ACUTE ADMINISTRATION OF SINGLE ORAL DOSE OF GRAPE SEED POLYPHENOLS RESTORES BLOOD PRESSURE IN A RAT MODEL OF METABOLIC SYNDROME: ROLE OF NITRIC OXIDE AND PROSTACYCLIN

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Abstract

Purpose. The aims of this study were to evaluate the antihypertensive effectiveness of different doses of grape seed polyphenols in cafeteria diet-fed hypertensive rats (CHRs) and to establish the mechanism involved in the blood pressure (BP) lowering effect of these compounds in this experimental model of metabolic syndrome (MS).

Methods. Male 8-week-old Wistar rats were fed cafeteria or standard (ST) diet for 10 weeks. After this, the antihypertensive effect of a single oral administration of a polyphenol grape seed extract (GSPE) was tested at different doses (250, 375 and 500mg/kg) in CHRs. BP was recorded before and 2, 4, 6, 8, 24 and 48h post-administration. The hypotensive effect of GSPE was also proved in ST diet-fed rats. Additionally, in other experiment CHRs were orally administered 375mg/kg GSPE. 4h post-administration, the rats were intraperitoneally administrated 30mg/Kg NG-nitro-L-arginine methyl ester (L-NAME) or 5mg/Kg indomethacin (inhibitors of nitric oxide (NO) and prostacyclin synthesis, respectively). BP was recorded initially and 6h post-administration.

Results. GSPE produced a decrease in SBP and DBP, the most effective dose (375mg/kg) showing an antihypertensive effect in CHRs similar to the drug captopril and did not affect BP of ST diet-fed rats. The antihypertensive effect was completely abolished by L-NAME, and partially inhibited by indomethacin.

Conclusions. GSPE acts as an antihypertensive agent in a rat model of hypertension associated with MS. The change in endothelium-derived NO availability is one of the mechanisms involved in the antihypertensive effect of GSPE in CHRs. Additionally, endothelial prostacyclin contributes to the effect of GSPE on arterial BP.

Keywords: cafeteria diet; hypertension; antihypertensive agent; endothelial-relaxing factors

Introduction

Hypertension (HTN) is a major risk factor for the development of cardiovascular disease (CVD)[1]. In fact, high blood pressure (BP) treatment has been associated with an approximately 40% reduction in the risk of stroke and an approximately 15% reduction in the risk of myocardial infarction [2]. Human HTN frequently occurs concurrently with many other CVD risk factors related to lifestyle, such as obesity, dyslipidaemia or impaired glucose tolerance (hyperglycemia) [3], resulting in metabolic syndrome (MS). Wistar rats fed cafeteria (CAF) diet, which consists of free access to highly palatable, energy dense, unhealthy human food, are considered a robust model of human MS [4], which is pandemic in western civilisation today [5]. CAF diet-fed rats present increased body weight (BW), additional abdominal fat and develop hyperinsulinemia, hyperglycaemia and hepatic steatosis (see review of animal model of MS in [6]). Additionally, elevated measures of BP in CAF diet-fed rats have been reported [7, 8]. Nevertheless, the use of CAF diet-fed hypertensive rats (CHRs) as an experimental model of HTN to assay antihypertensive compounds has not been widely explored. However, this dietary model present many of the complications associated with MS. Moreover, it is important to note that in humans diet is a major factor in the development of these pathologies.

Increasing evidence suggests that a vegetable and fruit-rich diet, which is abundant in polyphenolic compounds, helps to control BP. In fact, increased fruit and vegetable intake has been included in the guidelines for the management of arterial HTN [9]. Cocoa and grapes are a significant source of polyphenols, and particularly of flavanols. Our research group has previously demonstrated the antihypertensive effect of cocoa or grape seed flavanols in

spontaneously hypertensive rats (SHRs) [10–12]. The antihypertensive properties of cocoa and grape seed flavanols in SHRs have been demonstrated to be mostly mediated by changes in endothelium-derived nitric oxide (NO) availability [13, 14]. Additionally, prostacyclins could contribute also to this effect for grape seed polyphenols [14]. However, although SHRs are one of the well-established and most-used experimental model of HTN, these animals do not present other CVD risk factors frequently associated to human HTN, which result in MS.

