Research Article

Cancer Prevention Research

Sulindac Metabolites Induce Proteosomal and Lysosomal Degradation of the Epidermal Growth Factor Receptor

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Abstract

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. In response to ligand, EGFR is internalized and degraded by the ubiquitin-proteasome/lysosome pathway. We previously reported that metabolites of the nonsteroidal anti-inflammatory drug sulindac downregulate the expression of EGFR and inhibit basal and EGF-induced EGFR signaling through extracellular signal–regulated kinase 1/2. We now have evaluated the mechanisms of sulindac metabolite–induced downregulation of EGFR. EGF-induced downregulation of EGFR occurs within 10 minutes and lasts for 24 hours. By contrast, downregulation of EGFR by sulindac sulfide and sulindac sulfone was first evident at 4 and 24 hours, respectively, with maximal downregulation at 72 hours. Pretreatment with either the lysosomal inhibitor chloroquine or the proteosomal inhibitor MG132 blocked sulindac metabolite–induced downregulation of EGFR. Sulindac metabolites also increased the ubiquitination of EGFR. Whereas sulindac metabolites inhibited phosphorylation of EGFR pY1068, they increased phosphorylation of EGFR pY1045, the docking site where c-Cbl binds, thereby enabling receptor ubiquitination and degradation. Immunofluorescence analysis of EGF and EGFR distribution confirmed the biochemical observations that sulindac metabolites alter EGFR localization and EGFR internalization in a manner similar to that seen with EGF treatment. Expression of ErbB family members HER2 and HER3 was also downregulated by sulindac metabolites. We conclude that downregulation of EGFR expression by sulindac metabolites is mediated via lysosomal and proteosomal degradation that may be due to drug-induced phosphorylation at pY1045 with resultant ubiquitination of EGFR. Thus, sulindac metabolite–induced downregulation of EGFR seems to be mediated through mechanism(s) similar, at least in part, to those involved in EGF-induced downregulation of EGFR. *Cancer Prev Res; 3(4); 560–72. ©2010 AACR.*

Introduction

One of the most widely studied and promising groups of compounds for colorectal cancer prevention are the nonsteroidal anti-inflammatory drugs (NSAID). Sulindac is a NSAID that was originally developed as a prodrug (sulfoxide) in an effort to reduce its gastrointestinal toxicity, a limiting factor for chronic use of NSAIDs. Sulindac is rapidly metabolized in the liver into two active compounds: sulindac sulfide, an active NSAID that inhibits both cyclooxygenase (COX)-1 and COX-2, and sulindac sulfone, which does not inhibit either COX-1 or COX-2.

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Although substantial animal model as well as human observational and clinical evidence indicates that NSAIDs have chemopreventive activity, these studies have typically not examined the mechanisms responsible for the effect. Several biological mechanisms for the chemopreventive effects of NSAIDs have been proposed, including inhibition of proliferation, induction of apoptosis, and inhibition of angiogenesis. Our laboratory (1–3) and others (4) have reported that sulindac sulfide, as well as the non-NSAID sulindac sulfone and the structurally related compound OSI-461 (5, 6), induces apoptosis of cancer cells *in vitro*, prevents tumor formation in animal models, and causes regression of adenomas in familial adenomatous polyposis (7–11). Apoptosis seems to be the major biological mechanism for growth inhibition by these drugs both *in vitro* (4, 12) and *in vivo* (11).

The ErbB family contains four homologous receptors: the epidermal growth factor receptor (EGFR) also called ErbB1/HER1, ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. Under normal conditions, these receptors have essential roles in signaling pathways that modulate cell proliferation and differentiation and promote cell survival and angiogenesis. The ErbB receptors and their signaling pathways are genetically and/or biochemically altered in

many cancers (13, 14) due to overproduction of growth factor and/or overexpression of the receptors, but it can also be due to constitutive activation of downstream pathways including K-Ras or b-Raf mutations (15, 16). Uncontrolled activation of ErbB signaling may stimulate cell proliferation and promote cell survival in cancer cells (17).

Previous studies have shown that increased signaling through ErbB receptors is a common early event in colorectal carcinoma. Overexpression of the EGFR ligand transforming growth factor α and upregulation of EGFR, HER2, and HER3 are well documented in colorectal tumors (14, 18, 19) and seem to be related to the biological behavior of colorectal cancer (20–22). Several investigators have reported that expression of the EGFR family of receptors and their ligands correlates with more aggressive disease and a poorer prognosis (23–26); however, at present, there is not enough evidence to establish EGFR expression or signaling as an independent prognostic factor in colorectal cancer. The therapeutic success that anti-EGFR therapies have shown in colorectal cancer (cetuximab and panitumumab are Food and Drug Administration approved for the treatment of colorectal cancer), however, highlights the relevance of EGFR in colorectal cancer tumorigenesis (27–30).

Binding of stimulatory ligand to the extracellular domain of ErbB receptors induces the formation of homodimers and heterodimers, activation of the kinase domain, and subsequent phosphorylation on specific tyrosine residues within the cytoplasmic tail of the receptor. The phosphorylated residues serve as docking sites for signaling molecules containing SH2 or PTB domains, whose recruitment leads to the activation of several intracellular signaling pathways including Ras/Raf/extracellular signal–regulated kinase 1/2 (ERK1/2), phosphoinositide 3-kinase/Akt, signal transducer and activator of transcription, and other pathways.

Ligand binding to EGFR also leads to rapid internalization and proteosomal/lysosomal degradation of the receptors. This process results in a dramatic downregulation of both total and cell surface receptors. EGF-induced degradation of EGFR is thought to be initiated by phosphorylation of tyrosine 1045 of the receptor followed by binding of Cbl adaptor proteins and ubiquitination of the receptor. Internalized EGFR is transported to early endosomes where receptor-ligand complexes are sorted for either degradation or recycling to the cell surface. Complexes continuing along the degradation pathway are ultimately targeted to degradation by lysosomal proteases (31, 32). It is well documented that EGFR is processed from the cell surface via internalization leading to rapid lysosomal degradation (33, 34); however, proteasomes have also been proposed to regulate EGFR degradation (35).

We previously reported that both sulindac sulfide and sulindac sulfone downregulate EGFR expression and phosphorylation at the same doses that were found to inhibit ERK1/2 phosphorylation and induce apoptotic cell death (36). The ability of sulindac metabolites to inhibit EGFR levels and signaling suggested that these drug effects could be the result of decreased EGFR synthesis, increased EGFR degradation, or other mechanisms. Thus, the goal of this project was to determine the mechanism of EGFR downregulation by sulindac metabolites. We report that sulindac metabolite–induced downregulation of EGFR expression is due to degradation mediated by both lysosomal and proteosomal mechanisms in a manner that is biochemically and morphologically similar to EGF-induced degradation of the receptor.

Materials and Methods

Reagents

Cell culture media and fetal bovine serum (FBS) were purchased from Mediatech and tissue culture plates from Falcon. Eight-well culture slides were from BD Biosciences. Primary antibodies raised against total HER2, total HER3, ubiquitin, and total EGFR (1005 and 528) and EGFR-agarose conjugate for immunoprecipitation were purchased from Santa Cruz Biotechnology; primary antibody against phosphorylated EGFR (Tyr1068), horseradish peroxidase–conjugated antimouse and antirabbit secondary antibodies, and EGF were purchased from Biosource. Primary antibodies against phosphorylated EGFR (Tyr1045), phosphorylated HER2 (Tyr1221/1222), and phosphorylated HER3 (Tyr1289) were obtained from Cell Signaling Technology. Alexa Fluor 488–labeled EGF was from Molecular Probes. Cy3-goat anti-mouse IgG conjugate was purchased from Invitrogen Corporation. Hybond membranes and chemiluminescent visualization reagents were from Amersham. Primary antibodies against α-tubulin and MG132 were purchased from Calbiochem. Chloroquine diphosphate salt was purchased from Sigma-Aldrich and leupeptin hemisulfate from Fisher BioReagents. Sulindac sulfide was obtained from LKT Laboratories, Inc. Sulindac sulfone was a generous gift from Cell Pathways, Inc. (now OSI Pharmaceuticals).

