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Novel epicatechin derivatives with antioxidant activity modulate interleukin-1β release in lipopolysaccharide-stimulated human blood

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Abstract—We examine the potential antioxidant activity and the immune function of new epicatechin conjugates obtained by depolymerization of grape polymeric flavanols in the presence of cysteamine or cysteine. When incubated with an erythrocyte suspension, flavanols protected the erythrocyte membrane from hemolysis induced by 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH), an azo free radical initiator. The inhibitory effect was concentration-dependent and the IC_{50} was 119.8 μM for epicatechin, and 74.9 and 89.4 μM for the cysteine and cysteamine derivatives, respectively. These compounds were tested for their antioxidant activity and their capacity to modulate interleukin-1β (IL-1β), which is currently considered to be the major cytokine factor influencing the acute phase of the inflammatory response. At concentrations up to 20 μM, epicatechin and its derivatives inhibited the production of IL-1β in whole blood incubated in the presence of *E. coli* lipopolysaccharide (LPS), in a concentration-dependent manner. The most active compound was the cysteamine derivative.
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Much interest has arisen surrounding the possible beneficial effects of non-nutritive substances from plants.[1,2] For example, phytochemicals, such as flavanols are present in many frequently consumed fruit and vegetables and these may reduce lipid oxidation.[3] They also protect foods against oxidation. Among a wide range of natural compounds including vitamins, polyphenols have been reported to function as antioxidants by virtue of their hydrogen-donating properties.[4,5] Catechin and epicatechin are monomeric members of the flavanol family of polyphenols, components of green tea and red grapes, with powerful antioxidant properties in vitro.[6] It has been demonstrated that flavanols such as epicatechin, epigallocatechin, and their gallate esters scavenge both aqueous and lipophilic radicals and protect low-density lipoprotein from oxidation by acting as chain-breaking antioxidants.[7]

Because of their susceptibility to peroxidation, red blood cells (RBCs) have been used as a model to assess oxidative damage in biomembranes. Exposure of erythrocytes to oxidative conditions results in successive free radical-mediated reactions that ultimately lead to cell lysis.[8] The oxidative hemolysis in RBCs induced by various agents including hydrogen peroxide, dialuric acid, xantine oxidase, and, 2,2′-azo-bis(*a*-amidinopropane)dihydrochloride (AAPH) has been extensively studied as a model for the peroxidative damage of biomembranes.[9] Sato et al.[10] have modeled hemolysis induced by free radicals by competitive reaction between lipid peroxidation and protein oxidation, including the redistribution of oxidized band-3 proteins to form hemolytic holes. This model is based on two events: lipid peroxidation and protein oxidation causing hemolysis. Reactive oxygen species (ROS) are generated during electron-transfer reactions in aerobic cells, they are involved in pathological conditions and they can damage cell membranes and biological molecules when the endogenous antioxidant systems are insufficient,[6,8] and exogenous antioxidants to scavenge excess ROS are recommended.

Abbreviations: AAPH, 2,2′-azo-bis(*a*-amidinopropane)dihydrochloride; Ec, epicatechin; Cya-Ec, 4β-(2-aminoethylthio)epicatechin; Cys-Ec, 4β-(*S*-cysteinyl)epicatechin; LPS, lipopolysaccharide; RBCs, red blood cells; IL-1β, interleukin-1beta; TNFα, tumor necrosis factor alpha; ROS, reactive oxygen species.

Keywords: Epicatechin derivatives; Grape polymeric flavanols; Antioxidant; Interleukin-1β; Hemolysis.

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Dietary antioxidants and immune functions are also clearly correlated. Polyphenols in chocolate inhibit IL-2 secretion by T cells.[11] There is increasing evidence that green-tea polyphenols have anti-inflammatory effects, possibly mediated by their antioxidant properties. For instance, epigallocatechin gallate inhibits okadaic acid-induced TNFα production and gene expression in BALB/3T3 cells.[12] Green-tea polyphenols also inhibit NO production in peritoneal exudate (macrophage) cells,[13] inhibit lipopolysaccharide (LPS)-induced NO production and iNOS gene expression in isolated peritoneal macrophages by decreasing NF-κB activation,[14] block TNFα gene expression by inhibiting NF-κB activation, and reduce inflammatory responses.[15] Wine polyphenols such as quercetin and resveratrol has been demonstrated to inhibit LPS-induced production of TNFα in the macrophage cell line RAW 264.7.[16]

Grape skins and seeds are agricultural byproducts that mostly go to waste, although they are rich sources of antioxidant polyphenols. The aim of the present study is to determine the potential antioxidant and immunomodulatory activity of high added-value novel epicatechin derivatives obtained from white grape pomace and to assess their possible effects on human health.

