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Enterodiol and Enterolactone Modulate the Immune Response by Acting on Nuclear Factor-κB (NF-κB) Signaling

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Lignan-rich whole-grain cereals, beans, berries, and nuts show protective effects against a variety of chronic diseases, including cancer. Lignans are converted by intestinal microflora to enterolactone (EL) and its oxidation product enterodiol (ED). To investigate the immunomodulatory effect of EL and ED in human cells, peripheral blood lymphocytes were treated with increasing physiologically relevant concentrations of EL and ED (0−1000 μM) and stimulated with lipopolysaccharide (LPS) and anti-CD3 plus anti-CD28 monoclonal antibodies. A dose-related inhibition of cell proliferation and cytokine production was observed, with EL being the most active. Molecular investigations in THP-1 cells showed that both EL and ED prevented inhibitory-κB (I-κB) degradation and nuclear factor-κB (NF-κB) activation, which in turn resulted in decreased tumor necrosis factor-α (TNF-α) production. EL and ED were also able to pass the intestinal barrier and modulate cytokine production. The findings of the present study reveal potential mechanisms that could explain some *in vivo* beneficial effects of lignans.

KEYWORDS: Lignans; inflammation; enterodiol; enterolactone; NF-κB; cytokine; proliferation

INTRODUCTION

Lignans are a group of polyphenolic compounds found in plants, particularly in flaxseed. Plant lignans considered relevant for biological activity are secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol (*1*). Most of the plant lignans in human foods are converted in the upper part of the intestine by aerobic microflora to enterolactone (EL) and enterodiol (ED), also called mammalian lignans or enterolignans (*2*, *3*). The plant lignan secoisolariciresinol diglucoside is a precursor of enterolignans. During this conversion, secoisolariciresinol diglucoside first undergoes hydrolysis to the aglycone secoisolariciresinol, which is then dehydroxylated and demethylated to ED; ED can then be oxidized to EL. Literature data extimated that the potential concentration of secoisolariciresinol diglucoside within the colon subjects consuming 50 g of flaxseed would be approximately 666 μM (*30*). The conversion degree to ED and EL depends upon the precursor; it can vary from less than 15 to 100%. For secoisolariciresinol and matairesinol, a conversion degree of 55−100% has been estimated (*4*). Enterolignans can be found in human plasma and urine of subjects consuming semi-vegetarian or lignan-rich diets and were reported to possess biological activities, such as estrogenic, anti-estrogenic, and antioxidant action (*5*−*7*). Interest in the use of lignans as functional food or as nutraceutical ingredients continues to grow, as a result of the increasing body of evidence, suggesting that fiber- and lignan-rich whole-grain cereals, beans, berries, nuts, and various seeds may have beneficial health effects, including anti-tumor, anti-inflammatory, anti-viral, and hepatoprotective properties (*6*, *8*−*10*). Experimental evidence in animals has also shown anticarcinogenic effects of flaxseed or pure lignans in many types of cancer (*11*−*13*). The antioxidant effect of enterolignans is of particular interest because many acute and chronic diseases are characterized by an oxidative stress component in the disease etiology, such as the initiation of cancer (*14*), autoimmune disorders (*15*), low-density lipoprotein (LDL) peroxidation in atherosclerosis (*16*), etc. The generation of reactive species is associated with the release of inflammatory mediators, such as growth factors, cytokines, chemokines, and lipid mediators, as well as with DNA damage, activation of signal pathways, trascription factors, and alteration in genes that are involved in carcinogenesis. In particular, several lines of evidence demonstrated a role of reactive oxygen species (ROS) in nuclear factor-κB (NF-κB) activation, which therefore represents a possible target for enterolignans. Furthermore, a great number of plant-derived substances have been shown to inhibit the NF-κB pathway, including lignans (i.e., manassantins, saucernetin, and saucerneol methyl ester), sesquiterpenes (i.e., parthenolide from *Tanacetum parthenium* L.), diterpenes, triterpenes, polyphenols, etc. (*17*).

NF-κB is a transcription factor consisting of homo- or heterodimeric subunits of the Rel protein family (*18*). In normal cells, NF-κB is sequestered in the cytoplasm as an inactive complex bound to inhibitory κB (I-κB) proteins (*18*). Exposure to lipopolysaccharide (LPS) activates the cellular I-κB kinase (IKK) complex through TLR/MyD88 complex-dependent pathways (*19*−*21*). Phosphorylated I-κB would then undergo ubiquitination and proteolysis by the 26S proteosome; the release of I-κB unmasks the nuclear localization signal and results in the translocation of

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NF-κB to the nucleus, followed by the activation of specific target genes (*19*, *22*). The NF-κB signaling pathway is described to be activated by ROS, and regardless of the stimulus or agonist, it was found to activate, owing to the production of ROS (*23*). NF-κB mainly controls the expression of genes involved in inflammation and other immune responses.

We focused our attention on NF-κB activation, because its activation has been implicated in the induction of inflammatory reactions, immune cell activation, tumor angiogenesis, and metastasis. Only few studies have investigated the effects of lignans on immune cell activation (*24*, *25*).

