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Original Paper

Hydroxytyrosol Decreases Phosphatidylserine Exposure and Inhibits Suicidal Death Induced by Lysophosphatidic Acid in Human Erythrocytes

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Key Words
Erythrocytes • Lysophosphatidic acid • Hydroxytyrosol • Phosphatidylserine • Cardiovascular diseases

Abstract
Background/Aims: Lysophosphatidic acid (LPA) is a phospholipid signal molecule that regulates many cellular processes both physiological and pathological. Moreover, its high plasma concentrations are toxic for several cellular types, including erythrocytes (RBC), as it acts as a pro-thrombotic and pro-atherogenic agent. It is therefore essential to explore the potential protective role of nutrition in protecting cells from the possible toxic effects of high plasma concentrations of LPA by testing bioactive nutrients. In particular, our focus was on hydroxytyrosol (HT), a phenolic antioxidant occurring naturally in virgin olive oil, investigating its possible protective effect in preventing LPA-induced programmed cell death (eryptosis) in human RBC. ***Methods:*** Intact RBC were incubated in the presence of 2.5 μM LPA and increasing concentrations of HT. Phosphatidylserine (PS) exposure with cell shrinkage, influx of extracellular calcium (Ca^{2+}), adenosine triphosphate (ATP) and glutathione levels were measured by FACS analysis. In addition, confocal laser scanning microscopy was used to determine RBC morphological alterations, as well as microvesicle formation. ***Results:*** Our study confirms that LPA-induced eryptosis is characterized by PS exposure at the cell surface, with cell shrinkage and ATP and glutathione depletion; (Ca^{2+}) influx is also a key event that triggers eryptosis. Here we report for the first time that cell co-incubation with LPA and in quantities as low as 0.1 μM HT causes a significant decrease in PS-exposing RBC, in addition to providing significant protection from the decrease in cell volume. Moreover, treatment of RBC with HT counters the influx of extracellular Ca^{2+} and completely restores ATP and glutathione content

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at 1 μM. Finally, under the same experimental conditions, HT exerts a protective effect on RBC morphological changes and microvescicle release, completely restoring the typical biconcave shape at 1 μM. ***Conclusion:*** Taken together, the findings reported in this paper point to a novel biological effect for HT in preventing programmed suicidal death in anucleated cells and indicate that prevention from LPA toxic effects may represent an additional mechanism responsible for the health-promoting effect of this dietary phenol which has been claimed, particularly related to cardiovascular diseases.

Introduction

Lysophosphatidic acid (LPA) is an endogenous bioactive lipid, precursor in the biosynthesis of cellular phospholipids. In addition, LPA is an important extracellular signalling molecule and a second intracellular messenger [1, 2]. LPA performs various biological functions mainly via receptor-mediated mechanisms [2, 3]. These include, among others, the induction of platelet aggregation [4] and the regulation of embryonic growth and of blood pressure [2]. Furthermore, LPA also mediates many pathological processes, such as nephrotic diabetes [5], tumor progression [6] and various inflammatory processes [7], most notably of the skin [8].

Several mechanisms of LPA generation have been identified. In particular, its synthesis starts from membrane phospholipids through both extracellular and intracellular mechanisms. The enzyme phospholipase D, called autotaxin, which converts lysophosphatidylcholine into LPA is responsible for extracellular biosynthesis of this phospholipid [9]. LPA is also synthesized intracellularly in the endoplasmic reticulum and in the mitochondrial membrane, while several lipid phosphatases are in charge of LPA degradation [10]. LPA can be synthesized from various cell types, including fibroblasts, adipocytes and nerve cells following stimulation with cytokines or growth factors [11]. Moreover, thrombin-stimulated platelets also significantly contribute to LPA production [12]. Accordingly, serum LPA concentrations are higher than plasma. LPA is present in the plasma of healthy volunteers in the range of 0.14-1.64 μM but its concentration increases about 10 fold in coagulated serum [13, 14]. A higher concentration of up to 200 μM has also been found in plasma and malignant ascites fluid in ovarian and cervical cancer patients [15, 16]. The presence of LPA in plasma strongly influences all the hematic cellular types. Apart from the previously discussed effect on platelets, white cells are also responsive to LPA stimulation. LPA modulates monocytic cell migration directly and indirectly via its induced secretion of inflammatory cytokines [17]. Finally, the effects of LPA on erythrocytes (RBC) have also been reported in the literature. Chung *et al*. (2007) demonstrated that RBC respond to endogenous LPA expressing Phosphatidylserine (PS) on their surface, associated with severe morphological changes and the release of microvesicles (MV) [18].