Drug dosing

The concentrations of sulindac metabolites used *in vitro* have been discussed in previous publications (37). Downregulation of EGFR has previously been shown (36) to occur at doses and within a time course that are consistent with the ability of sulindac metabolites to induce apoptotic cell death (as determined by nuclear morphology and confirmed by caspase-3 cleavage); apoptotic doses of the drugs were therefore used in all experiments.

Cell culture

HT29 and Caco-2 human colon cancer cells were purchased from the American Type Culture Collection. HT29 and Caco-2 cells were respectively maintained in RPMI 1640 and DMEM, supplemented with 10% FBS. Cells were plated and grown on tissue culture plates as specified in Results and in figure legends.

Preparation of cell lysates

At time of harvest, cells were scraped from the plates, washed with ice-cold PBS, and pelleted at 2,400 × *g* for

5 min. Care was taken to keep the samples at 4°C during harvest. After aspirating the supernatant, the cells were washed twice with ice-cold PBS and centrifuged. The cell pellet was lysed in cell extraction buffer [10 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 60 μg/mL aprotinin, 10 μg/mL leupeptin, 1 μg/mL pepstatin] for 30 min on ice with vortexing at 10-min intervals. Lysates were then centrifuged at 18,000 × *g* for 10 min at 4°C, and supernatants collected. Protein concentrations of lysates were determined by the method of Lowry (38).

Western blot analysis

Lysate samples (50 μg total protein) were separated by SDS-PAGE and electrotransferred overnight onto Amersham Hybond-P membranes. Nonspecific binding was blocked by immersing the membranes in Tris-neutral saline with 5% (w/v) dry milk and 0.05% Tween 20 for 30 min at room temperature. The blots were then incubated with the indicated primary antibody while rocking at 37°C for 1 h. Immunoreactive protein was detected by incubating the blots with horseradish peroxidase–conjugated secondary antibody for 1 h, followed by chemiluminescent substrate for 1 min. Immunoreactive proteins were visualized by scanning on a Typhoon (Amersham). Quantitation of protein levels was completed by densitometry using a visual imaging system (Image Quant TL, Amersham); only specific bands (i.e., for EGFR 185 kDa) were included in the densitometric analyses. Phosphorylated receptor levels were calculated as the amount relative to the total receptor level to show what proportion of the total receptor was phosphorylated under each experimental condition. α-Tubulin immunoblots served as loading controls for each experiment.

Immunoprecipitation

Cell lysates were prepared as noted above. For immunoprecipitation, 500 μg of protein were brought to a final volume of 1 mL in radioimmunoprecipitation assay buffer, followed by overnight incubation at 4°C (with mixing) with 40 μL of anti-EGFR-agarose conjugate. The pellet was collected by centrifugation at 3,000 rpm (~1,000 × *g*) for 5 min at 4°C followed by aspiration of the supernatant. The pellet was washed three times with radioimmunoprecipitation assay buffer, centrifugating after each wash. After the final wash, supernatant was aspirated and pellet was resuspended in 60 μL of 2× electrophoresis sample buffer and boiled for 5 min. Thirty microliters of the sample were subjected to SDS-PAGE and immunoblotted as described above.

Immunocytochemistry

Cells were plated in BD BioCoat Poly-D-Lysine Cellware eight-well culture slides and grown to 50% to 60% confluence. Cells were then serum deprived for 48 h, followed by treatment with or without 120 μmol/L sulindac sulfide or 600 μmol/L sulindac sulfone for 24 h in fresh serum-free medium. After 24 h of drug treatment, cells were incubated with 500 ng/mL Alexa Fluor–labeled EGF for 0, 10, 30, or 60 min at 37°C and then fixed with paraformaldehyde for 30 min at room temperature. After blocking nonspecific binding with a 5% bovine serum albumin/0.2% Triton X-100/PBS solution for 1 h at room temperature and then with 10% goat serum for 1 h at room temperature, cells were incubated with total EGFR primary antibody (200 μg/mL) overnight at 4°C followed by antimouse Cy3-conjugated secondary antibody (1:100, Invitrogen) with 4′,6-diamidino-2-phenylindole incubation for 1 h at room temperature. In place of incubation with primary antibody overnight at 4°C, negative controls were incubated with isotype-specific normal mouse serum (IgG2a, 200 μg/mL) followed by antimouse Cy3-conjugated secondary antibody (Invitrogen) with 4′,6-diamidino-2-phenylindole incubation for 1 h at room temperature. Slides were coverslipped with antifade fluorescent mounting medium.

Fluorescently labeled cells were imaged using a digital deconvolution microscopy imaging system attached to an Axioplan 2 EPI fluorescence upright microscope (Carl Zeiss). Cells were viewed under a 63× oil immersion lens (final magnification, ×630). The microscope is configured with different fluorescence cube sets, including, 4,6-diamidino-2-phenylindole, Cy3, FITC, and color bright-field microscopy. The microscope, stage, filter wheels, and camera are controlled through Slidebook (version 4.1.0.11), a software interface (Intelligent Imaging Innovations). Images were digitally captured with a Cooke Sensicam QE high-resolution (1,376 × 1,024 resolution; Cooke Corporation), black and white, supercooled, charge-coupled device camera and assigned colors by the software interface.

Statistical analysis

Results of densitometry analyses are shown as a percentage of vehicle treatment group. Data were analyzed by one-way ANOVA using a Newman-Keuls multiple comparison test, and statistical significance was accepted at $P < 0.05$ (GraphPad Prism 4.0, GraphPad Software, Inc.).

Results

Inhibition of lysosomal and proteosomal activity blocks EGFR downregulation

HT29 colon cancer cells were used in all experiments except where otherwise noted. The HT29 cell line was selected for these experiments because we have previously described the ability of sulindac metabolites to inhibit EGFR signaling and expression in this line (36) with confirmation in HCT116 and Caco-2 human colon cancer cell lines (36). To confirm downregulation and degradation of EGFR on incubation with EGF in our cell system, HT29 cells were pretreated with or without the lysosomal inhibitor chloroquine for 1 hour followed by incubation with EGF for 1 hour. EGF treatment significantly decreased EGFR expression (Fig. 1A and B). Although chloroquine treatment alone decreased the expression of EGFR, it also

prevented the ability of EGF to downregulate EGFR (Fig. 1A and B).