The purpose of this study was to investigate the immunomodulatory effects of EL and ED in human cells and the molecular mechanisms underlying such effects. Both peripheral blood leukocytes and cell lines were used. We could demonstrate that both EL and ED, by virtue, at least in part, of their antioxidant capacity, prevented I-κB degradation and NF-κB activation, which in turn resulted in decreased activation of immune cells. We could also demonstrate in an *in vitro* co-colture system using CaCo-2 and THP-1 cells the ability of EL and ED to pass the intestinal barrier and modulate cytokine production.

MATERIALS AND METHODS

Chemicals. EL, ED, secoisolariciresinol, lipopolysaccharide from *Escherichia coli* serotype 0127:B8, antibodies against I-κB, and β-actin as all cell culture reagents were from Sigma (St Louis, MO). Reagents and primers for real-time polymerase chain reaction (PCR) were from Applied Biosystems (Foster City, CA). Electrophoresis reagents were from Bio-Rad (Richmond, CA). All reagents were purchased at the highest purity available.

Cells. For all experiments using THP-1, cells (Istituto Zooprofilattico di Brescia, Brescia, Italy) were diluted to 10^6 cells/mL in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 International Units (IU)/mL penicillin, and 50 μM 2-mercaptoethanol, supplemented with 10% heat-inactivated fetal calf serum (FCS, media) and cultured at 37 °C in a 5% CO_2 incubator. For tumor necrosis factor-α (TNF-α) release, 0.5×10^6 cells were seeded in a 24-well plate, while for western blot analysis and mRNA expression, 4×10^6 cells were plated in 15 mL polypropilene tubes. Cells were incubated for different times with or without different concentrations of LPS in the presence or absence of increasing concentrations of enterolignans or dimethyl sulphoxide (0.2% final concentration) as a vehicle control, as described in the figure captions.

For the experiment using the human colon adenocarcinoma CaCo-2 cells (Istituto Zooprofilattico di Brescia), cells were grown at 37 °C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FCS, 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Cells were seeded in Transwell cell culture inserts (BD Italy, Buccinasco, Italy) and grown for 18−22 days. This time allows cells to differentiate in enterocytes and the separation of the apical from the basolateral compartment, reproducing the *in vivo* organization of the intestinal mucosa. To evaluate the effect of different concentrations of EL and ED on monolayer integrity, the transepithelial electrical resistance (TEER) was measured with a volt−ohm meter (Evohm, World Precision Instruments, Sarasota, FL), and the paracellular flux of the extracellular marker phenol red was assessed 24 h after treatment. Ethanol (20%) was used as a positive control. In some experiments, the basolater culture medium of cells apically treated with EL and ED was recovered (conditioned medium) and used to treat THP-1 cells, to assess immunodulatory effects.

Cytokine Production. For cytokine release, 0.5 mL of cells was seeded in 24-well plates and incubated with increasing concentrations of enterolignans for 1 h and then LPS was added at different times. Cytokine release was measured in cell-free supernatants obtained by centrifugation at 1200 rpm for 5 min and stored at −80 °C until measurement. Cytokine production was assessed by sandwich enzyme-linked immunosorbent assay (ELISA) (ImmunoTools GmbH, Friesoythe, Germany). Results are expressed in pg/mL. The limit of detection was 15.6 pg/mL for both TNF-α and interleukin (IL)-10.

Whole Blood Assay. Healthy subjects, enrolled among colleagues of the researchers, were selected according to the guidelines of the Italian Health authorities and the Declaration of Helsinki principles. Criteria for exclusion were abnormal laboratory values, medication known to affect the immune system, i.e., steroids and non-steroideal anti-inflammatory drugs, or patients suffering from malignancies, inflammations, and infections. All subjects signed an informed consent and were informed about methods and aims of the study. Blood samples (5 mL) were taken by venous puncture with 0.5 M sodium citrate as an anticoagulant.

Functional assays were performed using whole blood with sodium citrate as an anticoagulant. Sodium citrate was chosen instead of heparin or ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, because functional assays were performed using the whole blood assay and heparin may be contaminated with endotoxin, while EDTA interferes with cell activation. Blood was diluted 1:10 with cell culture medium RPMI 1640 (Sigma, St. Louis, MO) containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 IU/mL penicillin. Microcultures for the evaluation of mitotic response were set up in triplicates with 150 μL of diluted whole blood in 96-well plates in medium alone or with increasing concentrations of EL or ED or DMSO as a vehicle control (0.1% final concentration) in the presence of 0.01 μg/mL anti-CD3 plus 0.5 μg/mL anti-CD28 monoclonal antibodies. Cells were cultured for 72 h at 37 °C in a humidified 5% CO_2 incubator and were pulsed during the final 18 h with 1 μCi/well of ^{3}H-thymidine (Amersham, Little Chalfont, U.K.). Cells were harvested using a cell harvester (Dynatech, PBI, Milan, Italy), and the uptake of ^{3}H-thymidine was measured in a scintillation counter (Packard, Meriden, CO). Results are expressed as stimulation index (SI).

