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Grape-Seed Procyanidins Act as Antiinflammatory Agents in Endotoxin-Stimulated RAW 264.7 Macrophages by Inhibiting NFkB Signaling Pathway

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Procyanindin extract (PE) is a mixture of polyphenols, mainly procyanidins, obtained from grape seed with putative antiinflammatory activity. We evaluated the PE effect on RAW 264.7 macrophages stimulated with lipopolysaccharide plus interferon-γ that show a rapid enhanced production of prostaglandin E_2 (PGE_2) and nitric oxide (NO). Our results demonstrated that PE significantly inhibited the overproduction of NO, dose and time dependently. PE caused a marked inhibition of PGE_2 synthesis when administered during activation. Moreover, PE pretreatment diminished iNOS mRNA and protein amount dose dependently (10−65 μg/mL). PE (65 μg/mL) pretreatment inhibited NFκB (p65) translocation to nucleus by nearly 40%. Trimeric and longer oligomeric-rich procyanidin fractions from PE (5−30 μg/mL) inhibited iNOS expression but not the monomeric forms catechin and epicatechin. Thus, we show that the degree of polymerization is important in determining procyanidin effects. PE was considerably a more effective inhibitor of NO biosynthesis (IC_{50} = 50 μg/mL) in comparison to other antiinflammatories, such as aspirin (3 mM), indomethacin (20 μM), and dexamethasone (9 nM). In conclusion, PE modulates inflammatory response in activated macrophages by the inhibition of NO and PGE_2 production, suppression of iNOS expression, and NFkB translocation. These results demonstrate an immunomodulatory role of grape seed procyanidins and thus a potential health-benefit in inflammatory conditions that exert an overproduction of NO and PGE_2.

KEYWORDS: Procyanidins; dimers; trimers; lipopolysaccharide; INF-γ; RAW 264.7; NO; iNOS; PGE_2; NFκB; p65

INTRODUCTION

Procyanidins are phenolic compounds from the flavonoid group that are widely found in cereals, vegetables, and fruits such as grapes, berries, cocoa, and apples. They are high molecular weight polymers of flavan-3-ol units (catechin, epichatechin) (**Figure 1A**).

They have a wide range of biological activities (*1*−*4*). They function as powerful antioxidants and could exert antiinflammatory activities (*5*). There is growing interest in the utilization of procyanidins for their dietary and pharmacological properties. The few *in vitro* studies into the effects of procyanidins on inflammation have shown different results (*6*). This is probably due to the different structural characteristics of the molecules tested and the different experimental designs used. Recent studies have revealed potent antiinflammatory properties of procyanidins on experimental inflammation in rats and mice (*7*, *8*). Their antiinflammatory mechanisms are still poorly understood (*9*). They are related to oxygen free radical scavenging, inhibition of the formation of inflammatory cytokines, and anti-lipid peroxidation (*9*, *10*).

Macrophage activation is crucial in the progression of multiple inflammatory diseases via the release of inflammatory mediators such as cytokines, nitric oxide, and prostaglandins (*11*). In macrophages, cytotoxicity and inflammation can all be promoted through cellular response to nitric oxide (*12*, *13*), a gaseous free radical and intercellular messenger that mediates a variety of biological functions. NO production is mediated by the inducible macrophage isoform iNOS, type II, which is a member of the nitric oxide synthase (NOS) family. iNOS catalyzes the oxidization of L-arginine to produce L-citrulline and NO, and it is expressed in macrophages upon stimulation by interferon-γ

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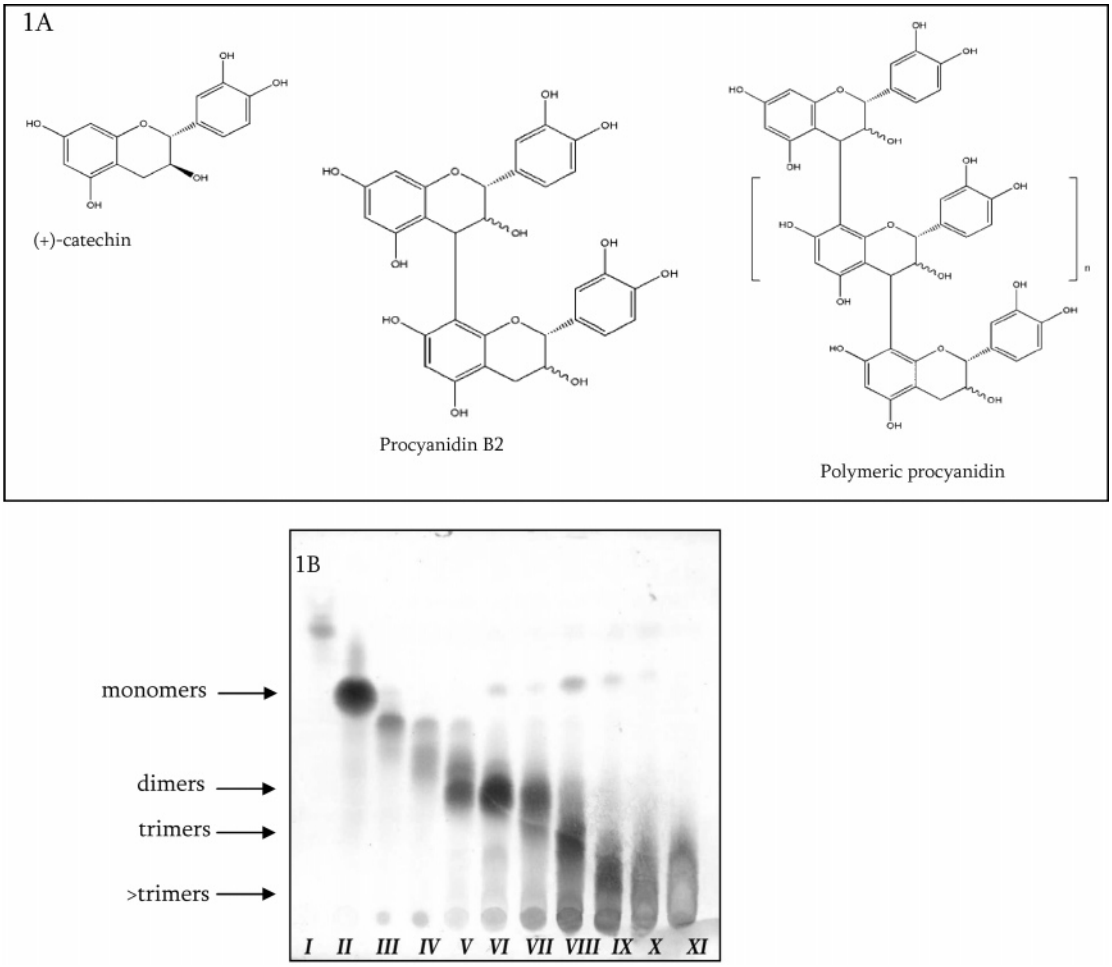

