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RESEARCH ARTICLE

Procyanidin dimer B1 and trimer C1 impair inflammatory response signalling in human monocytes

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Abstract

The way specific procyanidins exert their anti-inflammatory effects is not fully understood. This study has investigated the capacity of different procyanidins to modulate lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) production in THP1 human monocytes and their effects on the redox regulated protein kinases activity: IkB kinase beta (IKKb) and the extracellular signal-regulated kinase (ERK). LPS-triggered increase of ROS was prevented by cell pre-incubation with procyanidins. LPS induced ERK1/2 activation through phosphorylation, which was inhibited by all the compounds tested, the most active being epigallocatechin (EG), followed by epigallocatechin gallate (EGCG) and C1. Procyanidins inhibited IKKb activity *in vitro*. C1 and procyanidin extract (PE) exerted the maximal IKKb inhibition, followed by EGCG and dimer B1. Catechin exerted a slight but significant IKKb inhibition, in contrast to epicatechin, which was ineffective. In conclusion, procyanidins reduce the LPS-induced production of ROS and they exert their anti-inflammatory effects by inhibiting ERK1/2 and IKKb activity.

Keywords: *cell signaling, ERK, flavonoid, free radicals, lipopolysaccharide, NFkappaB*

Abbreviations: *B1, B2, B3, procyanidin dimers; CAT, catechin; CD14, cluster of differentiation 14; C1, procyanidin trimer; c-JNK, Jun amino terminal kinases; DCF, 2',7'-dichlorfluorescein; DCFH-DA, 2',7'- dichlorfluorescein-diacetate; EG, epigallocatechin; EGCG, epigallocatechin gallate; EPICAT, epicatechin; ERK1/2, extracellular response kinase ½; FCS, Foetal Calf Serum; GST, Glutathione S-transferase,* H_2O_2*, hydrogen peroxide; HPLC, high performance liquid chromatography; HRP, horserasish peroxidase; IkB, Inhibitor of kappa beta; IKK-beta, Inhibitor of kappa Beta kinase beta; LPS, lipopolysaccharide; LBP, LPS binding protein; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase kinase; NADPH, nicotin adenine dinucleotide hydrogen phosphate; NFkB, Nuclear factor kappa beta; NO, nitric oxide; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; O2−, superoxide anion; OH, hydroxyl radical; PE, procyanidin extract; p-ERK1/2, phospho-extracellular response kinase ½; PVDF, polyvinylidene fluoride; ROS, reactive oxygen species; SDS-PAGE, sodium dodecil sulphate poliacrylamide gel electrophoresis; TMB; Tetramethyl benzidine; THP1, human monocytic cell line; TLR-4, Toll-like receptor-4; TNF-α, Tumour necrosis factor alpha.*

Introduction

The major outer membrane component of gram-negative bacteria, lipopolysaccharide (LPS) or endotoxin, is a potent activator of monocyte function leading to responses that are both protective and injurious to the host [1]. LPS binds to LPS-binding protein (LBP) and then recruits CD14, which activates signal transduction pathways and transcription factors that induce gene expression [2]. LPS induces reactive oxygen species (ROS) production and the expression of inflammatory cytokines [3]. LPS has been shown to activate members of the mitogen-activated protein

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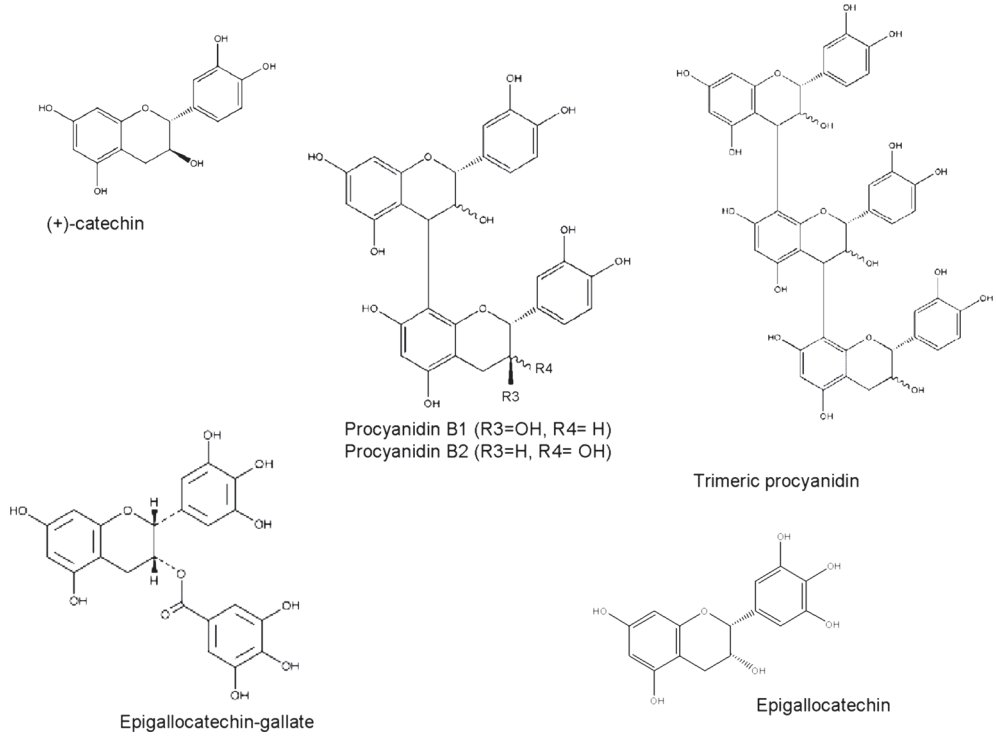

