Regulation of Oxidant-Induced Intestinal Permeability by Metalloprotease-Dependent Epidermal Growth Factor Receptor Signaling


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ABSTRACT

Inflammatory bowel disease (IBD) affects more than 1 million Americans, with more than 30,000 new cases diagnosed each year. IBD increases patient morbidity and susceptibility to colorectal cancer, yet its etiology remains unknown. Current models identify two key determinants of IBD pathogenesis: hyperpermeability of the gut epithelial barrier to bacterial products and an abnormal immune response to these products. Two factors seem critical for hyperpermeability: oxidant-induced stress and proinflammatory cytokines (e.g., tumor necrosis factor-α). The aim of this study was to investigate the role of oxidant stress-mediated transactivation of the epidermal growth factor receptor (EGFR) in intestinal hyperpermeability. We propose a new intestinal permeability model in which oxidant transactivates EGFR signaling by activation of TACE and cleavage of precursor TGF-α. These data could have a significant effect on our view of IBD pathogenesis and provide new therapeutic targets for IBD treatment.

We show that oxidant-induced intestinal hyperpermeability can be blocked by specific inhibitors of the EGFR, tumor necrosis factor convertase (TACE) metalloprotease, transforming growth factor (TGF)-α, and mitogen-activated protein kinases, especially extracellular signal-regulated kinase 1/2. We also show that oxidant initiates these signaling events, in part by causing translocation of TACE to cell-cell contact zones. In this study, our data identify a novel mechanism for oxidant-induced intestinal hyperpermeability relevant to IBD. We propose a new intestinal permeability model in which oxidant transactivates EGFR signaling by activation of TACE and cleavage of precursor TGF-α. These data could have a significant effect on our view of IBD pathogenesis and provide new therapeutic targets for IBD treatment.

Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory disorder that affects more than 1 million Americans, with more than 30,000 new cases diagnosed each year (Podolsky, 2002; Farhadi et al., 2003; Clayburgh et al., 2004). This disorder profoundly increases patient morbidity and decreases quality of life and increases patient susceptibility to colorectal cancer, although its etiology remains unknown. Current models of disease pathogenesis identify as the two principal determinants of IBD pathogenesis: increased permeability of the epithelial barrier of the intestinal mucosa, which results in penetration of luminal bacterial products into the mucosa; and an abnormal immune response to these products (Farhadi et al., 2003; Clayburgh et al., 2004). Regarding intestinal hyperpermeability in IBD,

ABBREVIATIONS: IBD, inflammatory bowel disease; TNF, tumor necrosis factor; TJ, tight junction; IEC, intestinal epithelial cell; EGFR, epidermal growth factor receptor; PKC, protein kinase C; EGF, epidermal growth factor; TGF, transforming growth factor; TACE, tumor necrosis factor-α converting enzyme; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TAPI-2, N-(R)-[2-(hydroxymethyl)-4-methylpentanoyl-1-t-butyl-alanyl-L-alanine, 2-aminoethyl amide, TNF-α protease inhibitor-2; GM6001, Ilomastat or N-[2R]-2-(hydroxymidocarbonylmethyl)-4-methylpentanoyl-L-tryptophan methylamide; AG1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline; GW2974, N4-(1-benzyl-1H-indazol-5-yl)-N6,N6-dimethyl-4-piperidone-4,6-diamine; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylmercapto)butadiene ethanolate; p38, 38-kDa protein (MAPK); SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)5-(4-pyridyl)-1H-imidazole; JNK, jun kinase; SP600125, 1,9-pyrazoloanthrone anthrapyrazolone; LY29004, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; TAPI, TNF-α protease inhibitor; Ab, antibody; HB-EGF, heparin binding epidermal growth factor; AKT, protein kinase B; DMEM, Dulbecco’s modified Eagle’s medium; FSA, fluorescein-5-(and-6)-sulfonic acid trisodium salt; FITC, fluorescein isothiocyanate; RT, reverse transcription; PCR, polymerase chain reaction; SIRNA, short inhibitory RNA; MMP, matrix metalloproteinase; DMSO, dimethyl sulfoxide; MLCK, myosin light chain kinase; ADAM, A disintegrin and metalloproteinase; MMPI, matrix metalloproteinase inhibitor.
two factors are thought to play critical roles: oxidant-induced stress and proinflammatory cytokines, especially TNF-α and interferon-γ (Bruwer et al., 2003; Podolsky, 2002). The principal permeability pathway in IBD is the paracellular route, which is ultimately regulated by tight junctions (TJs) between the intestinal epithelial cells (IECs) that constitute the intestinal barrier (Clayburgh et al., 2004). Clearly, a better understanding of the mechanisms underlying oxidant-induced barrier disruption and intestinal hyperpermeability seems to be a key to understanding the pathogenesis of IBD. Therefore, we studied these mechanisms in the hope that it would suggest targets for novel interventions that interrupt pathogenic inflammatory cascades in IBD.

The study being reported herein is part of our long-term goal of identifying mechanisms of oxidant-induced intestinal hyperpermeability using in vitro, animal, and clinical models of IBD (Keshavarzian et al., 1990; Banan et al., 2000a,b). Using in vitro models of human intestinal epithelium, this and other laboratories have identified key roles for specific intracellular signaling pathways mediating disruption of TJ permeability by oxidative stress. These pathways involve signaling via the epidermal growth factor receptor (EGFR) (Banan et al., 2001b, 2002; Basuroy et al., 2006).

