Critical Role of the Atypical $\lambda$ Isoform of Protein Kinase C (PKC-$\lambda$) in Oxidant-Induced Disruption of the Microtubule Cytoskeleton and Barrier Function of Intestinal Epithelium


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ABSTRACT

Oxidant injury to epithelial cells and gut barrier disruption are key factors in the pathogenesis of inflammatory bowel disease. Studying monolayers of intestinal (Caco-2) cells, we reported that oxidants disrupt the cytoskeleton and cause barrier dysfunction (hyperpermeability). Because the $\lambda$ isoform of protein kinase C (PKC-$\lambda$), an atypical diacylglycerol-independent isoform, is abundant in parental (wild type) Caco-2 cells and is translocated to the particulate fractions upon oxidant exposure, we hypothesized that PKC-$\lambda$ is critical to oxidative injury to the assembly and architecture of cytoskeleton and the intestinal barrier function. To this end, Caco-2 cells were transfected with an inducible plasmid, a tetracycline-responsive system, to create novel clones stably overexpressing native PKC-$\lambda$. Other cells were transfected with a dominant-negative plasmid to stably inhibit the activity of native PKC-$\lambda$. Cells were exposed to oxidant ($H_2O_2$) $\pm$ modulators. Parental Caco-2 cells were treated similarly. We then monitored barrier function (fluorescein sulfonic acid clearance), microtubule cytoskeletal stability (confocal microscopy, immunoblotting), subcellular distribution of PKC-$\lambda$ (immunofluorescence, immunoblotting, immunoprecipitation), and PKC-$\lambda$ isoform activity (in vitro kinase assay). Monolayers were also processed to assess alterations in tubulin assembly, polymerized tubulin (S2, an index of cytoskeletal integrity), and monomeric tubulin (S1, an index of cytoskeletal disassembly) (polyacrylamide gel electrophoresis fractionation and immunoblotting). In parental cells, oxidant caused: 1) translocation of PKC-$\lambda$ from the cytosol to the particulate (membrane + cytoskeletal) fractions, 2) activation of native PKC-$\lambda$, 3) tubulin pool instability (increased monomeric S1 and decreased polymerized S2), 4) disruption of cytoskeletal architecture, and 5) barrier dysfunction (hyperpermeability). In transfected clones, overexpression of the atypical (74 kDa) PKC-$\lambda$ isoform by itself (~3.2-fold increase) led to oxidant-like disruptive effects, including cytoskeletal and barrier hyperpermeability. Overexpressed PKC-$\lambda$ was mostly found in particulate cell fractions (with a smaller cytosolic distribution) indicating its activation. Disruption by PKC-$\lambda$ overexpression was also potentiated by oxidant challenge. Stable inactivation of endogenous PKC-$\lambda$ (~99.6%) by a dominant-negative protected against all measures of oxidant-induced disruption. We conclude that: 1) oxidant induces disruption of epithelial barrier integrity by disassembling the cytoskeleton, in large part, through the activation of PKC-$\lambda$ isoform; and 2) activation of PKC-$\lambda$ by itself appears to be sufficient for disruption of cellular cytoskeleton and monolayer barrier permeability. The unique ability to mediate an oxidant-like injury and cytoskeletal depolymerization and instability is a novel mechanism not previously attributed to the atypical subfamily of PKC isoforms.

A key discovery in recent years in inflammatory bowel disease (IBD) research was the understanding that a leaky gut barrier can lead to intestinal inflammation and that maintaining a normal mucosal barrier function is required for intestinal health (e.g., Hollander, 1988, 1992; Yamada et al., 1993; Hermiston and Gordon, 1995; Farhadi et al., 2003a,b). For instance, transgenic rodents with a hyperpermeable intestinal barrier show symptoms of intestinal mucosal inflammation (Hermiston and Gordon, 1995). The pathophysiology of mucosal barrier dysfunction in IBD remains, however, poorly understood. Nonetheless, it is known that chronic gut inflammation in IBD is associated with...

ABBREVIATIONS: IBD, inflammatory bowel disease; GI, gastrointestinal; PKC, protein kinase C; DMEM, Dulbecco’s minimum essential medium; TRE, tetracycline-responsive expression; FBS, fetal bovine serum; tTA, tetracycline-responsive transactivator; LSCM, laser scanning confocal microscopy; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; FSA, fluorescein sulfonic acid; TTX, tetracycline; INOS, inducible nitric-oxide synthase.
excessive amounts of oxidants (e.g., H₂O₂) and that this excess is a key contributor to mucosal inflammation and injury (Keshavarzian et al., 1992, 2003; McKenize et al., 1996; Banan et al., 2000a,b, 2001b). Oxidative stress disruption is of substantial clinical importance not only because oxidants are commonly produced during intestinal inflammation, but also because they can lead to mucosal barrier dysfunction and initiation and/or continuation of mucosal injury (Hollander, 1988, 1992; Keshavarzian et al., 1992, 1999; Yamada et al., 1993; Hermiston and Gordon, 1995). Accordingly, investigating how gut barrier integrity is lost as well as how it is protected against oxidative, proinflammatory conditions is of fundamental biologic and clinical value.

The gut mucosal epithelium contains the body's largest interface between man and his hostile external environment. An essential characteristic of the gut epithelium is to maintain a normal barrier function. This is based on the ability of the epithelium to function as a highly selective permeability barrier that normally permits the absorption from the lumen of nutrients and electrolytes, but prevents the passage of proinflammatory molecules into the mucosa. Disruption of this barrier function, in contrast, can allow the penetration of excluded luminal proinflammatory antigens (e.g., endotoxin, immunoreactive agents) into the mucosa and lead to the initiation or perpetuation of inflammatory processes (Hollander, 1988, 1992; Hermiston and Gordon, 1995; Banan et al., 1999). It is thus not surprising that disruption of gut barrier function (i.e., hyperpermeability) has been implicated in the pathogenesis of a wide range of gastrointestinal and systemic disorders, including IBD (e.g., Hollander, 1988, 1992; Unno et al., 1996; Keshavarzian et al., 1999). For instance, intestinal hyperpermeability (“leaky gut”) has often been reported and implicated in the pathogenesis of IBD (Hollander, 1988, 1992; Hermiston and Gordon, 1995).

