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1 **Soluble guanylate cyclase chronic stimulation effects on**
2 **cardiovascular reactivity in cafeteria diet-induced rat model of**
3 **metabolic syndrome**

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21 **Abstract**

22 Metabolic syndrome is linked to an increased risk of cardiovascular complications by a
23 mechanism involving mainly decreased nitric oxide (NO) bioavailability and impaired NO-
24 soluble guanylate cyclase (sGC)- cyclic guanosine monophosphate (cGMP) signalling (NO-
25 sGC-cGMP). To further develop this scientific point, this study aimed to investigate the
26 effects of long-term treatment with BAY 41-2272 (a sGC stimulator) on cardiovascular
27 reactivity of spontaneously hypertensive rats (SHR) as a model of metabolic syndrome. SHR
28 were randomly divided into 3 groups: Control group, cafeteria diet (CD)-fed group and CD-
29 fed group treated daily with BAY 41-2272 (5mg/Kg) by gastric gavage for 12 weeks. *In vivo*
30 measurements of body weight, abdominal circumference, blood pressure and glucose
31 tolerance test were performed. At the end of the feeding period, *ex vivo* cumulative
32 concentration-response curves were performed on isolated perfused heart (isoproterenol
33 (0.1nM - 1µM)) and thoracic aorta (phenylephrine (1nM – 10 µM), acetylcholine (1nM – 10
34 µM), and sodium nitroprusside (SNP) (0.1nM – 0.1µM)). We showed that chronic CD
35 feeding induced abdominal obesity, hypertriglyceridemia, glucose intolerance and
36 exacerbated arterial hypertension in SHR. Compared to control group, CD-fed group showed
37 a decrease in β -adrenoceptor-induced cardiac inotropy, in coronary perfusion pressure and in
38 aortic contraction to phenylephrine. While relaxing effects of acetylcholine and SNP were
39 unchanged. BAY 41-2272 long-term treatment prevented markedly arterial hypertension
40 development and glucose intolerance, enhanced the α_1 -adrenoceptor-induced
41 vasoconstriction, and restored cardiac inotropy and coronary vasodilation. These findings
42 suggest that BAY 41-2272 may be a potential novel drug for preventing metabolic and
43 cardiovascular complications of metabolic syndrome.

44 **Key words: metabolic syndrome, sGC stimulation, SHR, Cafeteria diet, cardiovascular**
45 **reactivity**

46 **1. Introduction**

47 Metabolic syndrome consists of a combination of cardiovascular risk factors such as obesity,
48 insulin resistance, arterial hypertension, dyslipidaemia and an impaired glucose tolerance
49 (O'Neill and O'Driscoll, 2015). Individuals with metabolic syndrome are at significant risk of
50 developing cardiovascular disease and type II diabetes mellitus (Galassi et al., 2006; Aschner,
51 2010). Endothelial dysfunction characterized by an impaired endothelium-dependent
52 vasodilation, is a predictive hallmark of later cardiovascular complications such stroke and
53 heart attack (Shayo et al., 2019). Currently, an association is clearly established between
54 endothelial dysfunction and metabolic syndrome (Abd El Aziz et al., 2018). The endothelium
55 generates several vasoactive compounds and signals which act locally to adjust blood flow
56 including nitric oxide (NO) (Fernandes et al., 2017; Khaddaj Mallat et al., 2017). Once
57 released, NO diffuses into the smooth muscle cell, activates soluble guanylate cyclase (sGC)
58 thereby generating cyclic guanosine monophosphate (cGMP) synthesis. cGMP induces
59 vasorelaxation via lowering intracellular calcium levels (Kim et al., 2019). It has been
60 reported that endothelial dysfunction associated with the metabolic syndrome is mainly due to
61 the reduced NO bioavailability and therefore to an impairment in NO-sGC-cGMP signalling
62 pathway (Matthews et al., 2018; Breitenstein et al., 2017). However, recent studies have
63 shown that chronic treatment with sildenafil citrate, a phosphodiesterase type 5 (PDE5)
64 inhibitor, improves energy balance contributing to weight loss in high fat-fed mice (Ayala et
65 al., 2007), reduces hyperinsulinemia and up-regulates endothelial nitric oxide synthase
66 (eNOS) expression in a rat model of insulin resistance (Oudot et al., 2010). Those findings
67 suggest that the NO-cGMP pathway modulation may constitute a key link between its
68 metabolic and vascular protective effects. Nonetheless, basal endogenous cGMP production is
69 a prerequisite for the PDE5 inhibitors action (Tobin et al., 2018). Currently, different classes
70 of drugs have been developed, which increase cGMP production independently of NO

71 availability, by targeting the NO receptor sGC (Breitenstein et al., 2017). Thus, sGC
72 stimulation may have a crucial advantage over PDE5 inhibition due to its NO-independent
73 mechanism of action (Chamorro et al., 2018), especially since sGC activity has been
74 described as reduced in spontaneously hypertensive rats (SHR) (Priviero et al., 2009). BAY
75 41-2272 is a sGC stimulator that has been shown to induce antihypertensive action, to
76 attenuate remodelling in models of systemic arterial hypertension and to reduce pulmonary
77 vascular resistance (Boerrigter and Burnett, 2007). Furthermore, potential antiobesity and
78 insulin sensitizing effects of cGMP signalling have been postulated (Mitschke et al., 2013).
79 However, the cardiovascular effects of BAY 41-2272 long-term treatment during metabolic
80 syndrome are not well known yet.

81 Several experimental models are now available for studying the pathogenesis and prevention
82 of metabolic syndrome (Miesel et al., 2010). The SHR are one of the most commonly animal
83 models studied (Oron-Herman et al., 2008). This strain is not only genetically hypertensive
84 but is also insulin resistant (Potenza et al., 2005), thus representing a relevant study model of
85 the metabolic syndrome in humans.

86 Thus, the present study was designed to investigate the effects of long-term sGC stimulation
87 with BAY 41-2272 on metabolic parameters and cardiovascular reactivity in a SHR
88 experimental model of metabolic syndrome.