Recently, a new pathway for oxidant (H₂O₂) signaling through EGFR activation was identified. The pathway is “metalloprotease-dependent transactivation of EGFR signaling” (Prenzel et al., 1999; Fischer et al., 2004). In this model (see Fig. 15), metalloproteases, especially from the ADAM family of membrane-bound metalloproteases, become activated by a variety of signals and cleave EGFR precursor ligands attached to the cell surface. These solubilized ligands then bind to the EGFR and initiate EGFR-mediated signaling pathways (Schafer et al., 2004). Several studies have shown that oxidants (e.g., H₂O₂) are one of several stimuli capable of triggering signaling via ADAM-dependent transactivation of the EGFR (Schafer et al., 2004). The ADAM involved in greater than 80% of all cases is the tumor necrosis factor-α convertase (TACE; ADAM-17) (Black et al., 1997; Blobel, 2005). In the intestine, TACE levels are increased in the inflamed mucosa of patients with IBD, and TACE inhibitors ameliorate colitis in animal models of IBD (Colon et al., 2001; Brynskov et al., 2002). We hypothesized that oxidant-induced metalloprotease-EGFR signaling could play a role in oxidant-induced increases in the intestinal permeability characteristic of IBD.

In the present study, we use an in vitro model of human intestinal epithelium to show that metalloprotease-EGFR signaling does play a key role regulating intestinal permeability in response to oxidant stress as well as baseline intestinal permeability. We identify TACE as the ADAM mediating EGFR transactivation through cleavage of TGF-α in response to oxidant stress. We also identify downstream EGFR-mediated mitogen-activated protein kinase (MAPK) signaling via ERK1/2 MAPKs to be necessary for oxidant-induced intestinal hyperpermeability. These data are the first demonstration of this EGFR transactivation mechanism regulating intestinal epithelial permeability. In addition, oxidant stress is known to exist in IBD epithelium, and TACE is required for TNF-α production so important in IBD pathogenesis. Therefore, stimulation of TACE activity by oxidant stress could clearly fuel IBD pathogenesis by stimulation of both TNF-α production and now we show through EGFR-mediated increased permeability resulting from TACE cleavage of TGF-α. This study could therefore have significant implications toward a new understanding of IBD pathogenesis in addition to providing new avenues for therapy of IBD.

### Materials and Methods

**Reagents.** H₂O₂ (Sigma, St. Louis, MO) was made fresh daily. TACE inhibitors used were TAPI-2 (Chemicon, Temecula, CA) and GM6001 (Calbiochem, San Diego, CA). EGFR inhibitors used were AG1478 and GW2974 (Sigma); they show similar results in permeability and signaling studies. Kinase inhibitors (Calbiochem) were as follows: for ERK1/2, PD98059 and U0126; for p38, SB203580; for JNK 1/2 inhibition, SP600125; and for phosphatidylinositol 3′-OH kinase inhibition, LY29004. Blocking antibodies (Abs) to EGFR (no. LA1; Upstate, Temecula, CA) and EGFR ligands used were: amphi- regulin (R&D Systems, Minneapolis, MN), TGF-α (R&D Systems), and the CRM197 HB-EGF blocker (diphtheria toxin) (Sigma). We also used Ab to ZO-1 (Invitrogen, Carlsbad, CA) and a rabbit polyclonal anti-TACE Ab (Sigma). EGFR (Ab-1), phosphotyrosine Ab (4G10), and EGFR-positive control were from Upstate Biotechnologies. All other phospho- and total MAPK Abs and controls were from Cell Signaling Technology (Danvers, MA). These comprised phosphospecific Abs, including ERK1/2, p38, JNK1/2, and AKT and total MAPK Abs (controls for loading), including ERK1/2, p38, JNK1/2, and AKT.

**Cell Culture.** We used confluent human colonic epithelial Caco-2 cells (number CRL2101, C2BBe1, colorectal adenocarcinoma; American Type Culture Collection, Manassas, VA) or IEC-6 (rat, nontransformed intestinal epithelial; American Type Culture Collection number CRL1592) between passages 15 and 25 grown on permeable insert Caco-2 monolayers was measured as apical to basolateral flux of the fluorescent marker fluorescein-5-(and-6)-sulfonic acid trisodium salt (FSA) (400 M) was added into the bottom (basolateral) chamber, and DME/M (300 μl) with FSA (400 nM) or FITC-dextran (1.25 μM) was added into the top (apical) chamber. At specific times, aliquots (50 μl) were transferred to opaque-bottom, 96-well fluorescence plates. Fluorescent signals were quantitated using a fluorescence plate reader and reported as “FSA or FITC-dextran clearance” (nanomoles per centimeter squared per...
hour). Excitation/emission spectra for FSA/FITC-dextran were 485/530 nm. Controls and a standard curve were done with each run. Inhibitors and blocking Abs were preincubated with cells for 1 h before adding H₂O₂.

Western Blotting Analysis. Western blotting was performed using the Bio-Rad Mini-Protein 3 system (Bio-Rad, Hercules, CA) as described previously (Forsyth et al., 2002). Cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma) and 1 mM phenylmethylsulfonyl fluoride with 1% Nonidet P-40. Lysates were assessed for total protein (Bio-Rad), and 20 μg was loaded per lane of 4/10% 1.0 mM Laemmli SDS-polyacrylamide gel electrophoresis gels. Protein was transferred to nitrocellulose and blocked with 5% bovine serum albumin for 1 h at 4°C and then incubated at 4°C overnight with primary Ab. Blots were then washed, incubated with horseradish peroxidase-conjugated 2°C Ab for 2 h at 4°C, washed, developed with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ), exposed to film (Fuji, Tokyo, Japan), and scanned for densitometry analysis with NIH Image J software (Banan et al., 2005).

Isolation of RNA and RT-PCR. Isolation of total RNA was done using the RNEasy kit (QIAGEN, Valencia, CA). RNA was quantitated in a spectrometer. Reverse transcription of RNA to cDNA was carried out using the High-Capacity cDNA RT kit (Applied Biosystems, Foster, CA). Real-time quantitative PCR was then performed using the resulting cDNA with the Sybr Green PCR Master Mix (Applied Biosystems) and 35 cycles of 95°C for 20 s, 60°C for 15 s, and 72°C for 15 s for PCR amplification. The reactions were performed in 96-well plates using an Applied Biosystems 7900HT Prism apparatus. Primers for RT-PCR used in the present study were: β-actin (157 bp), forward, 5'-GGCGAGTTCA-GACGCAGG-3' and reverse, 5'-TGCTATCCAGGCTGTGCTA-3'; and TACE (154 bp), forward, 5'-CTGTGGTGCAAAAGCAGAAA-3' and reverse, 5'-TGCCAAATGCCTCATATTCA-3'. Primers were designed with Oligoexpert software (Invitrogen) and purchased from Invitrogen. TACE data were normalized first to actin expression in the same sample and then normalized to the value of TACE expression in the "0 time" start cells to arrive at the -fold expression value.

