Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 1312–1319
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Interferon-γ-induced STAT1-mediated membrane retention of NHE1 and associated proteins ezrin, radixin and moesin in HT-29 cells

Fernando Magro [1], Sónia Fraga, Patrício Soares-da-Silva *

Institute of Pharmacology and Therapeutics, Faculty of Medicine, 4200 Porto, Portugal

Received 20 June 2005; accepted 18 July 2005

Abstract

This study evaluated the effect of interferon-γ (IFN-γ) upon the function and expression of type 1 Na^+/H^+ exchanger (NHE1) in human intestinal epithelial HT-29 cells, namely that concerning the abundance of surface NHE1 and NHE1 binding to the ezrin, radixin and moesin (ERM) family of proteins. HT-29 cells express endogenous NHE1 and the ERM family of proteins that retain the localization of NHE1 in the membrane. Long-term exposure (24 h) of HT-29 cells to IFN-γ resulted in a concentration-dependent decrease in NHE1 activity. Inhibition of NHE1 activity by IFN-γ was absent after pretreatment with cariporide. The long-term exposure to IFN-γ was accompanied by increase in surface NHE1 and ERM abundance and no changes in total NHE1 and ERM abundance. Inhibition of signal transducer and activator transcription factor 1 (STAT1) with epigallocatechin-3-gallate (EGCG) prevented the inhibitory effect of IFN-γ. Treatment with IFN-γ activated phospho-STAT1 was markedly attenuated by EGCG. The IFN-γ-induced increase in surface NHE1 and ERM abundance was prevented by EGCG. In conclusion, long-term inhibition of NHE1 activity by IFN-γ involves STAT1 phosphorylation and is accompanied by increased abundance of surface NHE1 and the NHE1 membrane anchoring ERM proteins.

Keywords: Interferon-γ; NHE1; ERM proteins; HT-29 cells

1. Introduction

Recent evidence suggests that diarrhea in inflammatory bowel disease (IBD) may relate to a reduction in electrolyte absorption rather than increases in electrolyte secretion [1]. This fits well the view that the colonic mucosa of IBD patients responds poorly to secretagogues and that Na^+ absorption is diminished [2]. Consistent with these findings is the observation that interferon-γ (IFN-γ), a cytokine suggested to be involved in the pathophysiology of IBD, reduces agonist-induced and cAMP-induced Cl^- intestinal secretion [3]. IFN-γ has also been shown to downregulate the cystic fibrosis conductance regulator [3], the Na^+-K^+-$2Cl^-$ cotransporter [4,5], the Na^+/H^+ exchanger [6] and the Na^+-K^+-ATPase [7,8].

In general, the signal transduction pathway initiated by binding of IFN-γ to its receptor leads first to Janus kinase (JAK) 1 and 2 activation and their association with the IFN-γ receptor [9]. JAK then phosphorylate IFN-γ receptor on specific tyrosines, which serve as docking sites for the signal transducer and activator transcription factor 1 (STAT1). Recently, we have reported on the effects of IFN-γ on intestinal Na^+-K^+-ATPase activity and the intracellular signaling pathways involved in cultured human intestinal epithelial Caco-2 cells, while using probes that interact with Raf-1, type 2 extracellular signal-regulated kinases (ERK2), p38 mitogen-activated protein kinase (p38 MAPK), MAPK/ERK kinase (MEK), protein kinase C (PKC) and STAT1, and measuring the degree of activation of p38 MAPK and STAT1. It was shown that the decrease in Na^+-K^+-ATPase activity by long-term exposure (24 h) to IFN-γ may involve the activation of PKC downstream STAT1 phosphorylation and Raf-1, MEK, ERK2 and p38 MAPK pathways, in a complex sequence of events [10]. The IFN-γ-induced decrease in Na^+-K^+-ATPase activity was accompanied by no changes in the abundance

* Corresponding author. Tel.: +351 22 5519147; fax: +351 22 5502402.
E-mail address: pss@med.up.pt (P. Soares-da-Silva).
[1] On leave from Department of Gastroenterology, Faculty of Medicine, 4200 Porto, Portugal.

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.07.015

of α_1 subunit Na^+-K^+-ATPase [10]. In the same cell line, however, long-term exposure (24 h) to IFN-γ was found to decrease isoform 1 Na^+/H^+ exchanger (NHE1) activity that was accompanied by an increase in surface abundance of NHE1 [11]. The transduction mechanisms set into motion by long-term exposure to IFN-γ involved STAT1 activation with p38 MAPK playing a minor role in SER701 phosphorylation of STAT1 [11]. The increase in surface abundance of NHE1 after long-term exposure to IFN-γ in parallel with inhibition of NHE1 activity is a most unusual observation. In fact, the most prevalent findings with IFN-γ indicate that inhibitory effects by the cytokine are usually accompanied by down-regulation of the target proteins [3–8]. However, in the case of Caco-2 cells the inhibitory effects of IFN-γ upon NHE1 and Na^+-K^+-ATPase activities did not follow that pattern [10,11].

The aim of the present study was to examine the effects of IFN-γ upon the function and expression of NHE1 in human intestinal epithelial HT-29 cells, in particular in what concerns the abundance of surface NHE1 after IFN-γ treatment and evaluate the role of mechanisms restricting NHE1 at the cell membrane, namely those involving direct NHE1 binding to the ezrin, radixin and moesin (ERM) family of proteins [12–14]. The main reason to use HT-29 cells, a subclone of human colorectal cancer cell line, is because they grow as highly differentiated polarized epithelial monolayers with properties of Cl^-, and mucous-secreting cells [15] and express NHE1 in the both apical and basal lateral membranes of polarized HT-29 cells in culture [16,17]. It is reported a decrease in NHE1 activity and increase in NHE1 and ERM surface abundance after long-term exposure to IFN-γ. The transduction mechanisms set into motion by long-term exposure to IFN-γ involves STAT1 phosphorylation and the STAT1 inhibitor (-)-epigallocatechin-3-gallate (EGCG) prevented the IFN-γ-induced inhibition of NHE1 activity and the increase in NHE1 and ERM surface abundance.