89 **2. Materials and methods**

90 2.1. Animals and experimental protocol

91 All the experiments were performed in accordance with institutional guidelines from the
92 ethical committee of Pays de la Loire, France (Ministry authorisation, APAFIS N° 6445).

93 Nine-week old, male SHR obtained from Janvier Labs (Le Genest St Isle, France), were used
94 for this study. All rats were housed under a 12-hour light/dark cycle, at a controlled

95 temperature (22°C) and humidity (50%) and were allowed free access to standard chow
96 (KLIBA NAFAG®, Kaiseraugst, Germany) and drinking water. An acclimatisation period of
97 1 week was allowed before any experiment was initiated. Rats were randomly divided into 3
98 groups that received respectively for 12 weeks: standard chow, Cafeteria diet (CD) and CD
99 with BAY 41-2272 administered orally (5mg/kg/day). CD included different commercial
100 variety of chocolate, cookie and cereal bars. The resulting CD provided an average of 68.3%
101 energy from carbohydrates, 5.83% from protein and 18.2% from total fat. The foods provided
102 were changed daily to stimulate hyperphagia.

103 2.2. Physiological parameters

104 Body weight and abdominal circumference of all groups were monitored weekly during the
105 feeding period.

106 Measurements of systolic (SBP) and diastolic (DBP) arterial blood pressures were assessed by
107 non-invasive tail cuff plethysmography method in awake rats (CODA, Kent Scientific Co.,
108 Torrington, CT, USA). In order to limit stress-related variations in blood pressure, all
109 measurements were performed by the same person and in a quiet room and rats were
110 subjected to an adaptation period of one week before data collection.

111 Before starting measurements, rats were placed in a restraining box, preheated at 37° C in
112 order to dilate the tail arteries. Ten consecutive pressure measurements were recorded for
113 each rat and averaged to obtain a representative value of SBP and DBP (mmHg).

114 2.3. Glucose tolerance test and biochemical measurements

115 At the 12th week, rats were fasted overnight. An intraperitoneal glucose tolerance test was
116 carried out by means of glucose solution injection (1g/kg body weight, intraperitoneally).
117 Blood sampling (one drop) was performed from tail vein (under ointment lidocaine

118 application) before and at 15, 30, 45, 60 and 90 min after glucose injection. The concentration
119 of blood glucose was determined with a blood glucose meter (Glucometer, Pura ®).

120 At the end of the experimental protocol, animals were anesthetized with pentobarbital
121 (54mg/kg i.p). Anaesthesia of the rat was checked by the paw withdrawal reflex. Blood
122 samples were obtained via cardiac puncture and centrifuged at 5000g for 10 min at 4°C.

123 Plasma was extracted and stored at -80°C. Total cholesterol and triglycerides plasma
124 concentrations were assayed using an automatic biochemical analyser in the Veterinary
125 University Hospital Centre of ONIRIS, Nantes, France. Insulin plasma concentrations were
126 determined using a rat insulin ELISA kit (Thermo Fisher Scientific, France),

127 2.4. *Ex vivo* cardiac function

128 Immediately after blood sampling, rats were killed by exsanguination of abdominal aorta. The
129 hearts were thoroughly excised and immersed in a cold *Krebs-Henseleit* solution (in mM):
130 NaCl, 118.3; KCL, 4.7; MgSO₄, 1.2 ; KH₂PO₄, 1.2 ; NaHCO₃, 20 ; EDTA, 0.016 ; Glucose,
131 11.1 and CaCl₂, 2.5 ; pH 7.4) previously filtered (0.2 µm filter funnel) and aerated with 95%
132 O₂- 5% CO₂ gas mixture. The heart is cannulated through the aorta in order to allow a
133 retrograde perfusion at a constant flow rate of 12 ml/min, according to the Langendorff
134 method (Skrzypiec-Spring et al., 2007). To assess left ventricular function, a water-filled latex
135 balloon was inserted into the left ventricle through the mitral valve. An equilibration period of
136 30 min was required to ensure the stability of the parameters recorded before any molecule
137 addition. Left ventricular developed pressure (LVDevP) was determined as the difference
138 between left ventricular systolic pressure and left ventricular end-diastolic pressure. Coronary
139 vasodilation was determined by perfusion pressure variation. Each parameter was recorded
140 initially and after the addition of increasing concentrations of isoproterenol (a non-selective β-
141 adrenoceptor agonist (0.1 nM- 1µM)).

142 All the parameters recorded were analysed by LabChart ®Pro software (V7, ADInstruments,
143 France).

144 2.5.Vascular reactivity experiments

145 Immediately after the sacrifice, thoracic aorta was rapidly removed, dissected, cleaned of fat
146 and connective tissue and cut into rings (2-3 mm long). Thoracic aortic rings were then
147 suspended on stainless-steel hooks in individual organ baths (Emka Technologies, Paris,
148 France), containing 10 ml of *Krebs-Henseleit* solution heated to 37°C and continuously
149 aerated with a 95% O₂ and 5% CO₂ gas mixture. Thoracic aortic rings were progressively
150 stretched to a resting tension of 2g. Isometric tension was detected using isometric force
151 transducers of a myograph (Emka Technologies, Paris, France) and recorded by data
152 acquisition software (iOX, Paris, France). Briefly, after 1h of equilibration at a resting tension
153 of 2g, the endothelium viability was verified by the observation of at least 60% relaxation to
154 acetylcholine (1µM) in thoracic aortic rings after phenylephrine (1µM, a selective α1-
155 adrenoceptor agonist) precontraction (Sauvaget et al., 2010). Cumulative concentration-
156 response curves (CCRCs) to phenylephrine (1nM-10µM), acetylcholine (1nM-10µM) and
157 sodium nitroprusside (a nitric oxide donor, 0.1nM- 0.1µM) were constructed. To evaluate the
158 role of inducible nitric oxide synthase (iNOS) in aortic contraction, Thoracic aortic rings were
159 incubated with aminoguanidine (100µM, an iNOS inhibitor) for 30 min. The agonist
160 maximum response and sensitivity were determined by E_{max} and pD₂ = -log EC₅₀ values
161 respectively.