In our efforts to better understand endogenous disruptive mechanisms in gut inflammation, we have been investigating molecular mechanisms underlying oxidant-induced cytoskeletal and barrier disruption in the gut mucosal epithelium. Our hope has been to devise a rational basis for the development of potentially more effective treatment regimens for inflammatory disorders of the GI tract, especially IBD. Utilizing monolayers of human intestinal (Caco-2) cells exposed to oxidants, a widely validated model for cytoskeletal and barrier dysfunction, we previously showed that oxidants (e.g., H₂O₂) induce disruption of intestinal barrier function by disrupting the assembly of the cytoskeletal network, especially the microtubules (Banan et al., 1999, 2000a,b). More recently, we showed that oxidants cause barrier hyperpermeability, apparently in large part, through activation of proinflammatory (e.g., H₂O₂) induce disruption of intestinal barrier function by disrupting the assembly of the cytoskeletal network, especially the microtubules (Banan et al., 1999, 2000a,b). More recently, we showed that oxidants cause barrier hyperpermeability, apparently in large part, through activation of proinflammatory isoforms of PKC (Banan et al., 2000a,b). For our studies, we used isolated clonal cell lines from the 74-kDa λ isoform of PKC in oxidant-induced disruption because 1) it is the only other isoform of PKC that is translocated to the membranes in parental Caco-2 cells by oxidant challenge; 2) unlike novel PKC-δ, PKC-λ is an “atypical” PKC isoform; 3) it would more fully establish the idea that specific “injurious isoforms of PKC” play fundamental roles in disruptive mechanisms within cells; 4) it should lead to a better understanding of the pathophysiology of intestinal barrier hyperpermeability; and 5) developing agents that suppress injurious PKC isoforms could lead to the development of unique and more effective therapeutics for inflammatory diseases of the GI tract that are related to oxidative injury.

Accordingly, we explored the role of the atypical PKC-λ isoform utilizing targeted molecular interventions that enabled us to develop novel and stably transduced intestinal cell lines. In several clones, the 74-kDa isoform PKC-λ was reliably overexpressed; in the other clones, PKC-λ activity was inhibited. Using these new models, we tested the hypothesis that oxidant-induced disruption of microtubule cytoskeletal assembly and barrier function of epithelial monolayer depends on translocation and activation of the λ isoform of PKC. Herein, we report substantial mediation of oxidant-induced barrier hyperpermeability and cytoskeletal disassembly and instability by a member of the atypical subfamily of PKC isoforms in intestinal epithelium. To our knowledge, this is the first report that PKC-λ is shown to influence the dynamics of cytoskeletal and barrier function in cells.

Materials and Methods

Cell Culture. Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) at passage 15. This cell line was chosen because it forms monolayers that morphologically resemble small intestinal cells with defined apical brush borders and a highly organized microtubule network upon differentiation (Gilbert et al., 1991; Banan et al., 1998). Cells were maintained at 37°C in complete Dulbecco’s minimum essential medium (DMEM) in an atmosphere of 5% CO₂ and 100% relative humidity. Parental or stably transfected cells (see below) were split at a ratio of 1:6 upon reaching confluence and set up in either 6- or 24-well plates for experiments or T-75 flasks for propagation. Cells grown for barrier function experiments were split at a ratio of 1:2 and seeded at a density of 200,000 cells/cm² into 0.4 μM Bicocat collagen I cell culture inserts (0.3 cm² growth surface; BD Biosciences Discovery Labware, Bedford, MA), and experiments were performed at least 7 days postconfluence. The media were changed every 2 days. The utility and characterization of this cell line has been previously reported (Gilbert et al., 1991; Banan et al., 1998).

Plasmids and Stable Transfection. The sense and dominant-negative plasmids of PKC-λ were constructed as described (Banan et al., 2000a,b). A unique tetracycline-responsive expression (TRE) system was used to overexpress the native PKC-λ. cDNA encoding the entire reading frame of PKC-λ was subcloned into the TRE vector creating a TRE system encoding atypical PKC-λ (TRE PKC-λ). The dominant-negative PKC-λ plasmid was also constructed (Banan et al., 2000a,b). For our studies, we used isolated clonal cell lines from the
populations of cells transfected with the various concentrations of DNA for PKC-\(\lambda\).

Cultures of Caco-2 cells grown to 50 to 60% confluency were cotransfected with hygromycin resistance plasmid (\(p\)-hyguro) and expression plasmids encoding either sense PKC-\(\lambda\) or dominant-negative PKC-\(\lambda\) by Lipofectin (Lipofectin reagent; Invitrogen, Carlsbad, CA) (Banan et al., 2001a, 2002a). In brief, cells were incubated for 16 h at 37°C with the plasmid DNA in serum-free medium in the presence of Lipofectamine (25 \(\mu\)l/25 cm\(^2\) flask). Subsequently, the DNA-containing solution was removed and replaced by fresh medium containing 10% FBS to relieve cells from the shock of exposure to serum-free medium. Following transfection, cells were subjected to hygromycin selection (1 mg/ml) over 4 weeks. Cells were maintained in DMEM/FBS and 0.2 mg/ml hygromycin (selection medium).

For inducible overexpression of PKC-\(\lambda\), Caco-2 cells were initially transfected with a plasmid expressing the tetracycline-responsive transactivator (\(\mathrm{tTA}\), or so-called \(\mathrm{pTEToff}\), as it encodes a tetracycline-responsive PKC-\(\lambda\)-hygro) and a second plasmid conferring resistance to hygromycin (selection medium). More specifically, we isolated (picked) clonal cell lines from the populations of cells transfected with the various amounts (1, 2, 3, 4, or 5 \(\mu\)g) of plasmid for TRE PKC-\(\lambda\). Subconfluent cultures were then cotransfected with hygromycin selection plasmid (\(p\)-hyguro) (for colony picking) and expression plasmid encoding TRE PKC-\(\lambda\) (e.g., 4 \(\mu\)g of cDNA for the 4-\(\mu\)g clone of TRE PKC-\(\lambda\)) by Lipofectin (Banan et al., 2001a, 2002a). Control conditions included the same amount of vector DNA (\(\mathrm{Tre}\)-\(\lambda\)) alone. Cells were then subjected to hygromycin selection (1 mg/ml) over 1 month allowing colony selection. Cells in each colony were maintained in culture medium/FBS and 0.2 mg/ml hygromycin (selection medium). Repeating this approach for different colonies (e.g., the 1-\(\mu\)g TRE PKC-\(\lambda\)-sense clone, etc.), we obtained various clones stably overexpressing PKC-\(\lambda\) (by induction in the absence of tetracycline in culture medium). PKC-\(\lambda\) protein expression and activity and/or distribution were verified, respectively, by Western blot analysis of cell lysates, in vitro kinase activity assay, or immunofluorescence staining. Clones were subsequently plated on cell culture inserts, allowed to form confluent monolayers, and then used for experiments. When tetracycline was present in the medium, its concentration was 1 \(\mu\)g/ml. In the tetracycline studies, cells were grown for 48 h in the presence of tetracycline (used to prevent overexpression) prior to experiments.

