"Among Plant Lignans, Pinoresinol Has the Strongest Antiinflammatory Properties in Human Intestinal Caco-2 Cells"

During, Alexandrine ; Debouche, Céline ; Raas, Thomas ; Larondelle, Yvan

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Dietary lignans show some promising health benefits, but little is known about their fate and activities in the small intestine. The purpose of this study was thus to investigate whether plant lignans are taken up by intestinal cells and modulate the intestinal inflammatory response using the Caco-2 cell model. Six lignan standards [secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), pinoresinol (PINO), lariciresinol, matairesinol (MAT), and hydroxymatairesinol] and their colonic metabolites [enterolactone (ENL) and enterodiol] were studied. First, differentiated cells were exposed to SDG, SECO, PINO, or ENL at increasing concentrations for 4 h, and their cellular contents (before and after deconjugation) were determined by HPLC. Second, in IL-1β-stimulated confluent and/or differentiated cells, lignan effects were tested on different soluble proinflammatory mediators quantified by enzyme immunoassays and on the NF-κB activation pathway by using cells transiently t...

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QUERIES - jn162453q

[AG1] UCLouvain correct as expanded?

[AG2] HMR defined according to definition in text. Please check. Please also define PGE2.

[AG3] Should the phrase "whether plant lignans are taken up" be clarified? E.g., "taken up by cells in the small intestine..."

[AG4] “which is a saturable process” ok as edited?

[AG5] Please spell out PGE2.

[AG6] “mg” ok as edited for LARI value?

[AG7] “mice with induced” ok as edited?

[AG8] If the “respective” cell values correspond to “confluence” and “full differentiation”, please place “, respectively,” after “(21 d)” accordingly.

[AG9] Are all brand products listed in sentence beginning “The Thermo Separation HPLC…”? If so, add “all from” before “ThermoFinnigan” in parentheses. If not, please provide name of manufacturer for each brand product.

[AG10] A and B appear to refer to the 2 mobile phases; however, please amend wording to clarify “B increase from ...”.

[AG11] “were well separated after the following times” or similar intended? Please check wording for clarity.

[AG12] Check edits in sentences beginning “After treatment with or without ... of the polyphenol ...”.

[AG13] “performed” instead of “carried on” ok as edited?

[AG14] “form” (x2) ok as added?

[AG15] “continuously renewing” ok as edited?

[AG16] “with our study” ok as added? Also, “human” ok as added?

[AG17] Please provide journal volume number in ref 32.

[AG18] Please define PGE.

[AG19] There does not appear to be a 3-asterisk callout (*** in Figure 5. Please check legend.
Among Plant Lignans, Pinoresinol Has the Strongest Antiinflammatory Properties in Human Intestinal Caco-2 Cells

Alexandrine During, Céline Debouche, Thomas Raas, and Yvan Larondelle

Abstract

Dietary lignans show some promising health benefits, but little is known about their fate and activities in the small intestine. The purpose of this study was thus to investigate whether plant lignans are taken up and modulate the intestinal inflammatory response by using human intestinal Caco-2 cells. Six lignan standards [secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), pinoresinol (PINO), lariciresinol, matairesinol (MAT), and hydroxymatairesinol] and their colonic metabolites [enterolactone (ENL) and enterodiol] were studied. First, differentiated cells were exposed to SDG, SECO, PINO, or ENL at increasing concentrations for 4 h, and their cellular contents (before and after deconjugation) were determined by HPLC. Second, in IL-1β-stimulated confluent and/or differentiated cells, lignan effects were tested on different soluble proinflammatory mediators quantified by enzyme immunoassays and on the NF-κB activation pathway by using cells transiently transfected. SECO, PINO, and ENL, but not SDG, were taken up and partly conjugated by cells, which is a saturable conjugation process. PINO was the most efficiently conjugated (75% of total in cells). In inflamed cells, PINO significantly reduced IL-6 by 65% and 30% in confluent and differentiated cells, respectively, and cyclooxygenase (COX)-2–derived PGE2 by 62% in confluent cells. In contrast, MAT increased significantly COX-2–derived PGE2 in confluent cells. Moreover, PINO dose-dependently decreased IL-6 and macrophage chemoattractant protein-1 secretions and NF-κB activity. Our findings suggest that plant lignans can be absorbed and metabolized in the small intestine and, among the plant lignans tested, PINO exhibited the strongest antiinflammatory properties by acting on the NF-κB signaling pathway, possibly in relation to its furofuran structure and/or its intestinal metabolism. J. Nutr. 142: 1–8, 2012.

