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Effects of pomace olive oil-enriched diets on endothelial function of small mesenteric arteries from spontaneously hypertensive rats

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Pomace olive oil (POM), an olive oil subproduct traditionally used in Spain, is a good source of minor components from the unsaponifiable fraction such as triterpenoids, mainly in the form of oleanolic acid, which induces vascular protection and vasodilatation. Our aim was to evaluate the effects of long-term intake of diets enriched in POM with high concentration in oleanolic acid on endothelial dysfunction associated to hypertension in small mesenteric arteries (SMA) from spontaneously hypertensive rats (SHR). During 12 weeks, rats (six rats per group) were fed either a control 2 % maize oil diet (BD), or high-fat diets containing 15 % refined olive oil (OL), pomace olive oil (POM), or pomace olive oil supplemented in oleanolic acid (POMO; up to 800 parts per million). Endothelial and vascular functions were assessed by relaxing or contracting responses to acetylcholine (ACh) or phenylephrine, respectively. The involvement of endothelium-derived relaxing factors in these responses was evaluated. In contrast to BD, SHR fed high-fat diets showed a biphasic response to ACh related to changes in eicosanoid metabolism. POM enhanced the endothelial function in SMA from SHR by increasing the endothelium-derived hyperpolarising factor (EDHF)-type component, whereas administration of POMO resulted in a similar contribution of NO/EDHF in the endothelial response to ACh. The present study shows that despite the lack of changes in blood pressure, consumption of POM improves endothelial function in SMA from SHR by improving the agonist-mediated EDHF/NO response. Thus, triterpenoids confer a protective role to POM against endothelial dysfunction in hypertension.

Pomace olive oil: Endothelium: Hypertension: Oleanolic acid: Small mesenteric arteries

Arterial hypertension is the most common cause of cardiovascular and cerebrovascular complications in human subjects. Although effective pharmacological strategies for the treatment of hypertension exist, there is a great deal of interest in using dietary approaches to attenuate the cardiovascular disorders associated to hypertension. Over the years, a number of epidemiological and clinical studies developed in different countries constitute a firm and reliable basis supporting the beneficial effect of the Mediterranean diet pattern, containing olive oil as the main source of fat, against CVD[1]. An increasing number of evidences associate the healthful effects of olive oil consumption on cardiovascular risk factors with the presence of bioactive components in this oil[2]. Despite the attention paid to its high content in MUFA such as oleic acid, minor components of olive oil found in the non-glyceridic fraction may also explain the cardioprotective effects of olive oil intake[3–5].

Among minor components from olive oil, interest on its triterpenic fraction has been gaining importance in recent years. The triterpenic fraction in olive oil and olive skin is mainly constituted by pentacyclic terpenic acids (oleanolic and maslinic acid) and diols (erythrodiol and uvaol)[6]. The procedure applied for extraction of olive oil is important for the content in triterpenoids and other minor constituents. Pomace olive oil (POM; called in Spain as 'orujo' olive oil) is obtained from the residue that remains after virgin olive oil mechanical extraction. As a consequence of the extraction processes needed to obtain both refined and POM, the hydrosoluble fraction including phenolic compounds is lost. In spite of the lack of phenolic compounds, POM, extracted using a new procedure (patent number 200400755) contains higher concentrations of triterpenic compounds than virgin olive oil, the latter components exceeding values of 120 mg/kg. Among olive and POM triterpenoids, oleanolic acid has been the most widely studied and distributed in the vegetable kingdom. In fact, a wide range of biological activities has been attributed to this triterpenoid[3]. Despite the lack of studies on CVD, recent and novel data have suggested the vasoactive

Abbreviations: ACh, acetylcholine; AUC, area under curves; BD, baseline diet; COX, cyclo-oxygense; EDHF, endothelium-derived hyperpolarising factor; L-NAME, N^{ω}-nitro-L-arginine; NOS, NO synthase; OL, refined olive oil diet; Phe, phenylephrine; POM, pomace olive oil diet; POMO, POM supplemented in oleanolic acid diet; PSS, physiological salt solution; SHR, spontaneously hypertensive rats; SMA, small mesenteric arteries; TRL, TAG-rich protein particles; WKY, Wistar–Kyoto.

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effects provided by POM and its triterpenoids in terms of vasodilatation and endothelial function improvement[7–10].

The vascular endothelium is a major regulator of local vascular homeostasis by the synthesis and release of vasoactive factors promoting vasodilatation such as NO, prostacyclin and endothelial derived hyperpolarising factor (EDHF), or vasoconstriction, such as thromboxane A_2, isoprostanes and superoxide anion. Upsetting the balance between the production of these substances leads to endothelial dysfunction, frequently associated to hypertension[11]. Our *in vitro* data revealed that olive oil triterpenoids induce vasodilatation in isolated conductance and resistance arteries from normotensive and hypertensive rats[7–10]. The vasodilatation response was endothelium-dependent and mediated by mechanisms involving NO release via phosphorylation of endothelial NO (eNOS) at Ser^{1177} residues through Akt kinase activation[10]. Recently, we have shown that long-term intake of a POM-rich diet in spontaneously hypertensive rats (SHR) resulted in an improved endothelial function in large conductance arteries without affecting blood pressure *in vivo*[7]. It was further determined that the POM-rich diet increased aortic eNOS expression and plasmatic levels of NO metabolites and the impaired endothelial function was reversed in animals fed a POM-enriched diet for 3 months[7].

It has long been recognised that hypertension is strongly linked to both functional and structural changes in microcirculation, which includes resistance arteries. Due to the fact that changes in small resistance arteries are the hallmark of hypertension, the present study was designed to evaluate the effects of long-term intake of POM rich in triterpenoids on hypertension-related endothelial dysfunction in small mesenteric arteries (SMA) of SHR. We further examined the contribution of endothelial-derived factors to these responses.