To determine if sulindac metabolite–induced EGFR downregulation was also due to proteosomal and/or lysosomal degradation, HT29 cells were pretreated (1 hour) with the lysosomal inhibitor chloroquine or the proteosomal inhibitor MG132, followed by treatment with vehicle, sulindac sulfide, or sulindac sulfone for 4 hours. The 25 μmol/L concentration of MG132 was selected because this dose was previously shown to be sufficient to increase c-Jun NH_2-terminal kinase phosphorylation (37), consistent with inhibition of proteasome activity, as previously reported (39). Sulindac sulfide and sulindac sulfone both downregulated total EGFR (Figs. 1C and D and 2A and B). However, in the presence of chloroquine (Fig. 1C and D) or MG132 (Fig. 2A and B), sulindac metabolites were no longer able to downregulate EGFR. Similar results were obtained in Caco-2 (Supplementary Fig. S1) and SW480 cell lines (Supplementary Fig. S2). As has previously been shown (36), evidence of apoptosis was determined by the presence of caspase-3 cleavage products (first detected at 24 hours) and confirmed by examination of nuclear morphology after staining with acridine orange and ethidium bromide (apparent at 48 hours, the earliest time point examined in this manner; data not shown). We have previously shown that pretreatment of HCT116 and SW480 cells with MG132 did not affect cleavage of caspase-3 by sulindac metabolites (37). Similar experiments done in HT29 cells were not interpretable because pretreatment with either chloroquine or MG132 alone caused antiproliferative (as measured by MTS) and apoptotic (as measured by caspase-3 cleavage and examination of nuclear morphology) effects that could not be separated from the effects of sulindac metabolites.

EGFR is ubiquitinated following sulindac sulfide treatment

Degradation of EGFR is thought to be initiated by phosphorylation of tyrosine 1045, leading to binding of Cbl and ubiquitination of the receptor that targets the protein for proteosomal and lysosomal degradation (35, 40, 41). To determine if sulindac metabolites increase EGFR ubiquitination, HT29 cells were treated with sulindac metabolites and EGFR was immunoprecipitated from the cell lysates. The immunoprecipitates were analyzed by immunoblotting with a polyclonal anti-ubiquitin (Fig. 3A and B, top) or anti-EGFR antibody (Fig. 3A and B, bottom). Sulindac sulfide and sulfone treatment increased the appearance of a broad smear of anti-ubiquitin immunoreactivity in the EGFR immunoprecipitates as early as 12 and 48 hours, respectively, after treatment, indicative of polyubiquitination of EGFR (Fig. 3A and B, top). This pattern of EGFR ubiquitination is similar to that previously reported in the literature (42, 43). Sulindac metabolites significantly decreased the expression of EGFR (Fig. 3A and B, bottom); therefore, the amount of ubiquitination was corrected for total EGFR in the immunoprecipitate (Fig. 3C and D). These analyses showed more than 3- and 10-fold increases in ubiquitination of EGFR by sulindac sulfide and sulfone, respectively (Fig. 3C and D).

Phosphorylation of EGFR tyrosine 1068 is inhibited but phosphorylation of tyrosine 1045 is activated by sulindac metabolites

EGF-induced signaling and degradation are both thought to be initiated by phosphorylation events. Signaling through ERK1/2 is mediated by phosphorylation of tyrosine 1068 (pY1068) of EGFR, whereas degradation of the receptor is thought to be initiated by phosphorylation of tyrosine 1045 (pY1045). Phosphorylation of pY1045 leads to binding of Cbl and ubiquitination of the receptor that targets the protein for proteosomal and lysosomal degradation (35, 40, 41). To examine the effects of sulindac metabolites on phosphorylation of EGFR tyrosine 1045, Caco-2 human colon cancer cells were used because they express detectable levels of EGFR pY1045 compared with HT29 cells and have been observed to undergo downregulation of EGFR in a manner similar to HT29 cells (Supplementary Fig. S3). Caco-2 cells were treated with sulindac metabolites for 1 or 48 hours, and lysates assayed by immunoblotting to determine the phosphorylation status of EGFR pY1045. At 1 hour after treatment, total EGFR is moderately but significantly decreased by sulindac sulfide (Fig. 4A and B). At this same time point, in contrast to the inhibitory effect of these drugs on EGFR phosphorylation at tyrosine 1068 [Fig. 4A and C and Supplementary Figs. S1A and S1C (4 hours)], sulindac sulfide led to a significant increase (1.6-fold) in the relative amount of total receptor phosphorylated at tyrosine 1045 (and before substantial downregulation of total receptor levels; Fig. 4A and D), and this effect persisted through 48 hours. By 48 hours after treatment with sulfide, total EGFR was significantly decreased (Fig. 4A and B), inhibition of phosphorylation at tyrosine 1068 intensified (Fig. 4A and C), and the relative amount of total receptor phosphorylated at tyrosine 1045 remained significantly increased (Fig. 4A and D). The effect of sulindac sulfone on EGFR phosphorylation at tyrosine 1068 and tyrosine 1045 was similar to that seen with sulfide (Supplementary Fig. S4).

Cellular localization of EGFR on incubation with EGF

The effects of sulindac metabolites on the cellular distribution of EGFR and on the processing of EGF ligand were determined by fluorescence microscopy using Cy3-labeled anti-EGFR (red) and Alexa Fluor 488–labeled EGF (green). EGFR was mainly localized as punctuate intracellular clusters on or near the plasma membrane and/or diffusely through the cytoplasm in vehicle/control cells (Fig. 5A-b); this pattern of staining is similar to that previously reported for human colon carcinoma cells in the literature (23). On incubation with sulindac sulfide (Fig. 5A-d) or sulindac sulfone (Fig. 5A-f; Supplementary Fig. S5) for 24 hours, total immunoreactive EGFR decreased dramatically, confirming our immunoblotting results. Treatment with Alexa Fluor–labeled EGF decreased EGFR at 10 minutes

