CYP450 dietary inhibitors attenuate TNF-α-stimulated endothelial molecule expression and leukocyte adhesion

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Sasaki, Makoto, John W. Elrod, Paul Jordan, Makoto Itoh, Takashi Joh, Alireza Minagar, and J. Steven Alexander. CYP450 dietary inhibitors attenuate TNF-α-stimulated endothelial molecule expression and leukocyte adhesion. Am J Physiol Cell Physiol 286: C931–C939, 2004; 10.1152/ajpcell.00351.2003.—Enhanced expression of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and other endothelial cell adhesion molecules (ECAMs) are associated with the onset and progression of inflammatory bowel disease (IBD). We show in this study that two cytochrome P-450 (CYP450) inhibitors from Citrus paradisi (grapefruit), bergamottin, and 6,7-dihydroxybergamottin (DHB) block tumor necrosis factor (TNF-α)-stimulated expression of MAdCAM-1 in cultured endothelial cells and also reduce αβ-dependent lymphocyte adhesion. Bergamottin (20–50 μM) or DHB (10–30 μM) pretreatment dose-dependently reduced TNF-α-mediated expression of MAdCAM-1 and lymphocyte adhesion. Bergamottin and DHB also prevented expression of two other ECAMs, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (but not E-selectin). SKF-525a, a specific CYP450 inhibitor, also blocked the expression of MAdCAM-1 mediated by TNF-α. Similar to SKF-525a (20 μM), bergamottin (20 μM) and DHB (20 μM) directly inhibited the activity of CYP450 3A4. These results suggest that natural CYP450 inhibitors may be effective in reducing ECAM expression and leukocyte adhesion and therefore be useful in the clinical treatment of inflammatory states like IBD.

cytokine P-450; inflammatory bowel disease; lymphocytes; mucosal cell adhesion molecule-1

GRAPEFRUIT PRODUCTS HAVE RECENTLY received increasing clinical interest because of their ability to maintain the bioavailability of many drugs, including calcium channel blockers (3, 50), ethinylestradiol (47), cyclosporin A (13), midazolam (29), triazolam (24), and terfenadine (5). It has now been established that biopersistence of these compounds when taken with grapefruit juice reflects a diminished clearance of these agents by the liver and prove stability of these compounds in grapefruit. Recent in vitro studies have characterized two furanocoumarin compounds in grapefruit and peel oil [bergamottin and 6, 7-dihydroxybergamottin (DHB)] as potent CYP450 inhibitors that reduce microsomal CYP3A4 activity (16). Consequently, drug incompatibilities with grapefruit based on CYP450 metabolism are now recognized as an important clinical contraindication.

With respect to inflammation, the induction of endothelial cell adhesion molecules or “ECAMs” recruit leukocytes to the tissues during inflammatory responses by promoting rolling, arrest, and extravasation of leukocytes (18). ECAM-dependent leukocyte infiltration of tissues is a classic hallmark of chronic inflammatory states that include inflammatory bowel diseases (IBD; ulcerative colitis and Crohn’s disease) and several other forms of chronic inflammation (arthritis, lupus, and diabetes; see Refs. 20, 45, 59). Synthesis of multiple ECAMs [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin] and the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is increased in human colitis (6, 51) and in animal models of IBD (12, 48). MAdCAM-1 is a 60-kDa endothelial cell surface molecule selectively expressed on high endothelial venule cells that appears to be necessary for lymphocyte homing, especially to mucosa-associated lymphoid tissue (8, 52). MAdCAM-1 is strongly expressed by mucosal endothelial cells, particularly after exposure of these cells to proinflammatory cytokines such as tumor necrosis factor (TNF-α) (37, 49). Because MAdCAM-1 is normally expressed in the gut, but is dramatically increased during IBD (6, 51), it has been suggested that increased MAdCAM-1 expression plays an important role in the etiology of IBD. Interactions mediated by MAdCAM-1 and its receptor, αβ, mediate the lymphocyte homing that promotes inflammation in IBD, a notion well supported by the observations that antibodies directed against either MAdCAM-1 or its lymphocyte ligand, αβ, will attenuate the development of experimental models of colitis (27, 39). Although MAdCAM-1 is dramatically increased in the gut during IBD, MAdCAM-1 is probably necessary but insufficient to achieve complete IBD disease activity, since antibodies for other ECAMs (e.g., VCAM-1) can also effectively reduce disease activity (4, 19, 43, 54). Therefore, an enhanced understanding of mechanisms regulating ECAM expression, especially that of MAdCAM-1 and VCAM-1, could improve therapeutic treatments for colitis and chronic inflammation.

