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ARTICLE

# Olive Oil Phenolic Compounds Inhibit Homocysteine-Induced Endothelial Cell Adhesion Regardless of Their Different Antioxidant Activity

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In this study, we examine the effect of extra virgin olive oil phenolic compounds on homocysteine-induced endothelial dysfunction and whether the protective effects are related to their different scavenging activities. Structurally related compounds have been assayed for their ability to reduce homocysteine-induced monocyte adhesion as well as the cell surface expression of intercellular adhesion molecule-1 (ICAM-1) in EA.hy.926 cells. As well-known, among the selected phenolic compounds, hydroxytyrosol, homovanillyl alcohol, and the hydroxycinnamic acid derivatives caffeic and ferulic acid display high scavenging activities, while tyrosol and *p*-coumaric acid are poorly active. All of the tested compounds, approaching potential in vivo concentrations, significantly reduce homocysteine-induced cell adhesion and ICAM-1 expression. Interestingly, we report the first evidence that monophenols tyrosol and *p*-coumaric acid are selectively protective only in homocysteine-activated cells, while they are ineffective in reducing ICAM-1 expression induced by TNFα. Finally, we report the synergistic effect of *o*-diphenolic and monophenolic compounds.

**KEYWORDS: Adhesion molecules; homocysteine; endothelial cell adhesion; cardiovascular diseases; extra virgin olive oil; phenolic compounds**

## INTRODUCTION

Numerous epidemiological data indicate that extra virgin olive oil modulates a number of cardiovascular risk factors and contributes to the low incidence of cardiovascular diseases (CVDs) associated with the Mediterranean diet (*1*−*3*). Biomolecular and clinical studies have focused on the impact of the different extra virgin olive oil components on the cardiovascular system (*4*). In particular, recent studies have emphasized the importance of phenolic compounds as modulators of key mechanisms implicated in the development of atherosclerosis, including plaque formation (*5*). These compounds play a key role in improving cardiovascular health, due to their antioxidant activity, presumably counteracting the oxidative stress-induced endothelial dysfunction (*6*). Moreover, they are endowed with significant antithrombotic, antiatherogenic, and anti-inflammatory activities (*7*). Mechanisms underlying these biological effects include inhibition of platelet aggregation (*8*) and lipoxygenase and cycloxygenase activities (*9*). Among the different phenolic compounds, particular attention has been devoted to hydroxytyrosol (3,4-dihydroxyphenylethanol; HT), naturally occurring in high concentrations in extra virgin olive oil, either as a simple phenol or in esterified forms (*10*). This compound, recalling the structure of cathecol, is endowed with a potent free radical scavenging activity (*7*). Accordingly, tyrosol (*p*-hydroxyphenylethanol, Tyr), the HT monophenolic analogue that lacks the *o*-diphenolic structure, is unable to counteract the oxidative stress-induced cytotoxic effects in Caco-2 cells (*11*).

Homocysteine (Hcy) is a nonprotein sulfur amino acid and an important intermediary of methionine metabolism. Alterations of Hcy metabolism, due to genetic anomalies as well as to nutritional factors, result in an intracellular accumulation of this amino acid, which in turn is released into blood, causing a condition of hyperhomocysteinemia (HHcy) (*12*, *13*). During recent years, the association between elevated plasma levels of Hcy and increased risk of CVDs has been proven (*14*, *15*). It is still uncertain, however, whether HHcy is a causative factor or a marker of vascular disease.

As far as the mechanisms responsible for the cardiovascular system impairment observed in HHcy, one of the most important aspects concerns the existing relationship between elevated plasma level of Hcy and increased adhesiveness of the endothelium. In particular, data from our and others laboratories indicate that the treatment with Hcy induces monocyte adhesion to different types of endothelial cells in culture (*16*−*18*).

Several reports have investigated the potential Hcy metabolic alterations and mechanisms underlying the endothelial dysfunction, which include Hcy-induced expression of adhesion molecules (*16*, *19*). Moreover, Carluccio et al. have recently demonstrated that Hcy-induced vascular cell adhesion

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molecule 1 (VCAM-1) expression is mediated by nuclear factor-kB (NF-kB) and NAD(P)H oxidase activation in human umbilical vein endothelial cells (HUVEC) (*20*).