Experimental methods

Animals and diets

Three-week-old male Wistar–Kyoto (WKY) rats and SHR were obtained from Charles River Laboratories (Barcelona, Spain) and acclimatised for 1 week. The animals were randomly distributed into four groups of six rats each, according to diet and strain. During 12 weeks, animals were fed either a control low-fat diet (baseline diet, BD) containing 2 % (w/w) of maize oil, or high-fat diets containing 15 % (w/w) refined olive oil (OL), POM, and POM supplemented in oleanolic acid (800 ppm; POMO). The original POM and OL contained approximately 200 and 25 parts per million of oleanolic acid, respectively. According to the American Institute for Nutrition, the BD contained sucrose 50 %, casein 23 %, maize starch 15 %, α-cellulose 5 %, DL-methionine 0·3 % and choline chloride 0·2 %, purchased from Musal & Chemical (Granada, Spain), AIN-93-MX mineral mix 3·5 % and AIN-93-VX vitamin mix 1 %, provided from ICN Nutritional Biochemicals (Cleveland, OH, USA). Olive and POM were provided from the olive oil-producing company Oleicola Oleotejar (Cordoba, Spain), and maize oil from Koipe S.A. (Jaen, Spain)[7]. Oleanolic acid was obtained by extraction from olive leaves, as previously described[7]. To minimise oxidation, all diets were prepared weekly and stored at 4°C as previously described[12]. The fatty and non-fatty acid composition of the oils was analysed by GC in the company Oleicola Oleotejar (see composition of diets in Rodriguez-Rodriguez *et al.* [7]).

Food intake and corporal weight of the animals were weekly determined. Systolic blood pressure and heart rate were measured every week in conscious, prewarmed, restrained rats by tail-cuff plethysmography with a pressure meter (Niprem 645, Cibertec, Spain). The average of three separate measurements was taken as the final reading. At the end of the administration period, animals were anaesthetised with pentobarbitone (60 mg/kg) and tissue was collected. All protocols were approved by the Institutional Committee on Investigation in Animals (Universidad de Sevilla, Seville, Spain).

Arterial preparation

Segments of SMA (1·6–2 mm length) from WKY and SHR rats were mounted in wire-myographs filled with physiological salt solution (PSS; composition in mmol/l: NaCl 119, KCl 4·7, $NaHCO_3$ 14·9, $MgSO_4$ 1·47, $CaCl_2$ 2·5, KH_2PO_4 0·4 and glucose 5·5). Mechanical activity was measured isometrically by a force transducer Multi Myograph System-610M (JP-Trading I/S, Aarhus, Denmark) coupled to a Powerlab data acquisition system (AD-Instruments, Bella Vista, New South Wales, Australia) as previously described[13]. After stabilisation, challenges with 10 μmol/l noradrenaline were performed in SMA to test their maximal contractile response and to elicit a reproducible contraction. The presence of functional endothelium was determined by the ability of 1 μmol/l acetylcholine (ACh) to induce more than 50 % of precontracted vessels. In some arteries, endothelium was removed immediately after dissection by intraluminal perfusion with 0·5 % 3-[(3-cholamidopropyl) dimethylammonio]-1 propane sulphonate (Sigma, St Louis, MO, USA) in PSS. The lack of relaxation in response of ACh indicated a successful endothelial removal.

Measurement of vascular reactivity

The contractile responses evoked by cumulative addition of phenylephrine (Phe; 0·01–100 μmol/l) were studied in SMA preparations with or without functional endothelium. Concentration–response curves were referred to as contractions previously elicited by noradrenaline. To test the influence of endothelial NO on contractile responses to Phe, the same experimental protocol was performed in the presence of the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NAME; 300 μmol/l). Evaluation of endothelium-dependent relaxation was also evaluated in SMA precontracted at 80 % of their maximal contraction with Phe. When the contraction reached a plateau, cumulative addition of ACh (0·01–100 μmol/l) was performed. Concentration–response curves for ACh were constructed in the absence or presence of the following inhibitors at a maximally active concentration[14]: the NOS inhibitor L-NAME (300 μmol/l), the non-selective cyclo-oxygenase inhibitor indomethacin (10 μmol/l), a combination of indomethacin (10 μmol/l) + L-NAME (300 μmol/l; Sigma), PSS containing depolarising concentrations of KCl (25 mmol/l) in the presence of indomethacin and a combination of indomethacin + L-NAME + PSS KCl 25 mmol/l to block the EDHF-type relaxation. All the inhibitors were incubated for 15 min before the precontraction with Phe.

Statistical analysis

Relaxations were expressed as a percentage from the initial precontraction level and as means with their standard errors for *n* experiments; *n* represents the number of rats. Area under curves (AUC) were calculated from concentration–response curves for the agonist. The maximum vasoconstrictor response expressed as negative log molar ($pD_2 = -\log EC_{50}$) was calculated by nonlinear regression analysis of each individual concentration–response curve using GraphPad Prism Software version 3.0 (San Diego, CA, USA). Data were analysed using two-way ANOVA to compare concentration–response curves to agonists. Differences between means were assessed with Student's *t* test for unpaired data. Differences were considered significant when $P<0{\cdot}05$. A StatView Software package version 5.0 (SAS Institute Inc., NY, USA) was used for statistical analysis.

Results

Weights, food consumption and blood pressure

At the end of the 12-week diet administration, body weight and food intake values (weekly determined) were compared between the experimental groups, and no differences were appreciated. SHR developed hypertension at the age of 7 weeks, reaching values of systolic blood pressure significantly higher (196·95 (SEM 3·57) mmHg) than in WKY (157·63 (SEM 3·45) mmHg) after 12 weeks of treatment. None of the experimental diets attenuated this systolic blood pressure increase.