Therefore, the purpose of the present study was to investigate the antihypertensive effect of different doses of grape seed polyphenols in an experimental model of HTN associated with MS. In addition, the involvement of endothelial-relaxing factors as BP regulating mechanism of grape seed polyphenols in this dietary model has also been studied using CHRs treated alternatively with NG-Nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis or with indomethacin, an inhibitor of prostacyclin synthesis.

1. Methods

1.1. Grape-derived product and reagents

GSPE is a low molecular-weight polyphenol rich extract obtained from white grape seeds and was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The total polyphenol content was determined according to the Folin-Ciocalteu spectrophotometric method and it was expressed by using gallic acid as the standard [15]. Individual phenolic compounds were characterised by an high performance liquid chromatography

(HPLC) Agilent 1200 Series coupled to a time-of-flight mass spectrometer Agilent TOF 6210 as previously was described [12]. The antioxidant capacity of this extract was determined by the hydrophilic oxygen radical absorbance capacity (ORAC) assay according to the methodology reported previously [16]. The ORAC values were calculated by using the area-under-curve (AUC) results for Trolox, expressed as micromoles of Trolox equivalents (TE) per gram of GSPE. All the analyses were performed in triplicate and the results are reported on dry basis (Table 1).

Captopril, L-NAME and indomethacin were purchased from Sigma (Barcelona, Spain).

1.2. Experimental Procedure in Rats

1.2.1. General procedure

6-week-old male Wistar rats Crl:WI were purchased from Charles River Laboratories (Barcelona, Spain) and were singly housed in animal quarters in at 22ºC with a light/dark period of 12h. After quarantine and a training period of two weeks, the animals were divided into two dietary groups (Figure 1). The control group were fed with the standard (ST) chow Panlab A04 (Panlab, Barcelona, Spain) and tap water *ad libidum*. The second group had free access to a fresh CAF diet consisting of bacon (10-12g), frankfurter (8–12 g); biscuit with pâté (12–15 g); biscuit with cheese (10–12 g); *ensaïmada* (pastry) or muffins (4-5 g); carrots (8-10 g); milk with sugar (220 g/L; 50 mL); water (*ad libitum*) and ST diet. The ST chow had a calorie breakdown of 14% protein, 8% fat and 73% carbohydrates and 0,3% of Na, whereas the calorie breakdown of the CAF diet was: 14% proteins; 35% fat; and 51% carbohydrates and 0,2% of

Na. The animals were fed *ad libitum*, and the food was renewed daily. The CAF administration was maintained to the end of the experiment.

Values of BP, BW and abdominal circumference (AC) were recorded at the beginning, middle (5^{th} week) and final (10^{th} week) of feeding period in both groups of rats (Figure 1). Systolic BP (SBP) and diastolic BP (DBP) were recorded between 9 and 11 am in the rats by the tail-cuff method [17] as previously was described[8]. To minimize stress-induced variations in BP, all measurements were taken by the same person in the same peaceful environment. After the quarantine period, the BP method was performed as a training period to acclimatise the animals to the procedure.

AC was assessed on the largest zone of the rat abdomen, vertically immobilized, using a non-extensible measuring tape with an accuracy of 0.1 cm, without applying pressure to the body surface. Initially and after 5 and 10 weeks of feeding period the animals were housed for 24h in metabolic cages to estimate the daily food and liquid intake and to collect faeces and urine samples.

The animal protocol followed in this study was approved by the Bioethical Committee of Universitat Rovira i Virgili (European Commission Directive 86/609 and Spanish Royal Decree 223/1988).

1.2.2. Biochemical assays

Plasma biochemical assays were measured 10 weeks after the beginning of the experiment using tail vein blood draws after fasting overnight.

Glucose, total cholesterol and triglyceride plasma concentration were assayed using enzymatic colorimetric kits (GOD-PAP method for glucose, CHOD-POD method for total cholesterol and GPO method for triglycerides;

QCA, Tarragona, Spain). Insulin plasma concentrations were determined using a Rat Insulin ELISA/Ultrasensitive kit (Mercodia, Uppsala, Sweden).