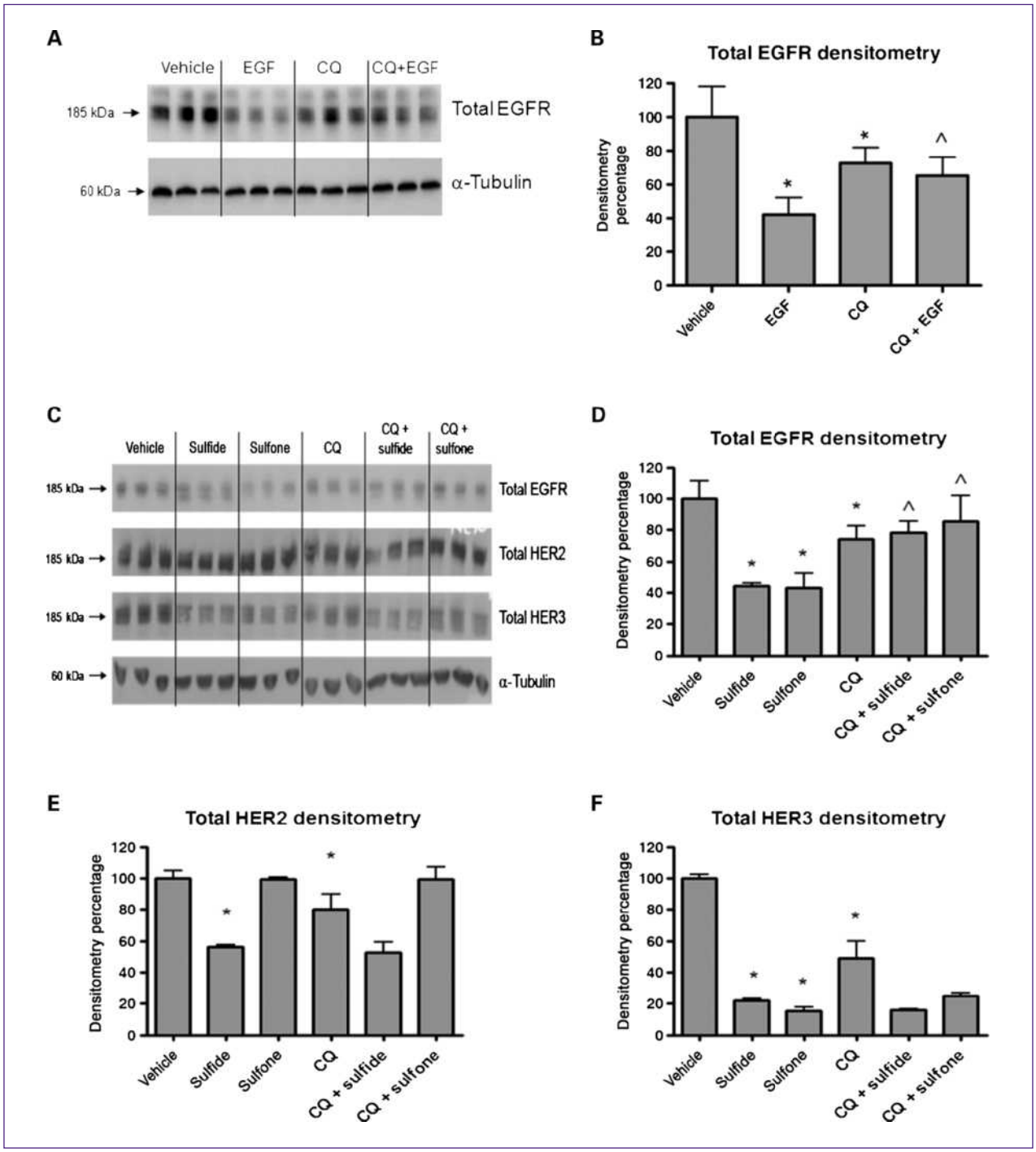

Fig. 1. Effects of the lysosomal inhibitor chloroquine on EGF- and sulindac metabolite–induced downregulation of EGFR, HER2, and HER3. A and B, HT29 cells were grown to 80% confluence in RPMI containing 10% FBS and then serum deprived for 48 h, followed by pretreatment with or without 100 μmol/L chloroquine (CQ) for 1 h. Cells were then treated with vehicle (water) or 10 ng/mL EGF for 1 h. Lysates were prepared and immunoblots performed with antibody raised against total EGFR; α-tubulin immunoblots of the same lysates served as loading controls. Immunoblots (A) and densitometry (B) of total EGFR bands. C to F, HT29 cells were grown to confluence in medium containing 10% FBS, followed by pretreatment with or without 100 μmol/L chloroquine for 1 h. Cells were then treated with vehicle (0.2% DMSO), 180 μmol/L sulindac sulfide (sulfide), or 600 μmol/L sulindac sulfone (sulfone) for 4 h. Lysates were prepared and immunoblots done with antibodies raised against total EGFR, total HER2, and total HER3; α-tubulin immunoblots of the same lysates served as loading controls. Immunoblots (C) and densitometry of total EGFR (D), total HER2 (E), and total HER3 (F) bands. B, D, E, and F, columns, mean of three independent samples; bars, SD. *, statistical significance between vehicle and single treatment groups (EGF, sulfide, sulfone, or chloroquine); ^, statistical significance between EGF, sulfide, or sulfone alone and combination treatment. Representative results of two independent experiments, each with triplicate samples.

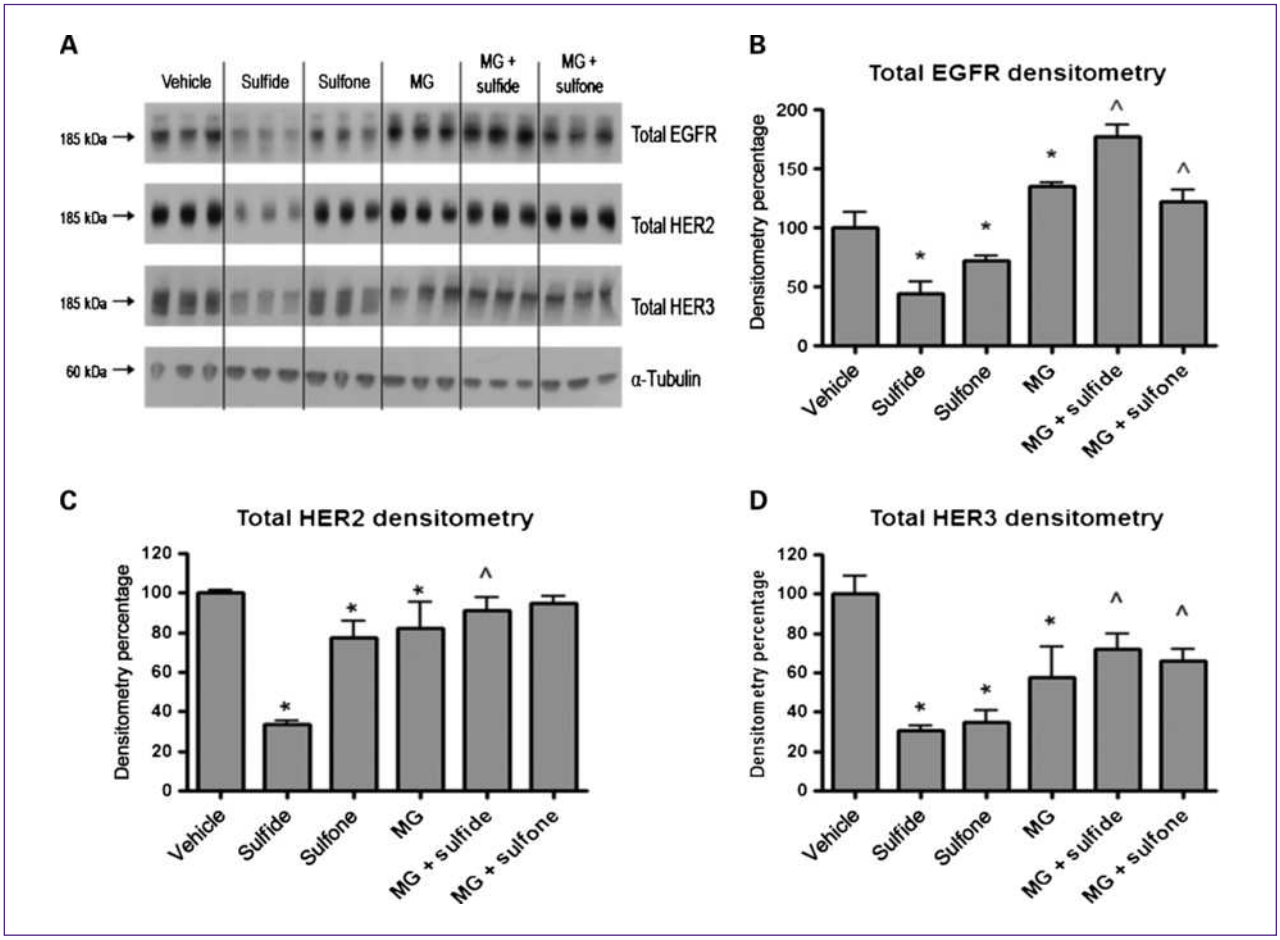

Fig. 2. Effects of the proteosomal inhibitor MG132 on sulindac metabolite–induced downregulation of EGFR, HER2, and HER3. A to D, HT29 cells were grown to confluence in medium containing 10% FBS, followed by pretreatment with or without 25 μmol/L MG132 (MG) for 1 h. Cells were then treated with vehicle (0.2% DMSO), 180 μmol/L sulindac sulfide, or 600 μmol/L sulindac sulfone for 4 h. Lysates were prepared and immunoblots performed with antibodies raised against total EGFR, total HER2, and total HER3; α-tubulin immunoblots of the same lysates served as loading controls. Immunoblots (A) and densitometry of total EGFR (B), total HER2 (C), and total HER3 (D) bands. B to D, columns, mean of three independent samples; bars, SD. *, statistical significance between vehicle and single treatment groups (sulfide, sulfone, or MG); ^, statistical significance between sulfide or sulfone alone and combination treatment. Representative results of two independent experiments, each with triplicate samples.