Figure 1. (**A**) Chemical structure of flavonoids. Procyanidins include from dimers to oligomers of (+)-catechin, (–)-epicatechin, and (–)-epicatechin gallate up to 10 units; further polymerized structures are classified as condensed tannins. (**B**) Polymerization degree of grape-seed procyanidin extract confirmed by TLC analysis.

(IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-1 and -6, or lipopolysaccaride (LPS) causing an increase in iNOS mRNA, protein, and activity levels that is followed by a significant increase in NO production over a long period of time. iNOS activation is regulated mainly at the transcriptional level but also at the translational and post-translational levels through effects on protein stability, dimerization, phosphorylation, cofactor binding, and availability of substrates (*14*, *15*).

The control of macrophage overproduction of inflammatory mediators such as PGE_2 and NO should greatly facilitate the treatment of inflammatory diseases. PGE_2 synthesis is catalyzed by cyclooxygenase-2 (COX-2), which is induced in macrophages by the same stimulus as iNOS (*14*, *16*).

Antiinflammatory molecules, such as aspirin and its derivatives (and other NSAIDs) at low therapeutic doses, irreversibly inhibit the activity of cyclooxygenases (COX-1 and COX-2) and the subsequent formation of prostaglandins, mainly PGE_2 (*17*). Other antiinflammatory drugs, such as indomethacin and dexamethasone, have been shown to inhibit NO and PGE_2 synthesis at different levels (*17*, *18*). The expression of iNOS and COX-2 is closely related to the up-regulation of nuclear factor kappa B (NFκB). NFκB, inducible transcription factor, is activated in response to various extracellular stimuli, including INF-γ, LPS, and oxidative stress. NFκB sites have been identified in the promoter region of the iNOS gene and the COX-2 gene. NFκB is present in the cytosol as an inactive complex IkB-NFκB. The IkB complex includes IkBα, -β, and -γ forms. The IkB−NFκB complex is phosphorylated by IkB kinase (IKK), which facilitate the translocation of free NFκB from cytosol to the nucleus and the induction of iNOS and COX-2 gene expression (*19*). The combination of LPS with IFN-γ induces iNOS and COX-2 expression synergistically. The regulation of iNOS and COX-2 via the NFκB pathway is an important mechanism in inflammatory processes and a potential site for intervention in inflammatory diseases (*13*, *20*).

The aim of this study was to examine the putative antiinflammatory effect of procyanidins in macrophages. We used RAW 264.7 cells stimulated with LPS and IFN-γ to mimic inflammation and specifically assessed the procyanidin effect in this model. Their effect on NO and PGE_2 production as well as their mechanism of action in these cells was studied.

MATERIALS AND METHODS

Chemicals. Grape-seed procyanidin extracts (PE) were provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the procyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%), and oligomeric (5−13 units) (31.7%) procyanidins and (4.7%) phenolic acids. Catechin, epicatechin, sulfanilamide, naphthylethylenediamide dihydrocloride, aspirin, indomethacin, dexamethasone, and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma-Aldrich, Inc. rmINF-γ was supplied by ProSpec-Tany TechnoGene Ltd. Anti-iNOS antibody and anti-p65 antibody were purchased from SantaCruz Biotechnology, Inc. Anti-beta actin antibody was purchased from Abcam. Monoclonal anti-rabbit IgG was purchased from Sigma-Aldrich, Inc. and ECL Advanced Western Blotting Detection Kit from Amersham Biosciences.

Chromatographic Separation of Procyanidin Extract. PE (0.5 g) was subjected to normal-column chromatography (35−70 mesh, Interchim, Monluçon, France) preconditioned with solvent A (acetone:hexane 65:35) as follows. PE components were separated according to size using an increasing gradient of solvent B (acetone:hexane 80:20). First, low molecular weight compounds were eluted with solvent A. Then, the proportion of solvent B was gradually increased until reaching 100% after 1 h. Then, further volume of solvent B was added, and fractions of 10 mL were collected using a fraction collector. Monitoring of the collected fractions was achieved by TLC on PolyGram silica gel 0.2 mm with fluorescent indicator UV_{254} (Macherey-Nagel, Hoerdt, France), in the mixture toluene:acetone:acetic acid (3:3:1, v/v). Revelation of TLC plates was done by spraying anisaldehyde reagent. Eleven major fractions with increasing degree of polymerization were identified, according to their R_f (**Figure 1B**). These fractions were lyophilized and kept at −20 °C until their utilization for the biological studies.

Mean degree of polymerization (mDP) for the fractions of study was determined by thiolysis according to the method described by Kennedy and Jones (*21*). Five representative fractions with different polymerization degree were selected. These fractions contained mainly monomers (fraction II mDP = 1.01 ± 0.02), dimers (fraction VI mDP = 2.13 ± 0.17), trimers (fraction VIII mDP = 2.71 ± 0.09 and IX mDP = 2.44 ± 0.02) and oligomeric procyanidins longer than trimers (fraction XI mDP = 2.98 ± 0.19).

Cell Culture. Murine macrophage cell line RAW 264.7 (European Tissue Culture Collection ECACC, ref 91062702, London, UK) was cultured in DMEM with or without phenol red containing 10% (v/v) fetal bovine serum, 2 mM D-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 25 mM HEPES. Cells were grown at 37 °C and with 5% CO_2 in fully humidified air and used for experiments between passages 5−14. At 80% of confluence, cells were stimulated with rIFN-γ (100 U/mL) and LPS (1 μg/mL).