Figure 1. Chemical structure of flavonoids. Procyanidins range from dimers to oligomers of (+)-catechin and (-)-epicatechin up to 10 units; further polymerized structures are classified as condensed tannins.

kinase (MAPK) family [4], including extracellular signal-regulated kinases (ERK), c-Jun amino terminal kinases (JNKs) and p38 MAPK. Inhibition of MAPK kinase kinase (MEK) in THP1 monocytes by U0126 reduced LPS induction of several inflammatory cytokines, including interleukin-1, interleukin-8 and TNF-α (tumour necrosis factor-alpha) as well as prostaglandin E2, which indicates a role for the ERK pathway in LPS signalling that is independent of the JNK and p38 pathways [5]. LPS stimulation of monocytes activates many transcription factors, including the nuclear factor NF-kB/Rel family, which induces genes that encode most of inflammatory mediators [6]. In unstimulated monocytes, the NF-kB/Rel proteins are retained in the cytoplasm by their interaction with the inhibitors IkBs [7]. LPS stimulation of monocytes leads to the phosphorylation of IkBs by IkB kinases (IKKs), which in turn leads to the rapid translocation of NF-kB/Rel proteins to the nucleus [7]. It has been demonstrated that LPS activation of IKK is required for kB-dependent transcription and TNF-α expression in THP-1 cells and that IKK is central in the control of the NF-kB pathway [2].

LPS induces ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), nitric oxide (NO) and hydroxyl radical ($OH^{\bullet}$) in the monocyte [8]. They are generated through multiple sources such as the electron transport chain in mitochondria and through enzymes, thus producing superoxide anions such as phagocytic and non-phagocytic NAD(P)H oxidases, xanthine oxidases, nitric oxide synthases, lipoxygenases and cyclooxygenases [9].

Upon LPS activation, cells produce ROS (which is a state of oxidative stress) leading to serious cellular injuries and contributing to the pathogenesis of several diseases [10,11]. In an inflammatory environment, hydrogen peroxide is produced by activated monocytes at an estimated concentration of 10–100 μM. This massive production of ROS in the inflammation site is called the 'oxidative burst' and plays an important role as a first line of defence against pathogens [10].

ROS act as second messengers in signal transduction and gene regulation in a variety of cell types and under several biological states [12]. It is now well established that H_2O_2 is the main ROS mediating cell signalling because of its capacity to inhibit tyrosine phosphatases through the oxidation of cysteine residues in their catalytic domain [13], which in turn activates the tyrosine kinases and downstream signalling that is common to other cell oxidants. Depending on the level of ROS, different redox-sensitive transcription factors are activated and coordinate distinct biological responses [14]. A low oxidative stress induces Nrf2, a transcription factor implicated in the transactivation of gene coding for antioxidant enzymes [13]. An intermediate amount of ROS triggers an inflammatory response through the activation of NF-κB (the first transcription factor shown to be redox-regulated) and a high level of oxidative stress induces perturbation of the mitochondrial permeability transition pore and disruption of the electron transfer, thereby resulting in apoptosis or necrosis [9,15].

Several foods of plant origin such as grapes, cocoa, cinnamon and apples are flavonoid (procyanidin)-rich and are composed of the monomeric flavanols (−)-epicatechin and (+)-catechin and of the resulting oligomeric and polymeric procyanidins that are formed from these monomeric units [16]. Some of these forms can be gallated [17]. Structurally, procyanidins consist of one or more aromatic rings with one or more hydroxyl groups, which can readily combine with free radicals to form resonance-stabilized phenoxyl radicals [11]. Moreover, the catechol structure of procyanidins enables them to chelate transition metals such as copper and iron, which play an important role in oxygen metabolism [18]. This structure confers strong antioxidant properties which mean that procyanidins exhibit antioxidant activity and can thus help to prevent peroxidation and cellular oxidant damage [19–21]. Recently, it has been shown that procyanidins modulate the activity of such regulatory enzymes as cyclooxygenase and lipoxygenase to limit free radical production. EGCG, the potent well known antioxidant [22–24], has been particularly well studied and shown to inhibit LPS-induced TNF-α production and the induction of inducible nitric-oxide synthase in mouse macrophages [25]. Several studies have focused on the potential anti-inflammatory and anti-carcinogenic mechanisms of EGCGs which inhibit the activation of NFkB and thus impair the induction of inflammatory cytokines and immune responses [22,24].

