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p-Coumaric acid reduces high glucose-mediated impairment of endothelium-dependent relaxation in rat aorta

[Ácido *p*-cumárico reduce el deterioro mediado por alta glucosa de la relajación dependiente de endotelio en aorta de rata]

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Abstract: *p*-Coumaric acid (*p*-CA) is an ubiquitous plant metabolite with antioxidant, anti-inflammatory, and anticancer properties. The present study was designed to evaluate the preventive effects of *p*-CA on endothelium-dependent impairment produced by high glucose (HG) (D-Glucose 25 mM) in isolated rat thoracic aorta. Aortic rings obtained from male Sprague-Dawley rats were mounted in an organ bath and pretreated for 3 h with D-Glucose 5 mM, HG and HG plus *p*-CA (1, 10 and 100 μM). After this period of time endothelium-dependent relaxation was tested by cumulative addition of acetylcholine (ACh) in pre-contracted rings with phenylephrine (PE) (0.1 μM). *p*-CA elicited a moderate endothelium-dependent vasodilatory effect (Emax= 29.28 ± 1.89%, N=6; pD_2= 6.075 ± 0.184, N=6). When aortic rings were pre-incubated for 3 h with HG, Emax for ACh decreased dramatically from 87.69 ± 2.59% (N=6) to 40.54 ± 1.78% (N=6). The negative effect of HG was partially reverted in rings co-incubated with *p*-CA in a concentration-dependent manner as shown for Emax values to each *p*-CA concentration: 1 μM (48.57 ± 1.82%), 10 μM (60.81 ± 1.80%) and 100 μM (64.51 ± 1.80%). The action of *p*-CA was associated with a significant change in Emax. No statistical difference in pD_2 was observed. Our results clearly show that *p*-CA protect ACh-induced endothelial-dependent relaxation affected by HG in isolated rat aortic rings.

Keywords: *p*-coumaric acid, rat aorta, endothelium, high glucose, acetylcholine

Resumen: El ácido *p*-cumárico (*p*-CA) es un metabolito ubicuo en plantas, con propiedades antioxidantes, anti-inflamatoria, y anticancerígenas. El presente estudio fue diseñado para evaluar los efectos preventivos de *p*-CA sobre la relajación dependiente de endotelio, deteriorada por niveles altos de glucosa (HG) (D-glucosa 25 mM) en aorta torácica aislada de rata. Los anillos aórticos obtenidos de ratas macho Sprague-Dawley se montaron en un baño de órganos y fueron pre-tratados durante 3 h con D-glucosa 5 mM, HG, y HG más de *p*-CA (1, 10 y 100 μM). Después de este período de tiempo se evaluó la relajación dependiente de endotelio mediante la adición acumulativa de acetilcolina (ACh) en anillos pre-contraídos con fenilefrina (PE) (0,1 μM). *p*-CA mostró un moderado efecto vasodilatador dependiente de endotelio (Emax = 29,28 ± 1,89%, N = 6; pD_2 = 6,075 ± 0,184, N = 6). Cuando los anillos aórticos se pre-incubaron durante 3 h con HG, la Emax para ACh se redujo drásticamente desde 87,69 ± 2,59% (N = 6) a 40,54 ± 1,78% (N = 6). El efecto negativo de HG se revirtió parcialmente, de manera dependiente de concentración, en los anillos co-incubados con *p*-CA tal como lo muestra el valor de Emax para cada concentración de *p*-CA: 1 μ M (48,57 ± 1,82%), 10 μM (60,81 ± 1,80%) y 100 μM (64,51 ± 1,80%). La acción de *p*-CA se asoció con un cambio significativo en la Emax. No se observó diferencia estadísticamente significativa en el pD_2. Nuestros resultados muestran claramente que *p*-CA protege la relajación dependiente de endotelio inducida por ACh, la cual es afectada por HG en anillos aislados de aorta de rata.

Palabras Clave: ácido *p*-cumárico, aorta de rata, endotelio, alta glucosa, acetilcolina.

