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Immunomodulatory Activity of a New Family of Antioxidants Obtained from Grape Polyphenols

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We examined the potential antioxidant activity and the immunopharmacological activity of new epicatechin conjugates obtained by depolymerization of grape polymeric flavanols in the presence of cysteamine or cysteine and with or without gallate. The compounds studied were (−)-epicatechin (**1**), cysteinyl-epicatechin (**2**), cysteamine-epicatechin (**3**), (−)-epicatechin gallate (**4**), cysteinyl-epicatechin gallate (**5**), and cysteamine-epicatechin gallate (**6**) When incubated with an erythrocyte suspension, flavanols protected the erythrocyte membrane from hemolysis induced by 2,2′-azobis-(2-amidinopropane) dihydrochloride, an azo free-radical initiator. All the epicatechin derivatives tested were more efficient as antioxidant than epicatechin. The most potent antioxidant was compound **6**. The compounds were tested for their capacity to modulate IL-1β and IL-6, which are the main cytokine factors influencing the acute phase of the inflammatory response. (−)-Epicatechin and its related compounds inhibited the production of IL-1β and IL-6 in whole blood incubated in the presence of *Escherichia coli* lipopolysaccharide. The most efficient inhibitor of cytokine formation was compound **3**.

KEYWORDS: Antioxidant; grape; polyphenols; immunomodulatory activity; red blood cells

INTRODUCTION

Free radicals cause degenerative human diseases such as cancer and heart and cerebrovascular diseases through multiple mechanisms. Natural foods and food-derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention because they are known to function as chemopreventive agents against oxidative damage (*1*).

Plant polyphenols act as antioxidants, reducing the speed and level at which our bodies suffer from oxidative stress, which in turn kills cells and triggers diseases, especially cancer. Oxidative stress is thought to play an important role in many physiological and pathological phenomena including the aging process itself. Important sources of polyphenols include fresh fruit and raw vegetables, tea, grains, and seeds (*2*).

Many byproducts and wastes generated in wine making, such as skins and seeds, contain polyphenols with potential applications as food antioxidants and preventive agents against cancer and other diseases. These products may be used as starting materials for the preparation of novel compounds with antioxidant properties. A new family of antioxidants of polymeric polyphenols from grape (*Vitis vinifera*) have been obtained by acid depolymerization in the presence of thiols such as cysteamine and cysteine (*3*, *4*). Because of their susceptibility to peroxidation, red blood cells (RBC) have been used as a model to investigate oxidative damage in biomembranes (*5*, *6*). 2,2′-Azobis(amidinopropane) dihydrochloride (AAPH), known to produce alkoxyl/peroxyl radicals in the presence of oxygen in the aqueous phase, initiates lipid peroxidation, which can be impaired in the presence of substrates that efficiently scavenge these radicals in the lipid phase or earlier in the aqueous phase (*7*). Lipid peroxidation and conformational change of band 3 together are responsible for hemolysis induced by peroxyl radicals released by treatment with AAPH (*8*).

Moreover, some antioxidants are potent inhibitors of the production of proinflammatory cytokines such as tumor necrosis factor α (TNFa), interleukin 1 beta (IL-1b), and interleukin 6 (IL-6) by human peripheral blood mononuclear cells stimulated by lipopolysaccharide (LPS) (*9*).

The aim of this study was to compare several new catechin derivatives obtained from grape polymeric polyphenols on the basis of their capacity to protect red blood cells for the hemolysis induced by AAPH and to inhibit the proinflammatory cytokine production induced by LPS.

MATERIALS AND METHODS

Chemicals. The biobased antioxidant compounds with putative application as food preservatives and dietary supplements were synthesized as described previously (*3*, *4*). These compounds were

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obtained by depolymerization of grape polymeric flavanols (proanthocyanidins) in the presence of cysteamine or cysteine. We aimed to generate new bio-based antioxidants with modified physicochemical and biological properties. The compounds studied were (−)-epicatechin (**1**), cysteinyl-epicatechin (**2**), cysteamine-epicatechin (**3**), (−)-epicatechin gallate (**4**), cysteinyl-epicatechin gallate (**5**), and cysteamine-epicatechin gallate (**6**). AAPH and LPS from *Escherichia coli* 055:B5 were purchased from Sigma (St. Louis, MO).

Blood Samples and Preparation of Red Blood Cells. 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 (RBCs) 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, and 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 cells/mL.

