °C for molecular analysis, and the other was fixed at 10 % buffered formaldehyde overnight, dehydrated, and paraffin-embedded oriented along its transversal axis for immunohistochemical assays.

Plasma adiponectin, soluble vascular cell adhesion protein-1, soluble E-selectin, and oxidized low-density lipoprotein quantification Adiponectin levels were measured by RIA method in rat plasma from all experimental groups, as previously described (Tomada et al. 2013a). Soluble vascular cell adhesion protein-1 (sVCAM-1), soluble E-selectin (sE-selectin), and oxidized low-density lipoprotein (OxLDL) were also quantified in plasma of all animals by ELISA (CSB-

E07275r, CSB-E07996r, and CSB-E07632r Immunoassay, Cusabio®, Hubei, China, respectively), according to the manufacturer's instructions. All samples were analyzed in the same series by an investigator blinded to the sources of the samples, and quantifications were performed by measurement of optical density at 450 and 550 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, Waltham, MA, USA).

Assessment of total nitrite levels in plasma The determination of the total amount of NO was evaluated in rat plasma by indirect method based on measuring the levels of nitrite, a stable end product of NO metabolism, using the Nitric Oxide Quantitation Kit (Active Motif Europe, Rixensart, Belgium). Briefly, total nitrite levels were determined as an index of NO generation in 70 μL of filtered rat plasma by the Griess reaction after conversion of nitrate into nitrite by nitrate dehydrogenase, according to the manufacturer's instructions. Quantifications were performed by measurement of optical density at 550 and 630 nm using a plate reader (Thermo Electron Corporation).

Immunofluorescence studies in rat cavernous tissue Immunofluorescence (IF) detections of eNOS or Sirt1 and α -actin were performed. Briefly, 4–6 μm thick tissue sections placed on 0.1 % poly-L-lysine-covered microscopy slides were deparaffinized, rehydrated, exposed to 1 M HCl for epitope retrieval, and neutralized with 0.1 M borax, followed by incubation for 1 h with blocking solution (1 % bovine serum albumin in phosphate-buffered saline, PBS). Sections were incubated overnight at 4 °C with a mixture of primary antibodies: rabbit anti-eNOS (Santa Cruz Biotechnology, CA, USA) or rabbit anti-Sirt1 (Santa Cruz Biotechnology) with mouse anti- α -actin (Chemicon International, Temecula, CA, USA). Negative controls were performed by primary antibodies omission. After washing twice with PBS, the sections were incubated at room temperature, with a suitable mix of secondary antibodies, anti-rabbit conjugated with AlexaTM 488 (green) and anti-mouse conjugated with AlexaTM 568 (red) (Molecular Probes, Leiden, Netherlands). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) (Molecular Probes). Sections were mounted in buffered solution of glycerol, observed in an ApoTome fluorescence microscope (Imager.Z1, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and images acquired with

AxionVision® software (Carl Zeiss MicroImaging GmbH).

Histomorphometric assessment of eNOS and Sirtuin-1 immunolabeled area in rat cavernous tissue In all experimental groups ($n=5$), two random slides of cavernous tissue sections for animal, immunolabeled for eNOS and Sirt1, were analyzed using the $\times 20$ objective of ApoTome fluorescence microscope (Imager.Z1, Carl Zeiss). Three to four randomly chosen immunostained fields, representative of the entire cavernosal tissue, were captured per slide, summing 30–40 fields per age group. The green fluorochrome (AlexaTM 488) intensity was detected at excitation and emission wavelengths of 495 and 519 nm, respectively, and all the images were captured in parallel with identical settings. Collected images were transferred to a computer and the cavernosal eNOS and Sirt1 immunolabeled area were quantified directly using the ImageJ® software (National Institutes of Health, Bethesda, MD, USA). In brief, the fluorochrome staining of each image was analyzed after setting thresholds for automated area stained positive. The number of pixels labeled with the fluorochrome in each image was determined as a percentage of the total image area. The mean value of the measurements was calculated per animal and then the results were used to calculate the average percentage for each experimental group. The image analysis was carried out blindly by a single researcher. Quantification of eNOS and Sirt1 was performed on raw images, which were unaltered throughout the analysis.

