TNF-α-induced endothelial cell adhesion molecule expression is cytochrome P-450 monooxygenase dependent


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Sasaki, Makoto, D. Ostain, J. W. Elrod, T. Oshima, P. Jordan, M. Itoh, T. Joh, A. Minagar, and J. S. Alexander. TNF-α-induced endothelial cell adhesion molecule expression is cytochrome P-450 monooxygenase dependent. Am J Physiol Cell Physiol 284:C422–C428, 2003.—It is strongly suspected that cytokine-induced gene expression in inflammation is oxidant mediated; however, the intracellular sources of signaling oxidants remain controversial. In inflammatory bowel disease (IBD) proinflammatory cytokines, such as TNF-α, trigger gene expression of endothelial adhesion molecules including mucosal addressin cell adhesion molecule-1 (MAdCAM-1). MAdCAM-1 plays an essential role in gut inflammation by governing the infiltration of leukocytes into the intestine. Several groups suggest that endothelial-derived reduced NADPH (NADPH) oxidase produces signaling oxidants that control the expression of adhesion molecules (E-selectin, ICAM-1, VCAM-1). In addition to NADPH oxidase, cytochrome P-450 (CYP450) monooxygenases have also been shown to trigger cytokine responses. We found that in high endothelial venular cells (SVEC4-10), multiple inhibitors of CYP450 monooxygenases (SKF-525a, ketoconazole, troleandomycin, itraconazole) attenuated TNF-α induction of MAdCAM-1, whereas NADPH oxidase inhibition (PR-39) did not. Conversely, E-selectin, ICAM-1, and VCAM-1 induction requires both NADPH oxidase and CYP450-derived oxidants. We show here that MAdCAM-1 induction may depend exclusively on CYP450-derived oxidants, suggesting that CYP450 blockers might represent a possible novel therapeutic treatment for human IBD.

cytochrome P-450 monooxygenase; reduced nicotinamide adenine dinucleotide phosphate oxygenase; mucosal addressin cell adhesion molecule-1; inflammatory bowel disease; endothelial cell adhesion molecules

MATERIALS AND METHODS

Reagents. N-acetyl-l-cysteine (NAC) and pyrrolidinedithiocarboxamide (PDTC) were purchased from Sigma (St. Louis, MO). 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). Recombinant mouse TNF-α was purchased from Endogen (Stough-

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Isolation of neutrophils. Human neutrophil polymorphonuclear leukocytes (PMN) were isolated from venous blood of healthy adults with standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma; Refs. 13, 36). 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. The superoxide anion produced by human PMN NADPH oxidase was determined from the visible absorption spectra, which is based on obtaining the reduction of cytochrome c by superoxide. PMN (2 × 10^6/ml) in HBSS with 0.45% (wt/wt) glucose and 0.1% (wt/wt) albumin (Sigma) were pretreated (20 min) with inhibitors (in µM: 20 SKF-525a, 10 ketoconazole, 20 trolean- domycin, 1 itraconazole, 10 fluconazole, 0.5 DPI, and 10 PR-39) or superoxide dismutase (SOD; 10 µg/ml) and then cotreated (30 min) with phorbol 12-myristate 13-acetate (PMA; 1 µM). Samples were centrifuged at 7,500 rpm for 5 min, and 200 µl of supernatant were transferred to 96-well plates and read on a Titerpak MCC340 plate reader (Titerpak Instruments, Huntsville, AL) with absorbance at a wavelength of 450 nm. For experiments, each treatment was performed at least in triplicate.

Chemicals and CYP450 (3A4) interaction. Direct effects of the different inhibitors used in this study on the activity of CYP450 3A4 (CYP3A4) activity was measured with the Vivid assay procedure in 96 wells according to the manufacturer's instructions. All reactions, 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 this maximum control fluorescence level. For each experiment, treatments were performed at least in triplicate.

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 the axillary lymph node vessels of an adult male C3H/Hej mouse (5). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 1% antibiotic-antimycotic. Cells were seeded into 24-well tissue culture plates at ~20,000 cells/cm², and experiments were performed immediately on cells reaching confluence.