On the basis of these results, it is conceivable that dietary components able to reduce the expression of adhesion proteins, and therefore to protect against the Hcy-induced endothelial cell adhesion, could reduce the severity of this cardiovascular risk factor in humans.

In recent years, several findings have revealed the capacity of antioxidant molecules, including HT and MHT, in preventing the expression of adhesion proteins induced by pro-inflammatory cytochine tumor necrosis factor α (TNFα) (*21*, *22*), through interfering with the activation of the redox-sensitive transcription factor NF-kB (*20*, *23*, *24*). Interestingly, Tyr has been reported to be completely ineffective in protection against the lipopolysaccharide-stimulated VCAM-1 expression in HUVEC (*21*).

To further examine the protective effect of extra virgin olive oil phenolic compounds on Hcy-induced endothelial dysfunction and to elucidate if their positive effects are strictly related to the antioxidant properties, structurally related compounds, endowed with different scavenging properties, have been selected. Their ability to reduce the Hcy-induced monocyte adhesion as well as cell surface expression of intercellular adhesion molecule 1 (ICAM-1) has been tested, using EA.hy 926 cells (*25*) as a model system.

## MATERIALS AND METHODS

**Materials.** L-Hcy thiolactone hydrochloride, recombinant human TNFα, L-glutammine, penicillin−streptomycin solution, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester (BCECF-AM), homovanillyl alcohol [2-(4-hydroxy-3-metoxyphenyl)-ethanol, MHT], Tyr, caffeic acid (3,4-dihydroxycinnamic acid), ferulic acid [3-(4-hydroxy-3-metoxyphenyl)-2-propenonic acid], and *p*-coumaric acid (4-hydroxycinnamic acid) were purchased from Sigma. Cell culture media [Dulbecco's modified Eagle's medium (DMEM), RPMI-1640] and fetal bovine serum (FBS) were from Gibco BRL. Black 96 well assay plates were from Microtech. The mouse antihuman ICAM-1 monoclonal antibody was purchased from R&D Systems. The anti-mouse IgG tetramethyl-rhodamine-isothiocyanate (TRITC) conjugate was from BioFX Laboratories. HT has been synthesized in our laboratory, as previously described (*26*). EA.hy 926 cells were a gift from Prof. Franco Dammacco, Department of Biomedical Sciences and Human Oncology, University of Bari. Human monocytic cells U937 used for the adhesion assay were from the American Type Culture Collection.

**Fluorescent Labeling of U937 Cells.** For quantitative cell adhesion assays, U937 cells ($4 \times 10^6$ cells/mL) were fluorescently labeled by incubation with 5 μM BCECF-AM in RPMI-1640 medium for 30 min at 37 °C. After incubation, the cells were washed twice with 1% FBS/phosphate-buffered saline (PBS) to remove nonincorporated dye and resuspended in DMEM medium at a density of $25 \times 10^4$ cells/mL.

**Endothelial Cells Treatment and Adhesion Assay.** L-Hcy was freshly prepared from its thiolactone as previously reported (*18*). EA.hy 926 cells were cultured to confluence in a 96 well plate ($17 \times 10^3$/well) and treated for 2 h with L-Hcy (100 μM) and TNFα (10 ng/mL) alone or in combination with an increasing concentration of the selected phenolic compounds. After incubation, EA.hy 926 cells were coincubated with BCEF-labeled U937 cells, and a cell adhesion assay was carried out according to a previous report (*18*). The fluorescence intensity of each well was measured using a fluorescence multiwell plate reader (VICTOR$^3$, Perkin-Elmer) set at excitation and emission wavelengths of 485 and 535 nm, respectively. Labeled U937 cells were used for a fluorescence standard curve.

