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Inhibition of Oxidant-Induced NF-κB Activation & I-κBα Degradation and Instability of F-actin Cytoskeletal Dynamics & Barrier Function by EGF: Key Role of PLC-γ Isoform

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Abbreviated Title: PLC-γ in NF-κB Inactivation in Gut Epithelium

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List of non-standard abbreviations: Wild type cells = [**WT**]; Cells transfected with PLC-γ sense = [**PLC-γ**]; Dominant negative inhibition of PLC-γ = [**PLC-z**]; Inflammatory bowel disease = IBD.

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ABSTRACT

Using monolayers of intestinal (Caco-2) cells as a model for studying IBD, we previously showed that NF-κB activation is required for oxidant-induced disruption of cytoskeletal and barrier integrity. EGF stabilizes the F-actin cytoskeleton and protects against oxidant damage, but the mechanism remains unclear. We hypothesized that the mechanism involves activation of phospholipase C-γ (PLC-γ) which prevents NF-κB activation and the consequences of this activation, namely cytoskeletal and barrier disruption. Methods: We studied wild type (WT) and transfected cells. The latter were transfected with varying levels (1-5 μg) of cDNA to either stably over-express PLC-γ or to inhibit its activation. Cells were pretreated with EGF before exposure to oxidant (H_2O_2). Results: Stably over-expressing PLC-γ (+2.0 fold) or pre-incubating with EGF protected against oxidant injury as indicated by 1) decreases in several NF-κB-related variables **[**NF-κB (p50 / p65 subunit) nuclear translocation, NF-κB subunit activity, I-κBα phosphorylation & degradation**]**; 2) increases in F-actin & decreases in G-actin; 3) stabilization of the actin cytoskeletal architecture; and 4) enhancement of barrier function. Over-expression induced inactivation of NF-κB was potentiated by EGF. PLC-γ was found mostly in membrane and cytoskeletal fractions (<9% in the cytosolic fractions), indicating its activation. Dominant negative inhibition of endogenous PLC-γ (-99%) substantially prevented all measures of EGF protection against NF-κB activation. Conclusions: 1) EGF protects against oxidant-induced barrier disruption through PLC-γ activation, which inactivates NF-κB; 2) Activation of PLC-γ by itself is protective against NF-κB activation; 3) The ability to modulate the dynamics of NF-κB / I-κBα is a novel mechanism not previously attributed to the PLC family of isoforms in cells; 4) Development of PLC-γ mimetics represents a possible new therapeutic strategy for IBD.

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The *epithelium* of the gastrointestinal (GI) mucosa functions as a selective permeability barrier that excludes the penetration of pro-inflammatory and immunoreactive antigens (e.g., LPS, endotoxin) while it allows the absorption from the lumen of water, nutrients and electrolytes into the mucosa. Loss of *barrier function* permits penetration of luminal antigens, endotoxin, and other harmful pro-inflammatory agents into the mucosa and systemic circulation and leads to the initiation or continuation of inflammatory processes and tissue injury (Hollander et al., 1998; Keshavarzian et al., 1992, 1999; Banan et al., 2000a). Indeed, disruption of barrier function (i.e., hyperpermeability) appears to contribute to the pathogenesis of a variety of GI and systemic illnesses (Hollander et al., 1998; Keshavarzian et al., 1999, 2003). Intestinal barrier hyperpermeability ("leaky gut") has often been reported and implicated in the pathogenesis of inflammatory bowel disease (IBD) (Hermitson and Gordon, 1995; Hollander et al., 1998; Keshavarzian et al., 2003; Yamada et al., 1993; Irvine and Marshall, 2000). For example, barrier hyperpermeability induced by the injection of peptidoglycan-polysaccharide (PG-PS) into the mucosa can elicit an inflammatory condition similar to IBD (Yamada et al., 1993). Transgenic animals with a constitutively *leaky* gut barrier function show symptoms of intestinal inflammation (Hermitson and Gordon, 1995).

Although the pathophysiology of mucosal barrier hyperpermeability in IBD remains unclear, it is known that chronic inflammation in IBD is dependent on high levels of oxidants such as H_2O_2 (Banan et al., 2000a,c; Keshavarzian et al., 1992, 2003; McKenizie et al., 1996) as well as on activation of NF-kappa B (NF-κB) (Neurath et al., 1999; Rogler et al., 1998; Shreiber et al., 1998; Banan et al., 2003a, b,d). In fact, both oxidant stress and NF-κB induction are important to the promotion of an inflammatory response and key in mucosal damage during IBD (Banan et al., 2003a,b,d, 2000a,c; Barnes and Karin, 1997; Keshavarzian et al., 2003; Neurath et al., 1999; Shreiber et al., 1998; Jobin et al., 1999). NF-κB is typically composed of p50 and p65 subunits and is tightly modulated by an endogenous cytosolic inhibitor, I-Kappa B-α (I-κBα) which complexes with the p65 protein and traps NF-κB in the cytosol in an inactive form. When activated NF-κB modulates key cellular processes involved in an inflammatory response such as the upregulation of iNOS-driven oxidative processes (Barnes and Karin, 1997; Banan et al., 2003a). Using monolayers of intestinal *cells*, we reported a new mechanism that NF-κB activation is crucial in oxidant-induced disruption of epithelial barrier function (Banan et al., 2003a). In view of these considerations, understanding how gut barrier function is *protected* against oxidative, proinflammatory conditions of NF-κB activation is of substantial clinical and biologic value.

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In an effort to better understand key endogenous defensive mechanisms, we have been investigating events underlying oxidant-induced mucosal damage and barrier disruption and protection against this damage mediated by growth factor-triggered signaling processes. Our hope has been to devise new strategies for the development of more effective treatment regimens for inflammatory disorders of the GI tract, in general, and IBD, in particular. For instance, using monolayers of intestinal cells as a well-established model of gut barrier function, we showed that cytoskeletal disassembly/instability is a key event in oxidant-induced injury (e.g., Banan et al., 2000a,b) and that growth factor (EGF or TGF-α) prevents injury by stabilizing the cytoskeleton in large part through a signaling pathway mediated by EGF-receptor and then phospholipase C-gamma (PLC-γ) (Banan et al., 2001c, 2003c). The involvement in protective mechanisms by PLC-γ in the GI epithelium as we originally reported was a novel concept (Banan et al., 2001c). We showed using wild type (naive) intestinal Caco-2 cells that following activation of EGF-receptor, EGF causes the distribution of the *native* PLC-γ isoform into the cell membrane and thus considered this PLC isoform as a potential contributor to EGF-mediated protection of the GI epithelial barrier function. Using targeted molecular interventions, we then found that maintaining an intact actin cytoskeleton is necessary for protection of intestinal barrier by EGF apparently through PLC-γ (Banan et al., 2002a). Despite the crucial importance of the -γ isoform of PLC to intestinal barrier function, the molecular mechanisms underlying PLC-γ-mediated, EGF-induced, protection of monolayer barrier and actin cytoskeletal integrity are poorly understood.

Accordingly, in the current investigation, we tested the hypothesis that PLC-γ not only prevents oxidant-induced NF-κB activation and I-κBα degradation, but also that it is key to EGF-mediated protection against the damaging consequences of this activation through the stabilization of I-κBα. We used both pharmacological and targeted molecular interventions employing several transfected intestinal cell lines. In several clones the full-length PLC-γ isoform was reliably over-expressed; in the other clones, endogenous PLC-γ activity was inhibited. We now report new mechanisms – suppression of the stress of NF-κB activation and of cytoskeletal instability– by the -γ isoform of PLC in epithelial monolayers. To the best of our knowledge, this is the first time that PLC-γ has been demonstrated to inhibit the dynamics of NF-κB and I-κBα in cells.

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MATERIALS AND METHODS

Cell culture. Caco-2 cells were obtained from ATCC (Rockville, MD) at passage 15. This cell line was chosen because they form monolayers that morphologically resemble small intestinal cells, with defined apical brush borders and a highly organized actin network upon differentiation (Banan et al., 2000b; Meunier et al., 1995). Cells were maintained at 37^{o}C in complete Dulbecco's minimum essential medium (DMEM) in an atmosphere of 5% CO_2 and 100% relative humidity. Wild type cells or stably transfected cells (see below) were split at a ratio of 1:6 upon reaching confluency, and set up in either 6 or 24 well plates for experiments, or T-75 flasks for propagation. The utility and characterization of this cell line has been previously reported (Meunier et al., 1995).

Plasmids and Stable Transfection. The sense and dominant negative plasmids of PLC-γ were constructed and then stably transfected by Lipofectin (Lipofectin reagent, GIBCO BRL) as we previously described (Banan et al., 2002a, 2001c). Expression was controlled by SV40 early promoter present in pXf vector. The dominant negative PLC-γ1 fragment from the Z region (designated as *PLCz*) of human PLC-γ1, which covers the SH2 and SH3 domains (amino acids 517-901), was isolated by reverse transcriptase/PCR and cloned into an eukaryotic expression vector, pXf (Chen et al., 1994; Turner et al., 1997). Control conditions included vector (pXf) alone. Multiple clones stably over-expressing PLC-γ or lacking PLC-γ activity were assessed by immunoblotting as well as tested for PLC-γ activity (see below). These cells were then plated on Biocoat Collagen I Cell Culture Inserts (Becton Dickinson, Bedford, MA) and subsequently used for experiments.

Experimental Design. In the first series of experiments, post-confluent monolayers of wild (naive) type cells were preincubated with EGF (1 to 10 ng/mL) or isotonic saline for 10 min and then exposed to oxidant (H_2O_2, 0 to 0.5 mM) or vehicle (saline) for 30 min. As we previously showed, H_2O_2 at 0.1 to 0.5 mM disrupts F-actin and barrier integrity and activates NF-κB (Banan et al., 2000a,b, 2003a). EGF at 10 ng/mL (but not 1 ng/mL) prevents both actin and barrier disruption. These experiments were then repeated using stably transfected cells. In all experiments we assessed actin cytoskeletal stability (cytoarchitecture, assembly, disassembly), actin molecular dynamics (F- and G-actin pools), I-κBα distribution (cytosolic expression and degradation), I-κBα phosphorylation, NF-κB p65 subunit activity (cytosolic levels, both nuclear translocation & activity), NF-κB p50 subunit activity (cytosolic levels, both nuclear translocation & activity), barrier integrity (permeability), and PLC-γ subcellular distribution as well as activity (immunoblotting, immunoprecipitation & *in vitro* assay).

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In the second series of experiments, cell clone monolayers that were stably over-expressing PLC-γ were preincubated (10 min) with EGF (1, 10 ng/mL) or vehicle prior to exposure (30 min) to damaging concentrations of oxidant (H_2O_2, 0.5 mM) or vehicle. Outcomes measured were as described above.

In the third series of experiments, monolayers of dominant negative, PLCz transfected cells stably lacking PLC-γ activity were treated with high (protective) doses of EGF and then oxidant. In corollary series of experiments, we investigated the effects of PLC-γ activation or inactivation on the state of (1) I-κBα degradation, phosphorylation, & stabilization, (2) NF-κB activation & inactivation, (3) actin assembly & disassembly, and (4) cytoarchitecture of the actin cytoskeleton.