Western blotting For protein analysis, penile cavernous tissue fragments were mechanically homogenized in 50 mM Tris–HCl pH 7.2, 0.1 M NaCl, 5 mM EDTA, 0.5 % Triton X-100, supplemented with 2 % Protease Inhibitor Cocktail P8340 (Sigma-Aldrich Co, Dorset, UK), and 0.2 % Phosphatase Inhibitor Cocktails 1 and 2 (P2850 and P5726, respectively; Sigma-Aldrich Co). After total protein levels determination by Bradford Protein assay (BioRad Laboratories, CA, USA), 20 μ g protein/lane from each sample was loaded on 8 % sodium dodecyl sulfate-polyacrylamide gel, separated using the Laemmli (1970) discontinuous buffer system (SDS-PAGE), and transferred to nitrocellulose membrane with 0.45 μ m pore (BioRad Laboratories) for 2 h. After incubation with blocking solution (5 % nonfat milk Molico® in 0.1 % Tween-20 Tris buffer

saline, TBS) for 1 h, the membranes were immunoblotted with the following primary antibodies: rabbit anti-phospho-eNOS (Ser-1177) (Cell Signaling Technology, MA, USA), rabbit anti-phospho-eNOS (Ser-615) (Antibodies-Online GmbH, Aachen, Germany), rabbit anti-phospho-Akt (Ser-473) (Cell Signaling Technology), rabbit anti-iNOS (Abcam, Cambridge, UK), and rabbit anti-Sirt1 (ProteinTech Inc., IL, USA), diluted in 5 % bovine serum albumin 0.1 % TBS, overnight at 4 °C with shaking. After extensive washing and incubation with appropriate secondary antibodies coupled to horseradish peroxidase, labeled bands were evidenced using chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, IL, USA). Membranes used for phospho-eNOS (Ser-1177 and Ser-615) and phospho-Akt (Ser-473) detection were then stripped and reprobed with antibodies for the index protein: rabbit anti-eNOS (Santa Cruz Biotechnology) and rabbit anti-Akt (Cell Signaling Technology), respectively. Phospho-eNOS (Ser-1177 or Ser-615) and phospho-Akt bands densities were normalized relative to their corresponding total protein forms, as phosphorylation of eNOS on Ser-1177 or Ser-615, and Akt on Ser-473 is coincident with the activation of the enzymes, and thus the ratio of phospho- to total enzyme expression represents relative content of activated forms. Normalization of total eNOS, total Akt, iNOS, and Sirt1 was performed using β -actin as loading control (rabbit anti- β -actin, Abcam). Each experiment was repeated three times and performed on all samples of each experimental group. The immunoblots were visualized in a ChemiDoc™ XRS apparatus (BioRad Laboratories), and intensity of the bands was quantified by densitometry using the Image Lab® software (BioRad Laboratories). Mean density values of each protein level in 6 and 18 months HFD, ER, and C groups are indicated in arbitrary units.

Statistical analysis All descriptive data are expressed as mean value $\pm$ SEM. Analysis of variance (ANOVA), followed by Tukey's post hoc test was used to compare biochemical data, as well as protein band densitometry, within and between groups. Unpaired Student's *t* test or Mann–Whitney *U* test was also used when appropriated. The correlation between plasma adiponectin with cavernous tissue protein levels of Phospho-eNOS at Ser-1177, Phospho-eNOS at Ser-615, total eNOS, iNOS, and Sirt1, was done based on correlation analysis (Pearson coefficients). Statistical analysis was performed using GraphPad Prism® software (GraphPad Software Inc.,

La Jolla, CA, USA). A probability value of $P<0.05$ was accepted as statistically significant.

Results

Circulating adiponectin, soluble vascular cell adhesion protein-1, soluble E-selectin, and oxidized low-density lipoprotein levels in rat plasma Plasma concentrations of adiponectin, and inflammatory markers, sVCAM-1, sE-selectin, and OxLDL from all experimental groups are depicted in the Fig. 1. Data are presented as a percentage of the respective age-matched controls (6- and 18-month C groups considered 100 %), since no significant variations were observed between 6- and 18-month controls for any of the studied molecules.

Energy-restricted animals presented the highest levels of plasma adiponectin comparatively to either age-matched HFD ($P<0.001$ and $P=0.005$, for 6 and 18 months, respectively) or controls ($P=0.002$ and $P=0.007$, for 6 and 18 months, respectively) (Fig. 1a). As previously verified (Tomada et al. 2013a), HFD consumption induced an important decrease in adiponectinemia compared to controls ($P<0.001$ and $P=0.003$, for 6 and 18 months, respectively).

