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A Citrus Extract Containing Flavanones Represses Plasminogen Activator Inhibitor-1 (PAI-1) Expression and Regulates Multiple Inflammatory, Tissue Repair, and Fibrosis Genes in Human Colon Fibroblasts

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The consumption of flavonoid-rich *Citrus* extracts has been associated with multiple beneficial effects including anti-inflammatory properties, but the potential effects on the inflammatory responses in the gut have not been thoroughly investigated. We used microarrays to search for molecular changes induced in human colon fibroblasts in response to the exposure to a flavanone-rich bitter orange extract under physiologically representative conditions. Dietary nontoxic levels of the predigested extract induced moderate but significant changes in the expression of genes associated with tissue repair and inflammation. Among the top regulated genes, plasminogen activator inhibitor 1 (PAI-1) was downregulated, and the matrix metallopeptidase 12 (MMP-12) was upregulated (mRNA and protein levels). Both proteins are involved in extracellular matrix (ECM) remodeling and fibroblast migration. The extract also affected the fibroblast migration and reduced monocyte adhesion, but the response was different in unstimulated cells and in cells pretreated with TNF-α. Collectively, these results were indicative of a moderate activation of the colon fibroblast inflammation-related function after exposure to the extract. Further investigations are required to identify the in vivo role of this *Citrus* derived extract in the maintenance of the normal balance in the intestine and in the pathogenesis of inflammatory diseases.

KEYWORDS: In vitro digestion; intestinal stability; microarrays; MMP-12; migration; monocytes adhesion

INTRODUCTION

Inflammation is a normal protective attempt by the organism to eliminate harmful stimuli, such as pathogens or toxins, as well as to initiate the healing and repair process of the damaged tissue. Inflammation is a very complex response that involves different types of cells (neutrophils, mononuclear cells, and fibroblasts) as well as a cascade of molecular mediators which are all closely regulated by the organism. Uncontrolled inflammation can lead to chronic diseases such as allergy, rheumatoid arthritis, and bowel inflammatory diseases (*1*). In these cases, anti-inflammatory compounds are therapeutically administered to control the inflammation response. Plants rich in certain flavonoids have been traditionally used for their anti-inflammatory properties, and recently, attention has been given to flavonoids isolated from *Citrus* (*2*). Flavanones are the flavonoids predominant in *Citrus*, where they are usually found as glycosides (naringin, neohesperidin, hesperidin, etc.) (*3*).These compounds are of commercial interest because of their multitude of applications in the food and pharmaceutical industries. Significantly, much of the bioactivity of *Citrus* flavonoids appears to impact blood and microvascular endothelial cells, and thus, inflammation has been an important area of research among the biological actions of *Citrus* flavanones. Hesperidin has been commercialized as a product that improves the permeability and integrity of the capillary lining (*4*). It has also been reported to exert noticeable in vivo anti-inflammatory systemic effects in mice models of lipopolysaccharide (LPS)-induced lung inflammation (*5*) and of endotoxin-induced infection (*6*), in rat models of rheumatoid arthritis (*7*, *8*) and against inflammation in mouse skin (*9*). Naringin also reduces LPS- or infection-induced endotoxin shock in mice (*10*, *11*) and, its aglycone, naringenin, exerts anti-inflammatory properties in macrophages and in human blood (*12*).

There are only a few reports looking at the anti-inflammatory effects of *Citrus* flavonoids in the intestine. Hesperidin has been shown to ameliorate colonic inflammation by reducing colonic damage and colonic mieloperoxidase activity in animal models of trinitrobenzenesulfonic acid (TNBS)- (*13*) and of dextran sulfate sodium (DSS)-induced experimental colitis (*14*). Bowel inflammatory diseases are characterized by chronic mucosal inflammation resulting from the transmural infiltration of immune cells (neutrophils, lymphocytes, and monocytes) accompanied by the

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overproduction of oxygen free radicals, ultimately leading to the disruption of the epithelial barrier (*1*). Colonic subepithelial fibroblasts are critically involved in wound healing and mucosal repair processes because of their ability to synthesize and secrete collagens and various other ECM components which are important molecules for ECM remodeling. It is becoming clear that colon fibroblasts also play an active role in the inflammation response in the gut since they affect the recruitment and activation of the immune cells through their synthesis of cytokines, chemokines, eicosanoids, adhesion proteins, and other inflammatory mediators (*15*).

To date, the potential effects of plant-derived flavanone-rich extracts in the inflammatory responses in the gut have not been investigated in great detail. In the present work, genome-wide microarray analyses followed by differential expression and functional analysis were used to investigate the response of human colon fibroblasts (CCD-18Co) to treat with a bitter orange extract enriched in several flavanones under conditions representative of those that may occur in vivo. This study also sought to examine the phenotypic response of the cells by looking at indications of improved migration and monocyte adhesion to colon fibroblasts after the treatment.

MATERIALS AND METHODS

Materials. The soluble extract from bitter orange kindly provided by Zoster S.A. (Murcia, Spain) was composed of flavonoids (45−55%), water (3−5%), proteins (11−13%), pectins (12−14%), cellulosic material (0−1%), ashes (2−3%), vitamins (1000−1200 ppm vitamin C and traces of vitamins B1, B2, and B6), β-carotene (3−5 ppm), and other components (3−5%). On arrival, the extract (a hygroscopic brown powder, 2.1% humidity) was kept in a tightly closed container within a desiccator at room temperature. Fresh solutions of the extract were prepared by dissolving 0.1 g of the powder extract in 50 mL of water (0.2%). Pepsin from porcine stomach mucosa (pepsin A, EC 3.4.23.1), pancreatin from porcine pancreas (4 g/L, 1.6×10^{-3} U.S. Pharmacopeia (USP) specifications), and bile extract mixture (25 g/L, containing a mixture of sodium cholate and sodium deoxycholate) were all from Sigma (Steinheim, Germany). Naringin (naringenin-7-*O*-neohesperidoside), hesperidin (hesperetin-7-*O*-rutinoside), neohesperidin (hesperetin-7-*O*-neohesperidoside), naringenin (4′,5,7-trihydroxyflavanone), hesperetin (3′,5,7-trihydroxy-4′-methoxy flavanone), and isosakuranetin (5,7-dihydroxy-4′-methoxy-flavanone) were purchased from Extrasynthese (Genay, France). Human recombinant TNF-α was from Sigma (Steinheim, Germany). All other chemicals were of analytical/HPLC grade. Ultrapure Millipore water was used for all solutions.