Contractile responses induced by phenylephrine

Concentration-dependent contractions evoked by Phe (0·01–100 μmol/l) in both WKY and SHR showed similar profile, with similar pD_2 values in endothelium-intact and -denuded arteries from both strains (Table 1). Inhibition of NOS with L-NAME enhanced Phe-induced contractions in SMA from BD groups from both strains nearly 0·3-fold more than those in control conditions (Fig. 1, Table 1). This fact unmasks the participation of vasoconstrictor agents in Phe-responses after NOS blockade in BD groups, in contrast to that observed in SMA from high-fat diet treated groups. A significant attenuation to Phe contractions was evidenced in the OL-fed SHR group compared to strain-matched BD group, even in the presence of L-NAME, whereas contractile responses to the agonist remained unaltered in POM- or POMO-treated groups (Fig. 1(B)).

Endothelium-dependent relaxations induced by acetylcholine

The muscarinic agonist ACh (0·01–100 μmol/l) induced relaxation in a concentration-dependent manner in SMA with functional endothelium precontracted with Phe (Fig. 2). Interestingly, the response curve to ACh in intact SMA from SHR was biphasic, showing a first phase of relaxation at ACh concentrations of 0·01–0·1 μmol/l, contraction at concentrations higher than 0·3 μmol/l, and a second phase of a lower extent of relaxation from 10 μmol/l ACh (Fig. 2(B)).

Relaxation curve of ACh (0·01–100 μmol/l) was notably enhanced in SMA from POM-treated WKY rats compared with strain-matched BD group ($P<0{\cdot}01$; Fig. 2(A)). Furthermore, POM diet clearly modified the biphasic profile of concentration–response curves for ACh in SMA from SHR ($P<0{\cdot}05$; Fig. 2(B)). In this regard, the maximal relaxant response to ACh 0·1 μmol/l (first phase of the curve) was increased from 23·42 (SEM 11·06) % in BD group to 52·59 (SEM 7·50) % in SHR fed a POM diet ($P<0{\cdot}05$). It is worthy to mention that the second phase of the concentration–response curve for ACh, from 3 to 100 μmol/l was altered in SMA from POM- and POMO-treated SHR, showing an evident relaxation, while this response was not detected in arteries from BD or OL groups (Fig. 2(B)).

The involvement of the main endothelial factors mediating the effect of ACh in resistance arteries, NO, cyclo-oxygense (COX)-derived metabolites and EDHF was considered.

Table 1. pD_2 and E_{max} values of concentration–response curves for phenylephrine (0·01–100 μmol/l) in isolated small mesenteric arteries with (E+) or without endothelium (E−), or in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NAME, 300 μmol/l) in arteries with endothelium

(Mean values with their standard errors)

	pD_2						E_{max} (% maximal contraction)					
	E+		L-NAME		E−		E+		L-NAME		E−	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
WKY (*n* 6)												
BD	5·55	0·05	5·77	0·04*	5·66	0·05	120·5	7·6	163·6	8·2	109·6	1·8
OL	5·68	0·13	5·77	0·09	5·74	0·05	104·9	5·2	112·2†	3·1	111·3	6·1
POM	5·74	0·08	5·77	0·06	5·77	0·09	112·2	3·2	116·9†	9·9	110·9	4·5
POMO	5·72	0·08	5·80	0·10	5·69	0·05	99·0†	6·2	92·7†	12·0	100·7	2·4
SHR (*n* 6)												
BD	5·73	0·13	6·22	0·30	6·02	0·14	117·6	6·6	121·5	4·9	118·5	2·0
OL	5·26†	0·16	5·10†	0·21	5·52	0·15	95·5	10·5	116·4†	9·9	116·6	7·2
POM	5·49	0·14	5·69	0·13	5·67	0·15	123·3	10·4	138·8	11·9	114·7	3·7
POMO	5·47	0·10	5·65	0·16	5·55	0·04	122·6	12·7	143·3	19·5	122·0	9·7

WKY, Wistar–Kyoto; BD, basal diet; OL, refined olive oil diet; POM, pomace olive oil diet; POMO, POM supplemented in oleanolic acid diet; SHR, spontaneously hypertensive rats.

* $P<0{\cdot}05$ *v.* E(+).

† $P<0{\cdot}05$ *v.* BD.

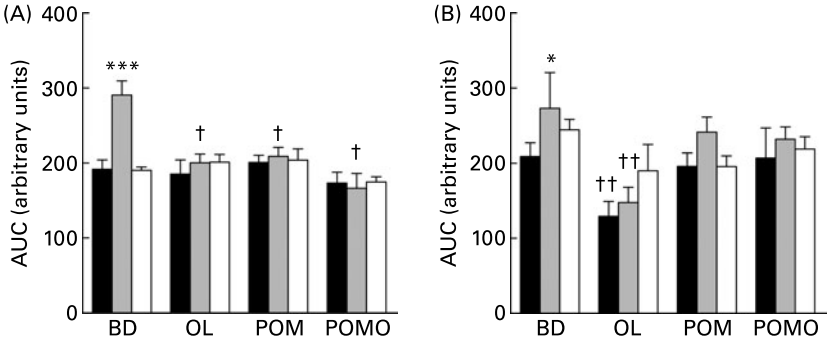

Fig. 1. Area under the concentration–response curves (AUC) for phenylephrine (0·01–0100 μmol/l) in isolated small mesenteric arteries (SMA) from either Wistar–Kyoto (A) or spontaneously hypertensive rats (B) fed a control basal diet (BD), refined olive oil (OL), pomace olive oil (POM) or POM supplemented in oleanolic acid (POMO) for 12 weeks. Curves were obtained in SMA with (E +, ■) or without endothelium (E −, □) or intact preparations in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NAME; 300 μmol/l; ▨). Values are means with their standard errors, *n* 6. Mean value was significantly different from the diet-matched control E + (* $P<0·05$, *** $P<0·001$). Mean value was significantly different from the BD group in the presence of L-NAME († $P<0·05$, †† $P<0·01$).

For this purpose, these factors were inhibited by incubation of the preparation with L-NAME, indomethacin and PSS containing 25 mM-KCl (Figs. 3 and 4), respectively.