Soluble VCAM-1 levels were relatively constant between groups (ANOVA, $P=0.258$) (Fig. 1b). However, at both 6 and 18-month endpoints, HFD animals tend to present an average increase of 20 % in sVCAM-1 levels relative to age-matched controls (119.7 ± 22.1 % and 120.1 ± 19.5 %, for 6 and 18 months, respectively). In opposition, sVCAM-1 levels exhibited by 6-month ER animals did not differ from those from HFD ($P=0.432$) and controls ($P=0.399$). However, 18-month ER animals had the lowest levels of this adhesion molecule, significantly different from those observed at age-matched controls ($P=0.002$). E-selectin circulating levels were significantly different between groups (ANOVA, $P<0.001$) (Fig. 1c). At 6-month endpoint, both ER and HFD rats presented an important decrease of sE-selectin concentrations compared to controls (70.8 ± 9.2 %, $P=0.043$ and 53.7 ± 5.2 %, $P=0.001$, for ER and HFD, respectively). However, the prolonged treatment with HFD increased sE-selectin levels about 2.6-fold relative to 6-month HFD rats ($P=0.001$), whereas long-term ER did not change the levels of this molecule in rat plasma in

comparison to 6-month ER treatment ($P=0.081$). The augmented levels of sE-selectin in older HFD animals were significantly different from those verified in age-matched ER animals (131.5 ± 20.0 versus 59.2 ± 5.9 %, $P=0.005$) and controls ($P<0.001$).

Oxidized LDL levels also vary between groups (ANOVA, $P=0.022$) (Fig. 1d). Broadly, HFD intake tends to induce an important increment in plasma concentrations of OxLDL compared to age-matched C rats (205.9 ± 41.8 % and 140.8 ± 19.5 %, for 6 and 18-month HFD animals, respectively). However, due to high intraindividual variations, the average increase did not reach statistical significance ($P=0.085$ and $P=0.503$, for 6 and 18 months, respectively). Conversely, 6-month ER animals presented low levels of OxLDL in comparison to either 6-month HFD ($P=0.004$) and 6-month C ($P<0.001$). Furthermore, along ER treatment, plasma OxLDL concentrations increase, as observed between 6- and 18-month ER rats ($P<0.001$).

Total nitrite plasma levels Total nitrite levels, an indirect index of NO production, assessed in rat plasma, were significantly different between groups (ANOVA, $P<0.001$) (Fig. 2). At 6 and 18 months, ER animals presented the highest levels of nitrites comparatively to either HFD ($P=0.032$ and $P=0.001$, for 6 and 18 months, respectively) and C rats ($P=0.017$ and $P=0.049$, for 6 and 18 months, respectively). No significant differences were registered neither within HFD ($P=0.081$) and controls groups ($P=0.101$) nor between HFD and C rats ($P=0.658$ and $P=0.263$, for 6 and 18 months, respectively). Conversely, long-term ER led to a significant increase in nitrite levels when compared to those found at 6-month endpoint ($P=0.001$).

Dual immunolabeling of eNOS/alpha-actin and Sirt1/alpha-actin Characterization of eNOS and Sirt1 expression in rat cavernous tissue of all experimental groups was done by IF detection of these proteins simultaneously with the specific SMC marker, alpha-actin. Endothelial NOS (green) and alpha-actin (red) dual-immunolabeling (Fig. 3a) revealed that besides eNOS expression in endothelial cells (labeled green in close proximity to VSMC layer) it was also detected in VSMC, as evidenced by the clear co-localization (yellow) verified in 6- and 18-month C rats. No noticeable differences in intensity of eNOS expression were

Fig. 1 Plasma adiponectin (**a**) (Tomada et al. 2013a), soluble vascular cell adhesion protein-1 (sVCAM-1) (**b**), soluble E-selectin (**c**), and oxidized low-density lipoprotein (OxLDL) (**d**) levels in rats from all experimental groups. Values represent the mean value of each molecule as a percentage of the respective

age-matched controls (6- and 18-month C groups considered 100 %). Error bars indicate the standard error of the mean. Controls, control group; HFD, high-fat diet group; ER, energy-restricted group; * $P<0.05$, ** $P<0.05$ versus age-matched C group, § $P<0.05$ versus age-matched HFD group

detected among HFD and ER groups. Sirt1 (green) and alpha-actin (red) IF detection (Fig. 3b) demonstrated that Sirt1 is clearly expressed both in endothelium and in cavernous VSMC, presenting co-localization with alpha-actin (yellow) in all experimental groups. The specificity of all the primary antibodies was confirmed by the absence of staining in the negative controls.

