Biochemical Pharmacology, Vol. 57, pp. 445–449, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00320-7

SHORT COMMUNICATION

Inhibition of Leukocyte 5-Lipoxygenase by Phenolics from Virgin Olive Oil

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ABSTRACT. Interest in the health-promoting effects of virgin olive oil, an important part of the 'Mediterranean diet', prompted us to determine the anti-eicosanoid and antioxidant effects in leukocytes of the principal phenolic compounds from the 'polar fraction': oleuropein, tyrosol, hydroxytyrosol, and caffeic acid. In intact rat peritoneal leukocytes stimulated with calcium ionophore, all four phenolics inhibited leukotriene B_4 generation at the 5-lipoxygenase level with effectiveness hydroxytyrosol > oleuropein > caffeic acid > tyrosol (approximate EC_{50} values: 15, 80, 200, and 500 μM, respectively). In contrast, none of these compounds caused substantial inhibition of thromboxane generation via the cyclo-oxygenase pathway. Hydroxytyrosol, caffeic acid, oleuropein, and tyrosol (decreasing order of effectiveness) also quenched the chemiluminescence signal due to reactive oxygen species generated by phorbol myristate acetate-stimulated rat leukocytes. None of these compounds were toxic to leukocytes at the concentrations tested. We conclude that the phenolics found in virgin olive oil possess an array of potentially beneficial lipoxygenase-inhibitory, prostaglandin-sparing, and antioxidant properties. BIOCHEM PHARMACOL **57**;4:445–449, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. virgin olive oil; polyphenolic compounds; natural antioxidants; free radical scavengers; eicosanoids; 5-lipoxygenase inhibitors; neutrophils

It is now well accepted that the 'Mediterranean diet' is correlated with a lower incidence of coronary heart disease [1, 2]. This diet is rich in fruit, vegetables, grain, and plant-derived oils, especially olive oil. Although the protective effect of such diets is likely to be multifactorial, epidemiological studies have stressed the importance of consuming dietary lipids possessing a high monounsaturated/saturated ratio [3–5], such as is the case with olive oil. Other recent studies of the Mediterranean and other diets have considered the likely protective effects of antioxidants which occur naturally in fruits, vegetables, and various beverages including red wine and tea [6–10].

In the case of olive oil, the major nutritionally relevant part is constituted of triglycerides with a high content of monounsaturated fatty acids, but there are also many other minor compounds, including various polyphenols [11–13], which are usually removed during the refining processes used in commercial oil production [14]. These substances, constituting the 'polar fraction' in virgin olive oil, prevent its autoxidation and underlie its exceptional thermal stability [11, 12, 15], and also contribute to its characteristic flavour and taste. They include simple phenols based on cinnamic acid (e.g. caffeic acid), the iridoid glycoside oleuropein and its hydrolysis product hydroxytyrosol, and tyrosol (Fig. 1).

In the context of coronary heart disease, it is already known that experimental feeding of olive oil enriched in polyphenols to humans or rabbits increases the resistance of low-density lipoprotein to oxidation *ex vivo* [16, 17], emphasising the known capacity of certain of the phenolics present within virgin olive oil to act as antioxidants when tested in chemical systems [18, 19].

There is evidence from human and animal studies that PMNs‖ may be involved in the development of coronary heart disease, angina, and other sequelae of atherosclerosis [20–22]. The recruitment of these cells to inflammatory sites and their subsequent activation is controlled by locally released mediators, including LTB_4, which is generated from arachidonic acid by 5-LO. This enzyme is known to be inhibited by other dietary phenolic compounds such as coumarins and flavonoids [23–25]. In this paper, we provide the first evidence that olive oil phenolics are capable of inhibiting leukocyte 5-LO whilst sparing the generation of prostaglandins, and also show that these compounds can reduce the generation of ROS by intact leukocytes.

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Received 25 May 1998; accepted 8 September 1998.

‖ *Abbreviations:* 5-LO, 5-Lipoxygenase; LTB_4, leukotriene B_4; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; and TXB_2, thromboxane B_2.

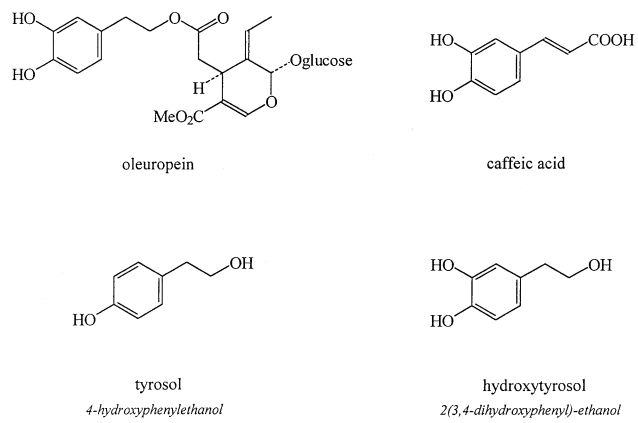

FIG. 1. Structures of the four phenolics from the polar fraction of virgin olive oil.