Short Inhibitory RNA Inhibition Studies. Smartpool short inhibitory RNA (siRNA) specific for human ADAM-17 (TACE), and control siRNA were purchased from Dharmacon, Inc. (Lafayette, CO). Caco-2 cells grown to 60% confluence in six-well plates were transfected with 60 nmol/ml ADAM-17 siRNA or control siRNA using Lipofectamine (Invitrogen) and Opti-MEM reduced serum media (Invitrogen) according to manufacturer's instructions. At 7 days post-treatment, cells were assessed for expression of TACE protein using Western blotting. Cells with greater than 80% knockdown of TACE expression were then treated with H₂O₂ and assessed for ERK1/2 signaling and TACE expression by Western blot.

Microscopy. Microscopic images were obtained of Caco-2 cells grown to confluence plus 7 days on glass coverslips using an Axiovert 100 microscope and Axiovision software (Carl Zeiss Inc., Thornwood, NY). Two-dimensional images (see Figs. 10–12) were obtained using an oil immersion 100× objective, whereas images for Figs. 13 and 14 were obtained using an oil immersion 40× objective. Figures 13 and 14 show images obtained by deconvolution of 2-stack images (15 at 1 μm each). Three-dimensional reconstruction of deconvoluted z-stacks was performed using the Zeiss Axiovision software (Carl Zeiss Inc.). Immunofluorescent microscopic analysis of Caco-2 monolayers has been described previously by us in greater detail (Banan et al., 2001a,b, 2004, 2005).

Statistical Analysis. Analysis of variance to compare outcomes for three or more groups with p = 0.05 being considered significant. Data were analyzed using SPSS software for Windows (SPSS, Chicago, IL).

Results

EGFR-Specific Inhibitors, TACE-Metalloprotease Inhibitors, and Inhibitors of MAPK ERK1/2 Block H₂O₂-Induced Hyperpermeability across Caco-2 Monolayers. Evidence summarized above supports oxidative stress as one of the principal causes of increased permeability in IBD that fuels the inflammatory response and tissue injury. Previous data from this and other laboratories suggests a role for pretreatment with EGFR ligands (EGF, TGF-α) in protection against IEC permeability caused by oxidative stress (Banan et al., 2000a,b; Basuroy et al., 2006). In addition, recent discoveries have shown that metalloprotease-EGFR signaling mediates cellular response to H₂O₂ in other cell types (Fischer et al., 2004). Therefore, we sought to determine whether metalloprotease-EGFR signaling could be playing a role in increased permeability caused by oxidative stress in IBD. We used the in vitro Caco-2 insert monolayer model that has been well established by this and other laboratories as a reliable in vitro model of intestinal permeability (Banan et al., 2000a,b; Basuroy et al., 2006). Permeability of monolayers was monitored by addition of the fluorescent dye fluorescein sulfonylic acid (FSA; molecular mass, 478 Da) added at a concentration of 400 nM to the top chamber and fluorescein of the bottom chamber (phenol red-free and serum-free DMEM) was assessed at 18 h, and the data were displayed in Fig. 1 as “FSA clearance” (change in FSA at 490/530 nM in the bottom well) in nanomoles per centimeter squared per hour (Sanders et al., 1995; Banan et al., 2005). All permeability data are means of triplicate wells from a representative experiment of more than three such experiments. Using H₂O₂ as a model stimulus for oxidative stress, we subjected cells to 500 μM H₂O₂. This dose of H₂O₂ is consistent with the 200 to 500 μM range of H₂O₂ used by ourselves in previous studies and other investigators as a model stimulus of oxidative stress (Fischer et al., 2004; Song et al., 2006). We have documented in previous studies that this dose does not significantly affect cell viability (Banan et al., 2000a,b). This treatment caused a dramatic increase (~5-fold/h) in monolayer permeability without affecting cell viability (Fig. 1) and confirmed prior studies that oxidant stress increases Caco-2 permeability (Banan et al., 2001a,b). The development of hyperpermeability was inhibited (~66%) by the EGFR-specific tyrosine kinase inhibitors AG1478 (500 nM) (Fig. 1a, H₂O₂ + EGFRi) and GW2901 (data not shown). It was inhibited to a somewhat greater extent (>70%) by the broad MMP-TACE inhibitor GM6001 (20 μM) (Fig. 1a, MMPi) and the TACE-specific inhibitor TAPI-2 (10 μM) (Fig. 1a, TACEi). The inhibition was significant (p < 0.05) compared with the H₂O₂ + DMSO control. These two inhibitors are known to markedly inhibit ADAM-17 (TACE) at the concentrations used (Mohler et al., 1994; Santiskulvong and Rozengurt, 2003). These data strongly support a TACE metalloprotease-EGFR transactivation mechanism, one requiring TACE cleavage of EGFR proligands such as EGF, TGF-α, or HB-EGF. Also shown in Fig. 1a, AG1478 (control + EGFRi) as well as GM6001 (control + MMPi) and TAPI-2 (control + TACEi) also caused small but significant (p < 0.05) increase in permeability relative to control wells treated only with media + vehicle (control + DMSO). These data support a role for TACE-EGFR signaling in regulation of baseline intestinal epithelial
EGFR and TACE metalloprotease inhibitors prevent oxidant-induced IEC permeability and changes in TJ protein ZO-1. a, permeability. Confluent human intestinal epithelial cells (Caco-2) grown on inserts were assessed for permeability to FSA after 18 h in serum-free (SF) media (see Materials and Methods). Permeability is expressed as FSA flux from apical to basal chamber (nano moles per centimeter squared per hour). Some cells were treated only with media or media + vehicle (DMSO) (hatched bars 1–5). Some cells were stimulated with oxidant (dark bars 6–10) at a dose determined by viability assay to have no effect on cell viability (0.5 mM H₂O₂). In addition, some cells were pretreated (1 h) with the EGFR-specific kinase inhibitor AG1478 (500 nM), the broad MMP-ADAM inhibitor GM6001 (20 μM), or the TACE-specific inhibitor TAPI-2 (10 μM). Data are means ± S.D. of triplicate wells from a representative experiment from more than three separate experiments. Means compared by analysis of variance were considered significant (*, p < 0.05), for bars 2 to 5 compared with bar 1, and for bars 7 to 10 compared with H₂O₂ control bar 6. b, ZO-1 staining in oxidant-induced increased barrier permeability shown in Fig. 1a. These above data support a requirement for EGFR signaling in oxidant-induced intestinal epithelial permeability. Therefore, we next sought to determine what key downstream EGFR signaling pathways might be mediating the increase in permeability.

