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Zinc inhibits calcium-mediated and nitric oxide-mediated ion secretion in human enterocytes

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ABSTRACT

Zn^{2+} is effective in the treatment of acute diarrhea, but its mechanisms are not completely understood. We previously demonstrated that Zn^{2+} inhibits the secretory effect of cyclic adenosine monophosphate but not of cyclic guanosine monophosphate in human enterocytes. The aim of the present study was to investigate whether Zn^{2+} inhibits intestinal ion secretion mediated by the Ca^{2+} or nitric oxide pathways. To investigate ion transport we evaluated the effect of Zn^{2+} (35 μM) on electrical parameters of human intestinal epithelial cell monolayers (Caco2 cells) mounted in Ussing chambers and exposed to ligands that selectively increased intracellular Ca^{2+} (carbachol 10^{-6}M) or nitric oxide (interferon-γ 300 UI/ml) concentrations. We also measured intracellular Ca^{2+} and nitric oxide concentrations. Zn^{2+} significantly reduced ion secretion elicited by carbachol (−87%) or by interferon-γ (−100%), and inhibited the increase of intracellular Ca^{2+} and nitric oxide concentrations. These data indicate that Zn^{2+} inhibits ion secretion elicited by Ca^{2+} and nitric oxide by directly interacting with the enterocyte. They also suggest that Zn^{2+} interferes with three of the four main intracellular pathways of intestinal ion secretion that are involved in acute diarrhea.

1. Introduction

Acute diarrhea is a major health problem especially for children in developing countries (Black et al., 2003). Microbial infection is the main cause of acute diarrheal diseases (Cheng et al., 2005). Oral rehydration solution (ORS) prevents dehydration but does not reduce the severity and duration of diarrhea (Guarino et al., 2001). The ideal treatment for acute diarrhea is replacement of water and electrolytes combined with medication that inhibits intestinal hypersecretion without affecting motility. Several randomized controlled trials and meta-analyses showed that Zn^{2+} reduces the severity and duration of diarrhea (Bahl et al., 2002; Bhatnagar et al., 2004; Bhutta et al., 2000; Hoque and Binder, 2006; INCLEN childnet Zinc Effectiveness for Diarrhea (IC-ZED) Group, 2006; Lazzerini and Ronfani, 2008; Roy et al., 2008). Moreover, the WHO and UNICEF recommend Zn^{2+} for the universal treatment of childhood acute diarrhea (WHO/UNICEF, 2004). However, the mechanisms of action of Zn^{2+} in the treatment of acute diarrhea are not completely known. Consequently, a major question is whether the information available about its mechanism of action is sufficient to support the universal use of Zn^{2+} in the treatment of acute diarrhea together with ORS (Bhatnagar et al., 2004; Lazzerini and Ronfani, 2008; Berni Canani and Ruotolo, 2006). It is generally agreed that enteric pathogens determine fluid secretion through 4 major intracellular signal transduction pathways: 1) cAMP; 2) cGMP; 3) intracellular Ca^{2+} ($[Ca^{2+}]_i$); and 4) nitric oxide (NO) (Fasano, 2002). Using an *in vitro* model, we previously demonstrated that $ZnCl_2$ stimulates ion absorption in human enterocytes (Berni Canani et al., 2005). To determine whether the pro-absorptive effects of $ZnCl_2$ were specifically related to zinc ion, we carried out parallel experiments with $ZnCl_2$, $Zn(C_2H_3O_2)_2$, $ZnSO_4$ and $MgCl_2$. All these zinc salts induced a decrease in Isc identical to that observed with $ZnCl_2$. In contrast, the addition of $MgCl_2$ at an equimolar concentration did not affect the electrical parameters, which indicates that the pro-absorptive effect was selectively related to zinc (Berni Canani et al., 2005). In addition, $ZnCl_2$ prevented *Cholera* toxin-induced active ion secretion by modulating intracellular cAMP concentration. On the contrary, $ZnCl_2$ did not affect *E. coli* heat-stable enterotoxin-cGMP-mediated ion secretion (Berni Canani et al., 2005). These findings suggest that Zn^{2+} exerts a selective effect against intestinal pathogens and raises the question: could Zn^{2+} interfere with other diarrheal mechanisms elicited by diverse pathogens?

