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Hydroxytyrosol and oleuropein of olive oil inhibit mast cell degranulation induced by immune and non-immune pathways

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ABSTRACT

The aim of this study was to determine whether hydroxytyrosol and oleuropein, the major phenols found in olives and olive oil, inhibit mast cell activation induced by immune and non-immune pathways. Purified peritoneal mast cells were preincubated in the presence of test compounds (hydroxytyrosol or oleuropein), before incubation with concanavalin A, compound 48/80 or calcium ionophore A23187. Dose–response and time-dependence studies were carried out. Comparative studies with sodium cromoglycate, a classical mast cell stabilizer, were also made. After incubation the supernatants and pellets were used to determine the β-hexosaminidase content by colorimetric reaction. The percentage of β-hexosaminidase release in each tube was calculated and taken as a measure of mast cell activation. Other samples of cell pellets were used for cell viability studies by the trypan blue dye exclusion test, or fixed for light and electron microscopy. Biochemical and morphological findings of the present study showed for the first time that hydroxytyrosol and oleuropein inhibit mast cell degranulation induced by both immune and non-immune pathways. These results suggest that olive phenols, particularly hydroxytyrosol and oleuropein, may provide insights into the development of useful tools for the prevention and treatment of mast cell-mediated disorders.

Introduction

Mast cells have been involved in the pathogenesis of a number of disorders including contact dermatitis, allergic rhinitis, asthma, cancer, rheumatoid arthritis, ulcerative colitis and peptic ulcer (Bischoff, 2007; Sur et al., 2007; Kumar and Sharma, 2010; Galli et al., 2011; Kraneveld et al., 2011; Weller et al., 2011; Theoharides et al., 2012). These cells play a key role in the pathophysiology of these disorders due to their ability to release a variety of inflammatory mediators in response to both immune and non-immune stimuli. Mast cell mediators include preformed molecules such as histamine and proteases stored in secretory granules (Kalesnikoff and Galli, 2008; Yamada et al., 2008; Tore and Tuncel, 2009). In this context, the exploration of interactions of mast cells with molecules capable of modulating mediator release from cell granules is a promising field for the treatment of mast cell-mediated diseases.

It has been shown that several plant products with antioxidant properties inhibit mast cell activation (Middleton et al., 2000; Penissi et al., 2009; Sakai et al., 2009; Vera et al., 2012). Recent scientific evidence has highlighted different nutritional interventions, such as dietary polyphenols, as promising agents able to alleviate symptoms associated with mast cell activation (Singh et al., 2011). The pulp of olives and olive oil contain dietary phenols, such as simple phenolic compounds like hydroxytyrosol and more complex compounds like oleuropein. Hydroxytyrosol and oleuropein are the major phenols found in olives (Tripoli et al., 2005; Jemai et al., 2008; Hagiwara et al., 2011). However, no studies have been published on the effects of these molecules on mast cell degranulation.

The goal of the present work was to determine the effects of both phenolic compounds on mast cell degranulation, and thus explore the possibility that oleuropein and hydroxytyrosol might inhibit *in vitro* mast cell activation.

Materials and methods

Chemicals and reagents

Hydroxytyrosol and oleuropein were supplied by Extrasynthèse (Lyon, France). The chemical structures of the polyphenols used in this study are shown in Fig. 1. These polyphenols were dissolved in

Abbreviations: ConA, concanavalin A; 48/80, compound 48/80; A23187, calcium ionophore A23187; FcεRI, high-affinity receptor for IgE; IgE, immunoglobulin E.

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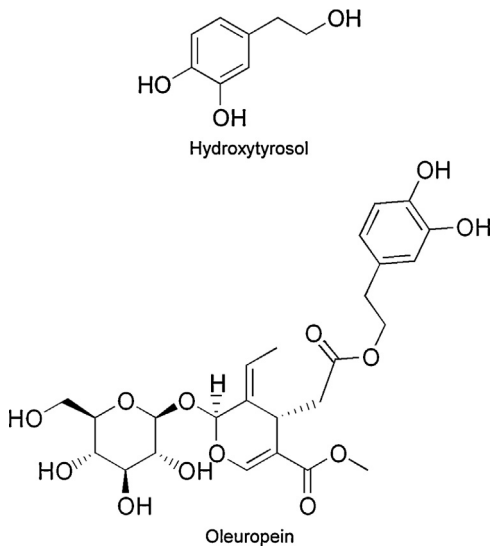