Fractionation and Immunoblotting of PLC-γ. Cell monolayers grown in 75 cm^2 flasks were processed for the isolation of the cytosolic, membrane, and cytoskeletal fractions (Banan et al., 2003c, 2002b). Protein content of the various cell fractions was assessed by the Bradford method (Bradford, 1976). For immunoblotting, samples (25 μg protein/lane) were added to a standard SDS buffer, boiled, and then separated on 7.5% SDS-PAGE. The immunoblotted proteins were incubated with the primary mouse monoclonal anti-PLC-γ (Santa Cruz Biotech, CA) at 1:1,000 dilution. A horse radish peroxidase-conjugated goat anti-mouse antibody (Molecular Probes, OR) was used as a secondary antibody at 1:4000 dilution. Proteins were visualized by enhanced chemiluminescence (ECL, Amersham, IL) and autoradiography, and subsequently analyzed. The identity of the PLC-γ bands were confirmed (Banan et al., 2001c) by {1} using a PLC-γ blocking peptide in combination with the anti-PLC-γ antibody that prevents the appearance of the corresponding "major" band in western blots. {2} Additionally, in the absence of the primary antibody to PLC-γ, no corresponding band for PLC-γ was observed. {3} The PLC-γ band ran at the expected molecular weight of 145 kDa as confirmed by a known positive control for PLC-γ (from rat brain lysates). {4} Pre-stained molecular weight markers (M_r 34,900 and 205,000) were run in adjacent lanes. We also confirmed (Banan et al., 2003c, 2001c) that over-expression of PLC-γ or dominant negative inhibition of PLC-γ did not affect the relative expression levels of other PLC isoforms nor did it injure the Caco-2 cells.

Immunoprecipitation and PLC-γ Activity. Immunoprecipitated PLC-γ was collected and processed for its ability to form [^{3}H]inositol phosphates (Banan et al., 2001c). Briefly, 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, anti-protease cocktail (10 μg/ml), 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, the lysates were incubated for 2 h at 4°C with monoclonal anti-PLC-γ (1:2,000

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dilution, in excess). The extracts were then incubated with protein G–Sepharose for 1 h at 4°C. The immunocomplexes were collected by centrifugation (2,500 g, 5 min) in a microfuge tube, washed 3 times with immunoprecipitation buffer containing 5 mM Tris-HCl, pH 7.4, and 0.2% triton X-100. They were then washed 1 time with sample buffer (20 mM Hepes, pH 7.5) and resuspended in 20 µL of buffer and 5 µL of reaction buffer **{**5 µCi/ml of [^{3}H]myoinositol**}** plus LiCl (10 mM, which inhibits inositol phosphate hydrolysis) and subsequently incubated for 5 min at 30°C. Reactions were then stopped by the addition of 8 µL of 5 X sample buffer, and the [^{3}H]inositol phosphates (IP) were recovered in the supernatant after centrifugation (16,000g, 5 min). The extracts were separated on a Dowex formate ion-exchange mini-columns (Bio-Rad, Hercules, CA). Radioactivity present (IP content) in samples was quantified by scintillation counting with aqueous counting scintillant. Counts for blanks were subtracted from the sample activity. Sample activity was also corrected for protein concentration (Bradford Method), and PLC-γ activity was reported as pmol/min/mg protein.

Analysis of NF-κB Activation. NF-κB (p65 and p50 subunit) activation was assessed by a unique ELISA procedure (Banan et al., 2003a). Monolayers of naive and transfected cells grown in 25 cm^2 flasks were processed for the isolation of the cytosolic and nuclear fractions. Cells fractions were added to a 96-well plate to which oligonucleotides containing a consensus-binding site for the NF-κB had been immobilized (Trans-Am, Active Motif, Carlsbad, CA). The NF-κB activity test is based on a validated ELISA principle in which NF-κB is captured by a double stranded oligonucleotide probe containing the consensus binding sequence for either NF-κB p65 or p50 subunits. Consequently, only the activated NF-κB is captured by the probe bound in microwell plates. The binding of NF-κB to its consensus sequence was then detected using a primary anti-NF-κB (p65 or p50) antibody (Santa Cruz Biotech, Santa Curz, CA), followed by a secondary antibody conjugated to horse radish peroxidase. The results were quantitated by a chromogenic reaction, which was then read for absorbance at 450 nm by a Seivers NOA 280 microplate analyzer (Sievers, CO).

Western Blot Analysis of Changes in NF-κB Subunit Levels and Nuclear Translocation. Cellular nuclear and cytosolic extracts from naive and transfected cells were prepared as above. Briefly, NF-κB nuclear translocation was determined by comparing the levels of NF-κB protein expression in the cytosolic *versus* nuclear extracts by anti-p65 and anti-p50 antibodies using a non-denaturing gel (6%) (Banan et al., 2003a). For immunoblotting, samples (20 µg protein per lane) were placed in a standard sample buffer, boiled, and then subjected to PAGE. Proteins were visualized by enhanced chemiluminescence (ECL, Amersham, IL)

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and autoradiography. For comparison of different blots, standard (positive) loading controls (20 μg of Hella cell extracts/lane) for NF-κB were run concurrently with each run.

Western Blot Analysis of I-κBα Degradation, Expression Levels, and Phosphorylation. I-κBα levels of expression in the cytosolic extracts as well as its degradation (i.e., disappearance from the cytosolic fractions) were assessed by anti-I-κB-α antibody (Santa Cruz Biotech) using a western blot protocol (10% gel) (Banan et al., 2003a). Samples (20 μg protein/lane) were added to a standard SDS buffer, boiled, and then separated on SDS-PAGE. For assessment of the I-κB-α phosphorylation levels an anti-phospho-I-κB-α (Ser 32/36) was utilized. Separate blots were done for I-κB-α phosphorylation and I-κB-α expression levels. As for NF-κB, proteins were visualized by enhanced chemiluminescence and subsequently autoradiographed. Standard (positive) loading controls (20 μg of Hella cell extracts/lane) for I-κB-α were included in each run. To quantify the relative levels of I-κBα (e.g., to monitor its degradation), the optical density of the bands corresponding to immunolabelled I-κBα were measured with a laser densitometer.

Immunofluorescent Staining and High-Resolution Laser Scanning Confocal Microscopy of Actin Cytoskeleton. Cells from monolayers were fixed in cytoskeletal stabilization buffer and then post-fixed in 95% ethanol at -20^{o}C (Banan et al., 2000b). Cells were subsequently processed for incubation with FITC-phalloidin (specific for F-actin staining, Sigma, St. Louis, MO), 1:40 dilution for 1 h at 37^{o}C. Following staining, cells were observed with an argon laser (λ = 488 nm) using a 63 X oil immersion plan-apochromat objective, NA 1.4 (Zeiss, Germany). The cytoskeletal elements were examined in a blinded fashion for their overall morphology, orientation, and disruption (Banan et al., 2001a, 2000a,b). The identity of the treatment groups for all slides was decoded only after examination was complete.

Actin Fractionation and Quantitative Western Immunoblotting of F-Actin and G-Actin. Polymerized (F-) actin and monomeric (G-) actin were isolated using a especially developed series of extraction and ultra-centrifugation steps (Banan et al., 2000b). Cells were gently scraped and pelleted with centrifugation at low speed (700 rpm, 7 min, 4^{o}C) and resuspended in Actin Stabilization-Extraction Buffer **[**0.1 M PIPES, pH 6.9, 30% glycerol, 5% DMSO, 1 mM $MgSO_4$, anti-protease cocktail (10 μg/mL), 1 mM EGTA, and 1% triton X-100**]** at room temperature for 20 min. Actin fractions were separated following a series of centrifugation and extraction steps. Cell lysates were centrifuged at 105,000 g for 45 min at 4°C and the supernatant containing the soluble monomeric pool of G-Actin (or S1) was gently removed. The remaining pellet was then resuspended in 0.3 mL Ca^{+2}-containing depolymerization buffer **[**0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, anti-protease cocktail (10 μg/mL), and 10 mM $CaCl_2$**]** and incubated on ice for 60

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min. Subsequently, samples were centrifuged at 48,000 g for 15 min at 4°C and the supernatant (S2 or F-fraction or cold/Ca^{+2}-soluble fraction) was removed. To ensure the complete removal of the F-fraction, the remaining pellet was treated with the Ca^{+2}-containing depolymerization buffer twice more by resuspension and centrifugation. The "actin" was recovered by separately incubating (at 37°C for 30 min) the S1 and S2 fractions with stabilizing agents, phalloidin (1 μM) and ATP (0.1 mM), in Actin Stabilization Buffer **[**0.1 M Pipes, pH 6.9, 30% glycerol, 5% DMSO, anti-protease cocktail (10 μg/mL), 1 mM EGTA, 1 mM $MgCl_2$, and 0.1 mM ATP**]** to promote polymerization of actin. Actin was then recovered by centrifugation and resuspended in the above stabilization buffer. Fractionated S1 and S2 samples were then flash frozen in liquid N_2 and stored at -70^{o}C until immunoblotting. For immunoblotting, samples (5 μg protein per lane) were placed in a standard SDS sample buffer, boiled, and then subjected to PAGE on 7.5% gels. Standard (purified) actin controls (5 μg/lane) were run concurrently with each run. To quantify the relative levels of actin, the optical density of the bands corresponding to immunolabelled actin were measured with a laser densitometer (Banan et al., 2000b).

Determination of barrier permeability by fluorometry. Status of the integrity of monolayer barrier function was *confirmed* 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 described (e.g., Banan et al., 2003a,c, 2001a,b,c, 2000a,b). Briefly, 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). 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/cm^2) = Fab/([FSA]a X S), where Fab is the apical to basolateral flux of FSA (light units/h), [FSA]a is the concentration at base line (light units/nL), and S is the surface area (0.3 cm^2). Simultaneous controls were performed with each experiment.

Statistical Analysis. Data are presented as mean ± SEM. All experiments were carried out with a sample size of at least 6 observations per treatment group. Statistical analysis comparing treatment groups was performed using analysis of variance followed by Dunnett's multiple range test (Harter, 1960). Correlational analyses were done using the Pearson test for parametric analysis or, when applicable, the Spearman test for non-parametric analysis. *P* values < 0.05 were deemed statistically significant.

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RESULTS

Our earlier findings (Banan et al., 2003c) showed that intestinal cells transfected with PLC-γ sense stably over-express the -γ {145 kDa} isoform of phospholipase C (~2.3 fold compared to wild type cells) and that this over-expression either by itself or in synergy with added growth factor (EGF) protects monolayer barrier integrity against oxidative stress. In the present investigation, using pharmacological and molecular biological interventions, we have investigated the molecular mechanisms underlying PLC-γ mediated protection.

Importance of PLC-γ isoform in protection against oxidant-induced NF-κB activation: Inhibition of both nuclear distribution and activation of NF-κB subunits, p65 and p50. We surmised that PLC-γ protection could be due to the suppression of oxidant triggered pathways such as the pro-inflammatory NF-κB. Using our wild type and transfected clones, we measured both the nuclear distribution of NF-κB subunits (p65 and p50) as well as their activation under conditions of oxidant challenge. In wild type cells (those not over-expressing PLC-γ), oxidant H_2O_2 alone induced substantial activation of both NF-κB subunits (*Figs. 1A and IB*). On the other hand, over-expression of PLC-γ [PLC-γ] by itself afforded substantial protection against NF-κB subunits activation. This inactivation did not necessitate the presence of growth factor, EGF. Although a low (non-protective) dose of EGF (e.g., 1 ng/mL) did not afford significant protection against NF-κB activation in wild type cells, this concentration of growth factor did potentiate NF-κB inactivation in PLC-γ over-expressing clones. In wild type cells, higher (protective) doses of EGF (e.g., 10 ng/mL) were required for NF-κB inactivation. Transfection of only the empty vector, as might be expected, did not suppress NF-κB activation. For instance, the level of p65 subunit that was activated was 0.09 ± 0.02 (OD 450 nm) for vector-transfected cells exposed to vehicle, 1.56 ± 0.20 for vector-transfected cells exposed to H_2O_2 alone and 0.27 ± 0.05 for PLC-γ sense transfected cells incubated in H_2O_2. Similarly, the level of p50 subunit that was activated was 0.10 ± 0.03 for vector-transfected cells exposed to vehicle, 1.46 ± 0.15 for vector-transfected cells exposed to H_2O_2 alone and 0.32 ± 0.06 for PLC-γ sense transfected cells incubated in H_2O_2. These alterations did not appear to be due to changes in the ability of oxidants to cause NF-κB activation as empty vector-transfected cells and wild-type cells responded in a similar manner to H_2O_2, both exhibiting comparable NF-κB activation.

