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Role of Fyn and PI3K in H₂O₂-induced inhibition of apical Cl⁻/OH⁻ exchange activity in human intestinal epithelial cells

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Abstract

H₂O₂ is a highly reactive oxygen metabolite that has been implicated as an important mediator of inflammation-induced intestinal injury associated with ischaemia/reperfusion, radiation and inflammatory bowel disease. Previous studies have shown that H₂O₂ inhibits NaCl absorption and activates Cl⁻ secretion in the rat and rabbit colon. To date, however, almost no information is available with respect to its effect on the human intestinal apical anion exchanger Cl⁻/OH⁻ (HCO₃⁻). The present studies were, therefore, undertaken to examine the direct effects of H₂O₂ on OH⁻ gradient-driven DIDS (4,4'-di-isothiocyanostilbene-2,2'-disulfonate)-sensitive ³⁶Cl⁻ uptake utilizing a post-confluent transformed human intestinal epithelial cell line, Caco-2. Our results demonstrate that H₂O₂ (1 mM for 60 min) significantly inhibited (approx. 60 %; *P* < 0.05) Cl⁻/OH⁻ exchange activity in Caco-2 cells. H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity involved the Src kinase Fyn and PI3K (phosphoinositide 3-kinase)-dependent pathways. H₂O₂ also induced phosphorylation of Fyn and p85 (the regulatory subunit of PI3K) in Caco-2 cells. Moreover, an increased association of Fyn and p85 was observed in response to H₂O₂, resulting in the activation of the downstream target PLCγ1 (phospholipase Cγ1). Elevated intracellular Ca²⁺ levels and PKCα (protein kinase Cα) functioned as downstream effectors of H₂O₂-induced PLCγ1 activation. Our results, for the first time, provide evidence for H₂O₂-induced Src kinase Fyn/PI3K complex association. This complex association resulted in the subsequent activation of PLCγ1 and Ca²⁺-dependent PKCα, resulting in the inhibition of Cl⁻/OH⁻ exchange activity. These findings suggest that H₂O₂-induced inhibition of the Cl⁻/OH⁻ exchange process may play an important role in the pathophysiology of diarrhoea associated with inflammatory disorders, where the amount of reactive oxygen species is markedly elevated.

Keywords

chloride absorption; down-regulated in adenoma (DRA); human intestine; putative anion transporter 1 (PAT1); phosphoinositide 3-kinase (PI3K); protein kinase C (PKC)

INTRODUCTION

ROS (reactive oxygen species), including H₂O₂, O₂⁻ (superoxide anion), OH⁻ (hydroxyl anion) and HOCl (hypochlorite), are generated in tissues as a consequence of normal cellular aerobic metabolism. These oxidants are normally detoxified by antioxidant defence enzymes such as catalase and glutathione peroxidase [1]. However, an excessive generation of oxidants and/or defect in antioxidant defence mechanisms may lead to tissue injury. The intestinal

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epithelium is exposed to ROMs (reactive oxygen metabolites) from multiple luminal and systemic sources. The potential sources of luminal oxidant generation are xenobiotics, toxins, mycoplasma, bile acids and cast-off mucosal cells [2]. The systemic sources of ROMs in the intestine include products of xanthine oxidases and COX (cyclo-oxygenase) and lipoxygenase enzymes of arachidonic acid metabolism and the respiratory burst of phagocytic cells. Intestinal ischaemia, ischaemic colitis and inflammatory bowel disease are associated with significantly higher levels of ROS [3], generated by activated neutrophils or inflammatory cells infiltrating the intestinal epithelial cells.

H₂O₂ plays an important role in the initiation and propagation of epithelial inflammatory injury. H₂O₂ is formed by the dismutation of O₂⁻ or the two-electron reduction of oxygen, and can react with lipids and sulfhydryl proteins to inactivate key enzymes. However, because of its relatively long life and lipophilic nature, H₂O₂ can also enter cells where it can react with transition metals, such as iron, to yield the extremely reactive OH[•] (hydroxyl radical), which in turn can exert important biological effects [4]. For these reasons, there is considerable interest in identifying the effects by which H₂O₂ alters ion transport across the intestinal epithelium.

In this regard, *in vitro* electrophysiological studies performed previously in the rat colon have shown that H₂O₂ stimulated electrogenic Cl⁻ secretion and inhibited neutral NaCl absorption through the release of prostaglandins [5]. In contrast, Mayol et al. [6] showed that the effects of H₂O₂ on electrogenic Cl⁻ secretion were independent of prostaglandin release in intact native rat colon. Moreover, addition of H₂O₂ to the apical or basolateral sides of T84 cells mounted in Ussing chambers increased Cl⁻ secretion in a transient manner [7], whereas, in another study, it attenuated the cAMP-induced Cl⁻ secretion in T84 cells [8]. Earlier studies have shown that H₂O₂ stimulated NHE1 (Na⁺/H⁺ exchanger 1) activation in adult rat cardiac myocytes [9]. In contrast, O₂⁻ was found to stimulate NaCl absorption in the rat thick ascending loop of Henle [10]. Thus these studies suggest an important role of H₂O₂ in modifying electrolyte transport process by altering either Cl⁻ secretion or Na⁺ and Cl⁻ absorption, which results in diarrhoea. However, the direct effects of H₂O₂ on the human intestinal apical Cl⁻/OH⁻ exchange activity and the apical anion exchangers SLC26A3 [also known as DRA (down-regulated in adenoma)] and SLC26A6 [also known as PAT1 (putative anion transporter 1)] are not known.

A number of studies have shown that ROS stimulate intracellular signalling events similar to those activated by growth factors, cytokines and agonists of G-protein-linked receptors [11]. H₂O₂ has also been reported to enhance the tyrosine phosphorylation of PDGFR (platelet-derived growth factor receptor), EGFR (epidermal growth factor receptor), Src kinases and MAPKs (mitogen-activated protein kinases), leading to activation of gene expression, including c-fos, c-myc, c-jun, NF-κB (nuclear factor κB) and AP-1 (activator protein-1) [12, 13]. Previous studies have demonstrated that H₂O₂ increases both PKC (protein kinase C) and PLD (phospholipase D) activity in endothelial cells [14], NIH 3T3 cells and Cos 7 cells [15, 16]. H₂O₂ also induced PLCγ1 (phospholipase Cγ1) activation in normal mouse embryonic fibroblasts [17]. However, the signal transduction pathways involved in H₂O₂-mediated alteration of electrolyte transport have not been delineated.

