Phospholipase C-γ inhibition prevents EGF protection of intestinal cytoskeleton and barrier against oxidants

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Banan, A., J. Z. Fields, Y. Zhang, and A. Keshavarzian. Phospholipase C-γ inhibition prevents EGF protection of intestinal cytoskeleton and barrier against oxidants. Am J Physiol Gastrointest Liver Physiol 281: G412–G423, 2001.—Loss of intestinal barrier integrity is associated with oxidative inflammatory GI disorders including inflammatory bowel disease. Using monolayers of human intestinal epithelial (Caco-2) cells, we recently reported that epidermal growth factor (EGF) protects barrier integrity against oxidants by stabilizing the microtubule cytoskeleton, but the mechanism downstream of the EGF receptor (EGFR) is not established. We hypothesized that phospholipase C (PLC)-γ is required. Caco-2 monolayers were exposed to oxidant (H₂O₂) with or without pretreatment with EGF or specific inhibitors of EGFR tyrosine kinase (AG-1478, tyrphostin 25) or of PLC (L-108, U-73122). Other Caco-2 cells were stably transfected with a dominant negative fragment for PLC-γ (PLCz) to inhibit PLC-γ activation. Doses of EGF that enhanced PLC activity also protected monolayers against oxidant-induced tubulin disassembly, disruption of the microtubule cytoskeleton, and barrier leakiness as assessed by radioimmunoassay, quantitative Western blots, high-resolution laser confocal microscopy, and fluorometry, respectively. Pretreatment with either type of inhibitor abolished EGF protection. Transfected cells also lost EGF protection and showed reduced PLC-γ phosphorylation and activity. We conclude that EGF protection requires PLC-γ signaling and that PLC-γ may be a useful therapeutic target.

phospholipase Cz transfection; tubulin; epidermal growth factor; barrier integrity; Caco-2 cells

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by the platelet-derived growth factor receptor and insulin-like growth factor-1 as well as by EGFR (19, 23, 39). It is also known that intestinal epithelial cells express the PLCγ isoform (48). In the current investigation, we sought to determine whether EGF-induced protection against oxidant injury to the microtubule cytoskeleton and intestinal epithelial barrier integrity occurs through activation of the PLCγ isoform. To this end, both pharmacological as well as targeted molecular biological techniques were used. The latter included the use of a dominant negative fragment of PLCγ, namely PLCz, which contains SH2, SH3, and PLC-gamma inhibitory domains, and which is known to specifically inhibit PLCγ activation but not other isoforms (23, 33, 56).

**MATERIALS AND METHODS**

_Cell culture._ Caco-2 cells, which were obtained from ATCC (Manassas, VA) at passage 15, were chosen because they form monolayers that morphologically resemble small intestinal cells, with defined apical brush borders, junctional complexes, and a highly organized microtubule network (11, 25, 44). Cells were maintained at 37°C in complete DMEM in an atmosphere of 5% CO2 with 100% relative humidity. Parental (wild type) cells and transfected cells (see Stable transfection of dominant negative PLCγ-1 in Caco-2 cells) were split at a ratio of 1:6 on reaching confluence and set up in M199, both pharmacological as well as targeted molecular biological techniques were used. The latter included the use of a dominant negative fragment of PLCγ, namely PLCz, which contains SH2, SH3, and PLC-gamma inhibitory domains, and which is known to specifically inhibit PLCγ activation but not other isoforms (23, 33, 56).

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**Stable transfection of dominant negative PLCγ-1 in Caco-2 cells.** The dominant negative PLCγ-1 fragment from the Z region (designated PLCz) was a generous gift from Dr. A. Wells (University of Pittsburgh, Pittsburgh, PA). The Z region of human PLCγ-1, which covers the SH2 and SH3 domains (amino acids 517–901), was isolated by RT-PCR and cloned into a eukaryotic expression vector, pXf (23, 56). Expression was controlled by the SV40 early promoter present in pXf vector. Cultures of Caco-2 cells grown to 50–60% confluence were cotransfected with G418 resistance plasmid and PLCz (pXf/PLCz) with LIPOFECTIN (GIBCO BRL). Control conditions included vector (pXf) alone. Stable transfected cells were selected in high-glucose DMEM-10% fetal bovine serum (FBS) supplemented with G418 (0.6 mg/ml). Stable expression of PLCz protein (~51 kDa) in these cells was demonstrated by Western blot analysis with monclonal anti-PLCγ-1. The PLCz-expressing cells were also tested for PLC activity (see Experimental design). Clones stably expressing PLCz were plated on Transwell cell culture inserts, allowed to form confluent monolayers, and used subsequently for experiments. In preliminary studies, we confirmed that PLCz expression did not injure the Caco-2 cell monolayer barrier and that it did not affect the expression levels of PLC γ.