In Vitro Digestion. The extract solution was subjected to successive in vitro salival, gastric, and pancreatic digestion. Gastric and pancreatic incubations were carried out following a previously published method (*16*) with some modifications. Briefly, 50 mL of extract solution was first incubated with 200 μL of fresh human saliva on a magnetic stirrer for 5 min. On the basis of in vivo results (*17*), the orange extract solution was then gradually adjusted to pH 2.0 by sequential addition of 0.1 N HCl and pepsin in a shaking bath as follows: time 0, initial pH 7.6, addition of 25% of pepsin; time 15 min, pH 5.5, addition of 25% of pepsin; time 30 min, pH 3.0, addition of the remaining 50% of pepsin; time 60 min, final pH 1.9 (total pepsin added: 15750 IU). A 20 mL sample of the pepsin digest was then neutralized with $NaHCO_3$ before the addition of 5 mL of the pancreatin−bile extract mixture and further incubation of the mixture (pH 7.5) for an additional 2.0 h. Control samples were run in parallel and consisted of an equivalent volume of ultrapure water subjected to the same digestion process (i.e., contains the mixture of enzymes + salts). At the end of each digestion stage, 1 mL samples were taken and filtered (0.2 μm) prior to analysis of the flavanones using high performance liquid chromatography−diode array detection (HPLC-DAD).

In Vivo Gastrointestinal Stability of the Flavanones. Male Sprague−Dawley rats ($n = 15$; Harlan Interfauna Ibérica, S.L., Barcelona, Spain) weighing 232 ± 14 g were kept in the Experimental Animal Facility of the University of Murcia (Spain). The study followed a protocol approved by the local animal Ethics Committee and the Local Government and was in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC). Rats were housed in a room with controlled temperature (22 ± 2 °C), 55 ± 10% relative humidity, and a 12 h light-dark cycle and had free access to tap water and a rat standard chow (Panlab, Barcelona, Spain). The animals were slightly anesthetized by intramuscular administration of a mixture (1:1 v/v; 0.32 mL/kg body weight) of xylazine (Calier Laboratories, Barcelona, Spain) and ketamine (Merial Laboratories, Barcelona, Spain) and 150 mg of the orange extract (63.15 mg of total flavanones) dissolved in 0.5 mL of water were administered via gastric gavage. At 1, 2, 6, 8, 12, and 24 h after administration, the animals were anesthetized (xylazine/ketamine 1:1 v/v; 1.0 mL/kg body weight), sacrificed by heart puncture exanguination and the stomach, small, and large intestine removed. The content of these organs were washed out with cold phosphate buffered saline (PBS), weighed into 15 mL sterile tubes, frozen in liquid nitrogen, and stored at −80 °C until extraction and analysis of the flavanones. Extractions were carried out following a reported protocol (*18*).

Analysis of Flavanones by HPLC-DAD. Samples (original extract, digested aliquots, culture medium, or rat lumen content) were all filtered (0.2 μm) and analyzed by HPLC-DAD to determine the composition and recovery of soluble flavanones. Separations were achieved on a 250 × 4 mm i.d., 5 μm, C_{18} Mediterranean Sea column (Teknokroma, Barcelona, Spain) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 mL/min. The linear gradient started with 7% solvent B in solvent A reaching 30% solvent B at 25 min, which was kept up to minute 30. At 36 min, the gradient reached 60% of solvent B and 90% at 37 min up to 42 min then back to the initial conditions again and kept in isocratic conditions up to 50 min. Flavanones were identified by their spectroscopic properties. Chromatograms were recorded at 280 nm, and quantification was done by comparison with authentic synthetic external standards dissolved in DMSO. Analyses were replicated (at least $n = 3$), and results are given as mean values ± SD.

Cell Culture and Cell Treatment. The myofibroblast-like cell line CCD-18Co, derived from a colonic mucosal biopsy, was obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Invitrogen S.A., Barcelona, Spain), and 10% v/v fetal bovine serum (FBS) at a final pH 7.2−7.4 and maintained at 37 °C under a 5% CO_2/95% air atmosphere at constant humidity. Cells were seeded at 6000 cells/cm^2 on 6-well plates (RNA extraction) or 10 cm plates (protein extracts) (Nunc, Roskilde, Denmark), allowed to adhere for 48 h and treated on day 5−6 after seeding (~80% confluence) as follows: (i) digested orange extract (~60 μM total flavanones in the culture medium); (ii) nondigested orange extract (~60 μM total flavanones in the culture medium); (iii) mixture of 6 major flavanones (~60 μM total flavanones in the culture medium). Cells were exposed to each of the extracts or compounds for 12 h, 24 h, and/or 48 h. The orange extract was dissolved in water, whereas the flavanones were dissolved in DMSO (< 0.1% in the culture medium) and filtered (0.2 μm) prior to addition to the culture media. Control cells were treated with control digesta (mix of enzymes and salts + water) or DMSO (< 0.1%). In a second set of experiments, the colon cells were deprived from serum for 24 h (0.1% FBS). Next, the cells were subjected to an inflammatory stimulus using TNF-α (20 ng/mL) for 8 h prior to treatment with the nondigested orange extract (~60 μM total flavanones in the culture medium) for a further 24 h. All treatments were carried out in triplicate and with cells between passages 30 and 35. To discard cell cytotoxicity caused by a pronounced shift of the osmolality and/or the pH of the culture medium when incubated with excessive quantities of digested extract, we measured the pH (Neutralit, pH 5.5−9.0, Merck) and the osmolality vapor pressure osmometer 5520 (VAPRO, Wescor) in the culture medium as well as cell proliferation and cell viability after 12 and 24 h of exposure to increasing amounts of the digested orange extract (2−470 μM total equivalent flavanone concentration in the culture media). The stability of the flavanones in the culture medium was determined by monitoring the concentration of each of the flavanones by HPLC-DAD at different time points during the incubation.

Human acute monocytic THP-1 cells were obtained from the European Collection of Cells Cultured (ECACC) (Salisbury, UK). Cells were maintained in RPMI 1640 culture media containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Invitrogen S.A., Barcelona, Spain) at a final pH of 7.2−7.4 and maintained at 37 °C under a 5% CO_2/95% air atmosphere at constant humidity. Cells were seeded at $3-5 \times 10^5$ cells/mL on either 25 or 75 cm^2 flasks (Nunc, Roskilde, Denmark). Cells were subcultivated every two days (cell concentration in the culture media was always below 8×10^5 cells/mL). All experiments were carried out in triplicate and with cells between passages 10 and 15.

Cell Proliferation and Viability. At the end of each treatment, trypsinized cells (2.5 g/L trypsin, 0.2 g/L EDTA) were suspended in culture medium, counted using a hemocytometer and viability measured using Trypan blue dye exclusion. Results of proliferation and viability in treated cells are presented in comparison to those of untreated cells. Data are presented as mean values ± SD from three independent experiments (n = 3 plates per experiment).

RNA Extraction. Total RNA was isolated using an RNeasy mini kit (Quiagen, Barcelona, Spain). Concentration and purity were checked using the Nanodrop spectrophotometer system. Only samples with a ratio of Abs_{260}/Abs_{280} between 1.8 and 2.1 were used for microarrays experiments. The integrity of the rRNA was further checked using agarose gel electrophoresis (1%). Treatments and extractions were done in triplicate.