2. Methods

2.1. Cell culture

HT-29 cells (ATCC – HTB-38; passages 3–22) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C. Cells were grown in McCoy's 5A medium (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 10^6 U/l penicillin G, 250 μg/l amphotericin B, 100 ng/l streptomycin (Sigma), 20% fetal bovine serum (Sigma) and 25 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanosulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.25% trypsin-EDTA, split 1:3 and subcultured in Costar Petri dishes with 21 cm^2 growth area (Costar, Badhoevedorp, The Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 5 days of initial seeding. For studies on Na^+/H^+ exchanger activity, the cells were seeded in 96 well plates. For 24 h prior to each experiment, the cell medium was free of fetal bovine serum. Experiments were generally performed 2 days after cells reached confluence, usually 7 days after the initial seeding.

2.2. NHE activity

NHE activity was assayed as the initial rate of intracellular pH (pH_i) recovery after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs' modified buffer solution (in mM: NaCl 140, KCl 5.4, $CaCl_2$ 2.8, $MgSO_4$ 1.2, NaH_2PO_4 0.3, HEPES 10, glucose 5, pH 7.4) in the absence of CO_2/HCO_3. In these experiments NaCl was replaced by an equimolar concentration of tetramethylammonium chloride (TMA). Intracellular pH measurements were performed in cells cultured in 96 well plates, as previously described [18–20]. Briefly, cells were loaded in serum-free medium with 5 μM BCECF/AM, the membrane-permeant acetoxymethyl ester derivative of 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF) for 40 min at 37 °C in 5% CO_2–95% air atmosphere. The cells were washed free of dye and, unless stated otherwise, the test compounds added to the extracellular fluid 0.5 h before starting the sodium-dependent pH_i recovery. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, USA) and fluorescence monitored every 17 s alternating between 440 and 490 nm excitation at 535 nm of emission with a cut-off filter of 530 nm. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to pH_i by comparison with values from an intracellular calibration curve using the nigericin 10 μM and high-K^+ method with pH_i ranging from pH 6.6 to 7.8 [18,19].

In experiments aimed to evaluate the sensitivity of the sodium-dependent pH_i recovery to selective inhibitors of NHE isoforms, cells were treated with increasing concentrations of amiloride, EIPA, cariporide or vehicle for 0.5 h before starting the sodium-dependent pH_i recovery. Amiloride and EIPA are both more effective in inhibiting NHE1 than NHE2 or NHE3, though EIPA is relatively more potent in inhibiting NHE1 [21]. On the other hand, cariporide is relatively selective for inhibition of the NHE1 isoform [22]. Differentiation between NHE1 and NHE2 can be achieved while employing selective concentrations of cariporide; NHE1 is fully inhibited by low concentrations (1 μM), whereas inhibition of NHE2 requires much higher concentrations of cariporide (25 μM) [23].

In experiments in which IFN-γ was tested upon NHE activity cells were treated with a given concentration of IFN-γ (from 10 to 1000 U/ml) or vehicle for 24 h before starting the sodium-dependent pH_i recovery. In some experiments, cariporide (300 nM) was co-incubated with

IFN-γ (1000 U/ml) for 0.5 h before starting the sodium-dependent pH_i recovery. Assessment of signaling pathways used by IFN-γ were conducted by using a single concentration (1000 U/ml) and 24 h exposure of HT-29 monolayers to human IFN-γ. Cells were pretreated with inhibitors of interest 0.5 h prior the addition of IFN-γ that was subsequently added to the culture well (inhibitor not washed out).

2.3. NHE immunoblotting

HT-29 cells cultured to 90% of confluence were washed twice with PBS and total cell protein extracted for NHE1 detection. Briefly, to obtain total cell extract, cells were lysed by brief sonication (15 s) in lysis buffer with protease inhibitors (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF aprotinin and leupeptin 2 μg/ml each) and incubated on ice for 1 h. After centrifugation (14,000 rpm, 30 min, 4 °C), the supernatant was mixed in 6× sample buffer (0.35 M Tris–HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, 0.01% bromphenol blue) and boiled for 5 min. The proteins were subjected to SDS-PAGE (10% SDS-polyacrylamide gel) and electrotransfered onto nitrocellulose membranes. The transblot sheets were blocked with 5% of non-fat dry milk in Tris–HCl 25 mM, pH 7.5, NaCl 150 mM and 0.1% Tween 20, overnight at 4 °C. Then, the membranes were incubated with appropriately diluted rabbit anti-NHE1 antibody (1:500) (Alpha Diagnostics, Autogenbioclear, Wiltshire, UK) or the rabbit anti-ERM antibody (1:1000) (Cell Signaling Technology™). The reaction was detected by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL system (Amersham Life, Arlington Heights, IL). The densities of the appropriate bands were determined using Quantity One (Bio-Rad Laboratories, Hercules, CA). Protein concentration was measured using the DC protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