**Experimental design.** In all experiments, barrier function, microtubule cytoskeletal integrity (intracellular architecture, tubulin assembly and disassembly), PLC activity, and PLCz protein expression were assessed. In the first series of experiments, postconfluent monolayers of wild-type Caco-2 cells were preincubated with EGF (1, 5, or 10 ng/ml) or isotonic saline for 10 min and then exposed to oxidant (0.5 mM H2O2) or vehicle (saline) for 30 min. These experiments were then repeated with cell monolayers stably expressing PLCz. Reagents, including inhibitors of EGFR-linked tyrosine kinase and of PLC, were applied on the apical side of monolayers unless otherwise indicated. The concentrations of oxidant or EGF used have been shown by our laboratory (5–8, 13, 14) to be effective at damaging and protecting, respectively, Caco-2 monolayers. Because our previous studies (6, 13) showed that the results were qualitatively similar regardless of whether apical or basolateral exposure of oxidants was used, all of the current studies used apical application.

In the second series of experiments, we further explored the possible importance of PLC signaling in EGF-mediated protection. For these studies, cells monolayers that were stably expressing PLCz were preincubated (10 min) with a high dose of EGF (10 ng/ml) or vehicle before the subsequent exposure (30 min) to damaging concentrations of oxidant (0.5 mM H2O2) or vehicle. In some experiments, expression of PLCz protein was determined by immunoblotting. Inhibition of PLC activity as well as the tyrosine phosphorylation of PLCγ-1 in PLCz-expressing cells was also assessed.

In the third series of experiments, we investigated the effects of PLCz expression on the state of both tubulin assembly and tubulin disassembly and on integrity of the microtubule cytoskeleton. Monomeric (S1) and polymerized (S2) fractions of tubulin (the structural subunit protein of microtubules) were isolated and then analyzed by quantitative immunoblotting (4, 11). In all experiments involving the microtubule cytoskeleton, microtubule integrity was assessed by 1) immunofluorescent labeling and fluorescence microscopy to determine the percentage of cells with normal microtubules, 2) detailed analysis by ultra high-resolution laser scanning confocal microscopy (LSCM), and 3) quantitative immunoblot analysis of monomeric and polymerized tubulin fractions.

In preliminary studies with two specific mitogen-activated protein (MAP) kinase (MAPK) inhibitors, PD-98059 (25 μM; inhibitor of MAPK kinase) and SB-203580 (10 μM; inhibitor of p38 MAPK) (23, 55), we confirmed that these inhibitors, alone or in combination, did not affect any measures of EGF-induced protection in our intestinal model.

**PLC activity in Caco-2 cells.** PLC activity was assayed by the formation of myo-[3H]inositol phosphates in cell monolayers as previously described (48, 56). Cells were labeled in serum-free medium containing 5 μCi/ml of myo-[3H]inositol for 24 h and subsequently washed three times to remove unincorporated label. LiCl (10 mM), which inhibits inositol phosphate hydrolysis by inositol phosphatases, was added 15 min before the addition of EGF and/or other treatments. Cells were then washed thrice with ice-cold PBS and lysed with 10% TCA. Cell lysates were collected, and the myo-[3H]inositol phosphates were recovered in the supernatant after centrifugation (16,000 g for 5 min). The extracts were separated on Dowex formate ion-exchange mini-columns (Bio-Rad, Hercules, CA). The radioactivity present (inositol phosphate content) in samples was quantified by scintillation counting with aqueous counting scintillant. Counts for blanks were subtracted from the sample activity. PLC activity was reported as counts per minute (cpm) per 10⁶ cells.

**Western immunoblotting of PLCz protein expression.** Differentiated cell monolayers grown in 75-cm² flasks were scraped and ultrasonically homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1%
Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, and 2 μg/ml each of aprotinin, pepstatin, leupeptin, and phenylmethylsulfonyl fluoride). The samples were then centrifuged (100,000 g for 40 min at 4°C), and the supernatant was used for bulk protein determination as assessed by the Bradford method (20). For immunoblotting, samples (100 μg protein/lane) were added to SDS buffer (250 mM Tris-HCl, pH 6.8, 2% glycerol, and 5% mercaptoethanol), boiled for 5 min, and separated on 7.5% SDS-polyacrylamide gels (7, 56). Subsequently, proteins were transferred to nitrocellulose membranes (0.2-μm pore size), blocked in 3% BSA for 1 h, and washed several times with Tris-buffered saline. The immobilblotted proteins were incubated for 2 h in Tween 20, Tris-buffered saline, 1% BSA, and the primary mouse monoclonal anti-PLC-γ1 (an antibody that recognizes PLCz; Transduction Laboratories and Santa Cruz Biotechnology) at a 1:2,000 dilution for 1 h at room temperature. A horseradish peroxidase-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) was used as a secondary antibody at 1:4,000 dilution. Proteins on membranes were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) and autoradiography. The identity of the PLCz band (~51 kDa) was ascertained with the use of a monoclonal antibody that recognizes PLCz. Additionally, in the absence of the primary antibody to PLCz, no corresponding band for PLCz was observed. Furthermore, prestained molecular weights (Mr 34,900 and 53,900) were run in adjacent lanes.