Microarray Analysis. A search for potential candidate genes for which transcription levels may have been modulated in the colon cells following exposure to the flavanone extract was performed using Affymetrix microarray analysis (GeneChip Affymetrix microarray HG U133 Plus 2.0) following the manufacturer's one-cycle protocol. Microarray data were Robust-Multi-Array (RMA)-normalized and tested for differential gene expression using a t-test and FDR adjustment for multiple testing implemented with software Gene Expression Pattern Analysis Suite (GEPAS) (*19*). Probes were considered to exhibit significant expression changes when they met the two following criteria: (1) FDR < 0.2 ($p < 0.005$); (2) changes were ≥ 1.25 or ≤ 0.80 (ratio treated/control). To identify significantly altered biological pathways and top regulated functions associated to responsive genes, Ingenuity Pathways Analysis (IPA) (*20*) was used. Minimum information about a microarray experiment (MIAME) compliant data from control and treated CCD-18Co cells after exposure to the flavanone enriched orange extract have been deposited in NCBIs Gene Expression Omnibus (GEO) (*21*) and are accessible through GEO Series accession number GSE15322.

RT-PCR. Changes in the expression of five selected genes responding to the treatment with the flavanone extract were further assessed by one-step quantitative RT-PCR (Taqman system, Applied Biosystems, ABI, Madrid, Spain). Primers and probes for the genes were selected from Assays-on-Demand (ABI, Madrid, Spain) and are as follows: *SERPINE1* (serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1) member 1), (Hs00167155_m1); *MMP12* (matrix metallopeptidase 12 (macrophage elastase)), (Hs00899662_m1); *SMAD3* (SMAD family member 3), (Hs00232219_m1); *TGFBR2* (transforming growth factor, β receptor II (70/80 kDa)), (Hs00234253_m1); SMURF2 (SMAD specific E3 ubiquitin protein ligase 2), (Hs00224203_m1) (_m indicates an assay whose probe spans an exon junction and will not detect genomic DNA). The one-step real-time RT-PCRs were run on the ABI 7500 system following manufacturer's conditions, using a total reaction volume of 25 μL in a MicroAmp Optical 96-well plate covered by optical adhesive covers and using Taqman Universal Master Mix (ABI, Madrid, Spain). All assays for a particular gene were undertaken at the same time under identical conditions and in triplicate. The expression levels of target genes were normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs99999905_m1) utilizing a standard curve method for quantification.

Western Blot Analysis. Following treatment, cells were washed twice with PBS and lysed with 0.5 or 1 mL of ice-cold lysis RIPA buffer containing a mixture of protease inhibitors (Roche). Lysates were centrifuged at 15000g for 15 min at 4 °C, and protein concentration was determined by Bradford's method. Equal amounts of protein (10 μg per lane) were separated on a 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to polyvinylidine diflouride membranes (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. The membranes were washed and incubated for 2 h with the primary antibodies: serpine1 (~40KDa), (Affinity Bioreagents, ABR, Madrid, Spain) at 1:1000; polyclonal antibody anti-MMP-12 (MBL international corporation, Woburn, MA) at 1:250; SMAD3 (48−52KDa) (ABR) at 1:500; TGFBR2 (~70KDa) (Abcam, Cambridge, UK) at 1:500; SMURF2 (~86KDa) (Abcam) at 1:1000 followed by 1 h with antimouse IgG (Sigma-Aldrich Química S.A., Madrid, Spain) or antirabbit IgG (Sigma-Aldrich) horseradish peroxidase (HRP)-linked secondary antibody at 1:5000. Membranes were developed with ECL Plus (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions and exposed to X-ray film. GAPDH antibody (~36KDa) (ABR) at 1:2500 was used to control for protein loading. Western blot analyses were done at least in triplicate.

Cell Migration: Cell Culture Wound Healing Assay. Confluent monolayers of CCD-18Co cells were scraped away horizontally with a sterile pipet tip. Media, dislodged cells, and debris were aspirated, washed once with PBS, and cells were incubated with fresh medium (0.1% FBS) for 24 h. Next, the cells were treated with TNF-α (20 ng/mL) for 8 h prior to treatment with the nondigested orange extract (~60 μM total flavanones in the culture medium) for a further 24 h. Randomly selected views along the scraped line were photographed on each well at the end of treatment, using a phase contrast inverted microscope and a CCD camera attached to the microscope. The change in the area of the experimental conditions was compared with that of the corresponding control. Results are representative of two to four experiments.

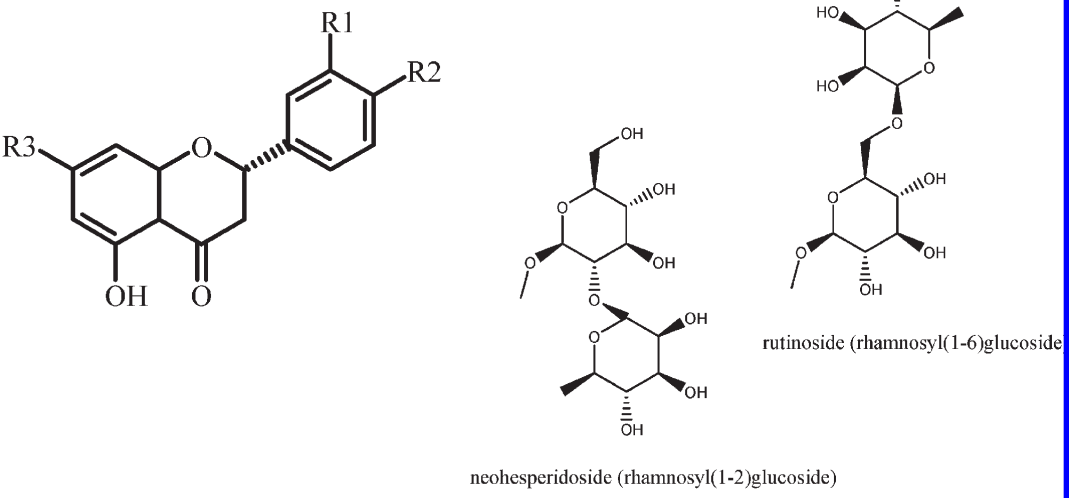
Monocyte-Fibroblast Cell Adhesion Assay. Monocyte-colon fibroblast cell adhesion was evaluated using the human leukemia monocytic THP-1 cells. Monocytes (1×10^6 cells/mL) were resuspended in PBS, labeled with 5 μM calcein (Sigma Aldrich, St. Louis, MO, USA) for 30 min at 37 °C, and washed twice with PBS before addition to the fibroblasts. Colon fibroblasts cultured in 96-well plates to confluence were incubated with fresh medium (10% or 0.1% FBS) for 24 h. Next, cells were inflamed using TNF-α (20 ng/mL) for 8 h prior to treatment with the nondigested orange extract (~60 μM total flavanones in the culture medium) for a further 24 h. After treatment, the colon fibroblasts were coincubated with calcein-prelabeled monocytes (2×10^5 cells per well) for 1 h at 37 °C. Nonadhering cells were removed and the cells washed twice with PBS before fluorescence was measured with a fluorescence-detecting microplate reader (Fluostar Galaxy, BMG Lab. Technologies v5.0) using excitation at 492 nm and emission at 520 nm.