Introduction

Lignans are found in a wide variety of plant-derived foods, including cereals, fruit, and vegetables, as well as beverages. Secoisolariciresinol (SECO) and matairesinol (MAT) were the first lignans isolated from plant foods (1), followed by pinoresinol (PINO) and lariciresinol (LARI) (2,3), and more recently, 7-hydroxymatairesinol (HMR), syringaresinol, and medioresinol (4–6). To date, lignan content and distribution in plant foods are under constant reevaluation as new dietary lignans are identified. Flaxseeds and sesame seeds present the highest lignan levels so far. Flaxseeds predominantly contain secoisolariciresinol diglucoside (SDG; 0.3–1.2 g/100 g dry weight), but also PINO (3 mg/100 g dry weight), LARI (3 mg/100 g dry weight), and MAT (0.5–9 mg/100 g dry weight) (3,7,8). The average daily intake of lignans (sum of SECO, MAT, PINO, and LARI) was estimated at 1 mg/d (9).

Plant lignans are thought to be bioactive through their metabolites, the 2 enterolignans enterodiol (END) and enterolactone (ENL), which are produced by intestinal bacteria in the colon. SDG, MAT, PINO, LARI, and HMR (Supplemental Fig. 1) as well as sesamin and syringaresinol are considered to be enterolignan precursors (10,11). ENL and END are efficiently absorbed as reported in vitro (12) and in vivo with high concentrations of enterolignans recovered in plasma and urine of individuals consuming lignan-rich foods (13,14). ENL and END appear in the blood circulation within 8–10 h after plant lignan ingestion (5,15). In contrast, low amounts of plant lignans (e.g., LARI and PINO) are detected in human plasma (16) and urine (17,18), and their appearance in blood occurs usually within 1 h after intake and with a half-life of 2–6 h (16),
suggesting that plant lignans could be quickly absorbed and used and/or eliminated by the body. Nevertheless, information on how plant lignans are absorbed at the molecular level is quite limited.

Lignans show promising beneficial effects for human health (19). Indeed, several dietary intervention studies have reported that high plant lignan intake results in a high plasma enterolignan concentration, associated with a reduced risk of developing certain diseases, such as breast, prostate, and colon cancers (20) and cardiovascular diseases (21). These purported health benefits of lignans may be related to their phytoestrogenic properties, free radical scavenging activities, and antimicrobial and/or anti-inflammatory effects (22–24). In inflammatory bowel diseases (IBD), which are characterized by an uncontrolled response of the intestinal immune system, the use of some “natural” preventive treatments in early life has been suggested in order to reduce or delay IBD development. In that regard, polyphenols could play this role by modulating intestinal inflammation (25). Different lignans that derive from nonedible plants have shown some anti-inflammatory properties in non-intestinal models (24). For instance, both PINO isolated from Coptis japonica rhizomes and LARI from Taxus baccata L. heartwood decreased TNF-α production in LPS-induced macrophages (26) and in mice with induced hind-paw edema (27). In vivo, flashseed-derived lignan preparations were shown to reduce a serum marker of inflammation (i.e., C-reactive protein) in healthy postmenopausal women and in type 2 diabetic patients (28,29). To our knowledge, only one recent study reported that lignan magnolol, a main constituent isolated from Magnolia officinalis bark, could prevent intestinal dysmobility and inflammation in rats with LPS-induced sepsis, by modulating various inflammatory mediators and suppressing NF-κB activity (30).

In view of the literature, plant lignans commonly present in our Western diet need more attention in regard to their fate and activities in the small intestine. Thus, in the present study, we investigated the following: 1) whether the plant lignans SDG, SECO, and PINO can be taken up and metabolized by intestinal cells and 2) whether plant lignans can attenuate the intestinal inflammatory response in intestinal Caco-2 cells stimulated with IL-1β.

Materials and Methods

**Chemicals.** SECO, MAT, ENL, END, and quercetin (QUER) were purchased from Sigma-Aldrich. HMR, PINO, and LARI were from ArboNova. SDG was a generous gift from Dr. Sam Posseniers from Ghent University, Belgium. All of these compounds presented a purity of ≥95%, except for MAT, which was of ≥85% purity (determined by HPLC). DMSO (≥99.8%) was obtained from ACROS Organics (Thermo Fisher Scientific). Human recombinant IL-1β (>98%), arachidonic acid from porcine liver (≥99%), taurocholic acid (≥95%), bisphenol A (≥99%), and β-glucuronidase from Helix pomatia (type H-2; glucuronidase ≥80 units/mL and sulfatase ≥7.5 units/mL) were also from Sigma-Aldrich. Caffeic acid phenethyl ester (CAPE) was purchased from Enzo.