Immunoprecipitation and Western blot analysis of EGFR and PLC-γ1 phosphorylation. After treatments, confluent cell monolayers were lysed by incubation for 20 min in 500 μl of cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 μg/ml of anti-protease cocktail, 10% glycerol, 1 mM sodium orthovanadate, 5 mM NaF, and 1% Triton X-100). The lysates were clarified by centrifugation at 14,000 g for 10 min at 4°C. For immunoprecipitation (48), the lysates were incubated for 90 min at 4°C with monoclonal anti-EGFR (1:1,000 dilution, in excess) or monoclonal anti-PLC-γ1 antibody (1:500 dilution, in excess). The extracts were then incubated with protein A Sepharose for 1 h at 4°C. The immunocomplexes were collected by centrifugation (2,500 g for 5 min) in a microfuge tube and washed three times with immunoprecipitation buffer containing 5 mM Tris-HCl, pH 7.4, and 0.2% Triton X-100. The resultant pellets were resuspended in a standard SDS sample buffer and boiled at 95°C for 5 min before separation by 7.5% PAGE. Prestained molecular weights (Mr 34,900 and 205,000) were also run. Gels were transferred to nitrocellulose membranes, blocked with 1% BSA and 0.01% Tween 20 in PBS for blotting by monoclonal anti-phosphotyrosine (1:5,000 dilution; Transduction Labs) and for detection of immune complexes by horseradish peroxidase-conjugated secondary antibody, incubated with chemiluminescence reagents, and autoradiographed.

Immunofluorescent staining and high-resolution LSCM of microtubules. Cell monolayers were fixed in cytoskeletal stabilization buffer and then postfixed in 95% ethanol at −20°C as we previously described (4, 5, 11). Cells were subsequently processed for incubation with a primary antibody, monoclonal mouse anti-β-tubulin (IgG1, rat/human reactive; Sigma, St. Louis, MO) at a 1:200 dilution for 1 h at 37°C. Slides were washed three times in Dulbecco’s PBS (D-PBS) and then incubated with a secondary antibody (FITC-conjugated goat anti-mouse; Sigma) at a 1:50 dilution for 1 h at room temperature. Slides were washed thrice in D-PBS and once with deionized H2O and subsequently mounted in Aquamount (Fisher). All antibodies were diluted with D-PBS containing 0.1% BSA. After staining, cells were observed with an argon laser (λ = 488 nm) equipped with a ×63 oil immersion plan-apochromat objective (NA 1.4, Zeiss). Single cells and/or a clump of two to three cells from desired areas of the monolayers were processed with the image processing software on a Zeiss ultra high-resolution laser scanning confocal microscope to create “near black” areas surrounding the cells. The cytoskeletal elements were examined in a blinded fashion for their overall morphology, orientation, and disruption as we previously described (5, 6, 11). Briefly, 200 cells/slide (culture well) were examined in four different fields by LSCM, and the percentage of cells displaying normal microtubules was determined. The microtubule cytoskeleton in Caco-2 cells was considered not normal on the basis of one or more of the following criteria: collapse, fragmentation, kinking, or disruption of the microtubule organizer center. Slides were examined in a blinded fashion to avoid bias. We coded them in such a way that the examiner had no knowledge of the experimental protocol. The slides were decoded only after examination was complete.

Microtubule (tubulin) fractionation and quantitative immunoblotting of tubulin. Polymerized (S2) and monomeric (S1) fractions of tubulin were isolated as we previously described (5, 11). Fractionated S1 and S2 samples were flash-frozen in liquid N2 and then stored at −70°C until being immunoblotted. For immunoblotting, samples (5 μg protein/lane) were placed in a standard SDS sample buffer, boiled for 5 min, and then subjected to PAGE on 7.5% gels. Procedures for Western blotting were performed as previously described (5, 6). To quantify the relative levels of tubulin, the optical density of the bands corresponding to immunodetected tubulin was measured with a laser densitometer.

Determination of barrier function by fluorometry. Barrier integrity was determined by measuring apical-to-basolateral flux of a membrane-impermeable fluorescent marker, fluorescein sulfonylic acid (FSA; 200 μg/ml; 478 Da) as we previously described (4, 5, 7). Briefly, fresh phenol-free DMEM (800 μl) was placed in the lower (basolateral) chamber, and phenol-free DMEM (300 μl) containing FSA was placed in the upper (apical) chamber. Aliquots (50 μl) were obtained from the upper and lower chambers at time 0 and at various subsequent time points and transferred to clear 96-well plates (clear bottom; Costar, Cambridge, MA). Fluorescent signals from the samples were quantitated with a fluorescence multichannel plate reader (FL 600, Bio-Tek Instruments). The spectra for FSA were 485 nm for excitation and 530 nm for emission. Clearance (Cl) was calculated with the following formula: Cl (nl h−1 cm−2) = Fab/[FSA]a × S, where Fab is the apical-to-basolateral flux of FSA (in light units/h), [FSA]a is the concentration at baseline (light units/ml), and S is the surface area (0.3 cm2). Simultaneous controls were performed with each experiment.