Statistical Analysis. Results are presented as the mean values ± SD (at least n = 3). Where indicated, differences between groups (control vs treated) were compared using an unpaired Student's t-test (except for microarray analysis; see specific section). Results with a two-sided p value < 0.05 or < 0.01 were considered statistically significant.

RESULTS

Stability of Flavanones Present in the Orange Extract During Gastro-Intestinal Digestion. HPLC-DAD analysis of the orange extract (0.2% in water) showed that the extract comprised approximately 42.1% soluble flavanones with the two most abundant compounds, naringin (24.5 ± 2.6%) and neohesperidin (11.4 ± 0.8%), followed by hesperidin (1.7 ± 0.4%), naringenin (0.4 ± 0.1%), hesperetin (0.4 ± 0.1%), and isosakuranetin (0.2 ± 0.1%) (% of the powder extract; mean ± SD; n = 7). The structures of the main flavanone constituents of the bitter orange extract are included in **Figure 1**. Other minor components with spectral properties similar to those of flavanones amounted to 3.5 ± 0.8% of the total compounds. The levels of soluble flavanones were not altered after the salival, gastric, or pancreatic phases of the in vitro digestion. These results show that the major flavanones present in the bitter orange extract are stable under the in vitro conditions assayed and suggest that in vivo, these compounds may remain constant throughout the small intestine and reach the large intestine in their original molecular form.

To validate the in vitro stability of the flavanones and to confirm the presence of these intact molecules in the gastrointestinal



Flavanone	R1	R2	R3	mg/g extract
Naringin	H	OH	ONeo[a]	245
Neohesperidin	OH	OCH_3	ONeo	114
Hesperidin	OH	OCH_3	ORut[b]	17
Naringenin	H	OH	OH	4
Hesperetin	OH	OCH_3	OH	4
Isosakuranetin	H	OCH_3	OH	2

[a]Neohesperidose; [b]rutinoside.

Figure 1. Chemical structures of the major flavanones present in the bitter orange extract.

tract, the luminal contents of the stomach, small and large intestines from rats dosed with the flavanone extract were analyzed by HPLC-DAD. The results are presented in **Table 1**. Around 33% and 18% of the administered flavanones were recovered, from the stomach and from the small intestine, respectively, 1 hour after dosing and were still detected in both sections of the gut even after 12 h of dose administration. The flavanones were first detected in the colon 2 h after dosing, and the levels of these compounds were found highest at 6 and 12 h after the dosing. At 24 h, some flavanones were still detected in the lumen of the stomach and of the small and large intestines (approximately 345 μM flavanones for an estimated 2 mL rat colon volume). The maximum percentage of total flavanones recovered was approximately 72% of the administered dose.

Expression Changes Induced in Human Colon Fibroblasts by the Digested Flavanone-Rich Orange Extract. Quantities of filtered (0.2 μm) digested orange extract equivalent to 2.0 to 60 μM of total flavanones added to the culture media or the corresponding control digestion mix did not appreciably alter the pH values (7.0−7.5) of the culture medium, and the osmolality was within the range tolerated by human cells (260−320 mOsm/L). Within this range of concentrations, the treated colon cells (12 or 24 h) exhibited viability (>90%) and proliferation values (>95%) similar to those of the control cells (treated with the mixture of salts + enzymes) or the untreated cells (EMEM). Also, the major flavanones present in the extract were stable in the culture conditions even after 24 h of incubation (results not shown).

Table 1. Total Flavanones Recovered from the Stomach, Small Intestine, and Colon of Rats Dosed with Orange Extract

time points[a]	stomach content	small intestine content	colon	total
1	20.6[b] (32.6%)[c]	11.4 (18.1%)	n.d.	32.0 (50.7%)
2	24.0 (38.0%)	9.0 (14.3%)	0.08 (0.13%)	33.0 (52.3%)
6	17.6 (27.9%)	10.8 (17.1%)	17.1 (27.1%)	45.5 (72.1%)
12	10.2 (16.2%)	0.8 (1.3%)	9.8 (15.5%)	20.8 (32.9%)
24	0.5 (0.80%)	0.04 (<0.06%)	0.4 (0.6%)	0.9 (1.5%)

[a] Hours after the dose. [b] In mg. [c] Percent of the administered dose of flavanones; n.d., not detected.

Microarray analyses were used to investigate gene expression changes induced in colon fibroblasts by the predigested orange extract at a dose that did not cause cytotoxicity or inhibition of

Table 2. Selection of Probes with Significant Expression Changes in Human Colon Fibroblasts Exposed to a Flavanone-Enriched Orange Extract and Involved in Immune Response and Tissue Repair

affymetrix probe	gene symbol	gene name	ratio T/C
		ECM Components	
211343_s_at	*COL13A1*	collagen, type XIII, α 1	0.6
217430_x_at	*COL1A1*	collagen, type I, α 1	0.7
202404_s_at	*COL1A2*	collagen, type I, α 2	0.8
212488_at	*COL5A1*	collagen, type V, α 1	0.7
37892_at	*COL11A1*	collagen, type XI, α 1	1.4
211981_at	*COL4A1*	collagen, type IV, α 1	1.6
214641_at	*COL4A3*	collagen, type IV, α 3 (Goodpasture antigen)	1.3
227048_at	*LAMA1*	laminin, α 1	1.6
204580_at	*MMP12*	matrix metallopeptidase 12 (macrophage elastase)	2.1
		Growth Factors and Related Genes	
210311_at	*FGF5*	fibroblast growth factor 5	0.6
203821_at	*HBEGF*	heparin-binding EGF-like growth factor	0.5
210095_s_at	*IGFBP3*	insulin-like growth factor binding protein 3	0.7
208944_at	*TGFBR2*	transforming growth factor, β receptor II (70/80 kDa)	0.7
209946_at	*VEGFC*	endothelial growth factor C	0.7
205239_at	*AREG*	amphiregulin (schwannoma-derived growth factor)	2.2
228121_at	*TGFB2*	transforming growth factor, β 2	1.3
204731_at	*TGFBR3*	transforming growth factor, β receptor III	1.5
		Interleukins	
206924_at	*IL11*	interleukin 11	1.3
205067_at	*IL1B*	interleukin 1, β	1.7
202948_at	*IL1R1*	interleukin 1 receptor, type I	1.7
206569_at	*IL24*	interleukin 24	1.6
205207_at	*IL6*	interleukin 6 (interferon, β 2)	1.6
202859_x_at,	*IL8*	interleukin 8	1.5
231779_at	*IRAK2*	interleukin-1 receptor-associated kinase 2	1.5
		Chemokines	
205476_at	*CCL20*	chemokine (C-C motif) ligand 20	1.6
204470_at	*CXCL1*	chemokine (C-X-C motif) ligand 1	1.7
204533_at	*CXCL10*	chemokine (C-X-C motif) ligand 10	2.2
210163_at	*CXCL11*	chemokine (C-X-C motif) ligand 11	1.9
209774_x_at	*CXCL2*	chemokine (C-X-C motif) ligand 2	2.2
207850_at	*CXCL3*	chemokine (C-X-C motif) ligand 3	2.4
215101_s_at	*CXCL5*	chemokine (C-X-C motif) ligand 5	3.1
206336_at	*CXCL6*	chemokine (C-X-C motif) ligand 6	1.9
		Protease Inhibitors	
202628_s_at	*SERPINE1*	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	0.4
212268_at	*SERPINB1*	serpin peptidase inhibitor, clade B (ovalbumin), member 1	1.4
212190_at	*SERPINE2*	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	1.7
		Prostaglandin Synthases	
207388_s_at	*PTGES*	prostaglandin E synthase	2.1
204748_at	*PTGS2*	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.7