**Cell culture.** Caco-2 cells (passage 18) derived from a human colon adenocarcinoma and obtained from American Type Culture Collection (ATCC no. HTB-37; LGC Promochem) were grown in the presence of DMEM containing 25 mmol/L glucose and 4 mmol/L glutamine and supplemented with 1% (v/v) of a nonessential amino acid (NEAA) solution at 10 mmol/L, 1% (v/v) of an L-glutamine solution at 200 mmol/L (Invitrogen SA), and 10% (v/v) of heat-inactivated FBS (Perbio Science) in cell culture conditions as described previously (31). For experiments, cells were seeded at a density of 4.10^4 and 2.10^4 cells/cm², respectively, to reach confluence (3 d) or full differentiation (21 d), and grown with the growth medium indicated above.

**Cytotoxicity assay.** Lignan cytotoxicity was determined by using a colorimetric lactate dehydrogenase (LDH) assay (Cytoxicity Detection kit (LDH); Roche Diagnostics).

**Preparation of polyphenol solutions.** All lignan standards (SDG, SECO, PINO, LARI, MAT, HMR, ENL, and END) and QUER were solubilized in DMSO at stock solutions ≥60 mmol/L and stored at −20°C.

**Protocol for cellular uptake and metabolism experiments.** Cells were grown on 6-well plates for 21 d. The growth medium was then replaced by DMEM supplemented with 1% NEAA (without FBS and phenol red) and incubated with SDG, SECO, PINO, or ENL at varying final concentrations (SECO: 9–274 μmol/L; PINO: 6–259 μmol/L; and ENL: 0.7–55 μmol/L) for 4 h. After incubation, the cell culture medium was removed, and cells were washed 3 times with 1 mL HBSS (pH 7.4) and detached from the plate surface with 1 mL of 0.1 mol/L sodium acetate buffer (pH 5). The resulting cell suspension was sonicated for 10 min to lyse cells, and an aliquot was kept on ice, prior to analysis for its free lignan content. Another aliquot was subjected to an enzymatic hydrolysis according to Jansen’s procedure (12) to free conjugated forms of lignans. Briefly, 3 μL Helix pomatia were mixed to the cell lysate (40 μL) and incubated at 37°C for 2 h. The reaction was stopped by cooling on ice, and kept on ice prior to analysis for its total lignan content.

**Lignan extractions and analyses by HPLC.** Lignans from cell samples and media were extracted with acetonitrile by mixing vigorously on a vortex for 1 min, followed by a 10-min incubation on ice and a centrifugation at 10,000 × g for 10 min at 4°C. An aliquot of the resulting supernatant was then mixed into Ultrapure water and filtered (0.45μm; Millipore) prior to injection into the HPLC system. The Thermo Separation HPLC system was equipped with a photo diode array detector (model UV6000LP), an autosampler (model AS100), a 100-μL injection loop, a column oven, and ChromQuest software (ThermoFinnigan). Lignan separation was achieved by using the Gemini C18 reverse-phase column (150 × 4.6 mm, 3 μm; Phenomenex) heated at 40°C and the 2 mobile phases: (A) water, 0.1% formic acid (100:0.1, by vol), and (B) acetonitrile, 0.1% formic acid (100:0.1, by vol), with the following gradient program: B increase from 20% to 50% in 15 min, and from 50% to 100% in 5 additional min. Flow rate was constant at 1 mL/min. Lignans were monitored at 280 nm and quantified from their peak areas by using external standard curves established for each lignan standard. Under these HPLC conditions, all of the standards were well separated as follows: 3.2 min (SDG), 6.9 min (HMR), 7.3 min (SECO), 7.9 min (LARI), 8.9 min (END), 10.4 min (PINO), 11.8 (MAT), and 12.3 min (ENL).

**Protocol for inflammatory experiments.** Cells were grown on 12-well plates for 3 or 21 d. The growth medium was then replaced by 1 mL DMEM supplemented with 1% NEAA and 0.5% FBS (without phenol red), and cells from 2 wells were exposed to one lignan standard or QUER at a fixed concentration (usually 50 μmol/L). After 1 h preincubation, inflammation was induced by adding IL-1β (25 μg/L); but only in 1 of the 2 wells pretreated with the polyphenol. Cells were further incubated for 24 h. Lignan effects were thus examined under unstimulated and IL-1β-stimulated conditions.