Toxins produced by Ciguatera fish, *Clostridium difficile*, *Cryptosporidium parvum*, *Rotavirus* (the non structural protein 4, NSP4), *Helicobacter pylori*, and *Vibrio parahaemolyticus* can exert a direct secretory effect on the intestine by modifying $[Ca^{2+}]_i$ (Berni Canani

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et al., 1999; Guarino et al., 1995; Guarino et al., 1998; Raimondi et al., 1995). We previously reported that the transactivator factor peptide (Tat) produced by HIV-1 induces ion secretion by a Ca^{2+}-mediated mechanism (Berni Canani et al., 2003a,b). Subsequently, we reported that pre-incubation of human enterocytes with Zn^{2+} resulted in an almost total inhibition of the Tat-induced ion secretion that triggers a specific mechanism of HIV-1-related diarrhea (Berni Canani et al., 2007). This finding could explain the reduction of diarrhea observed in patients with HIV-infection during Zn^{2+} supplementation (Bobat et al., 2005) and suggests that Zn^{2+} could play a role also in Ca^{2+}-mediated diarrhea. Finally, it has been demonstrated that Zn^{2+} can modulate $[Ca^{2+}]_i$ homeostasis in colonocyte cell culture in basal condition (Hershfinkel et al., 2001).

The importance of NO as a second messenger in intestinal ion secretion is well recognized. The pathogenic effects exerted by NO are concentration-dependent (Wingertzahn et al., 2003) and associated with increased NO production triggered by inducible NO synthase isoforms (iNOS) that are involved in some infectious and non-infectious diarrheal diseases (Fasano, 2002; Xu et al., 2002). The fact that zinc is an NO scavenger (Hershfinkel et al., 2001) supports the possibility that Zn^{2+} could be effective in NO-induced ion secretion.

In this study, we investigated the effect of Zn^{2+} on intestinal ion secretion mediated by the Ca^{2+} and NO intracellular signal transduction pathways. To this aim, we used a human enterocyte cell line model (Caco2) that has been validated in a study of intestinal transepithelial ion transport (Moltedo et al., 2000).

2. Materials and methods

2.1. Reagents and cell culture

Chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA). Culture medium was obtained from Life Technologies GIBCO BRL (Mascia e Brunelli, Milan, Italy). Transwell filters and supports were obtained from Costar (Costar Italia, Milan, Italy). Caco2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) that had a high glucose concentration (4.5 g/l) and that was supplemented with 10% fecal calf serum, 1% nonessential amino acids, penicillin (50 mU/ml), streptomycin (50 mg/ml) and were incubated in 5% CO_2–95% air. The medium was changed daily.

2.2. Ion transport studies

Caco2 cells were grown on uncoated polycarbonate transwell filters and were used in intestinal ion transport studies at 15 days post-confluence as previously described (Berni Canani et al., 1999). The filter area was 4.9 cm^2. Each filter was mounted in a Ussing chamber (WPI, Sarasota, FL, USA) as a flat sheet between the mucosal and the serosal compartments. Each compartment contained 5 ml of Ringer's solution (pH 7.4) with the following composition in mmol/l: NaCl (114), KCl (5), Na_2HPO_4 (1.65), NaH_2PO_4 (0.3), $CaCl_2$ (1.25), $MgCl_2$ (1.1), $NaHCO_3$ (25), glucose (10); the buffer was constantly gassed with 95% O_2–5% CO_2 and was connected to a thermostat-regulated circulating pump to maintain a temperature of 37 °C. The following electrical parameters were measured or calculated as described elsewhere (Berni Canani et al., 2005, Field et al., 1971), before and after the mucosal or serosal addition of the testing substance: transepithelial potential difference (PD), short-circuit current (Isc) and tissue ionic conductance (G). Isc was expressed as microamperes per square centimeter ($\mu A/cm^2$), G as millisiemens per square centimeter (mS/cm^2), and PD as millivolts (mV) (Field et al., 1971). Cell viability was evaluated by measuring the electrical response to the serosal addition of theophylline (5 mM) at the end of each experiment. In Ussing chambers, Caco2 cell monolayers were exposed to a well established agent of the Ca^{2+}-mediated ion secretion pathway, i.e., carbachol (10^{-6} M), to the serosal side, alone or in combination with $ZnCl_2$ (35 μM). The two agents were used at the maximal effective dose, as previously established in the same experimental model (Moltedo et al., 2000). To investigate the effect of Zn^{2+} on NO-mediated ion secretion, we added IFN-γ (300 UI/ml) to the serosal side of Caco2 cells. Interferon-γ is an agonist of iNOS and stimulates Cl^- secretion through the increase of NO (Chavez et al., 1999). To allow activation of iNOS-mediated NO production, the cells were mounted in Ussing chambers after 1 h of incubation with IFN-γ, and the electrical parameters were measured in the presence or absence of $ZnCl_2$ (35 μM). To obtain additional evidence of the effect of $ZnCl_2$ on NO-mediated ion secretion, we repeated these experiments using the NO donor sodium nitroprusside (10 mM) added to the serosal side.