For the evaluation of cytokine production, macrocultures were set up in a 24-well plate containing 1 mL of 1:10 diluted whole blood, in medium alone or with increasing concentrations of EL or ED or DMSO as a vehicle control (0.1% final concentration) in the presence of 1 μg/mL LPS. For TNF-α release, cells were incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator.

Lactate Dehydrogenase (LDH). LDH was determined in cell-free supernatants using a commercially available kit (Takara Bio, Inc., Japan). Results are expressed as optical density (OD).

Real-Time Reverse Transcriptase (RT)-PCR. Total RNA was isolated using a commercially available kit (TriReagent from Sigma) as described by the manufacturer. For the synthesis of cDNA, 2.0 μg of total RNA was retro-transcribed using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA) following the instructions of the supplier. TNF-α gene expression was evaluated by real-time RT-PCR. For PCR analysis, Taq-Man-PCR technology was used. PCRs were performed in duplicate and according to the standard protocol suggested by the manufacturer. For each PCR reaction, 100 ng of total RNA was used. The 18S rRNA transcription was used as an endogenous reference, and the quantification of the transcripts was performed by the ΔΔCt method.

Western Blot Analysis. The activation of NF-κB was assessed by measuring cytosolic degradation of I-κB by western blot analysis. Briefly, cells were treated, after 1 h of incubation at 37 °C, to acclimatize cells, with enterolignans for 1 h, and then LPS was added for 30 min. Cells were then collected, washed once with phosphate-buffered saline (PBS), centrifuged, lysed in 100 μL of homogeneization buffer [50 mM tris(hydroxymethyl)-aminomethane (TRIS), 150 mM NaCl, 5 mM EDTA at pH 7.5, 0.5% Triton X-100, 50 μM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 1 μg/mL leupeptin], and denatured for 10 min at 100 °C. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad). For cytosolic I-κB and β-actin expression, 10 μg of extracted proteins was electrophoresed in a 12% sodium dodecyl sulfate (SDS)−polyacrylamide gel under reducing conditions. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, Little Chalfont, U.K.). The different proteins were visualized using I-κB (1:2500) and β-actin (1:5000) primary antibodies and developed using enhanced chemiluminescence (ECL, Amersham, Little Chalfont, U.K.). The image of the blot was acquired with the Molecular Imager Gel Doc XR (BioRad). The optical density of the bands was calculated and analyzed by means of the Image 1.47 program for digital image processing (Wayne Rasband, Research Service Branch, National Institute of Mental Health (NIMH), National Institutes of Health (NIH), Bethesda, MD).

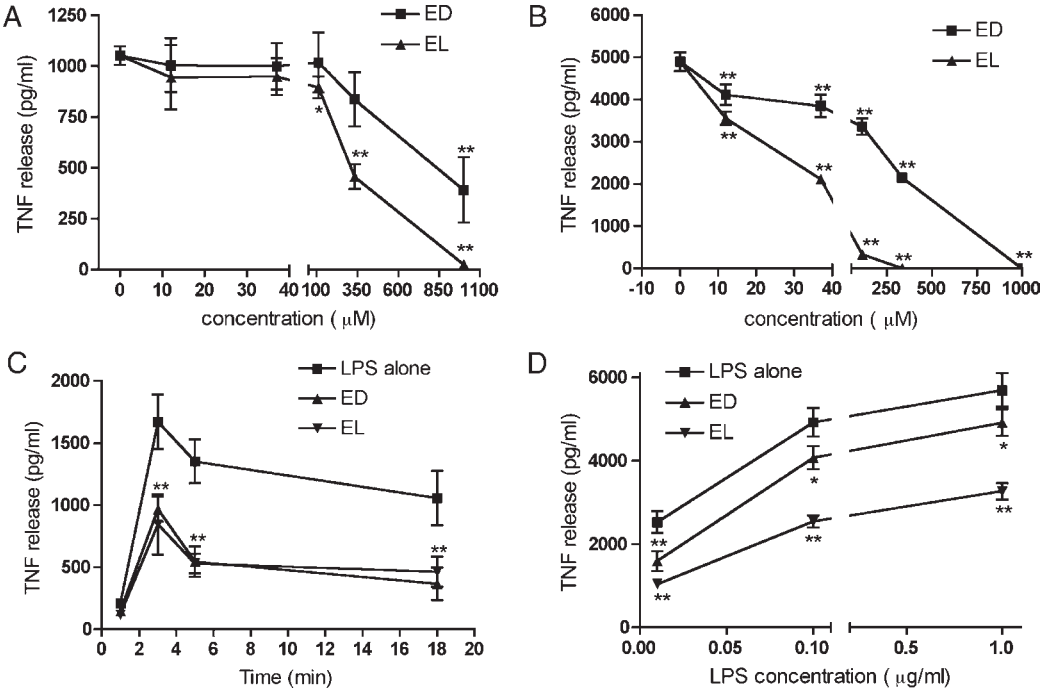

Figure 1. Enterolignans inhibit in a dose- and time-dependent manner LPS-induced TNF-α secretion. (A) Dose response in human whole blood: 1:10 diluted whole blood was treated for 1 h with increasing concentrations of ED, EL (1−1000 μM), or DMSO as a vehicle control, and then LPS (1 μg/mL) was added for 24 h. (B) Dose response in THP-1 cells: cells were treated for 1 h with ED, EL (0−1000 μM), or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added for 3 h. (C) Time course: THP-1 cells were treated for 1 h with ED, EL (110 μM), or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added at different times (1−18 h). (D) Effect of ED and EL on TNF-α release induced by increasing concentrations of LPS: THP-1 cells were treated for 1 h with ED, EL (110 μM), or DMSO as a vehicle control, and then LPS (0.01−1 μg/mL) was added for 3 h. TNF secretion was evaluated by specific ELISA. Each value represents the mean ± SD of three samples. Statistical analysis was performed with Dunnett's multiple comparison test, with (**) $p < 0.01$ versus cells treated with LPS alone.