The present studies were, therefore, undertaken to examine (i) the regulation of Cl⁻/OH⁻ exchange activity in Caco-2 cells by H₂O₂; (ii) the signal transduction pathways involved in this process; and (iii) whether H₂O₂ regulates Cl⁻/OH⁻ exchange activity by modulating the apical anion exchangers DRA and PAT1. Our present studies demonstrate that H₂O₂ inhibits apical Cl⁻/OH⁻ exchange activity in Caco-2 cells via Src kinase-, PI3K (phosphoinositide 3-kinase)- and Ca²⁺-dependent PKC-mediated pathways. Moreover, this inhibition of Cl⁻/OH⁻ exchange activity in response to H₂O₂ was independent of changes in the surface expression of both DRA and PAT1 at the membrane level.

EXPERIMENTAL

Materials

DIDS (4,4'-di-isothiocyanostilbene-2,2'-disulfonate), DPC (*N*-phenylanthranilic acid), H₂O₂ and indomethacin were obtained from Sigma. Radionuclide ³⁶Cl as HCl was obtained from NEN Life Science Products. Caco-2 cells were obtained from A.T.C.C. BAPTA-AM [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)] was obtained from Molecular Probes. Chelerythrine chloride, calphostin C, herbimycin, PP1 and ET-18 were obtained from Biomol. Ro318220, Go6796 and rottlerin were obtained from Calbiochem. Affinity-purified rabbit polyclonal antibodies against PKC α , PKC δ , c-Src, Fyn and phospho-PLC γ 1, goat anti-(rabbit IgG) antibody conjugated to HRP (horseradish peroxidase) and Protein A-agarose were purchased from Santa Cruz Biotechnology. The anti-PLC γ 1 antibody was purchased from Cell Signaling. The HRP-conjugated anti-phosphotyrosine (4G10) antibody, anti-p85 antibody (where p85 is the regulatory subunit of PI3K), and monoclonal anti-Fyn and anti-Src antibodies were obtained from Upstate Biotechnology. Rabbit anti-(phosphorylated protein) antibody was from Invitrogen. All other chemicals were of at least reagent grade and were obtained from either Sigma or Fisher Scientific.

Cell culture

Caco-2 cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 4.5 g/l glucose, 2 mM glutamine, 50 units/ml penicillin, 50 μ g/ml streptomycin, 10 mM Hepes, 1 % essential and non-essential amino acids and 20 % FBS (fetal bovine serum), pH 7.4, in 5 % CO₂/95 % O₂ at 37 °C. For the uptake experiments, cells from passages between 20 and 25 were plated in 24-well plates at a density of 2×10^4 cells/ml. Confluent monolayers were then used for transport experiments at day 10–12 post-plating. To study the effect of H₂O₂ on Cl[−]/OH[−] exchange activity, cells were acutely exposed to 1 mM H₂O₂ in cell culture medium for 60 min.

In separate sets of experiments, cells were pre-treated with the Ca²⁺-chelator BAPTA-AM (20 μ M), the general PKC inhibitors chelerythrine chloride (2 μ M) and calphostin C (200 nM), the PKC α inhibitor Go6976 (5 nM), the PKC ϵ inhibitor Ro318220 (100 nM), the specific PKC δ inhibitor rottlerin (10 μ M), the tyrosine kinase inhibitors herbimycin (1 μ M) and PP1 (10 μ M), the PLC inhibitor ET-18 (10 μ M) or the dual COX inhibitor indomethacin (0.1 and 1.0 μ M) for 60 min prior to the addition of H₂O₂ (1 mM). These inhibitors were also co-incubated along with H₂O₂ for another 60 min.

Cl[−]/OH[−] exchange activity

Cl[−]/OH[−] exchange activity was determined by measuring DIDS-sensitive ³⁶Cl[−] uptake in base-loaded cells, as described previously by Olsnes et al. [18] and established further by us with some modifications [19]. Caco-2 cells were incubated with DMEM base medium containing 20 mM Hepes/KOH (pH 8.5) for 30 min at room temperature (23–25 °C). The medium was removed and the cells were rapidly washed with 1 ml of tracer-free uptake buffer [260 mM mannitol and 20 mM Tris/Mes (pH 7.0)] and 100 μ M DPC to block Cl[−] channels. The cells were then incubated with uptake buffer for 5 min in the absence or presence of DIDS (300 μ M). This time period was chosen as it was within the linear range of Cl[−] uptake in this system. For ³⁶Cl[−] uptake studies, uptake buffer containing 1.68 μ Ci of ³⁶Cl[−] (2.9 mM) of HCl (specific radioactivity, 17.12 mCi/g) was used. Uptake was terminated by removing the buffer and washing the cells rapidly twice with 1 ml of ice-cold PBS (pH 7.2). Finally, the cells were solubilized by incubation with 0.5 M NaOH for 4 h. The protein concentration was measured by the method of Bradford [20], and radioactivity was counted using a liquid-scintillation

analyser (TRI-CARB 1600-TR; Packard Instrument). The $^{36}\text{Cl}^-$ uptake values were expressed as nmol/mg of protein per 5 min.

Cell lysates, immunoprecipitation and Western blotting

Caco-2 cells grown to confluence in 6-well plates (10×10^4 cells/ml; Corning Costar) were serum-starved overnight and treated with H_2O_2 (1 mM) for 15, 30 and 60 min, or the Src kinase inhibitor PP1 (10 μM) or the PI3K inhibitor LY294002 (5 μM). Cells were washed with ice-cold PBS three times and lysed in lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 1 mM EGTA and 1 $\times$ complete protease inhibitor cocktail]. The cells were homogenized by passing through a 26-gauge needle a total of ten times. The lysate was centrifuged at 5000 g for 5 min at 4 °C, and the protein concentration was determined by the method of Bradford [20]. To monitor the phosphorylation of Fyn, c-Src, p85 or PKC α , Fyn, cSrc, p85 or PKC α was immunoprecipitated by incubating the cell lysates (500 μg) with anti-Fyn, anti-c-Src, anti-p85 or anti-PKC α antibodies respectively, overnight at 4 °C with mixing. Protein G- or A-agarose was added [40 μl of a 50 % (w/v) solution] and mixed for an additional 2 h at 4 °C. Immunoprecipitates were washed three times in lysis buffer and solubilized in SDS-gel-loading buffer. To detect PLC γ 1 phosphorylation in treated Caco-2 cells, equal amounts (approx. 75 μg /sample) of whole-cell lysates were solubilized in SDS-gel-loading buffer and boiled for 5 min.