The exposure of PS on the outer membrane leaflet of RBC serves as a signal for eryptosis, a mechanism responsible for the clearance of aged cells from blood circulation, [19] as well as anemia in some pathological conditions, including cancer [20]. Similar to apoptosis of nucleated cells, RBC may undergo programmed cell death, also called eryptosis [21–23]. The aim of this present study was to evaluate the possible protective effect against LPA-induced eryptosis exerted by hydroxytyrosol (HT), a bioactive dietary phenol, present in elevated concentrations in virgin olive oil [24–26]. As we have already reported in a previous paper, HT is able to counteract the metabolic alterations associated with eryptosis induced by the heavy metal mercury [27]. Intact human RBC were incubated *in vitro* in the presence of LPA and the protective effect of HT against eryptosis was evaluated by measuring several hallmarks including increase in PS exposure and intracellular calcium (Ca^{2+}), cell shrinkage, ATP and glutathione (GSH) depletion. Moreover, we also analyzed the protective effect of HT on changes in cell morphology of LPA-treated RBC, using confocal laser scanning microscope.

Materials and Methods

Chemicals

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), LPA, and HT were from Sigma Chemical Co. Annexin V-FITC, Fluo-3/AM and 5-Chloromethylfluorescein diacetate (5- CMF) were from Immunotools (Friesoythe, Germany), Biotium (California,USA) and Santa Cruz Biotechnology (California,USA), respectively. All other chemicals used were of the purest grade available.

Preparation and treatment of red blood cells

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant were discarded. RBC were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO4, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37 °C for 4 h. In nominally Ca^{2+}-free solutions, 1 mM $CaCl_2$ was replaced by 1 mM Ethylene glycol-bis(2-aminoethylether)-*N, N, N′, N′*-tetraacetic acid (EGTA). RBC were co-incubated at 37 °C for 24 h with LPA 2.5 μM and increasing concentrations of HT (0.1-1 μM).

Annexin-V-binding and forward scatter

The percentage of annexin V and cell volume were determined as reported by Officioso *et al*. [27]. After incubation under respective experimental conditions, 100 μL of RBC samples were washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:200 dilution) in this solution at 37 °C for 20 min under protection from light. The annexin V abundance at the RBC surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Assay system for hemolysis

The extent of hemolysis was determined as previously described [27]. The samples were centrifuged at 1100 x *g* for 5 min after incubation under the respective experimental conditions and the supernatants were harvested. As a measure of hemolysis, the hemoglobin concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of RBC lysed in distilled water was defined as 100% hemolysis.

Intracellular calcium measurement

The Fluo 3 fluorescence was conducted according to Officioso *et al*. [27]. After incubation, 100 μL of RBC samples were washed in Ringer solution and then loaded with Fluo-3/AM in Ringer solution containing 5 mM Fluo-3/AM. The cells were incubated at 37°C for 30 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded RBC were resuspended in 200 μL Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Determination of Reactive oxygen species

Reactive oxygen species (ROS) formation was determined utilizing DCFH-DA as previously reported [27]. After incubation, a 100 μL suspension of RBC was washed in Ringer solution and then stained with DCFH-DA at a final concentration of 10 μM. RBC were incubated at 37 °C for 30 min in the dark and then washed three times in Ringer solution. The DCFH-DA-loaded RBC were resuspended in 200 μL Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Intracellular ATP measurement

The intracellular ATP content was evaluated utilizing a luciferin-luciferase assay kit (Roche Diagnostics). 80 μL of RBC pellets were incubated for 24 h at 37°C in Ringer solution with LPA and HT. All subsequent manipulations were performed at 4 °C to avoid ATP degradation. Cells were lysed in distilled water, and

proteins were precipitated by addition of $HClO_4$ (5%). After centrifugation, an aliquot of the supernatant (400 µL) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentrations of the samples were determined measuring the bioluminescence, according to the manufacturer's protocol (Berthold Biolumat LB9500, Bad Wildbad, Germany).