Multiple clones of intestinal cells transfected with 1 to 5 μg of PLC-γ sense plasmid demonstrated (*Table I*) a dose-dependent inhibition of oxidant (H_2O_2)-induced NF-κB activation. The cell clone

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transfected with 3 μg of PLC-γ sense (+γ3) provided maximum (~85%) suppression of NF-κB activity and thus was utilized in subsequent experiments.

Representative western blots of the changes in NF-κB subunit distribution in the cell nuclear fractions are shown in *figures 1C and 1D*. In transfected clones, PLC-γ over-expression inhibits oxidant-induced distribution of the p50 *(1C)* and p65 *(1D)* subunits of NF-κB into the cell nuclear fractions as shown by band densities that were reduced to a level comparable to the controls. In wild type cells, as for NF-κB inactivation, only high (protective) doses of EGF (e.g., 10 ng/mL) prevented NF-κB nuclear distribution. In these same cells, oxidant caused the distribution of NF-κB subunits to the nucleus, paralleling findings on NF-κB activation.

Figure 2 shows a time course for changes in NF-κB activation in wild type cells and its suppression in PLC-γ over-expressing clones. These data further corroborate the aforementioned findings. Maximal fold increase in NF-κB activation by H_2O_2 alone is ~20; this increase is suppressed by PLC-γ over-expression.

Key role of PLC-γ in stabilization of cytosolic I-κBα: inhibition of I-κBα degradation. Because oxidants increase both I-κBα degradation and NF-κB activation as well as disrupt monolayer barrier permeability (Banan et al., 2003a), we surmised that decreases in degradation of I-κBα (a 37 kDa endogenous inhibitor of NF-κB) is a critical mechanism underlying PLC-γ induced inactivation of NF-κB under conditions of oxidant challenge.

To this end, multiple clones of intestinal cells transfected with 1 to 5 μg of PLC-γ sense cDNA demonstrated (*Table I*) a dose-dependent stabilization (absence of degradation) of cytosolic I-κBα against H_2O_2 exposure. As for NF-κB inactivation, the 3 μg clone of PLC-γ (+γ3) led to the highest level of protection against I-κBα degradation. For example, there was a substantial decrease in oxidant-induced I-κBα degradation (~70% less degradation) in the 3 μg PLC-γ over-expressing clone, which is similar to the steady-state levels of I-κBα seen in the controls. PLC-γ expression induced stabilization of I-κBα did not require EGF. But, a low concentration (1 ng/mL) of EGF, which by itself did not afford stabilization of I-κBα in wild type cells, potentiated I-κBα stability in clones over-expressing PLC-γ (not shown). In wild type cells, similar to NF-κB inactivation, I-κBα stabilization required a higher dose of EGF (10 ng/mL, *Table I*).

Figure 3A is a representative immunoblot showing the stabilization of cytosolic I-κBα levels by PLC-γ over-expression using the 3 μg sense transfected clone (a clone which also protects barrier function,

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Banan et al., 2003c). H_2O_2 induces I-κBα degradation in wild type cells, whereas PLC-γ over-expressing clones show almost steady-state levels of I-κBα. The corresponding O.D. for control was 5000 ± 32; 0.5 mM H_2O_2, 690 ± 175; and PLC-γ sense transfected clones incubated in H_2O_2, 4187 ± 124, indicating I-κBα stabilization in the PLC-γ clones. Transfection of the empty vector alone, as expected, did not confer protection to I-κBα (not shown).

Stabilization of I-κBα by PLC-γ isoform involves prevention of I-κBα phosphorylation (ser 32/36). We then determined the mechanism underlying PLC-γ induced I-κBα stabilization (i.e., decreased I-κBα degradation) and consequent NF-κB inactivation. Because alteration in phosphorylation is a key mechanism for modulating I-κBα protein stability and function (Jobin et al., 1999), we surmised that reduction of I-κBα phosphorylation is a key mechanism for PLC-γ induced decreases in I-κBα degradation and NF-κB inactivation.

I-κBα phosphorylation levels from both transfected and wild type monolayers exposed to H_2O_2 are shown in *Figure 3B*. PLC-γ expression reduced the I-κBα phosphorylation (Ser32/36 phospho-I-κBα) in transfected clones. In wild type cells, inhibition of I-κBα phosphorylation was seen only by high doses (e.g., 10 ng/mL) of EGF. Oxidant, in contrast, markedly increased I-κBα phosphorylation in these wild type cells. As before, transfection of empty vector was ineffective (not shown).

To further study the mechanism underlying the protective affect of PLC-γ on the I-κBα, we utilized immunoprecipitation analysis *(Figs. 4A and 4B)*. Thus, cells were initially immunoprecipitated with an anti-PLC-γ antibody and then the immune complexes were analyzed for the presence of I-κBα (to see whether this PLC isoform physically associates with I-κBα). Wild type (resting) vehicle treated cells did not show any association between these proteins (*Fig. 4A*), whereas a small amount of I-κBα co-precipitated with PLC-γ in EGF pretreated wild type cells. The amount of I-κBα co-precipitation was markedly increased in transfected clones over-expressing PLC-γ, demonstrating enhanced formation of a PLC-γ/I-κBα complex. Using an opposite protocol (*Fig. 4B*), we further corroborated the aforementioned co-association findings. Here, an anti-I-κBα antibody was utilized and immune complexes were then analyzed for the presence of PLC-γ. Not surprisingly, PLC-γ was not detectable in the complex in wild type (vehicle) treated cells (indicating no co-precipitation with I-κBα). Stable over-expression of PLC-γ led to an accumulation of I-κBα/PLC-γ complex, paralleling findings in *4A*. In a third protocol, we further assessed the specificity of the formation of the PLC-γ/I-κBα complex. We thus probed lysates

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from another PLC isoform clone, namely the PLC-beta that exhibited no physical association with I-κBα (not shown).

NF-κB inactivation in PLC-γ transfected clones stabilizes both the assembly and cytoarchitecture of F-actin cytoskeleton. In parallel with the suppression of oxidant-induced affects, PLC-γ expression conferred protection to the assembly of actin pool (*Fig. 5A*, % polymerized actin fraction) and the architecture of actin cytoskeleton *(Fig. 5B, panels a to c,* laser confocal microscopy*)*. Fluorescent images in *figure 5B* show that clones over-expressing PLC-γ *(Panel c)* exhibit a preserved and smooth cytoarchitecture of the actin cytoskeleton under H_2O_2 exposure, which is comparable to the normal and intact organization of actin in control (untreated) cells (*Panel a*). Wild type cells, which were challenged with H_2O_2, show instability, beading, and collapse of the actin cytoskeleton *(Panel b).* These findings parallel the stabilizing effects of PLC-γ against NF-κB activation and I-κBα degradation.

PLC-γ subcellular distribution and activation correlates with multiple indices of NF-κB inactivation. Following over-expression of the PLC-γ isoform (~145 kDa) it is mostly distributed into the particulate cell fractions (particulate = membrane + cytoskeletal fractions) with only a small distribution in the cytosolic cell fractions (Banan et al., 2003c), indicating the activation of this isoform. Pretreatment with EGF enhances the fraction of PLC-γ found in the particulate fractions. In wild type cells, PLC-γ is present in a mostly cytosolic distribution with a smaller pool in the particulate fractions, indicating its inactivity. EGF also increased membrane and cytoskeletal distribution of *native* PLC-γ in these wild type cells.

Table II depicts the activity measurements of PLC-γ isoform (by *in vitro* assay) from immunoprecipitated particulate cell fractions. In stably transfected clones exposed to vehicle, PLC-γ over-expression markedly increases the activity of -γ isoform. EGF additionally activates PLC-γ in these transfected clones, climbing to near maximal activation levels. Basal PLC-γ activity levels are seen in wild type cells exposed to vehicle. In these wild type cells, EGF further activates *native* PLC-γ, however as might be expected, at lower levels as compared to the transfected clones under similar conditions.

Using data across all-experimental conditions, we report significant inverse correlations (e.g., $r = -0.92$; $p<0.05$) between PLC-γ activity (*in vitro* kinase assay or optical density from the particulate fraction) and NF-κB inactivation, further suggesting that activation of PLC-γ is critical in protection against NF-κB activation. Additional robust correlations were found between either NF-κB nuclear distribution or I-κBα degradation and the PLC-γ levels ($r = -0.91, -0.95$, respectively, $p<0.05$ for each). Similarly, when markers of monolayer stability such as either actin integrity or actin assembly were correlated with the PLC-γ levels, other robust correlations were seen ($r = 0.90, 0.89$, respectively, $p<0.05$ for

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each). We found still other supporting correlations such as those between I-κBα phosphorylation or I-κBα stabilization and PLC-γ activation (r = -0.88, 0.93, respectively, $p<0.05$ for each), furthermore indicating that activation of PLC-γ isoform is important in NF-κB inactivation via stabilization of I-κBα.

Inactivation of native PLC-γ isoform by dominant-negative Inhibition: Prevention of EGF-induced NF-κB inactivation and I-κBα stabilization. The aforementioned findings together indicate that PLC-γ could play a key intracellular function in protection against oxidant-induced NF-κB activation. To further assess the possible role of PLC-γ in EGF-mediated suppression of NF-κB activation, we utilized stable dominant-negative transfected, "PLCz mutant" clones of Caco-2 cells we have developed (Banan et al., 2001c). To this end, cDNA encoding a PLCz dominant-negative fragment from the Z region of human PLC-γ1 was utilized. Using this targeted molecular approach, we are capable of substantially reducing the steady-state activity levels for *native* PLC-γ isoform by approximately 99% (*Table II*, 3 μg dominant clone / -γ3). Indeed, EGF can no longer enhance the *native* PLC-γ isoform activity in these dominant negative PLCz mutants.

Tables I also demonstrates the dose-dependent effects of varying amounts (1 to 5 μg) of PLC-γ dominant-negative plasmid (i.e., PLCz) on prevention of EGF-induced NF-κB inactivation and I-κBα stabilization in intestinal cells. Clone that was stably transfected with 3 μg of PLCz plasmid led to maximum suppression of EGF's effects and thus was subsequently utilized for other inhibition experiments.

Figure 6 shows that stable dominant-negative inhibition of *native* PLC-γ activity largely prevents the protection afforded by high (protective) doses (e.g., 10 ng/mL) of EGF against NF-κB subunits activation, p50 (*Fig. 6A*) and p65 (*Fig. 6B*). In contrast, this same dose of EGF completely abrogates oxidant-induced NF-κB subunits activation in wild type cells. Indeed, a large proportion of EGF-induced NF-κB inactivation (~89%) is dependent on the PLC-γ. Inactivation of PLC-γ by itself had no affect on basal NF-κB activity.

Assessment of the I-κBα levels *(Table I)* and I-κBα phosphorylation *(Fig. 7)* from these dominant-negative clones additionally demonstrates that inactivation of *native* PLC-γ isoform largely attenuates both EGF's enhancement of I-κBα stabilization and reduction of I-κBα phosphorylation. A large proportion (~80%) of EGF-induced I-κBα stabilization is PLC-γ dependent.

In parallel, immunoblotting assessment of the *molecular* assembly of F-actin from these same dominant-negative clones further demonstrates *(Fig. 8)* that suppression of *native* PLC-γ also prevents protection against actin disassembly by high (protective) doses of EGF. As might be expected, EGF can

not prevent oxidant-induced actin depolymerization in these mutant clones. PLC-γ inactivation by itself had no affect on actin assembly.