Histomorphometric evaluation of the effects of high-fat diet consumption and energy restriction on the eNOS and Sirtuin-1 expression in the rat cavernous tissue Subsequent to immunolabeling of eNOS and Sirt1 by IF (panel a and b of Fig. 3, respectively), the differences in their expression among experimental groups were assessed by histomorphometry (Fig. 3c, d). As illustrated by representative images (Fig. 3a) and quantitative analyses (Fig. 3c), no significant differences in the percentage of eNOS staining density among groups (ANOVA, $P=0.528$) were noted. Conversely, the density of

Sirt1 staining varied noticeably (ANOVA, $P<0.001$) (Fig. 3d) among groups. At identical settings, the percentage of the Sirt1 immunolabeled area of cavernous tissue from HFD animals was markedly increased when compared with controls ($P=0.042$ and $P=0.002$, for 6 and 18 months, respectively). In ER groups, values were higher when compared to age-matched C ($P=0.014$ and $P=0.043$, for 6 and 18 months, respectively). No differences were observed between 6- and 18-month C animals ($P=0.876$), in contrast with the increase in HFD animals (from 1.9 ± 0.2 to 4.2 ± 0.9 %, $P=0.038$) and the decrease observed in ER animals (from 2.1 ± 0.2 to 1.4 ± 0.1 %, $P=0.014$).

Long-term high-fat diet and energy restriction effects on phosphorylation of eNOS and Akt in rat cavernous tissue Phosphorylation and total protein levels of eNOS and Akt were assessed by WB (Fig. 4a). Representative blots of the variations obtained in the content of these proteins are presented, and relatively densitometric means

Fig. 2 Plasma total nitrite levels in rats from all experimental groups. Error bars indicate the standard error of the mean. Controls, control group; HFD, high-fat diet group; ER, energy-restricted group; * $P<0.05$, ** $P<0.05$ versus age-matched C group, § $P<0.05$ versus age-matched HFD group

are detailed in the graphs (Fig. 4b, c). No noticeable variations were observed between 6- and 18-month C groups for any of the studied proteins; thus, we considered values relative to expression of each protein in HFD and ER groups as a percentage of the respective age-matched controls (6- and 18-month C groups, 100 %).

It was observed a significant increase in phosphorylation of eNOS at Ser-1177 in the cavernous tissue of 6-month ER animals (177.1 %) compared with age-matched C ($P=0.007$). However, at 18-month endpoint, both ER and HFD rats presented a considerable diminution in phospho-eNOS levels comparatively to C animals (21.8 %, $P=0.026$, and 21.7 %, $P=0.004$, for 18-month ER and HFD, respectively). Over the time, HFD and ER experimental conditions also led to the decrement of phosphorylated eNOS levels ($P=0.014$ and $P=0.008$, for 6- versus 18-month ER and HFD, respectively). Regarding the other putative site of phosphorylation of eNOS, Ser-615, no significant differences were observed within HFD and ER experimental groups ($P=0.570$ and $P=0.097$, for 6- versus 18-month HFD and ER, respectively), despite the tendency to increase along aging in ER-treated rats. In fact, the older ER rats presented the highest levels of this phosphorylated enzyme that, however, only significantly differ from those observed in 18-month controls ($P=0.042$). Additional dissimilarities between groups

exist (ANOVA, $P=0.018$). Six-month HFD animals presented an important increment in phospho-eNOS-Ser-615 levels (227.0 %) compared to age-matched ER (128.1 %, $P=0.029$) and C rats (100.0 %, $P=0.042$). Although long-term consumption of HFD tended to reduce slightly the levels of phospho-eNOS at Ser-615 (a reduction of 12.5 %), they have remained 2.2-fold high by comparison with those observed in C rats ($P=0.030$), but reduced when compared to that observed in 18-month ER group (218.5 versus 273.3 %, $P=0.544$). No statistical differences were observed between experimental groups of both ages in total eNOS protein expression (ANOVA, $P=0.629$).