Key signaling pathways activated by EGFR signaling in other cell types include MAPK and AKT signaling (Fischer et al., 2004; Schafer et al., 2004). Thus, we determined the effect on H₂O₂-induced hyperpermeability of inhibitors of three principal MAPK pathways: ERK1/2, JNK1/2, and p38 as well as the effect of inhibition of phosphatidylinositide 3'-OH kinase activation of AKT (Fig. 1c). Of the inhibitors tested, Fig. 1c shows only the ERK1/2 inhibitors PD98059 (20 μM) and U0126 (20 μM) (data not shown) inhibited oxidant-induced permeability in a manner as great as the EGFR or MMP inhibitors in Fig. 1a. These data suggest that ERK1/2 activation is one major downstream signaling mechanism for EGFR-mediated hyperpermeability in response to H₂O₂ stimulation. Finally, we sought to validate the FSA permeability data with another marker of IEC permeability. We used the well validated 4-kDa FITC-dextran marker (1.25 μM) added to the apical surface of Transwells and assessed movement to the bottom chamber over 18 h as for FSA above (Fig. 1d). As for FSA, oxidant-induced permeability to 4-kDa FITC-dextran was substantially increased over 18 h, and this...
increase could be virtually eliminated by pretreatment with the EGFR, metalloprotease, and ERK inhibitors.

**EGFR-Specific Inhibitors and TACE-Metalloprotease Inhibitors Block H\(_2\)O\(_2\)-Induced EGFR and ERK1/2 Phosphorylation in Caco-2 Monolayers.** To further elucidate TACE-EGFR signaling mechanisms, we performed phosphoprotein analysis of cell lysates from cells stimulated with H\(_2\)O\(_2\) with or without inhibitors of EGFR, TACE, or ERK1/2 (Fig. 2, a and b). From Fig. 2a, it is clear that inhibitors of EGFR (AG1478) and TACE (TAPI-2) substantially block H\(_2\)O\(_2\)-stimulated EGFR phosphorylation (>90%). The effect of the TACE inhibitor supports a TACE-dependent transactivation mechanism involving cleavage of EGFR proligands as a requirement for H\(_2\)O\(_2\)-induced EGFR phosphorylation. In addition, the ERK1/2 inhibitor (PD98059) had no significant effect on EGFR phosphorylation. This suggests EGFR phosphorylation is upstream of ERK1/2 activation.

Besides EGFR phosphorylation, the parameter most often used to assess EGFR activation/signaling is phosphorylation (activation) of the MAPKs ERK1 and ERK 2 (Prenzel et al., 1999; Schafer et al., 2004). Figure 2b shows that both EGFR and TACE inhibitors reduce ERK1/2 activation to below control levels. As expected, the mitogen-activated protein kinase kinase-1 inhibitor PD98059 had no significant effect on EGFR phosphorylation. This suggests EGFR phosphorylation is upstream of ERK1/2 activation.

The activity of TACE or another ADAM metalloprotease (inhibited by TAPI-2) is required for oxidant-induced EGFR phosphorylation. We also wished to establish that H\(_2\)O\(_2\)-induced signaling via EGFR-metalloprotease activation could be demonstrated in other IEC cell lines and not only in Caco-2 cells. To do this, we used IEC-6 cells, a nontransformed rat cell line widely used in intestinal epithelial models (Fig. 3). Confluent cells were treated with H\(_2\)O\(_2\) ± an EGFR inhibitor (AG1478) or TACE inhibitor (TAPI-2) and assessed for ERK1/2 activation with phosphospecific Abs as in Fig. 2b. As with the Caco-2 cells, oxidant induced a greater than 5-fold increase in ERK1/2 phosphorylation that was inhibited by both the EGFR inhibitor (75%) and the TACE inhibitor (82%). These data further support the EGFR-metalloprotease model of H\(_2\)O\(_2\)-induced signaling in IECs and that it is also true for other intestinal epithelial cells.