cell proliferation (i.e., we were not investigating antiproliferative effects) and that was representative of the quantities that may be found in the colon lumen from the diet. We therefore chose to treat the colon cells with the highest quantity of digested orange extract that did not inhibit cell proliferation (~60 μM total flavanones in the culture media) and for the largest period of time at which the flavanones were proved to be stable in the culture media, i.e., 24 h. After exposure, a total of 705 probes were shown to exhibit moderate but significant expression changes in treated cells against cells treated with the control digesta. The significantly regulated probes and their ratios were uploaded into the IPA software in order to identify genes that could be potentially involved in specific diseases, molecular cell functions, and physiological processes and to pinpoint canonical pathways in which those genes are involved and may have been affected in the colon cells by the treatment. Modulated genes were primarily associated with inflammatory diseases, immune response, and processes of tissue repair and fibrosis. The principal molecular and cell functions that may have been affected in the treated cells were cell migration and adhesion and recruitment of immune cells. A selection of genes exhibiting significant expression changes in colon fibroblasts after exposure to the digested orange extract and that are associated with the role of intestinal subepithelial fibroblasts on inflammation and repair processes in the gut is presented in **Table 2**. A number of interleukins, chemokines, and prostaglandin synthases were all found to be upregulated, whereas several extracellular components, collagens, and growth factor related genes were both down- and upregulated in the

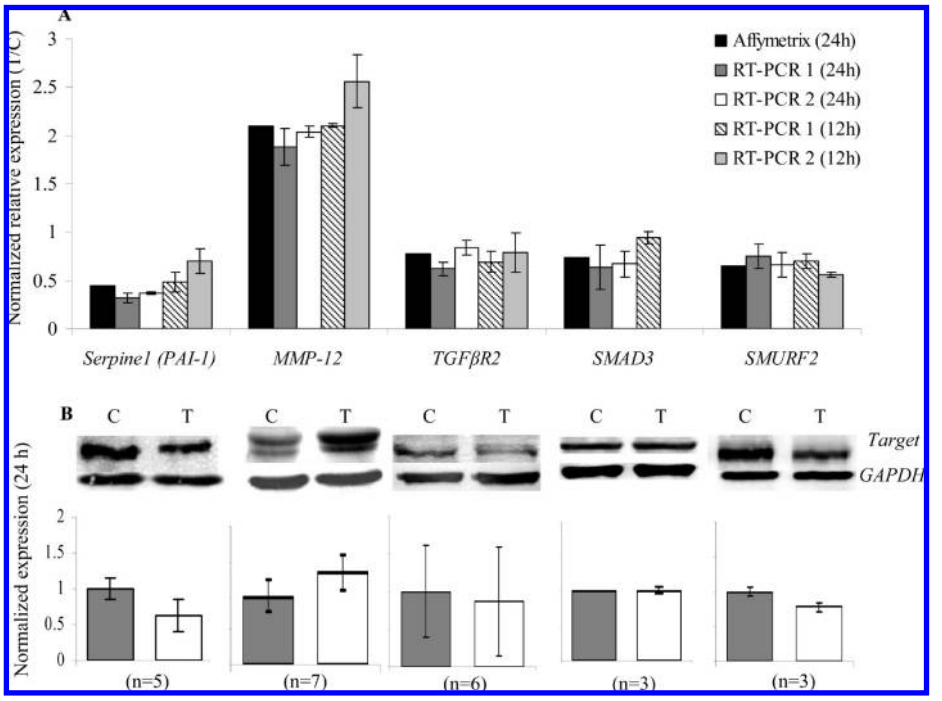


Figure 2. Expression levels of selected genes modified in colon fibroblasts after 12 or 24 h of exposure to the digested bitter orange extract rich in flavanones. (**A**) *GAPDH* normalized relative gene expression changes (T/C, treated/control) by Affymetrix microarrays and by RT-PCR (means ± SD; $n = 3$). (**B**) GAPDH normalized protein levels of the lysates from control and treated cells using Western blots. Values are the means ± SD ($n = 3$ to $n = 7$).

treated cells. Among the top regulated molecules, we detected a significant downregulation of the serine protease inhibitor *Serpine1* or *PAI-1* as well as an upregulation of *MMP-12* (macrophage elastase).

We selected *PAI-1* and *MMP-12* as well as several related signaling molecules, *TGFβR2*, *SMAD3*, and *SMURF2* to further confirm expression changes in response to the digested orange extract exposure. For each gene, RT-PCR reactions were performed in aliquots of the same mRNA used for microarrays (cells treated for 24 h) and in mRNA prepared from independent experiments (cells treated for 12 and 24 h; each experiment consisted of triplicate mRNA samples extracted from three separate wells). RT-PCR results are presented as relative expression changes (T/C) in **Figure 2A** and were in good agreement with the Affymetrix microarrays results. We also compared the protein levels between the lysates from control and treated cells using Western blots (**Figure 2B**). We found that the protein levels of PAI-1 and SMURF2 were consistently, although not significantly, downregulated (0.6 ± 0.2 ($n = 5$) and 0.7 ± 0.1 ($n = 3$), respectively), and the levels of MMP-12 were augmented (1.3 ± 0.1, $n = 7$) after 24 h exposure to the digested orange extract. Since no differences in the protein levels of TGFβR2 or SMAD3 were detected between the control and treated cells, we did not continue the analysis of these two genes.