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INTRODUCTION

Since the early 50's isolated thoracic aorta have been a fecund model to evaluate the bioactivity of many compounds, including phytochemicals (Vinet *et al.*, 2012). After the milestone discovery of the role that plays endothelium in the vasodilatation induced by acetylcholine (Furchgott and Zawadzki, 1980), isolated aorta -specially as rings- have been extensively used to assess the activity of natural compounds that cause endothelium - dependent vasorelaxation (Andriambeloson *et al.*, 1998; Vinet *et al.*, 2012).

A large number of studies have been performed to evaluate whether some phytochemicals prevent oxidation process associated with vascular dysfunction and progression of atherosclerosis (Howard and Kritchevsky, 1997; Traka and Mithen, 2011) A positive, relationship between antioxidant activity and total phenolic content shows that phenolic compounds are the dominant antioxidant components in medicinal herbs (Cai *et al.*, 2004). In relation to cardiovascular diseases, an increasing importance has been given to nutrition and specifically to plant products in the diet (Howard and Kritchevski, 1997; Traka and Mithen, 2011).

p-Coumaric acid (*p*-CA) (3-[4-hydroxyphenyl]-2-propenoic acid), a phenolic acid, is a hydroxyl derivative of cinnamic acid. In plants, *p*-CA is an intermediate product of the phenylpropanoid pathway, widely distributed in fruits, such as apples and pears, and in vegetables and plant products, such as beans, tomatoes, potatoes and tea. *p*-CA has been suggested to exhibit antioxidant, anti-inflammatory, and anticancer activities (Zang *et al.*, 2000; Zhang *et al.*, 2007; Lee *et al.*, 2009; Kilic and Yesiloglu, 2013; Kong *et al.*, 2013; Stojkovic *et al.*, 2013; Yoon *et al.*, 2013). In this work we evaluate the effect of *p*-CA as a protector against the impairment endothelium-dependent relaxation induced when aortic rings were pre-incubated with high glucose (HG, D-Glucose 25 mM).

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats weighing 240-280 g were used. Rats were housed in a standard environmental condition. Food and water were freely available. Animals were used in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011).

Preparation of rat thoracic aorta rings and recording

Rats were killed by decapitation and the thoracic aorta was carefully removed and mounted on a tissue chamber as previously described (Vinet *et al.*, 1991). Briefly, aorta was carefully dissected, clean of connective tissue and divided into three rings segments of 5 mm. Caution was taken not to touch the endothelial surface to preserve the functional endothelium. In some experiments the endothelium was mechanically removed. The rings were suspended between two L-shaped stainless steel hooks and placed in a 30 mL organ chambers containing a modified Krebs-Henseleit buffer (KHB) with D-Glucose 5 mM, maintained at 37° C and oxygenated continuously with a 95% O_2 - 5% CO_2 gas mixture.

Isometric tensions were measured using a Myobath II multi-channel isolated tissue bath system combined with Lab-Trax Data Acquisition System with a 4-channel amplifier and analyzed using the Data-Trax 2 software (World Precision Instruments, USA). Rings were allowed to equilibrate in the tissue bath for 60 min under an optimal resting tension of 1.5 g. Mechanical stability of the system was accomplished by adding KCl 70 mM, three times. Integrity of endothelium was evaluated by testing the relaxation induced by ACh (1 μM) in pre-contracted rings with phenylephrine (PE) (0.1 μM). Aortic rings were repeatedly washed and allowed to re-equilibrate for additional 30 min.

The first ring was incubated in KHB (Control); the second ring was incubated in KHB with HG; and the third ring was incubated in KHB buffer with HG plus *p*-CA (1, 10, 100 μM). All rings were incubated for 3 h. After this period, rings were pre-contracted with phenylephrine (0.1 μM) and once a stable contraction was achieved, cumulative concentration-response curves to ACh were obtained. Maximum relaxation (Emax) and concentration producing the half-maximal effect (EC_{50}) were determined from each concentration-response curve and expressed as pD_2 (-log EC_{50}). The relaxation from the pre-contracted level to the baseline was considered as 100% relaxation.

Statistical analysis

All statistics were run using GraphPad Prism version 6. A one-way analysis of variance (ANOVA) with

Newman-Keuls Multiple Comparison Test or a paired t-test was used to analyze the data as appropriate. All data are expressed as means ± SEM. Differences were considered significant at $P < 0.05$.