Antioxidant Activity. We measured the hemolysis of RBCs mediated by AAPH using a modification of the method described previously (*10*). 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. A 250 μL amount of the erythrocyte suspension 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, i.e., the hemoglobin release, at 540 nm in a Shimadzu spectrophotometer.

The antihemolytic activity of (−)-epicatechin and related compounds 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.

Cytokine Release and Determination. Human whole blood aliquots of 200 μL (containing 1400×10^6 cells/tube) were incubated with 70 μM (−)-epicatechin and related compounds. To induce the release of cytokines, 200 μL of human whole blood (HWB) was incubated for 18 h at 37 °C in the presence of 100 mL of a solution of LPS from *E. coli* (10 mg/mL). Each individual treatment was incubated in the presence and absence of LPS from *E. coli*. (−)-Epicatechin controls were also used in both assays at the same concentration as its derivatives. All samples were assayed in duplicate.

Levels of IL-1β and IL-6 were quantified in the supernatants using an IL-1β and IL-6 ELISA Kit, respectively (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 (11) to discard possible toxic effects of epicatechin derivatives.

A negative control was made to measure the spontaneous production of cytokines by the human whole blood in the absence of LPS.

RESULTS AND DISCUSSION

The antioxidant activity of the compounds studied (**Figure 1**) is presented in **Table 1** and expressed as the IC_{50} or concentration inducing 50% inhibition of the hemolysis induced by AAPH. Antioxidant activity increased, as shown by a lower IC_{50}, following the introduction of cysteine or cysteamine in the molecule of epicatechin. The most potent antioxidant was compound **6**, with 3-fold higher activity than (−)-epicatechin. In all cases the presence of a gallate group produced an increase in the antioxidant activity, which was more significant in the case of the epicatechin gallate or product **4**. Our results are consistent with other reports in the literature showing the role of the galloyl group in oxidative activity (*12*−*15*). The hydroxyl groups on the galloyl moieties contribute to the antioxidative activity, making the compounds capable of not only donating more hydrogen atoms but also providing more chelating sites for scavenging catalytic cations.

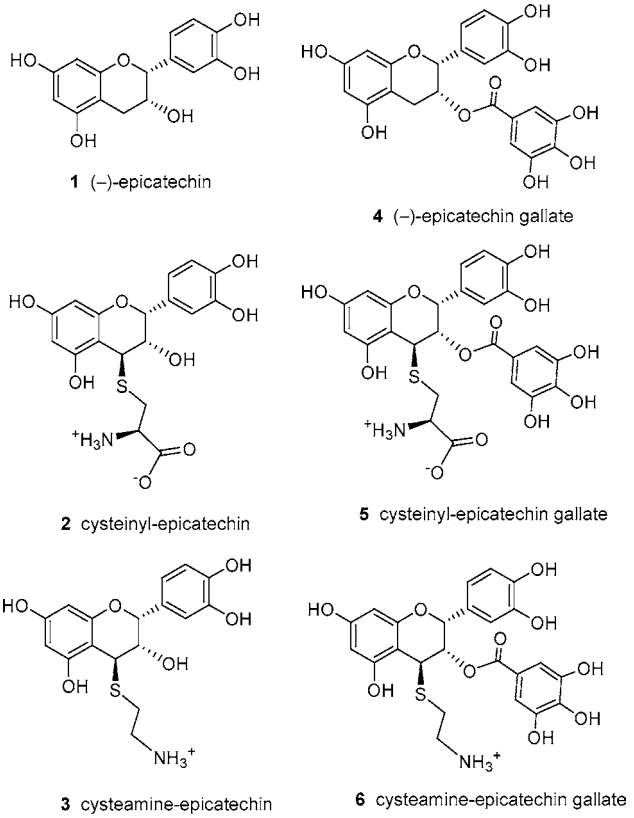

Figure 1. Chemical structures of epicatechin and epicatechin derivatives.