Fig. 1. Structural formulas of polyphenols used in this study.

a solution containing 6.7 mM Na_2HPO_4, 6.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.8 mM $CaCl_2$, 0.5 g/l albumin and 1 g/l glucose, adjusted to pH 7.2, and stored at −20 °C until required. The stock solutions were then diluted with the same solution to the desired final concentration. Bovine serum albumin (fraction V), concanavalin A, compound 48/80, calcium ionophore A23187, sodium cromoglycate, 4-nitrophenyl-N-acetyl-β-D-glucosaminide, toluidine blue, trypan blue, glutaraldehyde, formaldehyde and osmium tetroxide were purchased from Sigma (St. Louis, MO, USA). Percoll was obtained from GE Healthcare (Munich, Germany). All other substances were supplied by Merck (Darmstadt, Germany). All the chemicals used in these studies were of the highest grade available.

Animals

Male Wistar adult rats weighing approximately 300–500 g, infection free and maintained under a 12-h dark/light cycle in a temperature-controlled room (24–25 °C) with free access to drinking water and laboratory food, were used for the study. All animal experiments were carried out according to the standards included in the *Guide for the Care and Use of Laboratory Animals* (published by the National Academy of Science, National Academy Press, Washington, DC), and approved by the Institutional Committee for Care and Use of Laboratory Animals (CICUAL, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina).

Isolation and purification of mast cells

Mast cells were isolated by peritoneal lavage as previously described (Mousli et al., 1989) with some modifications. Rats were killed by CO_2 inhalation prior to an injection of 20 ml of a solution containing 6.7 mM Na_2HPO_4, 6.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.8 mM $CaCl_2$, 0.5 g/l albumin and 1 g/l glucose, adjusted to pH 7.2 into the peritoneal cavity. The abdomen was gently massaged for about 3 min. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. Peritoneal mast cells were then purified by centrifugation through a discontinuous gradient of Percoll as reported by MacGlashan and Guo (1991). Harvesting of the mast cells was simple since these cells gathered in a layer at the bottom of the tube whereas other cells formed a rather compact layer on top of the gradient and could easily be removed by aspiration. Cells were stained metachromatically with toluidine blue (0.1% (w/v), pH 1.0) and quantified by using a Neubauer hemocytometer under a Nikon microscope (magnification ×200). Crude peritoneal cell suspensions contained 3% mast cells, and the purity of the mast cells after gradient centrifugation was over 95%. Purified mast cells were washed, resuspended in a balanced salt solution containing 6.7 mM Na_2HPO_4, 6.7 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.8 mM $CaCl_2$, 0.5 g/l albumin and 1 g/l glucose, adjusted to pH 7.2 (cell density of 1×10^6/ml), and maintained for a maximum of 30 min at 4 °C. The viability of the mast cells was determined by their ability to exclude trypan blue and by the measurement of β-hexosaminidase in the supernatant. The trypan blue-exclusion test indicated viability over 95%. Nonspecific, spontaneous β-hexosaminidase release was always <4%.

General protocol

Purified peritoneal mast cells (cell density of 1×10^6/ml) were equilibrated at 37 °C for 10 min. Thirty microliter aliquots of the equilibrated cells were then preincubated in polypropylene tubes at 37 °C in the presence of the test compounds (hydroxytyrosol or oleuropein), before incubation with concanavalin A (final concentration 200 μg/ml, with 50 μg/ml phosphatidylserine added as a co-stimulator), compound 48/80 (final concentration 10 μg/ml), or calcium ionophore A23187 (final concentration 50 μg/ml), for 10 min at 37 °C. Negative (no stimulation with the mast cell secretagogues) and positive (stimulation with the mast cell secretagogues) controls were included in all the experiments. Dose–response (hydroxytyrosol and oleuropein concentrations of 10, 50, 100, 200 and 400 μM) and time-dependence (hydroxytyrosol and oleuropein preincubation for 5, 10, 20 and 45 min) studies were carried out. Comparative studies with sodium cromoglycate, a classical mast cell stabilizer, were also made within the same concentrations and time ranges. The final incubation volume in each tube was 100 μl. The mean total number of mast cells during incubations was 4×10^4/ml *per* tube. The secretion was stopped by cooling the tubes in an ice-cold water bath. Cells and supernatants were separated by centrifugation (180 × *g*, 5 min, 4 °C). The supernatants were used to determine the β-hexosaminidase content by colorimetric reaction, which was taken as a measure of β-hexosaminidase release. The cell pellets were lysated with 1% Triton X-100 to liberate the residual β-hexosaminidase, which was quantified by colorimetric reaction and taken as a measure of the remaining β-hexosaminidase. Other samples of cell pellets were used for cell viability studies by the trypan blue dye exclusion test, or fixed for light and electron microscopy. Cell viability studies were carried out in order to ensure that changes in β-hexosaminidase release were not due to cell death. The percentage of β-hexosaminidase release in each tube was calculated. All the experiments were repeated at least five times in duplicate.

