Due to the success of therapeutic anti-inflammatory compounds to inhibit, retard, and reverse the development of colon cancer, the identification of dietary compounds as chemopreventives is being vigorously pursued. However, an important factor often overlooked is the metabolic transformation of the food-derived compounds in the gut that may affect their bioactivity. Commonly consumed dietary phenolics (esterified ferulic acid and its 5,5′-linked dimer), which have the potential to undergo predominant microbial transformations (de-esterification, hydrogenation, demethylation, dehydroxylation, and dimer cleavage), were incubated with human microbiota. The metabolites were identified (high-performance liquid chromatography and nuclear magnetic resonance) and confirmed to be present in fresh fecal samples from 4 human volunteers. The potential anti-inflammatory properties were compared by measuring the ability of the parent compounds and their metabolites to modulate prostanoid production in a cell line in which the inflammatory pathways were stimulated following a cytokine-induced insult. The compounds were readily de-esterified and hydrogenated, but no dimer cleavage occurred. Only the monomer underwent demethylation and selective de-hydroxylation. The resultant metabolites had differing effects on prostanoid production ranging from a slight increase to a significant reduction in magnitude. This suggests that the microbial transformation of dietary compounds will have important inflammatory implications in the chemoprevention of colon cancer.

INTRODUCTION

Large numbers of epidemiological studies have suggested a protective effect of fruit and vegetable consumption against the development of diseases such as colorectal cancer (1,2). There is increasing evidence that inflammatory pathways are a key step in carcinogenesis (3), and studies have shown that there is potential for dietary compounds from plant sources to interfere at various stages during the development of colorectal cancer (4). However, such in vitro studies invariably assess the anti-inflammatory effect of the parent molecules, which are readily extracted from plant sources. Their relevance in vivo is uncertain, as the parent compounds are likely to undergo transformation by colonic microflora to form numerous metabolites.

Despite the obvious association between the colonic environment with inflammation (5–7), very few studies have addressed the involvement of gut bacteria and their ability to modulate inflammatory compounds derived from “non-nutritive” phytochemicals consumed in the diet. It is now evident that anti-inflammatory therapeutics such as the nonsteroidal anti-inflammatory drugs have an effect on development, retardation, progression, and recurrence of colorectal cancer (8). It is also likely that the metabolites of dietary components, which are similar in structure, will have an analogous effect. Although there appear to be many potential mechanisms of action, the pathway leading to the upregulation of prostanoids is undoubtedly implicated. Recently, we demonstrated that prostanoid production in normal colon fibroblast cells was modulated to varying degrees by a group of structurally related phenolic compounds following cytokine-induced upregulation of prostaglandin...
FIG. 1. Major pathway (highlighted in bold) for the metabolic transformation of methyl ferulate (compound 1) to ferulic acid [3-(4-hydroxy-3-methoxyphenyl) acrylic acid; compound 2] and its hydrogenated metabolites: 3-(4-hydroxy-3-methoxyphenyl) propionic acid (compound 3), 3-(3,4-dihydroxyphenyl) propionic acid (compound 4), and 3-(3-hydroxyphenyl) propionic acid (compound 5). The other potential metabolites of ferulic acid are also shown: 3-(4-hydroxyphenyl) propionic acid (compound 6), 3-(3,4-dihydroxyphenyl) acrylic acid (compound 7), 3-(3-hydroxyphenyl) acrylic acid (compound 8), 3-(4-hydroxyphenyl) acrylic acid (compound 9), 3-phenylacrylic acid (compound 10), and 3-phenylpropionic acid (compound 11).

H synthase-2 (9). This demonstrated that certain dietary abundant phytochemicals, derived via the phenylpropanoid pathway, could both enhance and inhibit prostanoid production. However, it is likely that these parent compounds will be transformed by the colonic microflora, requiring assessment of the structure of the metabolites and the overall effect of metabolism on inflammatory processes.