RESULTS

Prior to investigate the effects of *p*-CA on rat aortic rings pre-incubated with HG, we evaluated the direct effect of *p*-CA on aortic rings with (+E) and without endothelium (–E) pre-contracted with PE (0.1 µM). As shown the concentration-response curve in Figure 1A, *p*-CA displays a moderate endothelium-dependent vasodilatory effect. Emax (%) was 29.28 ± 1.89 (N=6), and pD_2 was 6.075 ± 0.184 (N=6).

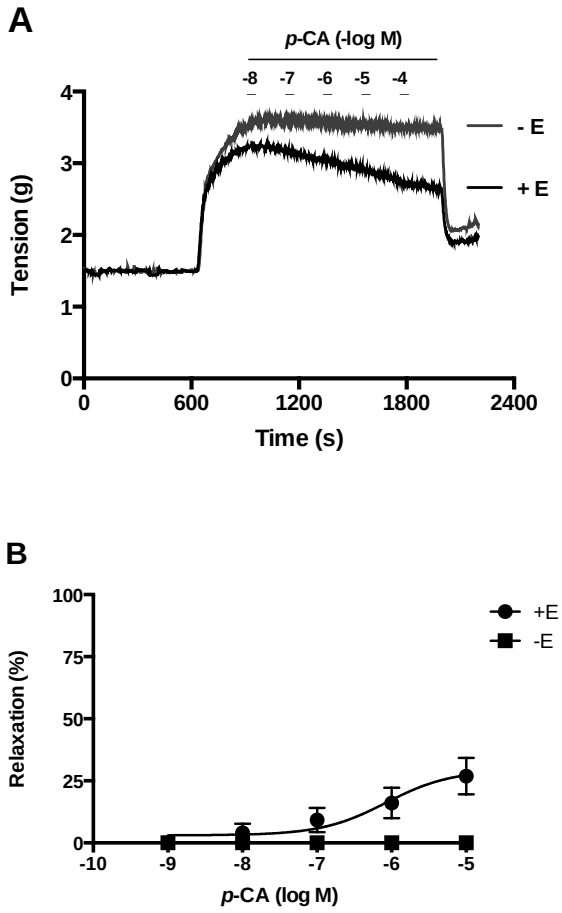

Figure 1
Relaxation induced by *p*-CA on rat aortic rings pre-contracted with PE (0.1 µM). (A) Representative record showing an endothelium-dependent relaxation induced by cumulative concentration of *p*-CA. *p*-CA produced moderated relaxation in aortic rings with endothelium (+E). No effect in aortic rings without endothelium (-E) was observed. (B) Concentration-response curve for *p*-CA. Results (means + SEM) are expressed as percentage of inhibition of contraction induced by PE (0.1 µM). Emax (%) and pD_2 were 29.28 ± 1.89 (N = 6) and 6.075 ± 0.184 (N = 6), respectively.

The next experiment was designed to evaluate the effect of *p*-CA as a protector of the impairment of endothelium-dependent relaxation induced by HG. As shows in Figure 2 and Table 1, when aortic rings

were pre-incubated for 3 h with HG, the maximum vasodilatory effect (Emax) of ACh decreased dramatically from 87.69 ± 2.59% (N = 6) to 40.54 ± 1.78% (N = 6).

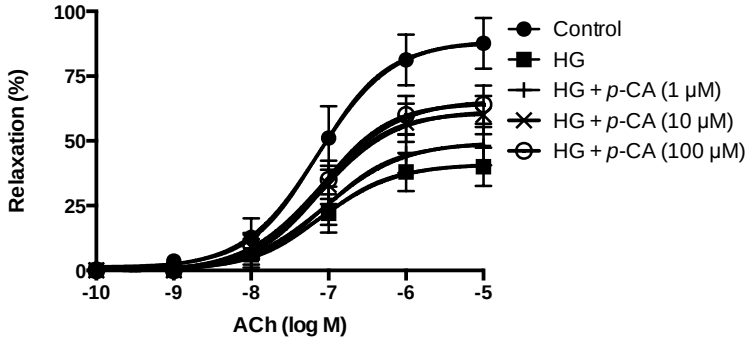

Figure 2

Relaxation induced by ACh on PE (0.1 µM)-contracted rat aortic rings pre-incubated with HG and *p*-CA. Cumulative concentration-response curve for ACh (10^{-10} – 10^{-5} M) in aortic rings pre-incubated for 3 h in the presence of D-Glucose 5 mM (Control), D-Glucose 25 mM (HG), and HG plus *p*-CA (1, 10, 100 µM). Results (means + SEM) are expressed as percentage of inhibition of contraction induced by PE (0.1 µM). Emax (%) and pD_2 are shown in Table 1.