Quantification of intracellular Glutathione levels

The measurement of cellular content of nonprotein thiols (glutathione) was made with 1 mM 5- CMF dissolved in phosphate buffered saline pH 7.4 (PBS) 1x [28]. Briefly, after incubation under the respective experimental condition, 100 µL of RBC samples were washed were centrifuged at 272 x *g* for 3 min at 22 °C, the supernatant was discarded and the cells were stained with 5-CMF at 37 °C for 45 min under protection from light and washed once in PBS. Finally, 200 µL PBS were added to the cells and GSH dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 510-530 nm on a FACS Calibur.

Morphological analysis of erythrocytes

To investigate the possible protective effect of HT on the alterations in the RBC shape induced by LPA, cells were co-incubated with 2.5 µM $HgCl_2$ and 1 µM HT for 24 h. After incubation, RBC were washed twice with PBS and counted in a Burker chamber. The Confocal Laser Scanning Microscope analyses were performed according to Nguyen *et al*. [29] with minor modifications. In brief, the cells were fixed with 2% formaldehyde for 1 h at 4°C, washed several times with PBS, and then incubated with anti-human anti glycophorin A-FITC antibody for 30 min at 4°C in the dark. Afterwards, the samples were placed on glass slides and air-dried for 1 h. For dehydration the slides were dipped quickly and gently washed stepwise with ethanol from 50 to 75, 90, 100% and then cells were fixed in 2% formaldehyde and finally washed three times with PBS. For Confocal Laser Scanning Microscope imaging, several randomly selected frames from each sample were captured for morphological observation and statistical strength. Excitation and emission filters were set at 488 nm and 550–600 nm, respectively.

Statistics

Data were expressed as means ± SEM. The significance of differences was determined by one-way ANOVA followed by a post hoc Tukey's multiple comparisons test. GraphPad Prism 5 was utilized for statistical analysis.

Results

Effect of LPA treatment on PS exposure and cell volume in RBC

In order to investigate the ability of HT to prevent LPA-induced programmed cell death in human RBC, intact cells were exposed for 24 h *in vitro* to increasing LPA concentrations and several markers of eryptosis were evaluated, including PS translocation towards the cell surface. PS exposure on the RBC membrane was identified by binding with annexin-V conjugated to a FITC fluorophore, as determined by flow cytometry. As reported in Fig. 1, and according to the data in the literature, exposure of the cells to LPA determines an increase in the percentage of annexin-V-binding RBC, reaching a statistically significant effect starting from a concentration of 1 µM and reaching a 40% increase at 2.5 µM. Under the same experimental conditions, treatment with LPA induces a decrease in forward scattering RBC, indicating cell shrinkage, a phenomenon known to be associated with eryptosis.

Effect of HT on LPA-induced PS exposure and cell volume in RBC

Based on these results, we used 2.5 µM LPA to evaluate the possible protective effect of HT on eryptosis. Fig. 2 reports the dose-dependency of the effect of the phenolic compound on LPA-induced PS exposure. The treatment of RBC with increasing concentrations of HT significantly reduced the percentage of annexin-V-binding RBC in a dose- dependent manner, starting from very low concentrations such as 0.1 µM, and showing a complete protection at