The expressions of ICAM-1, VCAM-1, E-selectin, P-selectin, and MAdCAM-1 are typically regulated by several proinflammatory Th1 cytokines like TNF-α (18). Cytokines regulate ECAM gene transcription through several transcription factors, including nuclear factor-κB (NF-κB), a member of the Rel family of transcription factors (11, 41). After cytokine stimulation, IκB, (inhibitor of κB) is phosphorylated, dissociating it from NF-κB, whereupon active NF-κB dimers enter the nucleus and help activate transcription of ECAMs (e.g., ICAM-1, VCAM-1, E-selectin).

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VCAM-1, E-selectin, and MAdCAM-1; see Refs. 11, 37, 38, 56) and other inflammatory genes such as inducible nitric oxide synthases (1).

Studies with several antioxidants support a role for reactive oxygen species (ROS) as important second messages linking cytokine exposure to NF-κB-mediated activation of inflammatory genes (10, 17, 32, 46). Although several groups have reported that NADPH oxidase may be the main source of these signal oxidants, there are other sources of oxidants in endothelial cells. These sources include, mitochondria, enzymes involved in eicosanoid metabolism, and CYP450 monoxygenases. These systems all have the capacity to form oxidants and can contribute to the total pool of “signal oxidants.” Pietersama et al. (40) have recently suggested that, of these, CYP450 may be a very significant source of oxidants after cytokine exposure in endothelial cells. Subsequently, we also showed that cytokine-dependent ECAM expression also appears to depend, at least in part, on CYP450 monoxygenase (44).

On the basis of these observations, our present study examines whether CYP450 inhibitor properties of the grapefruit compounds bergamottin and DHB might also be effective for limiting cytokine induction of ECAMs and lymphocyte-endothelial adhesion in vitro. Our data show that these naturally

### Fig. 1. Bergamottin/6,7'-dihydroxybergamottin (DHB) inhibits cytochrome P-450 (CYP450) activity. Bergamottin (20 μM) and DHB (20 μM) significantly inhibit the activity of CYP3A4 by 92 and 96%, respectively, as well as the specific CYP450 inhibitor, SKF-525a (20 μM). Data were assessed with the Vivid CYP3A4 Green Screening Kit. *P < 0.05 vs. control. One-way ANOVA with Fisher’s protected least significant difference (PLSD) test was used; n, no. of experiments.

### Fig. 2. Bergamottin blocks tumor necrosis factor (TNF)-α-induced mucosal addressin cell adhesion molecule (MAdCAM)-1 expression. Bergamottin significantly reduced the expression of MAdCAM-1 in response to TNF-α (1 ng/ml, 24 h) in a concentration-dependent fashion (20–50 μM). Alone, bergamottin had no effect on the expression of MAdCAM-1 (A). *P < 0.05 vs. TNF, #P < 0.05 vs. control. One-way ANOVA with Fisher’s PLSD test was used.
occurring CYP450 inhibitors might help limit tissue infiltration by leukocytes and help block leukocyte-dependent events in various forms of chronic inflammation, for instance, IBD.

MATERIALS AND METHODS

Reagents. Recombinant mouse TNF-α was purchased from Endogen (Woburn, MA), and bergamottin and DHB were purchased from Ultrafine (Manchester, UK). Antibodies to VCAM-1 (clone MK1.9) and ICAM-1 (clone YN1.7.4) were purchased from Southern Biotechnology Associates (Birmingham, AL), and antibodies to E-selectin (clone 10Eg.6) and MAdCAM-1 (clone MECA 367) were purchased from Pharmingen (San Diego, CA). SKF-525a was purchased from Biomol (Plymouth meeting, PA). Trypan blue was purchased from Sigma-Aldrich (St. Louis, MO).

