The triterpenoid alpha, beta-amyrin prevents the impaired aortic vascular reactivity in high-fat diet-induced obese mice

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Abstract To characterize the protective effects of the triterpenoid mixture alpha, beta-amyrin (AMY, 20 mg/kg, during 15 days) on the reactivity of isolated aorta of high-fat diet (HFD)-induced obese mice. Male Swiss mice were fed with HFD or normal diet (ND) for 15 weeks. Contractions of thoracic aorta in response to KCl or phenylephrine (PHE) and relaxation by acetylcholine (ACh) or sodium nitroprusside (SNP) were analyzed. HFD-fed mice developed hyperglycemia, hyperlipidemia, and significant body weight gain, parameters prevented by AMY treatment. Whereas aortic contractility did not differ in response to KCl, contractions induced by PHE (1 μ M) as well as relaxation induced by ACh (1–30 μ M) or SNP (1 nM–0.1 mM) on PHE-contracted aorta were decreased ($p < 0.05$) in tissues of HFD compared to ND mice, phenomenon significantly ($p < 0.05$) diminished in HFD mice treated with AMY. The relaxant actions of ACh and SNP were inhibited ($p < 0.05$) by tetraethylammonium (TEA, 5 mM), apamin (0.1 μ M), and 4-aminopyridine (4-AP; 3 mM) in aortae from ND group, but not from HFD. Treatment of HFD mice with AMY rescued the inhibitory effect of TEA ($p < 0.05$) on vasorelaxant

actions of ACh and SNP. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) inhibited similarly the relaxant effects of SNP in all groups. 8-Br-cGMP relaxed with similar profile aortae of all groups. By preventing HFD-induced obesity in mice, AMY rescued the blunted contractile response to PHE, and the attenuated vasorelaxation and K⁺ channel activation (opening) induced by ACh and SNP in isolated aorta.

Keywords Alpha, beta-amyrin · High fat diet · Obesity · Aortic vascular reactivity · Thoracic aortic rings

Abbreviations

ACh	Acetylcholine
4-AP	4-aminopyridine
AMY	Alpha, beta-amyrin
ANOVA	Analysis of variance
BKCa	Large-conductance Ca ²⁺ -activated potassium channel
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GLIB	Glibenclamide
HFD	High-fat diet
K _{ATP}	ATP-sensitive potassium channel
K _v	Voltage-dependent potassium channel
MKHS	Modified Krebs-Henseleit solution
ND	Normal diet
NO	Nitric oxide
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

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PHE	Phenylephrine
PSS	Physiological salt solution
SD	Standard deviation
SEM	Standard error of mean
SK	Small conductance Ca^{2+} -activated potassium channel
SNP	Sodium nitroprusside
TEA	Tetraethylammonium

Introduction

Past studies have suggested that a high-fat diet (HFD) is likely involved in the development of vascular disease caused by obesity (Barton and Furrer 2003; Mundy et al. 2007; Costa et al. 2011; Nascimento et al. 2011). Obesity increases the risk of the development of metabolic and cardiovascular diseases due to a disruption in adipose tissue function, leading to a chronic inflammatory state (Van de Voorde et al. 2014). Pro-inflammatory cytokines locally produced by perivascular fat and the dysregulation of the endocrine and paracrine actions of adipocyte-derived factors such as leptin, adiponectin, and resistin (Kougiyas et al. 2005; Liu et al. 2010) contribute to endothelial dysfunction, smooth muscle cell dysfunction, and the pathogenesis of vascular disease in obese individuals (Iantorno et al. 2014). Studies have shown an association between low adiponectin and high leptin levels with obesity-related metabolic disturbances and the incidence of coronary heart disease (Shanker et al. 2012). Therefore, new compounds that could ameliorate visceral adiposity and vascular alterations are desired to improve the quality of treatment for obesity.

Presently, there is a growing interest in and awareness of the roles of herbal medicines in the treatment and prevention of cardiovascular diseases (Walden and Tomlinson 2011). Herbal compounds rich in triterpenoids are well-known regulators of glucose and lipid metabolism and have beneficial effects on metabolic disorders and the prevention of obesity (Sudhahar et al. 2006; de Melo et al. 2009; de Melo et al. 2010; Rao et al. 2011; Castellano et al. 2013; Cai et al. 2014). Studies have demonstrated the potential of triterpenoids from olive oil as a therapeutic strategy in preventing vascular injury and the progression of cardiovascular diseases (Xiang et al. 2012). We have previously reported on the anti-obesity and hypolipidemic properties of the resin from *Protium heptaphyllum* and its major triterpenoid constituents, alpha- and beta-amyrin (AMY), in mice subjected to an HFD for 15 weeks (Carvalho et al. 2015; Carvalho et al. 2017). We have previously demonstrated the anti-obesity and pro-weight-loss potential of AMY, largely attributed to its modulatory effects on various hormonal and enzymatic secretions related to fat and carbohydrate metabolism and to the regulation of obesity-associated inflammation (Carvalho et al. 2015); however,

whether AMY can offer protective effects against the influences of obesity on vascular reactivity has not been established. It is well known that endothelial dysfunction plays an essential role in the initiation of the cascade of events leading to cardiovascular risk factors (Reriani et al. 2010), and a number of pathological conditions are associated with endothelial dysfunction, including diabetes, dyslipidemia, and obesity (Boger et al. 1997; Virdis et al. 2013; Wilk et al. 2013). Porto et al. (2010) reported that maternal diabetes produced alterations in K^+ channel activity on the endothelium-dependent aortic relaxation induced by acetylcholine in adult offspring rats. In the present study, we aimed to analyze the contractile and relaxant vascular responses of thoracic aortas collected from mice fed with a normal diet or an HFD and treated or not with AMY for 15 weeks, with an emphasis on the involvement of the guanylate cyclase- K^+ channel pathway in the vascular responses.