162 2.6.Immunofluorescence and quantification of iNOS expression

163 Thoracic aortic rings were fixed with 4% paraformaldehyde in PBS for 4 h at 4°C. After 3
164 washings in PBS, the aortic rings were incubated in PBS containing 20% sucrose for one
165 night. Then, they were embedded in tissue Tek OCT medium ®(Sakura, USA), frozen by

166 immersion in liquid isopentane and conserved at -80°C . Frozen section ($10\ \mu\text{m}$) were
167 permeabilized with 0.5 % Triton 100X in PBS for 5min and treated with a PBS solution
168 containing 0.5% Triton 100X and 2% bovine serum albumin (BSA) for 1 h to block the
169 nonspecific antigen binding.

170 Sections were then incubated with a rabbit polyclonal antibody against iNOS (1 :100 in the
171 blocking buffer, Abcam, Cambridge, United Kingdom) for one night. After 5 washings of 3
172 min each in PBS, the sections were incubated with secondary antibody, AlexaFluor 555-
173 conjugated donkey anti-rabbit (1 :300, life Technologie, Saint Aubin, France) for 1 h at room
174 temperature. The slides were covered with mounting medium (Mowiol, Calbiochem, San
175 Diego, CA, USA) and coverslip before being viewed using a spectral confocal microscope
176 (Zeiss LSM 780, Zeiss, France). 488 nm argon laser line was used to observe elastic lamina
177 autofluorescence while 561 nm solid state laser line was used for AlexaFluor 555 detection
178 (iNOS immunolabellings). Image analysis was performed to evaluate iNOS expression level
179 in the thoracic aorta of all rats by using Fiji Software. Mean Fluorescence Intensity (MFI)
180 values were determined from 5 different fields of immunolabeled thoracic aortic sections in
181 each group. The same threshold value was applicated on the sum intensity projections
182 obtained from z stacks in each section. Finally, the MFI was reported to total area of analysed
183 section.

184 2.7.cGMP levels in thoracic aorta, heart and epididymal fat samples

185 Quickly after the sacrifice, the remaining thoracic aorta, part of the heart and epididymal fat
186 were frozen in liquid nitrogen to avoid cGMP degradation and were stored at -80°C . The
187 cGMP content was measured colorimetrically using an immunoenzymatic assay kit (Cayman
188 Chemical Company). A spectrophotometer at 405nm was used to read absorbance. The mean
189 value was calculated from duplicate measurements of each sample and related to total cell

190 protein levels previously measured using a protein assay reagent kit (micro BCA-Pierce)
191 (Kanso et al., 2014).

192 2.8. Drugs

193 Phenylephrine hydrochloride, acetylcholine chloride, sodium nitroprusside and isoproterenol
194 were obtained from Sigma-Aldrich (Saint Quentin-Fallavier, France) and sodium
195 pentobarbital solution from Ceva Santé Animale (Libourne, France). BAY 41-2272 was
196 provided from Bayer (La Garenne-Colombes, France). All drugs were prepared in distilled
197 water.

198 2.9. Statistical analysis

199 All the results were expressed as mean \pm S.E.M of n experiments where n represents the
200 number of rats. The results were compared using a One-way ANOVA followed by Tukey
201 *post-hoc* test when needed. Statistical analysis were performed using GraphPad PRISM [®]
202 software version 5.

203 CCRCs were compared using either a non-linear mixed effect (NLME) model for complete
204 curves or a linear mixed effect (LME) model for incomplete curves on R software (Thorin et
205 al. 2010). $P < 0.05$ was considered statistically significant.

206 3. Results

207 3.1. Effect of CD and BAY 41-2272 treatment on body weight and metabolic parameters

208 As shown in Table 1, at the end of the feeding period, body weight gain increased
209 significantly more among SHR fed a CD diet than among those fed a normal diet ($P < 0.001$).
210 This weight gain was correlated to a significant increase in abdominal circumference
211 ($P < 0.001$) (Fig. 1A.1B) and epididymal fat ($P < 0.01$). In this study, we examined the effect of
212 long-term treatment with BAY 41-2272 on body weight in rats fed with CD. BAY 41-2272

213 treatment prevented excessive weight gain and increase in abdominal circumference and was
 214 well tolerated by SHR as well. Moreover, CD feeding induced a rise in SBP compared to the
 215 control SHR ($P<0.01$) (Table 1). Similar results were observed for DBP. The elevation in both
 216 SBP and DBP was significantly attenuated by BAY 41-2272 treatment ($P<0.001$). The plasma
 217 triglycerides level was significantly increased in CD-fed rats ($P<0.05$), whereas total
 218 cholesterol level was reduced compared to control rats. Plasma triglycerides levels remained
 219 elevated in CD-fed group treated with BAY 41-2272. However, no significant differences in
 220 glucose and insulin levels among groups were noticed (Table 1).