For Western blotting, the samples obtained above were subjected to SDS/PAGE [8 % or 10 % (w/v) acrylamide] and the proteins were transferred on to nitrocellulose membranes. To detect an association between Fyn and p85, protein-bound nitro-cellulose membranes were first incubated for 60 min at room temperature in blocking buffer containing 1 $\times$ PBS, 0.1 % Tween 20 and 5 % (w/v) non-fat dried milk. Nitrocellulose membranes were then incubated with the polyclonal antibody specific to p85 (1:500 dilution) in buffer containing 1 $\times$ PBS, 0.1 % Tween 20 and 1 % (w/v) non-fat dried milk for 60 min at room temperature and rinsed for 30 min with wash buffer containing 1 $\times$ PBS and 0.1 % Tween 20. Finally, the membranes were incubated with an HRP-conjugated goat anti-(rabbit IgG) antibody (1:2000 dilution) for 60 min at room temperature and washed for 45 min with agitation, during which the wash buffer was changed every 5 min. Bands were visualized with ECL[®] (enhanced chemiluminescence) detection reagents. To detect tyrosine phosphorylated Fyn or p85, the protein-bound nitrocellulose membranes were first incubated for 20 min at room temperature in blocking buffer containing 1 $\times$ PBS and 3 % (w/v) non-fat dry milk. Nitrocellulose membranes were then incubated with the HRP-conjugated anti-phosphotyrosine antibody (1:1000 dilution) in the blocking buffer overnight at 4 °C. The membranes were rinsed in water and washed with the wash buffer containing 1 $\times$ PBS and 0.05 % Tween 20 for 45 min with agitation. Tyrosine-phosphorylated bands were visualized with ECL[®] detection reagents. PLC γ 1 phosphorylation was detected as described previously [21]. c-Src or PKC α phosphorylation was detected by incubating protein-bound nitrocellulose membranes in blocking buffer containing 1 $\times$ TBS [Tris-buffered saline; 20 mM Tris/HCl (pH 7.5) and 500 mM NaCl], 0.1 % Tween 20 and 5 % (w/v) non-fat dried milk for 60 min at room temperature. Membranes were then incubated with the polyclonal anti-(phospho-Src Tyr⁴¹⁶) antibody (1:1000 dilution) or the anti-(phosphorylated protein) antibody (1:100 dilution) in 1 $\times$ TBS, 0.1 % Tween 20 and 2.5 % (w/v) BSA overnight at 4 °C, followed by washes for 45 min with wash buffer containing 1 $\times$ TBS and 0.1 % Tween 20. Phosphorylated bands were visualized with ECL[®] detection reagents.

Membrane translocation of PKC α and PKC δ

In a separate set of experiments, H_2O_2 -treated cells or untreated cells were washed with ice-cold PBS three times and scraped into 400 μl of ice-cold homogenization buffer [20 mM Tris/HCl, (pH 7.5), 250 mM sucrose, 4 mM EDTA, 2 mM EGTa and 1 $\times$ complete protease inhibitor cocktail]. The cells were homogenized on ice with 25 strokes of a glass tissue homogenizer.

The resulting homogenate was ultracentrifuged at 100 000 *g* for 50 min at 4 °C (Optima™ TLX Ultracentrifuge; Beckman). The supernatant was designated as the cytosolic fraction. The pellet was resuspended in 150 µl of the homogenization buffer containing 0.5 % (v/v) Triton X-100 by brief sonication and incubated on ice for 30 min. At the end of the incubation period, the samples were centrifuged at 14 000 *g* for 20 min at 4 °C. The resulting supernatant was designated as the membrane fraction. Activation of PKC α or PKC δ was detected as described previously [21].

Cloning of hPAT1 (human PAT1) for transfection in Caco-2 cells

Full-length cDNA of hPAT1 was amplified from small intestine by RT-PCR (reverse transcription-PCR). Briefly, 5 µg of total RNA was used for reverse transcription with random primers using SuperScript II reverse transcriptase kit (Invitrogen). The full-length cDNA of hPAT1 was then amplified by PCR utilizing gene-specific primers and the proof reading Elongase enzyme mix (Invitrogen), according to the manufacturer's instructions. The primer sequences (designed based on Genbank® accession number AF279265) are: 5' primer, CACCATGGACCTGCGGAGGCGAGACTAC (Kozak sequence is underlined); and 3' primer, GAGTCTGGTGACCGAAACAGGGCT.

PCR products were excised from a 1 % (w/v) agarose gel and purified utilizing Sephaglas™ BandPrep Kit (Amersham Biosciences). The amplified fragment was cloned into the pcDNA3.1D/V5-His-TOPO expression mammalian vector (Invitrogen) in-frame with the V5/His tag. The orientation and the sequence of the insert were confirmed by sequencing, and the expression of hPAT1-V5 fusion protein was examined by Western blotting using anti-V5 antibodies (Invitrogen).

Transient transfection

For transfection studies, Caco-2 cells were transfected using the Nucleofector System (Amaxa), according to manufacturer's instructions. Briefly, approx. 10×10^6 cells were harvested and were electroporated in 100 µl of solution T (supplied by Amaxa) along with 30 µg of the PAT1-V5 cDNA construct. The cells were transferred to full medium and plated on 12 wells of a 24-well plate. After 24 h, transfected cells were used for cell-surface biotinylation.

Cell-surface biotinylation

Cell-surface biotinylation was performed using sulfo-NHS-biotin (sulfo-*N*-hydroxysuccinimidobiotin; 0.5 mg/ml; Pierce) in borate buffer [154 mM NaCl, 7.2 mM KCl, 1.8 mM CaCl₂ and 10 mM H₃BO₃ (pH 9.0)], as described previously [22], with labelling for 60 min at 4 °C to stop endocytosis and internalization of antigens. Anti-DRA (custom synthesized against peptide corresponding to C-terminal sequence) or anti-V5-purified (C-terminus of PAT1 tagged with V5 epitope) antibodies were utilized. Surface DRA or PAT1 was compared with total cell antigen as determined by immunoblotting in solubilized cell extracts and with the amount of DRA or PAT1 not removed by avidin precipitation method (intracellular pool). To determine the efficiency of biotinylation, the blots were also probed with an avidin peroxidase antibody (1:1000 dilution for 60 min).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Each independent set represents the means $\pm$ S.E.M. of data from at least nine wells used on three separate occasions. A Student's *t* test was used for statistical analysis. *P* < 0.05 was considered statistically significant.