**SiRNA Studies Identify TACE as the Principal Metalloprotease Mediating H\(_2\)O\(_2\)-Induced EGFR Signaling in Caco-2.** Because chemical inhibition of metalloproteases is not gene specific, we next sought to establish whether TACE or another metalloprotease was mediating H\(_2\)O\(_2\)-induced EGFR signaling via EGFR-metalloprotease activation could be demonstrated in other IEC cell lines and not only in Caco-2 cells. To do this, we used IEC-6 cells, a nontransformed rat cell line widely used in intestinal epithelial models (Fig. 3). Confluent cells were treated with H\(_2\)O\(_2\) ± an EGFR inhibitor (AG1478) or TACE inhibitor (TAPI-2) and assessed for ERK1/2 activation with phosphospecific Abs as in Fig. 2b. As with the Caco-2 cells, oxidant induced a greater than 5-fold increase in ERK1/2 phosphorylation that was inhibited by both the EGFR inhibitor (75%) and the TACE inhibitor (82%). These data further support the EGFR-metalloprotease model of H\(_2\)O\(_2\)-induced signaling in IECs and that it is also true for other intestinal epithelial cells.
ERK1/2 phosphorylation. Blots were also stripped and re-probed with antibody to TACE to assess expression of TACE 110- and 90-kDa forms. The data shown in Fig. 4 reveal that in cells in which TACE protein expression was knocked down by 84% (lane 3, TACE SiRNA) compared with the control SiRNA-treated cells (lane 4), the H2O2-induced ERK1/2 activation was also reduced by 85% (p < 0.05) compared with the control SiRNA-treated cells. Our previous data above showed that H2O2-induced ERK1/2 activation in Caco-2 is EGFR- and metalloprotease-dependent and that ERK1/2 activation is required for H2O2-induced hyperpermeability. Therefore, although these data do not rule out participation of other metalloproteases, they do demonstrate in a gene-specific manner that TACE is the principal ADAM metalloprotease mediating H2O2-induced EGFR-ERK1/2 signaling and therefore increased permeability by this mechanism in Caco-2 cells.

TACE Protein Is Abundantly Expressed by Caco-2 Cells, and EGFR and TACE Inhibitors Do Not Act by Inhibiting TACE Protein Expression. Because the above data point to a role for TACE in H2O2-induced signaling and permeability, we wished to determine whether sufficient quantities of TACE were expressed by Caco-2 cells and how EGFR, TACE, and ERK1/2 inhibitors affected TACE protein expression. TACE protein was assessed using Western blotting of cell lysates with polyclonal antibody to the TACE cytoplasmic domain (Fig. 5). Studies of other cell types including Caco-2 cells have shown that production of TACE is relatively constant and that TACE exists in the cell as an intracellular, 110-kDa, inactive zymogen, “pro-TACE” form and an activated 90-kDa form predominantly found on the cell surface or near the cell surface in intracellular vesicles (Schlondorff et al., 2000; Soond et al., 2005). The 110-kDa pro-TACE seems to be associated with the cytoskeleton in a perinuclear location (Soond et al., 2005). The data from Caco-2 cells in Fig. 3 are in lanes 2 to 6. Lane 1 "+ Con" contains a TACE-positive control cell lysate (Chemicon). The 15-min time point chosen was the same as that for the EGFR and ERK1/2 signaling data for comparison. No significant differences in TACE protein expression from these data were seen with the EGFR and TACE inhibitors in 18-h lysates (data not shown). Clearly, Caco-2 cells even at rest express abundant TACE in both the pro-110-kDa as well as the activated 90-kDa forms (lane 2). In addition, treatment with H2O2 results in only a slight increase (but not statistically significant) in both the 110- and 90-kDa forms of TACE (lane 3). However, in the presence of H2O2, treatment with the EGFR inhibitor (lane 4), TACE inhibitor (lane 5), or the ERK1/2 inhibitor (lane 6) did not significantly alter the amount of pro- or activated TACE to an extent that would account for complete blocking of EGFR or ERK1/2 phosphorylation as seen in Fig. 2. In fact the amount of active 90-kDa TACE in Caco-2 cell lysates was evaluated by Western blotting for TACE protein expression using a polyclonal antibody to TACE (Chemicon).

Fig. 3. EGFR and TACE inhibitors also block H2O2-induced ERK1/2 activation in IEC-6 cells. IEC-6 (rat, nontransformed) intestinal epithelial cells were also assessed for EGFR-metalloprotease signaling in response to stimulation with H2O2. Confluent cells grown on inserts were treated as in Fig. 2.

Fig. 4. SiRNA knockdown of TACE blocks H2O2-stimulated ERK1/2 activation. Caco-2 cells grown to 60% confluence were transfected with Dharmacon Smartpool SiRNA (60 pM) to TACE (human ADAM-17) as described in Materials and Methods, allowed to rest for 24 h, and transfected with another round of Dharmacon Smartpool SiRNA (60 pM) to TACE. After 7 days, for ERK1/2 activation assessment, cells were treated as in Fig. 2, and cell lysates were evaluated by Western blot for ERK1/2 phosphorylation and total ERK1/2. Blots were stripped and reprobed for TACE 110-kDa precursor and TACE 90-kDa activated protein expression (top). Densitometry with Image J revealed 84% knockdown of TACE protein and 85% reduction of ERK1/2 activation compared with cells treated with control scrambled RNA (Dharmacon).
TACE is relatively constant in lanes 2 to 6, with no statistical difference detected. The blot was stripped and reprobed with Ab to actin as a protein loading control. Taken together, these data show that strong expression of TACE protein by Caco-2 in our system is consistent with other data above pointing to a role for TACE in oxidant-induced EGFR-mediated signaling and permeability changes. However, activation of TACE-mediated EGFR signaling does not seem to be related to changes in the active form of TACE protein.

Although no changes in TACE protein expression were seen either at 15 min or 18 h, we wished to assess whether changes in TACE gene expression (mRNA) could account for increased TACE signaling required for \( \text{H}_2\text{O}_2 \)-induced permeability (Fig. 6). We also wished to determine whether gene expression (mRNA) of TACE was altered in cells after \( \text{H}_2\text{O}_2 \) treatment for 18 h, we wished to assess whether changes in TACE gene expression (mRNA) could account for increased TACE signaling required for \( \text{H}_2\text{O}_2 \)-induced permeability. In addition, cells were treated with EGF, which we have shown in previous studies to prevent \( \text{H}_2\text{O}_2 \)-induced permeability in Caco-2 inserts (Banan et al., 2000a,b). In Fig. 6, data from quantitative real-time RT-PCR for TACE are normalized in each lane to actin expression and that ratio is expressed as a \(-fold\) expression of the start control (histogram). Treatment with \( \text{H}_2\text{O}_2 \) alone resulted in reduced TACE expression, although treatment with the EGFR inhibitor resulted in increased TACE expression. Interestingly, treatment with EGF also resulted in down-regulation of TACE expression. These data suggest a negative feedback mechanism that regulates TACE expression when EGFR signaling is stimulated by oxidants or EGF. Clearly, however, these data do not support increased TACE gene expression as the probable mechanism for oxidant-induced hyperpermeability through EGFR-TACE signaling.