Western analysis of cell lysates. Monolayers were either pretreated (1 h) with pharmacological blockers and then cotreated with TNF-α (20 ng/ml; 24 h) or not treated with these test agents and then treated with TNF-α (24 h). All cell samples were harvested at 24 h. Equal quantities of protein (75 µg) from each of the samples 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 twice for 10 min with wash buffer (0.1% milk powder in PBS). Primary rat anti-mouse MadCAM-1 antibody was added at a concentration of 10 µg/ml and incubated at room temperature for 2 h. These membranes were washed twice with wash buffer. Secondary goat anti-rat horseradish peroxidase-conjugated antibody (Sigma) was added at a 1:2000 dilution for 2 h. Finally, membranes were washed three times and developed with the enhanced chemiluminescence (ECL) detection system (Amersham, La Jolla, CA). The density of MadCAM-1 staining was measured by scanning the 60-kDa band with an HP ScanJet flatbed scanner. Images were analyzed for density with Image Pro Plus image analysis software (Media Cybernetics). The data are expressed as a percentage of TNF-α-induced level of density. In each protocol, treatments were performed at least in triplicate (n = 3).

Endothelial cell adhesion expression assay. The surface expression of ECAMs was measured with the method of Khan et al. (15). SVEC monolayers were grown in 48-well plates as described by Khan et al. and pretreated (1 h) with inhibitors (in µM: 20 SKF-525a, 10 ketoconazole, 20 trolean- domycin, 1 itraconazole, and 10 fluconazole, and 10 PR-39 with 500 nM DPI) 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 of 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 twice with 0.5 ml HBSS-PBS and incubated with horseradish peroxidase-conjugated rabbit anti-rat IgG (1:2,000 dilution; Sigma) in HBSS-PBS + 5% FCS at 37°C for 30 min. Monolayers were 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'-tetramethylbenzidine (Sigma) at 37°C for 60 min in the dark. The color reaction was stopped by adding 75 µl of 8 N H4SO4, and the samples were transferred to 96-well plates. Plates were read on a Titerpak MCC340 plate reader (Titerpak Instruments) at 450 nm. Blanking (i.e., background) was performed on monolayers stained only with secondary antibody. In each protocol, treatments were performed at least in triplicate (n = 3).

Statistical analysis. Results are expressed as means ± SE. Significant differences were assessed by one-way ANOVA plus Fisher's paired least significant difference (PLSD) test. P values ≤0.05 were accepted as statistically significant.

RESULTS

TNF-α-induced MadCAM-1 expression is oxidant sensitive. To show the dependence of TNF-α-induced endothelial MadCAM-1 expression on intracellular oxidants (as previously shown in the upregulation of other ECAMs like E-selectin, VCAM-1, and ICAM-1), we pretreated monolayers with NAC and PDTC, two potent intracellular antioxidants, before TNF-α treatment to block MadCAM-1 induction. SVEC endothelial cells pretreated with NAC (Fig. 1A; 10 mM) or with PDTC (Fig. 1B; 20 µM) showed significantly reduced TNF-α-induced MadCAM-1 expression (measured by

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Western blot analysis), supporting oxidants as key signals in TNF-α-induced MAdCAM-1 expression.

**Tests for enzyme inhibitor specificity.** In this study, we wanted to identify the sources of the signaling oxidants induced by TNF-α in the regulation of MAdCAM-1. Because the studies in Fig. 1 showed a strong dependence of MAdCAM-1 induction on intracellular oxidants, we next tried to distinguish whether these effects were due to NADPH oxidase-derived oxidants or to CYP450 monooxygenase-derived oxidants. Therefore, a panel of highly specific inhibitors for NADPH oxidase and for CYP450 were selected and their abilities to block these enzymes were analyzed by two assays for these respective systems: neutrophil superoxide formation (NADPH oxidase) and a direct assay for CYP3A4 monooxygenase.

**Effect of blockers used in this study on neutrophil NADPH oxidase.** To screen the drugs used in this study for their ability to block NADPH oxidase-dependent superoxide production, human PMN were used as a model NADPH oxidase-dependent superoxide-generating system, because PMN will form superoxide by the action of NADPH oxidase. Superoxide production and inhibition in this experiment were measured spectrophotometrically by the reduction of cytochrome c. PMA (1 μM; n = 6)-stimulated superoxide formation was considered to be “100%” or maximal activation. The production of superoxide by PMN was prevented by superoxide dismutase (10 μg/ml; n = 6), which demonstrates the specificity of this assay for the superoxide anion. DPI (0.5 μM; n = 4), a nonselective inhibitor for NADPH oxidase, and PR-39 (10 μM; n = 4), a specific NADPH oxidase blocker, almost completely inhibited NADPH oxidase-dependent superoxide formation. Troleandomycin (20 μM; n = 6) and fluconazole (10 μM; n = 6) slightly increased superoxide formation over PMA-stimulated levels. In contrast, ketoconazole (10 μM; n = 6), SKF-525a (20 μM; n = 6), and itraconazole (1 μM; n = 6) slightly decreased superoxide formation (Fig. 2).