RESULTS

H₂O₂ inhibits Cl⁻/OH⁻ exchange activity

To examine the effects of H₂O₂ on the Cl⁻/OH⁻ exchange activity, Caco-2 cells were incubated with H₂O₂ in cell culture medium at a concentration of 1 mM for 60 min and DIDS-sensitive (300 μM) Cl⁻/OH⁻ exchange activity was assessed. A significant decrease in Cl⁻/OH⁻ exchange activity (approx. 65 %) was observed at 60 min (3.008 ± 0.18 and 1.056 ± 0.22 nmol/mg of protein per 5 min in control cells and H₂O₂-treated cells respectively). Analysis of the effects of H₂O₂ on Cl⁻/OH⁻ exchange activity at higher concentrations of DIDS (600 μM and 1 mM) also showed almost similar inhibition by H₂O₂ (results not shown). The observed decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells by H₂O₂ was in agreement with the previous *in vitro* electrophysiological studies showing that H₂O₂ inhibited neutral NaCl absorption and stimulated electrogenic Cl⁻ secretion [5]. It should be noted that the concentration of H₂O₂ used (1 mM for 60 min) in our studies has been shown previously not to alter transepithelial electrical resistance in Caco-2 cells [23]. As, in these studies, the observed response was due to the release of prostaglandins, we next examined the role of prostaglandins. However, the dual COX inhibitor indomethacin (0.1 or 1.0 μM for 60 min) failed to abolish the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells (Table 1a).

Role of Src kinases in H₂O₂-induced inhibition of Cl⁻/OH⁻ exchange activity

Previous studies have shown that H₂O₂ triggers tyrosine phosphorylation of cellular proteins in T84 and Caco-2 cells [24]. Therefore it was of interest to determine the role of tyrosine kinases in the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity in Caco-2 cells. The effect of herbimycin A (a non-specific inhibitor; 1 μM for 60 min) and PP1 (a specific Src family kinase inhibitor; 10 μM for 60 min) on the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity was examined. Both of these inhibitors (Table 1b) blocked the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells, thereby suggesting the involvement of tyrosine kinases in the effects of H₂O₂ on Cl⁻/OH⁻ exchange activity.

H₂O₂ induces the tyrosine phosphorylation of Fyn

Previous studies have shown that the effects of H₂O₂ are mediated primarily through the activation of p60Src [25] and Fyn [26] in different cell types. Tyrosine kinases such as Lck and Syk have also been found to be activated by oxidative stress [12]. As our studies show the involvement of Src kinase members in H₂O₂-mediated cellular effects, we decided to identify the precise Src kinase involved in the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity. For this, the tyrosine phosphorylation profile of different Src kinase members, including c-Src, Fyn, Lyn, Yes and Lck, was studied in Caco-2 cells following stimulation with H₂O₂ for 15–60 min. H₂O₂ increased the tyrosine phosphorylation of Fyn (Figures 1A and 1B; 1.7-fold increase at 60 min compared with control), whereas it failed to have any effect on c-Src [Figure 1C; phosphorylated c-Src normalized to total c-Src expression, 2.68 ± 0.12 and 2.98 ± 0.095 arbitrary units for control cells and H₂O₂-treated cells (60 min) respectively; $n = 3$].

Interestingly, in the presence of PP1, this increased phosphorylation of Fyn was significantly inhibited. The other Src kinase members Lyn, Yes and Lck could not be detected in Caco-2 cells [19]. Thus our results show that Fyn might be the major Src kinase member involved in the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity in Caco-2 cells.

H₂O₂ induces Fyn/p85 association

Previous studies have shown that non-receptor protein tyrosine kinases physically associate with PI3K [27]. The SH3 (Src homology 3) domain of Src family kinases can directly interact

with the SH2 (Src homology 2) domain of p85, and this may be a mechanism to regulate PI3K activity [28]. In order to elucidate the potential role of PI3K in the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells, we examined the association of PI3K with Fyn in H₂O₂-treated Caco-2 cells. Cells were treated with H₂O₂ at different time points (15–60 min), lysed and immunoprecipitated with the anti-Fyn antibody and probed with the anti-p85 antibody. As shown in Figure 2(A), immunoprecipitation analysis showed a constitutive association of Fyn and p85, which was enhanced in the presence of H₂O₂ at 60 min (1.7-fold increase compared with control; Figure 2B). Moreover, tyrosine phosphorylation of p85 was also observed in response to H₂O₂ (Figures 3A and 3B; 1.6-fold increase at 60 min compared with control). Interestingly, the enhanced p85 phosphorylation at 60 min was abolished in the presence of PP1. The results indicated further that Fyn might be directly involved in the activation of the downstream signalling enzyme PI3K in the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells.

Role of PI3K

The increase in p85 phosphorylation and its association with Fyn (Figures 2A, 2B, 3A and 3B) was consistent with our functional data showing a significant prevention in the inhibition of Cl⁻/OH⁻ exchange activity in Caco-2 cells at similar time (60 min) by the PI3K inhibitor LY294002 (5 μM for 60 min; Table 1c). The findings confirm further the involvement of PI3K in mediating the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells.

H₂O₂ activates PLCγ1

Several studies have implicated the role of both Src family kinase members [29] and PI3K [30] in the activation of PLCγ1. We next investigated the effect of H₂O₂ on PLCγ1 phosphorylation in Caco-2 cells. Cells were treated with H₂O₂ (1 mM) for 60 min or the Src kinase inhibitor PP1 (10 μM), lysed and probed with the anti-(phospho-PLCγ1) antibody. As shown in Figure 4(A), H₂O₂ resulted in a significant increase in PLCγ1 phosphorylation at 60 min (2.5-fold increase compared with control; Figure 4B). However, in the presence of PP1, the increase in PLCγ1 phosphorylation observed at 60 min was abolished. Similar results were obtained with the PI3K inhibitor LY294002 (5 μM; Figures 4C and 4D). The results indicate that PLCγ1 acts as a downstream target to both the Src kinase Fyn and PI3K in the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells. As the findings suggested the involvement of PLCγ1 in H₂O₂-mediated effects, we directly examined the role of PLCγ1 in the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity. For this, the specific PLC inhibitor ET-18 (10 μM) was used. ET-18 (Table 1d) significantly inhibited the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells. These findings confirmed the involvement of PLCγ1 in mediating the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity.

