

Effects of the Olive Tree Leaf Constituents on Myocardial Oxidative Damage and Atherosclerosis

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Key words

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- LDL oxidation
- anti-atherosclerotic effects
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- *Olea europaea*
- Oleaceae

Abstract

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The olive (*Olea europaea*) leaf is considered an important traditional herbal medicine utilized against infectious diseases, and for the treatment of diabetes and hypertension. Moreover, olive leaf constituents have been related to cardioprotection, probably due to their association with cellular redox modulating effects. The pathogenesis of certain common diseases, including those of the cardiovascular system, involves oxidative stress

and tissue inflammation. Olive polyphenolic compounds, such as oleuropein, hydroxytyrosol, or tyrosol, possess antioxidant, anti-inflammatory, antiatherosclerotic, anti-ischemic, and hypolipidemic effects on the myocardium as demonstrated by various *in vitro* and *in vivo* studies. In this review article, we summarize the current knowledge on the role of the olive leaf constituents in the prevention of cardiac dysfunction and highlight future perspectives in their use as cardioprotective agents in therapeutics.

Introduction

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The beneficial effects of olive leaf extract are known since antiquity, with numerous records confirming its therapeutic use [1]. During the last two centuries, there has been increasing interest in investigating the pharmacological properties of the constituents of olive leaves, and it has been shown that they can exhibit a variety of biological actions, such as antioxidant [2,3], antimicrobial [3], antihypertensive [4–6], vasodilator [7], and hypoglycemic [8] properties. Clinical data on olive leaf extract are apparent since 1950 [1], with phenolic extract exhibiting the most promising beneficial effect. The wide use of oil, olives, and leaf extracts by the Mediterranean population for the alleviation of inflammatory diseases, gout, and blood pressure led to the incorporation of the 80% ethanolic olive leaf dry extract (OLE) in the European Pharmacopoeia (Ph. Eur.) [9]. According to the Ph. Eur., the most abundant substances in standardized dry leaf extracts are oleuropein, hydroxytyrosol, caffeic acid, tyrosol, apigenin, and verbascoside, with oleuropein being the major component of olive leaf extract [10].

Enzymatic hydrolysis of oleuropein leads to the formation of oleuropein aglycone, which is further hydrolyzed to hydroxytyrosol (also known as 3,4-dihydroxyphenylethanol or 3,4-DHPEA),

and other non-glycosidic secoiridoids (i.e., ellagic acid). Chemical hydrolysis of ligstroside, which has also been isolated from *O. europaea* L. leaf extract leads to the formation of tyrosol (also known as 4-hydroxyphenylethanol) [11]. Another derivative of oleuropein metabolic degradation is oleacein (also referred to as 3,4-DHPEA-E-DA or oleuropein aglycone decarboxymethylaldehyde form or noroleuropein aglycone).

The polyphenols that are present in olive leaves have been shown to be potent antioxidant and radical scavengers, exhibiting many therapeutic properties such as antitumor and anti-inflammatory ones [12]. Furthermore, the effects of olive polyphenols on scavenging lipid peroxyl radicals within membranes were associated with their beneficial effects for human skin protection [13]. In general, olive constituents exhibit a wide range of beneficial properties via antioxidant modulation in the management of diseases in which neuroprotective, gastroprotective, antidiabetic, or anti-ageing activities may play a beneficial role (reviewed in [14]).

Plant-derived compounds represent potential sources for molecules that may be used for the development of new drugs especially designed for the treatment and/or control of chronic inflammatory states, such as atherosclerosis and thrombogenicity [15], related to cardiovascular diseases

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(CVDs). The increasing number of patients around the world suffering from CVD indicates the need for innovative strategies for more effective CVD prevention and treatment [16–18]. In this review article, we summarize the current knowledge of the role of the major olive leaf constituents, oleuropein and its metabolites oleuropein aglycone, hydroxytyrosol, oleacein, elenolic acid as well as tyrosol, on the myocardium and highlight future perspectives of their use as cardioprotective agents. The above compounds have been selected as their presence in olives has been related to the unique bioactivity of olive-related foods, unlike constituents such as caffeic acid and apigenin, which are abundant in a wide range of plant extracts.