2.4. Cell surface biotinylation

Cell surface biotinylation was used to determine apical membrane NHE1 and ERM expression in HT-29 cells treated with IFN-γ (1000 U/ml) or vehicle for 24 h. Briefly, confluent cells were rinsed twice with ice-cold PBS with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS–Ca–Mg). The apical surface was then exposed to 500 μg/ml of Sulfo-NHS-Biotin (Pierce, Rockford, IL) in biotinylation buffer (10 mM triethanolamine, 2 mM $CaCl_2$ and 150 mM NaCl, pH 7.4) for 20 min with horizontal motion at 4 °C. After labeling, the cells were rinsed with quenching solution (PBS–Ca–Mg with 100 mM glycine) and cells lysed with RIPA buffer with protease inhibitors (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, and aprotinin and leupeptin 2 μg/ml each), briefly sonicated and incubated on ice for about 1 h. After centrifugation (16,000 × *g*, 30 min, 4 °C), the supernatant was adjusted to 3.4 mg/ml and the biotinylated protein precipitated overnight at 4 °C with 100 μl of streptavidin agarose beads (Pierce, Rockford, IL) in a total volume of 500 μl. The streptavidin agarose beads were washed twice with PBS and the bound proteins solubilized with SDS sample buffer (0.125 M Tris–HCl, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8) and subjected to SDS-PAGE and blotting for NHE1 and ERM as described in immunoblotting.

2.5. STAT1 immunoblotting

Total and phosphorylated STAT1 protein levels were detected in HT-29 cells exposed to IFN-γ (1000 U/ml) for 24 h using the PhosphoPlus® Stat1 (Tyr701) antibody kit (Cell Signaling Technology™) according to manufacturer's protocol. In some experiments, cells were pretreated with inhibitors of interest 0.5 h prior the addition of IFN-γ that was subsequently added to the culture well (inhibitor not washed out). Briefly, cells were washed twice with PBS, lysed with SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, 0.1% (w/v) bromphenol blue) and briefly sonicated (15 s). Samples were then denatured for 5 min at 95 °C, loaded onto 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The transblot sheets were blocked for 3 h with 5% of non-fat dry milk in Tris–HCl 25 mM, pH 7.5, NaCl 150 mM and 0.1% Tween 20, at room temperature. Then, the membranes were incubated with phospho-STAT1 or STAT1 antibody (1:1000) and the immunocomplexes detected with the Phototope®-HRP Western detection kit.

2.6. Data analysis

Geometric means are given with 95% confidence limits and arithmetic means are given with S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test or the Newman–Keuls test for multiple comparisons. A *P*-value less than 0.05 were assumed to denote a significant difference.

2.7. Drugs

(-)-Epigallocatechin-3-gallate (batch 022K1126, 95% purity by HPLC), (-)-epigallocatechin (batch 043K1442, 95% purity by HPLC) and human interferon-γ (batch 032K1368, 98% purity by SDS electrophoresis) was obtained from Sigma (Chemical Company, St. Louis, MO, USA). Cariporide (4-isopropyl-3-methylsulfonyl-benzoyl-guanidine methanesulfonate) was kindly provided Dr. H.J. Lang from Aventis Pharma Deutschland (Frankfurt, Germany). Stock solutions of (-)-epigalloca-

techin-3-gallate, (-)-epigallocatechin and cariporide were prepared in 100% DMSO and working solutions contained 0.1% DMSO. Stock solutions of interferon-γ were prepared in water.

3. Results

In the present study, NHE activity was assayed in HT-29 cells loaded with a pH-sensitive dye (BCECF), as the Na^+-dependent recovery of pH_i measured after an acid load imposed by 20 mM NH_4Cl followed by removal of Na^+ from the Krebs modified buffer solution, in the absence of CO_2/HCO_3. As shown in Fig. 1, after acidification, HT-29 cells showed a rapid pH_i recovery upon addition of 140 mM Na^+. This pH_i recovery was inhibited by amiloride (1 mM) and EIPA (10 μM), indicating that the endogenous NHE in HT-29 cells is both an amiloride- and EIPA-inhibitable exchanger. The sensitivity of NHE to inhibition by amiloride, EIPA and cariporide was also evaluated. Fig. 2A shows inhibition of NHE activity in HT-29 cells by amiloride and EIPA, being EIPA considerably more potent than amiloride (IC_{50} values: amiloride, IC_{50} = 24 μM [19,30]; EIPA, IC_{50} = 296 nM [235,373]). Fig. 2B shows inhibition of NHE activity by cariporide (IC_{50} = 11 nM [9,12]). Differences in sensitivity to inhibitors are in agreement with the observation that the cariporide-sensitive Na^+-dependent recovery of pH_i in HT-29 cells might be largely due to NHE1 [24,25].

As indicated in Fig. 3A, the long-term (24 h) treatment with increasing concentrations of human IFN-γ (10–1000 U/ml) resulted in a concentration-dependent Na^+-dependent recovery of pH_i. As observed in Caco-2 cells [11], exposure of HT-29 cells to 1000 U/ml IFN-γ for 24 h in the absence of fetal bovine serum did not reduce cell viability when compared to vehicle-treated cells (data not shown). To evaluate whether NHE isoform was involved in the inhibitory effect of IFN-γ, HT-29 cells were co-incubated with IFN-γ (1000 U/ml) plus cariporide (300 nM) for 30 min before starting the sodium-dependent pH_i recovery. As shown in Fig. 3B, inhibition of Na^+-dependent recovery of pH_i by IFN-γ (1000 U/ml) was prevented by cariporide (300 nM), suggesting that NHE1 might be involved in the inhibitory effect of IFN-γ.