Among procyanidins, dimers and trimers are more important because of their greater bioavailability and their biological activity compared to monomer forms. In the current study we compared EGCGs well established effect of reducing oxidative stress and inflammation with some pure oligomeric procyanidins and a grape-seed procyanidin extract.

Materials and methods

Reagents

HPLC analysis confirmed that the grape seed procyanidin extracts (PE) contained 1.63% of phenolic acids (mainly gallic acid), 20.92% of monomers (mainly catechin+epicatechin), 20.71% of dimers+EGCG, 17.33% of trimers and 39.41% of oligomerics longer than trimers. Pure procyanidins B1, B2, epicatechin gallate, epicatechin, catechin and epigallocatechin gallate, Trypan Blue and LPS were purchased from Sigma-Aldrich, Inc (Madrid, Spain). C1 was purified from a grape seed procyanidin extract and was kindly donated by Dr J. Valls (Tarragona, Spain). The cell-permeant dye 6-carboxy- 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) is from Molecular Probes, Inc. (Eugene, OR).

The anti-ERK1/2 (clone K-23, Cat. No. SC-94) and anti p-ERK1/2 (clone E-4, Cat. No. SC-7383) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-labelled secondary antibody was from Amersham Pharmacia Biotech (Arlington Heights, IL). Human IKK-2 activity *in vitro* assay (Cat. No. CBA044) was from Calbiochem (Nottingham, UK).

Cell culture and treatments

THP-1 is a human monocytic cell line isolated by Tsuchiya et al. in 1980 from a 1-year old Japanese boy with monocytic erytroleukaemia and was obtained from ATCC (American Type Culture Collection, Rockville, MD). THP-1 were grown in RPMI Dutch Modified (Sigma, Milan, Italy) supplemented with 10% FCS, non-essential amino acids, 2 mM glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 1 mM sodium pyruvate. Cells were maintained in log phase by seeding twice a week at a density of 3×10^5 cells/ml and at 37°C under 5% CO_2/air atmosphere. Experiments were routinely carried out on triplicate cultures.

The procyanidin extract or pure molecules were prepared in absolute ethanol. Appropriate dilutions were made to ensure a 0.01% (v/v) ethanol concentration in all control and treated wells.

To measure ROS production in THP-1 monocytes, cells were co-incubated for 24 h with PE (40 μg/mL) and LPS (100 ng/mL). After the treatment, DCFH-DA was added and the fluorescence measured. The incubation with H_2O_2 (15 min) was carried out after the DCFHDA was added. Values are relative change and expressed as a percentage of the maximum value.

For the procyanidin effect on LPS oxidative burst, cells were stimulated for 30 min with 1, 10 or 20 μg/mL of LPS. Cells were pre-incubated with the different procyanidins for 4 h and then stimulated for 30 min with LPS (10 μg/mL) and PE (40 μg/mL) or pure compounds (10 μg/mL): C1, trimer C1; B1, dimer B1; B2, dimer B2; EGCG, epigallocatechin gallate; EG, epigallocatechin.

For time course analysis of ERK1/2 activation in THP-1 monocytes, cells were pre-treated with PE (40 μg/mL) for 4 h and then stimulated with 1 μg/mL of LPS for 30 min, 1 h and 3 h.

Viability assay

The trypan blue dye exclusion method was used to evaluate the percentage of viable cells; 200 μL of cell suspension was placed in an appropriate tube with an equal volume of 0.4% Trypan blue and gently mixed for 5 min at room temperature. The number of viable (unstained) and dead (stained) cells was determined using an optical microscope in a Neubauer chamber. We calculated the percentage of unstained viable cells per mL. Thus, % viable cells = 100 × (live cells)/(dead+live cells).