Table 1. Antioxidant Activity of the Compounds Determined by Their Antihemolytic Action and Percentage of Inhibition of IL-1β and IL-6 Production in Whole Blood Incubated in the Presence of *E. coli* Lipopolysaccharide

product	IC_{50}, μM	% inhibition IL-1β mean (SE)	% inhibition IL-6 mean (SE)
(–)-epicatechin (**1**)	119.8	68.19 (4.35)	58.00 (7.28)
cysteinyl-epicatechin (**2**)	74.9	61.89 (1.10)	16.12 (11.01)
cysteamine-epicatechin (**3**)	89.4	82.10 (0.05)	66.31 (3.61)
(–)-epicatechin gallate (**4**)	61.0	66.14 (3.40)	50.02 (13.73)
cysteinyl-epicatechin gallate (**5**)	47.5	63.84 (3.26)	32.46 (3.20)
cysteamine-epicatechin gallate (**6**)	36.3	59.19 (1.24)	21.66 (5.38)

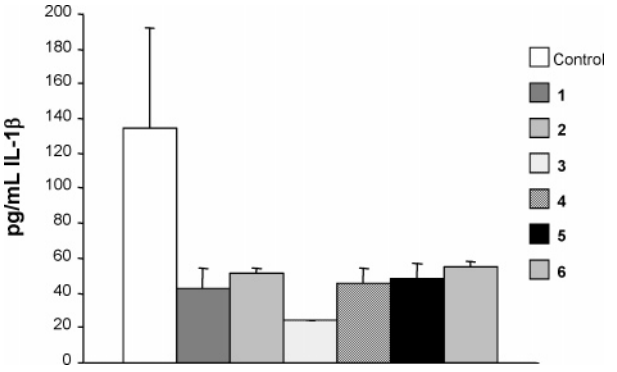

Figure 2. IL-1β production by human blood stimulated by LPS from *E. coli* and the effects of the treatment with 70 μM of epicatechin and epicatechin derivatives. The blood supernatants were collected and IL-1β determined by ELISA. Each bar represents the mean of three independent experiments.

Figures 2 and **3** show the production of IL-1β and IL-6 in human blood stimulated by LPS, respectively, and the effect of the previous incubation with the epicatechin derivatives. All

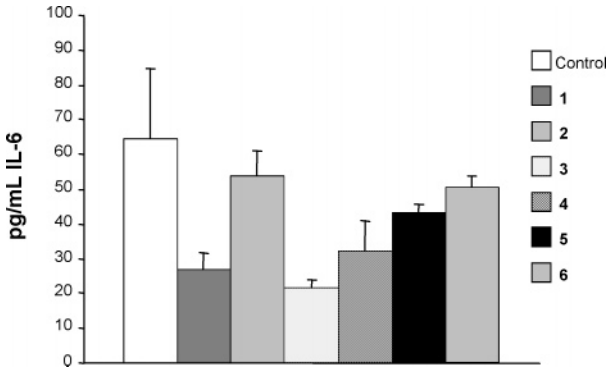

Figure 3. IL-6 production by human blood stimulated by LPS from *E. coli* and the effects of the treatment with 70 μM of epicatechin and epicatechin derivatives. The blood supernatants were collected and IL-6 determined by ELISA. Each bar represents the mean of three independent experiments.

the products reduce IL-1β and IL-6 production. It is unlikely that the compounds inhibited cytokine production through a cytotoxic effect because the results of trypan blue staining indicated no significant difference (data not shown). The percentage of inhibition induced by the different compounds is shown in **Table 1**. The most efficient inhibitor of IL-1β and IL-6 production was compound **3**. In general, the addition of a galloyl moiety did not modify the inhibitory effect. In contrast, compounds containing a galloyl group had a higher antioxidant effect. Some results in the literature show that phenolic compounds inhibit IL-1β production but not in IL-6 in whole blood (*16*). In contrast, studies of the activity of epicatechin on the stimulated production of IL-2 and IFN-γ in activated human peripheral blood cells did not show significant inhibition (*17*). Thus, antioxidants vary widely in their potency as cytokine inhibitors in a similar way to other compounds, which may be due to activation of different transcription factors and the transcription of genes encoding for proinflammatory cytokines, as suggested elsewhere (*9*). Curiously, the ionic moiety introduced through the thiol seems to exert more influence on cytokine production than the presence of the gallate moiety. This is in apparent contradiction with the results of other authors stressing the importance of the pyrogallol group in the interaction of catechins with the cell metabolism (*18*). The results presented here show that the compounds studied are promising products with antioxidant and immunomodulatory activity.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(α-amidinopropane) dihydrochloride; IC_{50}, inhibitory concentration 50; IL-1β, interleukin 1 beta; IL-6, interleukin 6; LPS, lipopolysaccharide; PBS, phosphate buffer solution; RBC, red blood cell; TNFα, tumor necrosis alpha.

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