The next series of experiments were intended to explore the involvement of STAT1 from IFN-γ receptor stimulation downstream to NHE. (-)-Epigallocatechin-3-gallate specifically inhibits the tyrosine phosphorylation of STAT1, but not of STAT3 [26]; (-)-epigallocatechin (EGC) does not interfere with the tyrosine phosphorylation

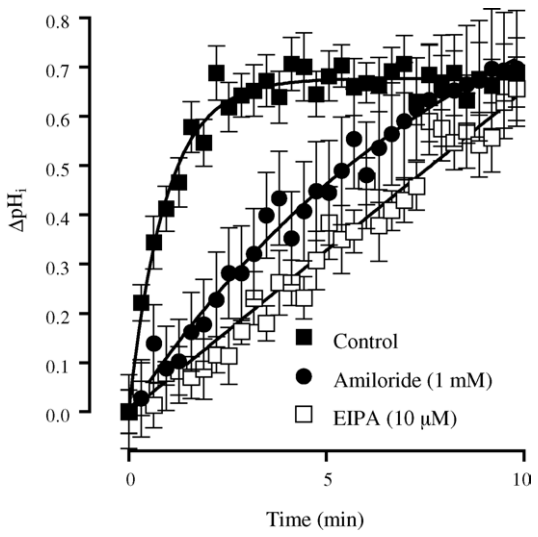

Fig. 1. HT-29 cells Na^+-dependent pH_i recovery after an acid load imposed by exposure to NH_4Cl. This pH_i recovery was inhibited by amiloride (1 mM) and by EIPA (10 μM). Symbols represent the mean of 14 experiments per group; vertical lines show S.E.M.

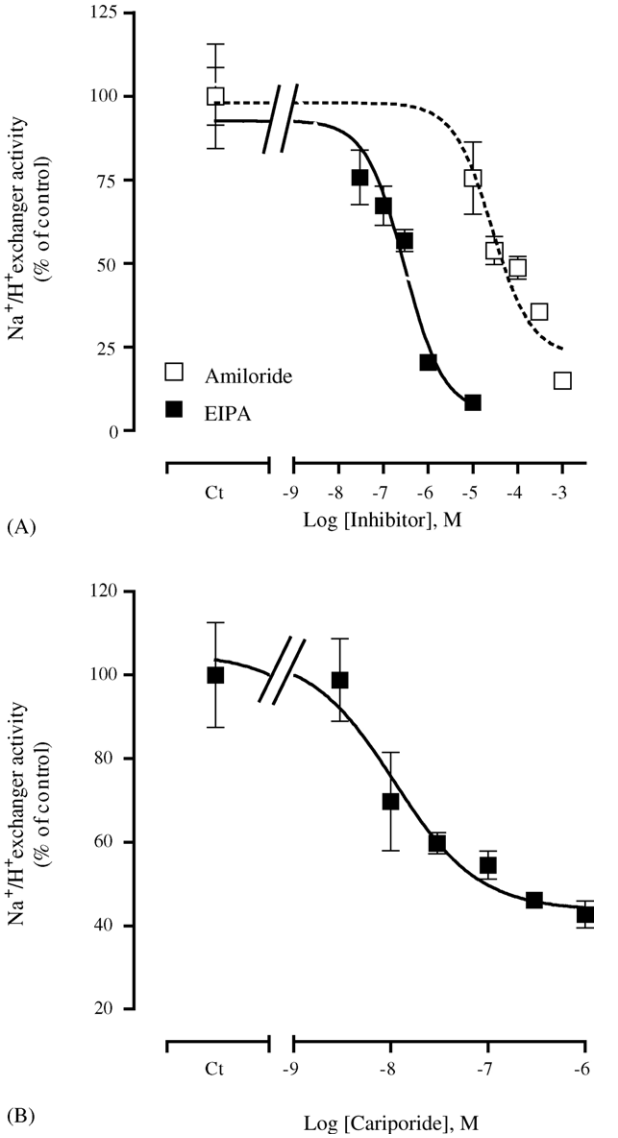

Fig. 2. (A) Concentration-dependent inhibitory effect of EIPA and amiloride and (B) cariporide on NHE activity in HT-29 cells. Symbols represent the mean of 12 experiments per group; vertical lines show S.E.M.

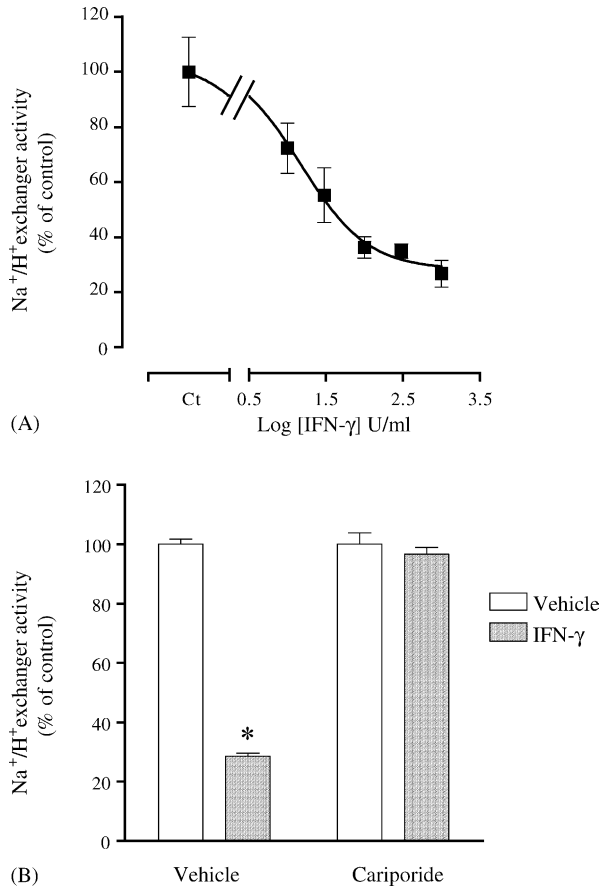

Fig. 3. (A) Concentration-dependent effect of IFN-γ (10–1000 U/ml) on NHE activity in cultured HT-29 cells after 24 h of exposure. (B) Effect of cariporide (300 nM) on the IFN-γ (1000 U/ml) induced inhibition of NHE activity in cultured HT-29 cells. Columns or symbols represent means of 12 experiments per group; vertical lines indicate S.E.M. values. Significantly different from control values ($^{*}P < 0.05$) using the Newman–Keuls test.