(compare Fig. 5A-b to B-b) and seemed to lead to a redistribution of EGFR (Fig. 5B-b and f) and EGF (Fig. 5B-c and g) from a small punctuate pattern to larger intracytoplasmic vesicular structures where EGF-EGFR colocalization (yellow) was observed (Fig. 5B-h). This translocation of EGF/EGFR complexes was essentially complete by 60 minutes after EGF treatment. The cellular distribution of EGF over time seemed to be similar between control cells (EGF treated) and those pretreated with 600 μmol/L sulindac sulfone (compare Fig. 5B-c and g to C-c and g). Colocalization of EGF-EGFR complexes was also visible in samples pretreated with sulindac sulfone for 60 minutes (Fig. 5C-h). The immunofluorescent studies suggest that the downregulation of EGFR by sulindac sulfone may involve an intracellular pathway similar to that induced by EGF-mediated receptor degradation. Similar experiments with sulindac sulfide were not interpretable because the drug decreased EGFR levels to the point where neither immunoreactive EGFR nor EGF binding could be detected. In addition, fewer cells remained attached to the tissue culture slides in sulfide- versus sulfone-treated cells, perhaps due to the repeated washings required for this assay and/or possible effects of sulfide on cell adhesion.

HER2 and HER3 are downregulated by sulindac metabolites

To determine the effects of sulindac metabolites on the protein levels and phosphorylation status of other ErbB family members that are known to dimerize with EGFR, HT29 cells were treated with sulindac metabolites for 4 and 12 hours and immunoblotting was done using antibodies to HER2 and HER3. Sulindac sulfide downregulated both total and phosphorylated forms of HER2 and HER3 (Fig. 6A-E) in a manner similar to its effects on EGFR. Treatment with sulindac sulfide was shown

to downregulate total HER2 and total HER3 by 40% to 50% and to downregulate pHER2 and pHER3 by 90%. Downregulation of both HER2 and HER3 persisted through at least 48 hours (data not shown). Similar results were seen with sulindac sulfone on HER2 and HER3 (Supplementary Fig. S6). To exclude the possibility that downregulation of HER2 and HER3 by sulindac metabolites was unique to HT29 cells, similar experiments were carried out in Caco-2 (Supplementary Fig. S7) and SW480 (data not shown) human colon cancer cells. Sulindac sulfide and sulfone downregulated the expression of both total and phosphorylated HER2 and HER3 in these cell lines as well.

Similar to its effects on EGFR, treatment with MG132 prevented sulindac metabolite–induced downregulation of total HER2 and HER3 (Fig. 2A, C, and D). However, the lysosomal inhibitors chloroquine (Fig. 1C, E, and F) and leupeptin (data not shown) failed to block sulindac metabolite–induced downregulation of total HER2 and total HER3 at any time point examined.

Discussion

Our laboratory previously reported that sulindac metabolites downregulate EGFR and inhibit its signaling through the ERK1/2 pathway (36). We now report that EGFR downregulation by these drugs is dependent on proteosomal and/or lysosomal degradation of the receptor in a manner similar to EGFR degradation mediated by the ligand EGF. The current results also show that sulindac metabolites are broadly effective in downregulating multiple members of the ErbB family and that degradation of these receptors seems to be mediated through both overlapping (proteosomal) and distinct (lysosomal) mechanisms.

Several studies have shown that degradation of EGFR occurs rapidly after exposure to EGF and that EGF-induced receptor downregulation is dependent on both lysosomal and proteosomal proteases (35, 42–45). The finding that chloroquine prevented sulindac-induced downregulation of EGFR indicates that the sulindac metabolite–induced downregulation of EGFR is also due to lysosomal/proteolytic degradation. In this regard, drug-induced degradation is similar to that induced by EGF in that both are prevented by lysosomal inhibition.

The importance of proteosomal activity for EGF- and sulindac metabolite–induced degradation of EGFR was suggested by the finding that MG132 inhibited degradation of EGFR induced by these compounds. MG132 is a low molecular weight molecule that is among the most widely used peptide aldehyde inhibitors of the proteasome. Thus, our results showing that MG132 blocked both EGF- (data not shown) and sulindac metabolite–induced degradation of EGFR suggests that the downregulation of EGFR by these compounds is due to degradation mediated by both the proteasome and lysosome. These findings agree with several previous studies where it was shown that MG132, chloroquine, and leupeptin all blocked EGF-induced EGFR degradation (44, 46, 47). However, it is also possible that EGFR is degraded by two independent catalytic pathways within the lysosome and proteasome or that another protein whose expression is regulated by the lysosome or proteasome is responsible for EGFR degradation by sulindac. Taken together, our results support the belief that both lysosomal and proteosomal activity may be important for degradation of EGFR by sulindac metabolites.

To place our biochemical results of EGFR downregulation in a morphologic context, we examined EGF-EGFR

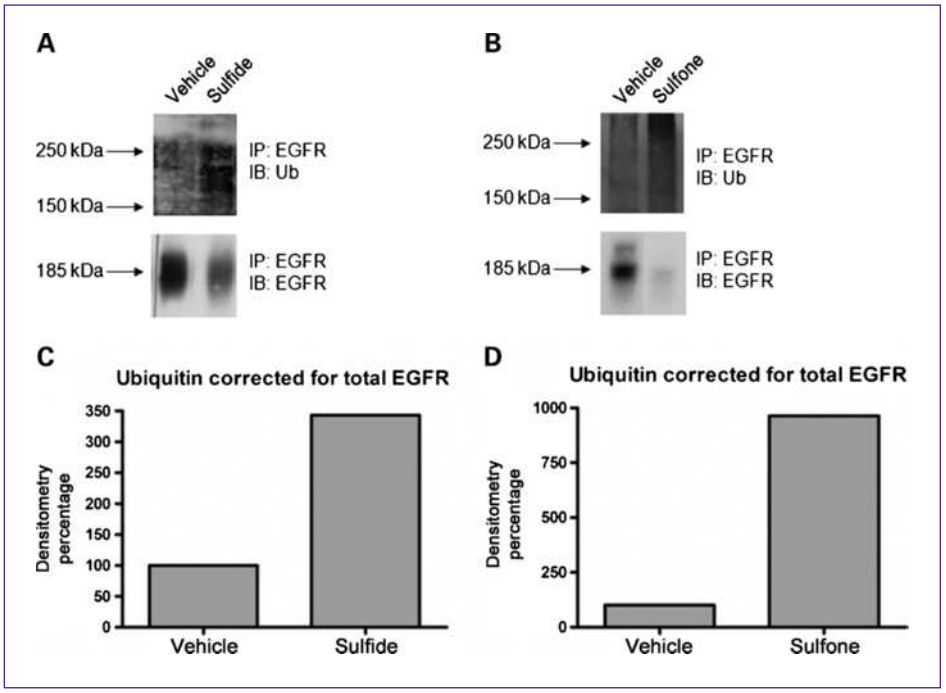

Fig. 3. EGFR is ubiquitinated on sulindac metabolite treatment. HT29 cells were grown to confluence in medium containing 10% FBS and treated with vehicle (0.2% DMSO), 160 μmol/L sulindac sulfide for 12 h (A and C), or 600 μmol/L sulindac sulfone for 48 h (B and D). Cells were then harvested and immunoprecipitated (IP) for EGFR. The immunoprecipitated protein was immunoblotted (IB) with an anti–poly-ubiquitin antibody (Ub) or anti–poly-EGFR antibody. Immunoblots (A and B) and densitometry (C and D) of the ubiquitin bands presented as an amount relative to total EGFR levels. Data represent single sample values. Representative results of two independent experiments.