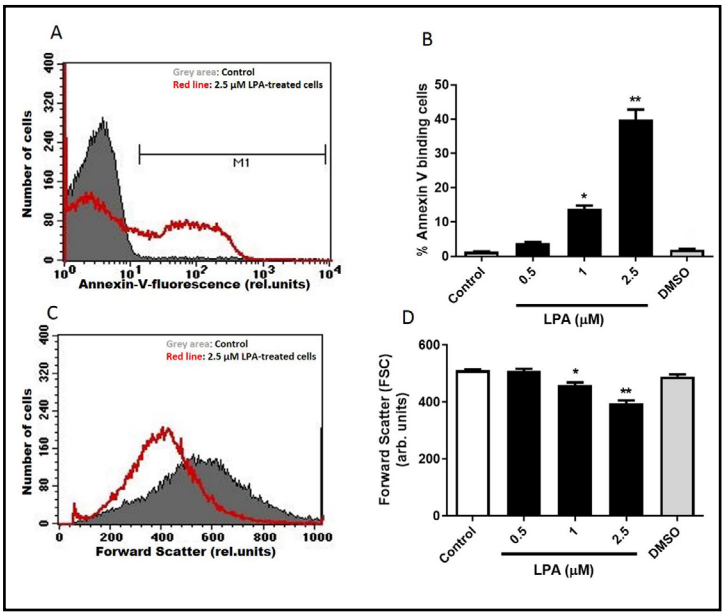


Fig. 1. Effect of LPA treatment on PS exposure and cell volume in RBC. Cells were treated in the presence of increasing concentrations of LPA. Original histogram of annexin-V-binding (A) and forward scatter (C) of RBC. Arithmetic means ± SEM (n = 8) of annexin-V-binding (B) and forward scatter (D) of RBC. Data are the means ± SEM (n = 8). Statistical significance was calculated by one-way ANOVA followed by Tukey's test. * ($p<0.05$) and ** ($p<0.01$) indicate a significant difference from cells lacking LPA treatment. For comparison, the effect of the solvent DMSO is shown (grey bar).

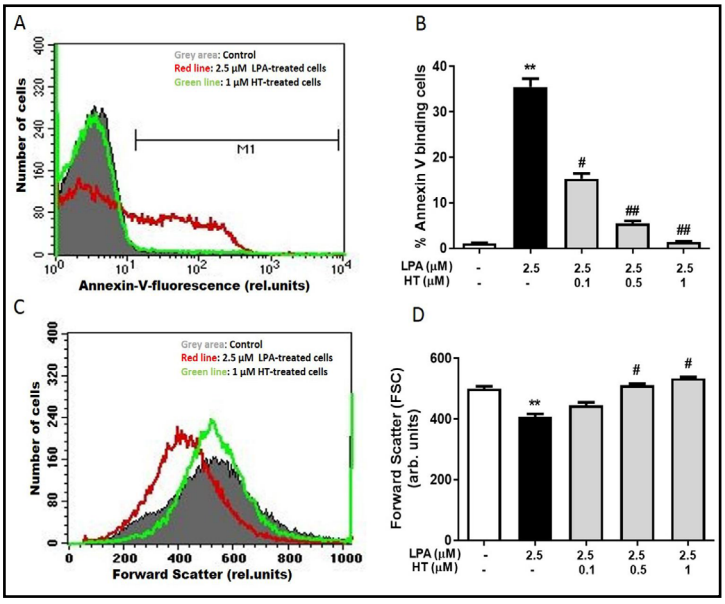


Fig. 2. Effect of HT on LPA-induced PS exposure and cell volume in RBC: Cells were treated with LPA for 24 h in the absence and presence of increasing concentrations of HT. Original histogram of annexin-V-binding (A) and forward scatter (C) of RBC. Arithmetic means ± SEM (n = 8) of annexin-V-binding (B) and forward scatter (D) of RBC. Data are the means ± SEM (n = 8). Statistical significance was calculated by one-way ANOVA followed by Tukey's test. ** ($p<0.05$) indicates a significant difference from cells lacking LPA treatment. # ($p<0.05$) and ## ($p<0.01$) indicate significant differences from cells lacking HT treatment.

1 µM. In the same concentration range, HT is also able to prevent the decrease in cell volume induced by LPA (Fig. 2).

Effect of HT on intracellular Ca^{2+} in LPA-treated RBC

The extracellular Ca^{2+} influx is considered a key event underlying the eryptotic process. Treatment with LPA induces an increase in the intracellular content of Ca^{2+}, measured as Fluo3 fluorescence. Treatment of RBC with HT completely inhibits the extracellular Ca^{2+} influx at a concentration of 1 µM (Fig. 3).