Measurement of Cell Viability. Uptake of the neutral red dye was used as a measure of cell viability in response to procyanidin treatment. Assays were performed in triplicates.

Cell Treatment. At 80% of confluence, adherent monocyte-RAW 264.7 cells were incubated with different PE concentrations (0−85 μg/mL), catechin (0−65 μg/mL) or epichatechin (0−65 μg/mL), and PE fractions of monomers (fraction II), dimers (fraction VI), trimers (fraction VIII and IX) and longer than trimers (fraction XI) at 5−30 μg/mL and with different antiinflammatories such as aspirin (3 mM), indomethacin (5−20 μM), or dexamethasone (9 nM).

Three different treatments were performed: (A) Cells were incubated for 4 h with different compounds, and the culture medium was removed and then activated with 1 μg/mL LPS and 100 U/mL rmIFN-γ for 19 h. (B) Cells were incubated with different compounds (PE, aspirin, indomethacin, or dexamethasone) and with 1 μg/mL LPS and 100 U/mL rmIFN-γ simultaneously for 19 h. (C) Cells were activated with 1 μg/mL LPS and 100 U/mL rmIFN-γ for 15 h, and the different compounds tested were then added for 4 h. The culture medium for control and treated cells was collected and tested.

Measurement of NO Production in RAW 264.7 Cells. The nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction (*22*). A standard procedure using Griess reagent [1% (w/v) sulfanilamide, 12.5 mM naphthylenediamide, and 6.5 M HCl] was used. Optical density was measured with a microplate reader at 540 nm (Anthos 2000, Pierce Laboratories). Nitrite production was normalized to protein content measured by Bradford method (Bio-Rad). Results were expressed as the percentage of NO production versus that produced by stimulated cells.

Measurement of PGE_2 Concentration. The level of PGE_2 released into culture medium was quantified using a competitive specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham Biosciences). PGE_2 production was normalized to protein content measured by Bradford method (Bio-Rad). Results were expressed as the percentage of PGE_2 production versus that produced by stimulated cells.

iNOS and IkBα mRNA Analysis by RT-PCR Real Time. RNA from treated cells was isolated with High Pure RNA Isolation Kit from Roche. cDNA was synthesized from 1 μg of total RNA using oligo-dT and Superscript II Rnase Reverse Trancriptase (Life Technologies). A 20 ng amount of cDNA was subjected to Quantitative RT-PCR amplification using Sybr Green Master Mix (Applied Biosystems). The forward and reverse primers for iNOS were 5′ GGATCTTCCCAGGCAACCA 3′ and 5′ AATCCACAACTCGCTCCAAGATT 3′, respectively. The forward and reverse primers for IkBα were 5′ CTTGGTGACTTTGGGTGCTGAT 3′ and 5′ GCGAAACCAGGTCAGGATTC 3′, respectively. The forward and reverse primers for G3PDH were 5′ CATGGCCTTCCGTGTTCCT 3′ and 5′ CCTGCTTCACCACCTTCTTGA 3′, respectively. Reactions were run on a quantitative PCR System, and the thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min.

Immunoblotting Analysis of iNOS Enzyme. The cells were lysed with buffer L (0.01 M NaOH, 0.01% SDS, 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM $NaHPO_4 \cdot 12H_2O$). Equal amounts of total cellular protein (20 μg) were resolved by SDS-PAGE 7% under reducing conditions (90 V, 2 h) and transferred to nitrocellulose membranes (400 A, 2.5 h) in buffer T consisting of 20% (v/v) methanol, 200 mM Gly, 25 mM Tris, pH 8.3. The membrane was blocked for 1 h at RT and then incubated with anti-iNOS rabbit polyclonal antibody (1:500) or with anti-actin rabbit polyclonal antibody (1:10000) for 1 h at RT. Horseradish peroxidase-conjugated anti-rabbit antibody was used and incubated for 1 h. Immunodetection was performed using an ECL Advanced Western Blotting Detection Kit chemiluminescence system (Amersham Biosciences). The autoradiograms were quantified using scanning densitometry (Quantity One, Bio-Rad).

Cell Fractionation. Nuclear extracts were prepared as described (*23*, *24*). Briefly, after cell activation and treatment for the times indicated, 4×10^6 RAW 264.7 macrophages were washed twice with ice-cold PBS. A 100 μL of ice-cold hypotonic buffer (20 mM HEPES, pH 7.9, 10 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5% (p/v) Nonidet p40 and 1% (v/v) protease inhibitors (Sigma)) was added and left at 4 °C for 10 min on a rocking platform. Cells were scraped from the plates, and the suspension was centrifuged at 14000*g* for 3 min at 4 °C. Aliquots of the supernatant (cytoplasmatic fraction) were collected and stored at −80 °C. Sedimented nuclei were gently resuspended in 20 μL of ice-cold saline buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 200 mM NaCl, 10% (p/v) glycerol, 1 mM DTT, and 1% (v/v) protease inhibitors), left on ice for 30 min, vortexed, and centrifuged at 14000*g* for 5 min at 4 °C. The supernatant was collected (nuclear fraction) and stored at −80 °C. Protein was determined by the Bradford method (Bio-Rad Protein Reagent).

Western Blotting Analysis of NFκB (p65) Translocation after Procyanidin Treatment. We assayed three different times 0, 30, and 60 min after cell stimulation. A 40 μg amount of cytosolic and a 20 μg amount of nuclear extracts from RAW 264.7 cells were electrophoresed in 12% SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes and blocked with NFDM 3% in PBS. Membranes were incubated with polyclonal antibodies to p65 (1/500) or anti-actin from rabbit. Secondary anti-rabbit peroxidase bound antibody was used (Sigma-Aldrich). The immunodetection was performed using an ECL Advanced Western Blotting Detection Kit chemiluminescence system. The autoradiograms were quantified by densitometry.

Calculations and 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 pairwise comparisons. All calculations were performed using SPSS software.