Statistical analysis. Data are presented as means ± SE. All experiments were carried out with a sample size of at least six observations per group. Statistical analysis was performed with ANOVA followed by Dunnett’s multiple-range test (29). Correlational analyses were done with the Pearson test for parametric analysis or, when applicable, the Spearman test for nonparametric analysis. P values <0.05 were deemed statistically significant.

RESULTS

Inhibitors of EGFR tyrosine kinase and of PLC prevent EGFr-induced protection of intestinal barrier function and of microtubule cytoskeletal integrity. First, we found that pretreatment with growth factor protects monolayer barrier integrity against oxidant-induced barrier hyperpermeability as assessed by reduced FSA
We then studied the effects of these various treatments on microtubule cytoskeletal integrity by quantitating, with immunofluorescent labeling, the percentage of Caco-2 cells in the monolayers that displayed normal intact microtubules. Preincubation of Caco-2 monolayers with EGF before H2O2 attenuated microtubule cytoskeletal disruption as shown by the high percentage of cells with normal microtubules, a number that was similar to the value obtained for control monolayers (vehicle) (Fig. 2). Similar to their effects on barrier function, preincubation with the EGFR-specific tyrosine kinase inhibitors AG-1478 and tyrphostin 25 abolished the protective action of EGF on the maintenance of the microtubule cytoskeleton. The specific PLC inhibitors U-73122 and L-108 attenuated the protection of microtubule integrity induced by EGF. As expected, the inactive analogs of the inhibitors were

clearance (Fig. 1), confirming our previous reports (6, 7). We also confirmed that this protection is EGFR-mediated because monoclonal anti-EGFR antibody (anti-EGFR, 1 μg/ml) abolished EGF-induced protection (data not shown).

Figure 1 also shows that pretreatment with the EGFR-specific tyrosine kinase inhibitors tyrphostin 25 and AG-1478 almost completely prevented EGF-induced protection. The inactive analog, tyrphostin A1, was ineffective. Preincubation with two different PLC-specific inhibitors, U-73122 and L-108, but not the inactive analog U-73343, also prevented the protective effects of EGF on monolayer barrier integrity. Inhibitors by themselves had no effect.

Fig. 1. Pretreatment with known epidermal growth factor (EGF) receptor (EGFR) tyrosine kinase inhibitors or phospholipase C (PLC) inhibitors prevents the protective effects of EGF on oxidant-induced disruption of intestinal monolayer barrier function as assessed by fluorescein sulfonic acid (FSA) clearance. Monolayers were preincubated (30 min) with the tyrosine kinase inhibitors tyrphostin 25 (25 μM) or AG-1478 (150 nM) or, as a control, the inactive analog tyrphostin A1 (iTyrphostin A1; 150 nM); others were treated with the PLC inhibitors U-73122 (1 μM) or L-108 (10 μM) or, as a control, the inactive form, U-73343 (1 μM) before treatment (+) with EGF and subsequent exposure (+) to oxidant (H2O2). Barrier permeability was calculated as apical-to-basolateral flux of the fluorescent probe FSA divided by the concentration of probe in the apical chamber, expressed as clearance; n = 6 observations/group. *P < 0.05 vs. vehicle; †P < 0.05 vs. H2O2; ‡P < 0.05 vs. EGF + H2O2; & P < 0.05 vs. active analog of inhibitors 1EGF 1H2O2.

Fig. 2. Percentage of monolayer Caco-2 cells with normal microtubule cytoskeletal architecture as determined by laser scanning confocal microscopy (LSCM). Conditions and treatments were described in Fig. 1. Cell monolayers were immunofluorescently stained for microtubules by incubation with a primary monoclonal anti-β-tubulin antibody, followed by a secondary FITC-conjugated antibody, and were subsequently analyzed by LSCM. Two hundred cells per slide (n = 6 slides/group) were examined in 4 different fields, and %cells displaying normal microtubules was determined. Note that both the EGFR tyrosine kinase inhibitors and the known PLC inhibitors (defined in Fig. 1), but not their inactive analogs, prevented protective effects of EGF on microtubules. *P < 0.05 vs. vehicle; †P < 0.05 vs. H2O2; ‡P < 0.05 vs. EGF + H2O2; & P < 0.05 vs. active analog of inhibitors 1EGF 1H2O2.
ineffective. In all cases, the inhibitors by themselves did not injuriously affect the microtubules.

Immunofluorescent images of the microtubule cytoskeleton as captured by ultra high-resolution LSCM (Fig. 3) supported the results shown in Fig. 2. Cells from control monolayers exhibited a normal and radial distribution of the microtubules throughout the cytosol, whereas cells exposed to 

H$_2$O$_2$ (b) display a fragmented, kinked, and collapsed organization of the microtubule cytoskeleton. In EGF-pretreated cells, normal filamentous appearance of microtubules is maintained and resembles the cytoarchitecture detected in controls. In cells of monolayers pretreated with U-73122 + EGF + H$_2$O$_2$ (d) (unlike the inactive U-73343 + EGF + H$_2$O$_2$; e), the microtubule filaments are highly fragmented and disrupted, similar in appearance to those of the oxidant-exposed group (b). Representative of n = 6 observations/group.