**Permeability and Signaling Data Support TGF-\( \alpha \) as the EGFR Ligand Mediating Increased Permeability in Response to Oxidant.** A key element in metalloprotease-EGFR transactivation is the EGFR proligand cleaved on the surface of the cell by TACE. To identify this ligand, we assessed the effects of blocking proteins to the EGFR and EGFR ligands on hyperpermeability induced by \( \text{H}_2\text{O}_2 \) (Fig. 7). Blocking antibodies to amphiregulin had no effect at several concentrations (data not shown). The blocking protein for HB-EGF (CRM197, 10 \( \mu \)g/ml, a diphtheria toxin) had a dramatic effect on resting cells (bar 3), increasing permeability to that of \( \text{H}_2\text{O}_2 \)-treated cells, but had little effect at blocking \( \text{H}_2\text{O}_2 \)-induced permeability (bar 7). In contrast, blocking Ab to TGF-\( \alpha \) (10 \( \mu \)g/ml) had little effect on resting permeability (bar 4) but blocked \( \text{H}_2\text{O}_2 \)-induced hyperpermeability by 96% (bar 8). Cells treated with blocking Ab to the EGFR caused some leakiness in control cells and blocked \( \text{H}_2\text{O}_2 \)-induced hyperpermeability by 80%. These data support a role for TGF-\( \alpha \) as the oxidant-stimulated proligand cleaved by TACE that subsequently binds to the EGFR to induce ERK1/2 and other signals required for increased permeability.
caused increased baseline permeability, these data also suggest a role for the EGFR in the mediation of baseline permeability.

To further evaluate TGF-α and HB-EGF signaling after oxidant stimulation, we carried out phosphoprotein analysis of cell lysates to assess EGFR phosphorylation (Fig. 8) and ERK1/2 activation (Fig. 9) in the presence of the above EGFR, TGF-α, and HB-EGF inhibitors when cells were at rest or stimulated with H₂O₂. The EGFR activation data (Fig. 8) agree closely with the ERK1/2 signaling data (Fig. 9). Figure 8, lane 3 shows cells that were treated with the EGFR inhibitor AG1478; all baseline EGFR phosphorylation was eliminated. The solid bars indicate treatment with H₂O₂; H₂O₂ greatly stimulated EGFR phosphorylation (lane 4). Pretreatment with EGFR kinase inhibitor (lane 5) and TACE inhibitor (lane 6) again reduced EGFR tyrosine phosphorylation to below control (baseline) levels seen in lane 2 despite H₂O₂ stimulation. Thus, as in Fig. 2a, virtually all H₂O₂-stimulated EGFR phosphorylation was found to be metalloprotease-dependent. Next, specific blocking proteins or Abs to the EGFR or EGFR ligands amphiregulin, HB-EGF, and TGF-α were tested for their effect on cell signaling at concentrations equal to those used in the Fig. 7 permeability studies. Blocking Ab to the EGFR significantly inhibited (to below control levels) EGFR phosphorylation stimulated by H₂O₂ (lane 7). Most important, treatment with blocking Ab to TGF-α eliminated EGFR phosphorylation to a level comparable with the effects of EGFR kinase and TACE inhibitors, whereas blocking protein to HB-EGF inhibited by only 40%. We next assessed ERK1/2 phosphorylation in the same cell lysates (Fig. 9). Lane 2 shows that virtually all ERK1/2 activation is due to EGFR signaling and is eliminated with EGFR kinase inhibitor treatment, even in control cells (baseline). H₂O₂ dramatically stimulated ERK1/2 activation (5-fold) (lane 3), and the EGFR kinase (lane 4) and TACE inhibitors (lane 5) blocked activation of downstream signaling, in this case ERK1/2, to below baseline control values. The blocking Ab to the EGFR (anti-ErbB1) also significantly (−88%) inhibited oxidant-induced ERK1/2 activation but not to the extent (≥95%) of the blocking TGF-α antibody. Finally, blocking protein to HB-EGF inhibited H₂O₂-induced ERK1/2 activation, but only by 30%. Blocking Ab to amphiregulin had no effect on EGFR or ERK1/2 phosphorylation at several concentrations (data not shown). Taken together, these permeability and phosphoprotein signaling data strongly support a metalloprotease-dependent EGFR transactivation mechanism regulating permeability of this in vitro model in response to oxidant stress. In addition, the data support a
model in which TGF-α is the proligand cleaved by TACE to initiate the EGFR-ERK1/2 signaling required for the increase in permeability.

The EGFR and the EGFR Proligands TGF-α and HB-EGF Are Primarily Localized to Peripheral Cell-Cell Junctions. We next sought to use immunofluorescence microscopy to identify the cellular locations of the EGFR and EGFR proligands TGF-α and HB-EGF. Figure 10 shows the results from staining unstimulated Caco-2 monolayers on coverslips with primary Ab to the EGFR (ErbB1) followed with FITC secondary Ab (green) as well as Ab to TACE followed by Texas Red secondary Ab. Clearly, the EGFR location is concentrated at the cell periphery in areas of cell-cell adhesion (arrows). No differences were seen after treatment with H₂O₂ (data not shown). Figure 11, a and b, show representative images resulting from Caco-2 on coverslips with primary Ab to either HB-EGF (Fig. 11a) or TGF-α (Fig. 11b) followed by FITC secondary Ab. Again, in both cases, the EGFR proligands are also primarily localized to cell-cell contact zones, which also did not change with oxidant stimulation (data not shown).