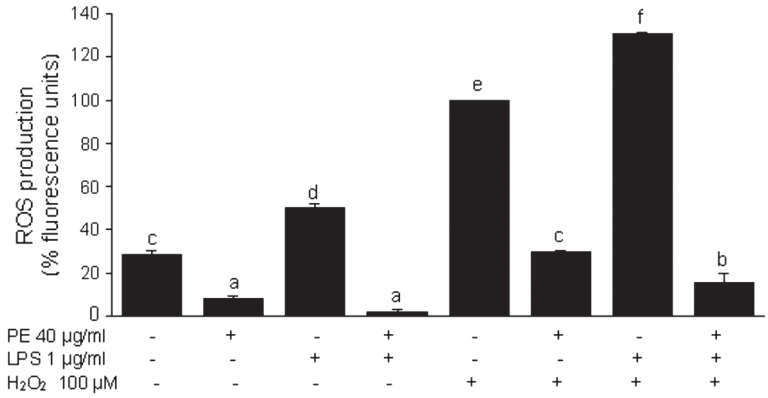

Figure 2. PE reduces ROS production in THP-1. Cells were co-incubated for 24 h with PE (40 µg/ml) and LPS (100 ng/mL). After the treatment, DCHF-DA was added and the fluorescence measured. The incubation with H_2O_2 (15 min) was performed after the DCHF-DA was added. Values are relative change and are expressed as a percentage of the maximum value. Values are expressed as mean ± SEM. The significance of difference among the groups was analysed by ANOVA. Bars showing a superscript letter are significantly different ($p > 0.05$).

DCF assay

We determined the LPS dose-dependent production of ROS by adding 1, 10 and 20 µg/mL of LPS; 2 × 10exp 6 cells were co-treated with 0.1 µg/mL of LPS and 40 µg/mL of PE for 24 h or pre-teated with PE (40 µg/mL) and the pure compounds (10 µg/mL) for 4 h and then stimulated with LPS 1 µg/mL for 30 min. Following LPS stimulation, cells were collected and washed with PBS to eliminate the amount of the procyanidins that were not cell associated. Cells were incubated with 2.5 mM DCFH-DA for 30 min at 37°C and 5% CO_2. A change in fluorescence was assessed with a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore Corp., Bedford, MA). The cell-permeant dye DCFH-DA was oxidized by hydrogen peroxide, peroxinitrite (ONOO_) and hydroxyl radicals (OH) to yield the fluorescent molecule 2′,7′-dichlorofluorescein (DCF). Thus, dye oxidation provided an indirect measure of the presence of these reactive oxygen intermediates, calculated by dividing the mean channel fluorescence of a treated sample by that of the untreated one and multiplying by 100 to obtain the relative change, expressed as a percentage.

Western blotting analysis of pERK1/2 and total ERK1/2

Cells (10×10^6) were pre-treated with the different procyanidins (PE, 40 µg/mL; pure compounds, 10 µg/mL) for 4 h and then stimulated with LPS (1 µg/mL) for 30 min. Cells were then harvested, washed once with ice-cold PBS and gently lysed for 30 min in ice-cold lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM Na_3VO_4, 20% glycerol, 1% NP40, 1 mM NaF). Lysates were centrifuged for 10 min at 4°C (10 000 g) to obtain the supernatants, which were used for Western blot analysis. Equal amounts of total cellular protein (100 µg) were resolved by SDS-PAGE 7% under reducing conditions (90 V, 2 h) and transferred to PVDF membranes (400 A, 2.5 h) in buffer T consisting of 20% (v/v) methanol, 200 mM Glicine, 25 mM Tris, pH 8.3. The membrane was blocked for 1 h at RT and then incubated overnight at 4°C with anti-ERK1/2 (1:100) or with anti p-ERK1/2 (1:100) monoclonal antibodies. The blots were washed and exposed to horseradish peroxidase-labelled secondary antibody (1:15000) for 1 h at room temperature. The blots were washed and the immunocomplexes were visualized by the enhanced chemiluminescence detection system ECL Advanced Western Blotting Detection Kit and quantified by densitometric scanning (Fluorchem F2, Alpha Innotech, CA, San Leandro).

Densitometric analysis

Density of bands in Western blotting was analysed by using Image Quant TL software (www.GElifesciences.com/western).

IKK-2 (beta) activity assay

The effects of various procyanidins on the activation of IKK-2 were assessed using an *in vitro* assay following the manufacturer's instructions (Cat. No. CBA044, Calbiochem). The assay was based on the colorimetric detection of the phosphorylated substrate IkBa. Briefly, the assay is an ELISA based activity assay that uses a 50-amino acid GST-IkBa fusion polypeptide substrate which includes the Ser32 and Ser36 IKKb phosphorylation sites. The GST-IkBa substrate and IKK-2 are incubated in the presence of IKK-2 inhibitors in the wells of a gluthatione-coated 96-well plate, which allows substrate phosphorylation and capture in a single step. The phosphorylated substrate

is detected using an anti-phospho-IkBa antibody, followed by the HRP-conjugate and colour development with TMB substrate. Absorbance is read at 450 nm and is directly related to the level of IKKb activity.

Statistical analysis

Results are expressed as mean value ± SEM. Effects were assessed using ANOVA or Student's *t*-test. We used Tukey's Test of honestly significant differences to make pair-wise comparisons. All calculations were performed using SPSS 15.0 software.