Role of Ca²⁺ and PKC in the H₂O₂-induced inhibition of Cl⁻/OH⁻ exchange activity

Activated PLCγ1 cleaves the membrane phospholipid PtdIns(4,5)P₂ generating two second messengers, DAG (diacyl-glycerol) and Ins(1,4,5)P₃. The former functions to activate PKC, whereas the latter stimulates the release of Ca²⁺ from internal stores. We thus examined the role of Ca²⁺ in mediating the ²⁺ chelator BAPTA-AM effects of H₂O₂ in our system using the Ca (20 μM for 60 min). As shown in Table 1(e), in the presence of BAPTA-AM, the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity was significantly abolished. The results suggested that the inhibitory effects of H₂O₂ on the Cl⁻/OH⁻ exchange activity in Caco-2 cells were dependent on the intracellular Ca²⁺ levels. Thus it appears that PLCγ1 might be one of the major effectors involved in stimulating the Ca²⁺ levels. We next investigated the role of PKC in the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells. As shown in Table 1(f), chelerythrine chloride (2 μM) or calphostin C (200 nM) completely abolished

the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity. These results suggest that the observed decrease in Cl⁻/OH⁻ exchange activity involved PKC activation.

PKC α is involved in the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity

As PKC was involved in the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells, we next examined which PKC isoform might be involved in the H₂O₂-mediated signalling pathway. The involvement of the nPKC (novel PKC) and aPKC (atypical PKC) isoforms was determined by using specific inhibitors for the nPKC isoforms, namely Ro318220 (PKC ϵ inhibitor; 100 nM for 60 min) and rottlerin (PKC δ inhibitor; 10 μ M for 60 min), and for the aPKC isoform, namely Go6983 (PKC ζ inhibitor; 10 μ M for 60 min); however, no change in the H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity was observed in Caco-2 cells when these inhibitors were used (Table 1g).

We then used the specific PKC α inhibitor Go6976 to study the role of PKC α in the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity. Go6976 (5 and 10 nM; Table 1g) significantly inhibited the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells. The results were confirmed further by studying the translocation and phosphorylation of PKC α in response to H₂O₂. H₂O₂ (1 mM) significantly increased PKC α in the membrane fraction of Caco-2 cells (Figures 5A and 5B; 2-fold increase compared with control) as well as PKC α phosphorylation at 60 min (Figures 5C and 5D; 1.6-fold increase compared with control). The increase in PKC α phosphorylation was completely blocked in the presence of the PI3K inhibitor LY294002, indicating that PI3K acts as the upstream mediator of PKC α . Thus PKC α activation at 60 min in response to H₂O₂ correlated well with the functional data, demonstrating a significant attenuation in H₂O₂-induced inhibition of Cl⁻/OH⁻ exchange activity by Go6976 (Table 1g). The results indicated further that PKC α might be the major isoform involved in mediating the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells. We also studied the translocation of PKC δ upon H₂O₂ treatment, as H₂O₂ is also known to activate PKC δ in Caco-2 cells [31]. As shown in Figure 5(E), H₂O₂ (1 mM for 60 min) did not increase PKC δ in the membrane fraction of Caco-2 cells [membrane PKC δ expression normalized to total PKC δ (sum of membrane and cytosol) expression, 0.532 ± 0.006 and 0.568 ± 0.040 arbitrary unit in control cells and H₂O₂-treated cells (at 60 min) respectively; $n = 3$].

Surface (plasma-membrane) expression of DRA and PAT1

As the observed inhibition of Cl⁻/OH⁻ exchange activity by H₂O₂ occurred within 1 hour (acute or short-term), this type of regulation might be due to a decrease in the number of active transporter molecules at the membrane (endocytic retrieval) or decreased insertion of the transporter into the plasma membrane and/or rapid degradation by changes in the turnover number of individual exchanger molecules [32,33]. We, therefore, examined the effect of H₂O₂ on DRA and PAT1 protein expression on the plasma membrane. DRA and PAT1, two members of SLC26 gene family, have been implicated as potential candidate proteins for apical Cl⁻/OH⁻ (HCO₃⁻) exchange in the human intestine [34,35]. It should be noted that PAT1 is endogenously expressed in Caco-2 cells; however, for determining PAT1 surface expression in Caco-2 cells, we used PAT1-V5-transfected cells as the commercially available anti-V5 antibody is more sensitive in detecting the PAT1 protein (V5 epitope-tagged to PAT1) compared with the antibodies raised against the native hPAT1 protein. Cell-surface biotinylation studies were performed to determine the changes in cell-surface antigen, defined as the biotin-accessible fraction of total cellular antigen. Biotinylated proteins from control and H₂O₂-treated cells were separated from the cell lysate by avidin, and proteins were probed with affinity-purified human anti-DRA or anti-V5 antibodies (for tagged PAT1). The expression of DRA (Figure 6A) and PAT1 (Figure 6B) on the surface was not significantly altered in response to H₂O₂. [surface expression normalized to total (sum of apical and

intracellular) expression, 0.583 ± 0.019 and 0.565 ± 0.031 arbitrary unit in control cells and H_2O_2 -treated cells (at 60 min) respectively, for DRA; and 0.314 ± 0.015 and 0.343 ± 0.026 arbitrary unit in control cells and H_2O_2 -treated cells (at 60 min) respectively, for PAT1; $n = 3$). We also performed biotinylation experiments in non-transfected cells (endogenous surface PAT1 expression). Similar results were obtained with the rabbit anti-PAT1 antibody [22] when compared with the anti-PAT1 V5 blot (results not shown). H_2O_2 did not alter the efficiency of biotinylation as determined by probing the surface proteins with an avidin–peroxidase antibody (results not shown). These results suggest that acute regulation of Cl^-/OH^- exchange activity in Caco-2 cells by H_2O_2 does not involve alterations in the surface level of DRA and PAT1, thereby ruling out the possibility that H_2O_2 -mediated inhibition of Cl^-/OH^- exchange activity is due to a decrease in the number of DRA and PAT1 molecules on the plasma membrane.