We next used immunofluorescent microscopy to identify the location of TACE before and after stimulation with oxidant (Figs. 12–14). To do this, we treated cells with Ab to the intracellular domain of TACE (+Texas Red 2°Ab) and the tight junction protein ZO-1 (+FITC 2°Ab), either as media-treated control (Fig. 12, a–c, top) cells or cells treated with H₂O₂ (Fig. 12, d–f, bottom). These images are representative of multiple experiments and images and clearly show several key points. First, note the characteristic deformation of ZO-1 staining in the H₂O₂-treated (bottom) panel as seen in Fig. 1 that is consistent with increased permeability. The next point is that TACE staining in unstimulated cells is characteristically without pattern and distributed diffusely within each cell as viewed two-dimensionally (arrows). However, in oxidant-stimulated cells, TACE staining consistently exhibits a strong distribution to the cell periphery and forms characteristic “ring-like” structures (arrows). Thus, stimulation with oxidant seems to result in dramatic TACE translocation to the cell-cell contact zones where the EGFR, TGF-α, and HB-EGF are already located. The translocation of TACE to colocalize with ZO-1 is shown in the x-z views compiled from deconvoluted z-stacks in Fig. 13. In the Fig. 13 x-z and y-z projections, note the large distribution of TACE (red) below the plane of ZO-1 (green) in control cells (unstimulated), whereas TACE can clearly be seen to colocalize with ZO-1 in the H₂O₂-stimulated cells. This redistribution of TACE to colocalize with ZO-1 is most clear in the 3-D reconstructions seen in Fig. 14. In Fig. 14, A and B, the TACE (red) pool is clearly located beneath ZO-1 (green) in a virtual bilayer arrangement. Dramatically, in the oxidant-stimulated cells, TACE translocates to colocalize and intersperse with ZO-1, and the bottom pool of TACE disappears (Fig. 14, C and D). In the unstimulated cells, TACE (red) exists pre-
dominantly in a pool beneath ZO-1 (green) that is perinuclear as reported by others in different cell types (Soond et al., 2005). Staining for nuclei with 4,6-diamidino-2-phenylindole has been left out of these figures because it obscured the view of TACE staining; however, the perinuclear (bottom) pool of TACE is still clear in the three-dimensional views in Fig. 14.
A and B. The bottom TACE pool is especially clear in Fig. 14A (orthogonal, from below), and this clearly becomes intercalated with ZO-1 in Fig. 14C. Taken together, these series of fluorescence microscopic images in Figs. 12 to 14 clearly demonstrate movement of TACE to colocalize with ZO-1 in oxidant-stimulated cells consistent with the proposed role for TACE in regulating TJ permeability in IECs.

**Discussion**

TNF-α and oxidants are widely acknowledged as key proinflammatory factors involved in tissue injury in both ulcerative colitis and Crohn’s disease (Podolsky, 2002; Farhadi et al., 2003). Indeed, anti-TNF-α (Zeissig et al., 2004) or antioxidant (Keshavarzian et al., 1992) therapies have been shown to be effective in the treatment of IBD. One of the mechanisms underlying the deleterious effects of oxidants and TNF-α in IBD is probably increased intestinal permeability (Bruewer et al., 2003; Clayburgh et al., 2004). However, the mechanisms of oxidant- and TNF-α-induced intestinal hyperpermeability in IBD are not fully understood. It is known that TACE is the metalloprotease required for TNF-α cleavage and biological activity (Black et al., 1997). Both TACE and TNF-α are up-regulated in active IBD as well as in normal-appearing mucosa of IBD patients (Brynskov et al., 2002; Zeissig et al., 2004). TNF-α is also known to stimulate TACE expression in a kind of vicious circle (Black et al., 1997). Our study now provides the novel finding that oxidant-induced barrier hyperpermeability is partly dependent on TACE metalloprotease-mediated transactivation of EGFR signaling. This study provides compelling data to support a new model (oxidant-induced metalloprotease-dependent EGFR transactivation) that could identify a new relationship between oxidants and TNF-α in inducing intestinal hyperpermeability and tissue injury in IBD. The key points of this model are summarized in Fig. 15. The model begins with oxidant stress (1), resulting in activation of the metalloprotease TACE (2). Activated TACE then cleaves membrane TGF-α to a soluble form (3) that binds to the EGFR. EGFR activation then results in downstream MAPK/ERK1/2 activation (4). ERK1/2 signaling (5) then results in increased intestinal permeability (6) by pathways that have yet to be identified.

Metalloprotease-EGFR signaling has been demonstrated in IECs in response to Substance-P (Koon et al., 2004), and Clostridium difficile toxin (Na et al., 2005) as a regulator of cell proliferation. Previous studies from this and other laboratories have shown a role for the EGFR in regulation of intestinal permeability in response to oxidative stress (Banazi et al., 2000b, 2001a; Basuroy et al., 2006). To our knowledge, however, ours is the first demonstration of metalloprotease-EGFR transactivation signaling in the regulation of intestinal barrier function by any stimulus and, in this case, in response to oxidative stress.

Several of our results strongly support TACE as the pri-
mary metalloprotease involved. First is potent inhibition of oxidant-induced intestinal hyperpermeability by GM6001 as well as by the TACE-specific inhibitor TAPI-2. TACE is not an MMP but is instead a member of the ADAM family of membrane metalloproteases. Although also capable of inhibiting MMP type metalloproteases, GM6001 has been shown by others to inhibit TACE but not necessarily other ADAM metalloproteases (Santiskulvong and Rozengurt, 2003). In contrast, TAPI-2 was originally identified by its ability to inhibit TACE but not MMPs; however, its specificity against other ADAM metalloproteases in the TACE family is not well defined (Mohler et al., 1994). Second, both the EGFR and TACE inhibitors also block oxidant-induced displacement in ZO-1 staining that is characteristic of increased permeability (Banan et al., 2001a; Basuroy et al., 2006). Third, RT-PCR and Western-blotting data show that Caco-2 cells in our model express abundant TACE, including the key 90-kDa activated form (Black et al., 1997). Fourth, gene-specific SiRNA knockdown studies of TACE reveal that TACE is the predominant metalloprotease mediating H₂O₂-induced ERK1/2 activation required for increased permeability in our model. Fifth and finally, our images also significantly show that with oxidant stimulation TACE is translocated to cell-cell junction areas and colocalizes with ZO-1 in proximity with the proposed ligand/receptor in this case: TGF-α/EGFR. These translocation images support a model proposed by others for translocation of TACE with specific proligand substrates as a key regulatory mechanism of TACE specificity (Werb and Yibing, 1998). Our TGF-α permeability and signaling blocking data clearly support that TGF-α is the principal EGFR proligand cleaved by TACE upon H₂O₂ stimulation, and these data are further supported by the dramatic peripheral colocalization of TACE:TGF-α in oxidant-stimulated cells. This is consistent with TACE being the major sheddase for TGF-α cleavage in EGFR transactivation in other cell types (Blobel, 2005).