Transient Transfections. A luciferase reporter plasmid with three NF-κB sites from the E-selectin promoter as described previously (*26*) and kindly provided by N. Marx (Department of Internal Medicine II-Cardiology, University of Ulm, Ulm, Germany) was used. THP-1 cells were transfected by the (diethylamino)ethyl (DEAE)−dextran method (*27*). Briefly, cells were exposed to a mixture of DNA−dextran (750 μg/mL final concentration) for 30 min, using 700 ng of NF-κB-luc reporter plasmid/1.5×10^6 cells. Cells were seeded in 12-well plates (1.5×10^6 cells/well) and then incubated for 48 h in complete medium (FCS-supplemented RPMI-1640). Cells were treated with increasing concentrations of enterolignans for 1 h and then in the presence or absence of LPS (0.1 μg/mL) for 3 h. At the end of the incubation, Britelite Plus reagent (Perkin-Elmer, Milan, Italy) was added (100 μL/well). The luciferase assay was performed using a luminometer (VictorX3, Perkin-Elmer, Milan, Italy). Results are expressed as luciferase activity normalized by protein content and represent the mean ± standard deviation (SD) values of three experiments performed in triplicate. Parthenolide was used as an inhibitor of NF-kB-driven transcription (82% inhibition at 20 μM).

NF-κB (p65) Translocation. Nuclear extracts were prepared essentially as described by Schreiber et al. Briefly, after treatment, 4×10^6 cells were resuspended in 0.4 mL of a hypotonic lysis buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1 mM PMSF]. Cells were incubated on ice for 15 min, and then 25 μL of a 10% Nonidet P-40 solution was added. Cells were mixed for 15 s and then centrifuged for 30 s at 12 000 rpm. Pelleted nuclei were suspended in 50 μL of buffer C (50 mM HEPES at pH 7.8, 50 mM KCl, 300 mM NaCl, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF), mixed for 20 min, and centrifuged for 5 min at 12 000 rpm. The supernatants represent the nuclear extracts. Protein concentrations in both fractions were measured using a commercial kit (Bio-Rad). Nuclear extracts were used to assess NF-κB (p65) translocation using a commercially available kit (Cayman Chemical, Ann Arbor, MI) that allows for the detection of specific transcription factor activities in cell extracts using an ELISA-based format.

Statistical Analysis. All experiments were reproduced at least 3 times, and where indicated, representative experiments are shown. Data are expressed as mean ± SD. Statistical analyses were determined with GraphPad Prism 4 software using the unpaired *t* test or a multiple comparison test, as indicated in the figure captions. Significance was set at $p \leq 0.05$.

RESULTS

Effect of Enterolignans on Human Peripheral Blood Leukocytes. The whole blood assay was used to assess the immunomodulatory effects of ED and EL. Peripheral blood obtained from health volunteers was 1:10 diluted and treated with increasing concentrations of ED and EL (0−1 mM) for 1 h, and then LPS (1 μg/mL) or anti-CD3 plus anti-CD28 monoclonal antibodies were added for 24 and 72 h, respectively. In **Figure 1A**, the effect of ED and EL on LPS-induced release of TNF-α is reported. Both enterolignans induced a dose-related decrease in TNF-α release, with EL being more active. The calculated IC_{50} values for TNF-α release were 800 and 430 μM for ED and EL, respectively. The inhibitory effect was not limited to TNF-α, because LPS-induced IL-10 release was similarly suppressed by both ED and EL: 197 ± 23 pg/mL in LPS-treated diluted whole blood, 91 ± 12 pg/mL in 333 μM ED-treated cells, and 14 ± 10 pg/mL in 333 μM EL-treated cells ($p < 0.01$). The immunomodulatory effect was not limited to cytokine production, because the proliferative response of T cells to the physiological stimuli anti-CD3 plus anti-CD28 was suppressed as well (**Table 1**). The calculated IC_{50} values for T cell proliferation were 530 and 300 μM for ED and EL, respectively.