Expression Changes Caused by the Whole Orange Extract and Not By Main Flavanone Constituents. We next wanted to discard the fact that the observed effects might have been caused by artifacts or other molecules formed during the in vitro digestion process. For this purpose, we examined whether the observed changes in the three selected markers (*PAI-1*, *MMP-12*, and *SMURF2*) were still induced in the colon cells exposed to undigested orange extract at the equivalent concentration of total flavanones (60 μM) (**Figure 3A** and **B**). We found that *PAI-1* gene expression was downregulated by 0.4 ± 0.1, 0.3 ± 0.1, and 0.6 ± 0.1 after 12, 24, and 48 h of exposure to the undigested orange extract ($n = 3$; $p < 0.01$ for 24 h). This was further confirmed at

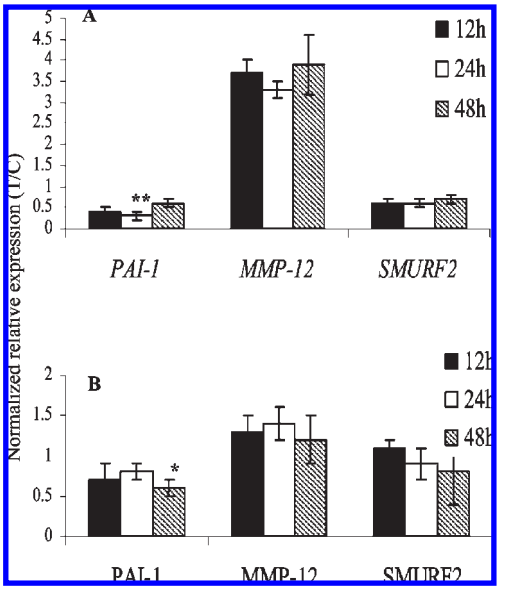


Figure 3. GAPDH normalized expression changes (T/C, treated/control) of PAI-1, MMP-12, and SMURF2 in colon fibroblasts after exposure to undigested bitter orange extract at the equivalent concentration of total flavanones (60 μM) for 12, 24, and 48 h. (**A**) Gene expression changes; (**B**) protein changes. Values are the means ± SD ($n = 3$), ** ($p < 0.01$), and * ($p < 0.05$).

the protein level by a small decrease of PAI-1 at the three time points tested significant after 48 h of treatment (0.5 ± 0.1, $n = 3$, $p < 0.05$). MMP-12 was also upregulated in the colon fibroblasts after exposure to the undigested extract at the mRNA level (3.7 ± 0.3, 3.3 ± 0.2, and 3.9 ± 0.7) and at the protein level (1.3 ± 0.2, 1.4 ± 0.2, and 1.2 ± 0.3) after 12, 24 and 48 h of treatment, respectively. *SMURF2* was downregulated at the mRNA levels, but no changes in the protein levels were detected.

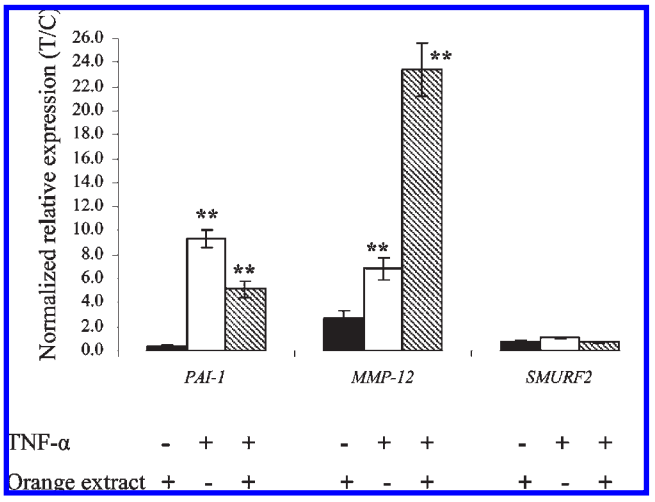


Figure 4. *GAPDH* normalized gene expression changes (T/C, treated/control) of *PAI-1*, *MMP-12*, and *SMURF2* in colon fibroblasts cultured in (i) EMEM culture medium (0.1% FBS) + orange extract added 32 h after serum withdrawal; (ii) EMEM culture medium (0.1% FBS) + TNF-α (20 ng/mL) added 24 h after serum withdrawal; (iii) EMEM culture medium (0.1% FBS) + TNF-α (20 ng/mL) added 24 h after serum withdrawal + orange extract added 8 h after TNF-α addition. RT-PCR analysis of control and treated cells were performed after 56 h of incubation. Values are the means ± SD ($n = 3$; ** ($p < 0.01$).

We next determined whether the observed changes in the levels of PAI-1, MMP-12, and SMURF2 were specifically caused by the main flavanones present in the extract. Exposure of the colon fibroblasts to a mixture of the six major flavanones present in the orange extract at an equivalent individual and total concentration in the culture medium (60 μM) induced the transcription levels of *MMP-12* by 2−3 times above the control cells but failed to produce any of the other observed changes, indicating that the main flavanones were not responsible for all the changes and that other components in the extract may be responsible for or contribute to them.

Changes Induced by the Orange Extract in PAI-1, MMP-12, and SMURF2 in TNF-α Stimulated Colon Fibroblasts. In a second set of experiments, TNF-α-treated colon fibroblasts (20 ng/mL, 8 h) were then exposed to the orange extract for 24 h, and the expression levels of *PAI-1*, *MMP-12*, and *SMURF2* were measured (**Figure 4**). TNF-α induced the expression levels of *PAI-1* (T/C, 9.3 ± 0.8, $n = 3$; $p < 0.01$), which were subsequently downregulated to approximately half the value (5.1 ± 0.7; $p < 0.01$) after the treatment with the orange extract. Treatment of the colon cells with TNF-α and the orange extract further upregulated the mRNA levels of *MMP-12* (23.4 ± 2.2; $p < 0.01$), whereas the cytokine had no effect on *SMURF2* expression (1.1 ± 0.1), which was still slightly downregulated (0.7 ± 0.1) in the inflamed fibroblasts by the orange extract.

Phenotypic Changes Induced in the Colon Fibroblasts by the Orange Extract. To determine whether the observed gene expression changes were associated with an effect in some of the modulated cell and molecular functions detected by the IPA analysis (i.e., cell migration and recruitment of immune cells), the phenotypic response of the colon fibroblasts to the treatment with the orange extract was further evaluated. Fibroblast migration is an important factor in wound repair, and we therefore investigated whether the orange extract affected the CCD-18Co cell migration using the cell culture wound healing assay. The data show that exposure of the colon fibroblasts to TNF-α (**Figure 5D**), to the orange extract (**Figure 5E**), or to the cytokine followed by the orange extract (**Figure 5F**) inhibited cell migration in comparison to that in control untreated cells (**Figure 5C**). However, treatment with the TNF-α followed by exposure to the orange extract (**Figure 5F**) slightly increased cell migration when compared to that in the treatment with cytokine or the extract alone (**Figure 5D** and **E**, respectively). In addition, and as a measurement of chemotaxis of immune cells, we determined effects of exposure to the orange extract on the capacity of the fibroblasts to adhere monocytes. Exposure of the cultured colon fibroblasts to the orange extract alone enhanced the adhesion of monocytes most significantly ($p < 0.01$) when the fibroblasts were cultured in 10% serum containing medium (**Figure 6**). TNF-α treatment greatly enhanced THP-1 cell adhesion, but exposure of the inflamed cells to the orange extract did not have any further effect on monocyte adhesion.