**Experimental Design.** In the first series of experiments, postconfluent monolayers of parental Caco-2 cells were preincubated with oxidant (H\(_2\)O\(_2\), 0 to 0.5 mM) or vehicle (isotonic saline) for 30 min. It is known that H\(_2\)O\(_2\) at 0.5 mM disrupts microtubules and barrier integrity in these cells (Banan et al., 2000a, 2001b). These experiments were then repeated using monolayers composed of transfected cells either overexpressing PKC-\(\lambda\) (TRE PKC-\(\lambda\)) or lacking PKC-\(\lambda\) activity (dominant-negative). Reagents were applied on the apical side of monolayers unless otherwise indicated. In all experiments, microtubule cytoskeletal stability (cytoarchitecture, disassembly), tubulin assembly (polymerization and depolymerization), PKC-\(\lambda\) subcellular (membrane/cytoskeletal/cytosolic) distribution (immunofluorescence, immunoblotting, immunoprecipitation), PKC-\(\lambda\) activity (in vitro kinase assay), and barrier function (permeability clearance) were assessed.

In the second series of experiments, cell monolayers that were overexpressing the atypical PKC-\(\lambda\) were incubated (30 min) with oxidant (H\(_2\)O\(_2\)) or vehicle. Outcomes measured were as described above.

In a third series of experiments, monolayers of dominant-negative transfected cells lacking PKC-\(\lambda\) activity were treated with oxidant. PKC-\(\lambda\) activity was determined in immunoprecipitated samples (see below). In corollary experiments, we investigated the effects of PKC-\(\lambda\) activation or inactivation on the state of tubulin assembly and disassembly and on stability of the cytoarchitecture of the microtubule cytoskeleton. Monomeric and polymerized fractions of tubulin (the structural protein subunit of microtubules) were isolated and then analyzed by immunoblotting (Banan et al., 2000a, 2001a). Cytoskeletal integrity was assessed by 1) immunofluorescence labeling and fluorescence microscopy to determine the percentage of cells with normal microtubules, 2) detailed analysis by high-resolution laser scanning confocal microscopy (LSCM), and 3) immunoblot analysis of monomeric and polymerized tubulin pools.

**Fractionation and Western Immunoblotting of PKC.** Cell monolayers grown in 75-cm\(^2\) flasks were processed for the isolation of the cytosolic, membrane, and cytoskeletal fractions (Banan et al., 2001a,c). In brief, following treatments, postconfluent monolayers were scraped and ultrasonically homogenized in Tris-HCl buffer (20 mM Tris-HCl (pH 7.5), 0.25 mM sucrose, 2 mM EDTA, 10 mM EGTA, 2 \(\mu\)g/ml aprotinin, 2 \(\mu\)g/ml leupeptin, and 2 \(\mu\)g/ml phenylmethylsulfonyl fluoride). The homogenates were then ultra-centrifuged (100,000g for 40 min at 4°C), and the supernatant was removed and used as a source of the cytosolic fraction. Next, pellets were washed with 0.2 ml of Tris-HCl buffer and resuspended in 0.8 ml of buffer containing 0.3% Triton X-100 and maintained on ice for 1 h. The samples were then centrifuged (100,000g for 1 h at 4°C), and the supernatant was used as the source of the membrane fraction. To this remaining pellet, 0.3 ml of cold (4°C) lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 2 \(\mu\)g/ml aprotinin, 2 \(\mu\)g/ml pepstatin, 2 \(\mu\)g/ml leupeptin, and 2 \(\mu\)g/ml phenylmethylsulfonyl fluoride) was added. The samples were then placed on ice for 1 h and ultracentrifuged as above. The remainder of the lysate or Triton X-100-insoluble cytoskeletal fraction was then removed. Protein content of the various cell fractions was assessed by the Bradford method (Bradford, 1976). For total PKC extraction, which provides the fraction used to confirm total PKC-\(\lambda\), scraped monolayers were placed directly into 1.5 ml of cold lysis buffer and subsequently ultracentrifuged as described above. The supernatant was used for bulk protein determination.

For immunoblotting, samples (25 \(\mu\)g of protein/lane) were added to SDS buffer (250 mM Tris-HCl, pH 6.8, 2% glycerol, 5% mercaptoethanol), boiled for 5 min, and then separated on 7.5% SDS-PAGE (Banan et al., 2001a). Subsequently, proteins were transferred to nitrocellulose membranes (0.2-\(\mu\)m pore size) and then blocked in 3% bovine serum albumin for 1 h followed by several washes with Tris-buffered saline. The immunoblotted proteins were incubated for 2 h in Tween 20, Tris-buffered saline, 1% bovine serum albumin, and the primary monoclonal anti-PKC-\(\lambda\) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:2000 dilution for 1 h at room temperature. A horseradish peroxidase-conjugated secondary antibody (MolecuProbes, Eugene, OR) was used at 1:3000 dilution. Proteins on membranes were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences Inc., Piscataway, NJ) and autoradiography and subsequently analyzed by densitometry. The identity of the PKC-\(\lambda\) band was assessed by 1) using the PKC-\(\lambda\) blocking peptide in combination with the anti-PKC-\(\lambda\) antibody that prevents the appearance of the corresponding “major” band in Western blots; 2) additionally, in the absence of the primary antibody to PKC-\(\lambda\), no corresponding band for PKC-\(\lambda\) was observed; 3) the PKC-\(\lambda\) band ran at the expected molecular weight of 74 kDa as confirmed by a known positive control for PKC-\(\lambda\) (from rat brain lysates); and 4) prestained molecular weight markers (\(M_\text{r}\) 67,000 and 93,000) were run in adjacent lanes. In preliminary studies using total PKC extracts, we confirmed that overexpression of PKC-\(\lambda\) or negative-dominant inhibition of PKC-\(\lambda\) did not affect the relative expression levels of other PKC isoforms.

**Immunofluorescent Staining of PKC-\(\lambda\) Intracellular Distribution.** Cell monolayers were fixed in a standard fixation buffer as described (Chang and Tepperman, 2001; Banan et al., 2004). Cells were subsequently processed for incubation with a PKC isotype-specific primary antibody, monoclonal anti-PKC-\(\lambda\) (Santa Cruz Bio-
technology, Inc.), 1:100 dilution for 1 h at 37°C and then with a secondary antibody (rhodamine-conjugated; Sigma-Aldrich, St. Louis, MO), 1:50 dilution for 1 h at room temperature. Following staining, cells were observed using a 63× oil immersion plan-apochromat objective, NA 1.4 (Carl Zeiss GmbH, Jena, Germany). The PKC-λ staining was examined in a blinded fashion for its intracellular distribution (e.g., cytosol versus membrane or cell-cell contact areas). The identity of the groups was decoded only after examination was done.