MATERIALS AND METHODS

Materials

Caffeic acid and tyrosol were purchased from Fluka. Oleuropein was extracted from the leaves of *Olea europea* (Instituto de la Grasa C.S.I.C., Seville, Spain). Hydroxytyrosol was prepared by acidic hydrolysis of oleuropein, according to the method described in Ref. 26. All of these substances were dissolved in DMSO.

Preparation of Rat Peritoneal Mixed Leukocytes

Leukocytes containing approximately 85% PMNs and 15% mononuclear cells were prepared as in Ref. 27 from male Wistar rats and resuspended in complete Hanks' balanced salt solution at 2.5×10^6 cells/mL containing 1.26 mM Ca^{2+} and 0.9 mM^{2+}. Cell viability based on trypan blue exclusion was greater than 95%.

Stimulation of the Release of Eicosanoids and Their Radioimmunoassay

Triplicate aliquots of 0.5 mL leukocytes were preincubated at 37° for 10 min with 2 μL DMSO containing the compounds of interest or an equivalent volume of the vehicle alone. After this, 5 μL of calcium ionophore A23187 was added in DMSO to give a final concentration of 1 μM for a further 10 min of incubation. The cells were pelleted by centrifugation at 2500 *g* for 10 min at 4°, and the supernatants were decanted and frozen. Aliquots (5–15 μL) of the thawed samples were subjected to radioimmunoassay for TXB_2 or LTB_4 by making up to 100 μL with 50 mM phosphate buffer pH 7.5 containing 0.1% human γ-globulin and 0.9% saline, adding 200 μL polyclonal rabbit anti-eicosanoid serum diluted 1:1500, 100 μL tracer containing 10 nCi^3H_8-TXB_2 or 4 nCi^3 H_8-LTB_4 (NEN or Amersham, respectively), mixing and incubating at 4° for 18 hr. After this, bound label was separated from free using 200 μL dextran-coated charcoal and the bound dpm counted in a Packard model 1900TR liquid scintillation analyser.

Chemiluminescence Generated from Activated Leukocytes

Rat mixed peritoneal leukocytes were isolated as described above. Briefly, 1.5 mL leukocyte suspension was preincubated with test drugs or vehicle in a plastic 5 mL reaction vial at 37°, and 50 μL lucigenin (250 μM) was then added prior to placing the vial in the light-tight cavity of an LKB-Wallac 1250 luminometer, and bubbled with 95% O_2/5% CO_2. The reaction was initiated after 3-min preincubation by addition of PMA (5 μM final concentration) through an auto-injector (LKB 1250-104). The changes in chemiluminescence were monitored by digital readout, and in analogue form using a Maclab®. Results were calculated as the peak value achieved or as total intensity, i.e. area under the curve (mVs).

RESULTS

Effect of Olive Oil Phenolics on Profile of Eicosanoids Released from Stimulated Rat Leukocytes

Treatment of elicited rat peritoneal leukocytes with calcium ionophore A23187 results in phospholipase A_2-dependent release of endogenous arachidonic acid from membrane phospholipids and its subsequent transformation via the cyclo-oxygenase and 5-LO pathways to thromboxane A_2/prostaglandin E_2 and LTB_4, respectively (Fig. 2). These pathways operate in both the PMNs and mononuclear leukocytes which comprise ca. 75–85% and 15–25% of the total elicited peritoneal leukocyte cell population [27].

The four phenolic compounds at 40–200 μM all inhibited leukotriene B_4 generation with effectiveness hydroxytyrosol > oleuropein > caffeic acid > tyrosol (100%–35% inhibition at 200 μM, approximate EC_{50} values: 15, 80, 200, and 500 μM, respectively). For comparison, two well-established inhibitors of the 5-LO pathway in leukocytes, ZM211965 [23] and the flavonoid phenolic quercetin, reduced LTB_4 generation by 98–100% at 20 μM. In contrast, none of the phenolic compounds derived from virgin olive oil affected TXB_2 generation in the range 40–200 μM (Fig. 2). For positive controls, two known potent cyclo-oxygenase inhibitors, indomethacin and piroxicam, and the thromboxane synthetase inhibitor dazoxiben produced 94–100% inhibition of TXB_2 generation at 20 μM. Taken together, these results show that the four phenolics cause a variable degree of inhibition of the generation of leukotriene B_4 by the activated leukocytes, and that they must be acting at the 5-LO level (rather than upstream at the phospholipase A_2 level, which would reduce TXB_2 generation as well).