CYP450 3A4 activity assay. To confirm the direct effect of bergamottin and DHB inhibition on cytochrome p450, CYP450 3A4 activity was measured using the VividCYP3A4 Green Screening Kit (PanVera, Madison, WI). This assay is a direct fluorometric test of CYP450 3A4 activity. Reaction tests that did not contain bergamottin or DHB but did contain CYP450 3A4 enzyme plus the fluorescent substrate were performed as the “100% activity” control (i.e., no inhibitory effect on CYP3A4 activity). Other reactions were identical to controls, except for the presence of test agent. Bergamottin (20 μM) and DHB (20 μM) were tested using the Vivid assay procedure in 96-well plates (according to the manufacturer’s instructions). SKF-525a (20 μM) was used as a known CYP450 inhibitor. After all incubations, CYP3A4 activity was read on a Fluoroskan Ascent (Labsystems, Helsinki, Finland), set for excitation at 485 nm and emission at 538 nm. The data are expressed as a percentage of the maximum (100%) control fluorescence level. For each experiment, treatments were performed in triplicate (n = 3).

Cell culture. The SVEC4–10 line is an endothelial cell line derived by SV40 (strain 4A) transformation of murine small vessel endothelial cells originally isolated from axillary lymph node vessels of an adult male C3H/Hej mouse (7, 8). These cell types were all maintained in DMEM with 10% FCS with 1% antibiotic/antimycotic, and seeded on 24-well tissue culture plates at ~20,000 cells/cm². These cultures were used immediately upon reaching confluence. Cell viability was analyzed using 0.1% trypan blue, as described by Oshima et al. (37).

Lymphocytes. The mouse CD8⁺ T cell lymphoma TK-1 cells (which constitutively express αβ-integrin) were obtained as a generous gift from Dr. Eugene Butcher (Stanford University). These cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 0.05 mM 2-mercaptoethanol (without antibiotic/antimycotic).

Western analysis of cell lysates. Monolayers were either pretreated (1 h) with blockers and then cotreated with cytokines (24 h) or treated without test agents and cotreated with cytokines (24 h). All cell samples were harvested at 24 h. Equal quantities of protein (75 μg) from each sample were electrophoretically separated on 7.5% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes (Sigma) and blocked with 5% milk powder in PBS at 4°C (overnight). These membranes were washed two times for 10 min with wash buffer.
(0.1% milk powder in PBS). Primary rat anti-mouse MAdCAM-1 monoclonal antibody was added at a concentration of 10 µg/ml and incubated at room temperature for 2 h. These membranes were washed two times with wash buffer. Secondary goat anti-rat horseradish peroxidase-conjugated secondary antibody (Sigma) was added at 1:2,000 dilution for 2 h. Last, membranes were washed three times and developed using the enhanced chemiluminescence detection system (Amersham, La Jolla, CA). The density of MAdCAM-1-stained monolayers was measured by scanning the 60-kDa band using an HP ScanJet flatbed scanner. Images were analyzed for density using Image Pro Plus image analysis software (Media Cybernetics). The data were expressed as a percentage of the TNF-α-induced level of density. In each protocol, treatments were performed at least in triplicate.

**TK-1 lymphocyte adhesion assay.** Briefly, TK-1 cells were suspended in culture medium and fluorescence labeled by incubating TK-1 cells at 2 × 10^6 cells/ml with 0.02 mg fluorescein diacetate (Sigma) at 37°C for 30 min. The cells were then washed two times with ice-cold HBSS, spun at 250 g for 5 min to remove unincorporated fluorescence, and suspended in HBSS. The TK-1 lymphocyte cell line used in this assay expresses high levels of the αβ7-integrin, which can interact with multiple ligands, including MAdCAM-1, as well as VCAM-1, L-selectin, and fibronectin (22). In this system, TNF-α-stimulated TK-1 adhesion to SVEC4–10 endothelial cells is at least 50% MAdCAM-1 dependent (37). SVEC monolayers were cultured in 48-well plates and, at confluency, pretreated with or without test agents (1 h) and then exposed to TNF-α (1 ng/ml) for 24 h. Cytokine-treated endothelial cells were washed three times with media. Labeled TK-1 cells were then added to the endothelium at a 5:1 lymphocyte-to-endothelial cell ratio (31) and allowed to bind for 30 min under static conditions. At the end of the incubation period, the supernatant was removed, and the monolayers were washed two times with HBSS. Plates were read on a Fluoroskan Ascent (excitation 485 nm, emission 515 nm; Labsystems). Blank wells (0% TK-1 adhesion) were run as controls that did not have TK-1 cells. The data are expressed as a percentage of the TNF-α-induced level of fluorescence. In each protocol, treatments were performed at least in triplicate.