**Determination of IL-8, IL-6, and MCP-1 secretion in the cell culture medium.** After treatment with or without IL-1β of the polyphenol solution, the cell culture media were harvested and centrifuged at 3000 × g for 10 min, and supernatants were analyzed for their contents of IL-8, IL-6, and monocytic chemotactic protein-1 (MCP-1) by using the human IL-8, IL-6, and MCP-1 ELISA Kits, respectively, according to the manufacturer’s instructions (BD). Values are expressed as picograms of IL-8, IL-6, or MCP-1 secreted per milligram of protein.
Determination of COX-2–derived PGE\(_2\) production by cells. After treatment with or without IL-1\(\beta\) of the phenolic solution, cells were assayed for cyclooxygenase-2 (COX-2)–derived PGE\(_2\) production as described previously (31). Briefly, cells were incubated with HBSS supplemented with arachidonic acid (10 \(\mu\)mol/L) and taurocholate (250 \(\mu\)mol/L) for 10 min at 37°C. PGE\(_2\) amounts in cell culture media were determined by using the PGE\(_2\) Enzyme Immunoassay ACE Competitive EIA Kit (Cayman Chemical, SPI-BIO). COX-2–derived PGE\(_2\) data were calculated according to the difference between the PGE\(_2\) amount produced by IL-1\(\beta\)-stimulated cells and the basal PGE\(_2\) amount produced by unstimulated cells and are expressed as picograms of PGE\(_2\) produced per milligram of protein.

Immune signaling pathway reporter luciferase assays. The effect of 4 phenolic compounds (CAPE, SECO, PINO, and MAT) on the NF-κB activation pathway by IL-1\(\beta\) were examined using the Signal NF-κB Reporter Luciferase Kit (CCS-0135; SA Biosciences, Tebu-bio). Cells (passages 27–33, viability of \(\geq 94\%\)) were seeded into 96-well, white, opaque, flat-bottomed microplates and allowed to reach \(\geq 80\%\) confluence. Cells were then transfected with 100 ng DNA reporter/well by using Lipofectamine LTX as a transfection reagent (Invitrogen SA). The DNA reporter consisted of a mixture of NF-κB–responsive firefly luciferase construct and a constitutively expressing [AQ15] Renilla luciferase construct (40:1). Transfection was performed for 24 h at 37°C. Cells were then treated with a lignan standard with or without IL-1\(\beta\) as described above. After 18 h incubation, firefly and Renilla luciferase activities were sequentially measured with the Dual-Glo Luciferase Assay system according to the manufacturer’s instructions (E2920; Promega Benelux BV) and by using a Fluoroskan Ascent Fluorometer (Thermo Scientific). For each experimental condition, the signal (S) firefly/Renilla was determined. The effect of a lignan on the NF-κB activation pathway was compared with that of the vehicle DMSO as follows:

\[
\frac{[S_{\text{IL-1\(\beta\)}} - S_{\text{basal}}]}{[S_{\text{basal}} - S_{\text{DMSO}}]} \text{ with}
\]

\(S_{\text{basal}}\) = the signal firefly/Renilla obtained in the presence of IL-1\(\beta\) and \(S_{\text{DMSO}}\) = the signal firefly/Renilla obtained in the absence of IL-1\(\beta\).

Cellular protein concentration assay. Cellular protein concentration was determined according to the bicinchoninic acid assay (Sigma-Aldrich) by using the bovine serum albumin as the standard.

Statistical analysis. Data are means ± SEM. For cellular lignan uptake experiments, relationships between the 2 variables were examined by simple or logarithmic regression analyses; the regression giving the highest regression coefficient \(R^2\) value was chosen. For inflammation experiments, all data showed homogeneous variances when tested by the Bartlett test and were thus analyzed by 1-way ANOVA, followed by the post hoc Tukey-Kramer test for multiple comparisons of means. For immune signaling pathway assays, differences between the 2 groups were assessed by using the unpaired Student’s t test. Statistical analyses were performed by using Statview, version 5.0, or JMP, version 10 (SAS Institute). Differences were considered significant at \(P < 0.05\).

Results

Cytotoxic effects of the lignan standards

Lignan standards did not show any cytotoxic effect (\(\leq 5\%\) of LDH activity released in cell culture media, compared with the positive control) at the different concentrations tested (\(\leq 275 \mu\text{mol/L}\)) and at both cellular stages (confluence and full differentiation) when applied to Caco-2 cells for 24 h.