Materials and methods

Drugs and chemicals

The following compounds were used in the experiments: phenylephrine hydrochloride (PHE), acetylcholine chloride (ACh), tetraethylammonium chloride (TEA), glibenclamide (GLIB), 4-aminopyridine (4-AP), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 8-Br-cGMP, apamin (Sigma Chemical Co., St. Louis, Missouri, USA), sodium pentobarbital (Fontoveter, Brazil), and sodium nitroprusside (SNP; Merck & Co., Inc., Rahway, NY, USA). Glibenclamide and ODQ were prepared in DMSO with a final concentration of 0.04 and 0.1%, respectively, in an organ bath. Apamin was prepared in acetic acid with a final concentration of 0.02% in an organ bath. Further dilutions of all stock solutions were prepared in distilled water. In the present study, a triterpenoid mixture composed of alpha- and beta-amyrin (63:37, AMY) was isolated from the resin of *P. heptaphyllum* per the methods described previously (Olea and Roque 1990; Mahato and Kundu 1994; Vieira-Junior et al. 2005). For experiments, AMY was suspended initially in 2% Tween 80 (v/v) and then further diluted in distilled water. Salts and reagents used were of analytical grade.

Animals and the HFD experimental protocol

Male Swiss mice aged 6 weeks and weighing 20–25 g obtained from the Central Animal House of the Federal University of Ceará were used. They were housed in a controlled environment ($24 \pm 2^\circ\text{C}$, $55 \pm 5\%$ relative humidity, 12 h light/dark cycle), and food (chow) and water were provided ad libitum. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the Federal University of

Ceará (Protocol Number 17/2011) in accordance with the Guidelines of National Institutes of Health (Bethesda, MD).

The standardized HFD used for the study (Estadella et al. 2004) was comprised of the following hypercaloric constituents: 15 g laboratory animal chow, 10 g roasted ground nut, 10 g milk chocolate, and 5 g maizen biscuits. These ingredients were ground and prepared in the form of pellets containing 20% protein, 48% carbohydrate, 20% lipids, 4% cellulose, 5% vitamins and minerals, and 3% moisture by weight. This diet has the net energy content of 21.40 kJ/g. To avoid the autoxidation of the fat components, the food was stored at ~24 °C. Laboratory pellet chow was used as the control diet (Nuvilab), which consisted of 19% protein, 56% carbohydrate, 3.5% lipids, 4.5% cellulose, 5.0% vitamins and minerals, and 12% humidity with a net energy content of 17.03 kJ/g.

The animals were randomly divided into three groups of eight ($n = 8$), matched for body weight, after 1 week of being fed the laboratory pellet chow. The normal control group continued to be fed with the laboratory pellet chow ad libitum and was designated ND. The remaining mice consumed a high-fat diet without (HFD control) or with AMY at 20 mg/kg (HFD + AMY 20) for 15 weeks. Since the effect of the 20 mg/kg AMY dose was more consistent than that of the 10 mg/kg dose, the 20 mg/kg dose was chosen for the current study. HFD-fed controls received the same vehicle in which AMY was suspended. AMY- or vehicle-containing water was changed twice per week, and weekly consumption of water (mL/week) was noted.

The body weight of each mouse was measured weekly, as were the consumption of food (g) and water (mL). At the end of this period, animals were starved for 6 h, and blood was taken by venous puncture under light anesthesia with diethyl ether. Blood samples were centrifuged at $2000 \times g$ for 10 min at 4 °C. The plasma was then removed and used within a few hours or immediately frozen at -70 °C until analysis. All mice were sacrificed by cervical dislocation. The thoracic aorta from each mouse was gently excised and equilibrated in physiological salt solution (PSS) buffer (pH 7.4) at 37 °C. The abdominal adipose tissues (epididymal and parametrial) were removed, weighed, and expressed in mg per 10 g body weight.

Plasma glucose (cat. no. 133), triglycerides (cat. no. 87) and total cholesterol (cat. no. 76) were determined using specific assay kits (Labtest, Minas Gerais, Brazil). Plasma insulin (cat. no. EZRMI-13K), adiponectin (cat. no. EZMADP-60K; Millipore, Billerica, MA, USA), and leptin (cat. no. MOB00; R&D Systems, Minneapolis, MN, USA) were measured using enzyme-linked immunosorbent assay (ELISA).