Results

Viability assay

The trypan blue exclusion method was used to evaluate the percentage of viable cells after the treatments with the different procyanidins and LPS in THP-1 cell line. Any significant change in THP1 viability was observed when cells were exposed to LPS (100–10 000 ng/mL) for 5–24 h with or without 10 µg/mL co-treatment with PE (40 µg/mL) or any procyanidin tested when compared to controls.

ROS production

The incubation of THP-1 cells with LPS at 100 ng/mL (0.1 µg/mL or 100 µg/L) for 24 h significantly increased intracellular ROS production, as shown by the increased fluorescence obtained by DCF compared to the control (vehicle alone) indicating the presence of intracellular H_2O_2, $ONOO^-$ or OH (Figure 2). However, the combined addition of PE (40 µg/mL) to cells treated with LPS prevented intracellular ROS production after 24 h of treatment. Cells treated with PE alone also showed a significant decrease in DCF fluorescence.

THP-1 human monocytes were also treated with H_2O_2 alone or in combination with LPS. ROS production H_2O_2-induced was higher than that induced by LPS alone; however the combination of both inducers produced the maximal response (Figure 2). In all the treatments PE was able to strongly reduce the intracellular ROS production.

To further investigate the PE action over ROS production we tested some pure compounds that are present in the extract. Firstly we tested the LPS dose-dependent production of ROS (Figure 3A) and we selected the minimal significant dose of LPS that produced an increase in ROS production after 30 min of incubation (10 µg/mL).

In these conditions, we showed that the PE produced the maximal reduction of ROS which inhibited ~ 60% of ROS compared to LPS, followed by the trimer C1

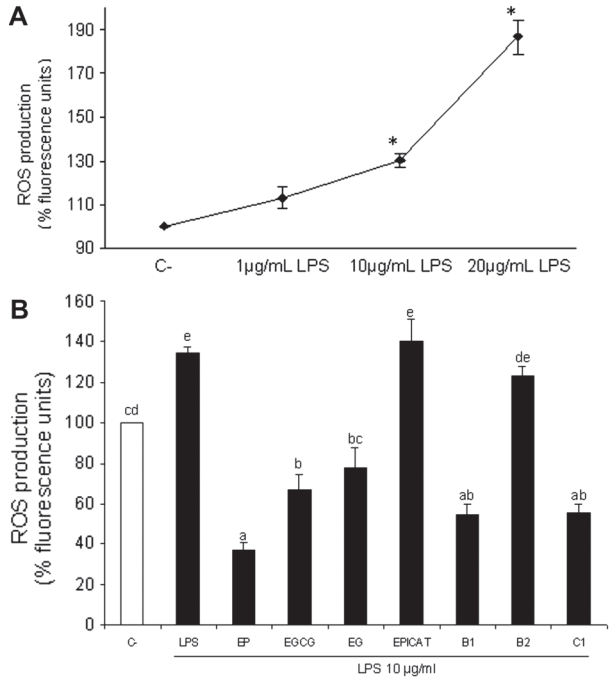

Figure 3. (A) LPS elicits a dose dependent oxidative burst. Cells were stimulated for 30 min with 1, 10 or 20 µg/mL of LPS. (B) Procyanidins reduced ROS production. Cells were pre-incubated with the different procyanidins for 4 h and then stimulated for 30 min with 10 µg/mL of LPS. PE (40 µg/mL), procyanidin extract; Pure compounds (10 µg/mL): C1, trimer C1; B1, dimer B1; B2, dimer B2; EGCG, epigallocatechin gallate; EG, epigallocatechin; EPICAT, epicatechin. Values are expressed as mean ± SEM. The significance of difference among the groups was analysed by ANOVA. Bars showing a superscript letter are significantly different ($p > 0.05$).

and the dimer B1. Surprisingly, dimer B2 did not produce any significant reduction (Figure 3B).

Procyanidins reduce ERK1/2 phosphorylation

We also examined the expression and degree of phosphorylation of the ERK1/2 MAP kinases (Figures 4 and 5). This kinase has been reported to be activated by various stress stimuli, including an over-production of ROS. LPS induced a remarkable increase in the level of the phosphorylated form ERK1/2 (p-ERK1/2) at 30 min and 3 h of incubation (Figure 4). Such an increase was prevented by the pre-treatment of the cells with the different compounds, with EG being (Figure 5).