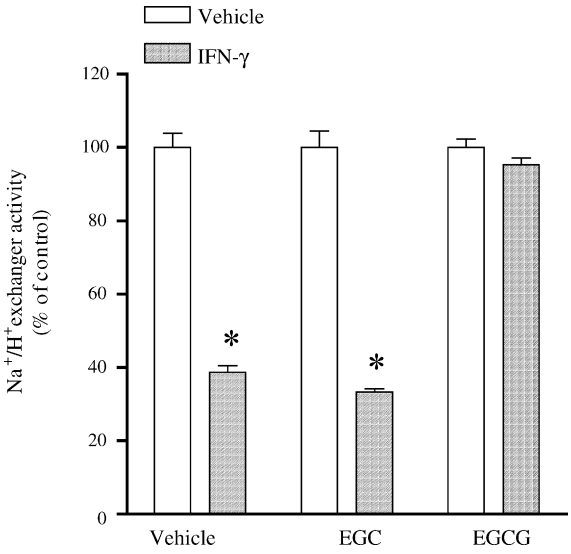

Fig. 4. Effect of epigallocatechin (EGC, 20 μM) and epigallocatechin-3-gallate (EGCG, 20 μM) on NHE activity after long-term (24 h) exposure to IFN-γ (1000 U/ml). Columns represent means of 10 experiments per group; vertical lines indicate S.E.M. values. Significantly different from control values ($^{*}P < 0.05$) using the Student's *t*-test.

of STAT1 [26]. IFN-γ was tested at a single concentration (1000 U/ml) and a 24 h exposure of HT-29 monolayers to human IFN-γ. Cells were pretreated with the inhibitors of interest 0.5 h prior the addition of IFN-γ that was subsequently added to the culture well (inhibitor not washed out). As shown in Fig. 4, the STAT1 inhibitor EGCG, but not the inactive analogue EGC, prevented the long-term inhibitory effect of IFN-γ (24 h exposure) upon NHE1 activity.

STAT and phospho-STAT activation was evaluated in HT-29 cells exposed to IFN-γ (1000 U/ml) for 24 h. Treatment with IFN-γ markedly increased total STAT and phospho-STAT (Fig. 5). Activation of phospho-STAT after 24 h exposure to IFN-γ (1000 U/ml) was significantly attenuated by EGCG (20 μM), but insensitive to EGC (20 μM) (Fig. 5).

To evaluate whether inhibition of NHE1 by IFN-γ was related to down-regulation of NHE1, HT-29 cells were treated with IFN-γ (1000 U/ml) for 24 h and the abundance of total NHE1 and biotinylated NHE1 was quantified. As shown in Fig. 6A, the abundance of total NHE1 was not altered after the long-term exposure (24 h) to IFN-γ, but the abundance of biotinylated NHE1 was significantly increased (Fig. 6B). The STAT1 inhibitor EGCG prevented the IFN-γ-induced increase in abundance of biotinylated NHE1 (Fig. 6B). Mechanisms restricting NHE1 at the cell membrane have been suggested to occur through direct binding of the ezrin, radixin and moesin family of proteins, which directly interact with actin filaments [12,13]. As shown in Fig. 7A, the abundance of total ERM was not altered after the long-term exposure (24 h) to IFN-γ, but the abundance of biotinylated ERM was significantly increased (Fig. 7B). The STAT1 inhibitor EGCG prevented the IFN-γ-induced increase in abundance of biotinylated ERM (Fig. 7B).

4. Discussion

The present study was aimed at evaluate the effect of IFN-γ on NHE1 expression and function in human intestinal epithelial HT-29 cells and identify the intracellular signaling pathways set into motion after IFN-γ receptor activation. It is reported a decrease in NHE1 activity after long-term exposure to IFN-γ that is accompanied by an increase in surface NHE1 and the NHE1 membrane anchoring ERM proteins. The transduction mechanisms set into motion by long-term exposure to IFN-γ involves a complex signaling pathway that mainly includes STAT1 phosphorylation.

The ubiquitously expressed NHE1 primarily serves the purpose of intracellular pH and cell volume regulation, cellular growth and differentiation [27,28]. Inhibition of NHE activity may also reduce the uptake of sodium