β-Hexosaminidase assay

β-Hexosaminidase release, as an index of mast cell degranulation, was assayed using a colorimetric assay as previously reported (Puri and Roche, 2008) with some modifications. Briefly, 50 μl of the supernatant was mixed with an equal volume of 2 mM substrate solution (p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.2 M citrate, pH 4.5) and then incubated for 3 h at 37 °C. The reaction was terminated by adding 250 μl of stopping buffer (0.4 M glycine in Na_2CO_3/$NaHCO_3$, pH 9). Absorbance was measured with a microplate reader at 405 nm (Thermo Scientific Multiskan FC, Helsinki, Finland). Results were expressed as the percentage of β-hexosaminidase activity released over the total (enzyme released plus intracellular enzyme).

Light microscopy and morphometry

Mast cells were fixed in 2% glutaraldehyde. After 2 h in the fixative, cell suspensions were stained with toluidine blue (0.1% (w/v), pH 3.0), placed between slides and coverslides, and examined under a Nikon Optiphot-2 microscope. Percentage of mast cells exhibiting degranulation was quantitated at a magnification of ×400. Mast cell activation was defined as the presence of extruded granules close to the surface of the cell in question or staining of about half or less of the cell with toluidine blue.

Transmission electron microscopy

Mast cells were fixed in Karnovsky's fixative (2% formaldehyde, freshly prepared from paraformaldehyde, 2.5% glutaraldehyde, 0.025% $CaCl_2$, 0.1 M cacodylate buffer, pH 7.4). After 1 h in the fixative at 20 °C, mast cells were rinsed in 0.2 M cacodylate buffer and postfixed in 1% OsO_4 in 0.1 M cacodylate buffer for at least 2 h at room temperature and dehydrated in ethanol. Then, cell suspensions were embedded in Spurr (Pelco, USA). Semithin transverse sections (1 μm) were cut with an automatic ultramicrotome (Leica Ultracut R, Austria) and stained with filtered 1% toluidine blue. Ultrathin sections (60 nm) were cut with diamond knives, stained with uranyl acetate and lead citrate, mounted on grids (Pelco, USA), and examined in a transmission electron microscope (Zeiss EM 902, Germany).

Statistical analysis

Results from biochemical and morphometric analyses are presented as means ± SEM. Differences between groups were determined using analysis of variance followed by Tukey–Kramer