**Endothelial cell adhesion expression assay.** Surface expression of ECAMs was measured using the method of Khan et al. (28). SVEC monolayers were cultured in 48 wells and were pretreated (1 h) either with bergamottin (20 μM) or DHB (20 μM) and then cotreated with TNF-α (20 ng/ml) at 37°C in medium for 24 h. The cells were washed three times with 0.5 ml HBSS/PBS (1:1) at 24 h, and monolayers were incubated with anti-mouse ICAM-1, anti-VCAM-1, anti-E-selectin, or anti-MAdCAM-1 antibody. All antibodies were added to cultures after treatment at a concentration of 1 μg/ml in HBSS/PBS + 5% FCS at 37°C for 30 min. Monolayers were then washed two times with 0.5 ml HBSS/PBS and incubated with horseradish peroxidase-conjugated rabbit anti-rat IgG (1:2,000 diluted; Sigma) in HBSS/PBS + 5% FCS at 37°C for 30 min. Monolayers were then washed four times with 0.5 ml HBSS/PBS followed by incubation with 0.25 ml of 0.003% hydrogen peroxide + 0.1 mg/ml 3,3',5,5'-tetrarmethylbenzidine (Sigma) at 37°C for 60 min in the dark. The color reaction was stopped by adding 75 µl of 8 N H₂SO₄, and the samples were transferred to 96-well plates. Plates were read on a Titertek MCC340 plate reader (Titertek Instruments, Huntsville, AL) at 450 nm. Blanking (i.e., background) was performed on monolayers stained only with secondary antibody. The data are expressed as a percentage of the TNF-α-induced level of density. In each protocol, treatments were performed at least in triplicate.

**Isolation of neutrophils.** Human neutrophilic polymorphonuclear leukocytes (PMN) were isolated from venous blood of healthy adults using standard dextran sedimentation and gradient separation on Histopaque 1077 (25, 60; Sigma). This procedure yields a PMN population that is 95–98% viable (by trypan blue exclusion) and 98% pure (by acetic acid-crystal violet staining).

**Measurement of NADPH oxidase activity.** Superoxide anion produced by human PMN NADPH oxidase was determined by spectrophotometry, based on the change in absorbance of cytochrome c-induced superoxide. We chose human PMN for this assay since they are easily collected in high volume and purity and are reasonably similar to murine PMN in terms of superoxide production. PMN (2 × 10⁶/ml) in HBSS with 0.45% (wt/wt) glucose and 0.1% (wt/wt) cytochrome c (Sigma) were treated in the presence of superoxide dismutase (10 μg/ml, negative control) or pretreated with bergamottin or DHB (20 μM). To activate PMN superoxide formation, PMN were then cotreated (30 min) with PMA (1 µM). Samples were then centrifuged at 7,500 rpm for 5 min to remove cells and 200 µl of supernatant read at a wavelength of 450 nm on a Titertek MCC340 plate reader (Titertek Instruments). Each experimental condition was performed at least in triplicate (n = 3).

**Statistical analysis.** All values are expressed as means ± SD. Data were analyzed using multiple comparisons. Probability (P) values <0.05 were considered significant.

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Fig. 4. Bergamottin/DHB reduces the adhesion of αβ7-expressing lymphocytes to TNF-α-stimulated endothelium. TNF-α stimulation (1 ng/ml, 24 h) significantly increased adhesion of TK-1 lymphocytes to SVEC monolayers. Both bergamottin (20 μM) and DHB (20 μM) significantly reduced TK-1 adhesion to SVEC in response to TNF-α stimulation at 24 h. *P < 0.05 vs. TNF. #P < 0.05 vs. control. One-way ANOVA with Fisher’s PLSD test was used.
RESULTS

Bergamottin or DHB interaction with CYP450. Because bergamottin and DHB have both been described as inhibitors of CYP450, we confirmed the inhibitory effect of bergamottin and DHB on CYP450 activity using the Vivid CYP3A4 Green Screening Kit. We confirmed a strong inhibition of our test agents on CYP450 activity using this system. Bergamottin (20 μM) and DHB (20 μM) significantly blocked the activity of recombinant CYP3A4 by 92 and 96%, respectively, and are nearly as effective as the CYP450 selective inhibitor SKF-525a at the same concentration (20 μM; Fig. 1). Cell viability was assessed by the trypan blue exclusion assay and found to be no different from controls (data not shown).