Vascular reactivity studies

The excised thoracic aortas were placed in Petri dishes containing modified Krebs-Henseleit solution (MKHS) at room

temperature (MKHS in mM 118.0 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.18 MgSO₄·7H₂O, 1.18 KH₂PO₄, 25.0 NaHCO₃, 11.0 glucose [pH 7.4]). After cleaning the loose connective tissue, each aorta was cut transversally into cylindrical ring-like segments (3–4 mm each) and attached to steel wire triangular pieces, which were suspended in 5-ml organ baths containing the perfusion medium (continuously aerated at 37 °C with 5% CO₂ in O₂). Endothelium-containing preparations were stretched with a passive tension of 1 g, and the tension was recorded using an isometric force transducer (ML870B60/C-V, AD Instruments, Australia) connected to a data acquisition system (PowerLab 8/30, AD Instruments). In all experiments, to evaluate the viability of tissues after an equilibration period of 60 min, control contractions were induced by adding a submaximal concentration (60 mM) of KCl to the bath. Aortic preparations were considered functionally equilibrated when two successive KCl-induced contractions had a similar magnitude. Tissues without reproducible contractions were discarded.

Concentration-response curves were constructed in response to the contractile agents KCl (10–120 mM) or phenylephrine (PHE, 0.001–10 μM). During the steady state of a PHE-induced contraction (1 μM), the relaxant effects of acetylcholine (ACh, 1–30 μM) or sodium nitroprusside (SNP, 1–1000 nM) were recorded. The relaxation responses to ACh and SNP were expressed as percentages of the maximal relaxant response. Further, to characterize the mechanisms involved in the relaxant effects of ACh and SNP on PHE-induced contractions, the aortic rings were incubated with glibenclamide (GLIB, 10 μM), an ATP-sensitive potassium channel (K_{ATP}) blocker; tetraethylammonium (TEA, 5 mM), a large-conductance Ca²⁺-activated potassium channel (BK_{Ca}) blocker; apamin (0.1 μM), a small conductance Ca²⁺-activated potassium channel (SK) blocker; 4-aminopyridine (4-AP, 3 mM), a voltage-dependent potassium channel (K_V) blocker; or with a soluble guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM). Each inhibitor was added to the bath for 10 min before PHE (1 μM). The concentrations of the inhibitors used in the present study were usually as recommended in the literature.

Statistical analyses

All data are reported as the mean ± standard deviation (SD) or standard error of mean (SEM), with n indicating the number of experiments. The EC₅₀ (i.e., the concentration of a relaxant compound at which 50% of a contractile response was inhibited) values were calculated by interpolation from semi-logarithmic plots and expressed as the geometric mean (95% confidence interval). Data were analyzed using a one- or two-way analysis of variance (ANOVA) followed by a Holm-Sidak multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Results

Effects of AMY treatment on obesity-related parameters in HFD mice

There was no significant difference in the initial body weights among the four groups of mice. At the end of 15 weeks, the values of final body weight and net food consumption were significantly higher ($p < 0.05$) in HFD group (55.57 g and 48.35 g, respectively) than in ND group (41.33 g and 33.36 g, respectively; Table 1). Compared to the ND group, the HFD mice revealed a 34.4% weight gain. Treatment with AMY (20 mg/kg) reduced the weight gain to 12.3% in the HFD + AMY 20 group, which was significantly lower in comparison with the HFD group ($p < 0.05$). There was a significant increase in the values of food consumption in the HFD group (44.9%) but not in the groups treated with AMY when compared to the ND group. AMY treatment, while it significantly decreased the HFD-induced increases in the levels of glucose, insulin, leptin, cholesterol, and triglycerides to the extent of 28, 72, 92, 27, and 41%, respectively, it increased the HFD-induced decreases in the level of adiponectin by 24%.

Vascular reactivity in mice fed a HFD and the effects of AMY treatment

Concentration-response curves constructed on mice aortic rings in response to KCl (10–120 mM) or PHE (0.001–10 μ M) are shown in Fig. 1 and Table 2. While the contractile response to KCl (Fig. 1a) did not reveal a significant difference between groups, the magnitude of the maximal contraction induced by PHE (Fig. 1b) was significantly lower in the

aortic rings of HFD mice in comparison with the ND group (at 10 μ M PHE: HFD = 0.44 ± 0.04 g [$n = 17$]; ND = 0.57 ± 0.06 g [$n = 12$]; $p < 0.05$). In contrast, the aortic rings of mice treated with AMY (20 mg/kg) showed no significant difference ($p > 0.05$) in their maximal values in comparison with the ND group (0.55 ± 0.02 g [$n = 9$]). Figure 2 shows the vasorelaxant responses to increasing concentrations of ACh (1–30 μ M; Fig. 2a) or SNP (1–1000 nM; Fig. 2b, c) applied during the steady state of a contraction previously induced by PHE (1 μ M; Fig. 2a, b) or KCl (60 mM; Fig. 2c). In the aortic rings of HFD mice, ACh-induced relaxation ($22.9 \pm 4.7\%$ [$n = 8$]) was significantly lower than in tissues of the ND group ($50.1 \pm 6.4\%$ [$n = 11$]; Fig. 2a). The relaxant responses to SNP (at 10 and 100 nM) in aortic rings pre-contracted by PHE (1 μ M) were found to be significantly ($p < 0.05$) attenuated in the HFD tissues (23.9 ± 3.0 and $57.4 \pm 6.4\%$, respectively [$n = 4$]) compared to the ND group (47.8 ± 5.6 and $77.3 \pm 1.5\%$, respectively, for SNP 10 and 100 nM [$n = 7$]; Fig. 2b). When the contractile stimulus was 60 mM KCl (Fig. 2c), the maximal relaxant response induced by 1000 nM SNP was significantly decreased in rings of the ND group ($47.1 \pm 9.0\%$ [$n = 7$]), but no significant difference was observed in comparison with responses in the HFD group ($35.9 \pm 3.7\%$ [$n = 6$]) or the HFD + AMY 20 group ($43.4 \pm 7.4\%$ [$n = 9$]; $p > 0.05$).