Myocardial Oxidative Stress

The damage of the cardiovascular endothelium is one of the first steps in the path to heart disease. The endothelium is a direct target of all of the major risk factors for heart disease such as diabetes, hyperlipidemia, hyperglycemia, inflammation, ageing, and hypertension. The common element in all of these pathophysiological conditions is the formation of reactive oxygen species (ROS) [19]. Many data have demonstrated that ROS production by endothelial mitochondria contributes to heart disease (reviewed in [20]). Moreover, oxidative stress within ventricular myocytes can also be detrimental to the heart. In fact, much of the contractile dysfunction and adverse myocardial remodeling, which has been observed in a wide range of cardiomyopathies, involves oxidative stress and endothelial nitric oxide synthase (eNOS) uncoupling leading to a decrease in NO production and an increase in ROS formation [21–23].

However, the oxidative damage occurs only when the oxidative stress levels exceed the antioxidant defense capacity. As already shown in patients with CVD, the activity of the endogenous antioxidant enzymes is reduced [24]. The manifestation and progression of CVDs are concomitant with a downregulation of enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase and proteins such as albumin and metallothionein, which are known to exhibit antioxidant protection in the myocardium [25]. Thus, the heart is one of the most susceptible organs to free radical-mediated oxidative stress [26]. Therefore, scavenging of ROS may have various protective and beneficial effects on the heart.

Atherosclerosis

Atherosclerosis is the primary cause of CVD and the underlying cause of more than 50% of all deaths in Westernized societies. CVD is considered to be a consequence of atherogenesis and is a result of a complex network of interactions between several risk factors and endogenous cell signaling between arterial wall cells. It is well established that dyslipidemia, diabetes, and platelet aggregation can lead to an impairment of the endothelial function and the formation of atheroma [27]. Atherosclerosis is a multifactorial disease in which inflammation and oxidative stress play an important role. Inflammatory and immunological responses contribute to the formation and rupture of atherosclerotic plaques and occlusion of the coronary artery [28,29]. The formation of ROS can be mediated through the activity of enzymes, such as NADH/NADPH and xanthine oxidase [30], reactions with products of the mitochondrial electron transport chain [31], and un-

coupling of eNOS [32–34]. The oxidation of low-density lipoproteins (LDL) by ROS is a prominent factor in the formation of atheroma in the beginning of the evolution of the disease [35]. Endothelial dysfunction is connected to the presence of oxidized LDL (ox-LDL), which leads to a signaling cascade that includes protein expression inducing atherogenesis and heart disease [36,37]. Furthermore, the aggregation and degranulation of platelets seem to trigger the formation of thrombus and coronary artery occlusion, with platelet activation factor (PAF) being a critical factor for platelet aggregation [38].

Oleuropein

Myocardial oxidative stress and low-density lipoprotein oxidation

Oleuropein has been shown to possess high antioxidant activity *in vitro* when it is compared to a water-soluble analog of tocopherol [39]. Furthermore, oleuropein scavenges superoxide anions and hydroxyl radicals, and inhibits the respiratory burst of neutrophils and hypochlorous acid-derived radicals [39–41]. *In vitro* studies have shown that oleuropein significantly reduced vitamin E consumption in an LDL-oxidation model, and it has been claimed that this component possesses high antioxidant properties [42]. In isolated rat hearts, significant protection against ischemia/reperfusion-induced oxidative stress was observed by oleuropein administration at a dose comparable to the average daily intake of biophenols from olive oil in the Mediterranean diet [43].

Furthermore, our group has shown that chronic treatment with oleuropein at a dose that was based on the average consumption of olive drupes and olive oil in the Mediterranean area significantly reduced circulatory oxidative stress biomarkers, such as malondialdehyde (MDA) and protein carbonyls, and enhanced SOD activity in normal and atheromatic rabbits that were subjected to ischemia/reperfusion [44]. We have also shown that oleuropein at a nutraceutical dose successfully treated doxorubicin (DXR)-induced cardiomyopathy in *in vivo* rat models by decreasing the oxidative stress biomarkers and reducing inducible nitric oxide synthase (iNOS) in cardiomyocytes [45,46], with a parallel restoration of the unbalanced metabolite profile in the myocardium [47].