1.2.3. Antihypertensive effects of grape seed polyphenols

After 10 weeks of diet feeding, the acute effect of GSPE on SBP and DBP were evaluated in CAF and ST diet-fed rats (Figure 1). GSPE was dissolved in water and orally administered by gastric intubation, between 9 and 10 am. Water was used as a negative control, and Captopril (50mg/kg), a known antihypertensive drug, as a positive control. Different doses of GSPE (250, 375 and 500mg/kg) were administered to CHRs and 375 mg/kg GSPE was also evaluated in ST diet-fed rats. The animals were always orally administered a single dose of either 1 mL of water or the appropriate solution of GSPE or Captopril. SBP and DBP were recorded at 0, 2, 4, 6, 8, 24 and 48h post-administration.

1.2.4. Cafeteria diet-fed rats treated with NG-Nitro-L-arginine methyl ester and indomethacin

Additional trials were conducted administering a single dose of 375mg/kg GSPE or water by gastric intubation between 9 and 10 am to CHRs. In addition, both groups were intraperitoneally treated with 1 mL of saline or 30mg/kg L-NAME or 5mg/kg indomethacin 4h after the oral treatment (n=5 per group). SBP and DBP were recorded initially and 6h post-administration (Figure 1).

1.3. Statistical analysis

Data were analysed by one-way ANOVA, two-way ANOVA (Tukey's test) or independent Student's T-test using IBM SPSS Statistics 20.0.0 for Mac as required. Kolmogorov-Smirnov and Levene's tests were used, respectively, to check for the normality and equality of variances of the data as required.

2. Results

2.1. *Effects of cafeteria diet on blood pressure, physiological and plasmatic parameters.*

At the beginning of the experiment, BW, AC and BP did not differ (Table 2). However, the animals fed CAF diet for 10 weeks presented increased BW, AC and BP (SBP and DBP) with respect to ST diet-fed rats. Compared to the control group, the animals fed a CAF diet had significantly increased BW throughout, with differences of 12.0% and 19.2% at the 5th and 10th week, respectively. Concerning the AC, the differences were of 7.8% at the 5th week and 16.4% at 10th week of the feeding period. CAF rats presented significant increase of SBP in both 5th (142±3 vs 122±2 mmHg) and 10th (140±2 vs 122±3 mmHg) week of feeding period compared with the ST group.

Pre-treatment fluid intake was not different between groups. The fluid intake by the CAF group was higher than for the ST group at the 5th week of the feeding period. Throughout the 10-week of CAF administration, the fluid intake by the CAF group was almost 3-times higher than of the ST group. It has to be taken into account that the CAF diet includes milk, which increases *per se* the total liquid intake of the CAF-fed animals. Thus, urine volume was 4-times higher in rat fed CAF diet than in the ST group.

Pre-treatment food total intake was not different between groups. However, the total food intake by the CAF group was significantly higher than that of the ST group at the 5th and 10th week of feeding period (3.5 and 4-fold higher, respectively). Consequently, the protein, fat, carbohydrate and energy intake were also higher in the CAF than in the ST group at the 5th and 10th week of diet. On the contrary, the amount of deposited faeces at the 5th and 10th week of

feeding period decreased in the CAF groups respect to ST diet-fed rats (Table 2).

The consumption of CAF diet for 10 weeks by rats resulted in a significant increase in plasma cholesterol, triglycerides and insulin concentrations under fasting conditions (Table 3).

3.2. Short-term effect of grape seed polyphenols on blood pressure in cafeteria diet-fed rats

All doses of GSPE produced an antihypertensive effect in rats fed CAF diet (Figure 2A and 2B). The maximum effect on SBP and DBP was caused by the dose of 375mg/kg GSPE at 6h post-administration (-21 ± 2 mmHg and -22 ± mmHg, respectively), statistically similar to those found for Captopril (-19 ± 4 mmHg and -26 ± 3 mmHg, respectively). Oral administration of 500mg/Kg GSPE showed a similar effect in SBP and DBP to the results observed with 250mg/kg. Water administration had no effect on SBP and DBP.

3.3. Effect of grape seed polyphenols on blood pressure in cafeteria diet-fed rats treated with NG-Nitro-L-arginine methyl ester and indomethacin

As expected, the animals that received only water and saline did not modify their SBP and DBP (Figures 3A and 3B). On the contrary, 375mg/kg GSPE and saline caused a significant decrease in SBP and DBP that could be appreciated 6h post-administration. Intraperitoneal administration of 30mg/kg of L-NAME caused an increase in SBP in the water group. The antihypertensive effect of GSPE was completely abolished by L-NAME treatment. Nevertheless, 5mg/kg indomethacin had no effect in the water-treated rats, but the effect of GSPE was slightly modified in the indomethacin treated rats (Figures 3C and 3D).