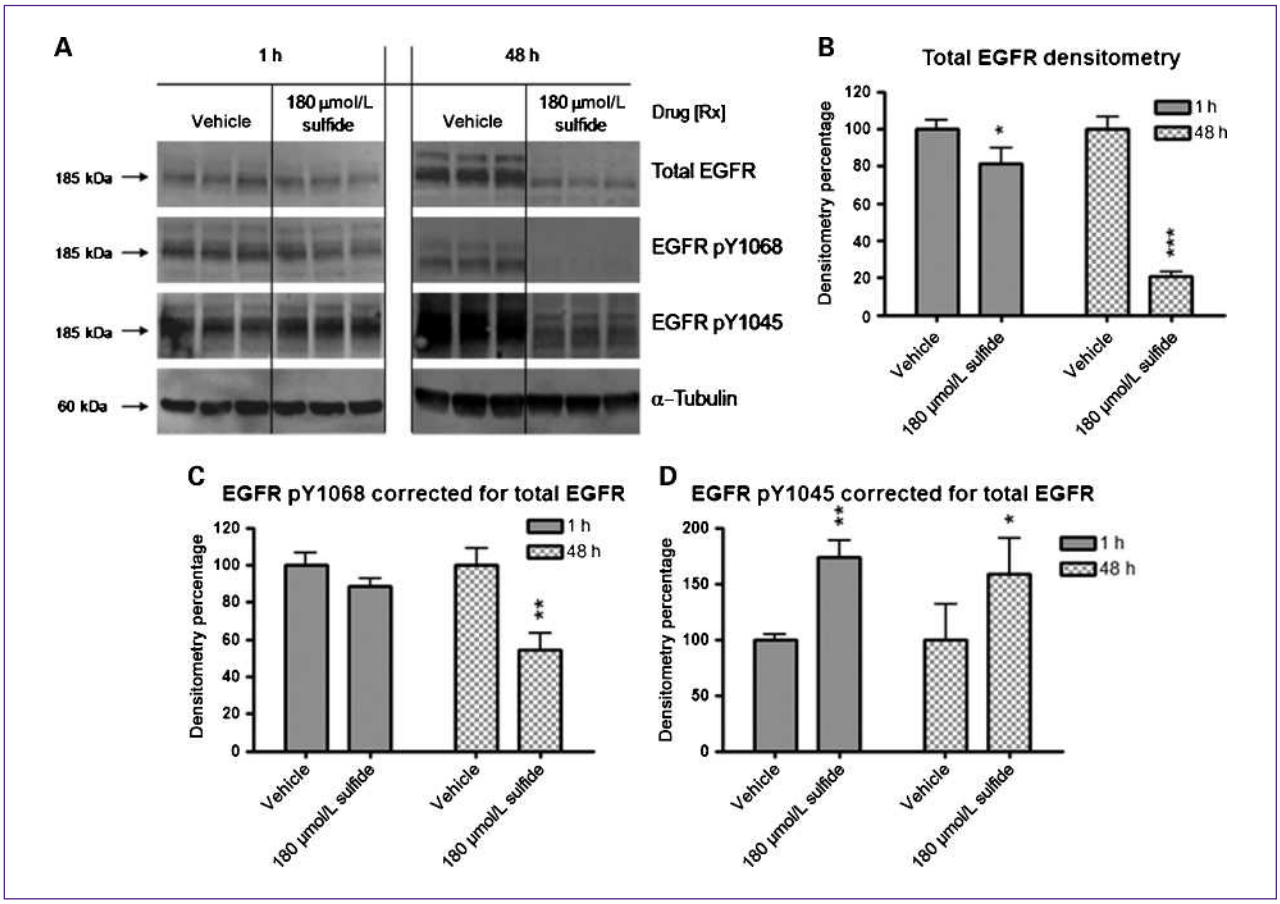

Fig. 4. Tyrosine 1045 of EGFR is activated by sulindac sulfide. Caco-2 cells were grown to confluence in medium containing 10% FBS, followed by treatment with vehicle (0.2% DMSO) or 180 μmol/L sulindac sulfide for 1 and 48 h. Cells were harvested and immunoblots done on cell lysates with antibodies raised against total EGFR, pEGFR (pY1068), and pEGFR (pY1045); α-tubulin immunoblots of the same lysates served as loading controls. Immunoblots (A) and densitometry of total EGFR bands (B), pEGFR (pY1068) bands presented as an amount relative to total EGFR levels (C), and pEGFR (pY1045) bands presented as an amount relative to total EGFR levels (D). Columns, mean of three independent samples; bars, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus respective time point vehicle. Representative results of two independent experiments, each with triplicate samples.

localization by immunofluorescence. EGF, sulindac sulfide, and sulindac sulfone all decreased total immunoreactive EGFR. The intracellular trafficking of EGF from the cell surface and small cytoplasmic vesicles to large intracellular vesicles and the intracellular association of EGF with EGFR did not seem to change in the presence of sulindac sulfone as compared with control (EGF)-treated cells. Sulindac sulfide decreased EGFR levels so dramatically that it did not allow detectable association of EGF with the EGFR. These results confirm that sulindac metabolites downregulate EGFR and suggest that the downregulation of EGFR is via a degradation pathway that is morphologically similar to that of EGF-induced downregulation and degradation.

Although our results suggest that sulindac metabolites downregulate EGFR via a degradation pathway that is biochemically and morphologically similar to that of EGF-induced downregulation and degradation, a substantial temporal discrepancy does exist between sulindac metabolite– and EGF-induced downregulation and degradation. EGF-induced downregulation of EGFR occurs as early as 10 minutes after treatment, lasting through 60 minutes and is no longer present at 24 hours. Sulindac metabolite–induced downregulation of EGFR occurs as early as 1 to 12 hours after treatment (1 hour is the earliest time point examined with the sulindac metabolites) and persists through at least 72 hours. The mechanism by which EGF binds to EGFR and stimulates internalization and degradation of the receptor is relatively well characterized (33–35). However, much less is known about the mechanism by which sulindac metabolites induce EGFR degradation. Sulindac may bind to EGFR, but with slower kinetics than EGF. Alternatively, sulindac metabolites may affect some other cellular processes that eventually affect EGFR internalization and/or ubiquitination, possibly through pY1045 or a ubiquitin ligase (35, 48). Numerous possible mechanisms for such an indirect effect could be postulated, including sulindac metabolites interfering with ligand binding and/or activating proteosomal mechanisms that eventually degrade the receptor, albeit somewhat less efficiently than EGF. These possibilities

could explain qualitative and quantitative differences between EGFR downregulation and degradation by sulindac metabolites compared with EGF.