**Effect of CYP450 monooxygenase blockers.** To screen the drugs used in this study for their ability to directly inhibit CYP450 monooxygenase, CYP3A4 activity was measured with the Vivid CYP3A4 Green
screening kit. SKF-525a (20 μM; n = 5), ketoconazole (10 μM; n = 5), troleandomycin (20 μM; n = 5), itraconazole (1 μM; n = 5), and DPI (0.5 μM; n = 5) all strongly inhibited CYP3A4 activity. Fluconazole (10 μM; n = 5), a CYP2C9 inhibitor, only slightly reduced CYP3A4 activity, whereas PR-39 (10 μM; n = 5), a selective NADPH oxidase inhibitor, slightly increased CYP3A4 activity (Fig. 3).

Effect of CYP450 and NADPH oxidase antagonists on MAdCAM-1 expression. To quantitate which oxidant contributes the most in the expression of MAdCAM-1 on SVEC endothelial cells, CYP450 and/or NADPH oxidase inhibitors were preincubated and coincubated with these blockers in the presence or absence of TNF-α. CYP450 inhibitors [SKF-525a (20 μM; n = 4), ketoconazole (10 μM; n = 3), troleandomycin (20 μM; n = 3), and itraconazole (1 μM; n = 3)] proven to have specific inhibition against CYP3A4 activity and DPI (0.5 μM; n = 3), a nonselective blocker of multiple CYP450 isoforms and NADPH oxidase, significantly decreased TNF-α-induced MAdCAM-1 expression. Fluconazole (10 μM; n = 3), a CYP2C9 blocker, and the NADPH oxidase inhibitor PR-39 (10 μM; n = 3) did not alter MAdCAM-1 expression by Western blotting. In addition, the phospholipase A2 inhibitor BPB (10 μM; n = 3) prevented the TNF-α-induced expression of MAdCAM-1 (Fig. 4).

Effect of blockers on expression of E-selectin, ICAM-1, and VCAM-1. To evaluate which oxidase is most important in the expression of other ECAMs CYP450 and/or NADPH oxidase inhibitors were incubated with SVEC in the presence (and absence) of TNF-α. Both a CYP450 inhibitor (SKF-525a, 20 μM; n = 4) and a NADPH oxidase inhibitor (PR-39, 10 μM; n = 8) prevented E-selectin expression assessed via surface expression assay (Fig. 5A). Both CYP450 and NADPH oxidase inhibition also prevented the TNF-α-induced expression of ICAM-1 (Fig. 5B). Similarly, both CYP450 and NADPH oxidase inhibition blocked the expression of VCAM-1 (Fig. 5C).
DISCUSSION

Cytokine-dependent leukocyte-mediated tissue injury is induced in large part by the adhesion and extravasation of several classes of leukocytes that infiltrate inflamed tissues. The motility of these leukocytes depends on the increased expression of several endothelial adhesion molecules whose synthesis depends on the formation of signaling oxidants formed in response to inflammatory cytokines. The expression of these molecules and tissue inflammatory injury appears to be linked to oxidants (16), because this type of injury is largely reversed by treatment with several antioxidants (11, 18, 25).

Regulating the hyperexpression of ECAMs associated with many chronic inflammatory states is now recognized as one of the most effective, novel approaches for limiting leukocyte-mediated tissue damage. Although it is well accepted that the expression of ECAMs (E-selectin, ICAM-1, and VCAM-1) in response to inflammatory cytokines is oxidant dependent (6, 20, 26, 28, 29, 34), there are a few reports that claim that oxidants may not be required for the activation of NF-κB, the major transcription factor activated by cytokines in inflammation (3) (2). Therefore, the first goal of this study was to confirm that TNF-α-induced MAdCAM-1 expression is, in fact, oxidant dependent. We showed that two different intracellular antioxidants, NAC and PDTC, each prevent TNF-α-induced MAdCAM-1 expression, suggesting for the first time that the regulation of MAdCAM-1 is oxidant mediated. These data cumulatively suggest that antioxidants and antioxidant source drugs are capable of downregulating ECAM expression and consequently blocking the hypermigration of leukocytes into tissues. On the basis of the effects of PDTC on intracellular glutathione, some investigators have reported that PDTC has prooxidant properties (4, 21). It is worthwhile noting that when PDTC was used in these studies as a prooxidant, it was used at high (>25 μM) concentrations. It may also be worth noting that in those studies where PDTC was a prooxidant, it was used in nonendothelial cells (EL4.NOB thymoma cells and E6.1 lymphoma cells). In the current study, we used PDTC at 20 μM and only in SVEC endothelial cells.