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DISCUSSION

Utilizing monolayers of intestinal epithelium as a widely used model for gut barrier integrity, we have shown in the current investigation that (1) the 145 kDa γ isoform of PLC is required for EGF-mediated protection against oxidant-induced activation of NF-κB and degradation of I-κBα as well as the instability of the actin cytoskeleton and barrier function in epithelial cells. Moreover, (2) PLC-γ by itself is important in protection of monolayer integrity against oxidant-induced stress and NF-κB activation. (3) The molecular mechanism underlying this unique effect of PLC-γ isoform appears to involve the stabilization of the 37 kDa I-κBα protein (an endogenous inhibitor of NF-κB activity).

These three conclusions are supported by independent lines of evidence. Activation of PLC-γ, which we reported to prevent disruption of barrier function, causes an EGF-like protection against oxidant-induced NF-κB stress. Specifically, PLC-γ expression, which leads to constitutive activation of this isoform in intestinal cells, induces a protective cascade of alterations:

↓ I-κBα phosphorylation ⇒ ↓ I-κBα degradation ⇒ ↑ inactivation of NF-κB subunits

Interestingly, we found a similar trend of changes when protein kinase C isoforms (-β1, -ζ) were expressed (Banan et al., 2003e,f). *Additionally*, PLC-γ activation promotes the stability of the molecular dynamics of the actin cytoskeleton (increased polymerized F-actin, reduced unstable G-actin) and protects monolayer integrity. *Moreover*, a non-protective dose of EGF (1 ng/mL) potentiates all measures of PLC-γ mediated protection against NF-κB stress and its consequences. *Furthermore*, dominant-negative inhibition of *native* PLC-γ (by PLCz) largely attenuates all of the stabilizing effects of EGF. In these PLCz mutant clones, EGF did not decrease I-κBα phosphorylation, enhance I-κBα stabilization, promote NF-κB inactivation, or increase the cytoarchitectural stability of F-actin. *Finally*, the high strength of the correlations among different outcome measures for PLC-γ and NF-κB/I-κBα further indicate that PLC-γ is key to protection against NF-κB activation. Accordingly, our findings are consistent with our hypothesis that activation of PLC-γ inhibits I-κBα instability, thereby suppressing NF-κB activation and its deleterious consequences in intestinal cell monolayers.

Our findings using molecular biological approaches are also consistent with other studies, including our previous reports. The PLC-γ isoform affects numerous cellular functions in both non-epithelial and epithelial cell models (Chen et al. 1994, 1996; Goldschmidt-Clermont, 1996; Homma and Takenawa, 1992; Rotin et al., 1992). For instance, modulation of the motogenic pathway in migrating

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intestinal cells that is mediated by growth factors is dependent on PLC-γ (Chen et al., 1994; Polk, 1998). Not surprisingly, PLC-γ is a key signal for reorganization of other cytoskeletal components including tubulin-based microtubules (Banan et al., 2001c) and actin remodeler gelsolin (Janmey et al., 1992; Goldschmidt-Clermont, 1996). We previously showed that PLC-γ is membrane bound and phosphorylated by the EGF-receptor (EGF-R) via the *src* homology 2 (SH2 or phosphotyrosine) domains (Banan et al., 2001c). Indeed, epithelial PLC-γ is the only isoform of PLC that contains *src* homologous 2 and 3 (SH2 and SH3) domains, and that is activated by growth factors (Chen et al. 1994; Polk, 1998; Banan et al., 2003c). The Z region of human PLC-γ1 (amino acids 517-901), the so-called PLC-z, which includes the SH2 and SH3 domains as well as the PLC-inhibitory domains, has been shown to specifically suppress PLC-γ1 and not any other PLC isoform in epithelial cells (Chen et al., 1996, 1994; Polk, 1998). More specifically, PLC-z contains the SH2-SH2-SH3 domains necessary for activation of PLC-γ1 by EGF-R as we and others showed (Chen et al. 1994; Banan et al., 2001c, 2003c; Homma and Takenawa, 1992; Turner et al., 1992). In the current study using this specific dominant-negative fragment for PLC-γ, PLC-z mutant expression not only prevented the activation of *native* PLC-γ but also abrogated EGF protection. Thus, it appears that activating PLC will have distinct beneficial effects on the intestinal epithelium. Our findings on the 145 kDa γ isoform of PLC now suggest a novel role among the PLC isoforms, namely modulation of NF-κB function via stabilization of its regulator I-κBα.

In epithelial cells, PLC-γ signal activation produces IP_3 and diacyl glycerol (DAG) (Chen et al., 1994; Polk, 1998; Reynolds et al., 1993; Homma and Takenawa, 1992). DAG is a key product of PLC-γ mediated PIP_2 hydrolysis which is known to induce the downstream activation of another key signal, namely Protein Kinase C (PKC) (Nishizuka, 1992; Balogh et al., 1995, Banan et al., 2001a,b,c). Indeed, using the same intestinal cell model, we showed that PKC signaling is also critical to EGF protection of barrier and cytoskeletal integrity against oxidant challenge (Banan et al., 2002b, 2001a,b). That PKC is a downstream signal from PLC-γ isoform in the cellular protective processes is further indicated by our previous findings that PKC activators such as OAG (a synthetic version of DAG) maintain cytoskeletal and barrier function while inhibiting NF-κB even in the presence of PLC inactivation (Banan et al., 2003e,f, 2001b). PKC was also shown to be downstream of PLC in other systems as well (Lindmark et al., 1998; Nishizuka, 1992; Polk, 1998; Reynolds et al., 1993). Thus, it appears that EGF protection of cytoskeletal and barrier function is mediated by PLC-γ and then PKC signaling.

Consistent with our current findings, hydrogen peroxide has been shown to activate NF-κB through degradation of I-κBα in a variety of cellular models. For example, activation of NF-κB induced

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by H_20_2 and TNF-α (and its effects on ICAM-1 expression) has been reported in endothelial cells (True et al., 2000; Barchowsky et al., 1995). Similarly, NF-κB activation induced by oxidative stress was seen in colonic circular smooth muscle cells (Shi et al., 2003) as well as in Jurkat T-cells (Brennan and O'Neill, 1995). Nevertheless, our present and previous studies on damage and protection together support a new model that a fundamental mechanism in the cascade of events that underlies growth factor-mediated protection of the GI epithelial barrier and cytoskeletal integrity against oxidant-induced injury involves direct interaction of PLC-γ with key elements of another, injurious, cascade of events that is initiated by oxidants. In the injurious cascade, similar to previous studies (e.g., True et al., 2000; Barchowsky et al., 1995; Brennan and O'Neill, 1995), oxidants induce I-κBα phosphorylation and degradation and then activate NF-κB– a crucial inflammatory mediator. We have expanded on these previous studies and shown that oxidants cause cytoskeletal and barrier instability through the activation of the NF-κB pathway. Furthermore, in the protective cascade, activation of PLC-γ suppresses I-κBα degradation and, in turn, inhibits NF-κB activation and its injurious consequences on cytoskeletal and barrier function in intestinal cells.

Phosphorylation of I-κBα is generally induced by the redox sensitive enzyme I-κB kinase (Iκ-K) which subsequently leads to the degradation of I-κBα and, in turn, results in activation of NF-κB (Jobin et al. 1999; Rogler et al., 1998). A question that remains to be answered is how I-κBα phosphorylation and degradation might be decreased in our intestinal model. We now suggest two mechanisms by which the I-κBα phosphorylation might be decreased**:** (1) inactivation of Iκ-K by increased PKC isoform activity; (2) inactivation of Iκ-K by increased PLC isoform activity and/or by PLC-γ -induced PKC activation. These mechanisms require that increased PKC isoform activity leads to decreased I-κBα phosphorylation and its consequences on NF-κB as was shown by two recent studies from our laboratory (Banan et al., 2003e,f). We showed that the classical -β1 isoform of PKC (PKC-β1) is required for EGF-mediated NF-κB inactivation through decreased I-κBα phosphorylation in intestinal cells. Similarly, the atypical -ζ isoform of PKC (PKC-ζ) inactivates NF-κB via reduced phosphorylation and degradation of I-κBα. Furthermore, these PKC isoforms, similar to PLC-γ, appear to be complexed (co-associated) with I-κBα, creating a novel "signalosome" modulating I-κBα (and NF-κB) in intestinal cells. Consistent with these proposed mechanisms, we previously showed that PLC-γ is phosphorylated and activated following activation of EGF-R by exogenously added EGF (Banan et al., 2001c) and that EGF-R activity is required for EGF-induced I-κBα degradation and NF-κB inactivation (Banan et al., 2003a). It remains to

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be seen whether Iκ-K activity is attenuated following increased PKC (or PLC) activity and whether Iκ-K complexes with PKC (or PLC) in this signalosome.

Our findings could be relevant for developing new treatment modalities for inflammation, in general, and inflammatory bowel disease or IBD, in particular. The manifestations of IBD, including Ulcerative Colitis and Crohn's Disease, wax and wane between active (symptomatic) phases of disease when oxidant-induced stress is prevalent, and inactive (asymptomatic) phases when oxidative stress is low. Our series of findings suggest a new anti-NF-κB defensive mechanism that could protect against the oxidative stress of NF-κB and suppress initiation, perpetuation or manifestation of the IBD attacks. This concept is consistent with recent characterizations of the pathophysiology of IBD and of the pro-inflammatory nature of NF-κB (Barnes and Karin, 1997; Schreiber et al., 1998; Banan et al., 2003a,b,d). NF-κB activation is a crucial event in the inflammatory response triggered by an array of conditions, especially oxidative stress in both non-GI (Chen et al. 1995) and GI models (Jobin et al., 1999; Rogler et al., 1998; Banan et al., 2003a,e,f). NF-κB activation occurs in the inflamed mucosa of patients with either Ulcerative Colitis or Crohn's Disease (Banan et al., 2003b,d; Neurath et al., 1999; Rogler et al., 1998; Schreiber et al., 1998) where both oxidant (H_2O_2) stress and mucosal barrier hyperpermeability were also found (Hollander 1998; Keshavarzian et al., 1992, 2003; Banan et al., 2000c; McKenizie et al., 1996; Irvine and Marshall, 2000). We showed that the amount of oxidant stress and NF-κB activation closely paralleled the degree of mucosal inflammation in patients with IBD (Keshavarzian et al., 2003; Banan et al., 2003b,d**,** 2000c). Interestingly, we also showed that the degree of mucosal cytoskeletal oxidation and instability correlated with the degree of inflammation and disease severity score in patients with IBD (Keshavarzian et al., 2003). The presence of activated NF-κB has also been shown in intestinal mucosal epithelial cells (IECs) from IBD patients (Rogler et al., 1998). Not surprisingly, induction of NF-κB appears to be crucial to the perpetuation of the *active,* symptomatic phase of IBD when intestinal oxidative stress creates a vicious inflammatory cycle dependent on sustained NF-κB activation, oxidant stress, cytoskeletal instability, and ultimately mucosal tissue damage. The defensive anti-NF-κB effects mediated by PLC-γ, as we have seen in intestinal cells, could be pivotal in suppressing the continuation of inflammatory processes.

Accordingly, developing a means of restoring mucosal barrier function during conditions of oxidant stress using endogenous protective factors (e.g., EGF) or agents that trigger defensive signaling pathways (e.g., PKC, PLC) could be beneficial in the treatment of IBD. Treating with mimetics of growth factors such as EGF might be a simple and effective strategy for the treatment of IBD. For instance,

mucosal barrier disruption induced by a wide variety of damaging conditions, including oxidant stress and NF-κB activation, is prevented by EGF, independent of its known anti-secretory properties (e.g., Bass and Luck, 1993; Banan et al., 2000a,b, 2003a; Wright et al., 1990). Not surprisingly, EGF is a crucial protective factor in the maintenance, growth, repair, and barrier function of gut mucosa. Previous studies have noted a marked enhancement in EGF-R immunoreactivity in the inflamed mucosa under IBD-like conditions (Wright et al., 1990). Indeed, pretreatment with EGF prior to the induction of IBD and daily thereafter accelerates healing of colonic mucosa in animals (Bass and Luck, 1993). Thus, EGF mimetics might synergize with the effects of agents triggering PLC-γ (or PKC) and/or the currently used anti-oxidants so that inflammatory processes are more effectively inhibited via the manipulation of both injurious and defensive intracellular mechanisms.