Involvement of cyclo-oxygense-derived metabolites

The presence of the COX inhibitor indomethacin significantly improved the relaxant response to ACh in SMA from WKY groups (Fig. 3). In these conditions, arteries from POMO WKY exhibited a moderate contraction phase at higher concentrations of ACh after the maximal relaxation peak. In SHR, COX inhibition also improved the relaxation to ACh and partially attenuated the contractile component evoked by the muscarinic agonist, indicating the potential participation of vasoconstrictor products derived from COX in that response (Fig. 4). Nevertheless, a residual contraction was still evident in SMA from SHR fed OL- or POM-rich diets at high concentrations of ACh in the presence of indomethacin (Fig. 4). This behaviour was much more evident in OL group. At this point, the level of contraction exhibited by ACh in OL SHR group was reached by an agonist concentration tenfold higher than in POM group in the presence of indomethacin. These findings suggest that different mediators rather than COX-derived metabolites might be responsible for this response. In addition, the improved relaxation observed in the presence of indomethacin was importantly stronger in SHR fed an OL diet, suggesting the important role of contracting factors derived from COX in this group.

Involvement of nitric oxide

In SMA from all groups of both WKY and SHR, the ACh-evoked relaxation was significantly attenuated by exposure to the NO synthesis inhibitor L-NAME (Figs. 3 and 4). This fact supports the notion that NO is one of the main mediators involved in the relaxation induced by ACh in SMA. Simultaneous blockade of COX and NOS by the combination of indomethacin + L-NAME attenuated the vasorelaxation for all the groups of WKY and SHR, but this attenuation was only partial and significantly lower than that exhibited in the presence of L-NAME alone (Figs. 3 and 4). The fact that ACh was able to induce relaxation in SMA in the presence of indomethacin + L-NAME even at greater extent than after incubation of L-NAME alone is explained by the release of an EDHF from the endothelium, which plays a major role in the resistance vasculature.

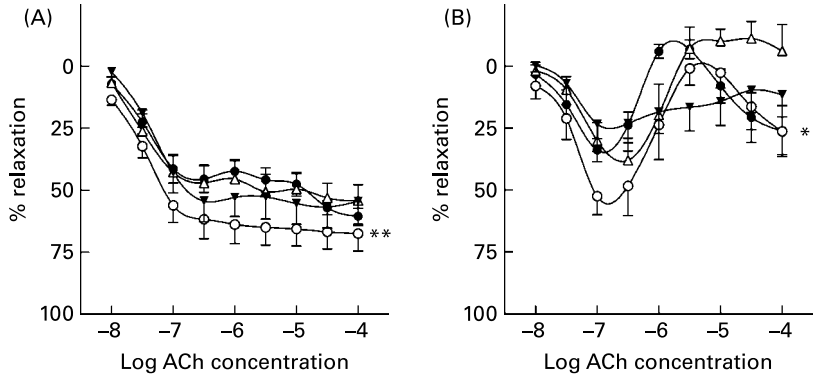

Fig. 2. Concentration–response curves for acetylcholine (ACh; 0·01–100 μmol/l) in endothelium-intact isolated small mesenteric arteries precontracted with phenylephrine from either Wistar–Kyoto (A) or spontaneously hypertensive rats (B) fed a control basal diet (BD, ▼), refined olive oil (OL, △), pomace olive oil (POM, ○) or POM supplemented in oleanolic acid (POMO, ●) for 12 weeks. Values are means with their standard errors, *n* 6. Mean value was significantly different from the BD group (* $P<0·05$, ** $P<0·01$).

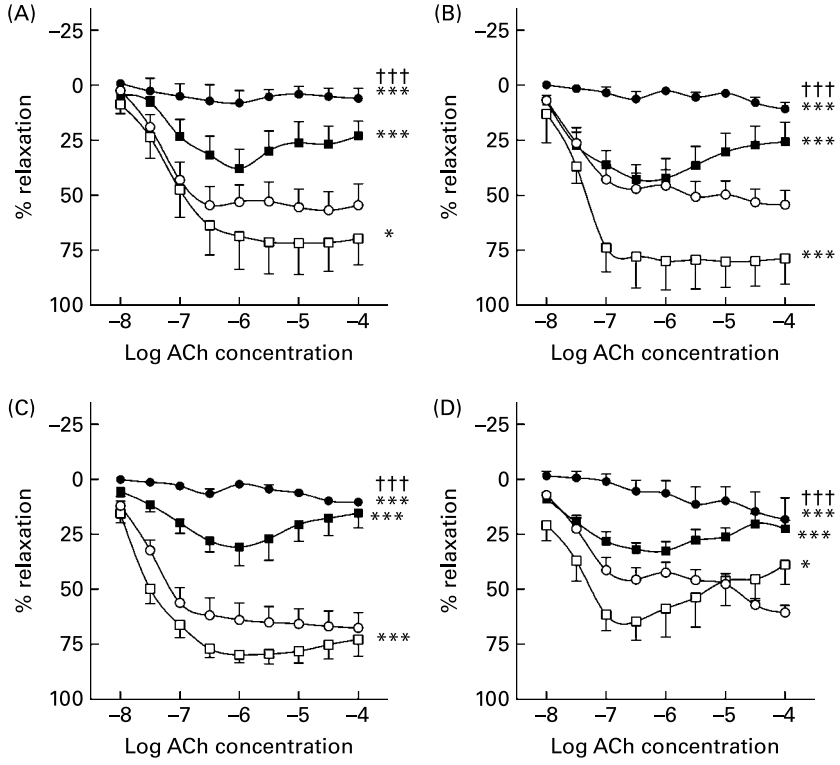

Fig. 3. Concentration–response curves for acetylcholine (ACh; 0·01–100 μmol/l) in endothelium-intact isolated small mesenteric arteries (SMA) precontracted with phenylephrine from Wistar–Kyoto rats fed a control basal diet (BD) (A), refined olive oil (OL) (B), pomace olive oil (POM) (C) or POM supplemented in oleanolic acid (POMO) (D) for 12 weeks. Curves were obtained in SMA in the absence (○, taken as control) or presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NAME; 300 μmol/l; ●), the cyclo-oxygenase inhibitor indomethacin (10 μmol/l; □) or indomethacin + L-NAME (■). Values are means with their standard errors, *n* 6. Mean value was significantly different from the control in the absence of inhibitors (* $P<0·05$, *** $P<0·001$). ††† Mean value was significantly different from that in the presence of indomethacin + L-NAME ($P<0·001$).