Concerning phosphorylation of Akt at Ser-473, densitometric analysis demonstrated a significant increase in cavernous tissue of 6-month ER rats (148.2 %) compared either with age-matched HFD (96.4 %, $P=0.007$) or C animals (100.0 %, $P=0.015$). Although no differences were observed between HFD and C groups in 6 months ($P=0.743$), a considerable decrease in HFD phospho-Akt levels were verified at 18 months (73.5 %, $P=0.006$) compared to controls. Conversely, 18-month ER rats presented a significantly higher expression of phospho-Akt than observed in 18-month HFD rats (107.7 versus 73.5 %, $P=0.007$). Total Akt protein expression did not exhibit statistical differences between groups (ANOVA, $P=0.127$).

High-fat diet and energy restriction affect cavernous tissue expression of iNOS Cavernous tissue levels of iNOS protein were assessed by WB. Representative bands of iNOS detection in all experimental groups are shown (Fig. 5a) and densitometric analysis is detailed in the graph (Fig. 5b). Taking into account that no variation between 6- and 18-month C groups was observed ($P=0.289$), a percentage of levels of iNOS expression in animals of different diet protocols relative to age-matched C groups (100 %) is indicated. Although not statistically significant, due to the interindividual variations, HFD animals presented higher levels of iNOS protein than age-matched C (175.7 %, $P=0.213$, and 182.2 %, $P=0.317$, for 6 and 18 months, respectively). Conversely, the lowest levels were verified in ER animals, with a significant decrease in 6-month ER animals (43.7 %) compared to HFD (175.7 %, $P=0.038$) and C (100.0 %, $P=0.016$) rats, and in 18-month ER compared to 18-month HFD rats (57.4 versus 182.2 %, $P=0.047$). No significant

differences were found within HFD- or ER-treated rats ($P=0.777$ and $P=0.835$, respectively).

Alterations in Sirtuin-1 levels profile on cavernous tissue from high-fat-fed and energy-restricted rats Western blot representative bands of Sirt1 detection in all experimental groups are depicted in Fig. 6a (densitometric analysis is presented in the graph, Fig. 6b). Although a drop in Sirt1 expression was found in control animals from 6 to 18 months, no significant differences

were observed ($P=0.087$). In opposition, HFD led to a very important increment in cavernous tissue Sirt1 levels, apparently in a time-dependent manner. When compared with age-matched C groups, the intensity of Sirt1 bands were 2.5-fold higher in 6-month HFD rats ($P=0.045$) and 29-fold higher in 18-month HFD animals ($P=0.007$). Concerning ER groups, we found a significant reduction in this protein from 6 to 18 months ($P=0.039$); nevertheless, levels were still high

Fig. 3 Dual immunolabeling by immunofluorescence of eNOS (green) and alpha-actin (red) (a), and Sirtuin-1 (green) and alpha-actin (red) (b) in the rat cavernous tissue of all experimental groups. The nucleus is stained blue (DAPI). The graphs show the quantification of the cavernosal eNOS (c) and Sirt1 (d) fluorochrome stained area (green labeled), performed in all samples of each experimental group. Values are presented as mean percentage of total image area for each experimental group. Error bars indicate the standard error of the mean. *Controls*, control group; *HFD*, high-fat diet group; *ER*, energy-restricted group; * $P<0.05$, ** $P<0.05$ versus age-matched C group, § $P<0.05$ versus age-matched HFD group

Fig. 4 Representative bands obtained by Western immunoblotting analysis of phospho-eNOS-Ser-1177, phospho-eNOS-Ser-615, total eNOS, phospho-Akt-Ser-473, and total Akt in the rat cavernous tissue homogenates of all experimental groups (a). The graphs represent the semiquantitative analysis of phospho-eNOS and total eNOS (b), and phospho-Akt and total-Akt proteins (c), performed in all samples of each experimental group. Phospho-eNOS (Ser-1177 and Ser-615) and total eNOS levels were normalized with total-eNOS and β -actin density in each

sample, respectively. Phospho-Akt-Ser473 and total-Akt levels were normalized with the correspondent Total-Akt and β -actin band, respectively. Values represent the mean value of each protein in rat cavernous tissue from all experimental conditions, indicated in arbitrary units. Error bars represent standard error of the mean. Controls, control group; HFD, high-fat diet group; ER, energy-restricted group; * $P<0.05$, ** $P<0.05$ versus age-matched C group, § $P<0.05$ versus age-matched HFD group

compared with age-matched of 6 months (3.9-fold, $P=0.004$) and 18 months (7.9-fold, $P=0.005$).