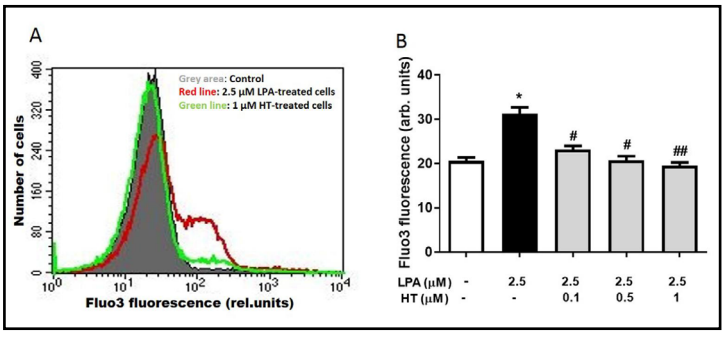


Fig. 3. Effect of HT on intracellular Ca^{2+} in LPA-treated RBC: Cells were treated with LPA for 24 h in the absence and presence of increasing concentrations of HT. A. Original histogram of the Fluo 3 fluorescence of RBC. B. Arithmetic means ± SEM (n = 8) of RBC. Statistical analysis was performed with one-way ANOVA followed by Tukey's test. * ($p<0.05$) indicates significant difference from the absence of LPA. # ($p<0.05$) and ## ($p<0.01$) indicate significant differences from cells lacking HT treatment.

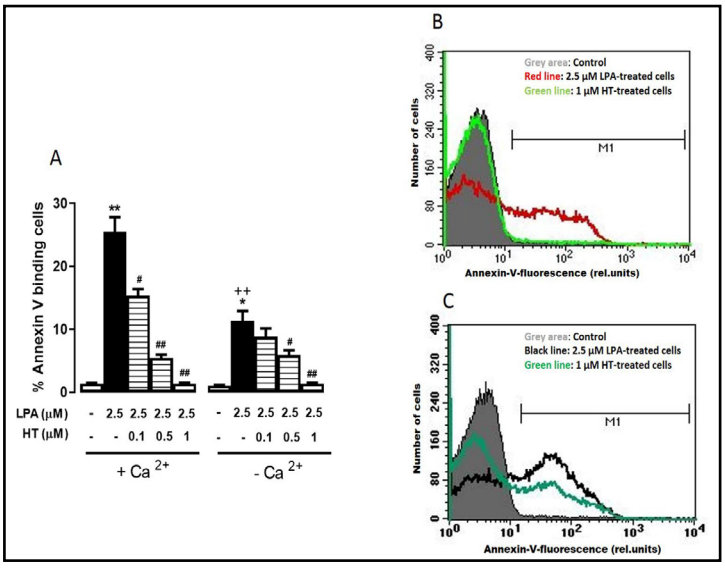


Fig. 4. Effect of HT on Ca^{2+} sensitivity of LPA-induced PS exposure in RBC: Cells were treated with LPA for 24 h in the absence and presence of increasing concentrations of HT both in absence or presence of Ca^{2+}. B, C. Original histogram of annexin-V-binding in RBC. A. Arithmetic means ± SEM (n = 8) of annexin-V-binding of RBC. Statistical analysis was performed with one-way ANOVA followed by Tukey's test. * ($p<0.05$) and ** ($p<0.01$) indicate significant difference from the absence of LPA. # ($p<0.05$) and ## ($p<0.01$) indicate significant differences from cells lacking HT treatment. ++ ($p<0.05$) indicates significant difference from corresponding value in the presence of Ca^{2+}.

Effect of HT on Ca^{2+} sensitivity of LPA-induced PS exposure in RBC

A series of experiments was then performed to further investigate the role of extracellular Ca^{2+} in LPA-induced PS exposure and the effect of HT. To this end, RBC were LPA-treated both in the absence and in the presence of extracellular Ca^{2+}. Incubation of cells in the absence of extracellular Ca^{2+} drastically attenuated the effect of LPA on PS exposure (Fig. 4), confirming that the mechanisms underlying eryptosis are both Ca-dependent and Ca-independent. It is interesting to note that HT affects both mechanisms.

Effect of HT on LPA-induced ATP depletion in RBC

Eryptosis is also associated with energy depletion, as indicated by the decrease in intracellular ATP levels, following exposure of cells to LPA (Fig. 5). Cell treatment with 1 µM HT completely restores the ATP levels of the control RBC.