**Detection of Cell Surface ICAM-1 Expression.** The expression of ICAM-1 on the cell surface of endothelial cells was conducted by cell enzyme-linked immunosorbent assay (ELISA) as described by Chen et al. (*27*), with minor modifications. Briefly, in 96 well microplates, the EA.hy 926 at confluence were incubated for 2 and 24 h with Hcy (100 μM) or TNFα (10 ng/mL) in the absence and in the presence of the selected phenolic compounds. The monolayers were then washed and incubated with mouse antihuman ICAM-1 monoclonal antibody at a final concentration of 0.5 μg/mL in PBS containing 1% BSA. After incubation for 1 h at 37 °C, the plates were washed twice with PBS containing 0.1% Tween-20 and then treated with 0.1 mL/well of TRITC-conjugated rabbit antimouse IgG (1:500 dilution in PBS containing 1% BSA). After 1 h of incubation at room temperature in the dark, the plates were washed twice with PBS containing 0.1% Tween-20, and the cells were lysed with Tris buffer 500 mM, pH 7.6, containing 1% sodium dodecyl sulfate. The fluorescence intensity was measured using a fluorescence multiwell plate reader (VICTOR$^3$, Perkin-Elmer) set at excitation and emission wavelengths of 550 and 570 nm, respectively. Because the cells are not permeabilized, this procedure allows the detection of cell surface-expressed proteins.

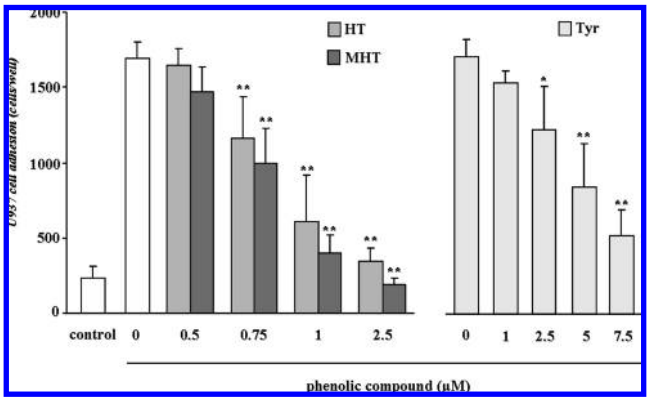


**Figure 1.** Effect of HT, MHT, and Tyr on Hcy-induced monocyte−endothelial cell adhesion. EA.hy 926 cells were incubated at 37 °C for 2 h with 100 μM Hcy in the absence or in the presence of increasing concentrations of the selected phenolic compounds, and U937 cell adhesion was measured. Data are the means ± SDs of three separate experiments, each performed in quadruplicate. *$P < 0.05$ and **$P < 0.01$ as compared to untreated cells.

**Data Analysis.** All tests were run in quadruplicate for each experiment, and each experiment was repeated three times. Data were expressed as means ± standard deviations (SDs). Statistical analysis was performed using Student's *t* test. A *P* value $< 0.05$ was considered statistically significant. $IC_{50}$ values were analyzed using Graph Pad Prism 5 software.

## RESULTS

**Effect of HT and Its Analogues on Hcy-Induced Endothelial Cell Adhesion and ICAM-1 Expression.** To investigate the protective effect of olive oil phenolic compounds on Hcy-induced endothelial dysfunction and the underlying mechanism(s), several phenolic compounds, endowed with different antioxidant activities, have been selected. Among them, HT and its in vivo metabolite MHT show comparable antioxidant properties (*28*−*30*), while Tyr, which lacks the *o*-diphenolic structure, is characterized by a weak scavenging activity (*31*).

In agreement with our previous findings (*18*), incubation of EA.hy 926 cells for 2 h with 100 μM Hcy results in a significant increase of monocyte adhesion to the endothelial cells (**Figure 1**). The Hcy-induced cell adhesion is dose dependently prevented by coincubating EA.hy 926 cells in the presence of either HT or MHT, a significant effect being observable starting from a concentration as low as 0.75 μM ($EC_{50}$, 1.06 and 0.76 μM, respectively). A remarkable protection against Hcy-induced cell adhesion was also observed in Tyr-treated samples, although this compound appears less effective as compared to HT and MHT ($EC_{50}$, 4.05 μM).