Quantitative immunoblotting (Fig. 4A) of polymerized tubulin (S2 fraction, index of stability) and monomeric tubulin (S1 fraction, index of disruption) in response to various treatment regimens was also consistent with the aforementioned findings. In monolayers exposed to oxidant, pretreatment with EGF increased the stable S2 tubulin fraction and decreased the monomeric S1 tubulin fraction to almost control levels. When the same cells were pretreated with inhibitors of EGFR tyrosine kinase or of PLC (but not with its inactive analogs), we noted a significant reduction in the S2 tubulin and an increase in the S1 tubulin, indicating microtubule disassembly.

Two representative Western blot photomicrographs of tubulin further confirm that pretreatment with EGF increases the stable polymerized S2 tubulin fraction and decreases the monomeric S1 tubulin fraction to almost control levels. When the same cells were pretreated with inhibitors of EGFR tyrosine kinase or of PLC (but not with its inactive analogs), we noted a significant reduction in the S2 tubulin and an increase in the S1 tubulin, indicating microtubule disassembly.

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EGF causes enhancement of PLC activity in monolayers. To further show that PLC is involved in EGF protection, we investigated whether EGF increases PLC activity in Caco-2 cells as assessed by radioimmunoassay of cell extracts from cells labeled with myo-[3H]inositol. EGF enhanced PLC activity in a dose-dependent manner (Fig. 5). Doses of EGF known to be protective (e.g., 10 ng/ml) significantly increased PLC activity; nonprotective doses of EGF (e.g., 1 ng/ml) did not. Figure 6 shows that pretreatment with the PLC inhibitors U-73122 and L-108 (but not the inactive U-73343) almost completely inhibited the ability of protective EGF to stimulate PLC activity. Preincubation with EGFR tyrosine kinase inhibitors caused similar effects. The drugs by themselves had no effect on basal PLC activity.

Fig. 5. Effects of varying doses of EGF on PLC activity in Caco-2 cells. Monolayers were exposed to oxidant (H$_2$O$_2$) for 30 min or were pretreated with varying doses of EGF for 10 min before subsequent exposure to H$_2$O$_2$. Controls (vehicle) were incubated in isotonic saline. PLC activity was assessed by radioimmunoassay of cell extracts from myo-[3H]inositol-labeled cells. *P < 0.05 vs. vehicle; †P < 0.05 vs. H$_2$O$_2$; ‡P < 0.05 vs. EGF (10 ng/ml) alone or EGF (10 ng/ml) + H$_2$O$_2$. n = 6 observations/group.

Fig. 4. A: quantitative immunoblotting analysis of the polymerized tubulin fraction (S2, index of stability) and the monomeric tubulin fraction (S1, index of disassembly) in Caco-2 monolayers. Conditions and treatments were similar to those in Figs. 1 and 2. Note that EGF prevented oxidant-induced tubulin (50 kDa) disassembly. Also, inhibitors of EGFR tyrosine kinase and of PLC prevented EGF enhancement of tubulin polymerization. %Polymerization of tubulin (backbone of microtubules) = ([S2]/[S2 + S1]) × 100, where S2 + S1 is the total cellular tubulin pool. *P < 0.05 vs. vehicle; †P < 0.05 vs. H$_2$O$_2$; ‡P < 0.05 vs. EGF + H$_2$O$_2$; &P < 0.05 vs. active inhibitor + EGF + H$_2$O$_2$. n = 6 Observations/group. B and C: representative Western immunoblot micrographs of the S2 (B) and S1 (C) tubulin extracts from Caco-2 cells after various treatments. Conditions were as in A. Tubulin fractions were analyzed by SDS-PAGE and Western immunoblot with the use of a monoclonal anti-β-tubulin antibody followed by a horseradish peroxidase-conjugated secondary antibody. Lanes: a, control (isotonic saline); b, 0.5 mM H$_2$O$_2$; c, EGF + 0.5 mM H$_2$O$_2$; d, AG-1478 + EGF + 0.5 mM H$_2$O$_2$; e, iTyrophostin A1 + EGF + 0.5 mM H$_2$O$_2$; f, U-73122 + EGF + 0.5 mM H$_2$O$_2$; g, inactive analog U-73343 + EGF + 0.5 mM H$_2$O$_2$; h, tubulin standard (50 kDa). EGF enhanced S2 tubulin band density (B) while reducing S1 tubulin (C), reversing the effect of oxidant. These effects of EGF were prevented by either specific inhibitors of EGFR tyrosine kinase or of PLC. Representative of n = 6 observations/group.
PLCz reduces EGF induction of PLC activity and EGF mediated protection of cell monolayers. To further demonstrate a key role for PLC in EGF induced protection, wild-type Caco-2 cells were stably transfected with cDNA encoding a PLCz dominant fragment from the Z region of human PLC-γ1 (i.e., pXf/PLCz). Figure 7 shows that PLCz (51 kDa) was detected by immunoblotting of the cell lysates from these transfected cells. Native PLC-γ1 (Fig. 7A) is demarcated as a protein with an apparent molecular weight of 145. Figure 7 also shows that the control vector (pXf) did not introduce the PLCz mutant. Normal parental (wild type) cells lacking PLCz are shown as an additional control.