Effect of HT on LPA-induced GSH depletion in RBC

To examine the relationship between LPA, cellular redox status and eryptosis, both cellular GSH content and ROS formation were evaluated. The data obtained indicate that treatment with LPA induces a significant depletion of GSH and that treatment with HT counteracts this depletion, restoring its basal levels at 1 µM (Fig. 6). Under our experimental

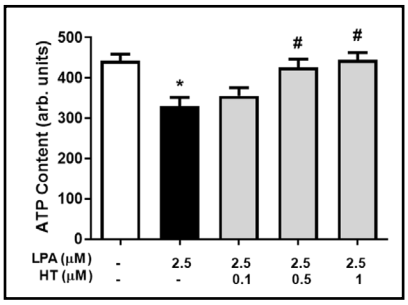


Fig. 5. Effect of HT on LPA-induced ATP depletion in RBC: Cells were treated with LPA for 24 h in the absence and presence of increasing concentrations of HT. Arithmetic means ± SEM (n = 8) of cellular ATP of RBC. Statistical analysis was performed with one-way ANOVA followed by Tukey's test. * ($p<0.05$) indicates significant difference from the absence of LPA. # ($p<0.05$) indicates significant difference from the absence of HT.

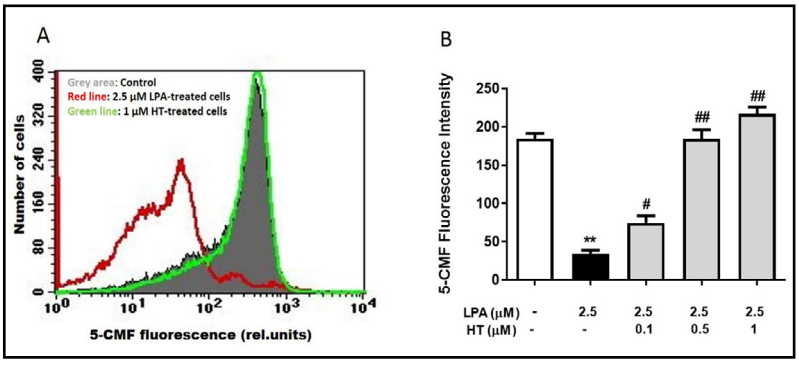
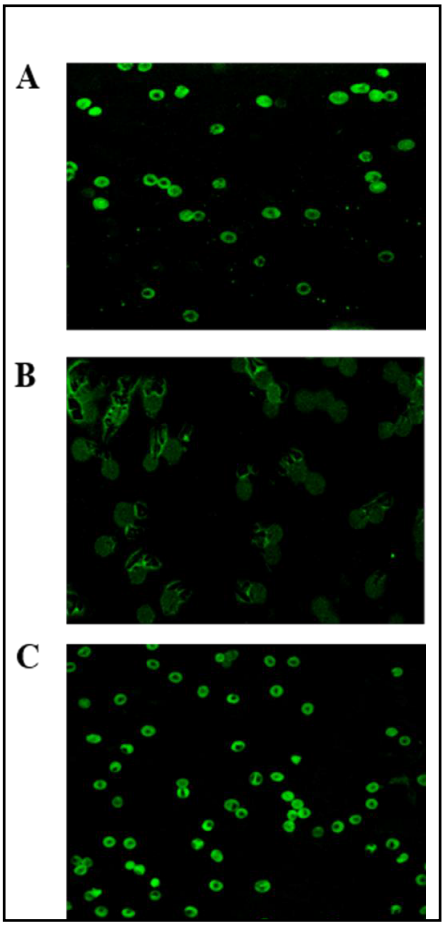


Fig. 6. Effect of HT on LPA-induced GSH depletion in RBC: Cells were treated with LPA for 24 h in the absence and presence of increasing concentrations of HT. A. Original histogram of 5-CMF of RBC. Arithmetic means ± SEM (n = 8) of the 5-CMF fluorescence of RBC. Statistical analysis was performed with one-way ANOVA followed by Tukey's test. ** ($p<0.05$) indicates significant difference from the absence of LPA. # ($p<0.05$) and ## ($p<0.01$) indicate significant differences from cells lacking HT treatment.