However, the negative effect of HG was partially reverted in vessels co-incubated with *p*-CA: 1 µM (48.57 ± 1.82%), 10 µM (60.81 ± 1.80%) and 100 µM (64.51 ± 1.80%). The protector effect of *p*-CA on HG endothelium-dependent relaxation induced by ACh was concentration-dependent. The action of *p*-CA was associated with a significant change in Emax. No statistical difference in pD_2 was observed.

Table 1
Effect of *p*-CA in the maximum response (Emax) and pD_2 (-log EC_{50}) to the ACh-induced endothelium-dependent relaxation in rat aortic rings pre-incubated with HG.

Group	Emax (%)	pD_2
Control	87.69 ± 2.59	7.139 ± 0.078
HG	40.54 ± 1.78*	7.097 ± 0.114
HG + *p*-CA (1 µM)	48.57 ± 1.82$^{*\#}$	7.046 ± 0.096
HG + *p*-CA (10 µM)	60.81 ± 1.80^{*+}	7.097 ± 0.076
HG + *p*-CA (100 µM)	64.51 ± 1.80^{*+}	7.094 ± 0.072

* *P* < 0.0001 vs. Control; $^{\#}$ P < 0.05 vs. HG; $^{+}$ P < 0.0001 vs. HG

DISCUSSION

Our results clearly show that *p*-CA protect ACh-induced endothelial-dependent relaxation affected by high glucose (D-glucose 25 nM) in rat aortic rings. This effect is in accordance with experimental results in bovine aortic endothelial cells under oxidative stress induced by high glucose; in these endothelial cells *p*-CA acts as antioxidant preventing lipid peroxidation and cell death without affecting the production of reactive oxygen species (Lee *et al.*, 2009). The moderate capacity of *p*-CA to induce endothelium-dependent relaxation, observed in our study, may also be explained on the basis of their antioxidant activity, protecting nitric oxide (NO) from degradation, and thereby increasing their bioavailability.

Recently, radical scavenging and antioxidant capacity of *p*-CA have been clarified using different analytical methodologies (Kilic and Yesiloglu, 2013). Evidences from *in vitro* studies show that *p*-CA effectively scavenge ·OH in a dose-dependent manner, suggesting that *p*-CA antioxidant properties may involve the direct scavenging of ROS (Zang *et al.*, 2000). According to an antioxidant mechanism *p*-

CA also protects rat hearts against doxorubicin (DOX), an anticancer antibiotic whose therapeutic use is limited by its cardiotoxicity, which is associated to oxidative stress in the heart (Abdel-Wahab *et al.*, 2003). Additional evidence supporting the free radical scavenging activity of *p*-CA shows it prevents lysosomal dysfunction against cardiac damage induced by isoproterenol (Roy *et al.*, 2013).

Interestingly, *p*-CA affects angiogenesis and glucose/lipid metabolisms. Thus, *p*-CA have been shown to inhibit the sprouting of endothelial cells in rat aortic rings, thereby inhibiting the tube formation and migration of endothelial cells; the mechanism of action of *p*-CA could be the downregulation of mRNA expression levels of key angiogenic factors, indicating that *p*-CA possesses potential anticancer properties due to the inhibition of angiogenesis *in vivo* (Kong *et al.*, 2013). Furthermore, *p*-CA modulates glucose and lipid metabolism via AMPK activation in L6 skeletal muscle cells; on this basis the authors have proposed that *p*-CA may overcome metabolic disorders (Yoon *et al.*, 2013). We are aware that *p*-CA effects indicated in this paragraph require an exposure time of several hours, so that does not explain our results that were obtained in a time frame of 3 h.

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