3.4. Short-term effect of grape seed polyphenols on blood pressure in standard diet-fed rats

The ST diet-fed animals that received water and 375 mg/kg GSPE did not modify their SBP and DBP (Figure 4), ruling out a hypotensive effect in normotensive rats.

4. Discussion

CAF diet-fed rats have been described as a robust model of human MS, this diet promotes voluntary hyperphagia that results in rapid weight gain [4]. Our study clearly confirms the development of hyperphagia, obesity and HTN in animals feeding on CAF diet for 10 weeks, mimicking the classical model of human MS (Table 2). In fact, HTN is one of the predominant complications associated with MS in humans, even more prevalent than obesity [18], although all of the factors are interlinked. In this study, the values of SBP reached in CHRs were not as high as the values reported for other experimental models of HTN such as SHRs, approximately 200 mmHg SBP at 17-20 weeks of age [19]. Nevertheless, the CAF diet-fed rats can be considered hypertensive, SBP 140 mm Hg or greater [20], at the 5th week of the CAF diet administration (Table 2). Moreover, in addition to the progressive increase in BW and BP, high plasma insulin levels in CAF diet-fed rats were observed (Table 3). Also, a significant elevation in HOMA-IR and HOMA-β levels has been reported for these animals respect to ST diet-fed rats (10.7 ± 1.0 vs 5.0 ± 0.5 and 1509± 389 vs 609 ± 93, respectively), indicating the presence of peripheral insulin resistance and an increase in pancreatic insulin secretion [21]. Additionally, the consumption of CAF diet induced dyslipidaemia in rats, as evidenced by an increase in plasma

249 total cholesterol and triglycerides at the 10th weeks of CAF diet administration
250 (Table 3).

251 To test the antihypertensive effect of grape seed polyphenols in HTN
252 associated to MS, *in vivo* studies were carried out in CHRs administered GSPE.
253 The administration of this extract produced a significant decrease in the SBP of
254 CHRs, the most effective dose (375mg/Kg) showing an antihypertensive effect
255 similar to captopril, which is a specific competitive angiotensin converting
256 enzyme inhibitor that is known to be a very effective antihypertensive treatment
257 in clinical practice. Interestingly and in contrast to SHRs [12], the
258 antihypertensive effects of GSPE was maintained 48h post-administration in
259 CAF diet-fed rats, indicating a more sustained antihypertensive effect in this
260 dietary model of HTN. Taking into account that HTN is a chronic pathology that
261 requires chronic treatment, and the use of strategies with long lasting
262 antihypertensive effects is always desirable, the antihypertensive properties of
263 these compounds would be more favourable in hypertensive rats with MS.
264 Paradoxically, the highest dose of GSPE (500mg/Kg) demonstrated a lower
265 antihypertensive effect than the medium dose (375mg/Kg). This apparently
266 surprising finding has also been described in SHRs administered grape seed
267 polyphenols [12] and other flavanol rich compounds such as cocoa [10]. These
268 results could be explained by the pro-oxidant properties and the excessive
269 production of reactive oxygen species caused by high doses of flavanols
270 described previously [10, 22]. In fact, endothelial tissue regulates vascular tone
271 and exerts finely tuned control over cardiovascular homeostasis, with NO being
272 one of the best-characterised vasodilator endothelial factors. However, NO is

273 also a precursor of potent pro-oxidant and nitrating compounds, such as
274 peroxynitrite and nitrogen dioxide [23].

275 Many different molecular targets and mechanisms have been proposed to
276 explain the cardiovascular effects of polyphenols, including participation of the
277 renin angiotensin aldosterone system (RAAS). In fact, angiotensin-converting
278 enzyme (ACE) inhibitory activity of flavanols has been demonstrated both *in*
279 *vitro* [24–27] and *in vivo* [8, 28]. However, no changes in plasma ACE activity
280 after the administration of flavanols from grape seed have been found [8, 12].
281 Nevertheless, these results did not eliminate the participation of this enzyme
282 before this moment or the participation of other RAAS system components in
283 the improvement of the BP.