In the aortic rings of mice treated with AMY (20 mg/kg) and subjected to the HFD, the vasorelaxant effects caused by ACh (at 30 μ M = $42.3 \pm 7.0\%$ [$n = 6$]) were significantly higher in comparison with the tissues of animals subjected only to the HFD (Fig. 2a). The vasorelaxant effects of 10 and 100 nM SNP were similarly increased (50.0 ± 3.7 and $85.2 \pm 1.8\%$, respectively [$n = 5$]) in the HFD + AMY 20 group than in the group subjected only to the HFD (Fig. 2b).

Table 1 Effects of alpha, beta-amyrin (AMY) treatment on HFD-induced obesity-related parameters in mice

Group	ND	HFD	HFD + AMY 20 mg/kg
Initial body weight (g)	22.10 $\pm$ 1.52	21.58 $\pm$ 2.64	22.43 $\pm$ 1.39
Final body weight (g)	41.33 $\pm$ 3.24	55.57 $\pm$ 7.45*	46.43 $\pm$ 2.64 [#]
Net food intake (g/week)	33.36 $\pm$ 6.48	48.35 $\pm$ 11.39*	27.81 $\pm$ 4.86 [#]
Net water intake (mL/week)	50.31 $\pm$ 3.28	48.40 $\pm$ 5.08	50.60 $\pm$ 5.13
Abdominal fat (mg/10 g)	174.50 $\pm$ 56.69	732.80 $\pm$ 238.40*	333.10 $\pm$ 117.10 [#]
Glucose (mg/dL)	118.80 $\pm$ 23.80	212.20 $\pm$ 44.31*	152.60 $\pm$ 40.19 [#]
Insulin (ng/mL)	1.385 $\pm$ 0.676	5.544 $\pm$ 3.256*	1.520 $\pm$ 0.835 [#]
Leptin (pg/mL)	156.4 $\pm$ 84.0	1514.0 $\pm$ 1194.0*	113.10 $\pm$ 76.66 [#]
Adiponectin (ng/mL)	7.650 $\pm$ 2.059	4.763 $\pm$ 1.165*	6.289 $\pm$ 1.048 [#]
Total cholesterol (mg/dL)	124.90 $\pm$ 11.22	214.20 $\pm$ 30.22*	156.20 $\pm$ 23.19 [#]
Triglycerides (mg/dL)	106.40 $\pm$ 34.69	197.10 $\pm$ 58.17*	115.60 $\pm$ 19.94 [#]

Values are means $\pm$ SD

ND normal diet, HFD high-fat diet, AMY alpha, beta-amyrin;

* $p < 0.05$ vs mice fed ND, [#] $p < 0.05$ vs mice fed HFD

Fig. 1 Concentration-effect curves of mice aortic rings in response to KCl and phenylephrine. Contractions were obtained by the addition of increasing concentrations of KCl (10–120 mM; **a**) or phenylephrine (PHE; 0.001–10 μ M; **b**) in aortic rings of mice subjected to normal diet [ND, white circle (KCl, $n = 12$; PHE, $n = 12$)], high-fat diet [HFD, black circle (KCl, $n = 17$; PHE, $n = 17$)] or HFD with alpha, beta amylin [AMY;

20 mg/kg; inverted triangle (KCl, $n = 13$; PHE, $n = 9$)]. Vasoconstriction is expressed in g. Values are mean and vertical bars indicate S.E.M. * $p < 0.05$ in comparison with the same concentration in ND group. # $p < 0.05$ comparing the same concentration in relation to HFD group (two-way ANOVA followed by Holm-Sidak test)

Involvement of the guanylate cyclase- K^+ channel pathway in the vascular responses to acetylcholine in mouse aortic rings contracted with phenylephrine

Recruitment of the guanylate cyclase- K^+ channel pathway in the vasorelaxant response to ACh (1–30 μ M) in PHE (1 μ M)

pre-contracted aortic rings was examined in the aortic rings of mice receiving the ND, HFD, and HFD + AMY 20 mg/kg. As shown in Fig. 3, the vasorelaxant effect caused by ACh was abolished in preparations treated with the guanylate cyclase inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) in all groups (Fig. 3a–c).