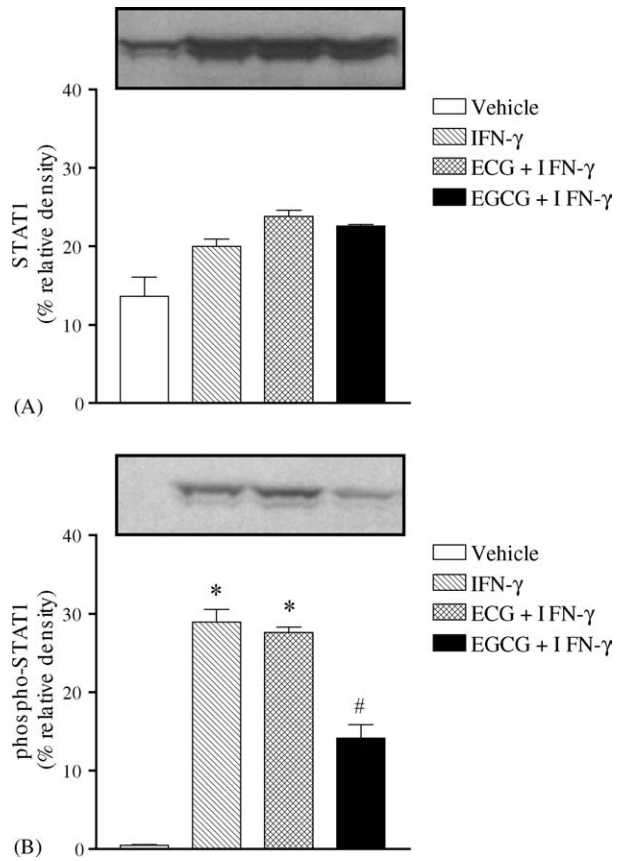

Fig. 5. Effect of epigallocatechin (EGC, 20 μM) and epigallocatechin-3-gallate (EGCG, 20 μM) on the abundance of (A) STAT1 and (B) phospho-STAT1 in Caco-2 cells treated for 24 h in the absence (vehicle) and in the presence of plus interferon-γ (IFN-γ, 1000 U/ml). Each lane contains equal amount of protein (50 μg). Columns indicate relative density and represent the mean of three–four separate experiments; vertical lines indicate S.E.M. Significantly different from control values ($^{*}P < 0.05$) using the Newman–Keuls test.

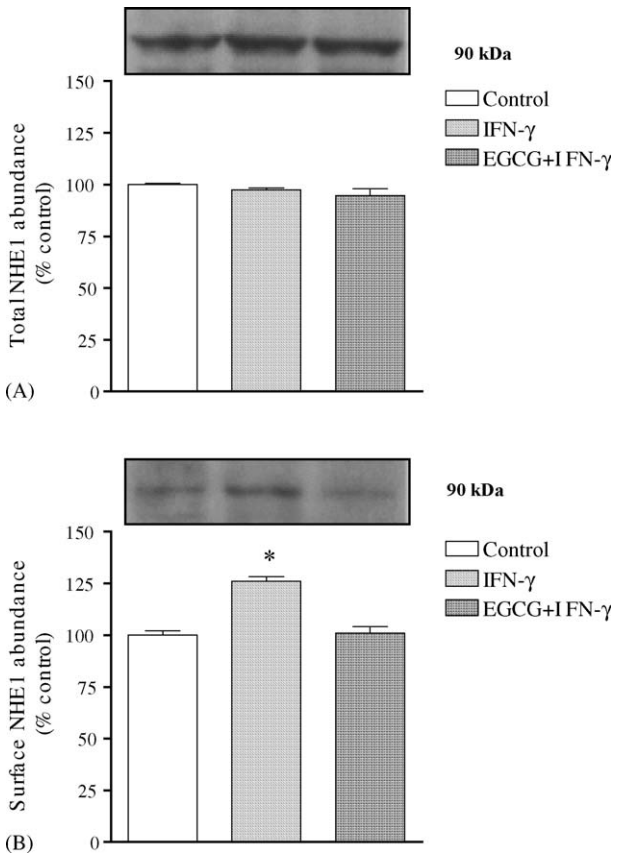

Fig. 6. Abundance of (A) total and (B) surface NHE1 in HT-29 cells treated for 24 h with IFN-γ (1000 U/ml). Each lane contains equal amount of protein (50 μg). Immunoblots were repeated four–six times. Columns indicate relative density and represent the mean of four–six separate experiments; vertical lines indicate S.E.M. values. Significantly different from values for corresponding control values ($^{*}P < 0.05$) using the Student's *t*-test.

chloride and water from the inflamed colonic lumen, and thus contribute to diarrhea. On the other hand, it is interesting to underline the observation that the level of NHE1 protein and mRNA was significantly decreased in the colonic mucosa of patients afflicted with both CD and UC [29]. This goes well with the suggestion that increases in NHE1 expression should contribute to intracellular pH and to cell growth and repair in colitis, and therefore, may be beneficial [30]. Another function of NHE1, apart its role in regulating pH_i and cell volume homeostasis is that of a structural anchor for actin filaments through its direct binding to the ERM family of actin-binding proteins [12,13]. It is possible that the IFN-γ-induced increase in NHE1 surface abundance may result from changes in cytoskeleton organization by the cytokine [31]. The finding that IFN-γ-induced NHE1 inhibition and increases in surface NHE1 and surface ERM were all antagonized by the STAT1 inhibitor EGCG strongly suggests a relationship between these two events. This is line with recent evidence suggesting that ERM binding and actin anchoring by NHE is necessary to retain the localization of NHE1 [12].

Transduction mechanisms set into motion during long-term activation of IFN-γ receptors in HT-29 cells appear to involve the activation of STAT1 pathway. This is evidenced by the sensitivity of IFN-γ-induced NHE1 inhibition to EGCG. The finding that the STAT1 inhibitor EGCG prevented the inhibition of NHE1 activity and the increase in NHE1 surface abundance associated with the long-term exposure to IFN-γ, strongly suggests that effects of the cytokine may correspond to events associated with the IFN-γ-induced STAT1 activation. The role of STAT1, which is well known for its effects as a tumor suppressor inhibiting cell growth and promoting apoptosis [32], in IFN-γ-induced NHE1 inhibition fits well the view that NHE1 may play an important role in cell growth [13].