**Immunoprecipitation and Atypical PKC-λ Activity Assay.** Immunoprecipitated PKC-λ was collected and processed for its ability to phosphorylate a synthetic peptide (Banan et al., 2003b). In brief, following 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 μM/ml antiprotease cocktail, 10% Triton X-100). The lysates were clarified by centrifugation at 14,000 g, pH 7.4, 150 mM NaCl, 10 μM/ml antiprotease cocktail, 10% Triton X-100. The remaining pellet was treated with the Ca2+-containing depolymerization buffer (0.1 M PIPES, pH 6.9, 30% glycerol, 5% dimethyl sulfoxide, 10 μg/ml antiprotease cocktail, 1 mM EGTA, 1 mM MgCl2, and 1 mM GTP) to promote polymerization of tubulin. Tubulin was then recovered by centrifugation and resuspended in the above stabilization buffer.

**Determination of Barrier Function by Fluorometry.** Status of the integrity of the monolayer barrier function was assessed by a widely used and validated technique that measures the apical to basolateral paracellular flux of fluorescent markers such as fluorescein sulfonic acid (FSA, 200 μg/ml; 0.478 kDa) as we (Banan et al., 1999, 2000a–c, 2001a–d, 2002c) and others (e.g., Sanders et al., 1995; Unno et al., 1996) have described. In brief, fresh phenol-free DMEM (800 μl) was placed into the lower (basolateral) chamber and phenol-free DMEM (300 μl) containing probe (FSA) was placed in the upper (apical) chamber. Aliquots (50 μl) were obtained from the upper and lower chambers at zero time and at subsequent time points and transferred into clear 96-well plates (clear bottom; Costar, Cambridge, MA). Fluorescent signals from samples were quantitated using a fluorescence multiplate reader (FL 600; Bio-Tek Instruments, Winoski, VT). The excitation and emission spectra for FSA were excitation = 485 nm, emission = 530 nm. Clearance (CL) was calculated using the following formula: CL (nl/h/cm2) = Fab/([FSA]e × S), where Fab is the apical to basolateral flux of FSA (light units/h), [FSA]e is the concentration at baseline (light units/ml), and S is the surface area (0.3 cm2). Simultaneous controls were performed with each experiment.

**Results**

**Transfection of Intestinal Cells to Stably Overexpress the PKC-λ Isoform.** Parental Caco-2 cells (tTA parental (or pTFET,m)) were cotransfected with complementary DNA (cDNA) for both hygromycin resistance (selection marker) and a TRE PKC-λ. In this inducible system, overex-
pression of PKC-λ is obtained in the absence of tetracycline (TTX) (Fig. 1A). In contrast, the presence of tetracycline reduces expression of PKC-λ to the levels seen in the parental cell line (Fig. 1B). Analysis of cell lysates in Fig. 1A shows overexpression of the PKC-λ isoform in the transfected cells. The PKC-λ isolated from these transfected cells comigrated with a known positive standard of ~74 kDa for the PKC-λ. The identity of the PKC-λ band was further ascertained by using the PKC-λ blocking peptide in combination with the anti-PKC-λ antibody that prevented the appearance of the corresponding major band in the Western blots. As expected, exclusion of the primary antibody also resulted in the disappearance of the corresponding PKC-λ band. Fig. 1B shows that total PKC-λ levels were increased by approximately 3.2-fold compared with that of parental cells. Overexpression of PKC-λ at this level caused neither any toxicity (0% cell death assessed by ethidium homodimer probe) nor any changes in Caco-2 growth (assessed by bromodeoxyuridine assay).

**Injurious Effects of Overexpression of Atypical PKC-λ Isoform on Monolayer Barrier Integrity.** In initial exploratory studies, multiple clones of intestinal cells transfected with 1, 2, 3, 4, or 5 µg of TRE PKC-λ cDNA showed a dose-dependent disruption of monolayer barrier integrity (increases in FSA clearance). Because the clone transfected with 3 µg of TRE PKC-λ led to disruption at a level comparable to that of oxidant (H₂O₂) in parental cell line, we used this clone for subsequent experiments.

In intestinal cells, PKC-λ overexpression by itself—without added oxidant—injuriously affected monolayer barrier function (Fig. 2). In the cells stably overexpressing PKC-λ (TRE PKC-λ and exposed to vehicle), monolayer barrier hyperpermeability was seen as demonstrated by increased FSA clearance (approximately 57-fold greater clearance than the control). When these same transfected cells overexpressing PKC-λ were incubated with tetracycline (i.e., TRE PKC-λ +

![Fig. 1. Overexpression of the atypical PKC-λ isoform in Caco-2 cells transfected with a TRE system for native PKC-λ (i.e., TRE PKC-λ) (A). Overexpressed PKC-λ (lane a) as well as a commercially obtained positive control for PKC-λ (+) comigrated as 74 kDa bands. In the absence of the monoclonal anti-PKC-λ antibody (b), the corresponding PKC-λ band is no longer seen. As expected, in the presence of the antipeptide (c) to primary antibody the PKC-λ band disappeared (“antipeptide” used is the target peptide for the anti-PKC-λ mAb—i.e., a competitive peptide). B, comparison of the total levels of PKC-λ protein expression in TRE PKC-λ-transfected cells versus parental cells with or without tetracycline in cell medium. In TRE PKC-λ-transfected cells, overexpression of native PKC-λ is seen in the absence of TTX. In contrast, the presence of tetracycline reduces expression of PKC-λ to the levels comparable with that of the parental cell line. In parental cells, the presence or absence of tetracycline has no effect on the steady-state levels of native PKC-λ. Analysis by densitometry showed a 3.2-fold elevation of the PKC-λ protein levels in TRE PKC-λ-transfected cells. Samples (25 µg of protein/lane) were processed for Western immunoblotting with a monoclonal anti-PKC-λ antibody followed by a horseradish peroxidase-conjugated secondary antibody. Prestained molecular weights, Mr = 67,000 and 93,000, were also run in adjacent lanes. Shown are representative blots. n = 6 per group.