In summary, our findings using targeted molecular interventions to stably over-express or inactivate PLC-γ demonstrate a new concept, that this “protective" PLC isoform appears to be crucial for a substantial portion of the endogenous protection of the intestinal epithelium against oxidant stress that is induced by NF-κB activation. PLC-γ perhaps is also key to suppressing amplification and establishment of an uncontrolled, oxidant initiated, inflammatory cascade that can be induced by free-radicals and other oxidants found under pathophysiological conditions in the GI tract.

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FIGURE LEGENDS

Figures 1A and 1B. Protective effects of phospholipase C-γ (PLC-γ) isoform expression against oxidant-induced activation of NF-κB subunits, p50 subunit (*Fig. 1A*) and p65 subunit (*Fig. 1B*) in intestinal cells. A unique transfected intestinal cell clone we have developed (see Methods) that stably over-expresses the PLC-γ by 2.3 fold was utilized. Intestinal monolayers over-expressing PLC-γ (3 μg clone) or wild type cells (not over-expressing PLC-γ) were incubated with growth factor (EGF, 10 min) prior to exposure to oxidant (H_2O_2, 30 min). Clones over-expressing "[PLC-γ]" show substantial inhibition of both NF-κB p50 and p65 subunit activities that was induced by oxidant. In wild type monolayers NF-κB was inactivated only by high (protective) doses of EGF (e.g., 10 ng/mL). NF-κB subunit activity was determined by an ELISA assay of nuclear extracts in especially coated multi-well plates which contained specific oligonucleotides containing a consensus-binding site for either the p50 or p65 subunit of NF-κB. *p <0.05 *vs.* vehicle. +p <0.05 *vs.* H_2O_2 in wild type. &p <0.05 *vs.* PLC-γ over-expressing "PLC-γ" cells exposed to H_2O_2 or pretreated with EGF prior to H_2O_2 in wild type cells. #p <0.05 *vs.* EGF (10 ng/mL) prior to H_2O_2 in wild type cells. [**WT**] = Wild type cells. [**PLC-γ**] = Cells transfected with PLC-γ sense. N = 6 per group in all figures, including subsequent ones, unless otherwise indicated.

Figures 1C and 1D. Representative blots of NF-κB protein subunits, p50 (*Fig. 1C*) and p65 (*Fig. 1D*) distribution in the nuclear fractions of the intestinal cells of wild type or transfected origin. The p50 or p65 protein bands are from the same treatments as described in figures 1A and 1B. In stably transfected clones (3 μg sense clone), PLC-γ expression prevents NF-κB subunits nuclear distribution against exposure to oxidant (H_2O_2, 30 min). This levels of distribution is comparable to the control (vehicle treated) cells which exhibit basal levels of NF-κB (p50 and p65) in the nuclear extracts. In wild type cells, only a high dose of EGF (10 ng/mL) suppresses NF-κB nuclear distribution. The region of gel shown was between the M_r 43,000 and 75,000 pre-stained molecular weights which were run in adjacent lanes. [**WT**] = Wild type cells. [**PLC-γ**] = Cells transfected with PLC-γ sense. Shown is a representative blot.

Figure 2. Time course for the inhibition of the activation of NF-κB (p65 subunit) in 3 μg transfected PLC-γ over-expressing clone, [PLC-γ]. Wild type [WT] cells are also shown where NF-κB is activated following H_2O_2 exposure. Cells were exposed to 0.5 mM H_2O_2 at zero time.

Figure 3A. Representative immunoblot for the protective (stabilizing) effects of PLC-γ over-expression on I-κBα levels in the cytosolic fractions of intestinal cells. The I-κBα bands are following the shown treatment regimes. Paralleling its inhibitory effects on NF-κB, PLC-γ over-expression (3 μg clone) stabilizes the 37 kDa I-κBα (an endogenous modulator of NF-κB) against oxidant insult (H_2O_2, 30 min). On the other hand, oxidant alone degrades I-κBα in wild type cells. In these wild type cells, EGF (10 ng/mL) protects the I-κBα. The region of gel shown was between the M_r 34,000 and 44,000 pre-stained molecular weights which were run in adjacent lanes. [**WT**] = Wild type cells. [**PLC-γ**] = Cells transfected with PLC-γ sense.

Figure 3B. Phosphorylation of I-κBα in the intestinal cells of either transfected or wild type origin assessed by immunoblotting. The I-κBα phosphorylation (Ser 32/36) bands are from the same treatment

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regimes as described in figure 3A. As for its protective effects on the I-κBα, PLC-γ over-expression in transfected cells (3 μg sense clone) prevents the phosphorylation of I-κBα. EGF (10 ng/mL), which stabilizes I-κBα (see fig 3A), also suppresses I-κBα phosphorylation in wild type cells. Wild type cells when exposed only to oxidant (H_2O_2, 30 min) exhibit increased phosphorylation of I-κBα. Here, separate blots were run for assessment of I-κBα phosphorylation than those for I-κBα expression levels. [**WT**] = Wild type cells. [**PLC-γ**] = Cells transfected with PLC-γ sense. Shown is a representative blot.

Figures 4A and 4B. PLC-γ over-expression leads to co-association of the 145 kDa PLC-γ isoform and the 37 kDa I-κBα in intestinal cells. Note the co-immunoprecipitation of the PLC-γ / I-κBα complexes in transfected cells (3 μg sense clone) over-expressing -γ isoform of PLC (*4A and 4B*). For *Figure 4A*, cleared cell lysates were incubated with excess (1:2000 dilution) of monoclonal anti-PLC-γ antibody bound to protein A beads and the immune complexes were then resolved by PGAE using the corresponding anti-PLC-γ or anti-I-κBα antibodies. Wild type (vehicle treated) cells display no PLC-γ/I-κBα complex. PLC-γ over-expressing clones or the EGF treated cells, in contrast, exhibit the PLC-γ/I-κBα complexes (see "*****"). For *Figure 4B*, an opposite protocol using anti-I-κBα antibody was performed for immunoprecipitation and the complexes formed were then examined. Thus, complexes seen were immunoprecipitated with anti-I-κBα prior to PAGE with the appropriate antibodies. As might be expected, a similar pattern of co-association is found between I-κBα / PLC-γ. [**WT**] = Wild type cells. [**PLC-γ**] = Cells transfected with PLC-γ sense. **WB** = western blot. **IP** = immunoprecipitated with the shown antibody.

Figure 5A. Analysis of the polymerized F-actin pool (an index of actin integrity) from the PLC-γ over-expressing clones as compared to wild type cells. PLC-γ over-expression (3 μg clone) enhances F-actin assembly against oxidant (H_2O_2, 30 min) challenge as shown by an increase in F-actin which is comparable to the control (vehicle). In wild type cells, in contrast, oxidant results in a large decrease in the assembly F-actin. This is prevented by EGF (10 ng/mL). F-actin (triton-insoluble) extracts were fractionated and processed for PAGE and subsequently analyzed for percent polymerized F-actin by laser densitometry. Percent polymerized F-actin = [(F) / (F + G)], where F + G is the total intracellular actin pool. *p <0.05 *vs*. vehicle. +p <0.05 *vs*. H_2O_2 in wild type. &p <0.05 *vs*. PLC-γ over-expressing "PLC-γ" cells exposed to H_2O_2 or pretreated with EGF prior to H_2O_2 in wild type cells. #p <0.05 *vs*. EGF (10 ng/mL) prior to H_2O_2 in wild type cells. [**WT**] = Wild type cells. [**PLC-γ**] = Cells transfected with PLC-γ sense.

Figure 5B. The cytoarchitecture of the F-actin cytoskeleton as revealed by laser scanning confocal microscopy (LSCM) of intestinal monolayers. *Panel a* is from untreated (control) cells exposed to vehicle. *Panel b* is from wild type Caco-2 cells exposed to 0.5 mM H_2O_2 (30 min incubation). *Panel c* is from PLC-γ over-expressing monolayers (3 μg clone) exposed to 0.5 mM H_2O_2 (30 min incubation). Only in clones over-expressing PLC-γ *(Panel c)* normal actin organization is preserved against oxidant challenge, which is indistinguishable from the control monolayers *(Panel a)*. F-actin cytoskeleton in these control cells is intact and smoothly distributed at areas of cell-to-cell contact *(Panel a)*, whereas wild type cells exhibit clear disruption and instability of F-actin "ring" under oxidant insult *(Panel b)*. Bar = 25 μm. Shown are representative photos.

Figures 6A and 6B. Stable dominant negative inactivation of the *native* PLC-γ isoform by the "PLCz" fragment inhibits the protective (suppressive) effects of growth factor (EGF) against oxidant-induced activation of NF-κB subunits, p50 (*6A*) and p65 (*6B*). A unique PLCz mutant cell line (3 μg dominant

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negative clone) that almost completely lacks *native* PLC-γ activity (see Table II) was utilized. Monolayers were preincubated for 10 min with a high (protective) dose of EGF (10 ng/mL) and then exposed for 30 min to H_2O_2 (0.5 mM). Wild type Caco-2 cells are also shown. EGF can not inactivate NF-κB subunits in the PLCz dominant negative mutants, but it does suppress NF-κB in the wild type cells. NF-κB subunits, p65 and p50 activities were assessed by an ELISA based assay of nuclear extracts using a specific consensus-binding site for either the p65 or p50 subunit of NF-κB. *p <0.05 *vs.* vehicle. +p <0.05 *vs*. H_2O_2. &p <0.05 *vs*. EGF + H_2O_2 in wild type cells. [**WT**] = Wild type cells. [**PLC-z mutant**] = Dominant negative inhibition of *native* PLC-γ activity.

Figure 7. Dominant negative inactivation of *native* PLC-γ isoform attenuates EGF's suppression of I-κBα phosphorylation in intestinal cells. Cell monolayers either lacking PLC-γ activity (3 μg PLCz mutant) or expressing *native* PLC-γ activity (wild type) were incubated with EGF before H_2O_2 as described in figure 6. The I-κBα phosphorylation is reduced by EGF in wild type cells, whereas this protective effect is substantially prevented in dominant negative PLCz clones. *p <0.05 *vs.* vehicle. +p <0.05 *vs*. H_2O_2. &p <0.05 *vs*. EGF + H_2O_2 in wild type cells. [**WT**] = Wild type cells. [**PLC-z mutant**] = Dominant negative inhibition of *native* PLC-γ activity.

Figure 8. Inhibitory effects of the dominant negative inactivation of PLC-γ isoform on EGF's enhancement of the assembly of F-actin cytoskeletal pool in Caco-2 cells assessed by immunoblotting. F-actin cytoskeletal extracts from Caco-2 cells were subjected to SDS-PAGE fractionation and immunoblotted using monoclonal anti-actin antibody followed by HRP conjugated-secondary antibody, and then autoradiographed. The F-actin (43 kDa) polymerization bands are from the same treatment conditions as described in figure 6. In 3 μg PLCz mutant clones, inactivation of *native* PLC-γ largely attenuates EGF's protection of F-actin assembly against oxidant insult. This is shown by a reduction of the band (lane) density for polymerized actin. In the wild type cells, in contrast, EGF preserves the polymerized actin pool. This level of assembly is comparable to the controls which exhibit steady-state levels of the polymerized actin. [**WT**] = Wild type cells. [**PLC-z mutant**] = Dominant negative inhibition of *native* PLC-γ activity.

