

Oral Administration of Polyphenolic Compounds from Cognac Decreases ADP-Induced Platelet Aggregation and Reduces Chronotropic Effect of Isoprenaline in Rats

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Summary

This study sought to evaluate whether consumption of polyphenol extract from Cognac (CPC) modulates platelet activation and cardiovascular reactivity in rats. Male Wistar rats were treated daily for 4 weeks by intra-gastric gavage receiving CPC at 80 mg/kg/day or vehicle (5 % glucose). Platelet adhesion and aggregation in response to different activators were assessed. Cardiac and vascular reactivity in response to various agonists as well as NO measurement by electron paramagnetic resonance technique were investigated in isolated heart and thoracic aorta. Oral administration of CPC decreased platelet aggregation induced by ADP but not by collagen. CPC did not affect adhesion to collagen. The chronotropic but not the inotropic response to isoprenaline was reduced without alteration of NO production in hearts from CPC-treated rats. CPC treatment did not affect *ex vivo* relaxation to acetylcholine nor NO content of rat aorta. CPC did not significantly alter the response to phenylephrine in aorta despite the participation of endothelial vasoconstrictor products. In summary, chronic treatment with CPC has no impact on *ex vivo* vascular and cardiac reactivity; however, it reduced heart work and platelet aggregation. These data suggest the existence of compounds in Cognac that may decrease the risk of coronary thrombosis and protect against some cardiac diseases.

Key words

Cognac • Polyphenols • Platelet aggregation • Isoprenaline • Nitric oxide

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Introduction

The regular and moderate consumption of alcoholic beverages including Cognac has been already described to decrease the risk of cardiovascular diseases, possibly due to ethanol content (Rimm *et al.* 1991, Carando *et al.* 1998). However, epidemiological studies reported that some beverages such as red wine, might impact mortality more favorably than other alcoholic beverages (Renaud and De Lorgeril 1992). The beneficial property of red wine could be attributed to the presence of polyphenols (Rotondo *et al.* 2000). Mechanisms that have been proposed to explain the prevention of cardiovascular diseases by polyphenols include reduced oxidation of low-density lipoprotein, inhibition of platelet aggregation and neutrophil adhesion. Polyphenols have also been reported to affect the activity of enzymes critically involved in pathways regulating cell proliferation and activation (for reviews see Curin and Andriantsitohaina 2005, Perez-Vizcaino *et al.* 2006). We have previously shown that polyphenols from red wine (RWPC) were able to produce *ex vivo* endothelium-dependent relaxation in rat aortic rings by enhanced nitric oxide (NO) synthesis (Andriambelason *et al.* 1997, 1998). In addition, short-term oral administration of RWPC produced a decrease in blood pressure in normotensive rats associated with an augmentation of endothelium-dependent relaxation (Diebolt *et al.* 2001). Finally, we provided evidence that Provinols™, another source of RWPC, partially prevents hypertension, cardiovascular remodeling and vascular dysfunction *via*

the increase of NO-synthase activity and inhibition of oxidative stress (Pecháňová *et al.* 2004).

Cognac belongs to alcoholic beverages rich in polyphenols and moderate consumption may have potential beneficial effects on health. Indeed, Cognac and its phenolic constituents have already been described to be antioxidant (Goldberg *et al.* 1999, Da Porto *et al.* 2000). Quantitatively, the most important phenolic constituent in this beverage is ellagic acid. This compound possesses antimutagenic and anticancer properties (Josephy *et al.* 1990) as well as *in vitro* and *in vivo* antioxidative effects (Majid *et al.* 1991).

Several studies demonstrated that polyphenols inhibit platelet aggregation and improve endothelial function (for review see Vita 2005, Perez-Vizcaino *et al.*, 2006). Recently, non-alcoholic polyphenolic extract from Armagnac, with qualitatively similar polyphenols found in Cognac, has been found to inhibit ADP-induced platelet aggregation *in vitro* (Umar *et al.* 2005). Moreover, we have previously reported that Cognac polyphenolic compounds (CPC) induce vasorelaxation *in vitro* and decrease infarct size subsequent to ischemia-reperfusion (Ralay Ranaivo *et al.* 2004). The present study was aimed to test the hypothesis that long-term oral administration of a non-alcoholic polyphenolic extract of CPC affects platelet aggregation, vascular reactivity to noradrenaline and acetylcholine in aortic rings and contraction force developed by isolated hearts. Finally, the hypothesis that CPC treatment alters aortic and cardiac production of NO was also investigated.