RESULTS

Cell Viability. To assess the potential antiinflammatory effect of procyanidins, we worked on cell line RAW 264.7 of mouse monocytes/macrophages. First we checked the cytotoxicity of procyanidin extract administration to cells by the neutral red assay. We found a range of non-cytotoxic concentration of 0−85 μg/mL of PE extract. Cell viability was >98% at the concentrations treated, which confirms that the observed effects were not

Table 1. Neutral Red Assay in RAW 264.7 Macrophages after Procyanidin Treatment

procyanidin treatment	PE μg/mL	% neutral red in lysosomes[a]
4 h	control	100.00 ± 1.36
	0.86	102.29 ± 10.94
	8.6	98.79 ± 9.72
	46	112.67 ± 9.89
	85	117.85 ± 2.90
	430	89.51 ± 3.07*
	850	65.62 ± 1.36*
19 h	control	100.00 ± 1.25
	45	111.03 ± 3.99
	65	102.21 ± 4.02
19 h + LPS+IFN-γ	0	95.51 ± 4.77
	45	95.12 ± 1.37
	65	98.15 ± 3.00

[a] Cells were plated in 12-well plates and cultured in medium containing 40 μg/mL of neutral red. They were then incubated at 37 °C with PE (0–850 μg/mL) or vehicle for 4 h, PE (0–65 μg/mL) or vehicle for 19 h, or PE (0–65 μg/mL) or vehicle for 19 h with LPS+IFN-γ (1 μg/mL; 100 U/mL, respectively). The medium was removed and neutral red was extracted with 50% (v/v) ethanol, 49% water, 1% glacial acetic acid. The absorbance was measured at 540 nm. All values were taken from three experiments performed in triplicate. Data are mean ± SEM ($n = 3$). *, significant difference from control (PE = 0 μg/mL) in each treatment; $p < 0.05$.

due to cell death by treatment. Higher incubation periods (19 h) were also non-cytotoxic at the concentration tested (45−65 μg/mL). We also checked the cytotoxicity of LPS+IFN-γ (19 h) to cells by the neutral red assay, and we found non-cytotoxic effects (**Table 1**). In the case of pure compounds, the nontoxic range in this cell line was obtained from the literature (*25*).

Procyanidin Extract Inhibits NO Production in RAW 264.7 Macrophages Induced by LPS and IFN-γ. The PE induced an inhibition of NO production in RAW 264.7 cell line that was dose- and time-dependent. A 4 h preincubation with PE at 25 μg/mL reduced 20% of NO that was present in media compared with a reduction of 55% when pretreatment was performed during 10 h with the same PE concentration. When 4 h preincubation with PE was performed, NO production was significantly inhibited after induction with LPS+INF-γ at all tested concentrations (10−85 μg/mL) (**Figure 2A**).

When PE was administered for 19h of stimulation period, NO was strongly inhibited (only 40% of NO was present in media at 10 μg/mL compared with 90% when pretreatment was performed) **(Figure 2B)**. When PE was administered at the final stage of stimulation for 4 h, the inhibition of NO was significant beginning from 45 μg/mL **(Figure 2C)**.

As PE contains several compounds that could explain this inhibitory effect, we tested some pure compounds available in the extract at the concentrations expected to be in. Monomeric procyanidins epicatechin and catechin (5−65 μg/mL) did not exert any significant inhibition of NO synthesis in these cells after preincubation for 4 h (See **Figure 3**).

Procyanidin Extract Inhibits PGE_2 Production in RAW 264.7 Macrophages Induced by LPS and IFN-γ. The PE inhibited PGE_2 production in the RAW 264.7 cell line when procyanidins were coincubated with LPS+INF-γ for 19 h **(Figure 4B)**. When they were incubated during the last 4 h of stimulation, 45 μg/mL dose of PE significantly inhibited PGE_2 production (**Figure 4C**). A 4 h preincubation period with PE did not inhibit PGE_2 cell production (**Figure 4A**).

Procyanidin Extract Inhibition of NO and PGE_2 Production Is Comparable to Indomethacin, Aspirin, and Dexamethasone. We then compared the capacities of procyanidins

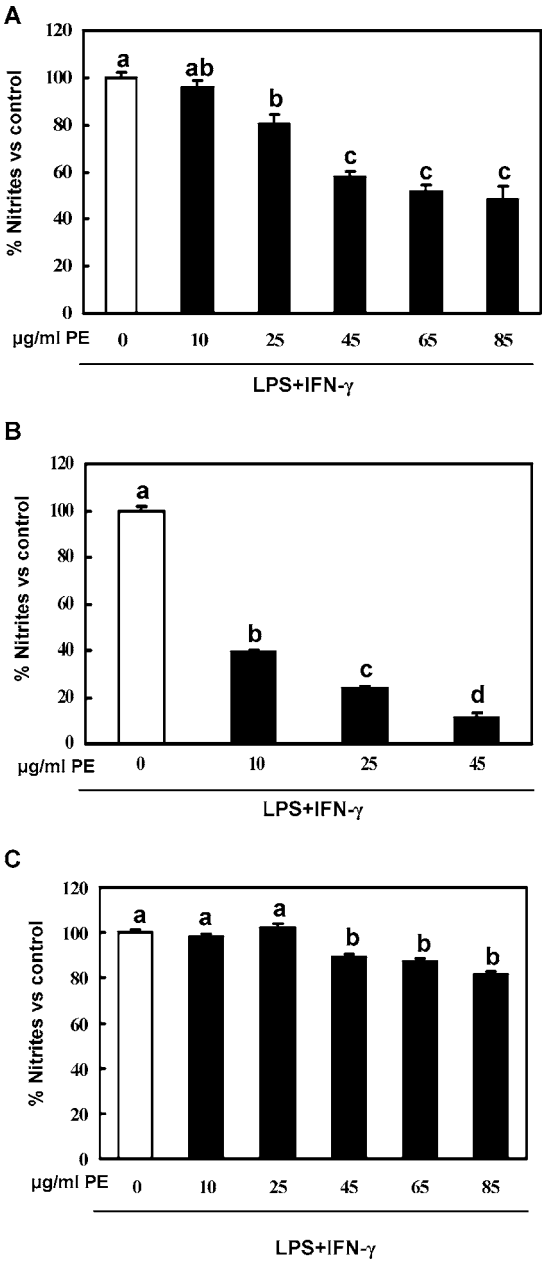

Figure 2. PE inhibition of NO production in LPS+IFN-γ induced RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 4 h (**A**), coincubated for 19 h (**B**) or incubated for the last 4 h of activation (**C**) with different PE concentrations. Cells were stimulated with LPS+INF-γ. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate.