Involvement of endothelium-derived hyperpolarising factor

To test the involvement of the hyperpolarisation in the persisting relaxation, concentration–response curves for ACh were performed in 25 mmol/l K^+ media. Under these conditions, relaxation to ACh was markedly inhibited in SMA from both WKY and SHR (Figs. 5 and 6). In arteries from BD- and OL-treated WKY rats, the combination of L-NAME + indomethacin in 25 mmol/l K^+ media attenuated the relaxant response to the same extent as observed in this K^+ media without inhibitors (Fig. 5(A), (B)). On the contrary, in SMA from WKY fed POM- or POMO-rich diets inhibition of the ACh vasorelaxation in 25 mmol/l K^+ media was significantly strengthened by the simultaneous presence of L-NAME and indomethacin in that media (Fig. 5(C), (D)). Regarding to SHR, in SMA from OL and POM groups vasorelaxation to ACh was similarly abolished by 25 mM-KCl either in the presence or absence of L-NAME + indomethacin (Fig. 6(B), (C)). In contrast, in BD and POMO-treated rats, the inhibition of ACh-induced relaxation by 25 mM-KCl was strongly augmented by the presence of both inhibitors (Fig. 6(A), (D)).

In addition, the differential participation of NO/COX-derived metabolites and EDHF to the experimental groups was further characterised determining the values derived from the equations: $AUC_{KCl+indo\ +\ L\text{-}NAME} - AUC_{KCl}$ and $AUC_{KCl+indo\ +\ L\text{-}NAME} - AUC_{indo+L\text{-}NAME}$ for NO/COX and EDHF contribution, respectively. As illustrated in Fig. 7, the contribution of EDHF in the endothelium-dependent vasorelaxation to ACh was significantly higher than NO/COX in SMA from normotensive rats in all the groups. No differences were appreciated in the participation of the EDHF-type relaxation between the WKY groups. However, the participation of NO/COX in the relaxant response was notably increased from 14·51 % in SMA from WKY rats fed a BD-rich diet to 54·98 % in the POM group. According to SHR, the EDHF-type vasorelaxation showed similar values of contribution than those exhibited in WKY, although this participation resulted significantly attenuated in arteries from SHR fed an OL diet. Interestingly, in resistance arteries from POM-treated SHR group, the contribution of EDHF was markedly higher than the NO/COX involvement (8·18 % of NO/COX contribution in relation to EDHF), whereas similar level of contribution for EDHF and NO/EDHF was detected in the POMO group.

Discussion

Numerous epidemiological studies have evidenced an inverse relationship between olive oil consumption and the incidence of cardiovascular risk factors including hypertension. The present study shows functional and vasoactive changes induced by long-term intake of olive oil and POM-enriched diets in small resistance arteries (often referred to as the 'hallmark' of hypertension[15]) from SHR.

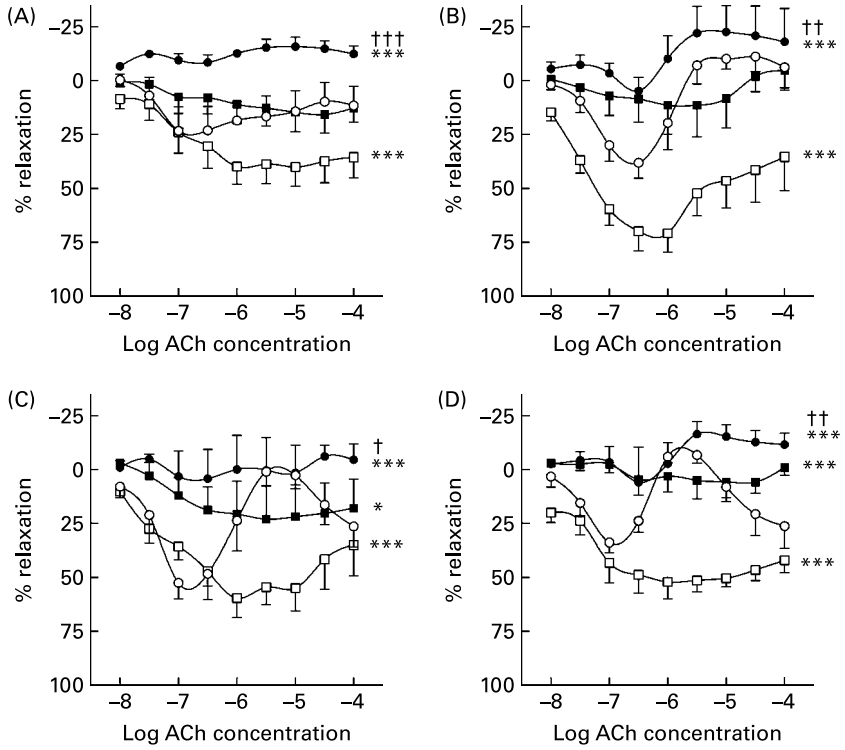

Fig. 4. Concentration–response curves for acetylcholine (ACh; 0·01–100 μmol/l) in endothelium-intact isolated small mesenteric arteries (SMA) precontracted with phenylephrine from spontaneously hypertensive rats fed a control basal diet (BD) (A), refined olive oil (OL) (B), pomace olive oil (POM) (C) or POM supplemented in oleanolic acid (POMO) (D) for 12 weeks. Curves were obtained in SMA in the absence (○, taken as control) or presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NAME; 300 μmol/l; ●), the cyclo-oxygenase inhibitor indomethacin (10 μmol/l; □) or indomethacin + L-NAME (■). Values are means with their standard errors, *n* 6. Mean value was significantly different from the control in the absence of inhibitors (* $P<0·05$, *** $P<0·001$). Mean value was significantly different from that in the presence of indomethacin + L-NAME († $P<0·05$, †† $P<0·01$, ††† $P<0·001$).