Table 2 Values of pD_2 and E_{max} for the vasoactive effects recorded on aortic rings from mice fed with ND, HFD, or HFD + AMY 20 mg/kg

Contractile stimulus	Group	pD_2	E_{max} (g)	Number
KCl	ND	1.53 (1.47–1.59)	0.42 ± 0.04	12
	HFD	1.57 (1.51–1.64)	0.39 ± 0.03	17
	HFD + AMY 20 mg/kg	1.51 (1.44–1.58)	0.39 ± 0.02	13
PHE	ND	7.46 (7.23–7.68)	0.57 ± 0.06	12
	HFD	6.95 (6.80–7.10)*	$0.44 \pm 0.04^*$	17
	HFD + AMY 20 mg/kg	7.20 (6.89–7.51)	0.55 ± 0.02	9
Relaxant stimulus	Group	pD_2	E_{max} (%)	n
ACh (PHE)	ND	—	50.13 ± 6.45	11
	HFD	—	$22.89 \pm 4.73^*$	8
	HFD + AMY 20 mg/kg	—	42.30 ± 7.00	6
SNP (PHE)	ND	7.92 (7.60–8.23)	100.0 ± 0.00	7
	HFD	7.23 (6.78–7.67)*	100.0 ± 0.00	4
	HFD + AMY 20 mg/kg	8.08 (7.79–8.37)	100.0 ± 0.00	5
SNP (KCl)	ND	—	47.12 ± 9.05	7
	HFD	—	33.20 ± 4.11	6
	HFD + AMY 20 mg/kg	—	43.43 ± 7.44	9
8-Br-cGMP (PHE)	ND	4.65 (4.46–4.67)	97.17 ± 2.27	7
	HFD	4.60 (4.44–4.75)	95.42 ± 2.54	7
	HFD + AMY 20 mg/kg	4.54 (4.44–4.64)	94.56 ± 2.82	6

pD_2 values are the negative logarithm of the EC_{50} shown as the geometric mean with 95% confidence intervals in parentheses. Relaxant stimulus was applied with acetylcholine (ACh), sodium nitroprusside (SNP), or 8-Br-cGMP added on the steady state of a contraction induced by PHE or KCl as indicated

— Not applicable, ND normal diet, HFD high-fat diet, AMY alpha, beta-amylin, E_{max} (g) maximal contraction induced by phenylephrine (PHE) or KCl, expressed in g, E_{max} (%) maximal relaxation induced by ACh, SNP, or 8-Br-cGMP expressed as % of the contraction induced by PHE or KCl

* $p < 0.05$ vs ND group

Fig. 2 Vasorelaxant responses to acetylcholine and sodium nitroprusside in mice aortic rings pre-contracted with phenylephrine or KCl. Aortic rings of mice subjected to normal diet (ND, white circle), high-fat diet (HFD, black circle) or HFD with alpha, beta amylin (AMY; 20 mg/kg; inverted triangle) were initially contracted with phenylephrine (PHE, 1 μM; a, b) or KCl (60 mM; c). On the steady-state contraction, acetylcholine [ACh, 1–30 μM; a (ND, $n = 11$; HFD, $n = 8$; HFD + AMY20, $n = 6$)] or sodium nitroprusside [SNP, 1–1000 nM; b (ND, $n = 7$; HFD,

$n = 4$; HFD + AMY20, $n = 5$) and c (ND, $n = 7$; HFD, $n = 6$; HFD + AMY20, $n = 9$)] were added to produce vasorelaxant responses, which were expressed as % of the control contraction induced by PHE (a and v) or KCl (60 mM, c) before the addition of ACh or SNP. Values are mean $\pm$ SEM. * $p < 0.05$ in comparison with the same concentration in ND group. # $p < 0.05$ comparing the same concentration in relation to HFD group (two-way ANOVA followed by Holm-Sidak test)

In contrast, pharmacological blockade of K^+ channels with tetraethylammonium (TEA, 5 mM), apamin (0.1 μM), or 4-aminopyridine (4-AP, 3 mM) significantly ($p < 0.05$) reduced the vasorelaxant response to ACh in the ND group (at 30 μM

from 50.1 ± 6.4 to $15.8 \pm 4.1\%$ [$n = 8$], $21.09 \pm 5.8\%$ [$n = 5$], and $21.9 \pm 3.9\%$ [$n = 5$], respectively; Fig. 3a). Glibenclamide (GLIB, 10 μM) did not interfere with the cholinergic relaxation in the ND group. Glibenclamide was similarly inert

Fig. 3 Involvement of guanylate cyclase- K^+ channel pathways in the vasorelaxant responses to acetylcholine in mice aortic rings pre-contracted with phenylephrine. Aortic rings of mice subjected to normal diet (ND, white symbols), high-fat diet (HFD, black symbols), or HFD with alpha, beta-amylin (AMY; 20 mg/kg; gray symbols) were initially contracted with phenylephrine (PHE, 1 μM). Preparations were maintained in the absence (ND, $n = 11$; HFD, $n = 8$; HFD + AMY20, $n = 7$) or in the presence of the K^+ channel blockers (apamin [APAM, 0.1 μM; squares; ND, $n = 5$; HFD, $n = 5$; HFD + AMY20, $n = 6$], tetraethylammonium [TEA, 5 mM; hexagons; ND, $n = 8$; HFD, $n = 8$;