DISCUSSION

The approach to use orally ingested flavonoids or flavonoid-rich extracts with preventive or therapeutic uses is controversial. Low bioavailability and loss of function during digestion and metabolism are two critical arguments against the efficacy of dietary supplementation with these compounds. Like for other flavonoids, the efficiency of absorption of flavanones has been shown to be poor, and relatively high quantities of the orally ingested flavanones can pass through the small intestine and reach the colon in their original molecular form (*22*). In the colon, the glycosides (e.g., naringin, hesperidin, and neohesperidin) may be deglycosylated to aglycones (e.g., naringenin and hesperetin) and further transformed into more simple phenolic compounds. Despite microbial metabolism, a proportion of the glycosides and aglycones may remain intact in the lumen for several hours (*23*, *24*). In this study, we were able to detect and quantify the major flavanones present in an orange extract both in the small intestine and in the colon of rats, even 12 and 24 h after oral administration of the extract. These results corroborate the fact that dietary flavanones can remain unaltered for several hours in the colon where they may be absorbed and/or interact with the mucosa and exert some of their biological effects by modifying gene and protein expression. We therefore investigated the effects of a flavanone-rich bitter orange extract on the transcriptomic profile of a human colon cell model (CCD-18Co) under conditions that were representative of the physiological situation. Colon fibroblasts were exposed for 12 and 24 h to the orange extract subjected to in vitro gastro-duodenal digestion at a final dose that did not exert any toxic effects on the cells (60 μM total flavanones). We considered this dose to be representative of the levels of flavanones that may be achieved in the colon lumen after oral intake since estimated colon concentrations of total dietary polyphenols may range from 100 μM to 3 mM (*25*, *26*). Also, we measured a concentration of ∼345 μM total flavanones in the colon of rats after an oral dose of 63.15 mg of flavanones (human equivalent dose (HED): ∼3 g for a 70 kg person). On the basis of all of the above, a concentration of approximately 60 μM of total flavanones in the colon is not unreasonable.

We examined the response of colon fibroblasts because they are a noncancerous cell line and because these cells constitute an important and active component in the maintenance of the intestinal mucosa. Although colon fibroblasts lie underneath the gut epithelium and are less likely to be in direct contact with the luminal digesta, transient increases in the permeability of the tight junction make possible the passage of many nutrients and small molecules (4000−5500 Da) through the paracellular route, allowing for direct contact of luminal content with the cells underneath the epithelium (*27*). Intestine fibroblasts are actively

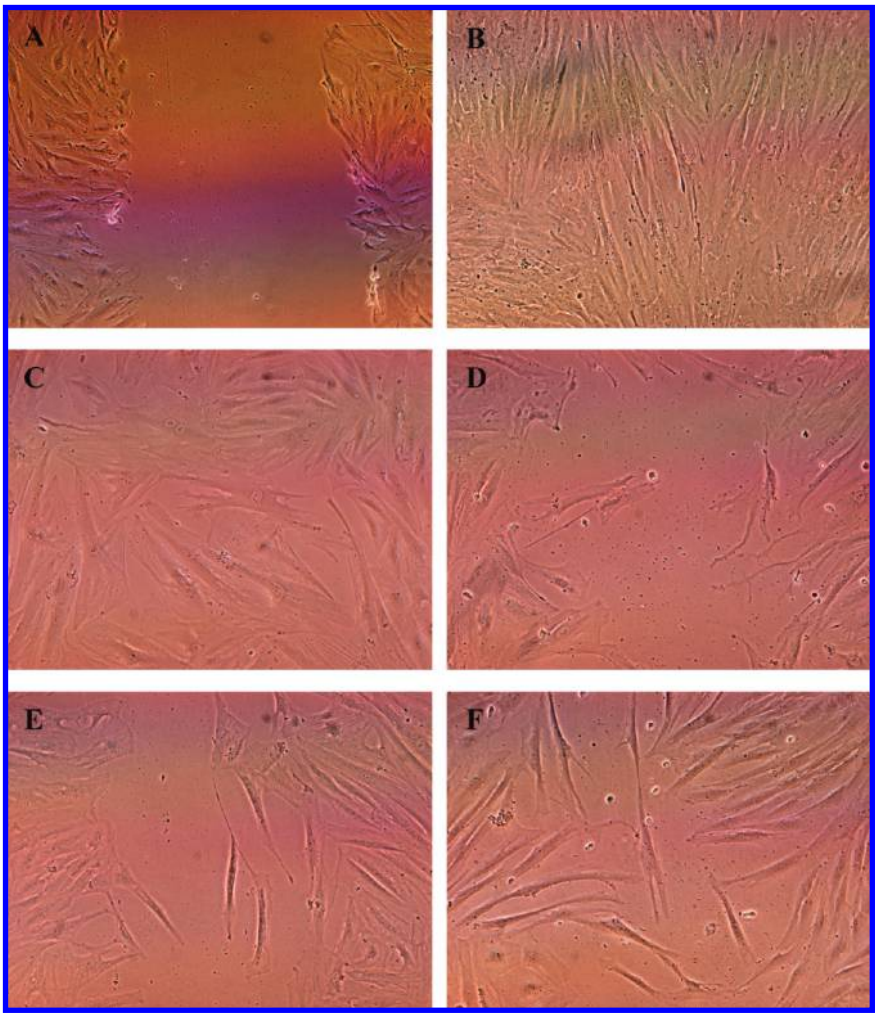


Figure 5. Effect of the flavanone-rich bitter orange extract on CCD-18Co migration examined by wound healing assay. Colon fibroblast monolayers were wounded at time 0 (**A**) and treated as follows: (**B**) EMEM culture medium (10% FBS); (**C**) EMEM culture medium (0.1% FBS); (**D**) EMEM culture medium (0.1% FBS) + TNF-α (20 ng/mL) added 24 h after serum withdrawal; (**E**) EMEM culture medium (0.1% FBS) + orange extract added 32 h after serum withdrawal; (**F**) EMEM culture medium (0.1% FBS) + TNF-α (20 ng/mL) added 24 h after serum withdrawal + orange extract added 8 h after TNF-α addition. Photographs of control and treated cells were taken after 56 h of incubation. Results are representative of 2 to 4 independent experiments.

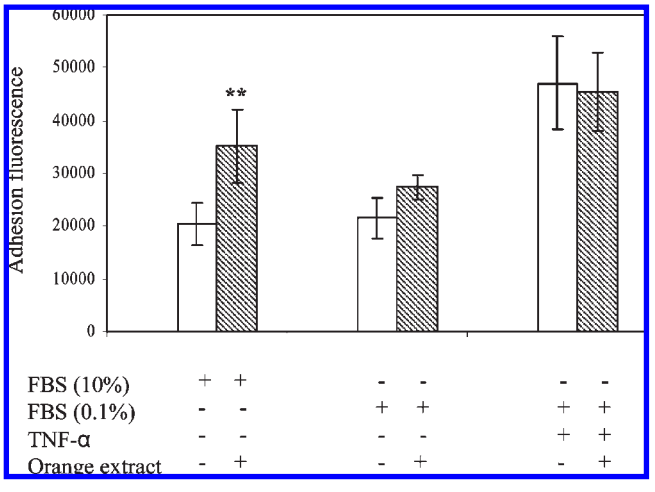


Figure 6. Effect of the flavanone-rich bitter orange extract on monocyte adhesion to the colon fibroblasts. The adhesion of calcein-labeled THP-1 monocytes to CCD-18Co colon fibroblasts was quantitated using a fluorescence-detecting microplate reader. Data are presented as the mean ± SD of at least three independent experiments; ** ($p < 0.01$).