Among all the environmental factors that affect arterial hypertension, dietary habits play a prominent role in blood pressure regulation and the associated endothelial dysfunction. Despite the ability of olive oil to modulate blood pressure is less known than its effects on lipid metabolism, epidemiological studies and feeding trials indicate that olive oil could favourably affect the hypertensive state[16–18]. Even though the mechanisms and contribution of the different components from olive oil affecting hypertension are not fully understood, several authors suggest that this action is mediated by improving endothelial function[4,12,19]. Human subjects' and experimental data propose that the beneficial effects of olive oil consumption in hypertension and endothelial activation are attributable to the content in oleic acid[17,20]. Nevertheless, olive oil is more than a monounsaturated fat, and minor constituents from olive oil such as phenolic compounds are also considered responsible for the cardiovascular protection[5,21]. In the present study, we have used OL that are lacking in phenolic compounds due to the refining process. These OL contain similar proportion in oleic acid, but different concentrations in minor components other than phenolic compounds (e.g. triterpenoids).

The potential vasoactive effects of triterpenoids found in the unsaponifiable fraction of olive oil are currently the subject of interest. Although their presence in olive oil is very low, they are found in important concentrations, up to 120 mg/kg, in POM, obtained from the residue that remains after virgin olive oil mechanical extraction[6]. The vasodilator activity of triterpenoids from olive and POM, mainly in the form of oleanolic acid, has been evidenced in isolated aorta from normo- and hypertensive rats[8,9]. Oleanolic acid is also able to substantially induce *in vitro* NO-mediated vasodilatation in resistance arteries from the rat mesenteric vascular bed, which are strongly involved in hypertension[10]. Regarding to *in vivo* evidences, SHR fed POM-enriched diets demonstrated an increased vasodilatation to ACh in isolated conductance arteries (aorta) compared to olive oil- or low fat-treated animals[7]. Those data support the notion than other components, different from oleic acid such as triterpenoids, found in the unsaponifiable fraction of the oils, might be responsible for the effects of POM intake on endothelial function. Supporting this background, the present investigation showed how long-term intake of POM-enriched diets containing substantial amounts of oleanolic acid, modified the vascular reactivity and endothelial responses in SMA, importantly affected by the hypertensive state.

Resistance arteries from SHR are characterised by endothelial dysfunction mainly associated to an impaired ACh-induced relaxation[11,22]. This is thought to be brought about by an increased release of endothelium-derived contracting factors, which are suggested to be a product of arachidonic acid cascade synthesised via COX pathway, since agents such as indomethacin can restore relaxation[22]. Therefore, the dysfunctional endothelium in SMA from SHR is often characterised by a substantial co-release of vasoconstrictor products derived from the COX pathway[13]. In our

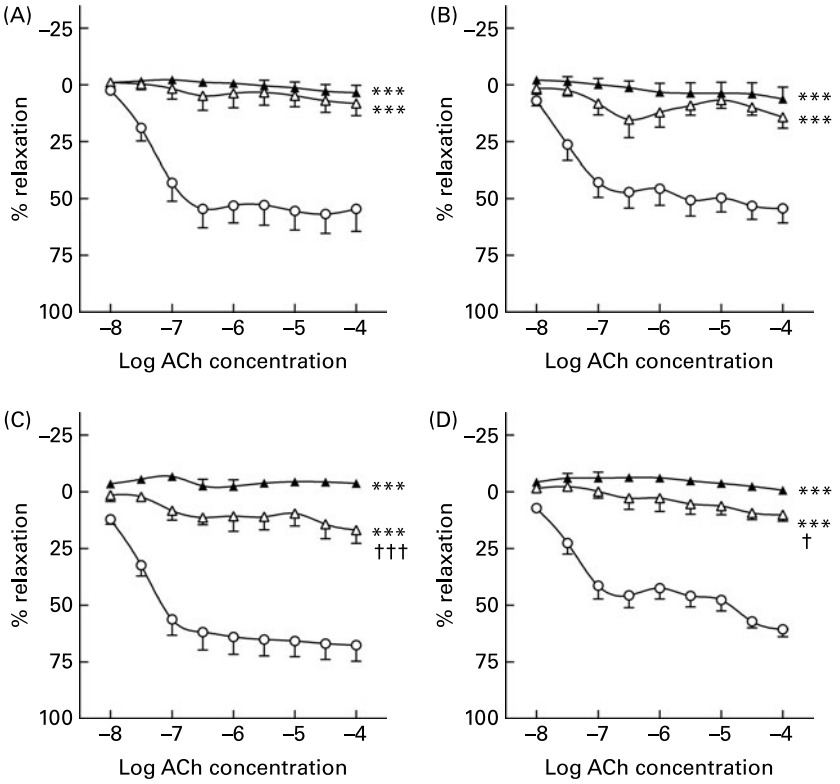

Fig. 5. Concentration–response curves for acetylcholine (ACh; 0·01–100 μmol/l) in endothelium-intact isolated small mesenteric arteries (SMA) precontracted with phenylephrine from Wistar–Kyoto rats fed a control basal diet (BD) (A), refined olive oil (OL) (B), pomace olive oil (POM) (C) or POM supplemented in oleanolic acid (POMO) (D) for 12 weeks. Curves were obtained in SMA in the absence (○, taken as control) or presence of KCl 25 mmol/l (△), or KCl 25 mmol/l + indomethacin (10 μmol/l) + L-NAME (300 μmol/l; ▲). Values are means with their standard errors, *n* 6. *** Mean value was significantly different from the control in the absence of inhibitors ($P<0·001$). Mean value was significantly different from that in the presence of KCl 25 mmol/l + indomethacin + L-NAME († $P<0·05$, ††† $P<0·001$).

investigation, BD control groups showed an improved Phe-induced vasoconstriction after inhibition of NO production by L-NAME, unmasking the participation of vasoconstrictor metabolites from COX. This finding is in line with previous evidence reporting that the reduced release of NO can potentiate the release or the actions of endothelium-derived contracting factors, which would increase sensitivity to Phe and impair the relaxation[23]. Our data also reported that all the WKY groups evidenced a significant improvement in the relaxant response to ACh in the presence of indomethacin, revealing similar contribution of contracting factors derived from COX to the vasoactive response.