Table I. Effects of transfection of varying amounts of Phospholipase C-gamma (PLC-γ) sense or dominant negative mutant DNA on both NF-kB activity and I-kB-α levels in intestinal Caco-2 monolayers

Treatment	NF-kB activity (OD 450 nm)	I-kB-α levels (% Maximum)
Vehicle	0.09 ± 0.01	100 ± 2.0
H_2O_2 [WT]	1.55 ± 0.25*	14 ± 7.0*
H_2O_2 [1 μg sense PLC-γ DNA]	1.28 ± 0.08*&	29 ± 0.6*&
H_2O_2 [2 μg sense PLC-γ DNA]	0.75 ± 0.09*+&	47 ± 3.0*+&
H_2O_2 [3 μg sense PLC-γ DNA]	0.27 ± 0.05*+	84 ± 4.0*+
H_2O_2 [5 μg sense PLC-γ DNA]	0.28 ± 0.09*+	82 ± 6.0*+
EGF (10 ng/mL) + H_2O_2 [WT]	0.12 ± 0.02+	95 ± 0.5+
EGF (10 ng/mL) + H_2O_2 [1 μg Dominant Neg. PLC-γ DNA]	0.23 ± 0.06*+&	80 ± 1.0*+&
EGF (10 ng/mL) + H_2O_2 [2 μg Dominant Neg. PLC-γ DNA]	0.89 ± 0.02*+&	56 ± 2.0*+&
EGF (10 ng/mL) + H_2O_2 [3 μg Dominant Neg. PLC-γ DNA]	1.39 ± 0.25*+	22 ± 5.0*+
EGF (10 ng/mL) + H_2O_2 [5 μg Dominant Neg. PLC-γ DNA]	1.35 ± 0.10*+	23 ± 3.0*+

Intestinal cells stably transfected with varying amounts of PLC-γ sense DNA (1, 2, 3, or 5 μg) were exposed to oxidant (H_2O_2, 0.5 mM). In separate studies, cells transfected with varying amounts of a PLC-γ dominant negative DNA , namely PLCz (1, 2, 3, or 5 μg) were treated with EGF (10 ng/mL) prior to exposure to oxidant. Wild type (WT) cells, which are not transfected, are also shown. Monolayer NF-kB (p65) activity and I-kB-α levels were assessed as described in the Methods. *$p < 0.05$ compared to the Vehicle. +$p < 0.05$ compared to H_2O_2 in WT cells. &$p < 0.05$ compared to corresponding cells transfected with 3 μg of sense PLC-γ DNA and exposed to H_2O_2 or cells transfected with 3 μg of PLC-γ dominant mutant DNA and treated with EGF and then H_2O_2 . Values are means ± SEM. N = 4 to 6 per group.

Table II. Activity levels of phospholipase C-gamma (PLC-γ) in intestinal cells of transfected and wild type origin

Treatment	PLC-γ activity (pmol/min/mg protein)
Vehicle[WT]	64 ± 6
Vehicle[PLC-γ]	303 ± 11*
Vehicle[PLCz mutant]	0.02 ± 0.01*+
H_2O_2 [WT]	60 ± 8
H_2O_2 [PLC-γ]	300 ± 16*+
H_2O_2 [PLCz mutant]	0.04 ± 0.01*+
EGF (10 ng/mL) + H_2O_2 [WT]	175 ± 14*+
EGF (10 ng/mL) + H_2O_2 [PLC-γ]	383 ± 7*+#
EGF (10 ng/mL) + H_2O_2 [PLCz mutant]	0.1 ± 0.05*+

Intestinal cells stably transfected with PLC-γ sense DNA (3 μg) were exposed to oxidant (H_2O_2, 0.5 mM). In corollary studies, cells transfected with a PLC-γ dominant negative DNA, PLCz (3 μg) were incubated with EGF prior to oxidant challenge. Wild type (WT) cells (not transfected) are also shown. PLC-γ isoform activity was assessed *in vitro* as described in the Methods. *$p < 0.05$ compared to the corresponding Vehicle. +$p < 0.05$ compared to H_2O_2 in WT cells. #$p < 0.05$ compared to the corresponding EGF prior to H_2O_2 in WT cells or in dominant negative PLCz clones . [PLC-γ] = Cells transfected with PLC-γ. [PLCz mutant] = Dominant negative inhibition of native PLC-γ. Values are means ± SEM. N = 4 to 6 per group.

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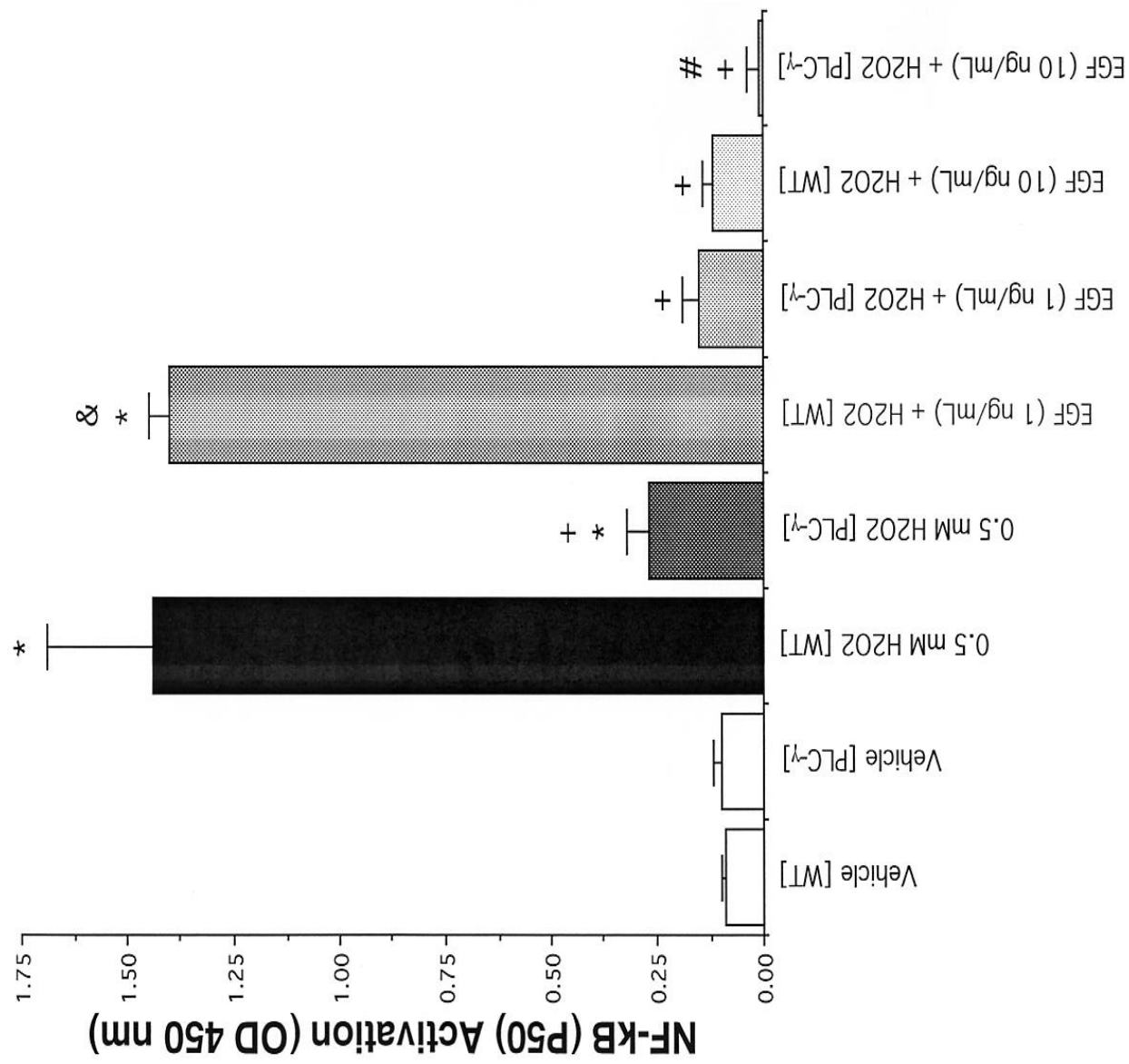
Fig. 1A



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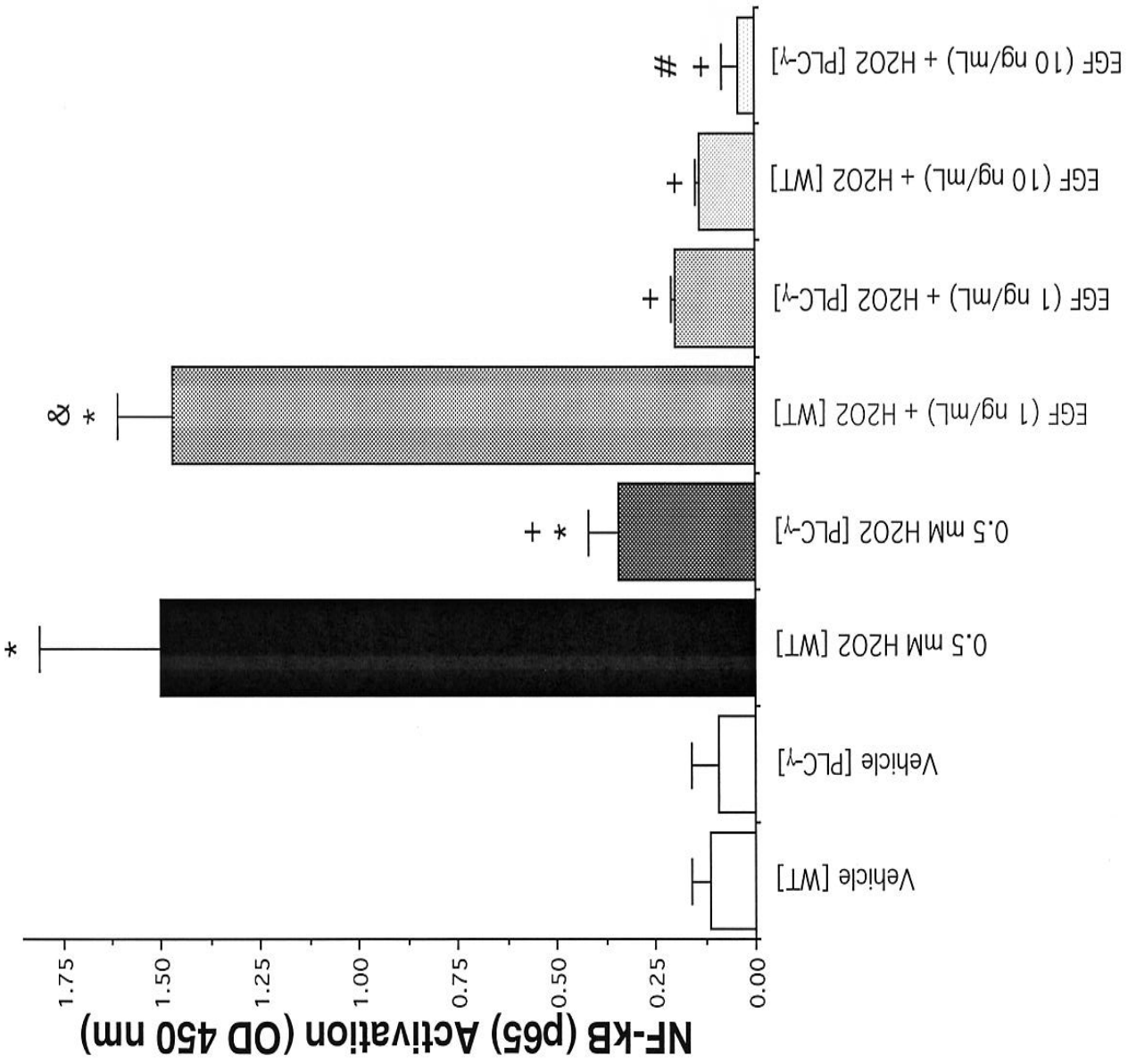