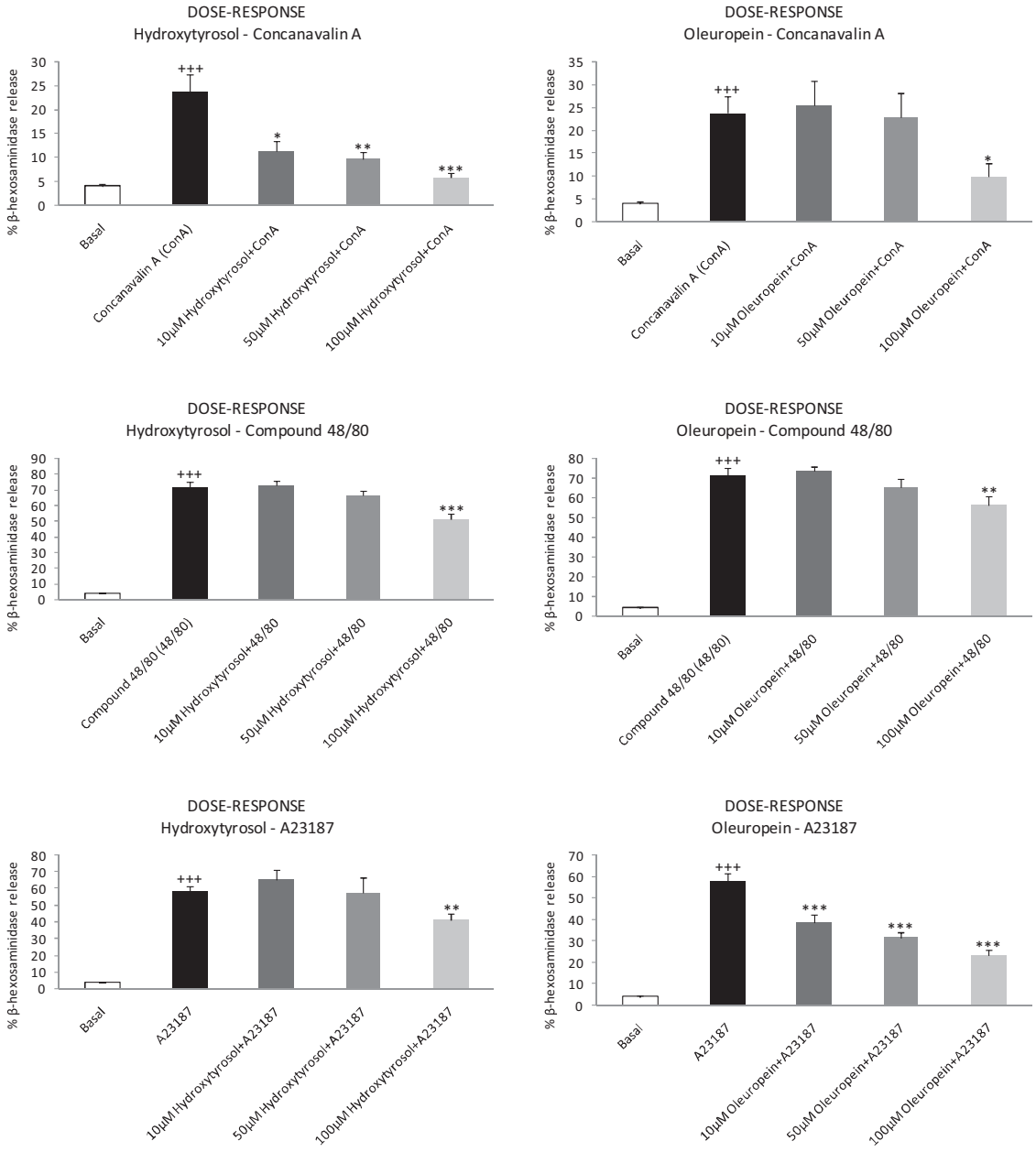

Fig. 2. Effect of varying concentrations of hydroxytyrosol and oleuropein on the concanavalin A-, compound 48/80- and calcium ionophore A23187-induced β-hexosaminidase release from rat peritoneal mast cells. Results are expressed as percentage release of β-hexosaminidase. Values are presented as means ± SEM. $^{+++}p < 0.001$ *versus* basal; $^{*}p < 0.05$ *versus* secretagogue; $^{**}p < 0.01$ *versus* secretagogue; $^{***}p < 0.001$ *versus* secretagogue.

multiple comparisons test. $p < 0.05$ was considered statistically significant.