Assessment of PLC activity (radioimmunoassay of cell extracts from myo-[3H]inositol labeled cells) in these PLCz-transfected cells shows that PLC was slightly lower than the basal levels seen in normal untransfected (wild type) cells (Fig. 8). In these transfected cells, PLCz completely inhibited the increases in PLC activity induced by a high dose of EGF. In contrast, in the wild-type cell line, this same dose of EGF significantly increased PLC activity. pXf vector alone did not affect PLC activity (data not shown).

FSA clearance measurements of cell monolayer barrier integrity (Fig. 9) indicate that dominant negative inhibition of PLC activity by PLCz prevented EGF induced protection. This same dose of EGF, however, almost completely protected monolayers of parental cells against injury by oxidant. PLCz protein expression by itself did not affect monolayer permeability.

Manipulation of PLC activity by PLCz transfection also abolished protection of the microtubule cytoskeleton by EGF as quantitated by the percentage of cells with normal microtubules (Fig. 10). PLCz by itself did not deleteriously affect the microtubules.
PLCz mutant prevents EGF induction of PLC-γ1 phosphorylation. Finally, both EGFR and PLC-γ1 were immunoprecipitated from both the PLCz-transfected and wild-type cells (those not expressing PLCz) and then subjected to Western immunoblotting to assess tyrosine phosphorylation (Fig. 11). In parental Caco-2 cells, the addition of EGF caused an increase in tyrosine phosphorylation of EGFR (Fig. 11A) and PLC-γ1 (Fig. 11B). Expression of PLCz prevented the tyrosine phosphorylation of PLC-γ1 by EGF, suggesting that PLCz mutant is an effective inhibitor of EGF ligand-induced PLC-γ1 phosphorylation and/or activation.

**DISCUSSION**

Identifying and characterizing the intracellular mechanisms underlying the ability of EGF to confer protection to the intestinal epithelial barrier is important because loss of GI barrier integrity is considered to be a key mechanism in the pathogenesis of IBD (32). Because oxidative tissue damage is also implicated in the pathophysiology of IBD (37, 42), we have been investigating mechanisms by which oxidants disrupt the intestinal barrier and growth factors protect it. Using monolayers of intestinal cells as a model of barrier function, our previous reports (5, 6) demonstrated that damage to the microtubule cytoskeleton is required for oxidant-induced damage to the intestinal barrier and that pretreatment with growth factors prevents this damage. We further showed that the EGFR and PKC activation mediate the ability of EGF to protect both microtubule cytoskeletal organization and intestinal barrier integrity (6–8). Continuing this probe into protective pathways, our current investigation demonstrates that PLC-γ is a critical downstream signal from the EGFR and is required for EGF-induced protection. To the best of our knowledge, this is the first demonstration of this mechanism for the defense and protection of intestinal epithelial barrier integrity.

Several independent lines of evidence support this conclusion. First, preincubation of oxidant-exposed monolayers with EGF not only increased PLC activity but also concomitantly prevented oxidant-induced intestinal monolayer hyperpermeability. In concert, EGF prevented oxidant-induced decreases in tubulin assembly (S2) and the percentage of cells with intact microtubule architecture and increases in tubulin disassembly (S1). Second, these protective effects of EGF were...
significantly attenuated by two different classes of inhibitors, those that inhibit EGFR tyrosine kinase and those that inhibit PLC, whereas the noninhibitory analogs of these same agents did not prevent protection by EGF.

Third, transfected cells in which PLC-\(\gamma_1\) activity was severely attenuated by PLCz expression were no longer protected by EGF against oxidant damage to tubulin assembly, microtubules, and barrier integrity. These transfection experiments were done, in part, because pharmacological inhibitors of PLC, namely U-73122 and L-108, are specific to PLC but not to any one PLC isoform. Moreover, PLCz, the dominant negative fragment of PLC-\(\gamma_1\) obtained from the Z region of PLC-\(\gamma_1\), is known to contain the SH2-SH2-SH3 domains necessary for activation and/or phosphorylation of PLC-\(\gamma_1\) by EGFR (23, 33, 56). The concordance of our findings resulting from the use of both pharmacological inhibition and molecular targeting supports a central role for PLC-\(\gamma_1\) in protection by EGF.