The outcome of the Masella et al. study showed that oleuropein completely prevented the murine J774 A.1 macrophage-like cell-mediated oxidation of LDL along with a reduction of oxygen species production while restoring the expression of glutathione-associated proteins [48]. The addition of 10% (w/w) extra virgin olive oil and 7 mg/kg oleuropein to the standard diet in rabbits increased the ability of LDL to resist oxidation [49].

Anti-atherosclerotic and anti-ischemic effects

In vitro results verify that various compounds originating from olives exhibit an antithrombotic effect with different potencies by inhibiting the phosphodiesterase (PDE) signaling pathway, whereas oleuropein was found to present the highest activity among phenols and flavonoids [50]. In addition, oleuropein exhibited the greatest potency as an anti-atherosclerotic agent among the other phenolic constituents originating from the olive tree [5].

One study has been performed in isolated rat hearts to investigate the direct cardioprotective effect of oleuropein in acute events that follow coronary occlusion. Pretreatment with oleuro-

pein before ischemia resulted in a significant decrease in creatine kinase and reduced glutathione release in the perfusate, likely because of its antioxidant properties [43]. We have also shown that oleuropein reduced the infarct size in normal rabbits and, at higher doses, in hypercholesterolemic rabbits *in vivo*. Furthermore, it reduced total cholesterol and triglyceride levels, altered the metabolic profile, and provided cardioprotection even before the onset of ischemia [44].

Oleuropein Aglycone

Myocardial oxidative stress and low-density lipoprotein oxidation

Other *O. europaea* metabolites also present beneficial actions. Oleuropein aglycone, also referred to as 3,4-DHPEA-EA, shows antioxidant activity comparable with that of caffeic acid, oleuropein, and hydroxytyrosol. An *in vitro* study evaluating the effect of oleuropein aglycone on the modification of Cu-stimulated human LDL revealed that this particular compound reduced the kinetic and extent of lipid peroxidation [51].

Hydroxytyrosol

Myocardial oxidative stress and low-density lipoprotein oxidation

The polyphenol hydroxytyrosol has been found to play a protective role against cardiovascular diseases and is considered a potent antioxidant factor [52]. *In vitro* studies on human neutrophils showed that this biophenol can eliminate N-formyl-methionyl-leucylphenylalanine (fMLP), phorbol myristate acetate (PMA), and opsonized zymosan-induced damage, which is mediated by hydrogen peroxide under oxidative conditions [53]. However, hydroxytyrosol had no effect on lucigenin-amplified chemiluminescence, suggesting that it does not inhibit NADPH oxidase activation or scavenge superoxide anions [53]. Additionally, experiments evaluating the cytotoxicity, apoptosis, and cell cycle-related effects of hydroxytyrosol in various cell lines showed that it causes an upregulation of numerous antioxidant proteins and enzymes, including heme-oxygenase-1, glutaredoxin, and glutathione peroxidase [54]. The antioxidant activity of hydroxytyrosol on H₂O₂-induced intracellular ROS has been demonstrated in porcine pulmonary artery endothelial cells (VECs). Herein, hydroxytyrosol showed its efficacy to regulate the antioxidant defense system in VECs by inducing the phosphorylation of 5'-adenosine monophosphate-activated protein kinase (AMPK) with subsequent activation of Forkhead box O3 (FOXO3a) and catalase expression [55]. Furthermore, it has also been shown to improve cardiac disturbances induced by DXR by significantly reducing the percentage of injured mitochondria and oxidative damage, leading to the conclusion that hydroxytyrosol improved the mitochondrial electron transport chain leading to cell survival [56].

Hydroxytyrosol also seems to be a potent inhibitor of LDL oxidation, as it inhibits the production of isoprostanes, which are formed during LDL oxidation [57].