![Fig. 2. Disruption of the barrier integrity of intestinal cell monolayers following overexpression of PKC-λ and potentiation of oxidant-induced disruption. Caco-2 monolayers overexpressing PKC-λ (TRE PKC-λ) were incubated with or without oxidant H₂O₂ (0.5 mM) or vehicle. Parental-type [tTA Parental] monolayers (those not overexpressing λ isoform) are also shown. Vehicle-treated cells overexpressing PKC-λ exhibit loss of monolayer barrier function (hyperpermeability). As might be expected, this disruption was inhibited in the presence of tetracycline in cell medium [TRE PKC-λ + TTX]. Also, note potentiation-induced disruption of barrier function in the PKC-λ-overexpressing cells, which were exposed to oxidant. Parental-type monolayers that were exposed to vehicle (with or without tetracycline) showed normal barrier function. These parental-type cells showed disruption of barrier permeability only after exposure to oxidant. Transfected cells, which were exposed to oxidant in the presence of tetracycline [TRE PKC-λ + TTX] as expected, responded comparably with that of the parental cells which were exposed to the same oxidant challenge. Barrier integrity (i.e., permeability) was expressed as flux of the fluorescent probe FSA from the apical to basolateral compartment of cell culture transwell inserts divided by the concentration of probe in the apical chamber. When normalized for the surface area of the monolayer, this expression has units of clearance. For tetracycline experiments, cells were grown for 48 h in the presence of tetracycline prior to experiments. *, p < 0.05 versus vehicle-treated parental cells. +, p < 0.05 versus H₂O₂-treated parental cells. &&, p < 0.05 versus corresponding PKC-λ-overexpressing [TRE PKC-λ] cells exposed to vehicle or PKC-λ-overexpressing cells incubated with tetracycline and exposed to oxidant [TRE PKC-λ + TTX]. [tTA Parental] = parental cells. [TRE PKC-λ] = cells transfected with the TRE system for native PKC-λ. n = 6 per group.
tetracycline where overexpression of \( \lambda \) is inhibited), we found maintenance of monolayer barrier integrity at near normal conditions. Similarly, parental-type cells (those not overexpressing PKC-\( \lambda \)) exposed to vehicle (with or without tetracycline) also demonstrated intact barrier function. These parental-type cells, however, had their barrier integrity disrupted by oxidant \( \text{H}_2\text{O}_2 \) (0.5 mM). Also, exposure to oxidant potentiated disruption of monolayer barrier permeability in the cells overexpressing PKC-\( \lambda \). As expected, this potentiation was prevented when tetracycline was present. Transfection of only the empty vector TRE-z, as expected, did not affect barrier function (FSA clearance = 20 \( \pm \) 7 nl/h/cm\(^2\) for vector-transfected cells exposed to vehicle and 17 \( \pm \) 6 for parental cells exposed to vehicle; 824 \( \pm \) 24 for vector-transfected cells exposed to \( \text{H}_2\text{O}_2 \) alone and 823 \( \pm \) 15 for parental cells incubated in \( \text{H}_2\text{O}_2 \)). Vector-transfected cells and parental cells, in fact, responded in a similar manner to either vehicle or \( \text{H}_2\text{O}_2 \).

The atypical PKC-\( \lambda \) overexpression also damaged the microtubule cytoskeleton as shown by a low percentage of cells with normal microtubules (Fig. 3). As for barrier hyperpermeability, this overexpression-induced disruption was inhibited in the presence of tetracycline (TRE PKC-\( \lambda \) + tetracycline). Moreover, exposure to oxidant \( \text{H}_2\text{O}_2 \) potentiated disruption of microtubule integrity in these transfected cells. As expected, tetracycline also completely inhibited this potentiation effect. In parental cells (tTA parental), microtubules were disrupted by oxidant, paralleling findings on loss of barrier integrity. Furthermore, transfection of empty vector TRE-z did not injure the microtubules (percentage of normal microtubules = 98 \( \pm \) 2\% for vector-transfected cells exposed to vehicle and 99 \( \pm \) 1\% for parental cells exposed to vehicle). Vector-transfected and parental cells also responded in a similar fashion to oxidant challenge (42 \( \pm \) 6\% for vector-transfected cells exposed to \( \text{H}_2\text{O}_2 \) and 43 \( \pm \) 4\% for parental cells exposed to \( \text{H}_2\text{O}_2 \)).

Laser scanning confocal microscopy of the microtubule cytoarchitecture corroborates the aforementioned findings (Fig. 4). Cell monolayers overexpressing PKC-\( \lambda \) (TRE PKC-\( \lambda \) without tetracycline) show a disrupted architecture of the cytoskeleton (panel C). This disruption is shown by the appearance of a beaded, damaged, and collapsed network of microtubules. On the other hand, when tetracycline is present (panel D) these cells exhibited a normal microtubule cytoskeleton as revealed by its preserved cytoarchitecture. This intact organization is indistinguishable from that of the parental cells, which were exposed to vehicle (panel A). Parental cells (panel B) show microtubule instability and disruption after exposure to oxidant alone; this is comparable with that of the PKC-\( \lambda \)-overexpressing cells (panel C).

We then determined any effects of PKC-\( \lambda \) overexpression on the molecular dynamics of the microtubule cytoskeleton (e.g., polymerization and depolymerization states) by performing immunoblotting analysis of its structural protein, tubulin. Polymerized tubulin (S2, an index of microtubule stability) and the monomeric tubulin (S1, an index of microtubule disruption) were isolated and then analyzed by SDS-PAGE. Immunoblotting analysis of tubulin shows (Fig. 5) that PKC-\( \lambda \)-overexpressing cells (TRE PKC-\( \lambda \), vehicle-treated) exhibited an abnormal tubulin assembly as demonstrated by a reduction in the polymerized tubulin and an increase in the monomeric tubulin. This abnormality in tubulin dynamics is comparable to that of the oxidant-exposed parental cells. In tetracycline-incubated TRE PKC-\( \lambda \) cells (where overexpression of \( \lambda \) is not seen), no changes in tubulin assembly (S2 or S1) were found, indicating maintenance of microtubule assembly. Indeed, the levels of tubulin polymerization seen were comparable to the normal levels seen in that of parental cells exposed to vehicle. In parental cells, exposure to oxidant led to increased tubulin disassembly. Furthermore, transfection of only the empty vector, as might be expected, did not disturb tubulin assembly (e.g., percentage of tubulin assembly = 65 \( \pm \) 0.7\% for vector alone transfected Caco-2 cells exposed to vehicle and 65 \( \pm \) 0.24\% for parental cells exposed to vehicle; 45 \( \pm \) 0.9\% for vector alone transfected cells exposed to \( \text{H}_2\text{O}_2 \) and 46 \( \pm \) 0.38\% for parental cells exposed to \( \text{H}_2\text{O}_2 \)). These findings on the molecular dynamics of tubulin parallel the deleterious effects of PKC-\( \lambda \) overexpression on intestinal microtubule integrity and barrier function.