Products of the plant phenylpropanoid pathway are abundant in our diet. These include the phenolic acids and compounds such as curcumin, resveratrol, and the green tea polyphenols, which are widely studied for their protective effect against colorectal cancer, with many studies having reported an anti-inflammatory mechanism of action (10–12). Cinnamic acid (3-phenylacrylic acid; Fig. 1, compound 10) is the first metabolite in this pathway, and in addition to being the precursor to other plant phenols, the hydroxylated and methoxylated analogues of this compound (Fig. 1; compounds 2, 7, and 9) are ubiquitous amongst vascular plants (13). Ferulic acid [3-(4-hydroxy-3-methoxyphenyl)acrylic acid; Fig. 1, compound 2] is a major and important component of plant-based foods where it is found esterified to polysaccharides (14) and provides cross-linking to other polysaccharides and to lignin (15). It is found in considerable amounts in most fruits, vegetables, and cereals (13), covalently linked through its carboxyl group and as a dimer (16). It contains structural features such as a carboxylic acid group, C3 side chain, olefinic double bond, aromatic hydroxyl, and methoxyl substituents, which are common to many of the phenylpropanoid-derived compounds of interest and is therefore an ideal candidate for metabolic studies. For these reasons, metabolism of ferulic acid was the subject of this study. The anti-inflammatory properties of the parent compound and its metabolites are compared.
**METHODS**

**Materials**

General laboratory reagents were purchased from Aldrich (Gillingham, England). Reported melting points are uncorrected, and evaporation was under reduced pressure at temperatures not exceeding 40°C. Compounds 2 and 7 through 11 (Fig. 1) were purchased from Aldrich. Compound 12 (Fig. 2) was prepared by the initial coupling of 4-hydroxy-3-methoxybenzaldehyde. The 4-hydroxyl substituent was then protected by acetylation and the side chain extended by a malonic acid condensation as published previously (17). Compound 13 (Fig. 2) was then prepared by hydrogenation of compound 12 (Fig. 2), as were compounds 3 through 6 (Fig. 1) from their corresponding cinnamic acids as published previously (17). To prepare compound 1 (Fig. 1), ferulic acid (Aldrich; 9.7 g, 50 mmol) was dissolved in methanol (600 cm³) to which HCl (0.6 cm³ of 10 mol dm⁻³) was added. The mixture was heated under reflux for 6 h and reduced under vacuum. The crude product was dissolved in ethyl acetate and extracted with NaHCO₃ (3% wt/vol). The solvent was dried over anhydrous Na₂SO₄, filtered and the solvent removed in vacuo. Crystallization from ether afforded [methyl 3-(4-hydroxy-3-methoxyphenyl) acrylate; Fig. 1, compound 1] as colorless plates. Yield was 90%; mp 62–63°C; NMR δH (CD3OD) 3.74 (3H, s, CO2CH3), 3.86 (3H, s, OCH3), 6.33 [1H, d, J 8.2 and 2.4, C(6)H], 7.14 [1H, d, J 2.4, C(2)H], 7.57 [1H, d, J 16, C(7)H] δC (CD3OD) 169.7 (CO2CH3), 150.5 (C4), 149.28 (C5), 146.7 (C7), 129.5 (C1), 124.1 (C2), 116.4 (C3), 115.1 (C8), 110.6 (C6), 56.4 (OCH3) 52.0 (CO2CH3) ppm.

**Microbial metabolism**

For isolation work, the parent compounds (100 mg) were dissolved in dimethyl sulfoxide (DMSO; 5 cm³) to obtain one anaerobic M2 medium/rumen inoculation (50:50; 95 cm³) and incubated under anaerobic conditions for 72 h at 37°C. For an analytical time-course study, smaller quantities in triplicate (2.5 mg) were dissolved in DMSO (0.5 cm³) added to both anaerobic M2 medium/rumen and M2 medium/human inoculation (50:50; 95 cm³). These were then incubated under anaerobic conditions for periods of 0, 24, and 72 h at 37°C.