Anti-atherosclerotic and anti-ischemic effects

Although the antioxidant properties of hydroxytyrosol have been confirmed in both *in vivo* and *in vitro* studies, data on the anti-atherosclerotic activity of hydroxytyrosol are less promising. Hy-

percholesterolemia decreases NO bioavailability via the down-regulation of eNOS in association with an increased production of oxygen-derived free radicals that may inactivate NO, thus the upregulation of eNOS contributes to the protection against atherosclerosis [58]. An *in vitro* study on human endothelial cells, which correlated eNOS upregulation with hydroxytyrosol protective activity, concluded that the treatment of cells with a wide range of hydroxytyrosol concentrations did not exhibit positive results as far as eNOS and NO bioavailability are concerned. This leads to the conclusion that the anti-atherosclerotic properties of this compound are not eNOS mediated [59]. However, another *in vitro* study showed that hydroxytyrosol exhibits anti-atherosclerotic properties by reducing monocyte cell adhesion to stimulated endothelium, as well as reducing the vascular cell adhesion molecule-1 (VCAM-1) [60].

The results originating from *in vivo* models are also divergent. The intake of hydroxytyrosol in an *in vivo* apo-E knockout mice model showed that this compound, when administered for 10 weeks, led to atherosclerotic lesions associated with the activation of monocytes and modification of the blood lipid profile. The study claimed that under certain conditions the phenol could be rather harmful than cytoprotective [61]. However, another animal study showed that a one-month intake of hydroxytyrosol by rabbits consuming an atherogenic diet resulted in an improved antioxidant capacity of the myocardium and a reduced size of atherosclerotic lesions compared to control animals [52]. The anti-ischemic properties of hydroxytyrosol have been proven for other tissues and organs apart from myocardium. The administration of hydroxytyrosol in mice protected hepatic cells from ischemia/reperfusion injury by inducing a parallel decrease in oxidative stress levels [62].

Elenolic Acid

Elenolic acid did not possess any significant effects on lipopolysaccharide (LPS)-stimulated expression of the adhesion molecule VCAM-1 in HUVECs and bovine aortic endothelial cells (BAECs) [60], indicating that the cardioprotective effects of elenolic acid are not evident. However, studies on its effect on the myocardium are scarce.

Oleacein

Myocardial oxidative stress and low-density lipoprotein oxidation

One of the most important derivatives of oleuropein metabolic degradation is oleacein. It has been shown to exhibit improved antioxidant properties compared to oleuropein [63]. It has been reported that the antioxidant activity of virgin olive oil is principally due to oleacein [64]. Oleacein exhibited anti-inflammatory activity on monocytes by significantly inhibiting the production of ROS and efficiently reducing cyclooxygenase-2 (COX-2) mRNA synthesis. These findings show an ability of oleacein to modulate proinflammatory molecule production [65]. Another *in vitro* study revealed that oleacein could permeate the cell membrane, thus exhibiting antioxidant activity inside the red blood cells (RBCs), fortifying the antioxidant capacity of the circulating blood [66]. The protective role of oleacein on erythrocytes was also proven by another group of investigators, who observed that it significantly protected RBCs from oxidative hemolysis induced by

H₂O₂, a protection motif exhibited by nearly all phenolic components found in olive extracts [67].

Anti-atherosclerotic and anti-ischemic effects

Oleacein has been proven to directly inhibit 5-lipoxygenase [68]. Moreover, oleacein inhibited neutral endopeptidase (NEP) activity, elastase, metalloproteinase-9 (MMP-9), and interleukin-8 (IL-8) release from neutrophils, thus protecting the endothelium from pathogenic alterations [69]. The protective actions of oleacein on the endothelium were also demonstrated by the fact that the pretreatment of cells with oleacein resulted in a dose-dependent inhibition of the proinflammatory CCL2 chemokine production and monocyte adhesion to human umbilical vein endothelial cells (HUVECs) [70].

However, to the best of our knowledge, there is no study, so far, investigating the anti-ischemic effects of oleacein.

Tyrosol

Myocardial oxidative stress and low-density lipoprotein oxidation

Tyrosol has been found to reduce lipid peroxidation products in cells, but to a significantly lesser extent than hydroxytyrosol, probably because of its lower scavenger ability. However, while accumulated intracellularly, it could preserve an antioxidant defense in cells [71]. Another *in vitro* study led to the conclusion that tyrosol strengthened the resistance to oxidative stress in the nematode *Caenorhabditis elegans* and extended its lifespan. This evidence was supported by proteomic studies identifying protein levels that were differentially expressed in nematodes grown in a medium containing tyrosol [72].