and the selected drugs to inhibit NO and PGE_2 production by iNOS and COX-2 in RAW 264.7 cells exposed to LPS+IFN-γ, as shown in **Tables 2** and **3**. We observed that a 4 h preincubation with PE, aspirin, and indomethacin (only at 20 μM) caused a decrease in NO when compared to stimulated control. Aspirin (3 mM), indomethacin (20 μM), and PE (65 μg/mL) inhibited NO production by about 19, 38, and 42%, respectively ($p < 0.001$) when compared to stimulated control. In contrast, dexamethasone (9 nM) did not exert any inhibition on NO production. All treatments showed similar results in NO inhibition by antiinflammatories (see **Table 2**). The most effective inhibition of NO with PE was observed in the coincubation treatment. We also tested lower doses of aspirin

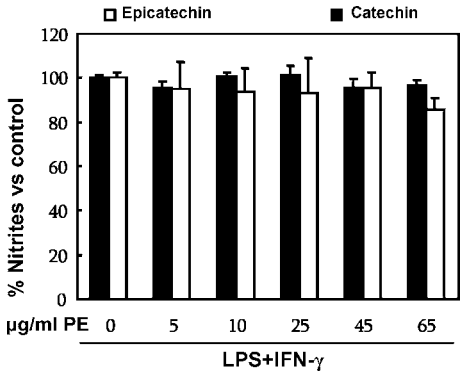

Figure 3. Monomeric procyanidins effect on NO production in LPS+IFN-γ induced RAW 264.7 macrophages. NO production after 4 h preincubation and subsequent stimulation with LPS+INF-γ for 19 h was measured. Each value represents mean ± SEM.

(0.015−1.5 mM), and we found the same inhibition as the high dose tested (3 mM).

We found inhibitory effects on PGE_2 production with all the selected antiinflamatories, except dexamethasone, in all the treatments performed. In contrast, PGE_2 production was inhibited when incubation with PE was performed during or at the end of the activation period but not by preincubation with PE (see **Table 3**). Although PE is an effective inhibitor of NO synthesis at tested concentrations, with respect to PGE_2 production it is not as effective as the drugs tested, which are more effective as PGE_2 inhibitors than are the NO inhibitors.

Modulation of iNOS Expression by Procyanidins. Several studies have shown that the induction of iNOS produces large amounts of NO during endotoxemia and under inflammatory conditions. Therefore, compounds that inhibit iNOS expression and/or enzyme activity decreased NO generation and may be beneficial in treating diseases caused by an overproduction of NO. In view of the involvement of iNOS in the inflammatory process, we monitored iNOS gene expression in macrophages exposed to PE.

We measured mRNA levels by real-time RT-PCR analysis. The expression of iNOS mRNA was hardly detectable in unstimulated cells. However, RAW 264.7 cells expressed high levels of iNOS mRNA when stimulated with LPS+IFN-γ for 19 h. Moreover, PE inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (**Figure 5A**). To determine the inhibitory mechanism of NO production from LPS-activated RAW 264.7 cells by PE, we checked the decrease in iNOS enzyme amount using the Western blotting technique. As **Figure 5B** shows, iNOS protein is barely detectable in unstimulated cells but it increased markedly 19 h after LPS (1 μg/mL) and IFN-γ (100 U/mL) treatment. Treatment with PE showed a concentration-dependent decrease in iNOS protein levels in LPS-stimulated RAW 264.7 cells that was statistically significant at the 45−85 μg/mL interval compared to control (**Figure 5**, table, lines 4−6).

Modulation of IkB-α mRNA Expression by Procyanidins. To assess the effect of PE on other NFκB target genes, other than iNOS, IkBα levels were measured by real-time RT-PCR analysis. The expression of IkBα mRNA was low in unstimulated cells. However, RAW 264.7 cells expressed high levels when stimulated with LPS+IFN-γ for 19 h. PE inhibited IkBα mRNA production in a dose-dependent manner (**Figure 5A**).

Differential Inhibitory Effects on NO Production and iNOS mRNA Expression Depending on the Procyanidin Fraction. The tested fractions induced an inhibition of NO production in RAW 264.7 cell line that was dose dependent.

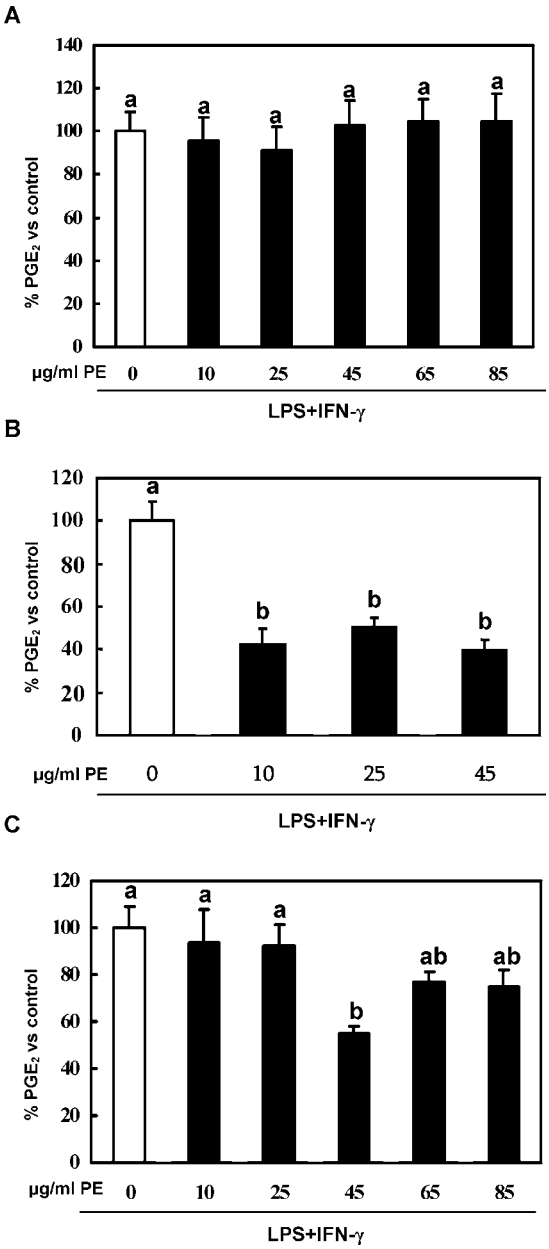

Figure 4. PE inhibition of PGE_2 production in LPS+IFN-γ induced RAW 264.7 macrophages. RAW 264.7 macrophages were preincubated for 4 h (**A**), coincubated for 19 h (**B**) or incubated for the last 4 h of activation (**C**) with different PE concentrations. Cells were stimulated with LPS+INF-γ and PGE_2 was measured after these treatments. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate.