The findings of other investigators are also consistent with the conclusions of our study. It is known that PLC-\(\gamma_1\) profoundly affects similar cellular functions in epithelial cells as well as in nonepithelial cells (23, 24, 27, 30, 33, 38, 53, 54, 57, 62). Several recent studies, for example, showed that migration of intestinal cells that is stimulated by growth factor receptors (i.e., a motogenic pathway) requires PLC-\(\gamma_1\) activity (15, 21, 45, 48, 55). Additionally, PLC-\(\gamma_1\) has been implicated in the pathway for EGF-mediated remodeling of the actin component of the cytoskeleton (16, 27, 34) and of EGF-induced regulation of profilin and gelsolin (34). Furthermore, several recent studies have suggested that PLC-\(\gamma_1\) is the only epithelial PLC isoform with SH2 and SH3 domains that may be activated by EGF ligands (23, 48, 51).

PLC-\(\gamma_1\) hydrolyzes PIP\(_2\) to produce not only inositol trisphosphate but also diacylglycerol (DAG) in epithelial cells (23, 48, 56). DAG is one of the best-characterized products of PLC-\(\gamma\)-mediated reactions and is known to activate serine/threonine protein kinase C (PKC) (3, 7, 46). This is consistent with the fact that we have recently shown, with the use of pharmacological and targeted molecular inhibition (i.e., antisense), that PKC, especially the PKC-\(\beta_1\) isoform, is an essential mediator of the EGF-enhanced signaling that leads to

![Fig. 10. Dominant negative inhibition of PLC activity by PLCz prevents the protective effects of EGF (10 ng/ml) on the microtubule cytoskeleton. Microtubule integrity was assessed by counting 200 cells/slide from 4 different fields and, subsequently, %Caco-2 cells of monolayers displaying normal microtubule cytoskeleton was assessed. Conditions were as described in Fig. 9. \(^*P < 0.05\) vs. vehicle; \(\dagger P < 0.05\) vs. H\(_2\)O\(_2\); \(+ P < 0.05\) vs. EGF + H\(_2\)O\(_2\) in parental cells. \(n = 6\) slides/group.](http://ajpgi.physiology.org/)

![Fig. 11. Immunoblotting assessment of the effects of EGF ligand on the tyrosine phosphorylation of immunoprecipitated EGFR (A) and PLC-\(\gamma\) (B) and its prevention by PLCz dominant mutant expression in intestinal cells. Cell monolayers were lysed and processed for immunoprecipitation of EGFR and PLC-\(\gamma\), and subsequently for Western blotting, with the monoclonal anti-phosphotyrosine antibody. Note that EGF caused an increase in tyrosine phosphorylation of both EGFR (A) and PLC-\(\gamma\) (B) in parental cells. In PLCz-transfected cells, dominant mutant expression prevented the tyrosine phosphorylation of PLC-\(\gamma\) by EGF (B).](http://ajpgi.physiology.org/)
the maintenance of intestinal barrier permeability (7, 8). Moreover, PKC activators (OAG or 12-O-tetradecanoylphorbol 13-acetate) can maintain both the cytoskeletal integrity and intestinal barrier function in the presence of PLC inhibitors, suggesting the integral role of PKC activation downstream of PLC-γ1 in protection. Indeed, PKC activity has also been shown to be PLC dependent in other systems (46, 61). Also, two recent studies (2, 40) proposed that a naturally occurring intracellular activator of PKC, namely DAG (OAG used in our recent studies is a synthetic version of this compound), modulates intestinal monolayer permeability in Caco-2 cells. Overall, it appears that growth factor-induced protection is mediated by PLC-γ1 and then PKC-β1. In our studies, specific inhibition of PLC-γ1 was required to demonstrate a causal relationship between this enzyme and EGF-mediated protection.

First, two different specific inhibitors of PLC activity, U-73122 (and its control condition, the inactive analog U-73343) and L-108, were used to determine whether inhibition of PLC would prevent protection. These drugs had no deleterious side effects on the “basal” rate of the parameters under consideration (e.g., microtubules, barrier, PLC). Numerous previous studies have used an identical pharmacological approach to answer similar kinds of questions in epithelial cells, including studies in colonic cells (23, 43, 48, 62, 63). For example, using identical PLC inhibitors, Polk (48) demonstrated the importance of PLC in EGF-induced motility of intestinal epithelial cells.

Second, pharmacological inhibitors of PLC do not necessarily prove that EGFR/PLC-γ signaling is critical to EGF-induced protection. To more convincingly identify PLC-γ as a required downstream signal for EGF protection, we used a previously validated and widely used molecular approach, PLCz mutant (22, 23, 33, 56). PLCz, which covers both the SH2 and SH3 domains (amino acids 517–901), is known to specifically inhibit PLC-γ activation and/or phosphorylation and not other PLC isoforms in epithelial cells (22, 23, 33, 56). The PLCz mutant is specific for inhibition of PLC-γ because extensive previous studies from several independent labs have shown that PLC-γ1 is the only epithelial PLC isoform that contains SH2 and SH3 domains and that is activated by EGF (23, 33, 43, 48, 51, 56). Indeed, in our own studies, PLCz dominant mutant expression prevented the tyrosine phosphorylation (activation) of PLC-γ1 (Fig. 11), while at the same time, it abrogated activation of PLC (Fig. 8) by EGF.