involved in wound healing and intestinal epithelium restitution through the controlled remodeling of ECM and the migration of activated fibroblasts to the site of the wound. Activated fibroblasts show upregulated expression and enhanced secretion of ECM components such as collagen IV, laminin as well as matrix metalloproteases, MMPs. In addition, intestinal fibroblasts are thought to mediate information between the intestinal epithelium and the immune system playing an active role both in the gut physiologic immune response and during pathological inflammation. Activated fibroblasts also produce multiple mediators of inflammation such as growth factors, adhesion molecules, chemokines, cytokines, and prostaglandins. These molecules are critical players in directing the balance between physiological and pathophysiological inflammation and in coordinating multiple cell types during the wound healing and epithelial barrier repair and maintenance (*15*, *28*). Under the experimental conditions chosen for our study, treated colon fibroblasts exhibited moderate changes in the expression of groups of genes associated with tissue repair and inflammation. The cells responded to treatment by modulating the levels of expression of multiple ECM components, e.g., upregulation of collagen type IV, laminin, and metallopeptidase MMP-12, and both up- and

downregulation of several protease inhibitors (serpins). The cells response also involved transcriptional changes for several chemokines, interleukins, growth factors, and prostaglandin synthases.

Among the top regulated genes in the treated colon CCD-18Co fibroblasts, we found downregulation of the expression of PAI-1 and upregulation of MMP-12 (both at the mRNA and protein levels). PAI-1, a member of the serine protease inhibitors, is a negative regulator of plasminogen conversion to plasmin (a fibrinolytic protein) (*29*), whereas the metallopeptidase MMP-12 (a macrophage elastase) is able to degrade a range of ECM components (elastin, type IV collagen, laminin, etc.) (*30*). Reduced levels of PAI-1 and increased levels of MMP-12 may contribute to focalized ECM degradation, favoring cell detachment and cell migration (*31*, *32*). Both PAI-1 and MMP-12 have been associated with inflammatory reactions in the intestine mucosa (*33*, *34*), and pro-inflammatory cytokines such as TNF-α are strong stimulators of PAI-1 (*35*) and of MMP-12 (*36*) while they reduce fibroblast migration (*37*). In our study, TNF-α greatly induced the expression levels of PAI-1 and MMP-12 in the CCD-18Co colon fibroblasts, and this was accompanied by a reduction in cell migration in comparison to that of untreated control cells. However, the cytokine-orange extract-treated cells showed a significant decrease in PAI-1 expression and a further induction of MMP-12 concomitant with a noticeable increase in cell migration in comparison to that of the cells treated with the cytokine alone. These results indicated that, in the presence of TNF-α, changes in the ability of colon fibroblasts to migrate may be associated with changes in the levels of PAI-1, whereas MMP-12 does not seem to have an effect. Reports on the effects of MMP-12 in migration are scarce. Whereas increased digestion of the ECM matrix by addition of soluble elastase contributes to lung fibroblast migration (*38*), MMP-12 does not seem to have any effects on smooth muscle cell (SMC) migration (*39*), suggesting that the effects may be cell dependent. Conversely, when colon fibroblasts were treated with the orange extract in the absence of TNF-α, although the expression levels of PAI-1 were diminished compared to that in the control cells, cell migration was also impaired. These results are in agreement with reported properties of PAI-1 for which both over- and underexpression have been associated with migration inhibition (*40*). The response may be associated with cell status (unstimulated vs inflamed cells). It has also been suggested that precise physiologic concentrations of PAI-1 are required for optimum migration (*40*). Additionally, the orange extract induced the adhesion of monocytes to the colon fibroblasts in the absence of the cytokine, whereas it did not show any effects on the TNF-α-induced monocyte adhesion. This response may be associated with the induction of MMP-12, which has been reported to induce the recruitment of neutrophils in association with a transient increase in cytokine and chemokine levels (*33*). Overall, these results were indicative of a general moderate activation of the colon fibroblasts and of an inflammatory-like response after exposure to the digested orange extract.

This study investigates the effects of a whole natural bitter orange extract. It is important to determine the effects on the intestine of mixtures of flavonoids or of mixtures of flavonoids with other compounds since complex mixtures of compounds rather than single compounds are the most likely and frequent forms in which these rich flavonoid natural extracts may be present in the intestinal lumen. Our study shows that the major flavanones present in bitter orange extract are not responsible for the downregulation of PAI-1 in the colon cells, although they contribute to the upregulation of MMP-12. It is interesting to note that, individually, flavanones such as hesperidin or naringenin do exert gene expression modulatory effects with specific anti-inflammatory and antifibrotic effects. For example, hesperidin inhibited the TNF-α-induced VCAM-1 expression in endothelial cells (*41*) and naringenin reduced TGF-β1-induced expression of profibrotic genes, including PAI-1, in rat hepatic stellate cells (*42*). The mixture of flavanones in the bitter orange extract tested here includes naringenin but did not induce any changes in the expression of PAI-1 in the colon fibroblasts. Although we cannot discard the fact that the different responses may be caused by differences in the cell model or the naringenin concentration used, these results indicate that it is necessary to determine the effects of individual bioactive compounds as part of the mixtures or extracts which are natural forms of consumption of these compounds. Our results also suggest that other citrus components with reported anti-inflammatory properties such as polymethoxylated flavones (*43*) may be present in the tested orange extract and may contribute to the observed effects.

Plant derived bioactive compounds and/or bioactive-rich nutritional extracts are, in principle, assumed to be of a preventive nature. Many studies looking at the anti-inflammatory effects of these compounds or extracts have been mostly investigated using in vitro and in vivo models of induced inflammation. However, the effects in healthy normal tissues may be very different from those induced under pathological conditions. One of the main physiological roles of intestine fibroblasts is to contribute to the maintenance of mucosal homeostasis, responding to daily challenge by exogenous inflammation mediators (e.g., toxins, bacteria, etc.) and leading to resolution of the inflammation and tissue damage to avoid inflammation from spreading and causing disease. The process is controlled by a complex spatiotemporal action of many inflammatory mediators leading through the progression of healing and resulting in restitution of the gut barrier functions (*15*). The results presented here suggest that the bitter orange extract tested in this study exerted moderate regulatory effects on the activation of colon fibroblasts which may be beneficial in healthy normal cells to prevent sustained inflammation. The ability of fibroblasts to migrate and monocyte adhesion were differentially affected by the treatment in unstimulated cells or in cells that had been inflamed with TNF-α, suggesting that this orange extract may exert different effects on healthy cells and those in disease status. Further investigations are required to identify the role in vivo of these *Citrus* derived extracts in the maintenance of the normal balance in the human intestine and in the pathogenesis of inflammatory diseases.

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Supporting Information Available: A complete listing of the probes with significant expression changes by microarray analysis with corresponding *p*-values, FDR-values, and expression levels (ratio cells treated with the extract/cells treated with control digesta; T/C) and a summary of the top functions and pathways detected by the ingenuity pathway analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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