HFD + AMY20, $n = 6$], or 4-aminopyridine [4-AP, 3 mM; diamonds; ND, $n = 8$; HFD, $n = 5$; HFD + AMY20, $n = 5$], or the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM; up triangles; ND, $n = 6$; HFD, $n = 6$; HFD + AMY20, $n = 4$). On the steady-state contraction, acetylcholine (ACh, 1–30 μM) was added to produce vasorelaxant responses, which were expressed as % of the control contraction induced by PHE before ACh addition. Values are mean $\pm$ SEM. * $p < 0.05$ in comparison with the same concentration in ND group (two-way ANOVA followed by Holm-Sidak test)

against ACh in the aortic rings of mice subjected to the HFD and the HFD + AMY 20 (data not shown). In the aortic rings of HFD mice (Fig. 3b), the K^+ -channel blockers TEA, apamin, and 4-AP revealed a tendency to inhibit the relaxant action of ACh, but their values did not achieve statistical significance in comparison with the HFD non-treated preparations ($p > 0.05$). In the HFD mice treated with AMY (20 mg/kg; Fig. 3c), the relaxation induced by ACh almost attained the level observed in the ND mice and was significantly reduced ($p < 0.05$) by TEA (at 30 μ M ACh, the effect was reduced to $11.3 \pm 2.5\%$ [$n = 8$] vs. $42.3 \pm 7.0\%$ [$n = 5$] in the HFD + AMY 20 preparations without TEA) but not by 4-AP or apamin.

Involvement of the guanylate cyclase- K^+ channel pathway in the vascular response to sodium nitroprusside in aortic rings contracted with phenylephrine

Figure 4 represents the vasorelaxant responses of mice aortic rings initially contracted with PHE (1 μ M) and subjected to increasing concentrations of sodium nitroprusside (SNP, 1–1000 nM) in the absence or in the presence of GLIB (10 μ M), TEA (5 mM), apamin (0.1 μ M), 4-AP (3 mM), or ODQ (10 μ M). In the aortic rings of ND mice (Fig. 4a), the relaxing effects of SNP were sensitive to pharmacological blockade of K^+ channels with TEA, apamin, and 4-AP, but not with GLIB. Values for the relaxant effects of SNP were

significantly reduced at 1, 10, and 100 nM and at 10 and 100 nM in preparations treated with TEA and 4-AP, respectively, and at 10 nM in preparations treated with apamin ($p < 0.05$). Such a reduction induced by the presence of these K^+ -channel blockers was not statistically evident in the aortic rings from the HFD group ($p > 0.05$; Fig. 4b). In the HFD mice treated with AMY (20 mg/kg; Fig. 4c), the relaxant responses induced by 10 and 100 nM SNP were significantly reduced by treatment with TEA (23.1 ± 5.0 and $59.3 \pm 6.2\%$ in TEA-treated preparations vs. 50.0 ± 3.7 and $85.2 \pm 1.8\%$ in non-treated preparations, respectively; $p < 0.05$), but not with GLIB or 4-AP. It should be highlighted that the inhibition of guanylate cyclase through treatment with ODQ significantly reduced the relaxant effect of SNP in all groups ($p < 0.05$).

Vasorelaxant response to 8-Br-cGMP in aortic rings pre-contracted with PHE in mice receiving a ND, HFD, or HFD with AMY

To evaluate the responsiveness of aortic smooth muscle cells to cGMP, the relaxant response caused by the cell-permeable cGMP analog 8-Br-cGMP (1–100 μ M) was measured in PHE (1 μ M)-contracted thoracic aortic preparations from mice fed a ND, HFD, or HFD + AMY 20 mg/kg. Figure 5 shows that the vasorelaxant response to 8-Br-cGMP in the HFD group ($pD_2 = 4.60$ [4.44–4.75]; $n = 7$) was not significantly different in comparison with the ND group ($pD_2 = 4.65$ [4.46–4.67];

Fig. 4 Involvement of guanylate cyclase- K^+ channel pathways in the vasorelaxant responses to sodium nitroprusside in mice aortic rings pre-contracted with phenylephrine. Aortic rings of mice subjected to normal diet (ND, white symbols), high-fat diet (HFD, black symbols), or HFD with alpha, beta amylin (AMY; 20 mg/kg; gray symbols) were initially contracted with phenylephrine (PHE, 1 μ M). Preparations were maintained in the absence (ND, $n = 7$; HFD, $n = 4$; HFD + AMY20, $n = 5$) or in the presence of the K^+ channel blockers (apamin [APAM, 0.1 μ M; squares; ND, $n = 10$; HFD, $n = 6$; HFD + AMY20, $n = 7$], tetraethylammonium [TEA, 5 mM; hexagons; ND, $n = 7$; HFD, $n = 5$;