Methods

Oral administration of CPC

Two groups of 10 male Wistar, rats (250-300 g) were treated daily for 4 weeks by intra-gastric gavage: the first group (control) received the vehicle (5 % glucose) and the second group received CPC at 80 mg kg⁻¹ day⁻¹. Blood was taken 24 h after the last dose of the treatment to study platelet adhesion and aggregation. Thoracic aorta and hearts from one half of the rats were isolated to study vascular and cardiac reactivity, and for NO measurements. Hearts from the other half of the rats were used for cardiac studies.

Platelet aggregation

Blood anticoagulated with acid-citrate-dextrose solution (1/6 blood volumes) was centrifuged at 1570 g for 2 min at 37 °C, after which the upper platelet-rich

plasma phase was removed and centrifuged at 1570 g for 15 min. The platelet pellet was washed twice in Tyrode's buffer in mM: NaCl 137, KCl 2, NaHCO₃ 12, NaH₂PO₄ 0.3, CaCl₂ 2, MgCl₂ 2, glucose 5.5, HEPES 5, pH 7.3, containing 0.35 % bovine serum albumin as previously described (Cazenave *et al.* 1983). Finally, it was resuspended at 2.5x10⁵ platelets μl⁻¹ in the same buffer, in the presence of 0.02 U/ml of the ADP scavenger apyrase (adenosine 5'-triphosphate diphosphohydrolase), a concentration sufficient to prevent desensitization of platelet ADP receptors during storage. This preparation of washed platelets was kept at 37 °C and used for platelet adhesion and aggregation experiments.

Platelet aggregation was studied using an aggregometer Chronolog CorporationTM. A volume of 225 μl of the platelet suspension was placed in a glass turbidity tube and warmed at 37 °C. Three minutes later, 25 μl of agonists solution was added to obtain a final concentration of ADP equal to 1, 2.5 and 5 μM or collagen equal to 25 μg ml⁻¹. The extent of aggregation was estimated by the percent of maximum increase in light transmission, with the buffer representing 100 % transmittance.

Platelet adhesion to fibrillar collagen

Ex vivo platelet adhesion was carried out as described by Cazenave *et al.* (1983). Briefly, washed platelets samples were incubated in duplicate in the same aggregometer at 37 °C and stirred at 900 rpm. EDTA (5 mM) was added to prevent platelet aggregation. After 5 min, collagen (50 μg ml⁻¹) was added and the samples were stirred for a further 15 min. Platelets were counted optically using a Bürker chamber, before and 15 min after collagen addition and a difference in platelet count was taken as an index of their adhesion to collagen.

Isolated perfused heart

Rats were anesthetized with pentobarbital (60 mg kg⁻¹, i.p.) and the heart was rapidly excised and placed in ice-cold Krebs-Henseleit buffer with the following composition in mM: NaCl 118, NaHCO₃ 24, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.7, glucose 10, gassed with 95 % O₂, 5 % CO₂ and maintained at 37 °C. Then, the heart was immediately mounted and cannulated in the retrograde mode according to Langendorff method as previously described (Gyorgy *et al.* 1999). Transducers were connected to a computer *via* an amplifier and a MacLabTM (ADI Castle Hill, Australia) for online recording and analysis of parameters. Cardiac parameters

(heart rate, contraction force) were recorded continuously. Concentration-response curves to isoprenaline were constructed to study the inotropic and chronotropic effects.

Aortic preparations and mounting

Thoracic aorta was removed, carefully cleaned of adhering fat and connective tissue and cut into rings (2-3 mm length). Aortic rings were mounted on myographs filled with a physiological salt solution (PSS) with the following composition in mM: NaCl 118, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24, glucose 11, at 37 °C and bubbled with a 95 % O₂, 5 % CO₂ mixture, under normalized tension as previously described (Diebolt *et al.* 2001). In some experiments, the endothelium was removed by gentle rubbing of the intima of the ring with curved forceps. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (1 µM) to induce more than 50 % relaxation of rings precontracted with noradrenaline (1 µM). The absence of relaxation to acetylcholine indicates that the vessels were functionally denuded of their endothelium. Concentration-response curves to noradrenaline were constructed in a cumulative manner in aorta with or without functional endothelium to study vascular contraction. Endothelium-dependent relaxation was studied in thoracic aorta with and without functional endothelium and precontracted to approximately 80 % of maximal tension with noradrenaline. Acetylcholine was applied in a cumulative fashion.