The monomeric fractions did not exert any inhibitory effect on NO production coinciding with the results obtained with pure catechin and epicatechin.

When 4 h preincubation with the different fractions was performed, NO production was significantly inhibited after induction with LPS+INF-γ at all tested concentrations (5−30 μg/mL). This inhibitory effect increased with the polymerization degree of the procyanidins up to trimer and was dose dependent (**Figure 6A**).

To assess the effect of the procyanidin fractions on iNOS mRNA levels, we measured mRNA levels by real-time RT-PCR analysis. Our results show that trimeric and longer

Table 2. Effect of Procyanidins on NO Production in LPS+INF-γ Activated Macrophages Compared to Other Antiinflammatories

	% NO versus control		
treatment[a]	before activation	coincubation	4 h after activation
control	100.00 ± 6.70	100.00 ± 1.07	100.00 ± 1.41
3 mM aspirin	81.07 ± 4.87*	87.48 ± 6.00*	91.27 ± 3.07
5 μM indomethacin	85.89 ± 6.47	96.27 ± 4.04	89.36 ± 9.35
20 μM indomethacin	62.69 ± 2.79*	86.49 ± 2.58*	95.74 ± 1.16
9 nM dexamethasone	91.16 ± 4.93	100.02 ± 9.17	89.65 ± 2.12
65 μg/mL PE	38.64 ± 6.30*	11.05 ± 2.16*[b]	84.63 ± 1.79*

[a] RAW 264.7 macrophages were preincubated for 4 h, coincubated for 19 h, or incubated for the last 4 h of activation with PE 65 μg/mL, 3 mM aspirin, 5 and 20 μM indomethacin, or 9 nM dexamethasone. Cells were stimulated with LPS+INF-γ and NO production was measured after these treatments. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate. *, $p < 0.05$ as compared to control. [b] 45 μg/mL PE.

Table 3. Effect of Procyanidins on PGE_2 Production in LPS+IFN-γ-Activated Macrophages Compared to Other Antiinflammatories

	% PGE_2 versus control		
treatment[a]	before activation	coincubation	4 h after activation
control	100.00 ± 13.18	100.00 ± 6.78	100.00 ± 8.18
3 mM aspirin	13.67 ± 1.98*	1.20 ± 0.20*	31.41 ± 5.24*
5 μM indomethacin	10.33 ± 2.67*	0.43 ± 0.10*	10.11 ± 1.84*
20 μM indomethacin	9.74 ± 4.66*	0.47 ± 0.16*	11.64 ± 1.81*
9 nM dexamethasone	68.22 ± 1.77*	0.68 ± 0.16*	98.09 ± 7.53
65 μg/mL PE	96.75 ± 2.00	39.64 ± 5.31*[b]	83.54 ± 3.77*

[a] RAW 264.7 macrophages were preincubated for 4 h, coincubated for 19 h, or incubated for the last 4 h of activation with PE 65 μg/mL, 3 mM aspirin, 5 and 20 μM indomethacin, or 9 nM dexamethasone. Cells were stimulated with LPS+INF-γ, and PGE_2 was measured after these treatments. Results were normalized to control levels (100%). Each value represents mean ± SEM of three experiments performed in triplicate. *, $p < 0.05$ as compared to control. [b] 45 μg/mL PE.

oligomeric fractions inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (**Figure 6B**).

Procyanidin Extract Modulates NFκB Translocation to Nucleus. Because NFκB plays a key role in the induction of iNOS by LPS, we examined the effect of PE on this transcription factor. We observed that the incubation of cells with LPS+INF-γ produced an increase in NFkB translocation (p65 subunit) to the nuclear compartment that was evident at 30 min of incubation time. **Figure 7** shows that this translocation process is inhibited in cells pretreated with PE (65 μg/mL) before stimulation, which is demonstrated by the immunoblotting of the p65 protein in nuclear extracts. Maximal inhibition was at 60 min.

DISCUSSION

The results in the literature about the effect of procyanidins on NO metabolism are controversial (*25*−*28*). In agreement with our results, previous studies have shown that pine bark procyanidin extract (Pycnogenol) inhibited NO generation in RAW 264.7 macrophages (*26*). On the other hand, other authors reported a proinflammatory effect of trimer C2 and Pycnogenol in IFN-γ stimulated RAW 264.7 cells and antiinflammatory effect of monomers and dimers (*26*, *29*).