Cellular uptake and conjugation of SDG, SECO, PINO, and ENL

After 4 h exposure at a final concentration of \(\approx 40 \mu\text{mol/L}\), lignans were taken up by cells according to the following decreasing order: ENL (7%) > SECO (2%), whereas SDG was found only in trace amounts in cells (\(< 0.1\%\)) (Fig. 1). From total amounts recovered in cells, the 3 lignans showed extents of conjugation as follows: PINO (75%) > SECO (42%) > ENL (16%), with significant differences between the 3 lignans (\(P < 0.05\)).

Effect of the initial concentration of SECO, PINO, and ENL on their cellular uptake and conjugation

After 4 h exposure with various lignan concentrations (PINO: 6–259 \(\mu\text{mol/L}\); SECO: 9–274 \(\mu\text{mol/L}\); or ENL: 0.7 to 55 \(\mu\text{mol/L}\) (experiments performed in triplicate), aglycone forms increased linearly in cells with the initial concentration \((R^2 \approx 0.98, P < 0.0001)\), indicating that they entered into cells by simple diffusion or with a low affinity transporter (Fig. 2). In contrast, conjugated forms in cells first increased proportionally to the initial concentration and then plateaued at \(\approx 30–40 \mu\text{mol/L}\) for PINO and SECO and at 10 \(\mu\text{mol/L}\) for ENL, indicating that lignan conjugation within the intestinal cell is a saturable process. By using double inverse linear Lineweaver-Burk representations (PINO: \(R^2 = 0.94, P < 0.002\); SECO: \(R^2 = 0.96, P = 0.08\); ENL: \(R^2 = 0.92, P < 0.01\)), \(V_{\text{max}}\) values were estimated at 310, 280, and 105 \(\mu\text{mol/h (mg protein)}\) and \(K_m\) values at 13.0, 9.0, and 3.4 \(\mu\text{mol/L}\) for PINO, SECO, and ENL, respectively (Figure 2).

Effects of lignan standards on IL-1\(\beta\)–induced secretion of IL-8 and IL-6

Secretion of IL-8. Under IL-1\(\beta\) stimulation, higher levels of IL-8 were produced by confluent cells (3.5-fold more) than by differentiated cells, indicating that undifferentiated intestinal cells responded better to the stimulus of inflammation (Table 1). As expected, the antiinflammatory polyphenol QUER decreased IL-1\(\beta\)–induced IL-8 levels by \(\approx 30\%\) at both cellular stages, with a significant effect only in 3-d cells compared with the control vehicle DMSO (\(P < 0.05\)) (Table 1). Plant and mammalian lignans did have any reducing effect on IL-8 secretion.
Secretion of IL-6. Under IL-1β stimulation, higher levels of IL-6 were secreted by confluent cells (2-fold more) than by differentiated cells, and those levels were not changed by DMSO (Table 1). QUER significantly reduced IL-1β–induced IL-6 secretion at both cellular stages (37% reduction), compared with DMSO (P < 0.05). Among lignan standards, PINO was again the only one to decrease COX-2–derived PGE2 levels in 3-d cells (P < 0.05) and by 38% in 21-d cells (P = 0.068). MAT behaved differently by significantly increasing COX-2–derived PGE2 levels in 3-d cells, compared with DMSO (P < 0.05). Note that MAT effect on PGE2 was significantly higher than that of HMR at both cellular stages (P > 0.05).

Dose-dependent effects of PINO on IL-8, IL-6, and MCP-1 secretion
PINO did not affect IL-1β–induced IL-8 levels up to 100 μmol/L, whereas it reduced those of IL-6 and MCP-1 in a dose-dependent manner with IC50 of ~12.5 and ~100 μmol/L, respectively (Fig. 4).

Effect of plant lignans on the IL-1β–induced NF-κB signaling pathway in Caco-2 cells
As expected, CAPE, a specific inhibitor of NF-κB, significantly reduced NF-κB activation by ~45% and ~70% for 0.1 and 10 μmol/L CAPE, respectively, compared with the DMSO control.