NO spin-trapping and electron paramagnetic resonance (EPR) spectroscopy

The NO content of aortic rings (6-8 mm length) and heart slices was assayed as described by Alencar *et al.* (2003) after formation of the paramagnetic adduct Fe(II)NO(diethyldithiocarbamate)₂ ([Fe(II)NO(DETC)₂]) detectable by EPR in rings treated for 30 min at 37 °C with 0.5 mM [Fe(II)(DETC)₂] complex as colloid (Kleschyov *et al.* 2000). Tissues were frozen in liquid nitrogen until EPR measurements. EPR spectra were recorded on a MS100 spectrometer (Magnettech, Berlin, Germany) under the following conditions: temperature, 77 K; microwave frequency, 9.34 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; and time constant, 100 ms. After EPR measurements, the tissue samples were dried and weighted. The relative [Fe(II)NO(DETC)₂]

concentrations ($A W_{ds}^{-1}$) were determined dividing the third component amplitude (A) of the three-lines EPR signal by the weight of the dried sample (W_{ds}).

Expression of results and statistical analysis

The extent of platelet aggregation was estimated quantitatively by measuring the maximum curve height above baseline level and expressed as percentage of light transmission. Cardiac parameters were monitored continuously and included contraction force in gram and heart rate in beats per minute (bpm) and expressed as percentage of basal values. Vascular contraction was expressed in gram and the relaxation was expressed as percentage of the decrease in the level of precontraction. The NO content was expressed in relative [Fe(II)NO(DETC)₂] concentrations ($A W_{ds}^{-1}$).

All results are expressed as mean ± S.E.M. of n experiments, where n represents the number of rats. Analysis of variance and Student's unpaired t -test were used for statistical analysis. $P < 0.05$ value was considered significant.

Drugs

The enriched and lyophilized CPC powder was provided by the "Bureau Interprofessionnel du Cognac" with the following composition in mg g⁻¹ of powder: gallic acid 2.28, ellagic acid 15.17, 5-hydroxymethylfurfural 2.11, furfural 0.35, vanillic acid 0.30, 5-methylfurfural 0.17, syringic acid 0.74, vanillin 0.45, syringaldehyde 2.02, scopoletin 0.03, coniferaldehyde 2.35, sinapaldehyde 8.21. The total polyphenols content was about 48.8 %, determined by colorimetric Folin-Ciocalteu method using gallic acid as standard. All drugs were from Sigma Chemical (Grenoble, France).

Results

Platelet aggregation and adhesion

As illustrated in Figure 1a, ADP induced platelet aggregation in concentration-dependent manner from 1 to 5 µM. Interestingly, platelet taken from rats treated with Cognac displayed lower response to ADP. The reduced response to ADP was significantly different at 2.5 and 5 µM when compared to the response obtained in platelets from control rats ($P < 0.05$). In contrast to ADP, CPC treatment did not affect platelet aggregation when collagen (25 µg ml⁻¹) was used as agonist (Fig. 1b). Furthermore, adhesion response to fibrillar collagen was the same in the two groups (Fig. 1c).

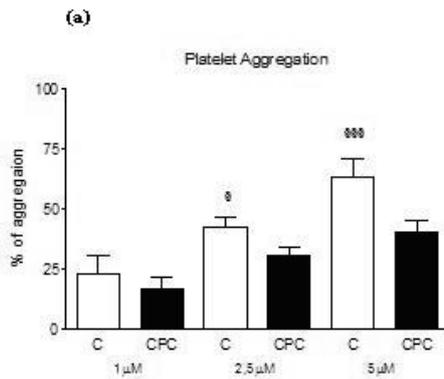


Fig. 1. Effects of cognac polyphenolic compounds (CPC) on platelet aggregation and adhesion. Percentage of platelet aggregation with ADP (1, 2.5 and 5 μM ($n=4$)) (a). Platelet aggregation and adhesion with collagen (25 $\mu\text{g ml}^{-1}$; 50 $\mu\text{g ml}^{-1}$ respectively ($n=5$)) (b and c). Aggregation and adhesion data are presented as mean \pm S.E.M. (* $P<0.05$, *** $P<0.001$ significantly different compared to response to ADP (1 μM), † $P<0.05$ Control vs CPC-treated rats); n represents the number of rats.