HFD + AMY20, $n = 5$], or 4-aminopyridine [4-AP, 3 mM; diamonds; ND, $n = 5$; HFD, $n = 4$; HFD + AMY20, $n = 5$], or the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μ M; up triangles; ND, $n = 4$; HFD, $n = 4$; HFD + AMY20, $n = 4$). On the steady-state contraction, sodium nitroprusside (SNP, 1–1000 nM) was added to produce vasorelaxant responses, which were expressed as % of the control contraction induced by PHE before SNP addition. Values are mean $\pm$ SEM. * $p < 0.05$ in comparison with the same concentration in ND group (two-way ANOVA followed by Holm-Sidak test)

Fig. 5 Vasorelaxant response to 8-Br-cGMP (1–100 μ M) in pre-contracted aortic rings with PHE in mice receiving a ND, HFD, or HFD with alpha, beta amylin diet. Values are mean and S.E.M. expressed as percent contraction of PHE (1 μ M). Vertical bars indicate SEM. (ND, $n = 7$; HFD, $n = 7$; HFD + AMY20, $n = 6$)

$n = 7$) or the HFD + AMY 20 mg/kg group ($pD_2 = 4.54$ [4.44–4.64]; $n = 6$) ($p > 0.05$).

Discussion

In this study, mice fed a HFD for 15 weeks showed increased body weights and abdominal fat mass, hyperglycemia, hyperinsulinemia, and hyperlipidemia, characterizing an insulin resistance and obesity state that was significantly abrogated in the group of animals treated with AMY, confirming our earlier findings (Santos et al. 2012; Carvalho et al. 2017). Obesity is a risk factor for cardiovascular disease, and evidence indicates that a high-fat diet induces endothelial dysfunction through the inhibition of nitric oxide (NO) produced by the endothelium (Yang et al. 2007; Magne et al. 2009). Moreover, pro-inflammatory cytokines produced by perivascular fat may also contribute to endothelial dysfunction and smooth muscle cell dysfunction and to the pathogenesis of vascular disease in obese states (Iantorno et al. 2014). Further, hyperlipidemia may alter the vascular response to vasoactive agents (Li et al. 2010). It has been suggested that the obesity-associated vascular dysfunction is, at least in part, reversible with weight-loss strategies (Ziccardi et al. 2002). Since AMY has exhibited anti-inflammatory (Melo et al. 2011) and weight-loss potential in HFD-induced obese mice, we examined AMY for the first time using a vascular reactivity assay in this ex vivo study, using the diet-induced mouse model of obesity.

The reactivity of the aortas isolated from obese and control mice to cumulative concentrations of contractile agents, such as PHE and KCl, was analyzed. In addition, emphasis was given to the changes caused by diet on the endothelium-

dependent relaxing properties of ACh and the endothelium-independent effects of sodium nitroprusside (SNP). Except for preparations contracted under the depolarizing stimulus induced by KCl, the functional effects induced by PHE, ACh, and SNP were biased at different extents in arteries of obese mice. Indeed, the PHE-induced contractions were significantly decreased in the aortas of mice subjected to a HFD, an observation that is consistent with the studies of Liu et al. (2014), which showed down-regulation of the α -adrenoceptor-mediated signaling cascade (with lesser involvement of inositol 1,4,5-trisphosphate and protein kinase C) resulting in decreased Ca^{2+} influx. Thus, reversal of the attenuated contraction induced by PHE in the obese mice reveals that AMY possesses potential effects to prevent detrimental changes in vascular reactivity, including the endothelial dysfunction associated with obesity.

There is evidence that impairment of NO synthesis represents a central defect that triggers many of the vascular abnormalities characteristic of obesity states (Deng et al. 2010; Martins et al. 2010). The endothelium modulates vascular tone through the release of vasoactive agents, such as NO. The relaxing role exerted by the endothelium was examined in aortic rings submaximally contracted with PHE and stimulated to relax using cumulative concentrations of ACh, which induces relaxation through the endothelium-dependent release of NO involving muscarinic receptor activation (Moncada et al. 1989). The results of the present study have demonstrated that in mice subjected to an experimental HFD regimen, vasorelaxation in response to ACh was attenuated, a phenomenon also prevented by AMY treatment, which significantly restored the diminished relaxant effects of ACh.

Similarly, the relaxing effects induced by SNP were reduced in the tissues of HFD mice. It is known that the NO donor SNP is largely an endothelium-independent relaxant agent that exerts a direct action on the vascular smooth muscle (Bonaventura et al. 2008). Like ACh, SNP induces relaxation in vascular smooth muscle mainly through a cGMP-dependent mechanism (Moncada et al. 1989). Once cGMP is produced as a result of the ACh- or SNP-induced soluble guanylate cyclase stimulation, cGMP targets proteins known as cGMP-dependent protein kinases (e.g., protein kinase G [PKG]; Lucas et al. 2000). Targeted PKG then modulates additional phenomena such as the gating of K^+ channels, which results in membrane hyperpolarization and smooth muscle relaxation (Kubo et al. 1994). In the present study, the vasorelaxant effects of SNP under a KCl stimulus were significantly reduced compared to the PHE-induced contractions, likely because the relaxant efficacy of compounds that recruit hyperpolarizing phenomena involving the opening of K^+ channels, such as SNP, is lost under depolarizing conditions imposed by a high extracellular (60 mM) K^+ concentration (Ribeiro-Filho et al. 2012). In addition, the relaxing effects of SNP did not differ between tissues obtained from mice

subjected to different diet regimens. Thus, it is reasonable to consider that the decreased vasorelaxation to both ACh and SNP observed in the aortic rings from HFD mice might somehow be a result of an impaired recruitment of K^+ channels imposed by obesity.