In the present study, the sodium-dependent pH_i recovery in HT-29 cells was largely sensitive to cariporide, a selective NHE1 inhibitor [22], suggesting that NHE1 might be the major isoform responsible for NHE activity. This fits well with previous evidence suggesting that HT-29 express NHE1 in the both apical and basal lateral membranes of

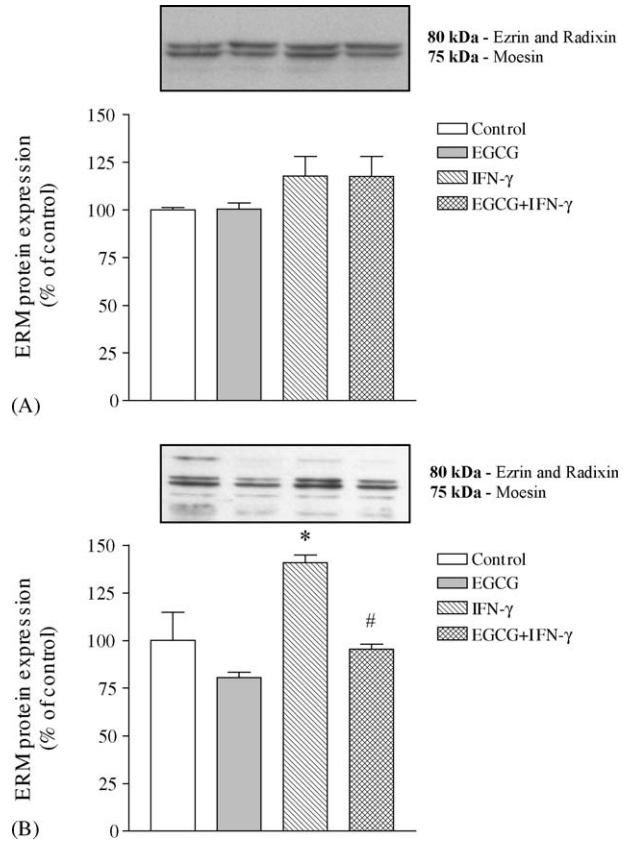

Fig. 7. Abundance of (A) total and (B) surface ezrin, radixin and moesin (ERM) in HT-29 cells treated for 24 h with vehicle, EGCG (20 μM), IFN-γ (1000 U/ml) and EGCG (20 μM) plus IFN-γ (1000 U/ml). Each lane contains equal amount of protein (50 μg). Western blots were repeated four times. Columns indicate relative density and represent the mean of three separate experiments; vertical lines indicate S.E.M. values. Significantly different from values for corresponding control values ($^{*}P < 0.05$) using the Student's *t*-test.

polarized HT-29 cells in culture [16]. The finding that inhibition of sodium-dependent pH_i recovery in HT-29 cells by IFN-γ is no longer observed in cells pretreated with cariporide, which results in 60% reduction in NHE activity, strongly suggests that NHE1 is the NHE isoform that is responsible for the IFN-γ-sensitive sodium-dependent pH_i recovery. In contrast, to that observed with other transporters involved in electrolyte transport that undergo down-regulation by IFN-γ, inhibition of NHE1 activity by the cytokine in HT-29 cells is accompanied by increases in surface NHE1. Similar findings were observed in Caco-2 cells [11], indicating that this type of response is not cell-line specific. In both cell lines (Caco-2 and HT-29), NHE1 activity assay was performed under V_{max} conditions for sodium, which suggests that changes in NHE1 activity might reflect differences in the number of operational exchanger units rather than differences in the affinity for Na^+ or H^+. Therefore, it is hypothesized that a considerable number of surface NHE1 units may be no longer operational after long-term exposure to IFN-γ. In contrast to these findings, inhibition of NHE2 and NHE3 activity after prolonged exposure to IFN-γ in Caco-2 has been suggested to occur in parallel with down-regulation of NHE2 and NHE3 [6]. It is likely, therefore, that mechanisms set into motion by IFN-γ may regulate distinct isoforms of NHE in a specific manner.

In conclusion, the IFN-γ-induced decrease in NHE1 activity after long-term exposure of HT-29 cells to the cytokine is accompanied by an increase in surface NHE1 and the NHE1 membrane anchoring ERM proteins. These findings are similar to those observed seen in Caco-2 cells [11], indicating that this type of response is not cell-line specific, and suggest that a considerable number of surface NHE1 units are no longer operational after long-term exposure to IFN-γ. The transduction mechanisms set into motion by long-term exposure to IFN-γ involves STAT1 phosphorylation.

Acknowledgment

Supported by grant POCI/SAU-FCF/59207/2004 from *Fundação para a Ciência e a Tecnologia*.

References

[1] Sundaram U, Wisel S, Rajendran V, West AB. Mechanism of inhibition of Na+-glucose cotransport in the chronically inflamed rabbit ileum. Am J Physiol 1997;273:G913–9.
[2] Sandle GI, Higgs N, Crowe P, Marsh NN, Venkatesan S, Peters TJ. Cellular basis for defective electrolyte transport in inflamed human colon. Gastroenterology 1990;99:97–105.
[3] Colgan SP, Parkos CA, Matthews JB, D'Andrea L, Awtrey CS, Lichtman AH, et al. Interferon-gamma induces a cell surface phenotype switch on T84 intestinal epithelial cells. Am J Physiol Cell Physiol 1994;267:C402–10.
[4] Fish SM, Proujansky R, Reenstra WW. Synergistic effects of interferon-gamma and tumor necrosis factor alpha on T84 cell function. Gut 1999;45:191–8.
[5] Zund G, Madara JL, Dzus AL, Awtrey CS, Colgan SP. Interleukin-4 and interleukin-13 differentially regulate epithelial chloride secretion. J Biol Chem 1996;271:7460–4.
[6] Rocha F, Musch MW, Lishanskiy L, Bookstein C, Sugi K, Xie Y, et al. IFN-gamma downregulates expression of Na^+/H^+ exchangers NHE2 and NHE3 in rat intestine and human Caco-2/bbe cells. Am J Physiol Cell Physiol 2001;280:C1224–32.
[7] Sugi K, Musch MW, Field M, Chang EB. Inhibition of Na^+,K^+-ATPase by interferon gamma down-regulates intestinal epithelial transport and barrier function. Gastroenterology 2001;120:1393–403.
[8] Magro F, Fraga S, Ribeiro T, Soares-da-Silva P. Regional intestinal adaptations in Na^+,K^+-ATPase in experimental colitis and the contrasting effects of interferon-gamma. Acta Physiol Scand 2005;183:191–9.
[9] Haque SJ, Williams BR. Signal transduction in the interferon system. Semin Oncol 1998;25:14–22.
[10] Magro F, Fraga S, Ribeiro T, Soares-da-Silva P. Intestinal Na^+-K^+-ATPase activity and molecular events downstream of interferon-gamma receptor stimulation. Br J Pharmacol 2004;142:1281–92.
[11] Magro F, Fraga S, Soares-da-Silva P. Signaling of short- and long-term regulation of intestinal epithelial type 1 Na^+/H^+ exchanger by interferon-gamma. Br J Pharmacol 2005;145:93–103.
[12] Baumgartner M, Patel H, Barber DL. Na^+/H^+ exchanger NHE1 as plasma membrane scaffold in the assembly of signaling complexes. Am J Physiol Cell Physiol 2004;287:C844–50.