The finding that sulindac metabolites increase the ubiquitination of EGFR provides further support for the hypothesis that the drugs increase EGFR degradation through the ubiquitin/proteasome/lysosome pathway. Additional indirect evidence to support ubiquitination was seen in the immunoblot analysis of nonimmunoprecipitated lysates (ref. 36; Figs. 1C and 2A). A variable increase in the molecular weight of EGFR, seen as an "up-smearing" on immunoblots, is indicative of ubiquitination (35, 44).

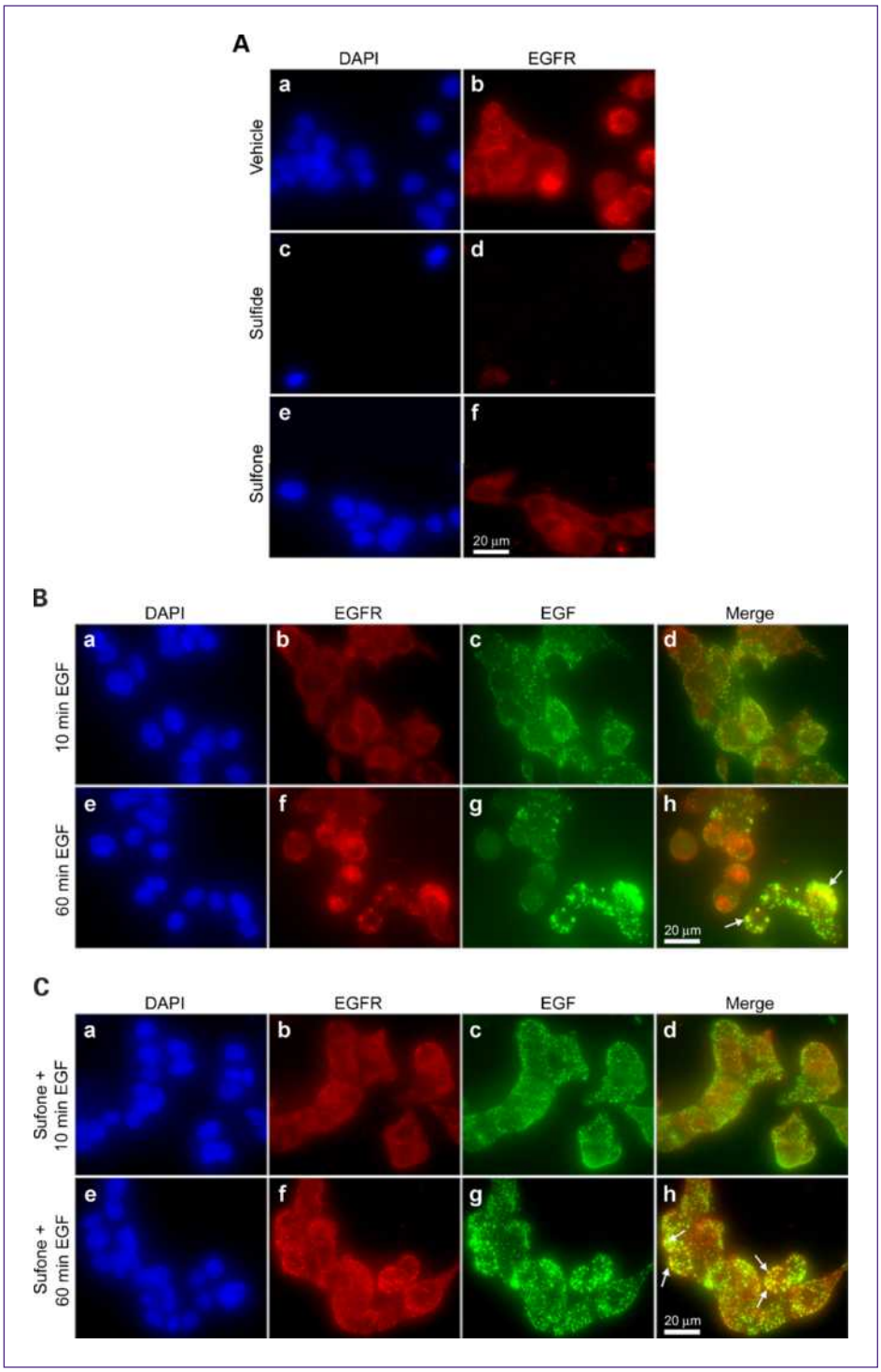

Fig. 5. Immunofluorescence analysis of EGFR localization in control and sulindac metabolite–treated cells on incubation with and without EGF. Cells were grown as described in Materials and Methods. A, HT29 cells were grown to 50% to 60% confluence and then serum deprived for 48 h, followed by pretreatment with vehicle (0.2% DMSO; a and b), 120 μmol/L sulindac sulfide (c and d), or 600 μmol/L sulindac sulfone (e and f) for 24 h. B, HT29 cells were grown to 50% to 60% confluence and then serum deprived for 48 h, followed by pretreatment with vehicle (0.2% DMSO) for 24 h. Cells were then treated with 500 ng/mL Alexa Fluor 488–labeled EGF (green) for 10 min (a-d) and 60 min (e-h) at 37°C. C, HT29 cells were grown to 50% to 60% confluence and then serum deprived for 48 h, followed by pretreatment with 600 μmol/L sulindac sulfone for 24 h. Cells were then treated with 500 ng/mL Alexa Fluor 488–labeled EGF (green) for 10 min (a-d) and 60 min (e-h) at 37°C. A to C, the cells were processed and immunostained as described in Materials and Methods using mouse anti-EGFR followed by Cy3-conjugated goat anti-mouse (red) with 4′,6-diamidino-2-phenylindole (DAPI; blue); colocalization of Alexa Fluor 488–labeled EGF and EGFR appears yellow (arrows in B-h and C-h). Representative results of two independent experiments.