It has been previously reported that TNFα specifically upregulates ICAM-1 expression in EA.hy 926 cells (*32*) and that HT and several others extra virgin olive oil phenolic

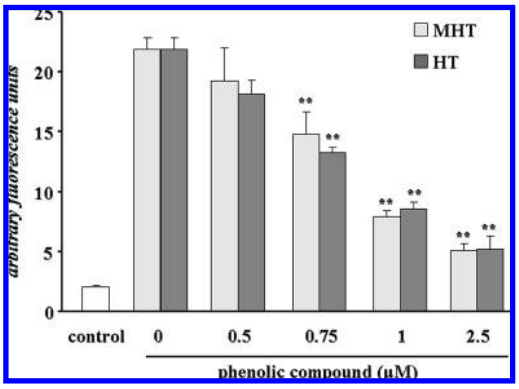


**Figure 2.** Effect of HT and MHT on Hcy-induced ICAM-1 expression. EA.hy 926 cells were incubated at 37 °C for 2 h with 100 $\mu$M Hcy in the absence or in the presence of increasing concentrations of the selected phenolic compounds, and cell surface ICAM-1 expression was measured. Data are the means ± SDs of three separate experiments, each performed in quadruplicate. $^{**}P < 0.01$ as compared to untreated cells.

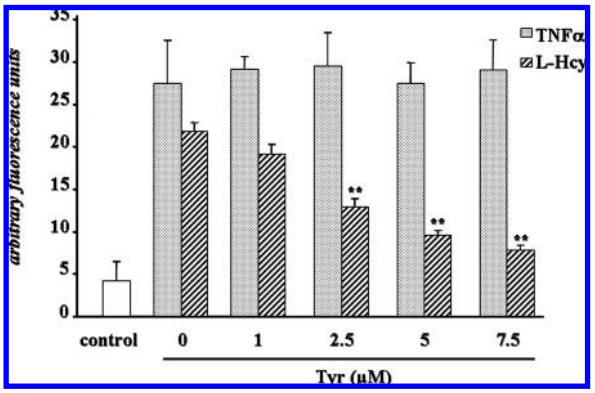


**Figure 3.** Effect of Tyr on Hcy- or TNFα-induced ICAM-1 expression. EA.hy 926 cells were incubated at 37 °C for 2 h with 100 $\mu$M Hcy or TNFα (10 ng/mL) in the absence or in the presence of increasing concentrations of Tyr, and cell surface ICAM-1 expression was measured. Data are the means ± SDs of three separate experiments, each performed in quadruplicate. $^{**}P < 0.01$ as compared to untreated cells.

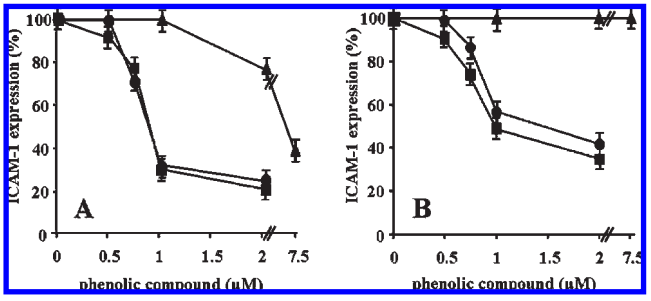


**Figure 4.** Effect of hydroxycinnamic acid derivatives on Hcy- or TNFα-induced ICAM-1 expression. EA.hy 926 cells were incubated at 37 °C for 2 h with 100 $\mu$M Hcy (**A**) or TNFα (10 ng/mL) (**B**) in the absence or in the presence of increasing concentrations of *p*-coumaric acid (▲), caffeic acid (■), and ferulic acid (●), and cell surface ICAM-1 expression was measured. All data have been plotted as a percentage of maximum control response (% of stimulated response without phenolic compounds). Data are the means of three separate experiments, each performed in quadruplicate.

compounds inhibit stimulated VCAM-1 expression (*21*). To verify the protection exerted by extra virgin olive oil phenolic compounds on specific events that ultimately result in increased endothelial cell adhesiveness, the effects of HT, MHT, and Tyr on Hcy-induced ICAM-1 expression at membrane level have been tested. As shown in **Figure 2**, Hcy treatment results in a significant increase in cell surface ICAM-1 expression as compared to control cells. HT and MHT appear equally protective against Hcy-induced expression of this adhesion protein with $EC_{50}$ values of 0.81 and 0.91 $\mu$M, respectively. As shown in **Figure 3**, also, Tyr significantly decreases Hcy-induced ICAM-1 expression, although to a lesser extent ($EC_{50}$, 3.23 $\mu$M) as compared to HT and MHT, while it is completely ineffective in reducing ICAM-1 expression in TNFα-stimulated cells (**Figure 3**).