Results

The effect of varying concentrations of hydroxytyrosol and oleuropein on the secretagogue-induced β-hexosaminidase release from mast cells is shown in Fig. 2. Incubation of mast cells with 200 μg/ml concanavalin A or 10 μg/ml compound 48/80 or 50 μg/ml calcium ionophore A23187 solutions significantly increased β-hexosaminidase release when compared with the corresponding value from the basal group. These effects were inhibited by preincubation of mast cells with hydroxytyrosol or oleuropein. Values of EC50 for each compound are shown in Table 1. Fig. 3 shows a dose–response comparative study with sodium cromoglycate, a classical mast cell stabilizer. The inhibitory effect of hydroxytyrosol was higher than that obtained with sodium cromoglycate at the same concentration (100 μM) when mast cells were challenged with concanavalin A. The inhibitory effect of oleuropein was higher than that obtained with sodium cromoglycate at the same concentrations (10, 50 and 100 μM) when mast cells were challenged with the calcium ionophore A23187. Fig. 4 shows the results from the kinetic study related to the effect of hydroxytyrosol and oleuropein on mast cell β-hexosaminidase release. The

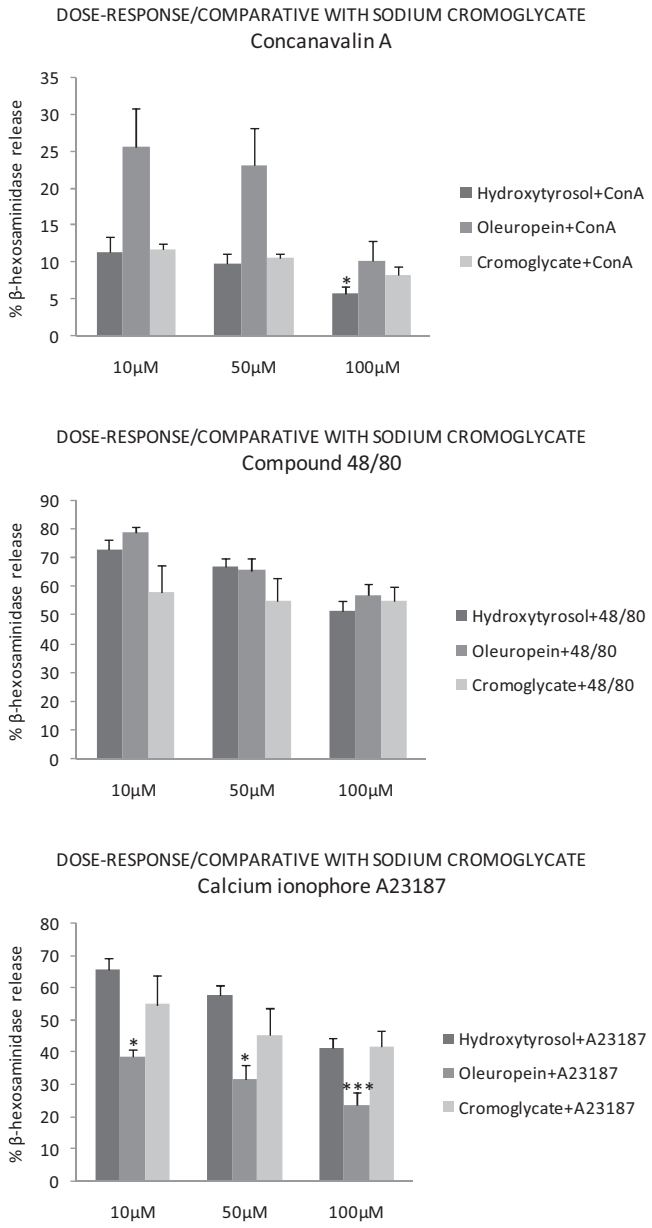

Fig. 3. Effect of hydroxytyrosol and oleuropein on β-hexosaminidase release from rat peritoneal mast cells, compared with a reference compound, the mast cell stabilizer sodium cromoglycate. Results are expressed as percentage release of β-hexosaminidase. Values are presented as means ± S.E.M. $^{*}p < 0.05$ *versus* sodium cromoglycate; $^{***}p < 0.001$ *versus* sodium cromoglycate.