Effect of plant and mammalian lignans on COX-2–derived PGE2 production
In the absence of inflammatory conditions, basal levels of PGE2 produced by Caco-2 cells were 347 and 73 pg PGE2/mg protein at d 3 and d 21 of maturation, respectively. These basal PGE2 levels were unchanged in the presence of the vehicle DMSO or in one of the lignan treatments (data not shown). After stimulation with IL-1β, PGE2 production increased by 6.1- and 2.4-fold in 3-d and 21-d cells, respectively, indicating that IL-1β indeed induced the COX-2 activity in Caco-2 cells (data not shown). These COX-2–related PGE2 levels were not changed by DMSO (Fig. 3). Compared with DMSO, QUER markedly reduced the induced PGE2 secretion at both cellular stages (88–98% reduction; P < 0.05). Among lignan standards, PINO was again the only one to decrease COX-2–derived PGE2 by 62% in 3-d cells (P < 0.05) and by 38% in 21-d cells (P = 0.068). MAT behaved differently by significantly increasing COX-2–derived PGE2 levels in 3-d cells, compared with DMSO (P < 0.05). Note that MAT effect on PGE2 was significantly higher than that of HMR at both cellular stages (P > 0.05).
The effects of lignan standards (50 μmol/L) on the secretion of proinflammatory cytokines IL-8 and IL-6 in 3-d and 21-d Caco-2 cells stimulated with IL-1β.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-8, pmol/mg protein</th>
<th>IL-6, pmol/mg protein</th>
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<tr>
<td>IL-1β+ DMSO</td>
<td>120 ± 20</td>
<td>19 ± 9</td>
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<td>IL-1β+ SECO</td>
<td>34 ± 2</td>
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<td>IL-1β+ SECO</td>
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<td>IL-1β+ PINO</td>
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<td>124 ± 10</td>
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Values are means ± SEM, N = 3–5 independent experiments. Labeled means in a row without a common letter differ, P < 0.05. End, enterodiol; ENL, enterolactone; HMR, 7-hydroxymatairesinol; LARI, laricresinol; MAT, matairesinol; SECO, secoisolariciresinol; SDG, secoisolariciresinol diglucoside; SECO, secoisolariresinol.

$P < 0.05$ (Fig. 5). However, at 1 μmol/L CAPE, the reduction in NF-κB activity was not significant compared with the control. PINO decreased IL-1β-induced NF-κB activity in a dose-dependent manner with reductions of 16%, 44%, and 75% for 10, 50, and 100 μmol/L PINO, respectively (with IC$_{50}$ of 60 μmol/L). This effect was significant for the highest concentration of PINO (100 μmol/L), compared with DMSO or the lowest PINO concentration (10 μmol/L) $(P < 0.05)$. Finally, both SECO and MAT at any of the 3 concentrations tested did not change IL-1β-induced NF-κB activity.

### Discussion

An understanding of the bioavailability of phytochemicals is crucial because it is a determinant factor for biological activities of these compounds. One of the purposes of this study was to examine the intestinal cellular uptake and metabolism of plant lignans with the use of fully differentiated Caco-2 cells that spontaneously differentiate into an enterocyte-like phenotype (32). Lignan concentrations used in the present study (≤275 μmol/L) were physiologic; indeed, polyphenols in the gastrointestinal tract can reach levels of up to several hundred micromoles per liter (33), and it was estimated that participants consuming 50 g flaxseeds would have a luminal SDG concentration of 666 μmol/L (22). These lignan concentrations were not cytotoxic for Caco-2 cells.

We showed here that the plant lignans SECO and PINO can be taken up by intestinal cells. Their cellular levels as aglycones increased linearly with the initial concentration, suggesting that both enter into Caco-2 cells by simple diffusion or through a low-affinity transporter. The extents of these 2 lignans in intestinal cells were relatively low (~2% of the initial dose) as seen for other polyphenols (33). However, the glycosylated lignan SDG with 2 additional glucosyl groups in its structure compared with SECO was not taken up by intestinal cells, suggesting that the glycosylation influences lignan bioavailability as has been reported previously for other polyphenols. Thus, for its passage across the small intestinal brush border, the main flax lignan SDG needs to be deglycosylated in the gut lumen via the participation of some enzyme activities. There are 2 β-glucosidases that could contribute in the hydrolysis of SDG into SECO: the lactate phlorizin hydrolase present on the apical brush border membrane and the cytosolic β-glucosidase. Both of these activities are expressed by Caco-2 cells (34,35). However, in our experimental conditions, we did not observe any SECO production in cell preparations or in the medium, suggesting that SDG is not a substrate of these enzymes or that these enzyme activities were not properly working in Caco-2 cells.