**Separation, isolation, and characterization of metabolites**

The large-scale samples were immediately centrifuged (3000 rpm; 10 min), the supernatant was subsampled (25 cm³) and extracted into ethyl acetate (3 × 50 cm³). The solvent was removed in vacuo and the components separated and isolated by preparative high-performance liquid chromatography (HPLC) and characterized by nuclear magnetic resonance (NMR). For metabolite quantification, an internal standard (3,4-dimethoxy benzoic acid) was immediately added to the small scale samples and the extraction volumes reduced 10-fold. Separation was performed on an analytical HPLC column and the components quantified by internal standardization and the response factor of the pure compounds. HPLC (preparative) was performed on a Genesis C18 column (4µ; 15 cm × 7 mm) and eluted with acetonitrile (AcCN), trifluoroacetic acid (0.05% vol/vol; pH 2.3), and methanol (which was constant at 1% in the eluent) at 1 cm³ min⁻¹ using the following gradient: 28% AcCN (10 min), 28–60% AcCN (10 min), 60% AcCN (2 min) and 60–28% AcCN (3 min). HPLC (analytical) was performed on a Genesis C18 column (3µ; 10 cm × 4.6 mm) and eluted with AcCN, trifluoroacetic acid (0.05% vol/vol; pH 2.3), and methanol (which was constant at 1% in the eluent) at 1 cm³ min⁻¹ using the following gradient: 5% AcCN (0 min), 5–7% AcCN (2 min), 7% AcCN (3 min), 7–13.5% AcCN (10 min), 13.5–23% AcCN (5 min), 23% AcCN (3 min), 23-34% AcCN (7 min), 33–49% AcCN (5 min), 49–59% AcCN (5 min), 59–70% AcCN (5 min) and 70–5% AcCN (10 min). NMR spectra were recorded using a Jeol LA-300 spectrometer (Jeol, Welwyn Garden City, United Kingdom), fitted with a 5 mm multinuclear, normal geometry TH5 probe. All samples were dissolved in (CD3)2SO (0.5 cm³), containing tetramethylsilane (0.03% vol/vol) internal reference and run with a probe temperature of 40°C.

**Identification of metabolites in fecal samples**

To determine the presence and concentration of the metabolites in the colon, fresh fecal samples were collected from human volunteers consuming an unrestricted, Western-style diet who had not consumed antibiotics in the previous 6 mo. Separation of solid matter from fecal waters was obtained by centrifugation (50,000 g; 2 h). Both the pellet and supernatant were then freeze-dried and the water content calculated. Both fractions
FIG. 3. Products obtained (mg) after incubation of methyl ferulate [methyl 3-(4-hydroxy-3-methoxyphenyl)acrylate, 1; 2.5 mg] with human and rumen microflora. Values are given as the mean ± SD (n = 3). Compounds detected were ferulic acid [3-(4-hydroxy-3-methoxyphenyl)acrylic acid, compound 2], hydrogenated ferulic acid [3-(4-hydroxy-3-methoxyphenyl)propionic acid, compound 3], hydrogenated caffeic acid [3-(3,4-dihydroxyphenyl)propionic acid, compound 4], and hydrogenated 3-hydroxycinnamic acid [3-(3-hydroxyphenyl)propionic acid, compound 5].

(≈100 mg) were suspended in HCl (0.2 mol dm⁻³; 10 cm³) and extracted into ethyl acetate (5 cm³), separating the layers by centrifugation (3,000 rpm; 5 min). This process was repeated 3 times and the organic layers combined, filtered through Na₂SO₄ and evaporated to dryness under reduced pressure at a temperature of less than 40°C. The extracts were then redissolved in methanol (0.2 cm³) and filtered through a 0.2 µm polyvinylidene fluoride membrane. Separation of the phenolic compounds was by HPLC employing 2 gradient elution methods using AcCN and trifluoroacetic acid (0.05% vol/vol; pH 2.3). Method 1 was 11–14% AcCN (35 min), 14–50% AcCN (5 min), 50% AcCN (10 min) and 50–11% AcCN (10 min) Method 2 was 11–40% AcCN (40 min), 40–50% AcCN (10 min) and 50–11% AcCN (5 min). Detection was at 215 and 280 nm, and the metabolites were quantified by internal standardization and use of response factors calculated from pure compounds.