Correlation between serum adiponectin with phosphorylation of eNOS at Ser-1177 and at Ser-615, total eNOS, iNOS, and Sirtuin-1 levels Independently of the diet and the age of the animals, a positive correlation was found between serum adiponectin concentrations and the levels of phospho-eNOS at Ser-1177 detected in rat cavernous tissue ($r=+0.723$, $P<0.001$). No correlation was verified neither for phospho-eNOS at Ser-615 ($r=+0.084$, $P=0.817$) nor for the total levels of the enzyme ($r=-0.175$, $P=0.437$).

When this statistical analysis was done considering the diet pattern, the values of phospho-eNOS at Ser-1177 and total eNOS correlate with adiponectin levels in blood in control rats ($r=+0.843$, $P=0.017$ and $r=-0.674$, $P=0.046$, for phospho-eNOS-Ser-1177 and total eNOS, respectively), but not in HFD or in ER animals. In opposition, phospho-eNOS at Ser-615 directly correlates with adiponectin levels only in HFD-treated rats ($r=+0.187$, $P=0.035$) (Table 1). Concerning Sirt1 levels in the cavernous tissue, it was also observed a significant positive correlation with adiponectin ($r=+0.597$, $P=0.004$), independently of the diet treatment and its duration (Table 1). Sirt1 levels were particularly

Fig. 5 Representative bands obtained by Western immunoblotting analysis of iNOS in the rat cavernous tissue homogenates of all experimental groups (a). The graph represents the semiquantitative analysis of iNOS performed in all samples of each experimental group, after normalization with respective β -actin density of each sample (b). Values represent the mean value of each protein in rat cavernous tissue from all experimental conditions, indicated in arbitrary units. Error bars represent standard error of the mean. Controls, control group; HFD, high-fat diet group; ER, energy-restricted group; ** $P < 0.05$ versus age-matched C group, § $P < 0.05$ versus age-matched HFD group

correlated with high levels of adiponectin in ER animals ($r = +0.885$, $P = 0.004$). Although a negative correlation between adiponectinemia and iNOS protein levels was observed ($r = -0.170$), it did not reach statistical significance ($P = 0.486$).

Discussion

Excessive dietary fat intake is an important contributor to the pathogenesis of endothelial dysfunction (Song et al. 2006). Although diets rich in simple carbohydrate, such as sucrose, promote cardiovascular complications (Bosse et al. 2013), HFD has traditionally been

Fig. 6 Representative bands obtained by Western immunoblotting analysis of Sirtuin-1 in the rat cavernous tissue homogenates of all experimental groups (a). The graph represents the semiquantitative analysis of Sirtuin-1 performed in all samples of each experimental group, after normalization with respective β -actin density of each sample (b). Values represent the mean value of each protein in rat cavernous tissue from all experimental conditions, indicated in arbitrary units. Error bars represent standard error of the mean. Controls, control group; HFD, high-fat diet group; ER, energy-restricted group; * $P < 0.05$, ** $P < 0.05$ versus age-matched C group, § $P < 0.05$ versus age-matched HFD group

associated with obesity and MetS development in rodent models (Buettner et al. 2007). In fact, the HFD-induced insulin resistance associated with dyslipidemia and hypertension, often present independently of the degree of body weight gain (Naderali et al. 2001; Tomada et al. 2012, 2013a), constitute key features of the MetS that impair endothelial NO bioavailability (Huang 2009). Although the effect is systemic, the early clinical manifestations occur in penile vascular bed; this finding led to consider vasculogenic ED as early sign, rather than a late consequence of advanced atherosclerosis (Yao et al. 2012). Hence, we employed cavernous tissue from a rat model of HFD-induced

Table 1 Correlation coefficients of serum adiponectin concentrations with phosphorylated eNOS at Ser-1177, phosphorylated eNOS at Ser-615, total eNOS, iNOS, and Sirtuin-1, evaluated by immunoblot densitometry

		Serum adiponectin			
		Age-independent		Age- and diet-independent	
		Pearson <i>r</i>	<i>P</i> value	Pearson <i>r</i>	<i>P</i> value
Phospho-eNOS (Ser-1177)	C	+0.843	0.017*	+0.723	<0.001*
	HFD	−0.251	0.588		
	ER	+0.236	0.523		
Phospho-eNOS (Ser-615)	C	+0.645	0.081	+0.084	0.817
	HFD	+0.187	0.035*		
	ER	+0.434	0.324		
Total eNOS	C	−0.674	0.046*	−0.175	0.437
	HFD	+0.261	0.572		
	ER	+0.619	0.190		
iNOS	C	−0.511	0.301	−0.170	0.486
	HFD	−0.639	0.172		
	ER	−0.017	0.972		
Sirtuin-1	C	−0.281	0.542	+0.597	0.004*
	HFD	+0.344	0.504		
	ER	+0.885	0.004*		