DISCUSSION

H_2O_2 has been widely studied as a potential regulator of intestinal epithelial function. The focus of several studies [5–10] has been to identify the role of H_2O_2 and ROS in modulating ion transport function. H_2O_2 has been shown to stimulate Cl^- secretion and inhibit NaCl absorption in rat colonic epithelium [5]; however, this response was attributed to the release of prostaglandins that subsequently acted on submucosal neurons to stimulate the release of neurotransmitters, rather than directly on the epithelium. Conversely, a recent study failed to demonstrate the role of prostaglandins in the effects of H_2O_2 on increased electrogenic Cl^- secretion in rat colon [6]. The direct effects of H_2O_2 on Cl^- secretion have been examined previously using cultured T84 cells [7]; however, to date the direct effects of H_2O_2 on the luminal Cl^-/OH^- exchanger in the human intestine remain unknown. Our present results, for the first time, demonstrate that the inhibitory effects of H_2O_2 on Cl^-/OH^- exchange activity in Caco-2 cells were independent of the prostaglandin-mediated COX-dependent pathway, but occurred via a novel and complex signalling pathway that involved the activation of the Src kinase Fyn, PI3K, PLC γ 1 and the Ca^{2+} -dependent PKC α .

The role of tyrosine kinases in the H_2O_2 -mediated decrease in Cl^-/OH^- exchange activity in Caco-2 cells was confirmed using specific tyrosine kinase inhibitors. The results are in agreement with the previous findings showing that H_2O_2 can trigger the tyrosine phosphorylation of cellular proteins in T84 and Caco-2 cells [24]. Our present results provide evidence, for the first time, for the involvement of Fyn in the H_2O_2 -mediated decrease in Cl^-/OH^- exchange activity in Caco-2 cells. However, H_2O_2 failed to induce the phosphorylation of c-Src.

A number of studies have shown that the Src family kinases, including p60Src and Fyn, act directly or indirectly to phosphorylate and/or activate the effector protein PI3K [27,36]. Binding to the SH2 domain of the PI3K regulatory subunit p85 is mediated through the SH3 domain of Src family kinases. Our present immunoprecipitation and tyrosine phosphorylation studies are in agreement with previous findings indicating enhanced association of Fyn and p85, as well as increased tyrosine phosphorylation of p85 at 60 min in response to H_2O_2 [28]. Interestingly, in the presence of the specific Src kinase inhibitor PP1, the observed phosphorylation was significantly abolished, thereby indicating that PI3K might act as a downstream target to Fyn in the H_2O_2 -mediated signalling pathway. In addition, the increase in p85 phosphorylation and its association with Fyn was consistent with our functional results, showing significant reversal in the inhibition of Cl^-/OH^- exchange activity by the specific PI3K inhibitor LY294002 ($5 \mu\text{M}$ for 60 min). The findings confirmed further the role of PI3K in the H_2O_2 -mediated inhibition of the Cl^-/OH^- exchange process.

The roles of both Src family kinases and PI3K have been suggested in the activation of PLC γ 1 [29]. Thus the increase in phosphatidylinositols [PtdIns(4,5) P_2 and PtdIns(3,4,5) P_3] by

Src tyrosine kinases and PI3K is essential for the activation and translocation of PLC γ 1 [30]. Our present studies have also demonstrated that the PLC inhibitor ET-18 (10 μ M for 60 min) significantly blocked H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity. It should be noted that the functional results were consistent with our phosphorylation findings showing a significant increase in PLC γ 1 phosphorylation at 60 min. This observed increase was attenuated by PP1, indicating that PLC γ 1 acts as a downstream target of Fyn and PI3K in the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells. Our present results are consistent with previous findings showing the phosphorylation of PLC γ 1 on tyrosine residues in response to H₂O₂ [17].

In epithelial cells, PLC γ 1 hydrolyses PtdIns(4,5) P_2 to produce Ins(1,4,5) P_3 and DAG [37]. Ins(1,4,5) P_3 stimulates the release of Ca²⁺ from internal stores, and DAG is known to activate the serine/threonine kinase PKC. In the present study, we have demonstrated the role of both Ca²⁺ and PKC in the inhibitory effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells. This was confirmed by using the specific PKC α inhibitor Go6976, which significantly abolished the H₂O₂-mediated decrease in Cl⁻/OH⁻ exchange activity in Caco-2 cells. We also observed enhanced PKC α association in the membrane fraction of Caco-2 cells, as well as increased PKC α phosphorylation at 60 min in response to H₂O₂, thereby indicating the role of PKC α in the H₂O₂-mediated signalling pathway. Our present results are consistent with the previous studies showing that H₂O₂ also induced PKC α activation in endothelial cells [38] and fibroblasts [16]. Moreover, H₂O₂-induced PKC α phosphorylation was abolished in the presence of the PI3K inhibitor LY294002, suggesting that PI3K acts as an upstream mediator of PKC α . We have shown previously the involvement of different PKC isoforms in the inhibition of apical Cl⁻/OH⁻ exchange activity in Caco-2 cells by different external stimuli, such as PMA (PKC ϵ) [39], 5-hydroxytryptamine (serotonin; PKC δ) [19] and taurodeoxycholate (PKC β 1) [40]. With respect to the effects of PMA, it appears that, although PKC α is activated by PMA, such an activation alone may not be sufficient to inhibit Cl⁻/OH⁻ exchange activity. It is possible that inhibition of Cl⁻/OH⁻ exchange activity by PKC α requires an additional accessory protein(s) that converges the signal to inhibit Cl⁻/OH⁻ exchange activity in response to H₂O₂, but these same factors may not be activated by PMA. Although previous studies have shown that H₂O₂ could activate c-Src and PKC δ [31,41], our present studies failed to show such an activation. We speculate that the observed discrepancy in the effects of H₂O₂ on PKC δ activation and c-Src phosphorylation in Caco-2 cells compared with previous studies may be due to different cell culture conditions, e.g. cell culture medium, differentiation status [31] and H₂O₂ treatment [41].

It should be noted that the effects of H₂O₂ on Cl⁻/OH⁻ exchange activity appear to be specific, involving complex signalling pathways, and are not secondary to changes in NHE activity, as parallel studies from our laboratory have demonstrated that H₂O₂ had no significant effects on NHE activity in Caco-2 cells (I. Syed, S. Saksena and P.K. Dudeja, unpublished work).