Effect of bergamottin or DHB on TNF-α-induced MAdCAM-1 expression on SVEC. TNF-α (1 ng/ml) dramatically increased expression of MAdCAM-1 at 24 h. Bergamottin significantly reduced the expression of MAdCAM-1 in response to TNF-α in a concentration-dependent fashion (20–50 μM), and bergamottin (50 μM) completely abolished the TNF-α-induced MAdCAM-1 expression (Fig. 2, A and B). DHB also significantly reduced the expression of MAdCAM-1 induced by TNF-α in a concentration-dependent manner (10–30 μM; Fig. 3, A and B). Alone, these compounds had no effect on the expression of MAdCAM-1 (Figs. 2A and 3A).

Adhesion of αββ*-expressing lymphocytes to TNF-α-stimulated endothelium. Having established a protective effect of bergamottin or DHB in TNF-α-stimulated endothelial MAdCAM-1 expression, we next examined the effects of bergamottin or DHB on the adhesion of the αββ*-expressing mouse lymphocyte cell line TK-1 to the TNF-α-treated endothelial cell monolayer. TNF-α stimulation (24 h) significantly increased adhesion of TK-1 lymphocytes to SVEC monolayers. Both bergamottin (20 μM) and DHB (20 μM) significantly reduced TK-1 adhesion in response to TNF-α stimulation at 24 h (Fig. 4, A and B). Either bergamottin or DHB alone had no effect on TK-1 adhesion.

Effect of bergamottin or DHB on TNF-α induced other ECAM expression on SVEC. Because bergamottin and DHB reduced MAdCAM-1 expression, we next considered whether these agents would also affect the TNF-α-stimulated expression of other ECAMs using a cell surface expression assay (ELISA). TNF-α stimulation (20 ng/ml, 24 h) increased the

Fig. 5. Effect of bergamottin or DHB on TNF-α-induced endothelial cell adhesion molecule (ECAM) expression. TNF-α stimulation (20 ng/ml, 24 h) increased the ECAMs [intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, and MAdCAM-1] on SVEC cells. Again, both bergamottin (20 μM) and DHB (20 μM) each significantly blocked TNF-α-induced expression of ICAM-1, VCAM-1, and MAdCAM-1, but not E-selectin. *P < 0.05 vs. TNF. #P < 0.05 vs. control. One-way ANOVA with Fisher’s PLSD test was used.
ECAMs: ICAM-1, VCAM-1, E-selectin, and MadCAM-1 on SVEC cells. Again, both bergamottin (20 μM) and DHB (20 μM) each significantly blocked TNF-α-induced expression of ICAM-1, VCAM-1, and MadCAM-1 but not E-selection (Fig. 5, A-D). There were no effects on expression of ECAMs treated with either bergamottin or DHB alone.

Effect of P450 inhibitor on TNF-α-induced MadCAM-1 expression on SVEC. To support a role for CYP450 in TNF-α-stimulated ECAM expression, we examined the ability of the CYP450 specific inhibitor SKF-525a to inhibit TNF-α-induced MadCAM-1 expression (using a cell surface expression assay). We found that SKF-525a (20 μM) significantly reduced MadCAM-1 expression in response to TNF-α (20 ng/ml 24 h; Fig. 6). Alone, this compound had no effect on expression of MadCAM-1.