Third, we note that although PLCz dominant negative mutant exerts its inhibitory effect by binding to receptor phosphoryrosine motifs (i.e., SH2 motifs), it is possible that other, non-PLC-γ SH2-mediated interactions may also be interrupted. However, this scenario seems unlikely because 1) the U-73122 and L-108 (pharmacological) data point to a phospholipase (PLC) being the target of action and 2) the PLCz mutant data specifically indicate an SH2 domain-containing molecule as a requirement for EGF-induced protection. PLC-γ is the only candidate that, once again, fulfills both requirements.

Several other reports, including our own, suggest that our proposed mechanism for EGF protection is both generalizable and relevant to IBD. We observed protective effects by EGF when hypochlorous acid (HOCl) or peroxynitrite (ONOO⁻) were the oxidative agents used to damage microtubules and the monolayer barrier (5–7). A key role for these oxidants, including the oxidant H₂O₂, in IBD is likely because the natural course of IBD involves recurrent episodes of an inactive phase (where there are no neutrophils) followed by acute flare-up, as characterized by mucosal infiltration of large numbers of leukocytes including neutrophils (37, 42). These leukocytes are capable of producing large quantities of reactive oxygen metabolites (e.g., H₂O₂ and HOCl) and reactive nitrogen metabolites (e.g., ONOO⁻), reactive species that create a vicious cycle and sustain an inflammatory cascade and tissue damage. Additionally, in recent studies (5, 6, 13), we noted similar protective effects for TGF-α, a structurally similar growth factor synthesized by the intestinal mucosa that acts through the same EGFR. In fact, no report has convincingly dissociated the biological activities of EGF and TGF-α in any cell population, including GI epithelium (28, 47). Indeed, it is likely that both growth factors play a role in protection. Moreover, salivary EGF and EGF contained within secretions of the Brunner’s glands and exocrine pancreas are the most important sources of gut EGF, and they play a major role in protection of both the small and large intestine (18, 47, 49, 50). For example, a previous study showed the presence of EGF-like immunoreactivity in a novel cell lineage derived from intestinal stem cells in the inflamed intestinal mucosa, such as occurs in IBD and peptic ulcers (58). EGF also prevents injury to the intestinal epithelium in animal models of IBD (trinitrobenzenesulfonic acid) (18, 49) as well as in vitro (Clostridium difficile toxin model of epithelial damage to human colonocytes) (52).

MAPK inhibitors did not prevent EGF-induced protection in our intestinal model of barrier function (data not shown). It is therefore likely that MAPKs are not involved directly in EGF-mediated protection. Not surprisingly, MAPKs regulate many deleterious (not protective) pathways in cells such as programmed cell death, oxidative stress, and the inflammatory response (e.g., cytokine production, immune cell degranulation, and oxidant stress by “extracellular signal-regulated kinase”-type MAPK) as well as mitogenesis and differentiation responses (e.g., by p38 MAPK) (1, 26, 35, 41). For example, MAPK can be activated by oxidants and is a key cellular response to oxidant stress (an upstream target of MAPK, p21 ras, is a target of reactive oxygen species) (1). Furthermore, although it is known that EGF can signal MAPK, different cells respond differently to EGF (1, 17, 26, 59). For instance, activation of EGF/EGFR does not necessarily cause MAPK signaling in lung epithelial cells (i.e., A549 cells, an adenocarcinoma epithelial cell line similar to our Caco-2 colon cancer cells) (26) as well as in several...
other cell types (23). Consistent with the above studies, several previous studies showed that MAPK is not always involved in EGF-induced effects (e.g., cell motility response) (23, 59).

In light of our new findings, it appears that an EGFR/PLC signaling pathway is responsible for a significant fraction of the normal protection of GI mucosal epithelium and perhaps is the key to preventing amplification and perpetuation of an uncontrolled oxidant-induced inflammatory cascade that can be ignited by free radicals and other oxidants that are ever-present in the GI tract. Our studies not only describe pathophysiological mechanisms, they also suggest possible ways to develop novel therapeutic strategies. In particular, our studies indicate that PLC-γ1 is a potential therapeutic target for pharmacological and perhaps genetic interventions against a wide variety of oxidative inflammatory conditions of the GI tract including IBD. For instance, one therapeutic approach (“targeted gene therapy”) might be the exogenous delivery of a sense vector for the PLC-γ1 isoform to the inflamed GI mucosa in vivo. If either the pharmacological or the gene therapy approach is successful, one should be able to protect and maintain epithelial integrity against oxidative stress, such as that which occurs during incipient or rampant inflammation, and subsequently limit the initiation and progression of GI mucosal inflammation and damage. Also, such therapies might synergize with the use of antioxidants so that inflammatory processes are attenuated through the manipulation of both the damaging and protective intracellular pathways. In summary, our findings demonstrate that PLC-γ1 is a key mediator in protection of the GI mucosal epithelium by growth factors.

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REFERENCES


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