None of these compounds interfered with the metabolic integrity of the leukocytes at the doses used. This was shown by incubating the cell suspensions for 30 min with the phenolics plus 0.15 mg/mL thiazolyl blue. In no cases was the blue colour generated by this marker of mitochondrial oxidative phosphorylation reduced by concomitant drug treatment (data not shown).

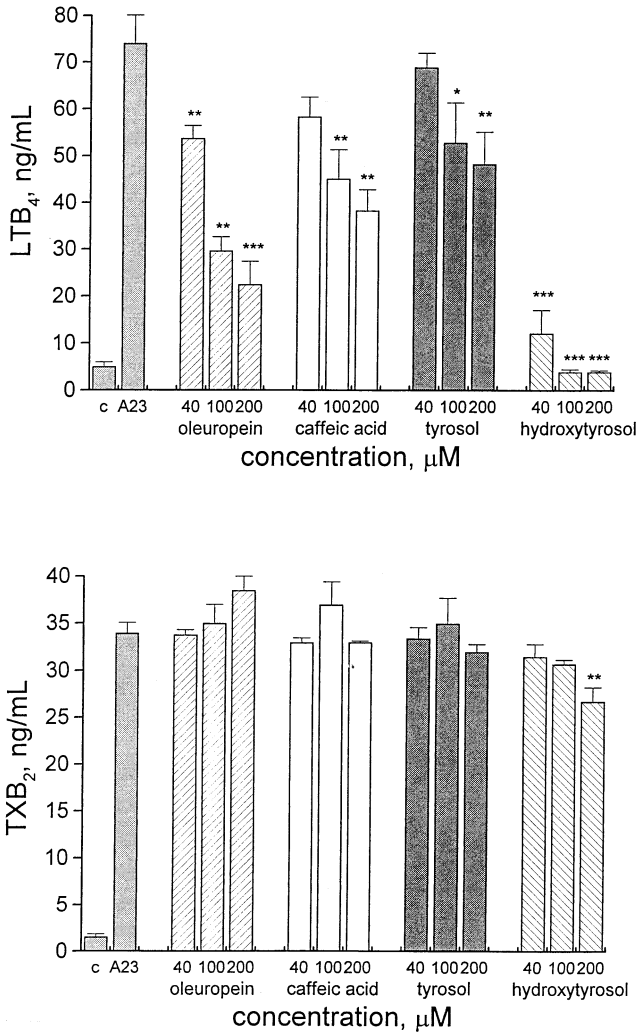

FIG. 2. Generation of LTB_4 and TXB_2 by ionophore-activated rat peritoneal leukocytes and effects of four phenolics from the polar fraction of olive oil. Results show means ± SEM for 3–9 tests and the symbols *, **, * indicate statistically significant differences with respect to control (Student's unpaired *t*-test), $P < 0.05$, 0.01, and 0.001, respectively.**

Effect of Olive Oil Components on ROS Generated by Rat Peritoneal Leukocytes During the Respiratory Burst, and Effects on ROS in Cell-Free Systems

As these phenolics have been shown previously to exert antioxidant activity in cell-free systems [18, 19], we tested their ability to scavenge or reduce the generation of ROS in intact leukocytes. This was achieved by stimulating the cells with PMA. This caused a prolonged respiratory burst involving the generation of superoxide anions and other species capable of inducing chemiluminescence of lucigenin. Table 1 (column A) shows that hydroxytyrosol, caffeic acid, oleuropein, and tyrosol reduced the peak luminescence signal by 67%, 66%, 51%, and 32%, respectively.

In view of the importance of superoxide for the respiratory burst, we also investigated the effects of the olive oil phenolics on superoxide generated by the hypoxanthine/xanthine oxidase cell-free system. Using the chemiluminescence method to detect ROS generation as before, we again found that all four phenolics reduced the ROS-dependent signal, with hydroxytyrosol having the greatest effect when tested at 100 μM and tyrosol the least (Table 1, column C). Cytochrome *c* reduction is also frequently used to assess superoxide generation and its scavenging, but we found that the three most active phenolics are capable of reducing cytochrome *c* themselves, thus invalidating the assay, although tyrosol again reduced the apparent amount of superoxide (Table 1, column B). Finally, we measured whether these phenolics were capable of scavenging hydrogen peroxide by using the guaiacol reaction; these tests showed oleuropein and hydroxytyrosol to have equivalent activity with lower activity for caffeic acid, but tyrosol was inactive (Table 1, column D).