284 A dysregulation between the vasodilator and vasoconstrictor secreted
285 factors in the endothelium has been described for MS and also for HTN [29].
286 NO is an important mediator of BP homeostasis and the increase in arterial tone
287 that characterizes the hypertensive state frequently implies an excess of free
288 radicals that destroy this mediator. Enhanced endothelial superoxide anion
289 production has been described in HTN and these effects are related to
290 impairment of endothelium-dependent relaxation [30]. In addition, a decreased
291 NO availability due to the increased oxidative stress in a rat model of obesity-
292 induced HTN has been reported [31]. In a recent study we reported a
293 decrement in hepatic lipid peroxidation products of rats fed CAF-diet
294 administered grape seed polyphenols, which indicates a decrease in oxidative
295 stress [8]. Hence, in this study we evaluated the involvement of NO as BP
296 regulating mechanism of GSPE in CHRs intraperitoneally injected L-NAME. L-
297 NAME is an *in vivo* and *in vitro* non-specific inhibitor of NO synthase [32], and in

298 the present study, a clear increase in SBP was observed in the L-NAME treated
299 CHRs. The inhibition of basal NO synthesis by L-NAME in these animals could
300 justify these results, but what was more important in order to fulfil the aim of the
301 present study was the observed impairment of GSPE antihypertensive effect in
302 the L-NAME-treated CHRs. These results suggest that facilitation of NO release
303 is a mechanism of action in the antihypertensive effect of grape seed
304 polyphenols in HTN associated with MS. Our results are in accordance with the
305 majority of available data, which support the evidence that flavanols are able to
306 improve NO availability [13, 14].

307 Nevertheless, the endothelium secretes other vasodilator agents in
308 addition to NO such as prostaglandin I2 (PGI2), also known as prostacyclin.
309 PGI2 is synthesised by the action of the cyclooxygenase (COX) enzyme and
310 prostacyclin synthase [33]. Therefore, we have also evaluated the effect of
311 GSPE in CHRs intraperitoneally injected indomethacin, which is an inhibitor of
312 COX enzyme and of the endothelial prostanoid biosynthesis. Indomethacin
313 treatment partially abolished the GSPE antihypertensive effect in CHRs (Figure
314 3b), indicating that endothelial prostacyclin could also contribute to the effect of
315 grape seed polyphenols on arterial BP in this animal model. In agreement,
316 increases in the release of PGI_2 in flavanol-treated human aortic endothelial
317 cells[34] and high plasmatic levels of prostacyclin in humans[34] and rats[14,
318 35] after flavanol consumption have been reported.

319 Finally, this study demonstrates the antihypertensive effect of GSPE in
320 hypertensive rats but not in normotensive rats, establishing the specific effects
321 of grape seed polyphenols on the hypertensive condition and ruling out a
322 hypotensive effect.

In conclusion, grape seed polyphenols exhibit a clear antihypertensive effect an in a rat model of HTN associated with MS. The change in endothelium-derived NO availability is one of the mechanisms involved in the antihypertensive effect of GSPE in CHRs. In particular, our results indicate that grape seed polyphenols affect endothelial NO synthesis in these animals. Additionally, endothelial prostacyclin contributes to the effect of GSPE on arterial BP. However, more studies are necessary to clarify the molecular mechanisms responsible for the antihypertensive properties of grape seed polyphenols in this animal model of MS and to demonstrate its long-term efficiency.

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6. Conflict of Interest

The authors declare no conflict of interest.

7. References

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Figure legends

Figure 1. Graphical representation of experimental design used in this study

Figure 2. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) in cafeteria-fed rats after the administration of water, Captopril or different doses of GSPE. Data expressed as MEAN ± SEM for 8 animals *per* group. Letters represent significant differences (two-way ANOVA; $p<0.05$).

Figure 3. Differences in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) in cafeteria-fed rats 6h post-administration of water or GSPE and L-NAME or saline. Differences in systolic blood pressure (SBP) (C) and diastolic blood pressure (DBP) (D) in cafeteria-fed rats 6h post-administration of water or GSPE and Indometacine or saline. Data expressed as MEAN ± SEM for 5-8 animals *per* group. Letters represent significant differences (one-way ANOVA; $p<0.05$).

Figure 4. Differences in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) in standard diet-fed rats after administration the administration of water or 375 mg/kg of GSPE. Data are expressed as mean ± SEM for 6 animals *per* group. No statistical differences were observed between groups (two-way ANOVA).