To evaluate such a hypothesis, the aortic rings were incubated with four inhibitors of the K^+ channel activity in smooth muscle cells: the large-conductance Ca^{2+} -activated potassium channel (BK_{Ca}) blocker TEA (Cook 1989), the small-conductance Ca^{2+} -activated K^+ channel (SK) blocker apamin (Murphy and Brayden 1995), the K_{ATP} channel blocker glibenclamide (Standen et al. 1989), and the voltage-dependent K_V channel inhibitor 4-AP (Satake et al. 1996). Whereas GLIB did not cause significant changes, TEA, apamin, and 4-AP decreased the relaxation of aortic rings from the ND mice, supporting the involvement of BK_{Ca} and K_V , but not K_{ATP} channels, as an underlying mechanism involved in the vasorelaxation effects of ACh and SNP in these tissues. In contrast, the vasorelaxant effects of ACh in aortic rings from the HFD mice were not influenced by the presence of 4-AP and apamin, while TEA was almost inert against the vascular response to ACh in the HFD tissues (only a single concentration was significantly decreased in comparison with the ND group). The relaxant response induced by SNP was refractory to the presence of TEA, 4-AP, and apamin. Such findings suggest a decrease in the essential role of K^+ channels mediating vascular tone in the aortic rings from HFD mice. Inhibited activity of BK_{Ca} has been attributed to the augmented vascular resistance in experimental models of obesity. In fact, reduced BK_{Ca} current was recorded in coronary artery smooth muscle cells obtained from a swine model of metabolic syndrome (Borbouse et al. 2009). In addition, the vasodilator effects of an adenosine analog were not affected by selective BK_{Ca} channel blockade with iberiotoxin in coronary arteries of obese animals, whereas it was reduced in the normal diet control animals (Borbouse et al. 2009). Franke et al. (2004) also reported reduced K_V channel activity in coronary arteries from HFD-fed pigs.

Aortic rings from HFD mice treated with AMY were susceptible to pharmacological blockade of K^+ channels, but only TEA was able to decrease the vasorelaxant effects of ACh or SNP. Such findings suggest, at least partially, that AMY preserved the ability of the smooth muscle cells to produce endothelium-dependent and endothelium-independent relaxant responses via guanylate cyclase-elicited recruitment of Ca^{2+} -sensitive BK_{Ca} , but not K_V channels. The experiments with apamin preclude the small-conductance Ca^{2+} -activated K^+ -channel (SK) as a target for the actions induced by AMY. The involvement of guanylate cyclase in the vasorelaxant effects of ACh and SNP was confirmed by the evident inhibition of preparations caused by treatment with the selective inhibitor of guanylate cyclase enzyme, ODQ (Garthwaite et al. 1995). Moreover, the lack of difference

significant difference between diet regimens in the vasorelaxant profile on ODQ-treated preparations (data not shown) reinforces the notion that a HFD is somehow able to disrupt the coupling between the guanylate cyclase-derived cGMP production and the subsequent recruitment of target proteins involved in the hyperpolarizing phenomena that ultimately determine the vasorelaxant phenotype in a mouse aorta.

The hypothesis above is reinforced by the results observed with 8-Br-cGMP. In fact, even in the aortic rings of HFD mice, exogenous administration of this stable cGMP analog was able to induce a vasorelaxant effect that did not differ from that observed in the tissues of ND mice, i.e., the aortic smooth muscle cells from the HFD mice maintained the native ability to respond to exogenous cGMP. However, whether cGMP production occurs via guanylate cyclase stimulation, the HFD regimen selectively determined an incomplete vasorelaxant response. Importantly, although the mechanism could not be precisely determined in the present approach, it is noteworthy that AMY impaired such disruption, suggesting a potential therapeutic role of this compound in obesity.

Although the family of K^+ channels is a main target of the cGMP-dependent protein kinases (PKG), K^+ channel activity can be modulated directly. Mistry and Garland (1998) showed that NO and NO donors can directly stimulate BK_{Ca} activity in cells isolated from the rat mesenteric artery, an action that was not affected by soluble guanylate cyclase inhibition. Thus, we hypothesize that the reduced K^+ channel activity observed in the present model is due to a decrease in NO production or a deficient action of NO, which requires a detailed further study.

In summary, the present study demonstrates that the HFD induces adiposity, increased body weight gain, and impaired vascular reactivity and that these effects could be ameliorated by AMY, a pentacyclic triterpenoid. AMY prevented the HFD-induced weight gain, insulin resistance, dyslipidemia, and endothelial dysfunction, and these results may, in part, contribute to the improvement of the impaired vascular function in aortic rings caused by a HFD, probably by a restoration in K^+ channel activity.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution (Federal University of Ceará).

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