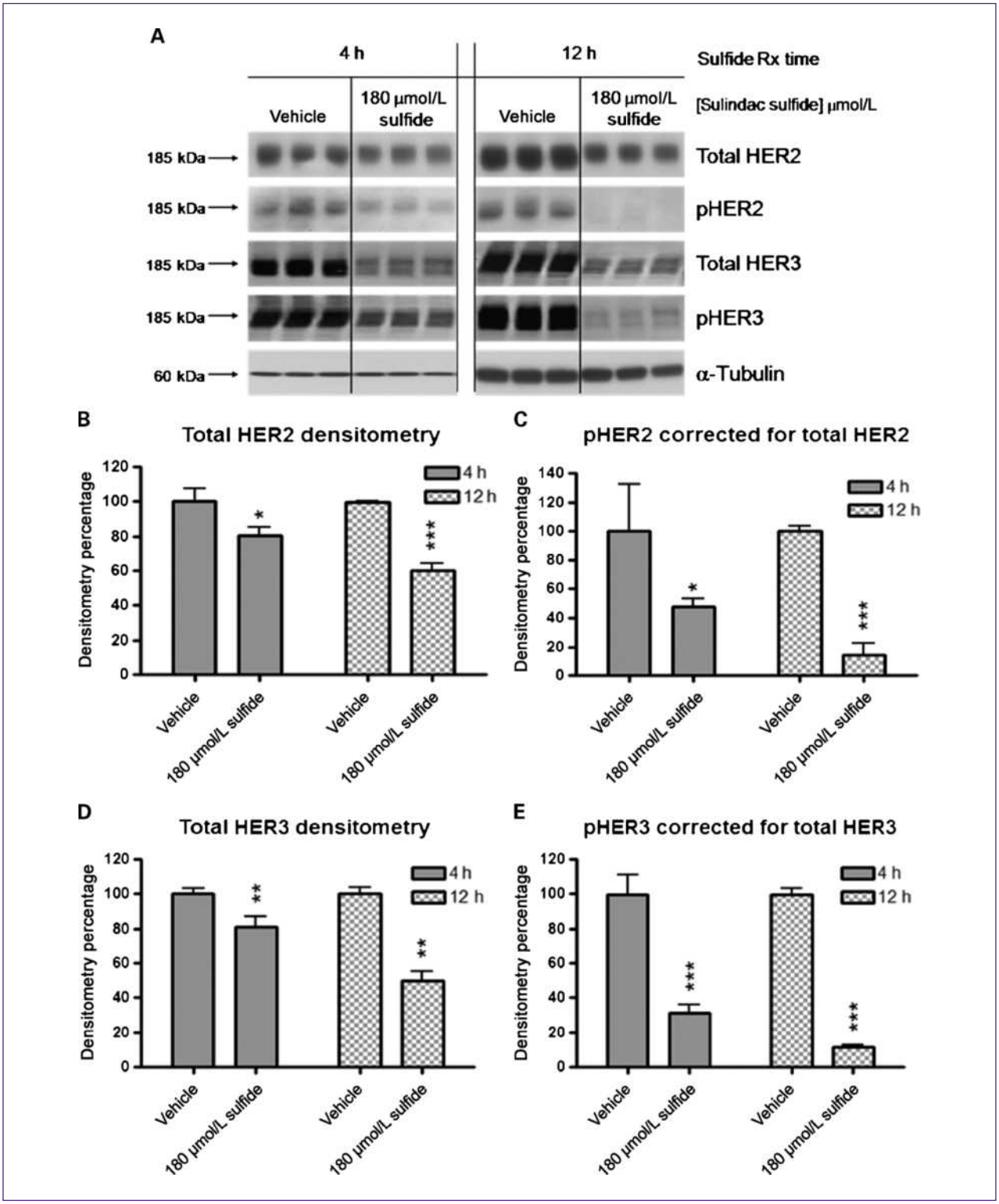

Fig. 6. HER2 and HER3 are downregulated by sulindac sulfide. HT29 cells were grown to confluence in medium containing 10% FBS, followed by treatment with vehicle (0.2% DMSO) or 180 μmol/L sulindac sulfide for 4 and 12 h. Cells were harvested and immunoblots performed on cell lysates with antibodies raised against total HER2, pHER2, total HER3, and pHER3; α-tubulin immunoblots of the same lysates served as loading controls. Immunoblots (A) and densitometry of total HER2 bands (B), pHER2 bands presented as an amount relative to total HER2 levels (C), total HER3 bands (D), and pHER3 bands presented as an amount relative to total HER3 levels (E). Columns, mean of three independent samples; bars, SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, versus respective time point vehicle. Representative results of two independent experiments, each with triplicate samples.

Conversely, it has been proposed that a variable decrease in the molecular weight of EGFR represents intermediate proteolytic EGFR products (35, 42, 44). Treatment of HT29 cells with sulindac metabolites led to an up-smearing of phosphorylated EGFR and a down-smearing of total EGFR (ref. 36; Figs. 1C and 2A), serving as an indirect indicator that the metabolites are increasing both receptor ubiquitination and proteolytic processing. Additional studies directly examining the effects of sulindac metabolites on EGFR association with Cbl, Grb2, LRIG1, SOCS4, and SOCS5 would further clarify the mechanism(s) by which sulindac metabolites increase lysosomal- and/or proteosomal-mediated degradation of EGFR.

Because EGFR downregulation by EGF and sulindac metabolites was similarly inhibited by lysosomal and proteosomal inhibitors, and similar alterations in the cellular distribution of EGFR and fluorescent EGF with these agents were observed, we assessed the effect of sulindac metabolites on earlier events in the known EGF-induced degradation pathway. EGF-induced tyrosine phosphorylation at pY1045 has been reported to be essential for stable association of EGFR with Cbl, which then leads to ubiquitin binding and targeting of the receptor for degradation. Our results indicate that phosphorylation of EGFR at sites such as pY1068, which are important in downstream signaling, were decreased or not affected by sulindac metabolites when assessed as total or as a proportion of the total EGFR present that was phosphorylated at that site. Thus, decreased EGFR phosphorylation by sulindac metabolites is an independent effect that is not due to reduced EGFR protein levels. In contrast, there was a significant increase in the proportion of EGFR that was phosphorylated at pY1045 with sulindac treatment at time points that preceded or were concurrent with major downregulation of total receptor. These data suggest that sulindac metabolites may have differential effects on EGFR phosphorylation sites and that induction of pY1045 could be promoting the critical association of receptor and Cbl required for EGFR ubiquitination and degradation.

Examination of other ErbB family members known to dimerize with EGFR showed that sulindac metabolites inhibited the phosphorylation of HER2 and HER3 and downregulated the expression of total HER2 and total HER3 in a manner that is similar to that seen with EGFR. Conversely, examination of HER2 and HER3 degradation showed that sulindac-mediated degradation is not the same for all ErbB receptors. It is known that all members of the ErbB receptor family display significant homology, but each of these receptors also has distinct properties such as ligand binding and receptor trafficking (49, 50); the EGFR is rapidly internalized and degraded on ligand binding (51), whereas the other ErbB receptors do not display significant ligand-induced internalization and/or recycle rapidly back to the cell surface after endocytosis (49, 52–54). Previous studies have established that HER2 degradation is mediated by proteosomal proteolysis (55, 56). In agreement with these findings, sulindac metabolite–induced downregulation of HER2 and HER3 seemed to be mediated via proteosomal, but not lysosomal, degradation. These results indicate that sulindac metabolites are broadly effective in downregulating multiple members of the ErbB family and that degradation of these receptors seems to be mediated through both overlapping (proteosomal) and distinct (lysosomal) mechanisms.

In summary, we have described for the first time that sulindac metabolites downregulate EGFR expression via proteosomal and lysosomal degradation. We also provide evidence that these degradation pathways may be induced by pY1045 phosphorylation and ubiquitination of the receptor in a manner similar to that seen with EGF-induced downregulation of the receptor. Sulindac-induced downregulation of EGFR was shown to be shared by other members of the ErbB family. To our knowledge, this study provides the first evidence that a NSAID can induce degradation of a family of cell surface receptors. Together, these results define a novel mechanism by which NSAIDs may function as chemopreventive agents. In the future, we would like to test whether the induction of apoptosis by sulindac metabolites is due to inhibition of ErbB through overexpression and knockdown of ErbB family members. This additional knowledge of the biochemical and biological mechanisms responsible for the growth inhibitory effects of sulindac metabolites could lead to the development of more effective and safer chemopreventive and chemotherapeutic agents.

The Food and Drug Administration has thus far approved two anti-EGFR drugs for the treatment of colorectal cancer, cetuximab and panitumumab, thereby establishing EGFR as an important and clinically relevant therapeutic target in colorectal cancer. Additionally, work from several laboratories has shown substantial interactions between the biochemical effects of NSAIDs and EGFR signaling (7, 57–59), wherein combination therapy of NSAIDs and ErbB antagonists was shown to cooperatively inhibit the survival of colorectal cancer cells (57) and displayed an additive effect against colon tumor development *in vivo* (7). These observations support our results that suggest that combining sulindac with other EGFR antagonists in a cocktail could enhance anti-EGFR effects and lead to greater inhibition of ErbB family signaling, thereby increasing the chemopreventive and possibly chemotherapeutic power of sulindac.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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