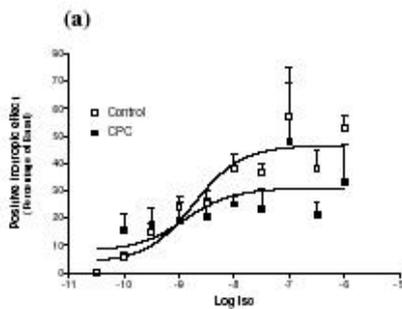
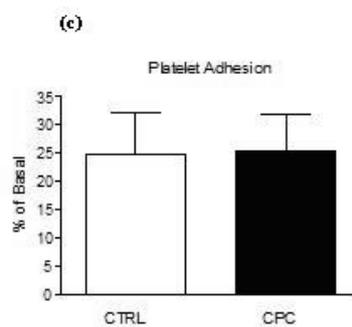
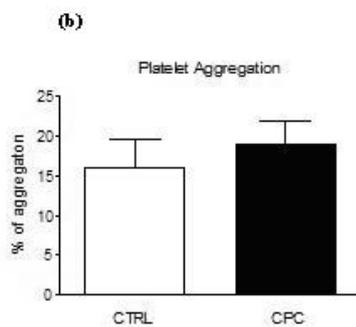
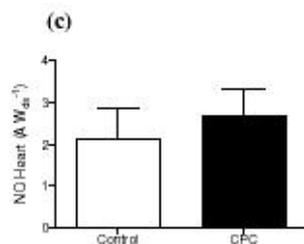
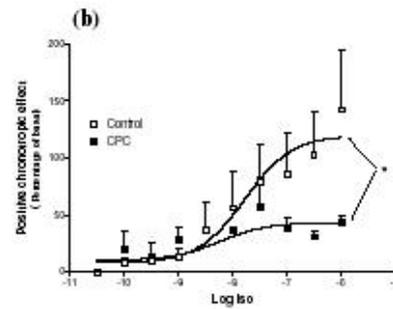


Fig. 2. Effects of cognac polyphenolic compounds (CPC) on cardiac reactivity. Control (\blacksquare ; $n=5$) and CPC treated (\square ; $n=6$) hearts were perfused in Langendorff mode. Changes in contractile force (a) and heart rate (b) in response to increasing concentrations of isoprenaline (Iso) are shown as percentage of baseline. NO content of heart measured by EPR in control (\square ; $n=5$) and CPC-treated rats (\blacksquare ; $n=5$) (c). Data are presented as mean \pm S.E.M. (* $P<0.05$), n represents the number of rats.



Effect of CPC on cardiac reactivity to isoprenaline and heart NO content

At 24 h after the end of treatment, cardiac parameters were recorded in isolated hearts from the two groups of rats. The basal forces and heart rate were 0.996 ± 0.06 g and 1.7 ± 0.28 g, and 118.4 ± 33.8 bpm and 211.6 ± 39.8 bpm for hearts from control and CPC-

treated rats, respectively. Isoprenaline produced concentration-dependent increase in force and heart rate (Fig. 2). The positive inotropic effect of isoprenaline was not significantly different in hearts from the two groups of rats (Fig. 2a). However, the positive chronotropic effect induced by isoprenaline was significantly reduced ($P<0.05$) in hearts from CPC-