In the present study, cellular uptake of the 3 flax lignans SDG, SECO, and PINO were compared with that of the enterolignan ENL because its uptake had already been examined by others (12). HT-29 cells were able to take up the enterolignans ENL and END, followed by a rapid intracellular conjugation (12). Similarly, we found that Caco-2 cells were able to accumulate and conjugate ENL. Nevertheless, differences in intestinal ENL fate between the 2 cell models (Caco-2 vs. HT-29 cells) were noticed. Indeed, for similar experimental conditions (4 h incubation with 10 μmol/L ENL), we found that cellular ENL level was an order of magnitude higher (800 vs. 60 nmol/L) and only partially conjugated (50% vs. 100%) in Caco-2 cells compared with HT-29 cells (12). Furthermore, ENL, like PINO and SECO, entered into cells by simple diffusion and its uptake was greater (7%) than that of PINO and SECO (2%).
Once in the enterocytes, polyphenols can be conjugated, and that process might affect their bioavailability. Our data indicate that a conjugation of the 3 lignans SECO, PINO, and ENL occurred in Caco-2 cells. Here, we did not attempt to identify the nature of the conjugates formed; however, glucuronidation has been described as the predominant conjugation pathway in the enterocytes for other polyphenols (36–38). Therefore, lignans are probably found mostly in glucuronidated form and possibly in sulfated form in our in vitro model as reported previously in HT-29 cells (12). In Caco-2 cells, PINO was the most efficiently conjugated (75% of total in cells), followed by SECO (42%) and ENL (16%) after 4 h exposure with ~50 μmol/L of each lignan. The relative low percentage of conjugated ENL could be attributed to the fact that there was a saturation of the conjugation process in Caco-2 cells, which was observed at a lower concentration for ENL than for PINO or SECO (10 vs. 30–40 μmol/L). In vivo saturation of the intestinal conjugation process was previously reported in rats given high doses or an acute administration of polyphenols (37,38), and it was suggested that, in some cases, newly absorbed unconjugated polyphenols could be recovered in the blood circulation as commonly described for drugs. The conjugation of polyphenols results in more hydrophilic compounds that might modify the biological activities of polyphenols in one or another way by triggering molecules/pathways other than their aglycone counterparts. To date, it is still unclear how conjugation of polyphenols affects their activities, and further investigation is clearly needed.

Under IL-1β stimulation, Caco-2 cells responded by increasing the secretion/production of the proinflammatory markers IL-8, IL-6, and MCP-1 and by enhancing COX-2 activity. All of these markers have been reported to be overexpressed in mucosal tissues of patients with IBDs. As reported previously (31), undifferentiated Caco-2 cells were more sensitive to the stimulus IL-1β than fully were differentiated cells. However, we thought it was important to investigate the inflammatory properties of polyphenols at both cellular stages (confluent and differentiated) because the small intestine is continuously renewing and thus intestinal cells are present at all stages of maturity.

Among the lignans tested, PINO exhibited the strongest antiinflammatory properties by reducing IL-6, MCP-1, and COX-2–derived PGE2 secretions in Caco-2 cells stimulated by IL-1β, and these effects were observed at relevant physiologic concentrations (with IC50 of 12.5 and 100 μmol/L PINO with IL-6 and MCP-1, respectively). In agreement with our study, a recent study (39) reported that PINO isolated from Forsythia koreana inhibited the production of several proinflammatory cytokines (i.e., IL-6 and PGE2) in LPS-activated microglial cells isolated from rat pup brains, but at concentrations (≥10 μmol/L) that are probably never met in the human brain, because maximum polyphenol concentrations in blood rarely exceed 1 μmol/L and only few polyphenols can selectively cross the blood-brain barrier (33). Moreover, both PINO and isolariciresinol isolated from Coptis japonica showed antiinflammatory effects in LPS-activated mouse macrophages, again at higher concentrations (IC50 of 39 and 124 μmol/L for PINO and isolariciresinol, respectively) than those expected to be seen in the blood (26). To our knowledge, this is the first report to indicate that PINO can diminish the inflammatory response in an intestinal cell model.

Both ENL and END at 50 μmol/L did not affect the secretion of proinflammatory markers in our in vitro model as reported previously in human leukocytes for enterolignan concentrations at 100 μmol/L (40). In another study (41), ENL was more efficient than END to reduce TNF-α release from 2 in vitro human peripheral blood cell models, although concentrations at which these effects were observed were high (IC50 ≥130 and 450 μmol/L for ENL and END, respectively). LARI did not show any antiinflammatory effects in Caco-2 cells, in contrast with a previous in vivo study that reported some antiinflammatory properties for LARI (27). Discrepancies between the 2 studies could be attributed to the model used (carrageenan-induced inflammation).