2.3. $[Ca^{2+}]_i$ measurement

The $[Ca^{2+}]_i$ was measured by single cell computer-assisted videoimaging (Secondo et al., 2000). Briefly, Caco2 cells, grown on glass coverslips, were loaded with 6 μM Fura-2 acetoxymethyl ester (Fura-2 AM) for 1 h at room temperature in Krebs solution containing the following substances (in mM): 5.5 KCl, 160 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes-NaOH, pH 7.4. At the end of Fura-2 AM loading, the coverslips were placed in a perfusion chamber (Medical System, Co. Greenvale, NY, USA) mounted into the stage of a Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a FLUAR 40× oil objective lens. The experiments were carried out with a digital imaging system consisting of a MicroMax 512BFT cooled CCD camera (Princeton Instruments, Trenton, NJ, USA), a LAMBDA 10-2 filter wheeler (Sutter Instruments, Novato, CA, USA) and Meta-Morph/MetaFluor Imaging System software (Universal Imaging, West Chester, PA, USA). After loading, cells were illuminated with light alternating between wavelengths of 340 and 380 nm using a xenon arc lamp. The emitted light was passed through a 512 nm barrier filter. Fura-2 AM fluorescence intensity was measured every 3 s. Ratiometric values were automatically converted by a specific software into $[Ca^{2+}]_i$ using a pre-loaded calibration curve obtained in preliminary experiments (Grynkiewicz et al., 1985; Secondo et al., 2000).

2.4. Nitrite/nitrate concentration measurement

The combined concentration of nitrite/nitrate (NO_2^-/NO_3^-), the end stable products of NO in the culture medium, was determined by the Griess reaction as previously described (Carlson et al., 2006). Therefore, total NO_2^-/NO_3^- production is referred to as "NO production" in the text. Caco2 cell monolayers were grown on plates and used at 15 days post-confluence. Cells were stimulated with IFN-γ (300 UI/ml) for 60 min in the absence or in the presence of $ZnCl_2$ (35 μM). Cells exposed to vehicle alone served as control.

2.5. Statistics

Each experiment was repeated 3 times. Continuous variables were expressed as mean ± standard deviation (SD). We used the *t*-test for equality of means to compare variables among the different groups. All tests of significance were two-sided. A *P* value of 0.05 was considered significant. Statistical analysis was performed using the SPSS software package for Windows (release 16.0.0; SPSS Inc., Chicago, IL, USA) and Stats direct (release 2.6.6).