Effect of Enterolignans in THP-1. As found in peripheral blood leukocytes and also in the human promyelocytic cell line THP-1, ED and EL were able to modulate in a dose- and time-dependent

Table 1. EL and ED Modulated Anti-CD3 plus Anti-CD28 Monoclonal Antibody-Induced Lymphocyte Proliferation[a]

treatment	ED[b]	EL[b]
vehicle control	34.9 ± 3.0	34.9 ± 3.0
4.1 μM	34.1 ± 6.9	34.8 ± 0.4
12.3 μM	32.8 ± 0.5	31.1 ± 1.6
37 μM	31.8 ± 1.2	27.0 ± 2.4[c]
111 μM	28.8 ± 1.0[c]	17.9 ± 2.6[c]
333 μM	21.8 ± 1.2[c]	3.6 ± 0.7[c]
1000 μM	4.1 ± 0.1[c]	0.1 ± 0.1[c]

[a] Whole blood obtained from healthy donors was diluted 1:10 in culture medium and treated with increasing concentrations of EL or ED or DMSO as a vehicle control (0.1% final concentration) in the presence of 0.01 μg/mL anti-CD3 plus 0.5 μg/mL anti-CD28 monoclonal antibodies for 72 h. Cell proliferation was assessed by ^{3}H-thymidine incorporation. [b] Results are expressed as SI. Each value represents the mean ± SD, with $n = 3$. [c] Dunnett's multiple comparison test, with $p < 0.01$ versus cells treated with anti-CD3 plus anti-CD28 monoclonal antibodies alone (vehicle control).

Table 2. Secoisolariciresinol Modulates, in a Concentration-Dependent Fashion, LPS-Induced TNF-α Release in THP-1 Cells[a]

treatment	TNF-α (pg/mL)
vehicle control	5 ± 8
LPS	1429 ± 241
125 μM secoisolariciresinol	355 ± 57[b]
500 μM secoisolariciresinol	255 ± 41[b]
1000 μM secoisolariciresinol	136 ± 27[b]
1000 μM EL	2 ± 4[b]
1000 μM ED	10 ± 3[b]

[a] Cells were treated with increasing concentrations of secoisolariciresinol or DMSO as a vehicle control (0.1% final concentration) in the presence or absence of LPS for 3 h. TNF-α release was assessed by ELISA. Results are expressed as pg/mL. Each value represents the mean ± SD, with $n = 3$. [b] Dunnett's multiple comparison test, with $p < 0.01$ versus cells treated with LPS alone.

manner LPS-induced TNF-α release (panels **B** and **C** of **Figure 1**), with EL being the most active compound. The calculated IC_{50} values for TNF-α release were 450 and 130 μM for ED and EL, respectively. As shown in **Figure 1D**, both ED and EL were able to significantly modulate the release of TNF-α induced by a wide range of LPS concentrations (10−1000 ng/mL). Similarly, the precursor of ED and EL, secoisolariciresinol, as shown in **Table 2**, was able to modulate LPS-induced TNF-α release. The calculated IC_{50} value for TNF-α release in this case was 230 μM.

The immunomodulatory effects observed in both peripheral blood leukocytes as well as THP-1 cells were not due to cytotoxicity, because no cytotoxicity was observed up to 72 h of treatment with ED and EL (1 mM) in both THP-1 and peripheral blood cells, as assessed by LDH leakage (data not shown).

Characterization of the Molecular Mechanism of Action of Enterolignans. Mirroring the data obtained with human peripheral leukocytes, THP-1 cells resulted in being a good *in vitro* model to study the molecular mechanisms underlying enterolignan-inhibited TNF-α production. To investigate if enterolignans acted at the pre- or post-transcriptional level, the LPS-induced TNF-α mRNA level was evaluated by real-time PCR. THP-1 cells were treated for 1 h in the presence or absence of ED and EL (333 and 1000 μM) or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added for 1 h. The TNF-α mRNA level was evaluated after 1 h, because in previous experiments, we found that, in THP-1 cells, TNF-α mRNA peaks at 1 h and declines thereafter (*28*). As shown in **Figure 2A**, in vehicle-treated cells, a significant increase in the TNF-α mRNA level was detected. On the contrary, in cells treated with both ED and EL, the LPS-induced TNF-α mRNA level was significantly reduced, indicating that the enterolignans acted at the pre-transcription level.