**Hydroxycinnamic Acid Derivatives and Hcy-Induced ICAM-1 Expression.** To confirm the observed protective effect of HT, MHT, and Tyr on Hcy-induced ICAM expression, several hydroxycinnamic acid derivatives have been selected and assayed. These include the *o*-diphenol caffeic acid and its methoxy analogue ferulic acid, sharing similar scavenging and antioxidant activities (*33*, *34*), and *p*-coumaric acid, the monophenolic analogue of caffeic acid (*31*). For comparison, their effect against the TNFα-induced ICAM-1 expression was also investigated.

As shown in **Figure 4A**, incubation of EA.hy 926 cells in the presence of either caffeic or ferulic acid significantly prevents Hcy-induced cell surface ICAM-1 expression. It is interesting to note that a comparable protection was also observed on TNFα-induced expression of this adhesion protein (**Figure 4B**). In contrast, when *p*-coumaric acid was assayed, a significant effect was observed only against Hcy-induced ICAM-1 expression (**Figure 4A**), while this monophenolic compound appears completely ineffective in preventing the expression of the adhesion protein induced by TNFα (**Figure 4B**). The experimental evidence that only potent antioxidant phenolic compounds are protective against TNFα-induced ICAM-1 expression confirms the literature view that the main mechanism by which the components of olive oil influence endothelial activation involves inhibition and/or scavenging of reactive oxygen species.

**Synergy of Olive Oil Phenolic Compounds in the Modulation of the Hcy-Induced ICAM-1 Expression.** To investigate if the combination of *o*-diphenolic and monophenolic compounds produces a synergistic effect on Hcy-induced ICAM-1 expression, EA.hy 926 cells were treated, for 2 and 24 h, with a mixture of 0.5 $\mu$M HT and 1 $\mu$M Tyr (**Figure 5**). The obtained results indicated that the association of the two compounds, which separately were only poorly protective, reduces ICAM-1 expression in more than an additive fashion (50 and 88% after 2 and 24 h, respectively). Similar results have been obtained when the cells were treated with a mixture of 0.5 $\mu$M caffeic acid and 1 $\mu$M *p*-coumaric acid (**Figure 5**). Interestingly, no synergistic effect has been observed when HT and Tyr are tested for their protective effects on ICAM-1 expression induced by TNFα (data not shown).

## DISCUSSION

CVDs are considered as a group of multifactorial pathological conditions associated with atherosclerosis, hypertension, and thrombosis, which represent the most frequent cause of death in western countries. These pathologies are closely related to both genetic factors as well as environmental influences, including diet. It is well-known, indeed, that almost all cardiovascular risk factors are influenced by dietary components (*1*−*3*). In this respect, a direct correlation between the Mediterranean diet, in which olive oil represents the main lipidic source, and the low incidence of CVDs has been proven (*1*−*3*). There is a general agreement that the health-promoting effect of olive oil "is not a question of fat alone" (*35*) and that oleic acid is not the only one responsible for its cardioprotective effects. Most of olive oil