Fig. 1B

Fig. 1C

Fig. 1D

Fig. 2

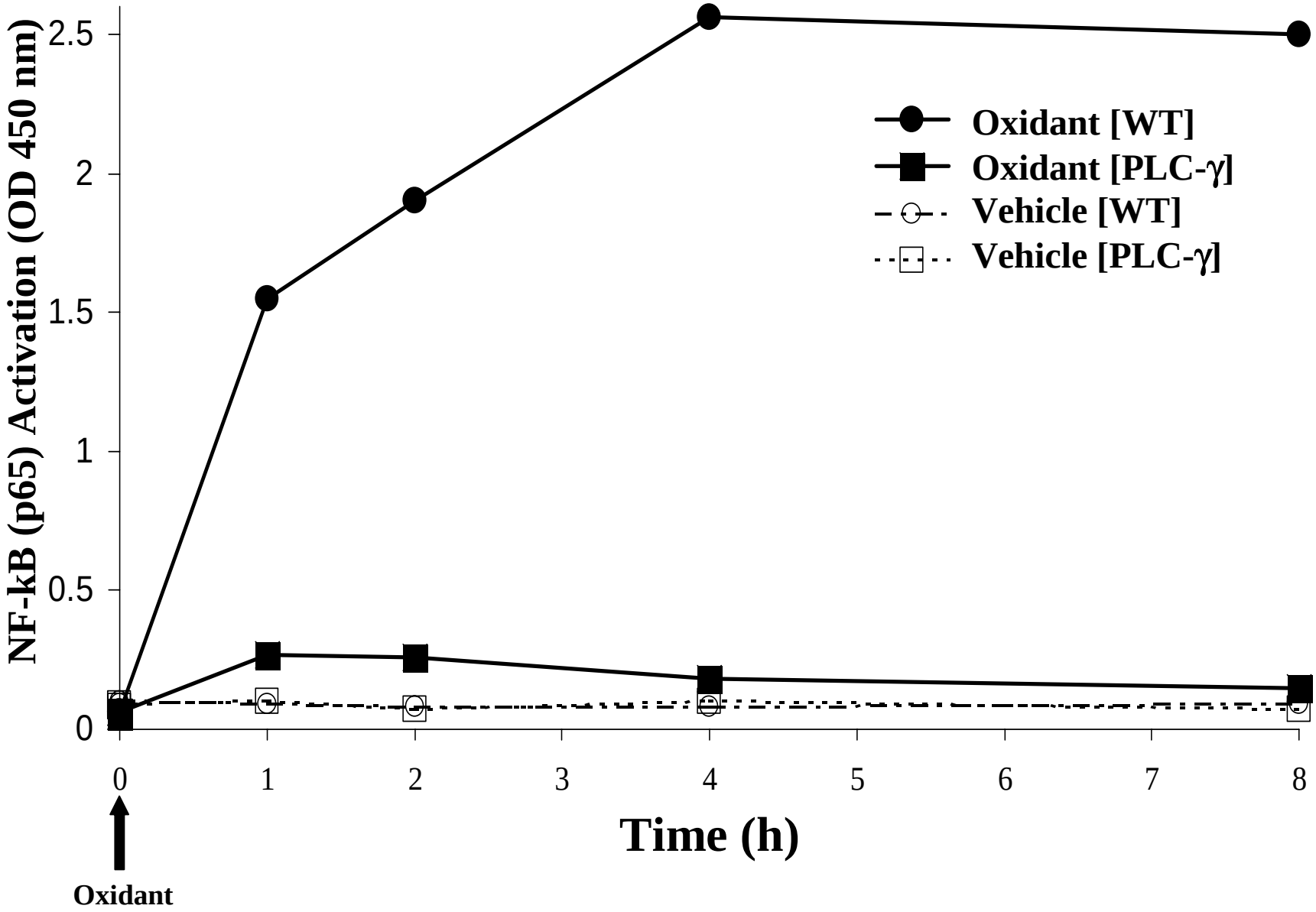
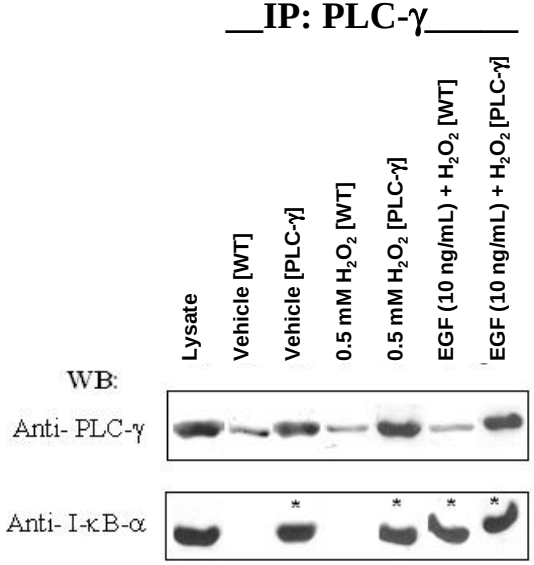


Fig. 3A

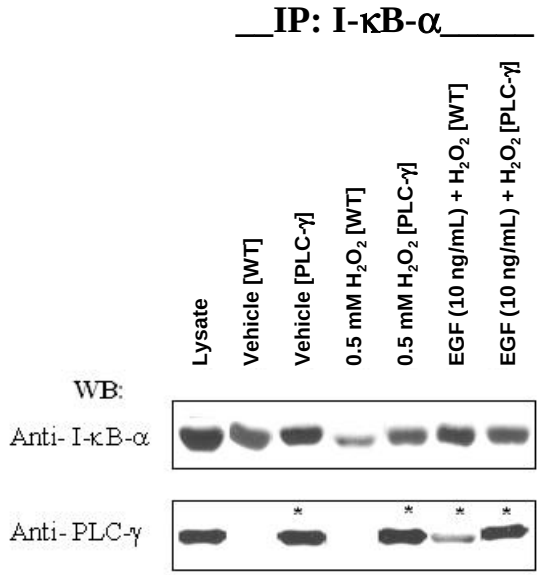
Fig. 3B

Fig. 4

Panel A



Panel B



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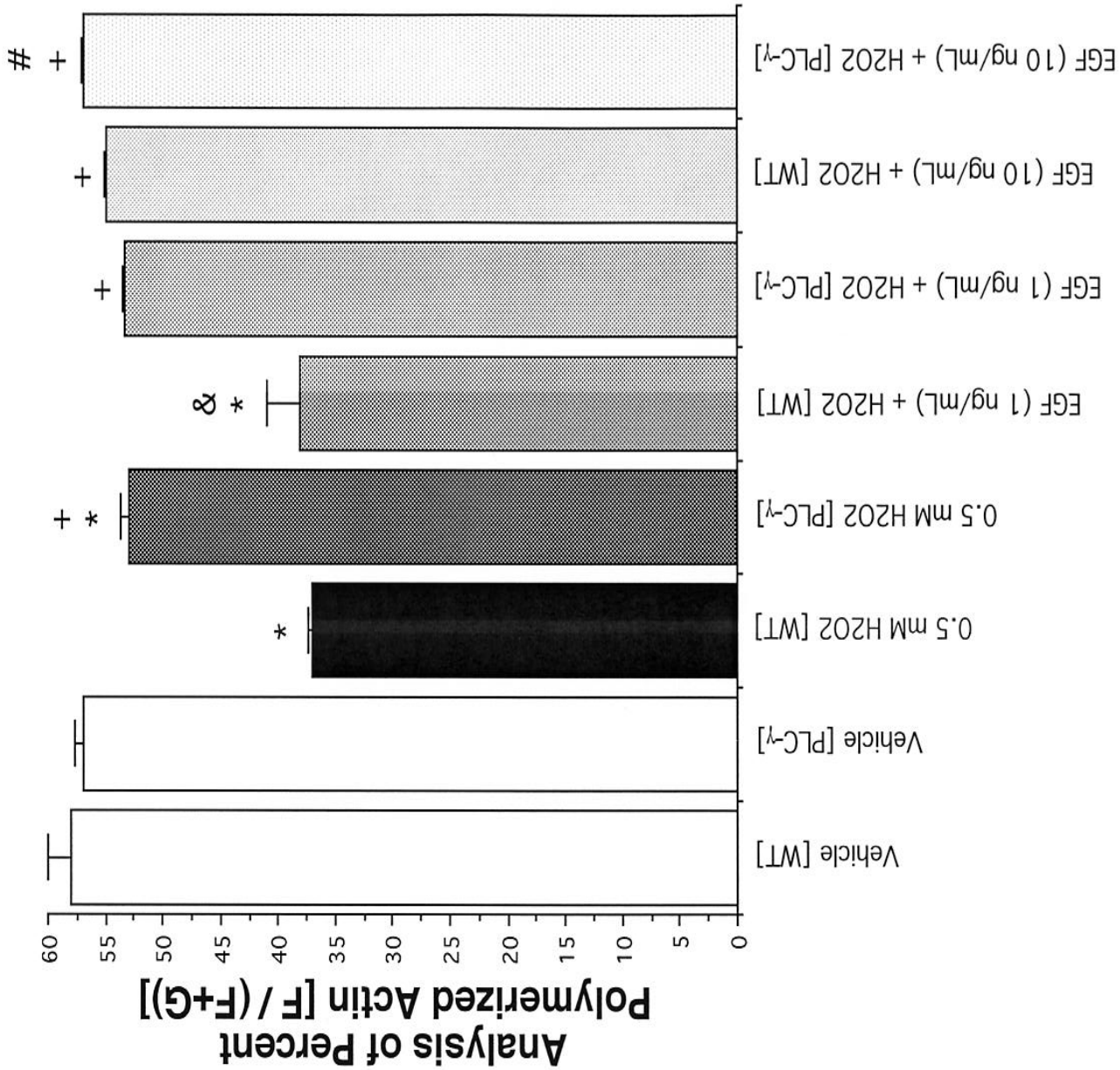

Fig. 5A

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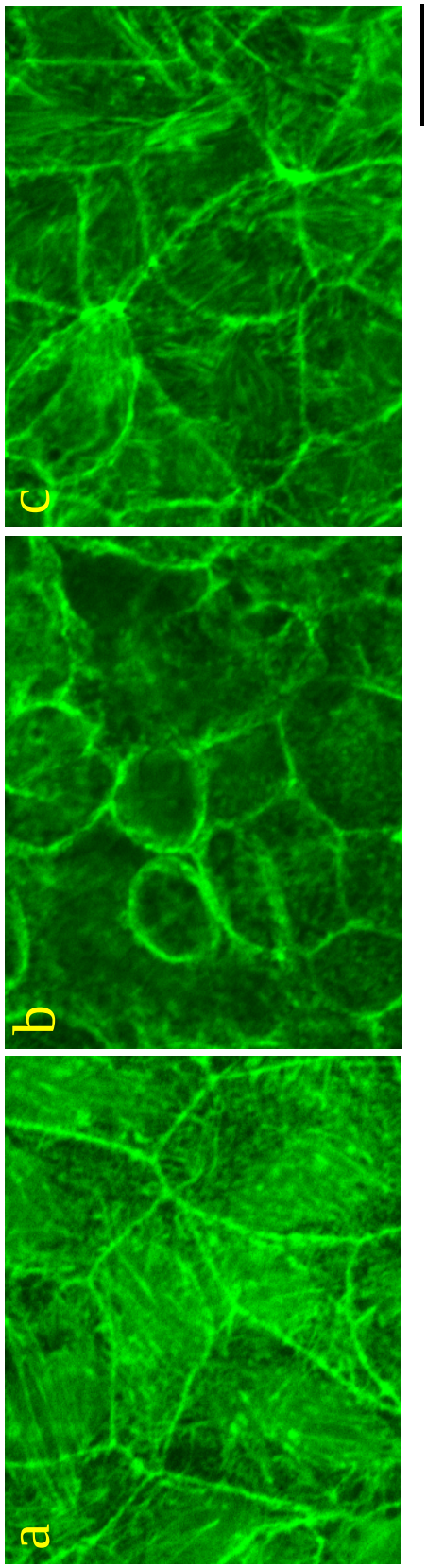