[13] Denker SP, Barber DL. Cell migration requires both ion translocation and cytoskeletal anchoring by the Na–H exchanger NHE1. J Cell Biol 2002;159:1087–96.
[14] Zachos NC, Tse M, Donowitz M. Molecular physiology of intestinal Na^+/H^+ exchange. Annu Rev Physiol 2005;67:411–43.
[15] Kreusel KM, Fromm M, Schulzke JD, Hegel U. Cl-secretion in ephitelial cells monolayers of mucus-formating human colon cells (HT-29/B6). Am J Physiol 1991;261:C574–82.
[16] Noel J, Roux D, Pouyssegur J. Differential localization of Na^+/H^+ exchanger isoforms (NHE1 and NHE3) in polarized cell lines. J Cell Sci 1996;109:929–39.
[17] Hecht G, Hodges K, Gill RK, Kear F, Tyagi S, Malakooti J, et al. Differential regulation of Na^+/H^+ exchange isoform activities by enteropathogenic *E. coli* in human intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 2004;287:G370–8.
[18] Gomes P, Vieira-Coelho MA, Soares-da-Silva P. Ouabain-insensitive acidification by dopamine in renal OK cells: primary control of the Na^+/H^+ exchanger. Am J Physiol Regul Integr Comp Physiol 2001; 281:R10–8.
[19] Gomes P, Soares-da-Silva P. Na^+/H^+ exchanger activity and dopamine D1-like receptor function in two opossum kidney cell clonal sublines. Cell Physiol Biochem 2002;12:259–68.
[20] Pedrosa R, Gomes P, Soares-Da-Silva P. Distinct signalling cascades downstream to G_{salpha} coupled dopamine D1-like NHE3 inhibition in rat and opossum renal epithelial cells. Cell Physiol Biochem 2004; 14:91–100.
[21] Noel J, Pouyssegur J. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. Am J Physiol 1995;268:C283–96.
[22] Schwark JR, Jansen HW, Lang HJ, Krick W, Burckhardt G, Hropot M. S3226, a novel inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types. Pflugers Arch 1998;436(5):797–800.
[23] Bachmann O, Sonnentag T, Siegel WK, Lamprecht G, Weichert A, Gregor M, et al. Different acid secretagogues activate different Na^+/H^+ exchanger isoforms in rabbit parietal cells. Am J Physiol 1998;275: G1085–93.
[24] Biemesderfer D, Rutherford PA, Nagy T, Pizzonia JH, Abu-Alfa AK, Aronson PS. Monoclonal antibodies for high-resolution localization of NHE3 in adult and neonatal rat kidney. Am J Physiol 1997;273:F289–99.
[25] Karim ZG, Chambrey R, Chalumeau C, Defontaine N, Warnock DG, Paillard M, et al. Regulation by PKC isoforms of Na^+/H^+ exchanger in luminal membrane vesicles isolated from cortical tubules. Am J Physiol 1999;277:F773–8.
[26] Menegazzi M, Tedeschi E, Dussin D, Prati AC, Cavalieri E, Mariotto S, et al. Anti-interferon gamma action of epigallocatechin-3-gallate mediated by specific inhibition of STAT1 activation. FASEB J 2001; 15:1309–11.
[27] Yun CH, Tse CM, Nath SK, Levine SA, Brant SR, Donowitz M. Mammalian Na^+/H^+ exchanger gene family: structure and function studies. Am J Physiol Gastrointest Liver Physiol 1995;269:G1–G11.
[28] Wakabayashi S, Shigekawa M, Pouyssegur J. Molecular physiology of vertebrate Na^+/H^+ exchangers. Physiol Rev 1997;77:51–74.
[29] Khan I, Siddique I, Al-Awadi FM, Mohan K. Role of Na^+/H^+ exchanger isoform-1 in human inflammatory bowel disease. Can J Gastroenterol 2003;17:31–6.
[30] Khan I, al-Awadi FM, Abul H. Colitis-induced changes in the expression of the Na^+/H^+ exchanger isoform NHE-1. J Pharmacol Exp Ther 1998;285:869–75.
[31] Flaishon L, Lantner F, Hershkoviz R, Levo Y, Shachar I. Low Levels of IFN-gamma down-regulate the integrin-dependent adhesion of B cells by activating a pathway that interferes with cytoskeleton rearrangement. J Biol Chem 2001;276:46701–6.
[32] Sironi JJ, Ouchi T. STAT1-induced apoptosis is mediated by caspases 2, 3 and 7. J Biol Chem 2004;279(6):4066–74.