* $P < 0.05$ (Significant correlation)

MetS previously characterized (Tomada et al. 2013a) to study the molecular mechanisms by which the chronic ingestion of a diet rich in saturated fats contributes to endothelial dysfunction. In addition, since ER delays the onset of age-associated diseases, including CVDs, we added this dietary regimen for comparison.

Endothelial-derived NO synthesis is ensured by eNOS, an essential enzyme for endothelial repair and regeneration, with known anti-inflammatory and anti-thrombotic effects (Cau et al. 2012). However, NO bioavailability does not depend exclusively on total levels of eNOS in tissues (El-Sakka et al. 1999; Felaco et al. 2001) but, instead, on its activity which, in vivo, is modulated by multiple mechanisms that include PI3K/Akt-dependent phosphorylation (Boo et al. 2002; Butt et al. 2000; Dimmeler et al. 1999; Lovren et al. 2009). Thus, considering the Akt-role in eNOS activation (Schleicher et al. 2009), we investigated the pattern of phosphorylation of both Akt and eNOS in cavernous tissue from HFD and ER animals.

No changes in cavernous tissue total eNOS and Akt levels were found among experimental groups. In contrast, phosphorylation levels of Akt and eNOS varied with diet conditions and the duration of treatment. In young adult rats (6 months), while

HFD induced an increase in phosphorylation of eNOS at Ser-1177 and Ser-615 residues, in older HFD animals (18 months) there was a reduction in phospho-Akt and phospho-eNOS-Ser-1177, but an enhancement of eNOS Ser-615 regulatory site activation. However, just as the upregulation of eNOS Ser-1177 phosphorylation alone has lesser ability to increase NO synthesis (when compared with simultaneous Ser-1177 and Ser-615 phosphorylation), the simple upregulation of phospho-eNOS-Ser-615 was not sufficient to enhance NO bioavailability under HFD conditions, as supported by total nitrite concentrations. Altogether, these findings are in agreement with the derangements previously found in metabolic status of HFD rats, as increased blood pressure, insulin resistance, hyperlipidemia, and adiposity (Tomada et al. 2013a), that independently compromise endothelial NO bioavailability (Huang 2009; Razny et al. 2011), and lead to ED onset (Kim et al. 2006).

High-fat-fed rats also present low levels of circulating adiponectin (Tomada et al. 2013a) which agrees with reduced eNOS phosphorylation, since adiponectinemia and eNOS activity, consequent to phosphorylation at Ser-1177, seem to be reciprocally related (Hattori et al. 2005). In fact, hypoadiponectinemia was noticed in HFD mice

with impaired endothelium-dependent vasodilation (Ouchi et al. 2003), possibly as a result of local ceramide accumulation that leads to disruption of the signaling kinases that phosphorylate eNOS at positive regulatory sites (Symons and Abel 2013).

The metabolic and molecular profiles of HFD animals contrast with the healthier cardiometabolic features of ER-treated rats, such as higher adiponectinemia (Tomada et al. 2013a) and higher index of NO production. In cavernous tissue, ER clearly activates Akt/eNOS axis. Even though a significant drop in phospho-eNOS-Ser1177 was found with prolonged ER treatment (18-month rats), the rate of production of NO seems to be compensated by the enhanced eNOS activation by phosphorylation at Ser-615, as reported by Bauer et al. (2003). In accordance, it is demonstrated that in conditions of higher adiponectin (ER groups), NO bioavailability is presumably greater, consistent with the protective role of adiponectin in the superoxide-mediated degradation of NO (Motoshima et al. 2004). These results highlight the vasculoprotective action of ER mediated by the adiponectin–eNOS axis (Iwashima et al. 2006; Lago et al. 2007).