Interestingly, we found a biphasic response induced by ACh in SHR treated with high-fat diets (OL, POM, POMO) in contrast to that observed in low-fat diet control (BD). This fact may be explained by the relationship between the type of oil consumed and the effect in the endothelial function. It has been demonstrated that dietary oils influence the lipid composition of postprandial TAG-rich protein particles (TRL) including chylomicrons, VLDL, and their remnants[24]. These TRL can cross the endothelial barrier and enter into the vascular wall, where they can differently modify the endothelial function and atherogenesis depending on the composition of the oil consumed in the diet[24,25]. TRL derived from virgin olive oil intake with high content in unsaponifiable fraction are known to reduce the release of proinflammatory and prothrombotic substances that play important roles in the dysfunction of the vascular reactivity[4]. Specifically, these TRL generated after virgin olive oil intake with high concentration in minor constituents decreased the production of COX-derived vasoconstrictor eicosanoids in vascular endothelial cells compared to dietary oils of different composition[4]. In line with these data, we may hypothesise that the different composition in MUFA and minor constituents between the oils tested in our investigation could be an important factor determining differences in the endothelial-dependent response to ACh. This might explain the biphasic response to the muscarinic agonist in SHR fed high-fat diets, but not in the control BD group. Furthermore, the fact that, in SHR, the contractile phase of the dose–response curves to ACh detected in high-fat dietary groups was clearly attenuated by indomethacin, support the idea that eicosanoids might be involved in the vasoactive effects of these olive and POM diets on the endothelial function. According to the earlier description, differential composition of the TRL derived from the experimental diets could differently alter vascular reactivity of SMA. In contrast, COX-derived metabolites did not play a major role in the endothelial-dependent response to ACh in conductance arteries (aorta) from SHR-fed POM-enriched diets[7], indicating that the vascular effect of these diets differs depending on the vascular arterial bed and vessel diameter considered. In the present study, we also observed a residual contraction resistant to indomethacin in SMA from SHR fed an OL- or POM-enriched diet, which

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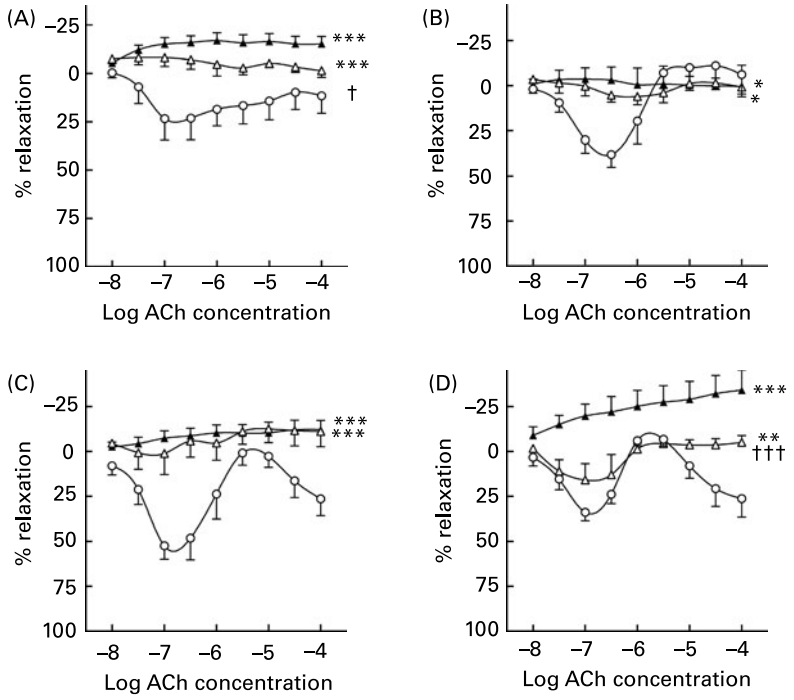

Fig. 6. Concentration–response curves for acetylcholine (ACh; 0·01–100 μmol/l) in endothelium-intact isolated small mesenteric arteries (SMA) precontracted with phenylephrine from spontaneously hypertensive rats fed a control basal diet (BD) (A), refined olive oil (OL) (B), pomace olive oil (POM) (C) or POM supplemented in oleanolic acid (POMO) (D) for 12 weeks. Curves were obtained in SMA in the absence (○, taken as control) or presence of KCl 25 mmol/l (△), or KCl 25 mmol/l + indomethacin (10 μmol/l) + L-NAME (300 μmol/l; ▲). Values are means with their standard errors, *n* 6. Mean value was significantly different from the control in the absence of inhibitors (* $P<0·05$, ** $P<0·01$, *** $P<0·001$). Mean value was significantly different from that in the presence of KCl 25 mmol/l + indomethacin + L-NAME († $P<0·05$, ††† $P<0·001$).

may be attributed to factors different from COX-derived metabolites. Nevertheless, further studies are required to better characterise the role of endothelial COX products in rats after administration of olive and POM diets.