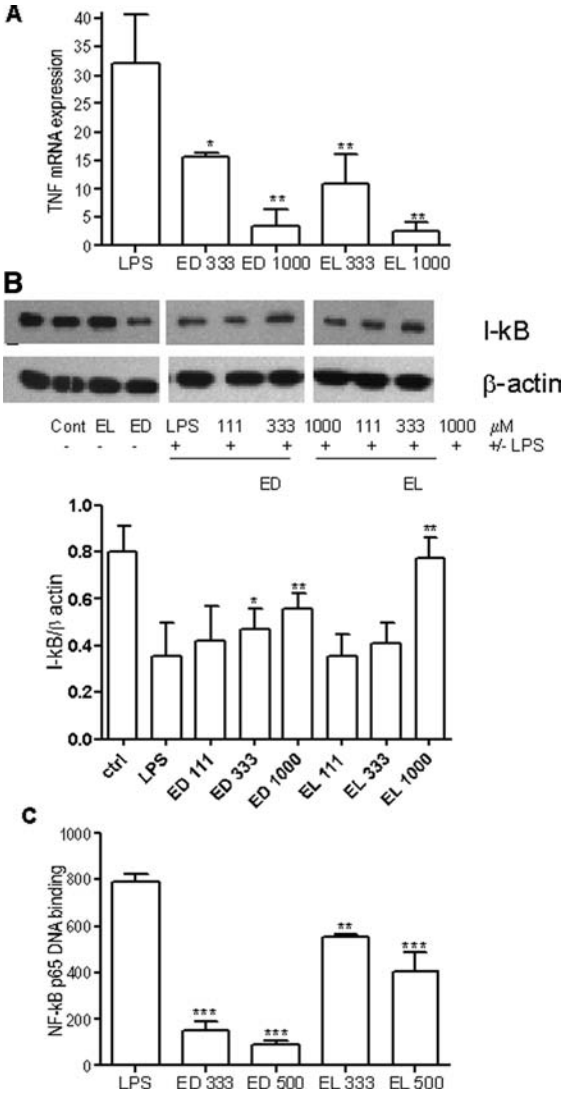

Figure 2. Enterolignans modulate LPS-induced TNF-α mRNA expression and NF-κB activation. (A) ED and EL modulated LPS-induced TNF-α mRNA expression: TNF-α mRNA expression was evaluated by real-time PCR. THP-1 cells were treated for 1 h with ED, EL (333−1000 μM), or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added for 1 h. Results are expressed as $2^{-\Delta\Delta Ct}$. Each value represents the mean ± SD of three independent experiments. (B) ED and EL modulated LPS-induced NF-κB activation, representative western blot of NF-κB activation as assessed by I-κB degradation, and the relative densitometric analysis: THP-1 cells were treated for 1 h with ED, EL (111−1000 μM), or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added for 30 min. Each value represents the mean ± SD of three independent experiments. (C) NF-κB p65 nuclear translocation: THP-1 cells were treated for 1 h with enterolignans (333−500 μM) or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added for 30 min. Each value represents the mean ± SD of four samples. Statistical analysis was performed with Dunnett's multiple comparison test, with (*) $p < 0.01$ versus untreated control cells and (**) $p < 0.01$ versus cells treated with LPS alone.

It is known that LPS-induced TNF-α production is dependent upon NF-κB activation. Thus, we then investigated the effect of ED and EL on LPS-induced NF-κB activation. Cells were treated for 1 h in the presence or absence of increasing concentrations of enterolignans (111, 333, and 1000 μM) or DMSO as a vehicle control, and then LPS (0.1 μg/mL) was added. As shown in **Figure 2B**, both ED and EL prevented in a dose-dependent manner LPS-induced I-κB degradation, the latest required for

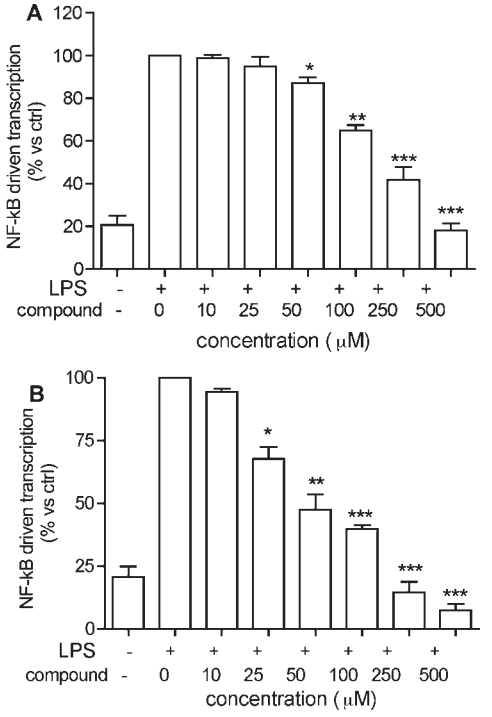

Figure 3. (A) ED and (B) EL reduce luciferase expression from a NF-κB-dependent reporter construct. THP-1 cells were transfected as described in the Materials and Methods and treated for 1 h with increasing concentrations of ED and EL (0−500 μM) and then in the presence or absence of LPS (0.1 μg/mL). Cells were lysed after 3 h, and luciferase activity was measured and normalized to the protein concentration. Results are expressed as normalized luciferase activity per microgram of protein. Each value represents the mean ± SD of three experiments performed in triplicate. Statistical analysis was performed with Dunnett's multiple comparison test, with (**) $p < 0.01$ versus cells treated with LPS alone.