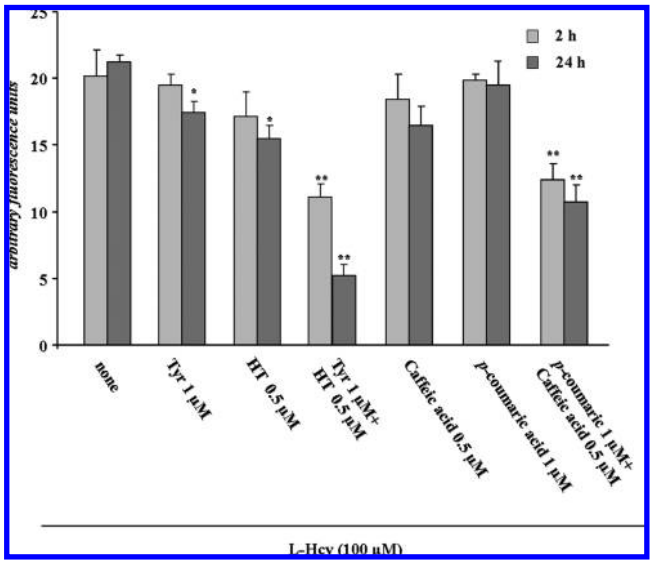


**Figure 5.** Synergistic effect of antioxidant and weak antioxidant phenolic compounds on Hcy-induced ICAM-1 expression. EA.hy 926 cells were incubated at 37 °C for 2 and 24 h with 100 $\mu$M Hcy in the presence of the indicated phenolic compounds either alone or in combination, and ICAM-1 expression was measured. Data are the means ± SDs of three separate experiments, each performed in quadruplicate. *$P < 0.05$ and **$P < 0.01$ as compared to untreated cells.

microcomponents, indeed, are endowed with significant biological effects that can be related to its cardioprotection (*4*, *5*). It is important to stress, in this respect, that the biological effects exerted by phenolic compounds have been generally related to their strong antioxidant activities (*6*).

In this paper, we demonstrate that olive oil phenolic compounds exert their protective effects on Hcy-induced cell adhesion through mechanisms not strictly related to their scavenging properties. In this context, it is interesting to note that also for vitamin E several alternative roles have been attributed, besides its chain-breaking activity, including modulation of gene expression and cellular signaling (*36*). In particular, our data represent the first experimental evidence that the monophenolic compounds Tyr and *p*-coumaric acid specifically down regulate ICAM-1 expression induced by Hcy in EA.hy 926 cells. On the contrary, they appear completely ineffective against the expression of this adhesion molecule induced by TNFα. The reported results suggest that these weak antioxidant compounds could affect the specific Hcy-activated signaling, which results in ICAM-1 expression, through redox-independent mechanisms that remain to be elucidated. It should be underlined, in this respect, that an increasing number of studies suggest the involvement of adenosine as a pathogenic factor in HHcy (*37*, *38*). In particular, we have recently demonstrated that the lowering of intracellular adenosine concentration represents the mechanism responsible for Hcy-induced increased adhesiveness of EA.hy 926 cells (*18*). It is well-known that this nucleoside plays a key role in the control of several inflammation-associated processes and that the vascular endothelium constitutes an important target for the anti-inflammatory action of this molecule. Interestingly, a recent paper reports that adenosine, via its A2B receptor, protects against inflammation and excessive vascular adhesion (*39*).

Finally, it is worth noting that the HT active concentration able to counteract the Hcy-induced cell adhesion ($EC_{50}$, 1.06 $\mu$M) is significantly lower as compared to that required to exert significant effects in inducing HL60 differentiation and apoptosis (50−200 $\mu$M) (*40*, *41*). Furthermore, similar concentration ranges have been utilized in studies on the effect of HT in modulation of cellular signaling (*42*). It is therefore conceivable that prevention of monocyte adhesion to endothelial cells represents one of the main mechanisms responsible for phenolic compounds health benefits in vivo. Also of importance are the findings that coincubation of *o*-diphenolic and monophenolic compounds results in a more than additive protection. The reported synergistic effects confirm the view that the benefits of bioactive compound-rich dietary patterns are primarily related to the specific interactions within the multicomponent mixture of nutrients (*43*) and suggest new and not yet explored interactions among the crossroads of the signaling patways of NF-kB.

In conclusion, the findings that olive oil phenolic compounds approaching potential in vivo concentrations are able to modulate the Hcy-induced expression of adhesion molecules, and likely delay plaque formation, confirm the beneficial role of extra virgin olive oil and the Mediterranean diet on human health, suggesting a possible therapeutic role of these compounds in Hcy-induced CVDs.

## ABBREVIATIONS USED

AdoHcy, *S*-adenosylhomocysteine; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein acetoxymethyl ester; CVDs, cardiovascular diseases; HT, hydroxytyrosol; Hcy, homocysteine; HHcy, hyperhomocysteinemia; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; MHT, homovanillyl alcohol; NF-kB, nuclear factor-kB; Tyr, tyrosol; TNFα, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1.

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