In addition to COX-derived mediators, endothelial cells release the vasodilators NO and EDHF. The relative contribution of both NO and EDHF varies among species, vascular bed and vessel size[26]. Particularly, in arterioles and small arteries such as SMA, EDHF appears to be of major importance, whereas in larger arteries such as aorta the role of NO is more pronounced[26]. Moreover, the EDHF-mediated hyperpolarisation and relaxation mainly detected in small arteries plays a pivotal role in the endothelial dysfunction in SHR model, since chronic treatment of these animals with antihypertensive agents markedly improved the EDHF system previously altered by hypertension[27,28]. Previous data in aorta from SHR showed the major contribution of NO in the improvement of endothelial function provided by POM-enriched diets consumption by increasing the aortic protein expression of eNOS[7]. In SMA, our findings showed that relaxation to ACh was inhibited by L-NAME indicating the participation of NO to the endothelial-dependent dilatation. However, simultaneous blockade of COX and NOS by indomethacin + L-NAME only evoked a partial inhibition of the relaxation to ACh, suggesting the substantial involvement of EDHF. These findings agree with those demonstrated by

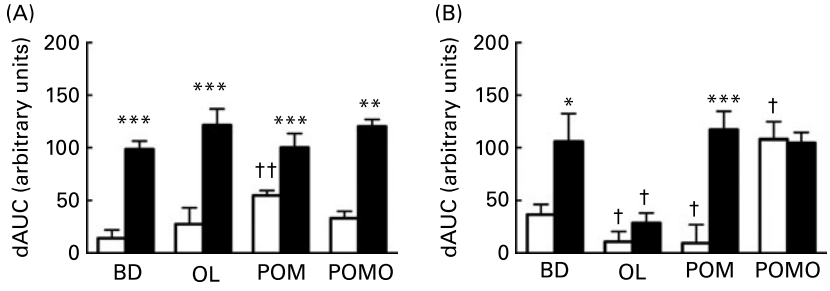

Fig. 7. Bars showing contribution of NO/cyclo-oxygense (COX; □) and endothelium-derived hyperpolarising factor (EDHF; ■) to the endothelial-dependent response curve to acetylcholine (0·01–100 μmol/l) in endothelium-intact isolated small mesenteric arteries precontracted with phenylephrine from Wistar–Kyoto (A) or spontaneously hypertensive rats (B) fed a control basal diet (BD), refined olive oil (OL), pomace olive oil (POM) or POM supplemented in oleanolic acid (POMO) for 12 weeks. Contributions were calculated from area under curves (AUC): NO/COX = $AUC_{KCl+indo\ +\ L\text{-}NAME} - AUC_{KCl}$ and EDHF = $AUC_{KCl+indo\ +\ L\text{-}NAME} - AUC_{indo+L\text{-}NAME}$. Mean value was significantly different from the NO/COX contribution (* $P<0·05$, ** $P<0·01$, *** $P<0·001$). Mean value was significantly different from that in the BD group († $P<0·05$, †† $P<0·01$).

Goto *et al.* [29] reporting that ACh evoked a fast depolarisation in SMA from SHR, which was responsible for a constriction that reduced EDHF-mediated relaxation. EDHF is abolished by K^+ concentrations >25 mmol/l and is resistant to the inhibitory action of indomethacin + L-NAME[30], supporting our observations. In SMA from SHR treated with POM, the maximal relaxant response to ACh was substantially improved compared with the rest of the dietary groups. Data generated in the presence of K^+ 25 mmol/l confirmed the important contribution of EDHF to the enhanced endothelium-dependent relaxation in resistance arteries from SHR fed a POM diet. In addition, and in contrast to the control group, the muscarinic agonist also evoked dilatation in the second phase of the dose–response curve in POM group. This phenomenon was also observed in arteries from SHR fed a POMO diet. Altogether, these observations support the notion that the modulator effects of POM-enriched diets in the endothelial dysfunction of resistance arteries from hypertensive rats are related to an increased participation of the EDHF-type component. Regarding to WKY rats fed a POM diet, the relative contribution of NO/COX was more important compared with SHR. This fact is in line with evidences that EDHF- and NO-mediated dilatation in SMA from hypertensive rats and mice showed a decreased NO and/or EDHF contributions to relaxation[23,31].

In WKY rats treated with POMO diet, relaxation response to ACh was enhanced by indomethacin, but there was a contraction that remained unaltered at high concentrations of ACh. This contraction was still remaining after indomethacin + L-NAME, but blocked by the additional presence of K^+ 25 mmol/l, suggesting that the contraction was related to contracting agents different from the COX pathway. Regarding to SHR fed a POMO diet, the participation of NO was notably increased compared with the control group, reaching similar levels of NO/EDHF contribution. The importance of NO exhibited in POMO groups compared with POM may be due to the higher concentration of oleanolic acid in the former. As previously described, this triterpenoid induces *in vitro* vasodilatation in conductance and resistance arteries with endothelial NO as the main mediator of the response[8–10]. In addition, an increased NO bioavailability was described as the major mechanism underlying the endothelial function improvement in aorta from rats fed a POMO diet. In this case, oleanolic acid was also suggested as a key component responsible for this effect[7].

In the present study, blood pressure values were not altered by any of the experimental diets, suggesting that changes in endothelial function associated to POM intake could not be explained by a secondary event to antihypertensive action, but by a protective activity in the vascular endothelium of resistance arteries. Diets were administered to SHR for 3 months, which may not have been long enough to observe the effects of POM in blood pressure; however, beneficial effects of these diets on endothelial function have been detected after this treatment period. Therefore, it remains to be determined whether a longer duration of treatment with POM/POMO would affect blood pressure.

Functional and structural changes of resistance arteries may represent the earliest type of hypertensive organ damage, and, as such, is a potential treatment target[32]. A number of very recent investigations evidence that either POM intake or some of its isolated triterpenoids substantially modify the endothelial dysfunction associated to hypertension and atherogenesis[7–10,33,34]. Our present study shows that these beneficial effects are further related to an improved endothelial function in small mesenteric resistance arteries from SHR after long-term intake of POM-enriched diets. The mechanisms underlying this effect involve enhancement of agonist-mediated EDHF/NO response, which is importantly altered in the dysfunctional endothelium with hypertension. The results also suggest that the triterpenic fraction may confer a protective role to POM against endothelial dysfunction in pathological situations such as hypertension and atherosclerosis.

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