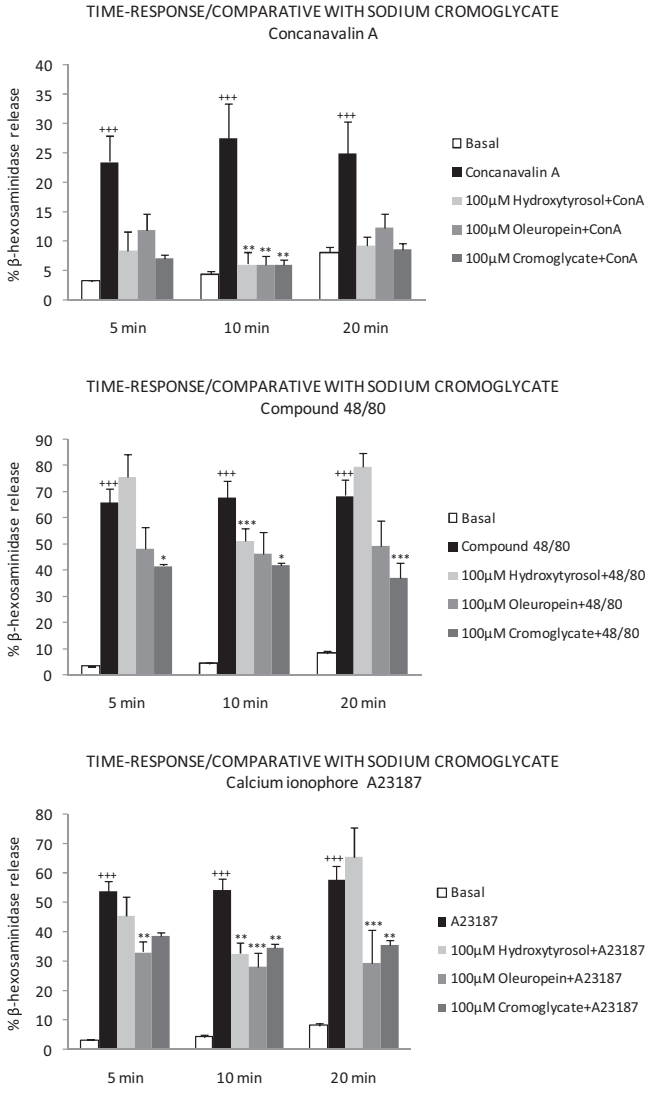

Fig. 4. Kinetic study related to the effect of hydroxytyrosol or oleuropein preincubation on mast cell β-hexosaminidase release induced by concanavalin A, compound 48/80 or calcium ionophore A23187. Results are expressed as percentage release of β-hexosaminidase. Values are presented as means ± S.E.M. $^{+++}p < 0.001$ *versus* basal; $^{*}p < 0.05$ *versus* secretagogue; $^{**}p < 0.01$ *versus* secretagogue; $^{***}p < 0.001$ *versus* secretagogue.

Table 1
EC50 (μM) ± SEM values for inhibitory activity of hydroxytyrosol, oleuropein and sodium cromoglycate on concanavalin A-, compound 48/80- and calcium ionophore A23187-induced mast cell activation.

	Hydroxytyrosol	Oleuropein	Sodium cromoglycate
Concanavalin A	7.58 ± 4.9	67 ± 5.1	95 ± 8.9
Compound 48/80	61 ± 5.0	59 ± 5.0	97 ± 9.1
Calcium ionophore A23187	64 ± 5.0	22 ± 1.8	54 ± 5.1