Fig. 7. Effect of HT on LPA-induced microvesicle release: Untreated cells are shown in (A). Cells were treated with 2.5 μM LPA for 24 h (B) and concurrently treated with 1 μM HT (C). RBC were stained with Annexin V-FITC.



conditions, no increase in ROS production was observed following exposure of RBC to LPA (data not shown).

Effect of HT on LPA-induced microvesicle release

To investigate the protective role of HT on RBC morphological changes and MV formation that are known to be induced by LPA treatment, cells were co-incubated with LPA and HT, as described in the section Materials and Methods, and analyzed with confocal microscopy. LPA treatment was associated with loss of the typical erythrocyte biconcave shape as well as the formation of MV, clearly discernible on cell membranes (not observable in the controls). Cell treatment with HT completely restores the typical biconcave shape at 1 μM (Fig. 7).

Discussion

LPA is not only a key metabolite in intracellular signalling but can also act as a potential endogenous toxic agent, as it can exacerbate inflammatory-related pathological conditions. It is therefore essential to explore new ways to protect cells from the possible toxic effects of high plasma LPA concentrations. In this regard, we considered that it was useful to verify the potential protective role of nutrition, by testing bioactive compounds normally present in our diet. Fruit and vegetables contain thousands of different phytochemicals, including polyphenols, some of which are able to strengthen biological functions with the aim of preventing and/or reducing the risk of diseases [30, 31]. Among these our attention has focused on HT, the olive oil phenol which is endowed with important biological properties [24] and which significantly contributes to the positive effect of the Mediterranean Diet on human health mechanism responsible for the health-promoting effect of this dietary phenol which has been claimed [32, 33].

Intact human RBC were exposed to 2.5 μM LPA and different markers of specific events and cellular toxicity were evaluated to test the protective effect of HT. Our study, in agreement with previous reports, confirms that LPA induces a suicidal death process in RBC through PS exposure at the cell surface, with a significant decrease in cell volume and energy depletion, associated with Ca^{2+} influx as well as a decrease in GSH content. Here we report for the first time experimental evidence of the efficacy of HT in preventing eryptosis in human RBC exposed *in vitro* to LPA treatment. Cell co-incubation with micromolar concentrations of HT and LPA causes a significant decrease in PS-exposing RBC, along with significant protection from the decrease in cell volume. Moreover, HT has countered the influx of extracellular Ca^{2+} and the decrease in cellular ATP. The protective effect of HT was further confirmed by microscopic observation of LPA-induced morphological changes. An interesting data is that HT is able to maintain cellular thiol homeostasis, counteracting GSH depletion in LPA-exposed RBC. As far as mechanisms underlying the observed effect are concerned, this dietary phenol is endowed with strong scavenging activity. However, although ROS accumulation has been implicated as a relevant cofactor contributing to the cascade of events leading to programmed cell death, in our model system no increase in ROS production is observed (data not shown), indicating that HT biological activities, which are different from the scavenging potential, are involved in the protective effect. In this respect, according to our data and that reported in the literature [27, 34] GSH enhancement may play a key role. Finally, it should be emphasized that the experimental evidence reported in this study was observed at a physiologically relevant concentration of LPA, slightly above plasma level [13]. Moreover, the protective effect of HT was observed starting from a concentration as low as 0.1 μM, which may be approached *in vivo* upon strict adherence to the Mediterranean dietary pattern [32, 33] by a daily intake of 20-25 g of phenol-rich virgin olive oil [35, 36].

Taken together, the findings reported in this paper, in agreement with our previous data on eryptosis induced by $HgCl_2$, point to a novel biological effect of HT in preventing programmed suicidal death in anucleated cells and indicate that prevention of the toxic effects of LPA may represent an additional mechanism responsible for the health-promoting effect of this dietary phenol, which has been claimed particularly related to Cardiovascular Diseases. As pointed out in the introduction, at a high concentration in the circulatory system, LPA can act as an endogenous atherogenic and thrombogenic molecule affecting the various phases of the complex process of coagulation and plaque formation [37–39]. Of possible pathophysiologic relevance is the LPA-induced platelet-monocyte aggregation in whole blood [40], which is considered an early event of acute myocardial infarction. The adherence of platelets to monocytes stimulates in these cells the expression of tissue factor, promotes fibrin formation, thus accelerating intravascular thrombosis. Moreover, in a recent paper, Ray and Rai reveal that LPA converts monocytes into macrophages in both mice and humans [41].