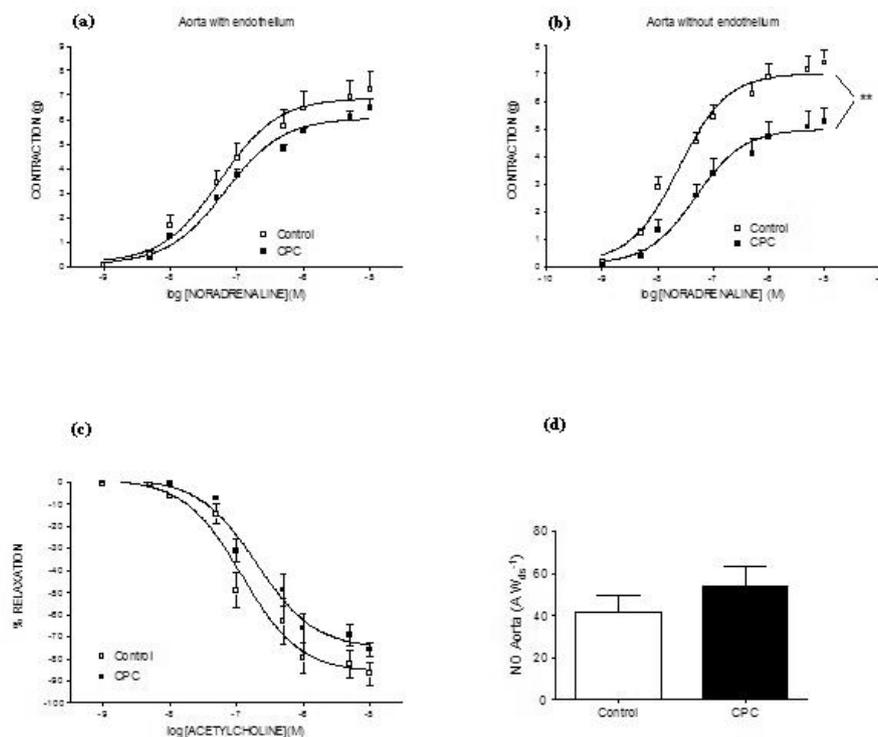


Fig. 3. Effect of Cognac polyphenolic compounds (CPC) treatment on vascular reactivity. Concentration-response curves to noradrenaline in aorta with (a) and without (b) endothelium taken from control (\square) and CPC (\blacksquare) treated rats. $**P < 0.01$ Control vs CPC-treated rats. (c) Concentration-response curves to acetylcholine in aortic rings precontracted with noradrenaline with endothelium from control and CPC-treated rats. (d) NO content of aorta measured by EPR in control (\square ; $n=5$) and CPC-treated rats (\blacksquare ; $n=5$). Data are presented as mean \pm S.E.M. n represents the number of rats.

treated rats compared to those from rats receiving vehicle (Fig. 2b). Finally, heart NO content measured by EPR technique was not significantly different between the two groups of rats (Fig. 2c).

Ex vivo vascular reactivity of aorta after CPC treatment

Noradrenaline produced concentration-dependent contraction of aorta with or without endothelium from both control and CPC-treated groups (Fig. 3). The contractile response to noradrenaline was not significantly different in aorta from control and CPC-treated rats in the presence of functional endothelium (Fig. 3a). However, CPC treatment significantly reduced noradrenaline-induced contraction in the absence of functional endothelium ($P < 0.01$; Fig. 3b). Acetylcholine produced a concentration-dependent relaxation in aorta with functional endothelium from both control and CPC-treated groups. Acetylcholine-induced relaxation was not significantly different in aortic rings from CPC-treated rats when compared with those from control group (Fig. 3c). In the absence of endothelium, acetylcholine failed to produce any relaxation response both in aortic rings from control and CPC-treated group (not shown). Finally, CPC treatment did not induce any significant change of basal aortic NO content (Fig. 3d).

Discussion

The present work showed that oral administration of CPC decreased platelet aggregation

induced by ADP but not by collagen. CPC did not also affect adhesion to collagen. The chronotropic, but not the inotropic response to isoprenaline was reduced without any alteration of NO production in isolated hearts from CPC-treated rats when compared with those from control rats. CPC treatment did not affect *ex vivo* responses to the endothelial relaxant agent, acetylcholine, nor the NO content of rat aorta. Interestingly, CPC induced subtle modifications which maintain unchanged agonist-induced contractility in the aorta as a result of participation of endothelial vasoconstrictor products which compensate the effects of vasodilator compounds, the nature of which remains to be determined. Thus, the *in vivo* effects of CPC with antiplatelet activity and its capacity in decreasing heart overload during chronotropic stress may be beneficial for preventing some cardiovascular diseases.

We have recently reported that *in vitro*, CPC had endothelial NO-dependent vasorelaxant effects and stimulated cardiac function. In addition, oral treatment of rats with CPC at $40 \text{ mg kg}^{-1} \text{ day}^{-1}$ for 2 weeks appeared to have no impact on blood pressure, heart rate or cardiac contractility *ex vivo*; however, it could decrease the infarct size after ischemia-reperfusion (Ralay Ranaivo *et al.* 2004). Indeed, we found that CPC possess antiplatelet properties and decrease inotropic effect to isoprenaline. CPC dose used in this study (80 mg kg^{-1}) represents a large dose and is not relevant in terms of Cognac consumption (i.e. CPC plus alcohol). It should be

noted that oral treatment of rats with CPC at 40 mg kg⁻¹ day⁻¹ for 2 weeks had no effect of ADP-induced platelet aggregation. Thus, higher dose of CPC and long-term treatment of CPC might be needed to affect the aggregation process. These differences require further investigation. However, the nature of polyphenols involved in the reported effect of *in vivo* CPC treatment is not known and it is out of the concern of the present work. Polyphenols found in CPC include mainly ellagic acid but also some phenolic acids (vanillic acid and gallic acid), furans and cinnamaldehydes (coniferaldehyde and sinapaldehyde). Ellagic acid has been found to possess antioxidant capacity and probably participate in part to the reported effect of oral CPC. Further studies are needed to identify the active components of CPC that produce the effects on both platelets and heart.