221 Table 1: Effect of CD with or without BAY 41-2272 treatment on metabolic disorders

	Control	CD	CD + BAY 41-2272
Body weight gain (g)	145±5.13	248±9.23 ^c	216±6.11 ^d
Abdominal circumference (cm)	15.48±0.29	18.07±0.17 ^c	17.46±0.14
Epididymal fat (g)	2.33±0.08	4.41±0.53 ^b	4.03±0.47
Systolic blood pressure (mmHg)	164.4±2.2	180.0±2.6 ^b	131.6±3.7 ^e
Diastolic blood pressure (mmHg)	127.5±2.8	151.6±2.4 ^c	106.9±3.2 ^e
Triglycerides (g/l)	0.79± 0.08	1.34± 0.16 ^a	1.37± 0.17
Total cholesterol (g/l)	0.85± 0.04	0.62± 0.06 ^b	0.75±0.04
Fasting glycemia (mg/dl)	103.60± 2.48	107.30± 4.52	96.71 ± 3.43
Fasting insulinemia (μUI/ml)	9.21± 1.32	11.83± 2.52	9.87 ± 2.37

223 All values are mean \pm S.E.M. ^a P<0.05, ^b P<0.01, ^c P<0.001 vs Control group, ^d P<0.05, ^e P<0.001 vs CD group;
224 ANOVA followed by the Tuckey's multiple comparison post-hoc test / NLME model, n=14-16 animals per
225 group. CD, Cafeteria Diet

226 Glucose tolerance was evaluated by intraperitoneal administration of glucose (1g/kg) to all
227 groups. CD feeding enhanced elevation of glucose blood level in comparison to the standard
228 chow diet (Fig. 2A). The area under the curve (AUC) was higher in the CD-fed group than in
229 the control group (P<0.001, Fig.2B). However, long-term treatment with BAY 41-2272
230 significantly improved glucose tolerance in CD-fed rats (P<0.001, Fig. 2A,2B).

231 3.2.Isolated heart data

232 To examine the effects of long-term treatment with BAY 41-2272 during CD feeding, cardiac
233 contractility and coronary perfusion pressure were evaluated using an isolated Langendorff
234 heart preparation. As shown in Table 2, both LVDevP and coronary perfusion pressure did
235 not differ between rats from the 3 groups at the basal level.

236 Table 2: Baseline cardiac parameters

	Control	CD	CD + BAY 41-2272
LVDevP (mmHg)	77.04 \pm 8.22	60.55 \pm 3.72	67.04 \pm 5.20
Coronary perfusion pressure (mmHg)	25.88 \pm 1.40	23.73 \pm 3.29	22.45 \pm 5.84

237 The hearts were perfused with aerated Krebs-Henseleit solution as previously described. LVDevP, Left
238 ventricular developed pressure. CD, Cafeteria Diet. n=6 animals/group. Values are expressed as mean \pm S.E.M
239 Moreover, in order to determine the effects of BAY 41-2272 on β -adrenergic response, we
240 evaluated cardiac function of the 3 groups under stimulation of increasing concentrations of
241 isoproterenol. β -adrenoceptor stimulation induced a marked increase in LV contractility

242 (determined by LVDevP and dP/dt (max)) in control rats. This positive inotropic effect was
243 significantly reduced in CD-fed group ($P < 0.001$) (Fig. 3A,3C). Similarly, LV relaxation
244 (dP/dt min) was also significantly depressed after isoproterenol stimulation in CD-fed group
245 compared to the control group ($P < 0.01$) (Fig. 3D). Thus, in our rat model of metabolic
246 syndrome, basal cardiac function was preserved but the inotropic and lusitropic effects in
247 response to β -adrenoceptor stimulation were altered suggesting an impairment in the β -
248 adrenoceptor signalling in this model. However, long-term treatment with BAY 41-2272
249 significantly restored the isoproterenol-induced lusitropy and the inotropy parameters. These
250 findings revealed that *in vivo* long-term sGC stimulation in CD-fed rats improved cardiac
251 systolic and diastolic functions (Fig. 3A,3C,3D). In parallel, isoproterenol-induced coronary
252 vasodilation was higher in control group than in CD-fed group ($P < 0.05$). This parameter was
253 also restored in CD group treated with BAY 41-2272 (Fig. 3B).

254 3.3. Vascular reactivity

255 Next, aortic reactivity was also evaluated. CCRCs to phenylephrine were then constructed in
256 aortic rings from all groups. The maximal contractile response (E_{max}) to phenylephrine, in
257 aortic rings from CD-fed group was significantly lower than that in the control group
258 ($P < 0.01$) (Fig.4A, Table 3). However, this response was normalized after adding an iNOS
259 inhibitor (Aminoguanidine, $100\mu\text{M}$) in the bathing solution (Fig.4B, Table 3). These findings
260 suggest an iNOS-dependent excessive NO release in the CD-fed group. On another side, BAY
261 41-2272 treatment completely restored the phenylephrine- concentration response curve in
262 CD-fed rats with significant increase in the maximal force of contraction (Fig. 4A, Table 3).

263

264

265

266 Table 3: pD₂ and E_{max} values of phenylephrine in the presence and in the absence of
 267 aminoguanidine in aortic rings isolated from all groups

	Control	CD	CD + BAY 41-2272
	Phe		
E _{max} (g)	4.16± 0.07 ^a	3.77± 0.09	4.30± 0.07 ^b
pD ₂	7.27± 0.04	7.30± 0.05	7.65± 0.06
	Phe + AMN		
E _{max} (g)	4.57± 0.21	4.46± 0.29 ^c	5.07± 0.10
pD ₂	7.28± 0.23	7.30± 0.08	7.59± 0.16

268
 269 Values are mean ± S.E.M. n=14-16 animals/group. ^aP<0.01 vs CD, ^bP <0.001 vs CD, ^cP<0.001 vs CD without
 270 AMN pretreatment determined by NLME model. Phe, Phenylephrine, AMN, Aminoguanidine, CD, Cafeteria
 271 diet

272 3.4. Quantification of iNOS expression in thoracic aorta

273 To test the hypothesis of a possible involvement of iNOS in the altered response to
 274 phenylephrine in CD-fed rats, immunofluorescence labelling was performed to determine the
 275 iNOS expression in thoracic aorta isolated from all groups. Our results did not show any
 276 significant difference between the control and CD-fed groups. However, the iNOS enzyme
 277 protein expression was significantly higher in CD-fed rats treated with BAY 41-2272
 278 (P<0.05) (Fig. 5A, 5B).