Figure 6 shows a time course for changes in barrier function (FSA clearance) and tubulin polymerization in transfected PKC-\( \lambda \)-overexpressing cells in the presence of tetracycline. The results indicate that tubulin polymerization is
increased prior to barrier function changes (i.e., decreased FSA clearance) (data reported as a percentage of maximum, which is arbitrarily assigned a value of 100).

Subcellular Distribution and Activation Levels of PKC-\(\lambda\) in Transfected and Parental Intestinal Cells. Figure 7, A–D shows immunoblots of the cytosolic, membrane, and cytoskeletal fractions from cells overexpressing PKC-\(\lambda\) (Fig. 7, C and D) demonstrating that the \(\lambda\) (74 kDa) isoform of PKC is distributed mostly in the particulate (i.e., membrane + cytoskeletal) cell fractions with a much smaller distribution in the cytosolic fractions, indicating the constitutive activation of atypical \(\lambda\) isoform. Overexpressed PKC-\(\lambda\) isoform is “constitutively active” because reaching this intracellular distribution did not require any stimulus (Fig. 7C). Exposure of these transfected cells to oxidant (Fig. 7D), however, further increased the levels of PKC-\(\lambda\) isoform in the particulate (e.g., membrane) fractions, achieving near total activation levels for \(\lambda\) isoform. Parental-type cells exposed to oxidant (Fig. 7B), as might be expected, showed increased membrane and cytoskeletal (particulate) distribution of native PKC-\(\lambda\), but at substantially lower levels than the transfected overexpressing clones under these conditions. In parental cells exposed to vehicle (Fig. 7A), in contrast, we found a mostly cytosolic distribution of native PKC-\(\lambda\) (indicating intracellular inactivity) with smaller levels in the particulate fractions.

Representative laser scanning confocal micrographs of immunofluorescently labeled PKC-\(\lambda\) corroborates (Fig. 7E, panels a–d) that PKC-\(\lambda\) is present mostly in the same membrane-bound areas in cells exposed to the oxidant (panel b) and cells overexpressing PKC-\(\lambda\) (panels c and d). This is seen in the intracellular appearance of PKC-\(\lambda\) at areas of cell-cell contact (i.e., inner side of plasma membrane, see arrows). In contrast, parental cells exposed to vehicle (panel a) show a mainly cytosolic staining of native PKC-\(\lambda\).

Figure 8 shows the activity levels of the atypical PKC-\(\lambda\) isoform (determined by a sensitive in vitro kinase assay) from immunoprecipitated particulate (membrane) cell fractions of both transfected and parental intestinal cells. There is a substantial increase in the activity levels of PKC-\(\lambda\) in the transfected TRE PKC-\(\lambda\) (vehicle-exposed) cells. Note that when tetracycline is present (TRE PKC-\(\lambda\) + tetracycline), a return to near basal activation levels for \(\lambda\) isoform is seen. As might be expected, the activation levels for the \(\lambda\) isoform in TRE PKC-\(\lambda\)-overexpressing cells is further increased in the presence of added oxidant. In contrast, parental cells exposed to vehicle (with or without tetracycline) show basal activity levels for native PKC-\(\lambda\) in these particulate fractions. In the same parental cells, oxidant further activates native PKC-\(\lambda\), however, at much lower levels than that of transfected overexpressing clones paralleling aforementioned findings in Fig. 7.

Intracellular Distribution and Activation of the PKC-\(\lambda\) Isoform Parallel Several Different Indices of Monolayer Barrier Disruption. Using data from all experimental conditions, we found significant positive correlations (e.g., \(r = 0.94; p < 0.05\)) between PKC-\(\lambda\) levels (in vitro kinase assay or optical density from the particulate fractions) and increased monolayer barrier permeability (FSA clearance), additionally indicating that activation of the PKC-\(\lambda\) isoform is key in intestinal monolayer barrier dysfunction. We note other robust (positive) correlations when other markers of instability such as either microtubule disruption or tubulin disassembly (increased S1 pool) were correlated with the PKC-\(\lambda\) levels (\(r = 0.90, 0.92\), respectively; \(p < 0.05\) for each). Reduced tubulin assembly (decreased S2 pool) and PKC-\(\lambda\) provide an additional robust correlation (\(r = 0.91, p < 0.05\)) further indicating that activation of the PKC-\(\lambda\) isoform is important in monolayer disruption.

Dominant-Negative Inhibition of PKC-\(\lambda\) Inactivates the Native \(\lambda\) Isoform and Attenuates Oxidant-Induced Disruption. Based on the aforementioned findings, PKC-\(\lambda\) appears to play an essential intracellular role in monolayer barrier disruption and possibly in oxidant-induced barrier hyperpermeability. To independently investigate a possible role for PKC-\(\lambda\) in oxidant-induced disruptive effects, we used a dominant-negative approach to stably reduce the steady-state activity of the native (endogenous) PKC-\(\lambda\) isoform. Figure 9 shows activity levels of native PKC-\(\lambda\) isoform from immunoprecipitated particulate fractions of parental type...
Caco-2 cells that were transfected with a PKC-λ dominant-negative cDNA and a plasmid-encoding hygromycin resistance. In these dominant-negative clones, we found a substantial decrease (−99.6%) in the activity of native PKC-λ isoform. Here, oxidant can no longer increase the native λ isoform activity.

In exploratory studies, we observed a dose-dependent effect of varying amounts (1, 2, 3, 4, or 5 μg) of PKC-λ dominant-negative cDNA on prevention of oxidant-induced disruption in intestinal monolayers. Because the clone transfected with 3 μg of dominant-negative plasmid for PKC-λ led to maximum prevention of oxidant-induced barrier disruption, we used this “protective” clone for subsequent inhibition studies.

Although PKC-λ inactivation did not injuriously affect monolayer barrier function (Fig. 10), dominant-negative inactivation of the native PKC-λ did substantially prevent monolayer barrier hyperpermeability induced by 0.5 mM oxidant challenge. This is an oxidant dose that substantially induced barrier dysfunction in parental Caco-2 cells. In fact, a large percentage (∼57 ± 8%) of oxidant-induced monolayer barrier hyperpermeability appears to be dependent on the atypical PKC-λ.

Analysis of the percentage of dominant-negative transfected cells with a normal microtubule cytoskeleton additionally demonstrates (Fig. 11) that dominant suppression of native PKC-λ activity protects against damage to microtubules induced by oxidant insult. As expected, PKC-λ isoform inactivation by itself did not damage the microtubules.

Immunoblotting analysis of tubulin from these same dominant-negative clones further demonstrates (Fig. 12) that without PKC-λ isoform activation, oxidant cannot cause any alterations in tubulin assembly (neither any reduction in the polymerized S2 tubulin nor any increases in monomeric S1 tubulin were seen), indicating protection against microtubule instability.