Procyanidins inhibit IKK-2 activity in vitro

IkB kinases are related kinases that play a major role in the activation and regulation of the transcription factor, NF-kB. In this study we assessed the inhibitory effect of some procyanidins on IKK-2 activity. We showed that the PE and the trimer C1 were the inhibitoriest compounds followed by EGCG. The dimer

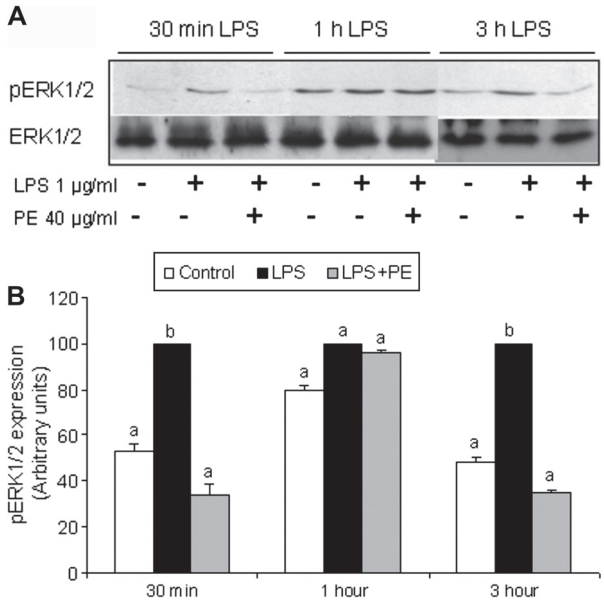

Figure 4. Time-dependent effects of PE on ERK1/2 in THP-1 human monocytes. Cells were pre-treated with PE (40 µg/mL) for 4 h and then stimulated with 1 µg/mL of LPS for the indicated times. (A) Representative western blot analysis. (B) Densitometric analyses of the immunoblots. Values are expressed as mean ± SEM. The significance of difference among the samples was analysed by ANOVA. Values showing a superscript letter are significantly different ($p > 0.05$).

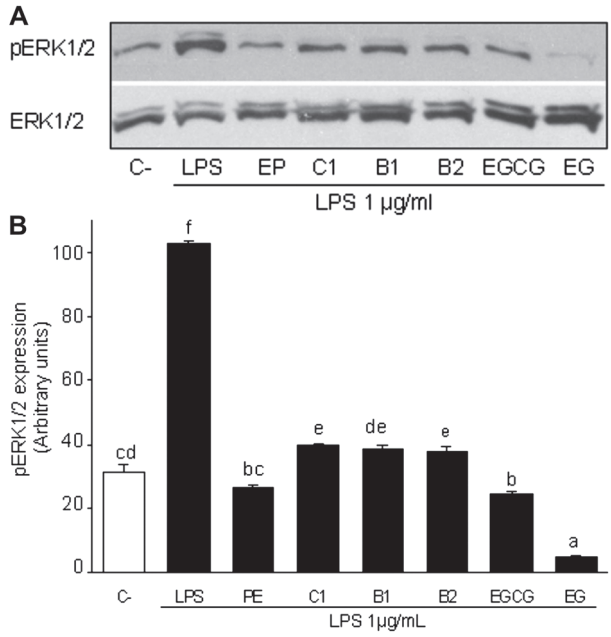

Figure 5. Effects of different procyanidins on ERK1/2 in THP-1 monocytes. Cells were pre-treated with PE (40 µg/mL), C1, B1, B2, EGCG or EG (10 µg/mL) for 4 h and then stimulated with 1 µg/mL of LPS for 30 min. (A) Representative western blot analysis. (B) Densitometric analyses of the immunoblots. Values are expressed as mean ± SEM. The significance of difference among the samples was analysed by ANOVA. Bars showing a superscript letter are significantly different ($p > 0.05$).

B3 and epicatechin monomer did not affect IKK-2 activity (Figure 6).

Discussion

While there is a substantial body of scientific literature which supports a positive role of flavonoids for health, the way specific flavonoids exert these benefits is still under intense investigation [16,26,27]. The underlying mechanisms responsible for the protective effects of procyanidins involve the antioxidant, and anti-inflammatory properties of polyphenols, of which EGCG is known as the most active catechin derivative. EGCG has the greatest antioxidative activity, the greatest inhibitory activity towards NF-KB and IkB degradation and also inhibits MAPK pathway [25,28, 29]. EGCG health benefits have been extensively studied. For this reason EGCG is an excellent natural compound for comparison with other oligomeric procyanidins. In the present study we investigated the anti-inflammatory effects of various oligomeric procyanidins in a human monocytic cell line activated with LPS.

The PE is a mixture of various monomeric and oligomeric procyanidins and phenolic acids from grape-seed which reduces intracellular ROS production induced by LPS and H_2O_2. We also demonstrate for the first time the same effects on LPS-induced ROS production with some pure oligomeric procyanidins, the trimer C1 and the dimer B1 being the most effective, followed by EGCG. Houde et al. [21] and Roychowdhury et al. [19] have previously reported the effect of EGCG and a GSPE on ROS production in RAW 264.7 macrophages activated with LPS. Some authors have attributed anti-inflammatory properties to the dimer B2 [30], but, in contrast to B1, in our conditions there was no apparent inhibition of the ROS production. Therefore, the structural differences between both dimers must be responsible for the differences in their effectiveness.