One of the novel findings of the present work was that CPC treatment reduced percentage and rate of ADP-induced aggregation, but it did not affect the same parameters and adhesion when collagen was used. These results suggest that CPC could not interfere with direct interaction between collagen and platelets, but they may lead to a decrease of aggregation induced by an agonist acting on either a specific receptor such as P2Y-receptors or shape-change process. Decrease in platelet aggregation is thought to be one of the mechanisms underlying the favorable effects of polyphenols in the prevention of coronary heart disease. These findings are in agreement with the others reported for Armagnac extract in rat arteriovenous shunt thrombosis model or *in vitro* treatments of human platelets (Umar *et al.* 2003, 2005). The precise composition of Armagnac extract is not known in these studies. It contains neither quercetin nor resveratrol, but it does contain polyphenol derivatives of ellagic acid such as those found in CPC. Thus, one can advance the hypothesis that ellagic acid might participate in the anti-platelets effect of CPC. Polyphenols have been shown to inhibit platelet function *in vitro* and *in vivo* (Hubbard *et al.* 2003). In particular, certain flavonoids have been shown to inhibit the generation of metabolites of arachidonic acid by cyclooxygenase and lipoxygenase (Beretz *et al.* 1982, You *et al.* 1999), and inhibit Ca²⁺-dependent isoforms of protein kinase C (Liu *et al.* 2000). Multiple signaling pathways controlled platelet functions and the exact mechanism of action has not been fully elucidated yet.

CPC administration has been reported to be able to reduce the infarct size after an ischemia-reperfusion injury, without affecting basal cardiovascular parameters

of the rats (Ralay Ranaivo *et al.* 2004). In the present study, we extend the observation of CPC treatment on cardiac reactivity to the full β -adrenoceptor agonist isoprenaline and found that it did not affect the inotropic effect of this agonist but interestingly reduced its chronotropic property. Thus, CPC could decrease the heart-work by its moderate bradycardial effect after isoprenaline administration; this effect could be related to the capacity of CPC to preserve a good oxygen supply/demand balance after stimuli. Moreover, this effect might partly explain the ability of CPC in decreasing infarct size measured in reperfused hearts *ex vivo* (Ralay Ranaivo *et al.* 2004). Indeed, cellular injury observed after ischemia-reperfusion is mainly attributable not only to the development of a reperfusion-induced oxidative stress but also to disturbances of ionic homeostasis (for review see Park 1999).

Turning now to the effect of CPC treatment on vascular reactivity in response to a vasoconstrictor agonist, we note that the contractions to phenylephrine were unchanged by CPC in aortas with functional endothelium. In endothelium-denuded aortic rings from CPC-treated rats, the phenylephrine-induced contraction was decreased. Even though the nature of this hyporeactivity is not known, it is compensated by vasoconstrictor components from endothelial sources. Similar observations have been reported for oral treatment with RWPC, although the nature of polyphenols present in both extracts is different. Indeed, it was found that RWPC treatment is associated with a subtle balance between extra-endothelial NO production, inducing vascular hyporeactivity, and increased release from endothelium of COX-dependent vasoconstrictor molecules in the aortic wall (Diebolt *et al.* 2001). However, it should be noted that CPC treatment was not able to increase NO production within the aorta.

Finally, we found that acetylcholine-induced relaxation was not affected in aortas taken from CPC-treated rats *in vivo*. Furthermore, as reported above, CPC treatment failed to modify NO production within the aorta. These results contrast with data obtained *in vitro* in which CPC induced endothelium-dependent relaxation sensitive to NO synthase inhibitor. The reasons of the different observations might be multiple. Firstly, the polyphenols contained in CPC that produce endothelial NO vasodilatation might not be absorbed and reached the circulation in a sufficient amount *in vivo*. Secondly, as discussed above, CPC treatment stimulated the release of vasoconstrictor products that may mask an increase in

endothelial relaxation.

In summary, we provide evidence that oral administration of CPC inhibits platelet aggregation mediated by ADP, reduces the chronotropic property of isoprenaline and induces subtle effects on vascular reactivity. This is independent from the alcohol content of Cognac. In addition to our previous studies showing that CPC treatment reduced the infarct size after an ischemia-reperfusion injury, the results of present study strengthen

the hypothesis that CPC may decrease the risk of coronary artery disease.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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