LPA is also an important factor favouring endothelial dysfunctions involved in the progression of atherosclerosis. LPA enhanced IL-8 and monocyte chemoattractant

protein-1 (MCP-1) expression in human umbilical vein endothelial cells [42] and stimulate the production of adhesion molecules on the cell surface, favoring monocyte-endothelial interactions, both key steps in the onset of the early atherosclerotic lesion [43, 44]. Moreover, LPA-induction of foam cells formation and oxidized low density lipoproteins (oxLDL) uptake in J774 macrophages have been reported [45, 46]. Interestingly, LPA concentration is particularly high in the atherosclerotic plaques, mainly bound to oxLDL [39]. In this respect it has been proposed that, following plaque rupture [47, 48], LPA accumulated in the lipid-rich core in the intima of the arterial wall may trigger platelet activation, thus contributing to thrombosis.

As indicated in the introduction, RBC can also be significantly modified by LPA, thus acquiring a pro-coagulant and thrombogenic activity. As clearly highlighted in a recent review by Weisel and Litvinov, RBC represent "a forgotten player in hemostasis and thrombosis" [49]. Beside the well-known role of platelets in blood coagulation, an increasing quantity of data has revealed that RBC metabolic and/or morphological alterations can play a clinically significant role in this pathophysiological process, mainly related to PS exposure [50] on the cell surface and PS-exposing MV formation. PS, an essential component of blood clotting, is normally located in the inner leaflet of the plasma membrane and its exposure on the cell surface provides a matrix for assembly of plasma coagulation factors. Accordingly, both the PS-bearing remnant cells and MV isolated from LPA-treated RBC actually potentiated prothrombinase activity, associated with increased thrombin generation in plasma [18]. Besides, after *in vitro* exposure of RBC to LPA, remnants of RBC showed enhanced adherence to endothelial cells [18] while MV can accelerate blood clotting [49] therefore promoting occlusion of microvasculature, associated with thrombotic events [29, 51]. Finally, an intracellular adhesion of RBC exposed to LPA have been reported [52].

Taken together, the reported literature data strongly indicate that LPA is a potential risk factor for Cardiovascular Diseases. In this respect, in a pilot study Cun-Shan *et al*. [53] found that people with one or two risk factors, namely hypertension and hyperlipidemia, tend to have higher plasma LPA levels compared to controls. Moreover, serum LPA levels have been reported to increase more than twofold in patients with acute myocardial infarction [54] and to increase in patients with acute coronary syndrome compared to both controls and patients with stable angina pectoris [55], confirming the involvement of LPA in the pathophysiology of the cardiovascular system.

Conclusion

Many pharmacological approaches have been designed for the prevention and treatment of LPA-related clinical outcomes, keeping as targets the enzymes of its synthesis and degradation as well as the LPA agonists [56–58]. The novel beneficial property reported in this paper regarding the efficacy of HT to reduce the LPA-induced metabolic as well as morphological alterations in human RBC opens a new horizon for innovative therapeutic interventions to prevent thrombotic events in the setting of atherosclerosis and provides a biochemical base for the use of bioactive nutrients-based functional foods or pharmacological preparations. In this respect HT, which is endowed with a broad spectrum of biological activity including anti-inflammatory, anti-atherogenic and anti-thrombotic properties demonstrated in several different model systems, appears as a promising candidate.

Abbreviations

5-CMF (5-Chloromethylfluorescein diacetate); ATP (Adenosine triphosphate); DCFH-DA (2',7'-Dichlorodihydrofluorescin diacetate); FSC (Forward scatter); GSH (Glutathione); HT (Hydroxytyrosol); LPA (Lysophosphatidic acid); MV (Microvesicles); oxLDL (Oxidized

low density lipoproteins); PBS (Phosphate-buffered saline); PS (Phosphatidylserine); RBC (Red blood cells); ROS (Reactive oxygen species); SSC (Side scatter).

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Disclosure Statement

All authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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