ER increases eNOS activity mainly through activation of the deacetylase Sirt1, a key regulator of vascular endothelial function (Mattagajasingh et al. 2007), implicated in ER-associated adjustment of insulin sensitivity (Csiszar et al. 2009), and in the inhibition of endothelial senescence by activation of PI3K/Akt pathway (Ota et al. 2010a). Previous studies in rat aorta evidenced that, in contrast with short-term treatment (Zanetti et al. 2010), long-term ER stimulates Sirt1-mediated eNOS expression and activity (Csiszar et al. 2009). In line with that, an important increment in Sirt1 that for the first time was shown in rat cavernous tissue in ER animals compared to controls was found at any age. Nevertheless, a considerable diminution was verified along ER treatment. This interesting result does not exclude Sirt1 enhancement of eNOS activity, but quantitative evaluation of acetyl-lysine residues on eNOS will be necessary to elucidate this aspect.

The exact role of Sirt1 in mammals remains largely controversial, particularly when its expression levels and/or activity are compared among different tissues and experimental models (including animals under different dietary patterns and knockout/transgenic mice). In the vascular system, the enhanced expression of Sirt1 protects against HFD-induced endothelial dysfunction (Zhang et al. 2008) and hyperglycemia-induced vascular

cell senescence in aorta (Chen et al. 2012). In mice hearts however, extremely high levels of Sirt1 (12.5-fold or higher) induced heart failure, local increase of oxidative stress, apoptosis, and fibrosis, outcomes that contrast to the protective effect of moderate levels of overexpression (2.5- to 7.5-fold) (Alcendor et al. 2007; Kawashima et al. 2011). In the rat's cavernous tissue, a similar regulation is likely to be operating. Indeed, while the 2.5-fold increase in the Sirt1 level of young HFD rats may be compensatory and protective (Banks et al. 2008; Pfluger et al. 2008), the near 30-fold Sirt1 level increase in the older HFD probably relates to serious tissue dysfunction. This point agrees with the lower circulating adiponectin and the trend of higher levels of systemic markers of endothelial activation/dysfunction (sVCAM-1, sE-selectin, and OxLDL). This hypothesis also agrees with previous demonstration of extensive connective tissue deposition in cavernous tissue and myocardium of rats in similar experimental conditions (Tomada et al. 2013a), and in the human cavernous tissue of androgen-depleted young individuals that present upregulation of Sirt1 (Tomada et al. 2013b).

An additional support to the HFD unwanted effects was provided by a noticeable upregulation of iNOS in cavernous tissue. In fact, while constitutively eNOS-derived NO affords endothelial protection, iNOS-derived NO is associated with tissue injury, inflammation, enhanced oxidative stress, and increased arterial vasoconstriction (Azadzi et al. 2004; Chung et al. 2002; van der Loo et al. 2000). The increment in iNOS expression is thus consistent with the increase in systemic inflammatory markers that are maintained in balance between circulation and tissues, accumulating in lesions, which strongly contribute to vascular dysfunction in cavernous tissue (Cau et al. 2012; Csiszar et al. 2008; Ungvari et al. 2008). In opposition to HFD, ER leads to a concomitant decrease in iNOS expression and inflammatory markers.

Hence, HFD contrasts with ER impact on rat cavernous tissue from an early age. The consumption of HFD induces inflammation and oxidative/nitrative stress, abnormal increase in Sirt1 levels, and likely a compensatory activation of eNOS. This pattern persisted in older animals, but the overall increase in phospho-eNOS was not followed by a proportional enhancement in NO bioavailability. In contrast, ER older rats evidenced low levels of pro-inflammatory markers, moderate increase of Sirt1, and higher index of NO. However, it is still uncertain how ER treatment

may prevent ED, considering that an extensive evaluation of endothelial function and intracavernous pressure to assess erectile capacity will be necessary to elucidate this aspect.

Conclusions

Taken together, our findings underscore that long-term HFD consumption leads to a considerable increase of systemic pro-inflammatory molecules (sVCAM-1, sE-selectin, and OxLDL). In the cavernous tissue, it leads to Akt-mediated eNOS activation without a corresponding NO production enhancement. That dietary pattern also induces overexpression of iNOS and of the deacetylase Sirt1 to levels considered harmful to cells and to metabolic regulation. These changes may be preventable through long-term ER as it activates eNOS by Ser-615 phosphorylation.

Overall, long-term nutritional conditions override the influence of age in the regulation of eNOS expression and activation in cavernous tissue. A clear understanding of aging-associated molecular modifications, and how it could be regulated by nutritional factors, certainly will contribute to defining specific diet-based recommendations that must begin well before the onset of any noticeable symptom.

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

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