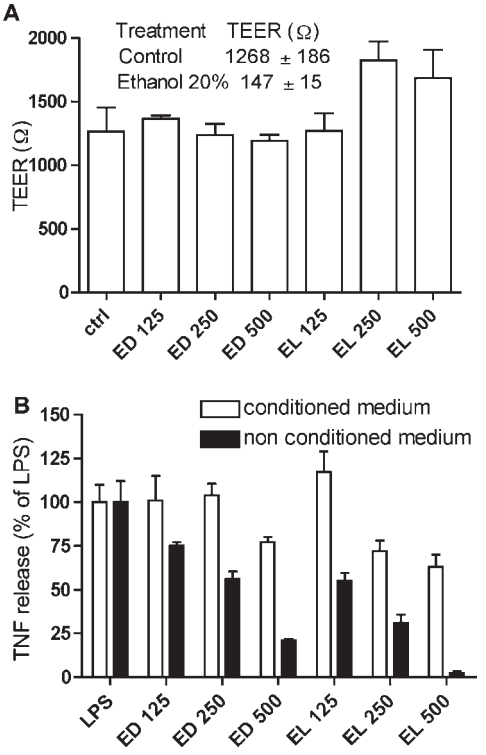

Figure 4. Effect of ED and EL on CaCo-2 integrity and their passage in the basolateral compartment. (A) Effect of ED and EL on the TEER in differentiated CaCo-2 cells: Ethanol (20%) was used as a positive control (inset). CaCo-2 cells were untreated (control) or treated with increasing concentrations of ED and EL. TEER, expressed as Ω, was measured after 24 h. (B) Immunomodulatory effect of CaCo-2 conditioned media on LPS-induced release of TNF-α in THP-1 cells: CaCo-2 cells were exposed through the apical side with different concentrations of ED and EL or DMSO as a vehicle control for 24 h. The basolateral medium was collected and used to treat THP-1 cells. THP-1 cells were cultured for 1 h with the CaCo-2 conditioned medium or with fresh medium containing increasing concentrations of ED or EL, and then LPS (0.1 μg/mL) was added. TNF-α release was evaluated 3 h later by ELISA. Each value represents the mean ± SD of three experiments performed in triplicate. Statistical analysis was performed with Dunnett's multiple comparison test, with (**) $p < 0.01$ versus cells treated with LPS alone.

NF-κB translocation from the cytosol to the nuclei. It is known that LPS-induced TNF production is dependent upon NF-κB activation. To strengthen the results of the modulatory effect of enterolignans on LPS-induced NF-κB activation, the NF-κB p65 DNA binding was assessed by ELISA. As shown in **Figure 2C**, LPS induced a significant NF-κB p65 DNA binding, which was modulated in a dose-related manner by both EL and ED. Parthenolide (20 μM), a selective NF-κB inhibitor, was used as a positive control (around 75% inhibition).

To determine if specific regulatory factors involved in TNF-α transcription, i.e., NF-kB, were attenuated by enterolignans, THP-1 cells were transiently transfected with luciferase reporter plasmid construct containing three NF-κB sites and the effect of enterolignans on luciferase activity, as an indicator of the promoter activity, was measured. Protein concentrations were determined and used to normalize luciferase activity for each sample. Cells transfected showed a significant dose-dependent decrease in LPS-induced luciferase activity relative to the control after treatment with both ED (**Figure 3A**) and EL (**Figure 3B**), confirming NF-κB as an intracellular target of enterolignans. Also, in this case, EL was more potent than ED. Parthenolide (20 μM), a selective NF-κB inhibitor, was used as a positive control (around 80% inhibition).

Taken together, our data indicate that enterolignans, interfering with LPS-induced NF-κB activation, prevented transcription and translation of TNF-α, resulting in a decrease in the release of this cytokine in monocytes.

Effect of Enterolignans on Intestinal Membrane Integrity. We used an *in vitro* model of human intestinal CaCo-2 cells to differentiate mature enterocytes after 18−21 days of culture, to evaluate the effect of enterolignans on membrane integrity. It has been shown that human colon epithelial cells, including CaCo-2 cells, can rapidly uptake, metabolize, and excrete the enterolignans ED and EL (*29*). In fact, EL-glucoronide, EL sulfate, and ED-glucoronide have been identified in cultured epithelial cells (*29*).

CaCo-2 cells were differentiated on Transwell filters, which allow for the separation of the apical from the basolateral compartment, to reproduce the *in vivo* organization of the intestinal mucosa. To evaluate the effect of ED and EL on monolayer integrity, the TEER and the paracellular flux of the extracellular marker phenol red were measured. As shown in **Figure 4A**, both ED and EL up to 500 μM did not affect the intestinal membrane integrity. Similar results were also obtained treating CaCo-2 cells with ED and EL at 1 mM concentration (data not shown). On the contrary, the positive control ethanol (20%) significantly affected membrane integrity and permeability, as assessed by TEER (inset in **Figure 4A**) and phenol passage (223 ± 52 μmol h^{-1} cm^{-2}), respectively. The passage of phenol red was absent for both ED and EL and in control cells (data not shown).

Modulatory Effects of CaCo-2 Conditioned Medium on LPS-Induced TNF-α Release in THP-1 Cells. We then investigated the effect of the basolateral conditioned medium of CaCo-2 cells treated on the apical side with increasing concentrations of ED and EL on LPS-induced TNF-α release. As shown in **Figure 4B**, at concentrations achievable in the colon lumen after intake of lignan-rich nutrients (10−1000 μM), conditioned medium was able to modulate LPS-induced release of TNF-α in THP-1 cells, indicating that some ED and EL present in the intestinal lumen can pass the enterocytes (differentiated CaCo-2) and modulate immune cell functions (i.e., TNF-α release in THP-1). In the same experiment, THP-1 cells were directly treated with the same solution used to treat the apical side of CaCo-2 cells (non-conditioned medium). As shown in **Figure 4**, in this case, the inhibitory effect achieved was significantly greater, suggesting that either only a small fraction of ED and EL can pass the intestinal mucosa in 24 h or, conversely, conjugated ED and EL by CaCo-2 lack immunomodulatory capacity.