A study on macrophages found that tyrosol inhibited H₂O₂ production induced by ox-LDL as well as arachidonic acid release and prostaglandin E₂ (PGE₂) synthesis. However, these effects of tyrosol were not mediated through interference of the compound with ox-LDL receptors, indicating that tyrosol abrogates the deleterious effects of ox-LDL [73]. Tyrosol possesses a binding capacity on LDL as exhibited by *in vitro* data and LDL composition is modified by one week administration of this phenolic compound through diet [74]. An antioxidant capacity of tyrosol has been evaluated neither *in vivo* nor *ex vivo*.

Anti-atherosclerotic and anti-ischemic effects

It has been shown *in vitro* that tyrosol could activate a molecular pathway, which includes phosphorylation of protein kinase B (PKB/Akt), expression of sirtuin 1 gene (SIRT1), and, as a result, the deactivation of transcription factor FOXO-3a activity, which is known to mediate apoptosis in cells. An additional element to the above results was the upregulation of eNOS, which contributes to the cardioprotective activity of tyrosol [75]. In an *ex vivo* study on Laggendorf perfused rat hearts, tyrosol was able to diminish infarct size when administered 30 min before the incidence of sustained ischemia. The underlying mechanism of the above cardioprotective effect of tyrosol was attributed to the activation of Akt and eNOS [76]. Experiments on H9c2 cardiomyocytes showed that tyrosol can ameliorate ischemia-reperfusion injury via inhibition of nuclear condensation, reduced caspase-3 activity, cytochrome c release, and c-Jun N-terminal kinases (JNK) activation [77].

Comparative and Synergistic Activities of Olive Leaf Extract Constituents

Myocardial oxidative damage

Oleuropein and hydroxytyrosol exhibited the greatest potency as anti-atherosclerotic agents among the other phenolic constituents originating from the olive tree. However, hydroxytyrosol possessed a lower EC₅₀ than oleuropein, indicating that this certain constituent is a better free-radical scavenger and possesses an improved protective factor against LDL oxidation [5]. A recent study compared the protective effect of the ethanolic and methanolic extracts of olive leaves with the effects of oleuropein, hydroxytyrosol, and quercetin as positive standards in a carbonyl compound (4-hydroxynonenal)-induced model of oxidative damage to rat cardiomyocytes (H9c2). The ethanolic extract of olive leaves, which contains larger amounts of oleuropein and hydroxytyrosol than the methanolic one, showed better protection on cardiomyocyte viability than the methanolic extract or each isolated phenolic compound against 4-hydroxynonenal-induced oxidative stress and toxicity [78]. Another study compared the capacity of four important olive polyphenolic compounds (oleuropein, hydroxytyrosol, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA) to protect red blood cells (RBCs) from oxidative hemolysis induced by H₂O₂. All compounds revealed significant protection to RBCs from oxidative hemolysis induced by H₂O₂ with the order of activity being:

oleacein > oleuropein-aglycone > hydroxytyrosol = oleuropein [79].

Treatment of the isolated rat aorta in *ex vivo* studies with oleuropein, tyrosol, and hydroxytyrosol showed that only hydroxytyrosol could provide protection against cumene hydroperoxide (CHP)-induced damage in the NO-mediated relaxation when CHP was used to stimulate oxidative stress [80]. Hydroxytyrosol exhibited the highest antioxidant activity compared to oleuropein and tyrosol in *ex vivo* experiments on the rat aorta [80].

Protection against oxidative stress induced by oleacein and other metabolites originating from olive leaves extract is also evident. Human peripheral blood mononuclear cells (PBMC) and promyelocytic leukemia cells (HL60) treated with hydroxytyrosol as well as 3,4-DHPEA-EA, oleuropein, and tyrosol, [p-hydroxyphenyl-ethanol (p-HPEA)] the dialdehydic form of elenolic acid linked to tyrosol, appeared to be protective against DNA damage [81]. Endothelial progenitor cells (EPCs) are responsible for neovascularization of ischemic tissue and may participate in the *de novo* formation of endothelium on an injured arterial wall. Oleacein along with oleuropein pretreatment of EPCs increased cell survival and telomerase activity, and decreased the percentage of senescent cells and intracellular ROS production. This effect was related to NF-E2-related factor 2 (Nrf2) transcription factor activation and to the increase of heme-oxygenase-1 (HO-1) expression [82].