In this study, we show that PE acts as an antiinflammatory substance *in vitro*. We demonstrate that procyanidins inhibit PGE_2 production and regulate, at the transcriptional level, the amount of iNOS mRNA and protein and, consequently, NO production. We administered the bioactive substances before, during, and in the last period of cell stimulation. We found that PE protected cells from overproduction of inflammatory mediators, mainly NO and PGE_2 generation. PE inhibition of NO production was more effective when treatment with PE was performed during cell stimulation and for a longer period of time (*17*). Nevertheless, it was also observed in the other treatments. As far as we know, the effects of procyanidins on PGE_2 production have not been studied before in this cell model

Treatment	Relative iNOS mRNA	iNOS protein	Relative IKBα mRNA
Control	1.07 ± 0.12	100.00 ± 1.87	1.01 ± 0.04
PE µg/mL 10	0.58 ± 0.05 **	90.12 ± 7.09	0.81 ± 0.07*
25	0.45 ± 0.03 **	45.92 ± 7.29*	0.66 ± 0.05*
45	0.33 ± 0.05 **	18.63 ± 1.43*	0.56 ± 0.02*
65	0.16 ± 0.05 **	9.03 ± 1.45*	0.43 ± 0.01*
85	0.06 ± 0.01 **		0.51 ± 0.03*

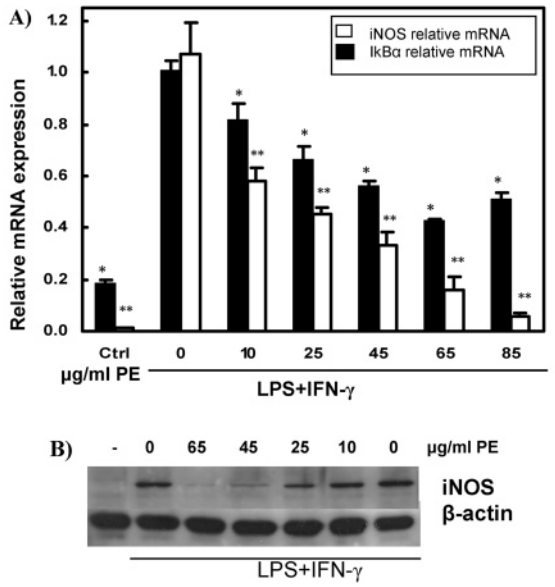

Figure 5. PE pretreatment of RAW 264.7 cells inhibits iNOS and IKBα gene expression and iNOS protein levels. Cells were preincubated with different PE concentrations for 4 h and then stimulated with LPS+INF-γ (1 μg/mL; 100 U/mL, respectively) for 19 h. After incubation, mRNA was extracted, cDNA was synthesized and measured by real-time RT-PCR (**A**). iNOS protein levels were determined by Western blotting in RAW 264.7 cells (**B**). Each value represents mean ± SEM of three experiments performed in triplicate. ∗, indicates significant differences from control group ($p < 0.05$). ∗∗, indicates significant differences from control group ($p < 0.0001$).

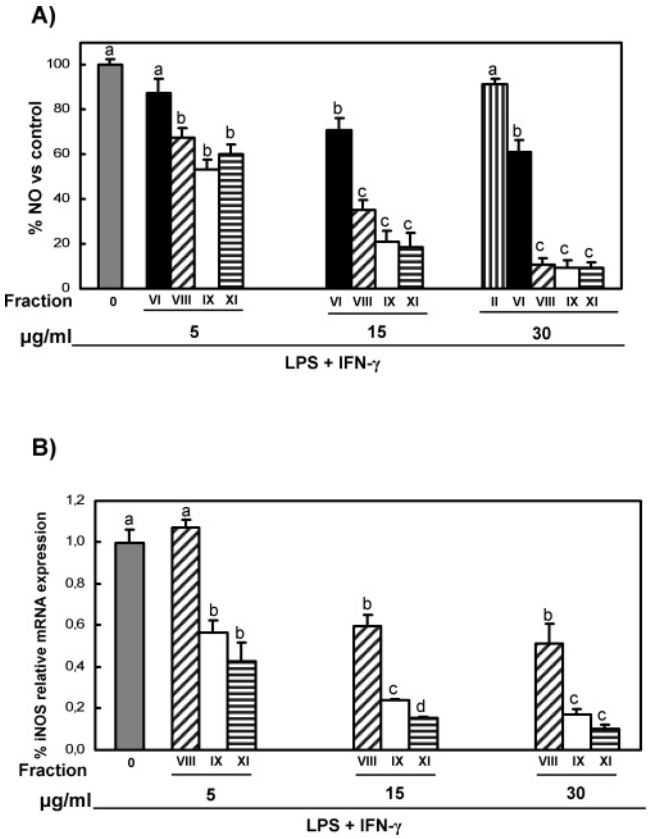

Figure 6. Effect of the procyanidin polymerization degree on NO production and iNOS mRNA expression. Cells were preincubated with the different fractions (5–30 μg/mL) for 4 h and then stimulated with LPS+INF-γ (1 μg/mL; 100 U/mL, respectively) for 19 h. Fractions tested were: II (monomers), VI (dimers), VIII (trimers), IX (trimers), and XI (oligomers longer than trimers). Results were normalized to control levels. Each value represents mean ± SEM of three experiments performed in triplicate. Effects were assessed using ANOVA ($p < 0.05$).

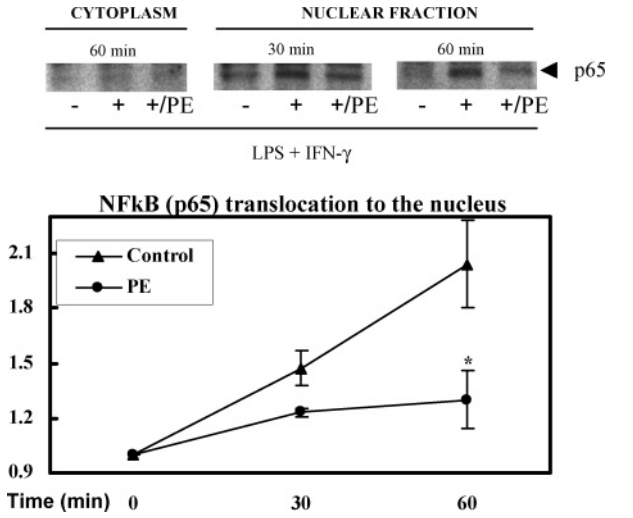

Figure 7. Effect of PE on p65 translocation. Cells were preincubated with PE (65 μg/mL) for 4 h and then stimulated with LPS+INF-γ (1 μg/mL; 100 U/mL, respectively) for 30 and 60 min. After cell fractionation, 20 μg of nuclear and 40 μg of cytosolic fractions were used for p65 Western blotting. Each value represents mean ± SEM of three experiments performed in triplicate.

although an inhibition of COX-2 expression by procyanidin dimer B2 has been recently described in human monocytes (*29*). We found that PE causes PGE_2 inhibition when it is administered during or at the end of the cell-stimulation period at concentrations of between 10 and 45 μg/mL but not with preincubation.