Discussion

Our main finding is that activation of the atypical λ isoform of PKC plays an important role in oxidant-induced damage to the microtubule cytoskeleton and intestinal monolayer barrier integrity. The mechanism appears to involve destabilization of the molecular dynamics of tubulin polymerization in intestinal epithelium. Moreover, PKC-λ by itself is sufficient to cause cytoskeletal and barrier instability. These conclusions were supported by several independent lines of evidence as discussed below.

First, exposure of parental monolayers to oxidant translates and activates native PKC-λ and evokes a sequence of changes that are consistent with our conclusions. Oxidant induces activation of a specific PKC isoform λ, increases the unstable monomeric tubulin pool while decreasing the polymerized tubulin pool, injures the microtubule cytoarchitecture, and disrupts monolayer barrier function.

Second, overexpression of atypical PKC-λ (in the absence of added oxidant) induces an oxidant-like instability and damage in sense-transfected cells. In these clones, PKC-λ results in an identical and consistent cascade of injurious alterations. These disruptive effects appear to require overexpression and activation of PKC-λ. For instance, loss of barrier function and cytoskeletal assembly required activation of PKC-λ, involving its redistribution into the particulate (cytoskeletal + membrane) fractions.

Third, transfected clones overexpressing PKC-λ appear to be more sensitive to damage induced by oxidant challenge. In these stable sense-transfected clones, induction of PKC-λ expression potentiates the injurious effects of added oxidant. This hypersensitivity requires not only overexpression, but also activation of PKC-λ.

Fourth, in dominant-negative transfected clones, which show inactivation of PKC-λ isoform, the ability of oxidants to cause disruption was substantially prevented. Indeed, in these mutant clones, significant protection was afforded against monolayer instability.

Finally, PKC-λ activation correlates positively and robustly with the changes in several markers of damage in both transfected and parent-type cells. These include correlations between 1) tubulin disassembly (increased S1 monomer pool) and PKC-λ activation; 2) microtubule instability (percent normal) and PKC-λ activation; 3) oxidant-induced decreases in tubulin assembly (i.e., decreased S2 polymer pool).
and PKC-λ activation; and 4) increased monolayer barrier permeability (FSA clearance) and PKC-λ activation. For each correlation, 90 to 95% of the variance was explained, further indicating that increased activation of PKC-λ is likely crucial to the disruptive effects of oxidant on the intestinal cytoskeletal and barrier function.

It should be noted that the pattern of microtubule disruption induced by oxidant in parental cells is similar, but not identical, to the pattern observed in cells overexpressing PKC-λ (Fig. 4). This finding suggests that the mechanisms involved overlap but are not necessarily the same. Indeed, the potentiation findings presented for microtubules in which incubation of PKC-λ-overexpressing cells with 0.5 mM oxidant led to a significantly greater number of cells with ab-
normal microtubules than either condition alone supports this view. Similarly, the partial protection against oxidant-induced hyperpermeability, provided by the dominant-negative mutant, further supports this view. Nonetheless, the fact remains that dominant mutant inhibition of PKC-ε substantially protects against oxidant-induced damage to both the barrier function and microtubules (e.g., 83 ± 5% of mutant cells display normal/protected microtubules under oxidant challenge compared with 39 ± 4% of parental cells under the same condition). Overall, these findings indicate that a significant portion, but not all, of the disruption is through a similar PKC-ε-mediated mechanism.

Activation of PKC-λ shares its disruptive ability with another PKC isoform, PKC-δ. We recently reported that PKC-δ, a 75-kDa member of the novel subfamily of PKC isoforms, mediates a portion (approximately 50 ± 10%) of oxidant-induced disruption (Banan et al., 2002a). In the current study, PKC-λ appeared to mediate approximately 50 ± 8% of oxidant-induced disruption. Based on these findings, it is reasonable that activation of these isoforms accounts for

100% of oxidant-induced disruption in intestinal epithelium. Although PKC-δ and PKC-λ share in common an ability to disrupt the intestinal barrier, there appear to be differences between them. For instance, PKC-δ requires cofactors such as ionized Ca²⁺, whereas the PKC-λ isoform does not. This difference is fully consistent with the fact that PKC-λ belongs to the atypical subfamily of PKC isoforms, whereas PKC-δ belongs to the novel subfamily of PKCs (Banan et al., 2002a). This also suggests that epithelium has developed more than one way to respond to oxidant challenge. Nevertheless, this is the first time PKC-λ-dependent mechanisms have been ascribed to the disruption of epithelial cells.

Our findings are also consistent with known biochemical properties of PKC (e.g., Goodnight et al., 1995; Wang et al., 1996; Mullin et al., 1998; Gopalakrishna and Jaken, 2000). PKC isoforms are typically inactive in resting cells. In this inactive conformation, PKC isoforms are typically distributed in the cytosol and only loosely bound to particulate cell fractions. Upon activation, in general, PKC isoforms redistribute into membrane/particulate fractions. For example, a pharmacological report (Chang and Tepperman, 2001) proposed that activation (translocation to the particulate fraction) is necessary for the observed effects of PKC-ε. Because
main of the PKC- plus C2 domain, which is different from the regulatory do-

gor site, which is involved in the activation of PKC isozymes with respect to these functional domains suggest (Gopalakrishna and Jaken, 2000). Thus, differences among

Banan et al., 2002c). For example, regulatory domain of the

PKC-1 isoform consists of zinc fingers (C1A + C1B)

plus C2 domain, which is different from the regulatory do-

main of the PKC-1 isoform from the classical subfamily

(Gopalakrishna and Jaken, 2000). Thus, differences among

isosymes with respect to these functional domains suggest

that each PKC isoform has a distinct activation mechanism

(Cho et al., 1998; Mullin et al., 1998; Gopalakrishna and

Jaken, 2000). Moreover, accumulated evidence suggests that

modifications of the regulatory domain of a PKC isoform can

lead to the activation of that particular isotype (Cho et al.,

1998, Gopalakrishna and Jaken, 2000). To achieve this for

example by PKC-1, binding of this isoform to phospholipids,

especially anionic phosphatidylserine, in membranes (i.e.,

translocation to membranes) is thought to be necessary to

cause conformational changes to its regulatory domain prior
to activation. Specifically, structural studies (Gopalakrishna

and Jaken, 2000) demonstrate that in the presence of induc-
ers (e.g., overexpression by transfection as we have done in

the current study), this regulatory domain (especially the

zinc finger portion) forms an automatic “hairpin-like” hydro-

phobic structure that mediates PKC interaction with the

membrane lipids and subsequent conformational changes

within the regulatory domain leading to “autoactivation”.