279 3.5. Endothelium-dependent and independent relaxations

280 In the present study, we evaluated the effects of both metabolic syndrome and long-term
281 treatment with BAY 41-2272 on endothelium-dependent vascular relaxation. Our results
282 showed that acetylcholine-induced endothelium-dependent relaxation was similar in aortic
283 rings from control and CD-fed group (Fig. 6A). This finding indicates that endothelial
284 function was not altered in our model of metabolic syndrome. Furthermore, maximal
285 relaxation in response to acetylcholine was not enhanced in aortic rings isolated from CD-fed
286 group treated with BAY 41-2272. Similarly, no significant difference in endothelium-
287 independent relaxation in response to SNP among the groups was noted (Fig. 6B).

288 3.6. Intracellular cGMP content in thoracic aorta, heart and epididymal fat samples

289 To further explore sGC-cGMP signalling pathway in our experimental conditions, we
290 assessed intracellular cGMP content in thoracic aorta, heart and epididymal fat samples
291 isolated from all groups. The results showed that compared to the CD-fed group, the cGMP
292 content was slightly but not significantly increased in CD-fed group treated with BAY 41-
293 2272 both in cardiac and vascular tissue. On the other hand, the cGMP content was
294 significantly higher in epididymal fat samples of CD-fed group treated with BAY 41-2272 in
295 comparison to the CD untreated group (Table 4).

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302 Table 4: intracellular cGMP level in thoracic aorta, heart and epididymal fat samples

cGMP (pmol/mg protein)	Control	CD	CD + BAY 41-2272
Thoracic aorta	1.20± 0.23	0.89± 0.12	1.40± 0.27
Heart	1.10± 0.12	1.25 ± 0.34	1.67± 0.16
Epididymal fat	0.33±0.07	0.16±0.04	1.15±0.28 ^a

303

304 Values are expressed as means ± S.E.M. CD, Cafeteria diet. n=8 animals/group. ^aP <0.01vs CD. ANOVA
 305 followed by the Tuckey's multiple comparison post-hoc test. CD, Cafeteria Diet

306 4. Discussion

307 In the present study, we showed that chronic CD feeding of SHR, induced abdominal obesity,
 308 hypertriglyceridemia, glucose intolerance and arterial hypertension, which are the main
 309 hallmarks of metabolic syndrome. These findings are consistent with those of La Russa et al.
 310 (2019) who demonstrated that CD is the most appropriate regime to induce severe obesity,
 311 glucose intolerance, insulin resistance, and high plasma triglyceride levels in rodents.
 312 However, they are partially inconsistent with the findings of Miesel et al. (2010) who
 313 reported that insulin resistance in SHR was more pronounced over the feeding period. It is
 314 well documented that SHR are already hypertensive and insulino-resistant (Reaven and
 315 Chang, 1991). Moreover, it is probable that CD-fed group presented insulin resistance, even if
 316 insulin levels were not modified, since obesity was associated to reduced glycemic tolerance
 317 in that group (Oliveira Junior et al., 2010).

318 One of the main findings of the present study is that long-term treatment with BAY 41-2272
319 reduced body weight gain and abdominal fat and improved glucose tolerance in CD-fed rats
320 in comparison to CD untreated rats. These results seem to be more related to increased energy
321 expenditure than to decreased energy intake since food consumption between untreated and
322 treated groups was not statistically different (data not shown). These results are consistent
323 with our previous work which showed that cGMP pathway activation through PDE5
324 inhibition, prevented weight gain in SHR fed a CD (Doghri et al., 2019). They are also in
325 agreement with the findings of Mitschke et al. (2013) who demonstrated that short-term
326 treatment with sildenafil in mice, increased the uncoupling protein-1 (UCP-1) expression and
327 promoted browning of white adipose tissue which is considered a primary site of energy
328 expenditure (Haas et al., 2009). Moreover, a recent study showed that pharmacological
329 stimulation of sGC induced weight loss and improved the metabolic phenotype in mice with
330 diet-induced obesity by enhancing brown adipocytes differentiation (Hoffmann et al., 2015).
331 Increasing evidence suggests that cGMP pathway modulation may regulate energy balance
332 via mechanisms that involve thermogenesis, by promoting mitochondrial biogenesis and
333 increasing the abundance of UCP-1 (Kim GW et al., 2014).

334 Although several mechanisms have already been described to better understand the metabolic
335 syndrome-induced cardiac dysfunction (Ilkun and Boudina, 2013; Tune et al., 2017), little
336 information is available on the β -adrenergic system changes during the metabolic syndrome in
337 SHR. The significant decrease in both inotropic and lusitropic effects of β -adrenoceptor
338 stimulation observed in CD-fed group could be related to either alteration in intracellular
339 calcium handling (Lima-Leopoldo et al., 2011; Nevelsteen et al., 2013) or to the down-
340 regulation of β_1 - and β_2 - adrenoceptors (Jiang et al., 2015). It is well known that sympathetic
341 nervous system plays a crucial role in maintaining cardiovascular homeostasis (Manolis et al.,
342 2014). In this regard, Li et al. (2015) have demonstrated an enhanced sympathetic activity in

343 SHR compared to normotensive rats. Moreover, several line of evidence show a link between
344 metabolic syndrome and sympathetic overactivity (Thorp and Schlaich, 2015). Therefore, it is
345 likely that sustained sympathetic overstimulation associated with metabolic syndrome may
346 contribute to the downregulation of myocardial β - adrenoceptors. On another side, previous
347 research has reported that the sympathetic overactivation associated with the development of
348 metabolic syndrome, resulted in vascular hyporeactivity to the α_1 -adrenoceptor stimulation in
349 rats (Battaut et al., 2018). These observations are in line with data from our study. In the
350 present study, CD-fed group showed a lower vasoconstrictor response to the α_1 -adrenoceptor
351 agonist, phenylephrine compared to their counterparts in the control group. Reduced aortic
352 contractility was also reported in aortic rings from other animal models of metabolic
353 syndrome such as obese zucker rats (Vendrame et al., 2014) and high sugar-fed mice (Silva et
354 al., 2016). However, one alternative explanation for such a result is the increase in iNOS-
355 induced NO production in CD-fed group. This hypothesis is supported by the fact that
356 addition of the iNOS inhibitor, aminoguanidine completely normalized the response to
357 phenylephrine in this group. Nonetheless, immunofluorescence assay did not show any
358 significant difference in iNOS expression in thoracic aorta between the control and CD-fed
359 groups. Data from literature concerning the iNOS expression modification in rat aorta during
360 metabolic syndrome are divergent (Araujo et al., 2018; Cebova et al., 2018). The possible
361 mechanisms explaining these divergent findings remain unclear and may be related to type of
362 diet and animal model used. Thus, further studies are needed to clarify the mechanisms
363 underlying these discrepancies.