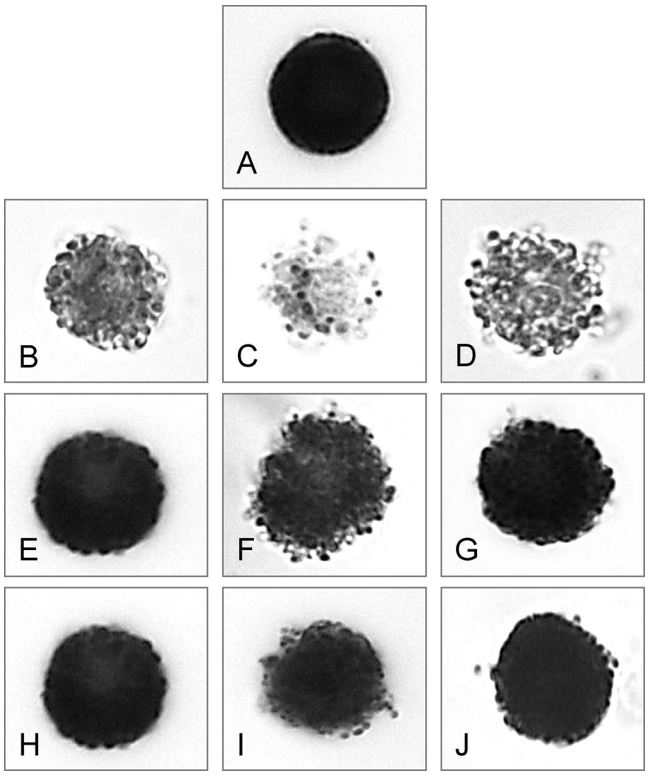

Fig. 5. Light-microscopic photographs of peritoneal mast cells (toluidine blue stain). (A) Basal. (B–D) Concanavalin A (ConA), compound 48/80 (48/80) and calcium ionophore A23187 (A23187), respectively. (E–G) Hydroxytyrosol + ConA, hydroxytyrosol + 48/80 and hydroxytyrosol + A23187, respectively. (H–J) Oleuropein + ConA, oleuropein + 48/80 and oleuropein + A23187, respectively. ×600.

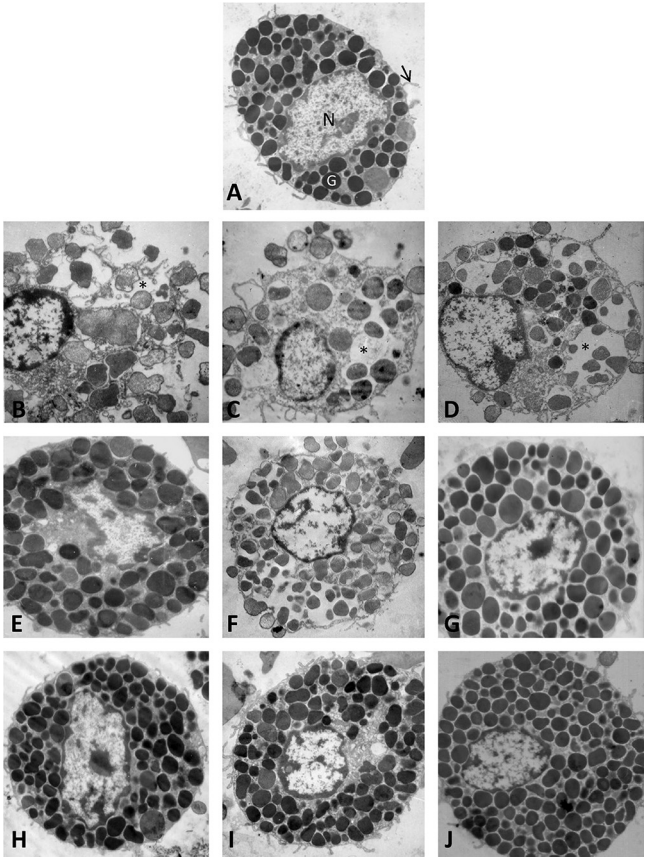

Fig. 6. Transmission electron micrographs showing peritoneal mast cells. (A) Basal. A non-segmented nucleus (N), narrow surface folds (arrow), and numerous secretory granules (G) distributed throughout the cell cytoplasm are observed. (B–D) Concanavalin A (ConA), compound 48/80 (48/80) and calcium ionophore A23187 (A23187), respectively. Granules surrounded by perigranular dilated and electron-lucent spaces are evident. Some granules are seen out of mast cell cytoplasm and others appear fused, forming cavities and intracytoplasmatic channels (asterisk). (E–G) Hydroxytyrosol + ConA, hydroxytyrosol + 48/80 and hydroxytyrosol + A23187, respectively. (H–J) Oleuropein + ConA, oleuropein + 48/80 and oleuropein + A23187, respectively. Polyphenol-treated cells show minimal degranulation. ×5000.

inhibitory action of the phenols was not accompanied by changes in cell viability, except for concentrations higher than 100 μM and times higher than 20 min (data not shown).