DISCUSSION

These studies add to our understanding of the biochemical properties of olive oil components by showing that the phenolic components exert selective inhibitory activity against the 5-LO (leukotriene) pathway of arachidonate

TABLE 1. Effect of olive oil phenolics on the chemiluminescence signal produced by reactive oxygen species generated by PMA-activated rat peritoneal leukocytes, and inhibitory actions on ROS generated in cell-free systems

Compound	(A) ROS generation by leukocytes. Maximum chemiluminescence intensity, mV	(B) Rate of superoxide generated by HX/XO (Cytochrome assay) mOD unit/min	(C) Amount of superoxide generated by HX/XO (Chemiluminescence assay) mV peak value	(D) H_2O_2 scavenging (IC_{50}, μM)
Control	13.68 ± 0.754	40.0 ± 0.4	39.6 ± 4.5	—
Oleuropein	6.18 ± 0.55*	†	4.9 ± 0.6*	145 ± 3.4
Caffeic acid	4.30 ± 0.32*	†	5.5 ± 2.3*	350 ± 10
Tyrosol	8.63 ± 0.91‡	25.5 ± 0.9	15.6 ± 3.8*	inactive at 250 μM
Hydroxytyrosol	4.16 ± 0.34*	†	1.7 ± 0.2*	186 ± 20
Conc tested (μM)	320	500	100	various

Results show means ± SEM. N = 4. Methods for experiments in columns (B) and (D) were as given in Ref. 24, those for column (C) were as in Ref. 36.
Symbols ‡ and * indicate statistically significant differences with respect to control (Student's unpaired *t*-test). $P < 0.01$ and 0.001, respectively.
Symbol † indicates that the assay could not be performed as the compound itself reduces the cytochrome *c*.

metabolism in activated leukocytes without affecting the cyclo-oxygenase pathway.

It is not surprising that hydroxytyrosol, oleuropein, caffeic acid, and tyrosol (ranked in descending order of potency) should all inhibit 5-LO in the ionophore-activated leukocytes, thus diminishing leukotriene B_4 generation, because all four compounds can be regarded as typical phenolic antioxidants, and antioxidants in general are known to often inhibit both animal and plant lipoxygenase enzymes. Other naturally occurring plant-derived phenolics such as flavonoids and coumarins also inhibit leukocyte 5-LO, and greatest potency is found in those possessing vicinal diol (catechol) functions [23, 25, 27], whereas those lacking this functionality are much less active, as seen here for tyrosol. Moreover, caffeic acid is already recognised and has been widely used as a relatively weak inhibitor of 5-LO devoid of activity against cyclo-oxygenase [28], although its potency is enhanced if its lipophilicity is increased [29, 30]. However, despite the fact that this order of inhibitory potency for the four phenolics is the same as for their activity against linoleic acid autoxidation [18], it should not be assumed that interception of arachidonyl peroxyl radicals is the mechanism; phenolic compounds may also bind iron ions [31, 32] and/or reduce them to the catalytically inactive ferrous form [33, 34], and both properties may be important for inhibition of lipoxygenase activity in leukocytes [25].

In relation to free radical scavenging, we have shown for the first time that the four phenolics are capable of preventing the generation of ROS by intact leukocytes, without evidence of toxicity. This amplifies a previous report which showed superoxide scavenging by oleuropein > caffeic acid > hydroxytyrosol (tyrosol inactive) in the xanthine/xanthine oxidase system as monitored by EPR [35]. Our results measuring the capacity of these four phenolics to quench the superoxide chemiluminescence in PMA-stimulated cells (as well as in the hypoxanthine/xanthine oxidase system) and to scavenge hydrogen peroxide (Table 1) suggest that of the four phenolics hydroxytyrosol is the most active with tyrosol the least potent, with intermediate activity for caffeic acid and oleuropein (which can be hydrolysed *in vivo* to hydroxytyrosol, see Fig. 1). Nevertheless, these comments are not based on full dose–response studies, and a more accurate investigation of the relative properties of these olive oil phenolics as biological antioxidants would be worthwhile.

In summary, the present results show that the principal phenolics present within the polar fraction of virgin olive oil possess an array of potentially beneficial lipoxygenase-inhibitory, prostaglandin-sparing, and antioxidant properties. This suggests that they may be able to reduce 5-lipoxygenase-driven cellular recruitment of leukocytes and the damaging consequences of their ability to release ROS whilst leaving unimpaired the generation of prostaglandins, which promote microvascular blood flow and act as immunomodulators. Phenolics from olive oil (as well as from other foodstuffs present within the Mediterranean diet) may in this way help to protect against those degenerative disorders of the cardiovascular system whose progression is accelerated by leukocyte involvement.

We thank the Spanish Ministry of Education for a Research Fellowship awarded to R. de la Puerta and the Spanish Interministerial Commission of Science and Technology (CICYT) for partial financial support of this work (Projects ALI95-0073 and ALI96-0456).

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