RESULTS AND DISCUSSION

Because ferulic acid (Fig. 1, compound 2) is most commonly found esterified to, or cross-linking cell wall components, the methyl ester (Fig. 1, compound 1) and the 5-5′ linked dimer (Fig. 2, compound 12) of this compound were selected as parent compounds for the microbial metabolism studies. Both of these compounds and the required potential microbial metabolites were synthesized and mechanisms of de-esterification, decarboxylation, demethylation, dehydroxylation, hydrogenation, and C-C dimer cleavage investigated. To obtain two diverse sources of gut microbial communities, the metabolism studies employed samples from both human and rumen microbiota. The rumen inoculant was selected, as in contrast to the human gut, ruminant microbiota are strictly adapted to an herbivorous diet, which is rich in ferulic acid. These studies showed that methyl ferulate was readily demethylated to ferulic acid. This was followed by hydrogenation of the olefinic double bond, demethylation of the methoxyl at C3, and selective dehydroxylation at C4 (Figs. 1 and 3). As well as human microbiota having the ability to perform these reactions, incubations with rumen microbes gave almost identical results, which were similar to transformations observed in earlier studies (18). For the 5-5′ linked dehydrodimer of ferulic acid (Fig. 2, compound 12), the main metabolite obtained was the α,β-unsaturated product (Fig. 2, compound 13). No cleavage of the 5-5′ linkage was observed, and no demethylation or dehydroxylation products were present. To confirm whether the α,β-unsaturated product was further metabolized, this product was treated under the same conditions as above and was fully recovered unchanged after 72 h incubation (Figs. 2 and 4).

Effect of compounds on prostanoid production

Anti-inflammatory properties were compared by measuring the ability of the parent compounds and their metabolites to inhibit prostanoid production in a cell system in which the inflammatory pathways were upregulated following a cytokine-induced insult as reported previously (9). In brief, fibroblast (CCD-Co18) cells (ATCC; Middlesex, United Kingdom) at P3-P5 (PDL 23-27) were seeded at a density 1 × 10⁴ well⁻¹ in a 96-well cell culture plate and treated with and without IL-1β. At the end of the 2-h stimulation period, aliquots were removed and screened with a prostaglandin screening enzyme immunoassay (Cayman Chemicals; Ann Arbor, MI) designed to measure a wide range of prostanoids. Samples were appropriately diluted and immediately assayed according to the manufacturers’ instructions. All compounds were added 30 min prior to stimulation at a concentration of 0.1 µmol dm⁻³, which is at least 10-fold less than the estimated mean concentration of ferulic acid in fecal water samples.
Once the structures of the metabolites were established, the presence of these compounds in the colon was confirmed by analysis of fresh fecal samples collected from 4 human volunteers consuming an unrestricted Western-style diet (Table 1).

Ferulic acid (Fig. 1, compound 2) was only detected in a very small quantity in a sample from one of the 4 volunteers. The 3 major metabolites were found in all samples, and the concentration in the fecal waters ranged from approximately 2 to 5 \(\mu\)mol dm\(^{-3}\).

Comparison of the anti-inflammatory properties was made by measuring the ability of the parent compounds and their metabolites to modulate prostanoid production in a cell system in which the inflammatory pathways were upregulated following a cytokine-induced insult (9). Following IL-1\(\beta\) treatment of these cells, all potential metabolites of ferulic acid decreased the total amount of prostanoids produced, with 7 compounds significantly inhibiting prostanoid production (40–74% inhibition; Table 2). Of the compounds that were identified to be the major microbial metabolites of ferulic acid, 3 showed a significant inhibitory effect. Hydrogenation of ferulic acid (Fig. 1, compound 3) resulted in an impairment of the inhibition of prostanoid production, whereas subsequent demethylation of the 3-methoxyl group (Fig. 1, compound 4) resulted in significant \((P = 0.007)\) inhibition of prostanoid production with slightly fewer prostanoids being produced. Dehydroxylation at C4 provided 3-(3-hydroxyphenyl) propionic acid (Fig. 1, compound 5), which also significantly \((P = 0.003)\) inhibited prostanoid production to the greatest extent of the metabolites identified. However, if this compound had undergone further dehydroxylation at C3 to 3-phenylpropionic acid (Fig. 1, compound 11), its ability to inhibit prostanoid production would be significantly decreased. Of the potential metabolites to which ferulic acid could have been transformed, p-coumaric acid (Fig. 1, compound 9) showed the highest significant inhibition of prostanoid production. Although, this compound is also found in considerable amounts in plant-based foods and could potentially have anti-inflammatory properties, it is possible that it could also undergo the same transformations as ferulic acid (hydrogenation and 4-dehydroxylation) to the less effective 3-phenylpropionic acid (Fig. 1, compound 11).