It has been reported that MAPKs play an important role in the expression of various genes because these kinases regulate some pro-inflammatory transcription factors. To further investigate the procyanidins' mechanism of action we assessed their modulation of ERK1/2 phosphorylation. We demonstrate that all the oligomeric procyanidins tested and the PE inhibited such phosphorylation and as a consequence ERK1/2 activation. Interestingly, EG showed the highest inhibition values. It decreased ERK1/2 phosphorylation by ~90%. EGCG showed a 70% inhibitory effect, followed by PE. However, these results are controversial. Ichikawa et al. [28] observed that EGCG and EG produced an increase in ERK1/2 phosphorylation after 24 h of treatment. These differences might be due to the duration of the treatment, because it is well established that the antioxidant effects of flavonoids are time- and dose-dependent [29]. Dimers B1 and B2 and the trimer C1 were equally effective (60%) at reducing ROS production.

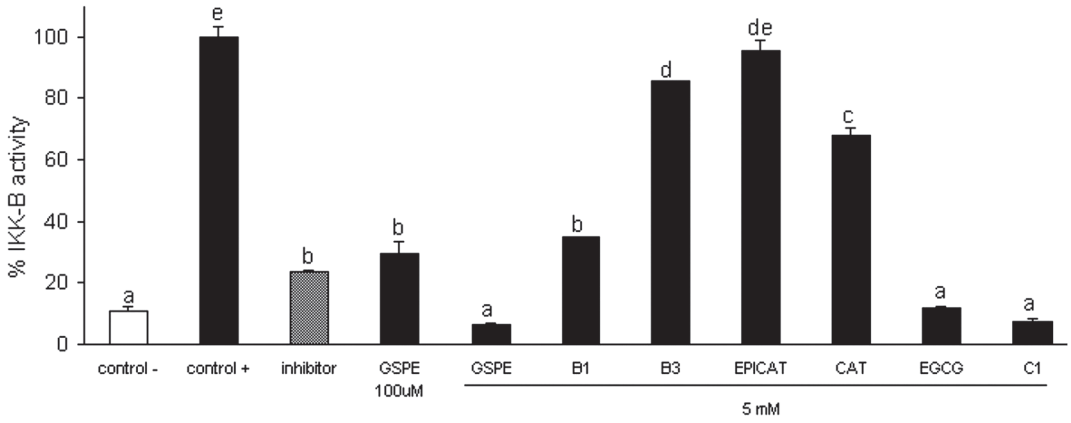

Figure 6. Inhibition of human IKK-B activity by various monomeric and oligomeric procyanidins. Kinase activity of human recombinant IKK-B was measured following the manufacturers instructions. PE 100 µM ~ 86 µg/mL. PE; procyanidin extract, B1; dimer B1, B3; dimer B3, EPICAT; epicatechin, CAT; catechin, EGCG; epigallocatechin gallate, C1; trimer C1. Control -, assay without the enzyme; Control+, assay including the enzyme but without the inhibitor.

Some authors have reported that EGCG and EG inhibited LPS-induced NF-kB activation by inhibiting IkB degradation. We have also demonstrated that the PE is able to inhibit p65 translocation to the nucleus, iNOS expression and NO production in RAW 264.7 [16]. However, procyanidin interaction with IKK has not yet been studied extensively. In this work we assessed the *in vitro* modulation of IKKb