The synthesis of the new bio-based antioxidant compounds (Fig. 1) with putative application as food preservatives and dietary supplements was performed as described previously.[17] They were obtained by depolymerization of grape polymeric flavanols (proanthocyanidines) in the presence of cysteamine or cysteine. We aimed to generate new bio-based antioxidants with modified physico-chemical and biological properties. The compounds studied are 4β-(*S*-cysteinyl)epicatechin (Cys-Ec) and 4β-(2-aminoethylthio)epicatechin (Cya-Ec) and epicatechin (Ec). 2,2′-Azo-bis(*a*-amidinopropane)dihydrochloride (AAPH) and lipopolysaccharide (LPS) from *E. coli* 055:B5 were purchased from Sigma (St. Louis, Missouri, USA).

Blood samples were obtained from healthy donors by venipuncture (Blood Bank of Hospital Clinic, Barcelona, Spain) following the ethical guidelines of the Hospital and collected in citrated tubes. Red blood cells (RBC) were separated from plasma and buffy coat by centrifugation at 1000*g* for 10 min. The erythrocyte layer was washed three times in phosphate buffer isotonic saline (PBS) containing: 22.2 mM Na_2HPO_4, 5.6 mM KH_2PO_4, 123.3 mM NaCl, glucose 10.0 mM in distilled water (pH = 7.4). The cells were then suspended in isotonic saline solution at a density of 8×10^9 cell/mL. We measured the hemolysis of RBC mediated by AAPH using a modification of the method described previously.[9] The addition of AAPH (a peroxyl radical initiator) to the suspension of RBCs induces the oxidation of cell membrane lipids and proteins, thereby resulting in hemolysis. The erythrocyte suspension (250 μL) was incubated in the presence of AAPH at a final concentration of 100 mM for 150 min at 37 °C to achieve 100% hemolysis. Hemolysis was assessed by measuring the absorbance of the supernatant fraction, that is the hemoglobin release, at 540 nm in a Shimadzu spectrophotometer. The antihemolytic activity of epicatechin and its derivatives was studied by adding several concentrations of the compounds, ranging from 40 to 200 μM, to the RBC suspension in the presence of 100 mM AAPH at 37 °C for 2.5 h. The IC_{50} (inhibitory concentration 50) of the hemolysis induced by AAPH was determined for each compound.

Human whole blood aliquots of 200 μL (containing 1400×10^6 cells/tube) were incubated with various concentrations of epicatechin and its derivatives (20, 70, 120, and 150 μM) for 18 h at 37 °C. Each individual treatment was incubated in the presence and absence of LPS from *E. coli* at 10 μg/mL. Epicatechin controls were also used in both assays at the same concentration as its derivatives. All samples were assayed in duplicate for each of the donor. Levels of IL-1β were quantified in the supernatants using an IL-1β ELISA Kit (Diaclone Research, France). The lower limit of detection for the ELISA system was less than 5.0 pg/mL. After exposure to the products, cell viability was evaluated by the trypan blue exclusion test[18] to discard possible toxic effects of epicatechin derivatives. Each experiment was performed at least three times and results are presented as mean (±SEM). The results for the IL-1β inhibition are presented as percentage respect to the LPS stimulated controls. Statistical analysis was performed using the two-tailed *t*-test. Differences were considered significant where $p < 0.05$.

Catechins may scavenge free radicals in living systems.[19] The antioxidant effect of red wine anthocyanins has been previously demonstrated.[20] Studies performed by

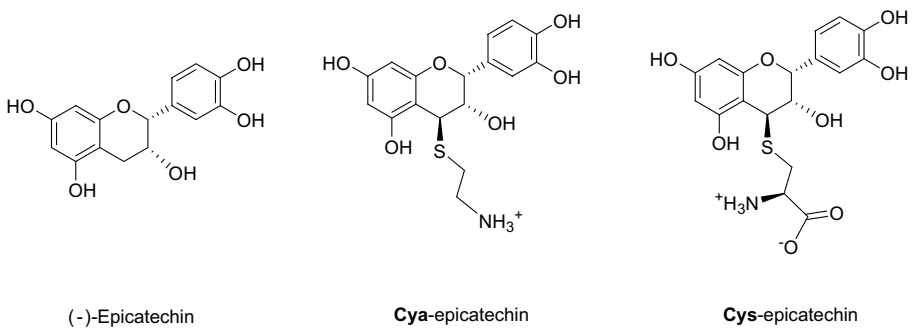

Figure 1. Chemical structure of epicatechin (Ec) and cysteamine (Cya-Ec) and cysteine (Cys-Ec) conjugates.

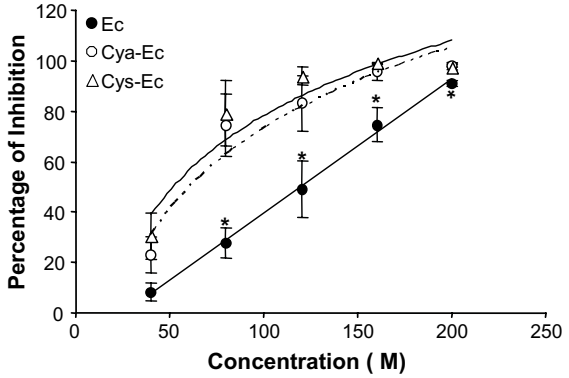

Figure 2. Protective effect of epicatechin and its derivatives on AAPH-induced lysis of human red blood cells (RBC) in vitro. An aliquot of RBC suspension was incubated with AAPH (100 mM) in the presence of different concentrations of the products during 2.5 h at 37 °C. Data are expressed as percentage of inhibition of control (mean ± SEM of four subjects). Ec has statistically the lowest antioxidant effect as its derivatives for all concentrations except for 40 μM as shown by the asterisk. No differences were found between Cya-Ec and Cys-Ec.