Regulation of multiple plasma membrane transport proteins under acute conditions has been shown to occur via recycling mechanisms (increased endocytic retrieval or decreased exocytic insertion) [32,33]. In addition, PI3K plays a crucial role in the trafficking of transporter proteins from the plasma membrane to the intracellular compartments and vice versa [42]. However, our present studies did not show any alteration in the surface expression of either of the apical Cl⁻/OH⁻ exchangers DRA or PAT1 in response to H₂O₂. Moreover, similar results were obtained with a rabbit anti-PAT1 antibody (detecting endogenous surface PAT1 expression) [22] when compared with the anti-V5 immunoblot (detecting exogenous surface PAT1 expression). These results suggested that H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity is independent of recycling events and may involve other events, such as protein phosphorylation of downstream effector molecules or accessory proteins, which may in turn phosphorylate Cl⁻/OH⁻ exchangers. Our low-stringent analysis of both DRA and PAT1 using

in silico analysis by Scansite2.0 [43] identified phosphorylation sites for various PKC isoforms (α , β , γ , δ , ϵ , μ and ζ), MAPK, Akt, calmodulin-dependent kinase II, casein kinase II etc. These phosphorylation sequence motifs may play an important role in the direct or indirect phosphorylation of DRA and PAT1. Future studies will address this important aspect regarding the role of PKC α in the phosphorylation of the apical Cl⁻/OH⁻ exchangers, e.g. DRA and/or PAT1, resulting in the attenuation of Cl⁻/OH⁻ exchange activity by H₂O₂.

On the basis of the results in the present study, we have proposed a model for the effects of H₂O₂ on Cl⁻/OH⁻ exchange activity in Caco-2 cells (Figure 7). H₂O₂ activates Src family kinase Fyn, which then activates PI3K. Both Fyn and PI3K associate to activate PLC γ 1. PLC γ 1 activation generates Ins(1,4,5)*P*₃ and DAG, which subsequently result in the increase in the levels of intracellular Ca²⁺ and activation of PKC α respectively, leading to inhibition of the apical membrane Cl⁻/OH⁻ exchange activity.

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Abbreviations used

BAPTA-AM

1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxymethyl ester)

COX

cyclo-oxygenase

DAG

diacylglycerol

DIDS

4,4'-di-isothiocyanostilbene-2,2'-disulfonate

DMEM

Dulbecco's modified Eagle's medium

DPC

N-phenylanthranilate

DRA

down-regulated in adenoma

HRP

horseradish peroxidase

MAPK

mitogen-activated protein kinase

NHE

Na⁺/H⁺ exchanger

PAT1

putative anion transporter 1

hPAT1

	human PAT1
PI3K	phosphoinositide 3-kinase
p85	regulatory subunit of PI3K
PKC	protein kinase C
aPKC	atypical PKC
nPKC	novel PKC
PLC	phospholipase C
ROM	reactive oxygen metabolite
ROS	reactive oxygen species
SH2	Src homology 2
SH3	Src homology 3
sulfo-NHS-biotin	sulfo- <i>N</i> -hydroxysuccinimidobiotin

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Figure 1. H₂O₂ causes the activation of Fyn but not c-Src in Caco-2 Cells

(A) For Fyn phosphorylation, Caco-2 cells were incubated with H₂O₂ (1 mM) in the cell culture medium for different time intervals, ranging from 15–60 min. Cells were also pre-treated with the Src kinase inhibitor PP1 (10 μ M) for 60 min and then co-incubated with H₂O₂ (1 mM) for another 60 min. Cells were washed with 1 $\times$ PBS, lysed and immunoprecipitated with the anti-Fyn antibody, and probed with the anti-phosphotyrosine antibody (pFyn). The blots were stripped and re-probed with the anti-Fyn antibody (Fyn) to indicate equal loading of protein in each lane. (B) Fyn phosphorylation was quantified by densitometric analysis. Values are means $\pm$ S.E.M. of three determinations and are expressed as arbitrary units (A.U.). * P < 0.05 compared with untreated control (0 min). H₂O₂-induced phosphorylation of Fyn at 60 min was

blocked by the Src family kinase inhibitor PP1. (C) For c-Src phosphorylation, Caco-2 cells treated with H₂O₂ (1 mM) for 15–60 min were immunoprecipitated with the anti-(c-Src) antibody and probed with the anti-(phospho-Src Tyr⁴¹⁶) antibody (pc-Src). The blots were stripped and re-probed with the anti-(c-Src) antibody (cSrc) to indicate equal loading of protein in each lane.

Figure 2. H₂O₂ induces Fyn/p85 association in Caco-2 cells

(A) Caco-2 cells were incubated with H₂O₂ (1 mM) in the cell culture medium for different time intervals, ranging from 5–60 min. Cells were washed with 1× PBS, lysed and immunoprecipitated with the anti-Fyn antibody. Immunoprecipitates were analysed by SDS/PAGE [8 % (w/v) acrylamide], followed by transfer of proteins to nitrocellulose and probed with anti-p85 antibody (p85). H₂O₂ enhanced the constitutive association of Fyn and p85 at 60 min. The blots were stripped and re-probed with the anti-Fyn antibody (Fyn) to indicate equal loading of protein in each lane. (B) The Fyn/p85 association was quantified by densitometric analysis. Values are means ± S.E.M. of three determinations and are expressed as arbitrary units (A.U.). **P* < 0.05 compared with the untreated control (0 min).

Figure 3. H₂O₂ induces tyrosine phosphorylation of p85 in Caco-2 cells

(A) Caco-2 cells were incubated with H₂O₂ (1 mM) in the cell culture medium for 15, 30 and 60 min (in duplicate). Cells were also pre-treated with the Src kinase inhibitor PP1 (10 μ M) for 60 min and then co-incubated with H₂O₂ (1 mM) for another 60 min. Cells were washed with 1 $\times$ PBS, lysed and immunoprecipitated with an anti-p85 antibody. Immunoprecipitates were analysed by SDS/PAGE [8 % (w/v) polyacrylamide gel], followed by transfer of proteins on to nitrocellulose and probed with anti-phosphotyrosine antibody (Tyr p85). H₂O₂-induced phosphorylation of p85 at 60 min was blocked by the Src family kinase inhibitor PP1. The blots were stripped and re-probed with the anti-p85 antibody (p85) to indicate equal loading of protein in each lane. (B) The tyrosine phosphorylation of p85 was quantified by densitometric analysis. Values are means $\pm$ S.E.M. of three determinations and are expressed as arbitrary units (A.U.). * P < 0.05 compared with the untreated control (0 min).