Lack of effect of bergamottin and DHB on neutrophil NADPH oxidase. Because cytokine-induced adhesion molecule expression depends on second-message signal oxidants (23, 44, 57), we hypothesized that bergamottin/DHB protection might reflect decreased production of oxidants like O2·- through either NADPH oxidase or CYP450. To help narrow possible sources and exclude bergamottin and DHB and NADPH oxidase inhibitors, we used a classical PMN O2·- assay (that detects only O2·- from NADPH oxidase) to assess NADPH oxidase activity. Activity was measured spectrophotometrically by the reduction of cytochrome c by 1 μM PMA-stimulated PMN (n = 6). O2·- formation in PMA-treated cells was set as “100%” (maximum activation). O2·- PMN was completely eliminated by superoxide dismutase (10 μg/ml, n = 6), confirming the specificity of this assay for O2·- anion. Neither bergamottin (20 μM, n = 6) nor DHB (20 μM, n = 6) significantly altered O2·- formation (Fig. 7), and it is therefore unlikely that these substances alter NADPH oxidase.

DISCUSSION

NF-κB is a key transcription factor that contributes to the increased expression of MadCAM-1, but also helps to regulate the expression of several other endothelial adhesion molecules in response to inflammatory cytokines (26, 37, 53). The activation of transcription factors like NF-κB has been linked with the formation of intracellular ROS. However, several other redox-sensitive transcription factors such as Sp-1 might also potentially be involved (58). Studies using antioxidants strongly support the idea that ROS are obligate signals in ECAM induction; these treatments also significantly block leukocyte infiltration into tissues, which depends on the expression of these ECAMs (30, 42). We showed that the expression of MadCAM-1 was ROS regulated (using antioxidants; see Ref. 44). Although it is generally agreed that oxidants constitute an important link between cytokines and ECAM expression, there is currently a great deal of controversy over the sources of oxidants within cells and the mechanisms through which these ROS generators are activated.

Among the possible sources of intracellular ROS formed in cells in response to cytokines, two, NADPH oxidase and CYP450 monoxygenase, appear to be major sources of ROS. Pietersma et al. (40) have suggested that cytokine-induced ECAM expression depends on oxidants formed through CYP450, rather than oxidants formed by NADPH oxidase. Therefore, although NADPH oxidase clearly does mediate some ROS responses in endothelial cells, CYP450 also appears to contribute to many activities, e.g., endothelial responses to cytokines.

Recently, we showed that TNF-α-induced ECAM expression on lymphoid endothelial cells (SVEC-40) was NADPH oxidase and/or CYP450 monoxygenase dependent using multiple selective inhibitors for CYP450 and NADPH oxidase (44). In our report, MadCAM-1 expression was specifically mediated by CYP450 monoxygenase but apparently unrelated to NADPH oxidase. Those data support CYP450 monoxygenase as an important link in the signal cascade activated by cytokines leading to ECAM induction. Although bergamottin and DHB are P450 inhibitors that do not inhibit NADPH oxidase, we have only indirect support for oxidant signals mediating these effects. For example, although antioxidants like N-acetylcysteine and pyrrolidinedithiocarbamate block ad-

![Fig. 7. Interaction between bergamottin/DHB and NADPH oxidase.](http://ajpcell.physiology.org/)
hesion molecule expression, we cannot yet measure CYP450-
derived oxidant formation in SVEC (using dihydrorhodamine
or hydroethidine). There are several possible reasons for this.
First, it is possible that, in response to cytokines, levels of
signal oxidants formed are too low to be detected by these
assays. Second, an oxidant signal intermediate that is not
reactive with these probes might be formed, and, last, some
nonoxidant CYP450 metabolite mediates these responses.
Therefore, although our data remain consistent with oxidant
signals from CYP450, future studies will be needed to establish
the identity of these signals.

The SVEC cell line was selected for study since MADCAM-1
expression is restricted to only a few tissues and has been
studied mainly using SVEC or bEnd3 endothelial lines in
vitro (37, 49). Similarly, α4β7-expressing lymphocytes are not
normally abundant enough to perform adhesion studies; there-
fore, we also selected the TK-1 cell line to evaluate α4β7/
MAdCAM-1 adhesion (2).

We found that two orally active CYP450 inhibitors de-
derived from Citrus paradisi (grapefruit) as well as a commer-
cial P450 selective inhibitor, SKF-525a, block the expres-
sion of MADCAM-1 and other ECAMs (ICAM-1 and
VCAM-1 but not E-selectin). Furthermore, bergamottin and
DHB also inhibit α4β7-integrin-dependent lymphocyte ad-
hesion to endothelium after TNF-α stimulation. The overall
levels of basal adhesion in this experiment were relatively
high (>60%) and likely reflect the static adhesion model
employed here. Most likely, shear forces in vivo would
reduce this level of background adhesion.