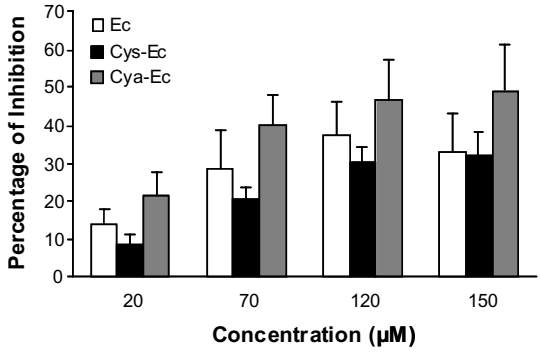

Figure 3. Effect of epicatechin and epicatechin derivatives on LPS-induced IL-1β production. Human whole blood was stimulated with LPS from *E. coli* (100 μg/mL) in the presence of different concentrations of epicatechin and epicatechin derivatives for 18 h. Data are compared with LPS alone and are expressed as percentage inhibition of control (means ± SEM of five subjects). No statistical differences were found between products at any concentration, although the Cya-Ec shows the highest inhibitory values.

our group[21] have demonstrated the antioxidant potential of these novel Ec derivatives by using a chemical reaction, the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. However, these results cannot be extrapolated to biological systems. Therefore, we evaluate the antioxidant effect of epicatechin and epicatechin derivatives by the inhibition of red blood cell lysis after addition of AAPH, a well-known peroxyl radical initiator. The protective effect of Ec, Cys-Ec, and Cya-Ec on the AAPH-induced hemolysis is presented in Figure 2 as percentage of inhibition. Moreover, Ec and its derivatives did not cause hemolysis, or affect the met-hemoglobin content in the absence of AAPH (data not reported). The inhibitory effect was proportional to the concentration of Ec and its derivatives. However, the novel Ec derivatives are more effective antioxidant protectors than Ec as shown by the statistical analysis. The IC_{50} for Ec, Cys-Ec, and Cya-Ec was 119.8, 74.9, and 89.4 μM, respectively.

The juices of red grapes and red wine have been proposed to have a variety of beneficial effects on health due in part to the presence of flavonoids in these beverages.[22] These recent findings, suggesting possible immunomodulatory functions, prompted us to examine the effects of epicatechin derivatives on the modulation of the pro-inflammatory cytokine interleukin-1β.

The inhibitory effect of epicatechin and its derivatives on the production of IL-1β was studied in whole blood treated with LPS from *E. coli*. In contrast to cell culture, this method has the advantage that all cells present in the circulation are incubated at the same ratios as in vivo, arguably representing a more physiological approach.[23] All three products showed concentration-dependent inhibition ($r = 0.96$; 0.99; 1.0 for Ec, Cys-Ec, and Cya-Ec, respectively), Cya-Ec being the most effective (Fig. 3). The percentage of inhibition at the maximal concentration reached a value of almost 50% in the case of Cya-Ec. Crouvezier et al.[24] reported a decrease of only 16% in the production of interleukin-1β in whole blood culture in the presence of LPS by epicatechin gallate, epigallocatechin, and epigallocatechin gallate. However, the highest concentration used by this author was only 20 μM, thus explaining the lower effect of these products. In addition, the presence of gallate could also explain these differences.[25]

Furthermore, the IL-1β production in response to LPS stimulation varied among individuals, as reported by other authors. The wide variation in any given immune response among individuals is in part related to genetic polymorphisms, which regulate the expression of cytokines, cytokine receptors, human leukocyte antigen, adhesion molecules, and so on. However, other factors may explain the variations.[26] Thus, antioxidants vary widely in their potency as cytokine inhibitors, which may be due to the activation of different transcription factors and the transcription of genes encoding for pro-inflammatory cytokines, as suggested elsewhere.[27]

In conclusion, the EC derivatives obtained by depolymerization of grape polymeric flavanols in the presence of cysteamine or cysteine have a profound protective effect on RBC challenged with exogenous oxidants and higher than epicatechin. We have demonstrated the immunomodulatory activity of novel Ec derivatives. The modifications produced in epicatechin during the recovery of agricultural byproducts not only maintain its properties but also enhance the potential uses. These results open the possibility of using raw materials as sources of high value added products with potential health benefits. In this sense studies of bioavailability are being performed in our laboratory to further characterize these novel antioxidant products to be used as nutraceuticals.

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