Olive phenols are correlated with antioxidant protection against ageing-induced damage. The administration of biophenols originating from olives could retain the rise of oxidative stress biomarkers in the heart, such as protein carbonyls, and raise the levels of Nrf2 and Nrf2-associated gene expression, indicating that the olive polyphenolic fraction is a promising factor of protection against ageing-induced oxidative stress [83]. Furthermore, when olive polyphenols are added to isolate LDL and treated with various oxidative agents, they achieve a significant reduction of lipoprotein oxidation *in vitro* [84]. These results indicate that olive oil phenolic compounds protect LDL against peroxy radical-de-

pendent and metal-induced oxidation and could associate with LDL after their incubation with plasma [84].

Anti-atherosclerotic and anti-ischemic effects

Olive leaf extract treatment attenuated the metabolic, structural, and functional changes in the heart in rats with a diet-induced metabolic syndrome through the antioxidant and anti-inflammatory effects of polyphenols, mainly oleuropein and hydroxytyrosol [85]. Olive leaf extract reduced serum levels of triglycerides, total cholesterol, LDL, HDL, and MDA in atherosclerotic rabbits with a parallel downregulation of monocyte chemoattractant protein-1 (MCP-1), VCAM-1, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and tumor necrosis factor- α (TNF- α) [86]. Moreover, olive leaf extract was found to reduce the formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS), protecting LDL against oxidation *in vitro*, deducing that it is a source of potent antioxidants and anti-atherosclerotic factors [87].

A clinical study was conducted in order to determine the effects of *O. europaea* L. leaf extract on platelet function in healthy human subjects. The product was stated to contain 5.40 mg/mL of oleuropein, which was considered its active ingredient. The results of the study clarified that oleuropein diminished the increased inflammation markers, mediated through phospholipase C and arachidonic acid metabolism, and reduced the elevated hydrogen peroxide concentrations together with platelet activation [88].

Conclusion

In this review we provided an overview of the recent advances and current knowledge on olive leaf constituents, namely oleuropein, oleuropein aglycone, hydroxytyrosol, oleacein, elenolic acid, and tyrosol, on myocardial oxidative stress and atherosclerosis.

It has been proven that phenolic compounds of olive leaf extracts are capable of reducing oxidative stress levels in animal experimental models. This effect is mainly ascribed to hydroxytyrosol and oleuropein. Defense against LDL oxidation is also induced by olive components *in vitro*, while a concomitant increase in glutathione-associated enzymes and hypolipidemic effects of olive derivatives has been observed *in vivo*. Although among the phenolic compounds, hydroxytyrosol, tyrosol, and oleuropein are endowed with significant antioxidant properties, the anti-atherosclerotic, hypocholesterolemic, and anti-ischemic activities of the above compounds have not been studied in detail. Protection against ischemia reperfusion is evident during chronic administration of oleuropein before the ischemic episode in an *in vivo* model. Findings concerning hydroxytyrosol are not that preferable, considering that the administration of the biophenol in an *in vivo* model led to an increased atherosclerotic incidence. Additionally, studies concerning the cytoprotective effects of hydroxytyrosol against ischemia-reperfusion injury are scarce.

In conclusion, oleuropein seems to be a promising molecule that may be used as a cardioprotective agent. However, the underlying signaling cascades of its cardioprotective effects remain to be elucidated.

Conclusively, *O. europaea* L. leaf constituents possess proven beneficial results on myocardial oxidative stress and atherosclerosis. Basic research is mandatory in order to investigate olive leaf constituents as pharmacological tools for the prevention and protec-

tion of heart diseases. Furthermore, there is a further need for research concerning the outcomes and clinical trials that have been carried out in order to obtain solid evidence for these claims that could be translated in clinical practice.

Conflict of Interest

None.

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