364 A major finding of this study is that the sCG stimulator BAY 41-2272, administered orally to
365 CD-fed SHR at the dose of 5 mg/kg for 12 weeks was able to attenuate significantly both
366 systolic and diastolic hypertension. Our results are in agreement with previous studies.
367 Geschka et al. (2011) showed that sGC stimulation by riociguat at dose of 3mg/kg for 14

368 weeks were sufficient to decrease markedly systemic hypertension and to improve survival in
369 Dahl salt-sensitive rats. Furthermore, a study established by Stasch et al. (2001) demonstrated
370 that oral administration of BAY 41-2272 (1-10 mg/kg) resulted in a strong decrease in blood
371 pressure in a low-NO rat model of hypertension. It is not unreasonable to postulate that
372 the BAY 41-2272 lowering blood pressure effect could play a role in the change of
373 cardiovascular reactivity observed in our study. Typically, the majority of antihypertensive
374 drugs may exert simultaneously both specific local and systemic effects; and it is not easy to
375 distinguish the drug-induced reduction of blood pressure from any local effect that may occur
376 at the cellular level. Ideally sub-antihypertensive doses of BAY 41-2272 should be tested in
377 order to accurately assess the effect of BAY 41-2272 on cardiovascular reactivity
378 independently of the change in blood pressure.

379 In addition to lowering blood pressure, long-term treatment with BAY 41-2272 improved β -
380 adrenoceptor responsiveness and restored the adrenoceptor-mediated vasoconstrictive
381 response in CD-fed rats. There is accumulating evidence that sGC stimulators possess anti-
382 fibrotic, anti-inflammatory and antioxidant properties (Tobin et al., 2018). Moreover, a study
383 conducted by Ferron et al. (2019) showed that improved β -adrenergic responsiveness in high
384 sugar-fat diet fed rats would be attributed to reduced oxidant status. In light of our results, we
385 suggest that improvement in the adrenergic response observed in CD-treated rats may involve
386 antioxidant properties of BAY 41-2272 in addition to its vasodilator potential. Unfortunately,
387 we were not able to get sufficient blood samples to analyze the oxidative status as we needed
388 to keep a good viability of the heart for *ex vivo* cardiac reactivity. Another plausible
389 explanation for this finding is a possible cross regulation between cyclic adenosine
390 monophosphate (cAMP)- and cGMP- mediated signalling pathways. It has been previously
391 shown that increasing levels of cGMP suppress cAMP hydrolysis rate by phosphodiesterase
392 1,2 and 3, leading to an amplified cAMP signalling (Zhao et al., 2015). This suggests that

393 long-term treatment with BAY 41-2272 improves β -adrenergic responsiveness probably
394 indirectly through a mechanism that may involve isoenzymes phosphodiesterase regulation.
395 According to other reports (Vendrame et al., 2014; Lyoussi et al., 2018), we showed that
396 acetylcholine-induced endothelium- dependent relaxation was similar in aortic rings from
397 control and CD-fed group. However, this result contrasts with other findings describing a
398 reduced endothelium-dependent relaxation in SHR (Anishchenko et al., 2015) and in other
399 animal models of metabolic syndrome (El-Bassossy et al., 2014; Bhatta et al., 2017). A
400 probable hypothesis to explain this divergence is that a compensatory mechanism likely
401 involving a role of NO was developed in CD-fed rats to offset the effects of arterial
402 hypertension. This hypothesis is consistent with findings of Berenyiova et al. (2018), who
403 showed that SHR develop adaptative mechanisms by preserving NOS activity level in order to
404 fight chronic NO deficiency. In addition, this is in line with the lack of difference between
405 groups concerning intracellular cGMP content in thoracic aorta observed in our experimental
406 conditions. It is widely documented that resistant arteries play an important role in the
407 regulation of blood pressure in SHR (Yu et al., 2016) and are less dependent on NO than
408 thoracic aorta (Lyoussi et al., 2018). A complementary study of the vascular reactivity in
409 resistance arteries would therefore be interesting to better assess the effects of CD and BAY
410 41-2272 treatment.

411 In conclusion, the present study demonstrated that long-term treatment with BAY 41-2272
412 prevented excessive weight gain, markedly attenuated arterial hypertension and improved
413 cardiovascular reactivity in CD-induced metabolic syndrome in SHR. These results need
414 deeper investigations to assess whether BAY 41-2272 might represent a promising potential
415 candidate in the management of the metabolic syndrome and associated cardiovascular
416 alterations.

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420 **Conflict of interest**

421 The authors declare that no competing interests exist

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639 **Figure captions**

640 Fig. 1. Follow up of body weight gain (A) and abdominal circumference (B) during 12 weeks
641 of diet. CD feeding significantly increase body weight gain and abdominal circumference in
642 comparison to the standard chow diet ($P < 0.001$). On the other hand, long-term treatment with
643 BAY 41-2272 limited excessive weight gain only at the 12th week of the treatment ($P < 0.05$ vs
644 CD) and tended to prevent increase in abdominal circumference in CD-fed group. Data are
645 expressed as mean \pm S.E.M (n=14-16 rats/group). * $P < 0.001$ vs Control determined by LME
646 model.