Procyanidins produced greater effect on NO production than on PGE_2 production in this cell model. In fact, the expression of COX-2 mRNA is regulated by several transcription factors including the cyclic-AMP response element binding protein (CREB), NFκB, AP-1, and the CCAAT-enhancer binding protein (C/EBP). COX-2 is also affected post-transcriptionaly, at the level of mRNA stability. Moreover, COX-2 can be affected directly at its enzymatic activity by nitric oxide. Then, each step of COX-2 regulation can be used as potential regulatory target (*30*). Despite our results showing an inhibitory effect of PE on NFκB translocation, there are many other regulatory targets for COX-2 expression and consequently, PGE_2 production, while, NFκB is the main regulatory step for iNOS expression and therefore NO production (*14*).

In this work, we compared the ability to inhibit NO and PGE_2 production in endotoxin-stimulated macrophages by PE (10−85 μg/mL), the classical NSAIDs (indomethacin, aspirin), and the glucocorticoid dexamethasone. Interestingly, the PE (65 μg/mL) inhibitory effect on NO production was substantially higher than the effect of indomethacin (5 and 20 μM), dexamethasone (9 nM), or aspirin (3 mM), which is at the upper range of plasma salicylate levels in individuals who are routinely taking high-dose aspirin. The mode of action of NSAIDs and dexamethasone is not yet fully understood (*31*) but their dose- and time-dependent inhibitory effect on iNOS and COX-2 activities has been established (*17*, *32*). Some studies indicate that the aspirin dose tested is probably not sufficient to block the transcription of iNOS gene (*17*). Furthermore, the NFκB-dependent transcription can be suppressed by NSAIDs at very high doses, which block IkB kinase activation (*33*).

Since iNOS is mainly regulated on the level of expression, the inhibitory effects of PE on NFκB translocation might be responsible at least in part of the inhibition of NO production. The concentrations tested of NSAIDs and dexamethasone might be ineffective on NFκB translocation and responsible for the difference found between commonly used antiinflammatories and PE on NO production.

On the contrary, NSAIDs exert a higher inhibition of PGE_2 production than PE. NSAIDs are traditional COX-2 activity inhibitors at pharmacological concentrations as shown in our results. Some authors have evaluated the role of NO on the activity of COX-2, and their data suggested that NO directly interacts with COX-2 to cause an increase in the enzymatic activity (*30*). Then, the PE inhibitory effect on PGE_2 production could be due to the diminished NO release, and consequently COX-2 activity is reduced. Moreover, this PE inhibitory effect of PGE_2 production was only shown when NO production was strongly inhibited in the coincubation treatment. Therefore, this may be one of the mechanisms of PE to reduce PGE_2 production. Further studies are needed to clarify the differences shown between PE and NSAIDs on NFkB, iNOS, and COX-2 regulation (*34*).

Since procyanidin extract is a complex mixture of different-sized monomers and polymers as well as phenolic acids, we determined which fraction in the extract is responsible for its effects. When trimeric or fractions of higher degree of polymerization were tested, we proved that dimers, trimers, and longer oligomers exerted an increasing degree of inhibition on NO production. We also tested some of the pure components of PE, including catechin and epicatechin, without any effect on NO synthesis.

To elucidate the mechanism by which PE inhibits NO production, we analyzed iNOS mRNA and total amount of iNOS enzyme. We found that PE strongly inhibited iNOS mRNA enzyme expression, which resulted in a decrease in iNOS protein. The iNOS enzyme inhibition mechanism was directly related to the reduction in the amount of NFκB(p65) in the cell nucleus. This resembles the mechanism described for other antioxidants such as anthocyanins and selenium, which are reported to suppress NO production in macrophages by mechanisms based on their ability to inhibit the activation of NFκB by reducing the intracellular redox state. This activation is critical in the induction of iNOS and is required to induce the expression of COX-2 in LPS-stimulated RAW 264.7 cells (*35*). Although no common second messenger has been identified, most NFκB activating signals can be inhibited by antioxidants because critical steps such as protein phosphorylation and binding of transcription factors to consensus sites on DNA are regulated by the intracellular redox status (*36*, *37*).

In RAW 264.7 macrophages, we propose procyanidins to act both by scavenging NO radicals (*38*) and by competition for TLR-4 (Toll Like Receptor-4) with LPS or by direct binding to LPS. This is reinforced by results shown in **Figure 2**, where coexistence of LPS and PE exert a higher inhibition of NO than that found when preincubation with PE alone was performed.

Procyanidins have been described to act by direct inhibiting NFκB target gene expression, although other sites of regulation of PE cannot be ignored. Our findings suggest that PE may exert antiinflammatory effect by inhibiting iNOS expression at transcriptional level by suppression of the NFκB signaling pathway.

Data presented herein show that PE treatment significantly inhibits NFκB translocation to the nucleus. These results suggest a possible inhibition of the degradation of IkB by regulation of IkB upstream proteins as IKK or 26S proteasome (*39*, *40*), but PE inhibition of NFκB DNA binding activity cannot be ruled out and has been described by others (*41*). We have found a down-regulation of IkBα that is a target gene of NFκB, thus reinforcing that PE could reduce NFκB activity.

The ability of procyanidins to ameliorate *in vivo* inflammation has been also reported in different animal models (*34*). The most of *in vivo* antiinflammatory effects are observed by intraperitoneal administration. Although, bioavailability of procyanidins *in vivo* is a matter of investigation, several studies indicate that some procyanidin dimers and trimers can be absorbed intact in rats and humans, but a possible oral effect is not characterized yet (*42*). Further studies are needed to confirm the role and pathophysiological implications of procyanidins *in vivo* in animal models of inflammation and in human disease.

ABBREVIATIONS USED

LPS, lipopolysaccharide; PE, procyanidin extract; NO, nitric oxide; iNOS, inducible nitric oxide synthase; INF-γ, interferon-γ; PGE_2, prostaglandin E_2; NFκB, nuclear factor-κB; COX-2, cyclooxygenase-2; NSAIDs, nonsteroidal antiinflammatory drugs; IkBα, alpha inhibitor of kappa beta.

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