Thus, a 3-fold overexpression of PKC-1 may promote a con-

formational change that releases the “inherent autoinhibi-
tion” present and triggers kinase catalytic activity.

Inducible nitric-oxide synthase (iNOS) is one of the major
hyperactivation in intestinal cells. Specifically, PKC-isoform (Banan et al., 2001b, 2002d, 2003a). We showed that the function as was shown by recent studies from our laboratory and its deleterious consequences on cytoskeleton and barrier PKC isoform activity leads to increased iNOS up-regulation isoform activity. This mechanism requires that increased enzyme (and its downstream events) by changes in PKC barrier instability: up-regulation of proinflammatory iNOS mechanism by which the PKC-isoform leads to cytoskeletal and barrier disruption. Based on the known disruptive mechanisms, we previously showed that the atypical ζ isoform of PKC (a protective PKC) targets iNOS (in an opposite direction) in the same intestinal model, leading to its down-regulation and monolayer protection (Banan et al., 2002d). It remains to be seen whether iNOS activity is altered following increased PKC-λ activity and whether PKC-λ up-regulates iNOS and its damaging oxidative consequences (reactive nitrogen metabolites, nitric oxide/peroxynitrite) in epithelial cells.

How oxidants activate PKC is not fully understood. It appears that tyrosine phosphorylation of PKC-δ by oxidants increases its enzymatic activity and promotes oxidative processes in a variety of cell lines including Caco-2 cells (Konishi et al., 1999; Otieno and Kensler, 2000; Sun et al., 2000; Cerda et al., 2001; Majumder et al., 2001). The consequences of this increased PKC activity also include loss of mitochondrial membrane potential, increased apoptosis, up-regulation of ornithine decarboxylase, loss of anchorage, and decreased cell growth (e.g., Otieno and Kensler, 2000; Cerda et al., 2001; Majumder et al., 2001). That the set of PKC isoforms have such wide-ranging effects is consistent with our suggestion above, that activating or mimicking a specific isoform of PKC will have a specific, i.e., unique effect on the GI epithelium. Apparently, this can include “protection” and as our study clearly shows “disruption” of cells as well. Nonetheless, our current study on PKC-λ isoform suggests a new role for this isoform among the atypical subfamily of PKC isoforms—mediation of oxidant-induced disruption of cytoskeletal and barrier integrity.

Our previous reports (e.g., Banan et al., 2001a, 2004) suggest a possible second mechanism (target) for PKC-λ-induced effects—protein phosphorylation of tubulin. We showed (Banan et al., 2001a) that the PKC activator OAG (a synthetic version of second messenger diacylglycerol) or PKC-β1 over-expression causes an increase in serine phosphorylation of the tubulin subunits of microtubules. This increase was prevented by antisense to PKC-β1. Moreover, the θ isoform of PKC appears to complex with tubulin as well as alter tubulin phosphorylation (and barrier function) in intestinal cells (Banan et al., 2004), suggesting that this PKC isoform may be acting directly or indirectly on these cytoskeletal protein subunits. This phosphorylation mechanism is consistent with previous studies. For example, PKC has been implicated in rearrangement of the cytoskeleton (Hartwig et al., 1992; Goodnight et al., 1995) although it is not clearly known which PKC isoforms are key in this process. Previous reports have proposed that PKC is capable of phosphorylating the cytoskeletal proteins, talin and vinculin (Goodnight et al., 1995). Furthermore, a major specific substrate for PKC, myristoylated alanine-rich PKC substrate, was proposed to be a cytoskeletal remodeler (Hartwig et al., 1992). Specifically, myristoylated alanine-rich PKC substrate cytoskeletal organizing activity is inhibited by PKC-mediated phosphorylation. Alternatively, PKC isoforms may target phosphorylation of one of the nontubulin- and/or tubulin-associated capping proteins (e.g., actin, microtubule-associated proteins). Further studies will be needed to explore the nature of the interactions between PKC isoforms and their targets, especially cytoskeleton in intestinal epithelial cells.

Our demonstration that PKC-λ mediates oxidant-induced injury is potentially relevant for developing new treatment strategies for IBD. It suggests a novel PKC-λ-dependent dis-

![Image](https://via.placeholder.com/150)

**Fig. 12.** Dominant-negative inactivation of PKC-λ protects against the deleterious changes in tubulin assembly induced by oxidant assessed by immunoblotting analysis of intracellular tubulin pools. The polymerized tubulin (S2) and monomeric tubulin (S1) were extracted and processed. Conditions were similar to those in Fig. 11. Percentage of polymerized tubulin = \([S2]/(S2 + S1)\). *p < 0.05 versus vehicle. +, p < 0.05 versus \(H_2O_2\) in parental cells. [tTA parental] = parental cells. [Negative Dom.] = dominant-negative inhibition of PKC-λ activity. n = 6 per group.
ruptive mechanism that might contribute to initiation or maintenance of the IBD attack. This mechanism is consistent with the current characterizations of the pathophysiology of the GI inflammation, in particular. For instance, high levels of oxidants, including H$_2$O$_2$ as well as loss of mucosal barrier integrity, have been reported in the intestinal mucosa of patients with ulcerative colitis and Crohn’s disease (Holland, 1988; Keshavarzian et al., 1992; 2003; McKenize et al., 1996; Banan et al., 2000c). We have shown (Keshavarzian et al., 1992, 2003; Banan et al., 2000c) that a high level of oxidant stress occurs in intestinal mucosa of these IBD patients. We reported (e.g., Keshavarzian et al., 2003) the amount of oxidant stress correlated with the degree of mucosal inflammation and disease severity index as well as with instability of key cellular organelles (e.g., cytoskeleton). Moreover, oxidative stress is thought to be especially important in the transitions from the inactive to the active (flare up) phase of inflammation in IBD in which intestinal oxidants generated during inflammation and the ensuing processes periodically create a vicious cycle that can lead to mucosal oxidative stress, barrier dysfunction, and, consequently, to structural tissue damage. The disruptive effects of PKC-α, such as the ones we report here, could play a pivotal role in promoting the establishment and continuation of such a vicious inflammatory cycle.

In summary, our findings demonstrate an original concept that the activation of the atypical PKC-α isoform can substantially disrupt the dynamics of cytoskeletal assembly and barrier function and that it appears to be essential for oxidant-induced monolayer damage in intestinal epithelium. These new insights may prove useful because targeting the inhibitory PKC isoforms could lead to development of new therapeutic agents for the treatment of a variety of inflammatory disorders of the GI tract (e.g., IBD) that are caused by oxidative injury.

References


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