Peritoneal mast cells were easily identified by the presence of a cytoplasm dominated by distinctive secretory granules which stain metachromatically (Fig. 5A–J). Mast cells from the basal group are dominated by tightly packed secretory granules. Disruption of the cell surface and degranulation may be seen in mast cells stimulated with the secretagogues. The morphology of the cells treated with the phenols shows a lower degree of degranulation than that of secretagogue samples. Fig. 6A–J shows mast cells observed under transmission electron microscope. Basal mast cells are characterized by narrow surface folds and numerous secretory granules regularly distributed throughout the cell cytoplasm. Almost all granules exhibit either round or oval profiles, and appear homogenously dense. Stimulated mast cells show obvious morphological changes, and evidence enhanced granule release by exocytosis compared to basal cells. These cells show a cytoplasm with irregular secretory granules exhibiting different degrees of electron densities. Some granules are surrounded by perigranular dilated and electron-lucent spaces. Some of these spaces appear fused, forming numerous cavities and intracytoplasmic channels. The morphology of the phenol-treated cells shows a lower degree of degranulation than that of secretagogue samples.

Discussion

Interestingly, the main findings of the present study showed that hydroxytyrosol and oleuropein, at non-cytotoxic concentrations, inhibit β-hexosaminidase release from peritoneal mast cells stimulated by different triggers, acting thus as mast cell stabilizers. Other *in vitro* studies have shown that several dietary polyphenols inhibit the activation of mast cells (Middleton et al., 2000; Singh et al., 2011). A well-studied immunological effect of polyphenols such as quercetin is their inhibitory action on degranulation, particularly histamine release from mast cells (Middleton et al., 2000; Singh et al., 2011; Weng et al., 2012). Quercetin is able to regulate calcium entry into mast cells; also the role of quercetin and polyphenols obtained from apples and grapes in inhibiting mast cell degranulation has been well documented using *in vitro* systems such as the RBL-2H3 assay (Middleton et al., 2000; Singh et al., 2011). Epigallocatechin gallate was found to be the active ingredient in green tea extracts that provided protection against cutaneous inflammation. This compound may also inhibit histamine release from mast cells stimulated both with a calcium ionophore and an IgE–antigen complex (Maeda-Yamamoto et al., 2012). Kaempferol, myricetin, phloretin and luteolin also proved to be effective inhibitors of histamine release (Middleton et al., 2000; Singh et al., 2011).

Furthermore, we have also shown that the inhibitory effects by hydroxytyrosol were stronger than those of oleuropein or the reference compound sodium cromoglycate when mast cells were activated by concanavalin A. On the contrary, oleuropein inhibited mast cell degranulation more efficiently than hydroxytyrosol or sodium cromoglycate when cells were challenged with the calcium ionophore A23187. These findings strongly suggest that the nature of the stimulus for β-hexosaminidase release and the

chemical structure of both polyphenols might determine the inhibitory activity. Each of the secretagogues use a different pathway of mast cell activation and these pathways may be differentially sensitive to the action of hydroxytyrosol or oleuropein.

The results of the present study suggest that hydroxytyrosol and oleuropein act at different molecular sites. It seems that the mechanism of the inhibitory action of hydroxytyrosol may be related, at least partially, to inhibition of the cross-linking of high-affinity receptors for IgE (FcεRI) or to a probable interaction of the polyphenol with concanavalin A. It has been reported that polyphenols form soluble and insoluble complexes with proteins and may render them hypoallergenic by either changing the structure of the allergenic protein or rendering it less bioavailable (Singh et al., 2011). Moreover, two mechanisms are postulated for the mast cell inhibitory action of quercetin and other polyphenols: one where polyphenols impact allergen–IgE complex formation and another where they impact on the binding of this complex to their receptor (FcεRI) on mast cells (Singh et al., 2011). Our results also suggest that oleuropein seems to block signaling pathways downstream of cytosolic calcium increase. However, further research is needed in order to explain the exact molecular mechanisms of these actions.

Conclusion

In conclusion, our current findings reveal for the first time that hydroxytyrosol and oleuropein, the major phenolic compounds in olive, inhibit mast cell degranulation induced by both immune and non-immune pathways. Our findings also suggest that olive phenols, particularly hydroxytyrosol and oleuropein, may provide insights into the development of useful tools to prevent and treat mast cell-mediated disorders. These results may be of interest in immunopharmacology or could even lead to establishing the ideal concentrations of each component in virgin olive oils to label them as healthy oils. However, further experimentation is needed in order to generate hypoallergenic products *via* polyphenol treatment.

Conflict of interest

There are no conflict of interest to declare.

Acknowledgments

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