**FIGURE 4** Dose effect of PINO (10–100 μmol/L) on the secretion of IL-8, IL-6, and MCP-1 by 3-d cells stimulated with IL-1β (25 μg/L). For each marker secretion, values are means ± SEM; n = 1 (means of 3 replicate wells). MCP-1, monocyte chemotactic protein-1; PINO, pinoresinol.

**FIGURE 5** Effects of phenolic compounds on NF-κB activation by IL-1β. Cells were transfected with the NF-κB reporter and then incubated with CAPE (a specific inhibitor of NF-κB), SECO, PINO, and MAT at 3 different concentrations (0.1, 1, and 10 μmol/L for CAPE and 1, 10, and 100 μmol/L for the 3 lignans) with or without IL-1β. Values are means ± SEM; n = 3 independent experiments (or n = 4 for the 3 concentrations of CAPE). *P < 0.05, **P < 0.01, and ***P < 0.005, compared with control DMSO. CAPE, caffeic acid phenylethyl ester; CTL, control; MAT, matairesinol; PINO, pinoresinol; SECO, secoisolariciresinol.
mouse hind-paw edema vs. IL-1β–stimulated human intestinal cells), to the molecule preparation tested (molecule extracted from Taxus baccata L. vs. pure standard), or to the inflammatory pathway endpoints analyzed. Finally, it is interesting to note that, in comparison with HMR, MAT (with one less hydroxyl group) enhanced PGE₂ production by inflamed Caco-2 cells, suggesting that the molecular structure of lignans could play a determinant role for their antiinflammatory properties. Nevertheless, one should note that standard MAT had the highest amount of impurities, which could contribute to those effects.

NF-κB is a key factor in the intestinal inflammatory response and controls the gene expression of many proinflammatory proteins, including the cytokines IL-1β, IL-6, IL-8, and MCP-1 and the enzyme COX-2 (42,43). In Caco-2 cells, we found that the main signaling pathway targeted by IL-1β is that of NF-κB after testing cells transiently transfected with 10 different NF-κB-luciferase constructs (data not shown). NF-κB activity increased by >5-fold in the presence of IL-1β, and PINO was able to reduce that NF-κB activation in a dose-dependent manner with an IC₅₀ of 60 μmol/L. Thus, PINO may attenuate the intestinal inflammatory response by blocking the NF-κB signaling pathway at one step, which in turn would result in the decrease of the expression of NF-κB–dependent IL-6, MCP-1, and COX-2 genes.

The fact that PINO effects were greater in undifferentiated cells than those seen in differentiated cells could be explained either by a difference in the “sensitivity” of cells (we reported that undifferentiated cells responded better to the stimulus of IL-1β than differentiated cells) and/or by a difference in the bioavailability of lignans between the 2 cellular stages. In the present study, PINO bioavailability was studied only in differentiated Caco-2 cells. Because lignans entered differentiated cells by a simple diffusion process, not requiring a specific transporter that would likely be expressed mainly in differentiated cells, it is probable that an identical mechanism occurs in undifferentiated cells. On the other hand, the extent of PINO conjugation might be expected to be lower in undifferentiated than in differentiated cells because the expression of most UDP-glucuronosyltransferases increases during Caco-2 differentiation (44). As such, a reduction in intracellular PINO conjugates would also be responsible for its greater antiinflammatory activities observed in undifferentiated cells. These antiinflammatory properties of PINO could be related to its “furofuran” structure, which is not present in the other lignan molecules tested (Supplemental Fig. 1). Nevertheless to validate the hypothesis of a structure-activity relationship, further studies would be necessary to examine other lignans with a furofuran structure (e.g., syringaresinol magnolin, eudesmin, or even sesamine) for their potential antiinflammatory activities in identical experimental conditions.

In summary, the present study indicates that plant lignans can be absorbed and metabolized in the small intestine. Among the plant lignans tested, PINO exhibited the strongest antiinflammatory properties in vitro by blocking the NF-κB signaling pathway, possibly in relation to its molecular structure and/or its conjugation efficacy in intestinal cells. Further studies are necessary to confirm these antiinflammatory properties of PINO in vivo and to more deeply explore the mechanisms by which this compound modulates the intestinal inflammatory response.

Acknowledgments
A.D. and Y.L. designed research and wrote the manuscript; A.D., C.D., and T.R. conducted research and analyzed data; A.D. performed statistical analysis; and Y.L. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited

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