The concentrations of bergamottin and DHB used here
(10–50 μM) are similar to those reported in other in vitro
studies and are near concentrations found in Seville oranges
and grapefruit juices. These concentrations are also near the
inhibitory constants reported for these substances toward
CYP3A4 (40 and 5.56 μM for bergamottin and DHB, respec-
tively; see Ref. 55). Bergamottin and DHB were recently
identified in grapefruit as CYP450 inhibitors based on their
ability to increase the bioavailability of several drugs, includ-
ing felodipine (3), diltiazem (33), and several other therapeutic
agents. We found a strong direct inhibition of CYP450
(CYP3A4) by bergamottin and DHB, as previously demon-
strated (14, 21, 36, 55). Furthermore, bergamottin and DHB
have been shown to inhibit many other P450 isoforms, which
include, but are not limited to, 1A2, 2C6, 2C9, 2C19, 2D6, and
2E4 (21, 55). We examined the isoforms present in our endo-
thelial cells by PCR and found at least two forms correspond-
ing to CYP3A11 and a novel CYP3A isoform. Therefore,
CYP450s are present in our cells and may mediate cytokine-
dependent effects.

CYP450 enzymes are now recognized in endothelial cells as
potent “endothelium-derived hyperpolarizing factor” (EDHF)
synthases. These enzymes apparently generate ROS; there-
fore, as we previously speculated, they could in this fashion
also serve as generators of oxidant signal in response to
stimuli (15).

Although oxidants are excellent candidate signal metabolites
produced by CYP450s, several eicosanoids are also produced
through CYP450, which include the epoxygenecosatetraenoic
acids (EETs) and hydroxyecosatetraenoic acids (HETEs).
These eicosanoids metabolic pathways are also thought to
represent the source of EDHF. Because these substances pro-
duced by CYP450 are arachidonic acid metabolites, it is
possible that either they or possibly some other oxidant species
might also modulate cytokine-induced ECAM expression.

However, when these eicosanoid agents were tested for their
effects on ECAM expression, they were not found to increase
expression but rather to decrease the expression of endothelial
adhesion molecules (35). Previously, we reported that a phos-
pholipase A2-specific inhibitor, bromophenacyl bromide
(BPB), could abolish TNF-α induction of MadCAM-1 (44).
Therefore, although CYP450-dependent MADCAM-1 induc-
tion might involve arachidonate as a substrate, perhaps in
oxidant signaling, the final EET or HETE eicosanoids derived
from these pathways do not appear to increase expression of
ECAMs.

Chen et al. (9) have reported that antioxidants attenuate
cytokine-induced ICAM-1 and VCAM-1 expression but not
E-selectin expression, suggesting that cytokine-induced E-se-
lectin expression may differ from that of other ECAMs. Sim-
ilarly, we also found that bergamottin and DHB failed to
prevent E-selectin expression induced by TNF-α stimulation.
Therefore, we presume that TNF-α-induced E-selectin expres-
sion may be regulated differently from that of MADCAM-1,
ICAM-1, and VCAM-1 in this and in other models of inflam-
mation.

The concept of ROS derived from CYP450 as inflammatory
signals is novel. Nevertheless, because both CYP450 content
and activity in tissues are often reduced after inflamma-
tion initiates, this scheme could be explained by CYP450 func-
tioning as an inflammatory “trigger,” which may need to be
downregulated to achieve restitution (34). The broad recogni-
tion of cytochrome isoforms as possible generators of signals,
which include ROS, in inflammation suggests that numerous
CYP450 inhibitors in addition to the few described here may
be therapeutic for inflammation. However, many problems
may still exist with the use of P450 inhibitors, particularly
increased biological lives of coadministered drugs. Currently,
our studies indicate that CYP450 isoforms could participate in
the inflammatory response but do not specifically identify the
oxidants or particular isoforms involved. CYP3A variants may
be good candidates, since bergamottin and DHB and SKF-525a
all demonstrate strong inhibition of CYP450 3A4 and also
block inflammation. Future studies with more specific inhibi-
tors will help determine the full complement of this potentially
new “signaling module.”

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