647 Fig. 2. Blood glucose concentrations (A) and AUC (B) of intraperitoneal glucose tolerance
648 test were measured at the end of experimental protocol in all groups. Long-term treatment
649 with BAY 41-2272 improved glucose tolerance in CD-fed rats. *** $P < 0.001$ vs Control, CD +
650 BAY 41-2272 by One-Way ANOVA followed by Tukey *post hoc* test (n=14-16 rats/group).

651 Fig. 3. Cardiac response to isoproterenol in CD-fed rats and CD-fed rats treated with BAY 41-
652 2272. The contractile function was evaluated by measuring the increase in left ventricular
653 developed pressure (LVDevP) (A) and time derivative of pressure during contraction (dP/dt
654 max) in response to isoproterenol (C). Coronary vasodilation was assessed through coronary
655 perfusion pressure variation (B). The diastolic function of the heart was evaluated by
656 measuring the increase in time derivative of pressure during relaxation (dP/dt min) (D). Each
657 value represents the mean \pm S.E.M (n=6 rats/group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs CD
658 determined by LME model.

659 Fig. 4. Cumulative-concentration response curves to phenylephrine in thoracic aortic rings
660 isolated from control rats, CD-fed rats and CD-fed rats treated with BAY 41-2272. Contractile
661 response to phenylephrine in the absence (A) or presence of aminoguanidine (100 μ M) (B).

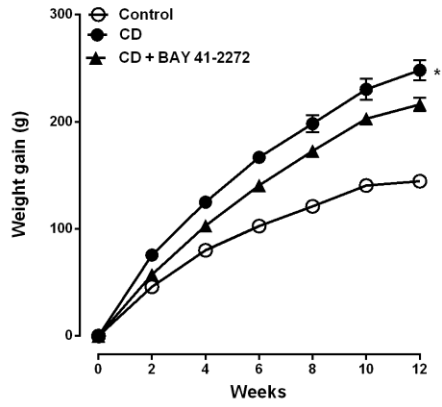
662 Each value corresponds to the mean \pm S.E.M (n=14-16 rats/group). **P<0.01, ***P<0.001 vs
663 CD determined by NLME model.

664 Fig. 5. Effects of CD and long-term treatment with BAY 41-2272 on iNOS enzyme protein
665 expression in thoracic aorta. (A) Fluorescence confocal microscopy of iNOS (red
666 fluorescence, λ_{exc} 561 nm) immunodetected in thoracic aorta (elastin with green
667 fluorescence, λ_{exc} 488 nm), scale bar 50 μ m. Control group (n = 4), CD-fed group (n = 3)
668 and CD-fed group treated with BAY 41-2272 (n=3). (B) Values were represented as mean \pm
669 S.E.M of mean fluorescence intensity (U.I.). *P<0.05 vs Control /CD group determined by
670 One Way ANOVA.

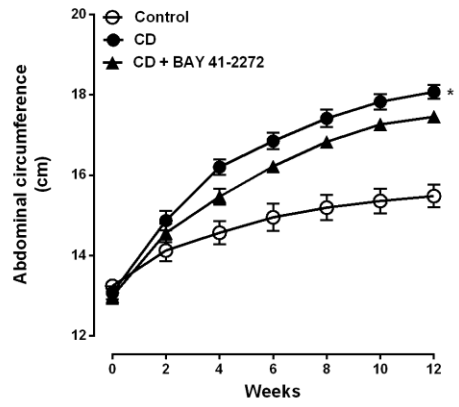
671 Fig. 6. Effects of chronic CD feeding and long-term treatment with BAY 41-2272 on
672 endothelium-dependent and independent relaxations in thoracic aorta. Cumulative
673 concentration response of acetylcholine-induced relaxation (A). Cumulative concentration
674 response curve to sodium nitroprusside (SNP) for the endothelium-independent relaxation
675 (B). values are expressed in percentage of the precontraction. Each value represents the mean
676 \pm S.E.M. Comparisons were performed using NLME model.

677

A

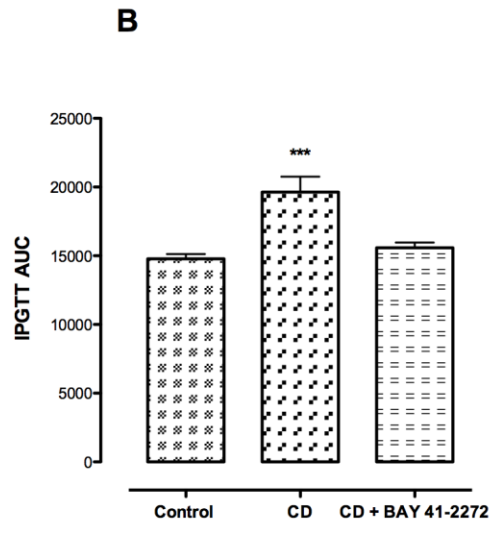
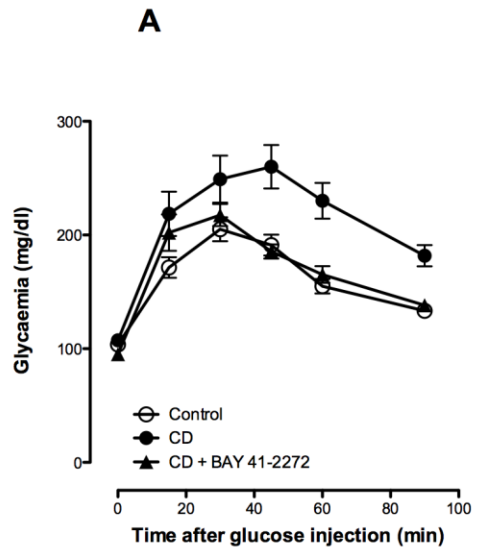