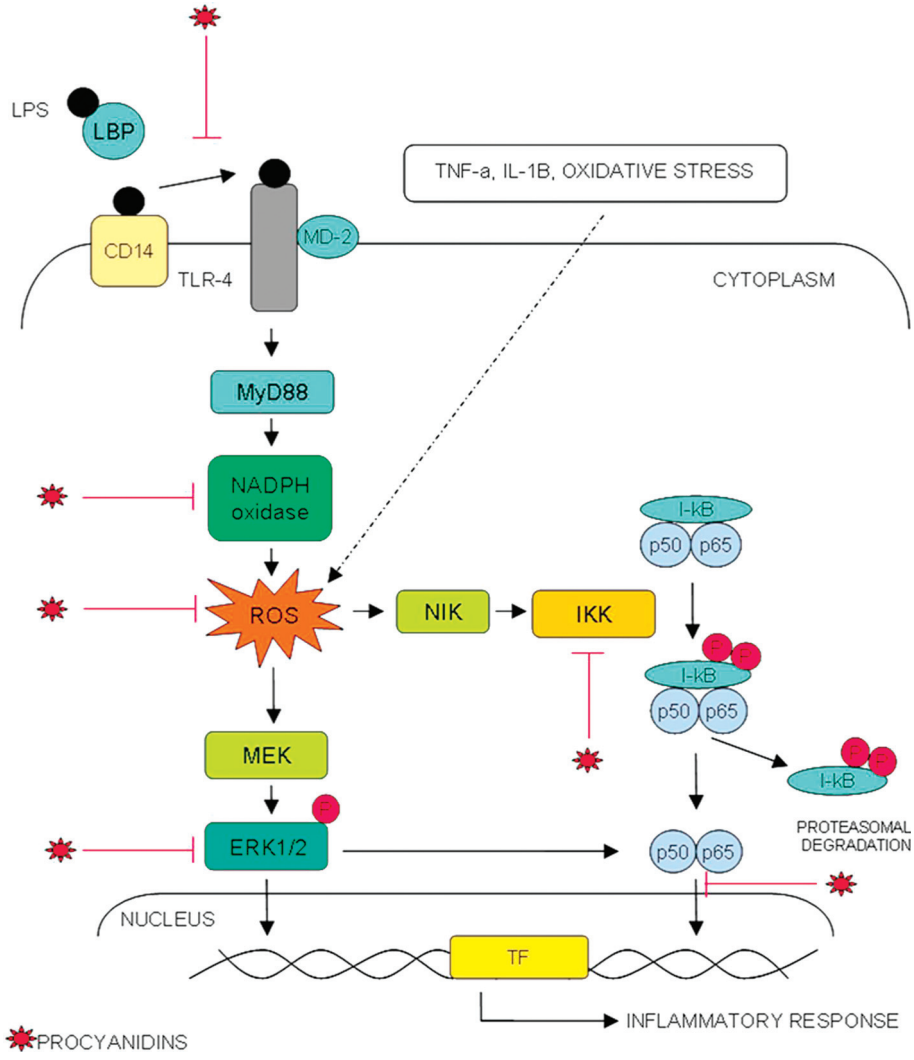

Figure 7. LPS signal transduction and activation of ROS production and pro-inflammatory transcription factors (TF). Procyanidin proposed model for its mechanism of action.

activation by procyanidins. We identified that the inhibition of IKK activity was an important target in the anti-inflammatory action of procyanidins and that their effectiveness was structure-related (Figure 1). C1 and PE exerted the maximal inhibition of IKK activation, followed by EGCG. Also the dimer B1 produced a significant reduction, in contrast to B3. Among the monomers tested catechin exerted a slight but significant inhibition (35%), differing from epicatechin, which was ineffective.

LPS induces oxidative stress and produces NO and ROS in macrophages [8]. It has long been known that ROS plays a role in intracellular signalling [9], but the various mechanisms by which the cellular redox state can influence signalling pathways are extremely complex. We hypothesize (Figure 7) that procyanidins, as potent antioxidants, prevent the production of reactive oxygen species (ROS), which have been suggested to be involved in the activation of the NF-κB signalling system [14,31] and MAPK signal pathway [10]. Furthermore, accumulating evidence indicates that there is a crosstalk between ERK and NF-kB pathways, where ERK seams to activate NF-KB [32–34]. This crosstalk might explain how procyanidins could inhibit both pathways.

ROS are important intracellular second messengers generated in response to pro-inflammatory agents including TNF-α [12], IL-1 and LPS. Although the precise intracellular source from which LPS induces ROS generation remains to be clarified, it has been reported that TLR-4 directly interacts with NADPH oxidase, which is required for induced H_2O_2 generation in HEK 293 cells [15]. A similar mechanism has been suggested for ROS generation by LPS in THP-1 cells. Also, in monocytic cells, the main source of ROS in IL-1b-induced NF-kB activation was shown to be the NADPH oxidase complex [15,35].

Therefore, the procyanidin inhibition of NADPH oxidase that has been extensively reported [36] might be responsible for these effects, although this point has not been addressed in this study. Other possible mechanisms of action of procyanidins have been proposed recently. Some authors [20,22] have proposed that procyanidins from different sources inhibit LPS-response by direct neutralization and binding to LPS molecules. Also, Mackenzie et al. [37] proposed that some dimeric procyanidins can act inhibiting the NF-kB binding to the DNA. All these conjectures are represented in Figure 7 and need to be further investigated.

In conclusion, we demonstrated that the oligomeric procyanidins B1 and C1 and the whole PE modulate the inflammatory response in human monocytes and that their effects are comparable to those exerted by the well known EGCG. The findings that flavanol-related chemical structures differentially inhibit monocyte pro-inflammatory pathways prompts the need to define not only the relevance of the chemical structure but also of the conformation of the procyanidins being studied. In future studies, molecular models should be constructed to better define the stereo-chemical possibilities for the interactions described.

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Declaration of interest

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