Our data show the mechanism for increased permeability due to H₂O₂ transactivation of EGFR signaling is mediated in part by MAPK signaling, specifically ERK1/2. Thus, another concept that emerges from this study is that ERK1/2 activation seems to be one critical pathway mediating intestinal hyperpermeability in response to oxidants through TACE-EGFR signaling. This is supported by the comparative permeability and signaling data since only the EGFR, TACE, and ERK1/2 inhibitors showed similar protection against increased permeability, and these three inhibitors exhibited the most significant inhibition of both EGFR and ERK1/2 activation by oxidants. These data agrees with that of others showing tyrosine kinase and Src inhibitors block H₂O₂-induced hyperpermeability in Caco-2 monolayers (Basuroy et al., 2003). This result also agrees with data from others showing that both TGF-α-induced hyperpermeability in Madin-Darby canine kidney cells (Chen et al., 2000) and H₂O₂-induced endothelial hyperpermeability (Fischer et al., 2005) can be blocked with the same ERK1/2 inhibitor. However, these data seem to differ from other studies showing ERK1/2 activation is required for EGF protection of Caco-2 monolayers from oxidant stress-induced hyperpermeability. The reason for this difference is not clear. One possibility is that the study referred to used very low concentrations of H₂O₂ (20 μM) that do not result in ERK1/2 activation in their data or in our laboratory. Therefore the mechanism and role of ERK1/2 may be different. The concentration of H₂O₂ used in the present study is consistent with the 200 to 500 μM concentration used by other investigators in the initial discovery of oxidant-induced EGFR transactivation (Prenzel et al., 1999) as well as more recent intestinal epithelial cell studies by others (Song et al., 2006). We have stated here and
in previous studies this concentration does not affect cell viability (Banan et al., 2000a,b).

Another key difference is the point we refer to as the "EGF-EGFR paradox." The paradox is as follows. Preincubation with EGFR ligands EGF or TGF-α protects against oxidant-induced permeability. However, paradoxically, EGFR signaling seems to be required for oxidant-induced permeability. The mechanism for this at present is unknown but has precedent in many of the preconditioning models in cell biology in which preconditioning with a stimulus "protects" against subsequent challenge with the same stimulus (e.g., heat shock, hypoxia). In fact, ERK1/2 have been shown to be important in oxidant preconditioning in other cell types (Hung et al., 2003). Although our own studies have shown previously that PLC-γ (Banan et al., 2001a) and PKC-β1 (Banan et al., 2002) are also critical for this EGF preconditioning protection, the complete mechanism for this paradox remains to be identified in future studies but seems to also require ERK1/2 signaling (Basuroy et al., 2006). Taken together, it is clear that EGFR signaling as well as ERK1/2 signaling play critical roles in oxidant-induced intestinal permeability and protection.

Our data showing that ERK inhibitors block oxidant-induced hyperpermeability agrees with studies with MDCK and endothelial cells. Therefore, key downstream targets of ERK seem to be critical in the regulation of paracellular permeability in many cell types. One possible candidate suggested in other studies is myosin light-chain kinase (MLCK) (Clayburgh et al., 2004). ERK1/2 directly phosphorylates MLCK (Nguyen et al., 1999), and MLCK activation of myosin light chain is one key mechanism regulating intestinal permeability and may play a role in IBD (Clayburgh et al., 2004). In addition, ERK1/2 can be immunoprecipitated with the TJ protein occludin in oxidant-treated IECs (Basuroy et al., 2006), suggesting phosphorylation of occludin by ERK1/2 as another mechanism for ERK1/2 regulation of TJ permeability. Finally, ERK1/2 have been shown to regulate TACE activation and phosphorylate TACE directly (Soond et al., 2005). Further studies will be needed to identify the specific role(s) for ERK1/2 in TACE/EGFR regulation of intestinal permeability.

In summary, we propose a new model in which metalloprotease-dependent EGFR transactivation in response to oxidant stress is one mechanism for intestinal hyperpermeability and tissue injury in IBD. In this model, the metalloprotease TACE plays a key role by cleavage of EGFR ligands and TNF-α. Indeed, metalloprotease inhibitors of TACE eliminate inflammation in animal models of IBD (Colon et al., 2001). Our permeability data with the CRM197-specific blocking protein for HB-EGF and our microscopy data showing it localized to cell-cell junction zones support a model in which HB-EGF’s signaling regulates baseline barrier integrity. This could occur possibly via juxtcrine interaction by HB-EGF proligands with EGFR on neighboring cells, which is known to inhibit mitosis (Singh et al., 2004).

Thus, our findings could have far-reaching implications for a better understanding of IBD pathogenesis using a new model of intestinal hyperpermeability. These data also identify potential targets for developing new therapies for IBD such as specific inhibitors of TACE-EGFR-initiated signaling and ERK1/2 inhibitors. Further in vivo animal studies are currently underway in our laboratory to determine whether modulation of the metalloprotease-dependent EGFR transactivation pathway can be safely and effectively targeted in IBD therapy.

References


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