B



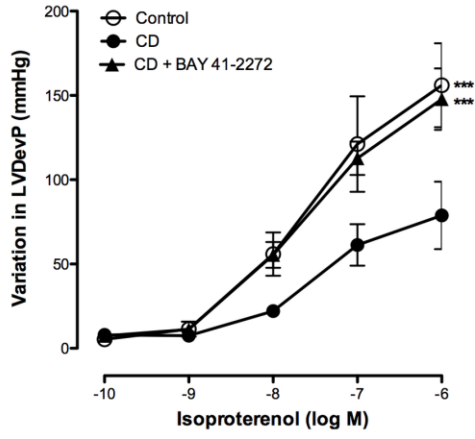
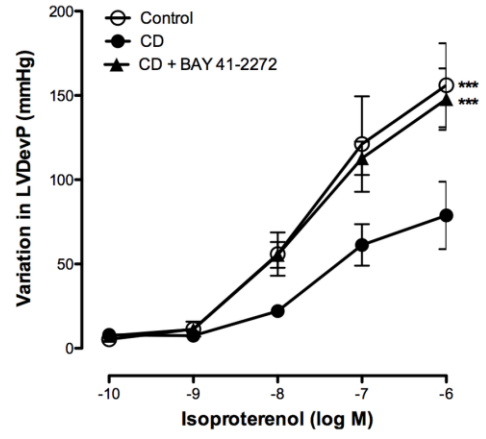
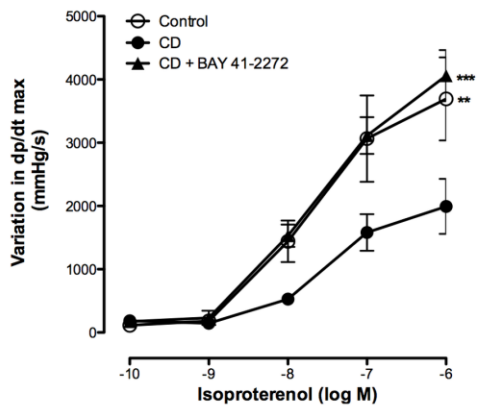
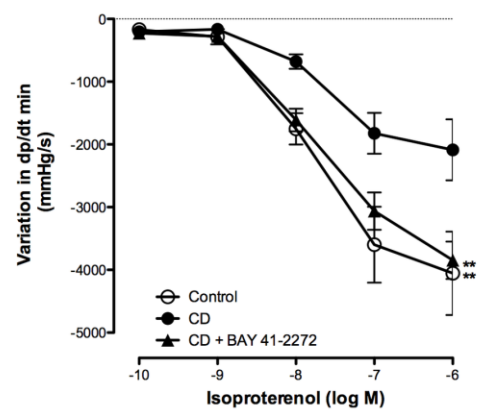
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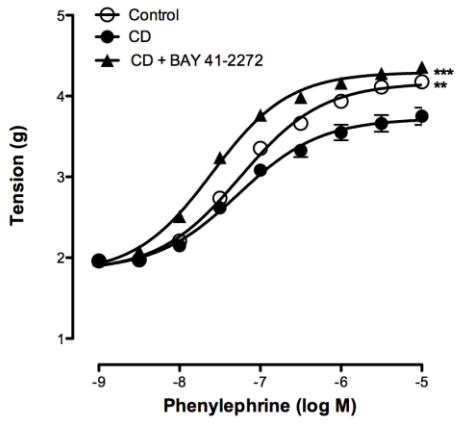
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A**B****C****D**

682

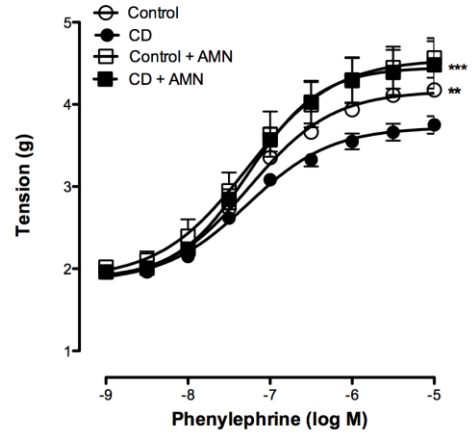
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A



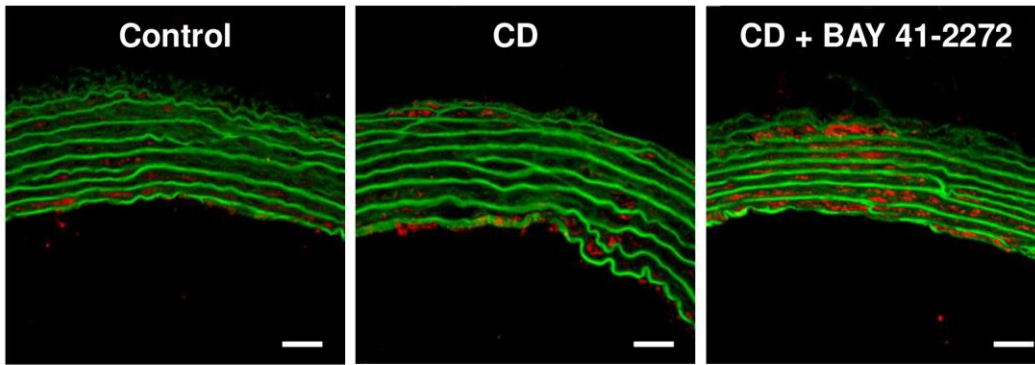
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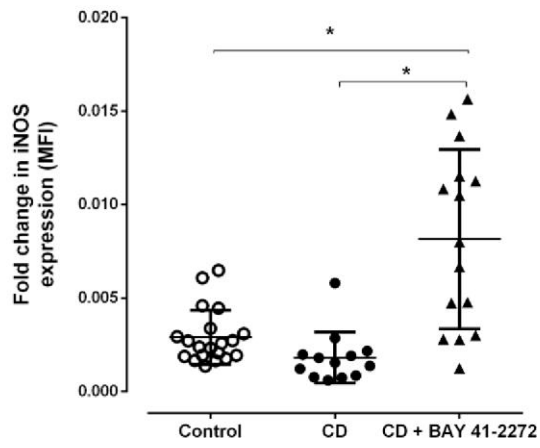


685

A



B



686

687