Figure 4. PLC γ 1 phosphorylation by H_2O_2 in Caco-2 Cells

(A, C) Caco-2 cells were incubated with H_2O_2 (1 mM) in the cell culture medium for 60 min. Cells were also pre-treated with the Src kinase inhibitor PP1 (10 μ M) (A) or the PI3K inhibitor LY294002 (5 μ M) (C) for 60 min and then co-incubated with H_2O_2 (1 mM) for another 60 min. After washing the cells with $1 \times$ PBS, extracted proteins (75 μ g) were subjected to Western blot analysis by SDS/PAGE [8 % (w/v) acrylamide gel] using the phospho-PLC γ 1 antibody (pPLC γ 1). H_2O_2 -induced phosphorylation of PLC γ 1 at 60 min was blocked by PP1 and LY294002. The blots were stripped and re-probed with the anti-PLC γ 1 antibody (PLC γ 1) to indicate equal loading of protein in each lane. (B, D) The phosphorylation of PLC γ 1 was

quantified by densitometric analysis. Values are means $\pm$ S.E.M. of three determinations and are expressed as arbitrary units (A.U.). * $P < 0.05$ compared with the untreated control (0 min).

Figure 5. Activation of PKC α , but not PKC δ , in Caco-2 cells in response to H₂O₂: time course of membrane translocation of PKC α or PKC δ

(A) Caco-2 cells were treated with 1 mM H₂O₂ for 15, 30 and 60 min. The membrane fraction was subjected to SDS/PAGE and probed with the specific PKC α antibody (PKC α) to examine its subcellular distribution. The PKC α blots were stripped and re-probed with the anti-actin antibody (actin) to indicate equal loading of protein in each lane. (C) Caco-2 cells were incubated with H₂O₂ (1 mM) in the cell culture medium for 60 min. Cells were also pre-treated with the PI3K inhibitor LY294002 (5 μ M; LY) for 60 min and then co-incubated with H₂O₂ (1 mM) for another 60 min. Cells were immunoprecipitated with the anti-PKC α antibody and probed with anti-(phosphorylated protein) antibody (pPKC α). H₂O₂-induced phosphorylation

of PKC α at 60 min was blocked by LY294002. The blots were stripped and re-probed with the anti-PKC α antibody (PKC α) to indicate equal loading of protein in each lane. The results for membrane (**B**) or phosphorylated (**D**) PKC α were quantified by densitometric analysis. Values are means $\pm$ S.E.M. of three determinations and are expressed as arbitrary units. * P < 0.05 compared with the untreated control (0 min). (**E**) Caco-2 cells were treated with 1 mM H₂O₂ for 15, 30 and 60 min. The membrane (M) and cytosolic (C) fractions were subjected to SDS/PAGE and probed with the specific PKC δ antibody (PKC δ) to examine its subcellular distribution.

Figure 6. Cell-surface expression of DRA or PAT1

Normal Caco-2 or PAT1-V5-cDNA-transfected cells grown on plastic supports were treated with H₂O₂ for 60 min and subjected to biotinylation at 4 °C using sulfo-NHS-biotin. After solubilization, biotinylated proteins were extracted with streptavidin–agarose from equal amounts of total cellular protein. Surface and intracellular fractions were separated by SDS/PAGE [10 % (w/v) acrylamide gel], followed by the transfer of proteins on to a nitrocellulose membrane. The blot was immunostained with a rabbit anti-DRA (A) or anti-V5 (B) antibody. A representative blot of three different experiments is shown.

Figure 7. Proposed model of H_2O_2 -induced inhibition of Cl^-/OH^- exchange activity in Caco-2 cells involving a Fyn/PI3K/PLC γ 1/PKC α -mediated pathway
 IP3, Ins(1,4,5) P_3 ; PIP2, PtdIns(4,5) P_2 .

Table 1Effect of various inhibitors on H₂O₂-mediated inhibition of Cl⁻/OH⁻ exchange activity in Caco-2 cells

(a) Role of dual COX inhibitor		
Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)		
Treatment	Control	H ₂ O ₂
Vehicle	3.26 ± 0.12	1.37 ± 0.17 *
Indomethacin (1 μM)	2.98 ± 0.17	1.06 ± 0.19 *
(b) Role of tyrosine kinases		
Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)		
Treatment	Control	H ₂ O ₂
Vehicle	3.12 ± 0.10	1.34 ± 0.05 *
Herbimycin A (1 μM)	3.06 ± 0.07	3.50 ± 0.07
PP1 (10 μM)	2.73 ± 0.31	2.48 ± 0.55
(c) Role of PI3K		
Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)		
Treatment	Control	H ₂ O ₂
Vehicle	3.57 ± 0.18	2.08 ± 0.04 *
LY294002 (5 μM)	3.08 ± 0.27	3.44 ± 0.19
(d) Role of PLC		
Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)		
Treatment	Control	H ₂ O ₂
Vehicle	3.76 ± 0.11	2.33 ± 0.11 *
ET-18 (10 μM)	3.45 ± 0.09	3.88 ± 0.07
(e) Role of Ca²⁺ chelator BAPTA-AM		
Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)		
Treatment	Control	H ₂ O ₂
Vehicle	2.93 ± 0.16	1.69 ± 0.18 *
BAPTA-AM (20 μM)	2.64 ± 0.31	2.79 ± 0.22
(f) Role of PKC antagonists		
Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)		
Treatment	Control	H ₂ O ₂

(a) Role of dual COX inhibitor

Treatment	Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)	
	Control	H ₂ O ₂
Vehicle	3.08 ± 0.26	1.76 ± 0.03 *
Chelerythrine Cl (2 μM)	2.96 ± 0.06	3.13 ± 0.02
Calphostin C (200 nM)	3.06 ± 0.06	3.30 ± 0.03

(g) Role of specific PKC isoform inhibitors

Treatment	Cl ⁻ /OH ⁻ exchange activity (nmol/mg of protein per 5 min)	
	Control	H ₂ O ₂
Vehicle	3.28 ± 0.14	2.23 ± 0.06 *
Go6976 (5 nM)	3.19 ± 0.12	2.68 ± 0.08
Go6976 (10 nM)	3.21 ± 0.06	3.07 ± 0.21
Ro318220 (100 nM)	3.31 ± 0.02	2.05 ± 0.14 *
Rottlerin (10 μM)	3.09 ± 0.17	2.16 ± 0.03 *
Go6983 (10 μM)	3.12 ± 0.15	2.13 ± 0.11 *

Values are means ± S.E.M. of 3–6 separate experiments performed in triplicate. Caco-2 cells were pre-incubated with the specific inhibitor for 60 min in the cell culture medium and then co-incubated with 1 mM H₂O₂ for another 60 min. Cl⁻/OH⁻ exchange activity was measured as DIDS-sensitive (300 μM) ³⁶Cl uptake at 5 min.

* $P < 0.05$ compared with control.