DISCUSSION

The mechanisms and the molecular targets of different plant-derived compounds that modulate the immune system can be different. Part of the beneficial effects of enterolignans can be ascribed to their antioxidant activity. We could demonstrate that enteroligans modulate the immune response by suppressing lymphocyte proliferation and cytokine production by targeting the activation of transcription factor NF-κB. In all instances, EL was more effective than ED. Overall, these data contribute to our understanding of the action of enterolignans, which could explain some of their *in vivo* beneficial effects.

The concentrations used in the present study are physiologically relevant; indeed, it has been calculated that the concentrations achievable in the colon lumen are 10−1000 μM (*30*). The concentrations of lignans observed in plasma, from either rodent model or clinical studies (*16*, *31*) are, however, below 1 μM. Nevertheless, lignans are abundant in whole-grain cereals, beans, berries, and nuts, which are widely consumed as dietary components; thus, it is reasonable to assume that plasma concentrations higher than 1 μM might be reached *in vivo*. In addition, one should also consider that typically lignans are consumed daily for long periods of time, which may lead to biological activities consistent with those described in the present paper. In our hands, concentrations close to 10 μM were able to significantly reduce cytokine release and T-cell proliferation. Most importantly, we found in an *in vitro* model of intestinal mucosa that ED and EL, at concentrations that can be achieved in the intestinal lumen, do not alter the membrane integrity, and by the ability of enterocytes to uptake and excrete enterolignans, we found that the immunomodulatory effects, observed directly treating immune cells, could be maintained in the basolateral conditioned medium obtained from CaCo-2 cells exposed to ED and EL on the apical side. This *in vitro* experiment clearly indicates that enterolignans can maintain their bioactivity once absorbed.

ED and EL have been reported to be effective inhibitors of lipid peroxidation at 10 and 100 μM concentrations, albeit EL was more effective than ED (*30*). ED and EL were also effective against DNA damage (*7*), whereas both compounds at 100 μM did not affect cytokine secretion in human leukocytes (*25*). In our experimental conditions, higher concentrations were tested, which resulted in a dose-related inhibition of cytokine production. The free-radical-scavenging activity of mammalian enterolignans can potentially confer protection against oxidative stress and lipid peroxidation, particularly in colon epithelial cells exposed to these compounds during microflora metabolism of plant lignans into mammalian lignans. The antioxidant activity can also account for the immunomodulatory effects described in the present paper.

LPS can trigger multiple signaling molecules, such as protein tyrosine kinase, protein kinase C, Ras, Raf-1, I-κB kinase, methyl ethyl ketone (MEK), mitogen-activated protein kinases, etc. Subsequently, the signals further transduce to downstream pathways and activate numerous transcription factors, including AP-1, NF-κB, and ATF-2. This in turn induces a large number of genes encoding for inflammatory mediators and cytokines. These pathways may converge or diverge or cross-talk, which makes signaling networks complicated. There are several indications that ROS may act as cellular second messengers, and it has been demonstrated that H_2O_2 can activate NF-κB (*23*, *32*), which regulates the expression of many inflammatory mediators, including TNF-α.

We could demonstrate by western blot analysis that enterolignans inhibited LPS-stimulated degradation of the cytosolic NF-κB inhibitor I-κB, and using a luciferase reporter plasmid constructs containing three NF-κB sites, a concentration-related inhibition in LPS induced promoter activity, confirming NF-κB as an intracellular target of enterolignans.

The following scenario can be proposed: enterolignans interfering with LPS-induced ROS generation block IKK activation and I-κB phosphorylation and degradation and prevent NF-κB nuclear translocation and cytokine gene expression and subsequent secretion. The inhibitory effect of enterolignans on NF-κB activation can also account for defective T-cell proliferation in response to anti-CD3 plus anti-CD28, because NF-κB activation has a pivotal role in the regulation of lymphocyte activation and proliferation (*33*, *34*). The ability of enterolignans to modulate NF-κB activation can provide a possible mechanism for their anti-inflammatory and anti-tumor actions. NF-κB can indeed control a large number of genes involved in the induction and maintenance of inflammatory reactions, angiogenesis, metastasis, and cell survival (*35*, *36*). Prolonged or imbalanced activation of NF-κB is also implicated in the pathogenesis of inflammatory disorders, such as asthma, arthritis, and inflammatory bowel disease (*37*−*39*). There is no doubt that NF-κB signaling cascade constitutes a promising therapeutic target for inflammatory and autoimmune disorders (*40*).

In summary, our data indicate that enterolignans interfering with the NF-κB pathway modulated immune cell activation. The characterization of specific interference with cell signaling ascribable to lignans can lead to a better understanding of the molecular mechanism of action, and in particular, these data contribute to highlight the action of enteroligans that could explain some of their *in vivo* beneficial effects.

ABBREVIATIONS USED

ED, enterodiol; EL, enterolactone; IL, interleukin; I-κB, inhibitory-κB; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; SI, stimulation index; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide.

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