Microbial transformation of methyl ferulate demonstrated that bacteria capable of performing both 3-demethylation and 4-dehydroxylation reactions are present in the gut microflora of both humans and ruminants. However, when ferulic acid is present as its dimer (5-5′ linked), neither the demethylation nor the dehydroxylation reaction proceeded, suggesting a structural specificity for these reactions. The 5-5′ dimer showed a slight (but not significant) increase in prostanoid production. Unlike for the hydrogenation of ferulic acid, hydrogenation of the double bond in the 5-5′ dimer resulted in a significant decrease \((P = 0.019)\) in production of prostanoids. This suggests that it is not the process of side-chain hydrogenation alone that affects the activity of the compounds but rather the overall structure of the compound produced.

<table>
<thead>
<tr>
<th>Compound: IUPAC Name</th>
<th>Total in Fecal Sample (mg kg(^{-1}) Dry Weight)</th>
<th>Fecal Water Concentration ((\mu)mol dm(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(4-hydroxy-3-methoxyphenyl) acrylic acid (synonym = ferulic acid); compound 2</td>
<td>0.00 0.00 0.00 0.01</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>3-(4-hydroxy-3-methoxyphenyl) propionic acid; compound 3</td>
<td>269.12 36.80 359.58 110.51</td>
<td>4.99 ± 9.40</td>
</tr>
<tr>
<td>3-(3,4-dihydroxyphenyl) propionic acid; compound 4</td>
<td>80.78 58.18 5.59 4.06</td>
<td>2.34 ± 4.10</td>
</tr>
<tr>
<td>3-(3-hydroxyphenyl) propionic acid; compound 5</td>
<td>32.23 14.49 74.76 5.89</td>
<td>2.01 ± 2.30</td>
</tr>
</tbody>
</table>

*Abbreviation is as follows: IUPAC, International Union of Pure and Applied Chemistry. Samples were obtained from 4 volunteers consuming an unrestricted Western-style diet. Concentration in fecal waters is given as mean ± SDs \((n = 4)\).*
As ferulic acid is found predominantly either as a monomer or dimer esterified to cell wall components, it will likely escape absorption in the small intestine and only become bioavailable in the colon if released by the gut microflora. These results show that the methyl ester of ferulic acid is rapidly de-esterified by the gut microflora, rendering it available for absorption in the colon. Ferulic acid was also further metabolized undergoing hydrogenation of the α,β-unsaturated bond, demethylation, and selective dehydroxylation at C4, and these metabolites were confirmed to be present in human fecal samples. The 5′-dimer of ferulic acid was not cleaved to give the monomers, and for the dimer, further demethylation and dehydroxylation reactions did not occur. This suggests that these reactions are structure specific, but despite this, the 5′-dimer still underwent hydrogenation of the α,β-unsaturated bond. In a cell model in which the inflammatory pathways have been upregulated, ferulic acid and the compounds to which it is transformed are all inhibitors of prostanoid production. Although ferulic acid has the potential to significantly \( (P = 0.011) \) inhibit prostanoid production in this system, hydrogenation of the α,β-unsaturated double bond decreases this ability (Fig. 1, compound 3). However, if the hydrogenated product of ferulic acid is further demethylated or 4-dehydroxylated, this ability is restored \( (P = 0.007 \) and \( 0.003 \), respectively). Unlike ferulic acid, the 5′-dimer does not inhibit the production of prostanoids and demonstrated a slight but nonsignificant increase in prostanoid production.
Acknowledgments

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References