Fig. 5B

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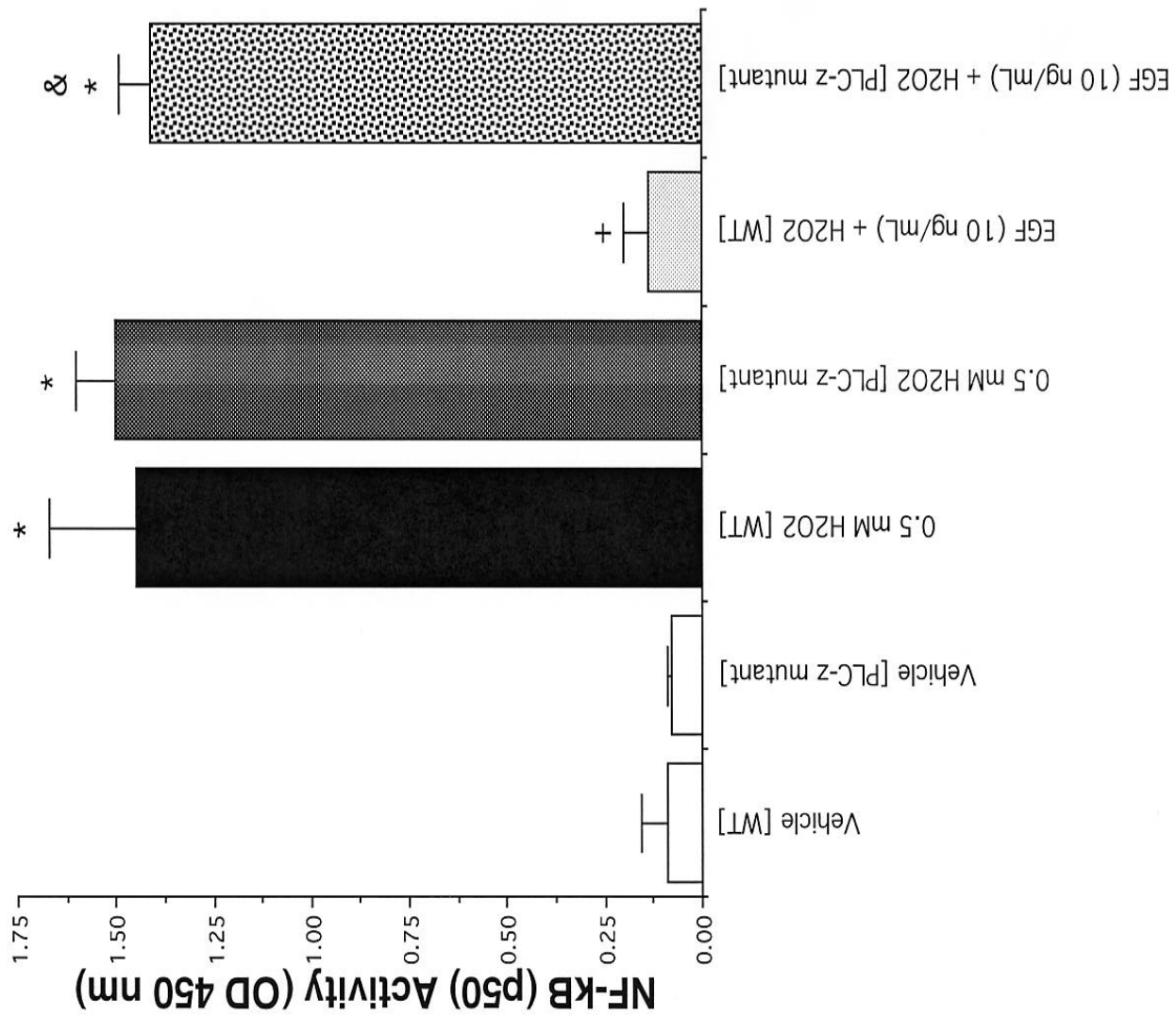

Fig. 6A

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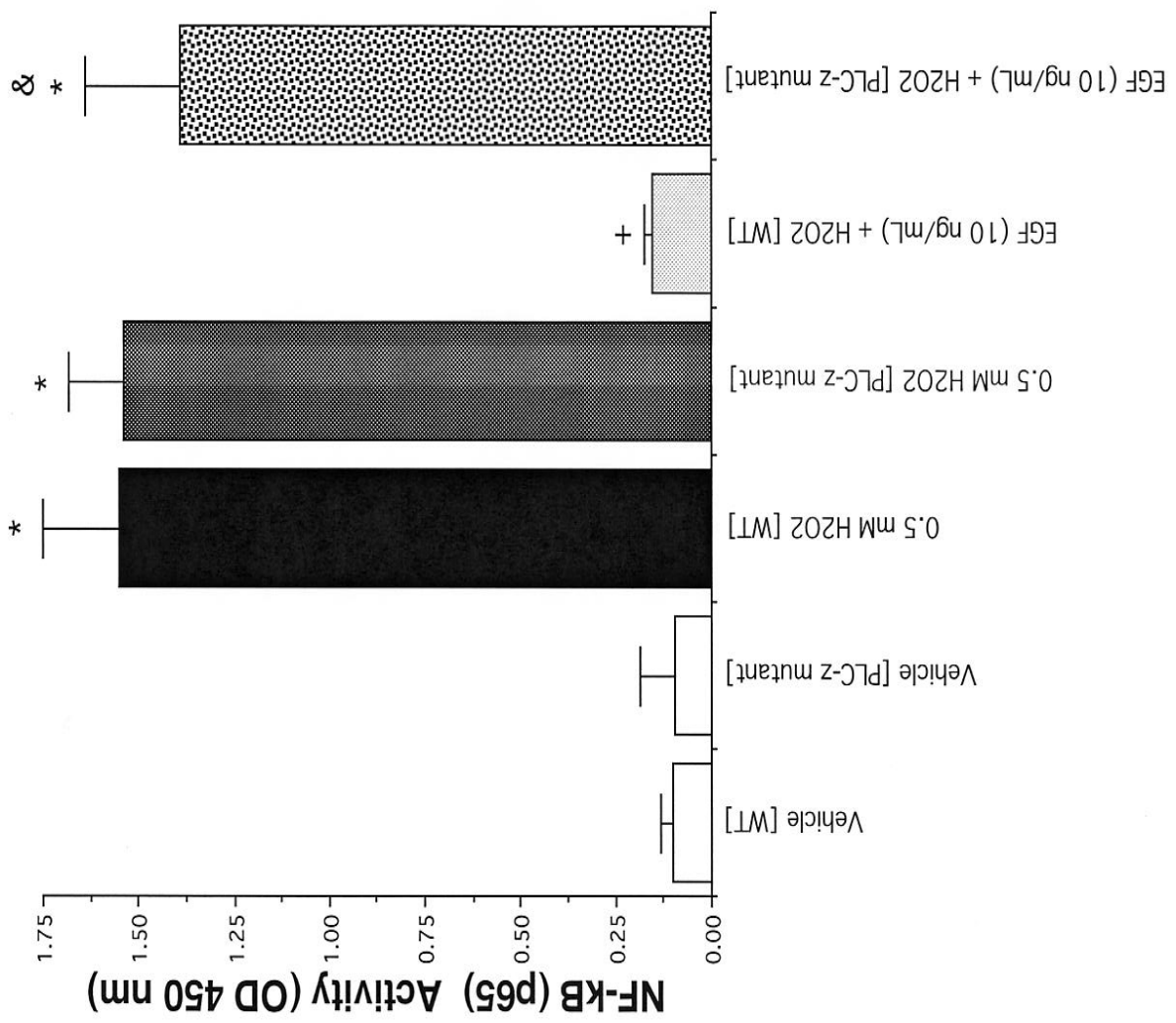

Fig. 6B

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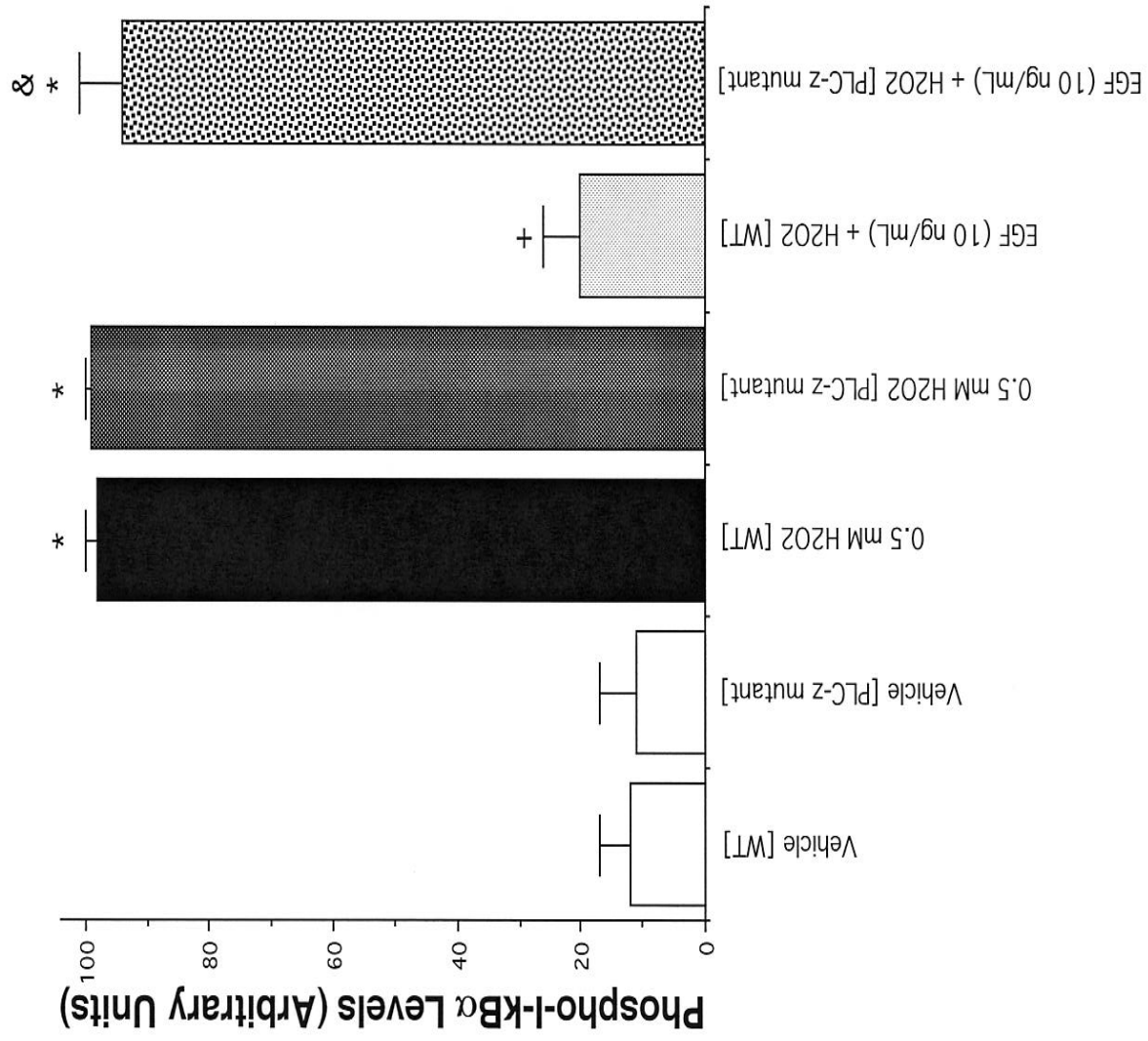

Fig. 7

Fig. 8