One of the major sources of endothelium-derived signaling oxidants appears to be an NADPH oxidase similar to that previously reported within PMN (10, 32). In endothelial cells, NADPH oxidase activity has also been implicated as a source of signaling oxidants that trigger NF-κB and AP-1 activation under ischemic conditions (35). However, CYP450 enzymes, which have now been identified as potential “endothelium-derived hyperpolarizing factor” (EDHF) synthases, have also recently been demonstrated to generate ROS and might therefore also function as oxidant signal generators in response to stimuli (9).

In this report, we show that CYP450-derived oxidant signaling plays a significant role in the expression of several ECAMs. Using selective inhibitors of CYP450 and NADPH oxidase, we show here that CYP450 is an
important source of ROS-dependent signals induced by cytokines. The effects of NADPH oxidase inhibitors and CYP450 inhibitors on the activity of these systems support the stated pharmacological specificity of the blockers used here. Our findings support the idea that PR-39 is a specific NADPH oxidase inhibitor and that SKF-525a, ketoconazole, troloxemycin, and itraconazole are specific blockers of various CYP450 isozymes.

Interestingly, the CYP450 blockers that displayed inhibition specific for CYP3A4, were most effective at blocking TNF-α-induced MAdCAM-1 expression, whereas the specific NADPH oxidase inhibitor (PR-39) did not. These results are consistent with CYP450-derived ROS, not NADPH-derived oxidants, as essential intermediates in TNF-α-stimulated expression of MAdCAM-1. With respect to cytokine signaling, our data corroborate and extend findings by Pietersma et al. (27), who stated that NADPH oxidase-derived ROS do not appear to contribute to cytokine induction of adhesion molecules in human umbilical vein endothelial cells (HUVEC). Rather, they suggested that cytokine-induced ECAM expression was due to oxidants formed by CYP450 activity. In that study, the authors were not able to implicate a specific CYP450 isoform in the activation of ECAM expression. It is also worthwhile noting that the opinion of this group was based on their presumption that HUVEC lacked most components of the phagocyte-type NADPH oxidase. However, because we find that PR-39 appears to block VCAM-1 expression, our data probably support both oxidant systems within HUVEC (data not shown). Because fluconazole, which is reported to be an inhibitor of CYP2C9, had no effect on CYP3A4 activity and also failed to prevent MAdCAM-1 expression, our data support the CYP3A4 isoform as a key cytochrome in cytokine signaling.

We also examined oxidant dependence in the induction of other ECAMs, (E-selectin, ICAM-1, and VCAM-1) using cell surface expression assays (ELISA). The expression of these three ECAMs appears to require the participation of both NADPH oxidase and CYP450 monooxygenase-derived ROS, because each class of inhibitor was able to reduce the expression of these molecules in response to cytokines.

Although oxidants are excellent candidate metabolites produced by cytochrome, several prostanoids are also produced by CYP450, including epoxyeicosatetraenoic acid (EET) and hydroxyeicosatetraenoic acid (HETE). The prostanoid metabolic pathways are also thought to represent a source of EDHF. Because these substances are metabolites of arachidonic acid, it is possible that they might also modulate cytokine-induced ECAM expression in addition to oxidants. However, when these agents were tested for their effects on ECAM expression, they were found not to increase, but rather to decrease, the expression of endothelial adhesion molecules (24). Paradoxically, we found that a specific phospholipase A2 inhibitor, BPB, abolished TNF-α induction of MAdCAM-1. Our current interpretation of this result is that CYP450 induction of MAdCAM-1 may require arachidonic acid as a substrate, which is important in the generation of oxidant signals, but the final prostanoids derived from these pathways do not promote ECAM expression.

In summary, ECAM expression in response to cytokines is complex and appears to require oxidants derived from several sources, including NADPH oxidase and CYP450. It is interesting that MAdCAM-1 regulation may be unique in its apparently strict requirement for oxidants derived from CYP450 monooxygenase. The exact reason(s) that MAdCAM-1 expression might show this type of specificity is not currently clear but could reflect interactions between prostanoids and oxidants in the activation of transcription factors, differential sensitivities of transcription factors to oxidants, or as yet unidentified posttranscriptional events. Importantly, the identification of CYP450 as an important oxidant signal generator in inflammation suggests that the large variety of safe and well-tolerated CYP450 antagonists may be effective, novel treatments for many forms of chronic inflammation mediated by cytokines, including arthritis, vasculitis, and some forms of IBD.

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