3. Results

3.1. Zn^{2+} effects on Ca^{2+}-mediated intestinal ion secretion

To investigate the effects of Zn^{2+} on Ca^{2+}-mediated intestinal ion secretion, we treated Caco2 cell monolayers to the maximal effective dose of carbachol (10^{-6} M) added to the serosal side in the absence or

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presence of $ZnCl_2$ (35 μM). As shown in Fig. 1, the addition of carbachol to the serosal side of Caco2 cell monolayers induced a prompt increase in Isc. This effect was entirely due to an increase of PD because G was unchanged. Pre-incubation with $ZnCl_2$ for 20 min resulted in a significant inhibition of carbachol-induced ion secretion (Fig. 1). To evaluate whether Zn^{2+} can modulate $[Ca^{2+}]_i$ homeostasis in human enterocytes, Caco2 cells, pre-loaded with Fura-2 AM, were superfused with carbachol (10^{-6}M) in normal Krebs solution containing physiological concentrations of Ca^{2+} (i.e., 1.2 mM). In response to this stimulus, approximately 50% of cells displayed a transient peak of $[Ca^{2+}]_i$, whereas the other 50% showed a slow increase of $[Ca^{2+}]_i$ that reached a plateau after about 8 min (Fig. 2, panels A and B, respectively). Despite the difference in the pattern of response, the maximum increase of $[Ca^{2+}]_i$ was similar in both subpopulations of cells (Fig. 2, panels D and E). Pre-incubation with 35 μM $ZnCl_2$ for 20 min significantly inhibited the carbachol-induced increase of $[Ca^{2+}]_i$ (Fig. 2: panels C and F).

3.2. Effects of Zn^{2+} on NO-mediated intestinal ion secretion

To investigate the effects of Zn^{2+} on NO-mediated intestinal ion secretion, we treated Caco2 cell monolayers with the maximal effective dose of IFN-γ (300 UI/ml) added to the serosal side in the absence or presence of 35 μM $ZnCl_2$. As shown in Fig. 3, the addition of IFN-γ to the serosal side of Caco2 cell monolayers induced an increase in Isc. This effect was entirely due to an increase of PD because G remained unchanged. As shown in Fig. 3, exposure to $ZnCl_2$ for 20 min completely inhibited IFN-γ-induced ion secretion. We next evaluated the effect of Zn^{2+} on NO production. Basal NO_2^-/NO_3^- concentrations were reduced consequent to the addition of $ZnCl_2$ (Fig. 4). Pre-treatment with $ZnCl_2$ (35 μM) inhibited the increase in NO concentration induced by IFN-γ (300 UI/ml) (Fig. 4). The addition of sodium nitroprusside to Caco2 cell monolayers induced an increase in Isc. The maximal effect was observed 20 min after the addition of sodium nitroprusside (ΔIsc 2.42 ± 0.4 μA/cm^2). The effect was entirely due to an increase of PD because G remained unchanged. Pre-incubation with $ZnCl_2$ (35 μM) for 20 min resulted in a significant inhibition of ion secretion elicited by this NO donor (10 mM) (−69.5%, $P<0.01$).

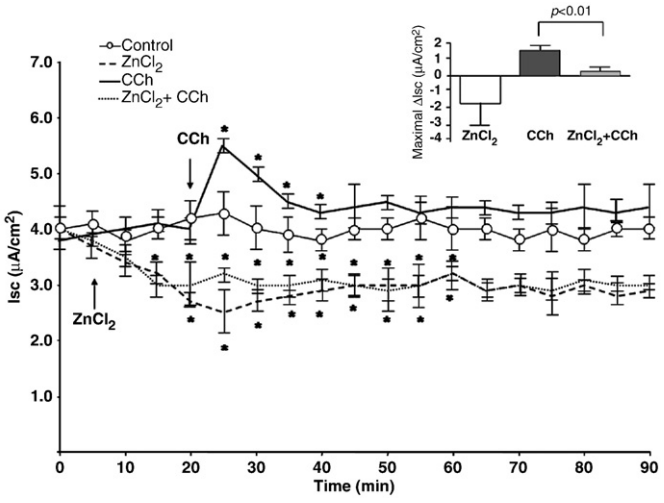

Fig. 1. Effects of Zn^{2+} on carbachol-induced intestinal ion secretion. Time course effects of carbachol (10^{-6}M) and $ZnCl_2$ (35 μM), alone or in combination, on short-circuit current (Isc) in Caco2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent. Carbachol induced a significant increase in Isc, consistent with Cl^- secretion (*$P<0.01$ vs. control). $ZnCl_2$ induced a significant decrease in Isc, consistent with chloride absorption (*$P<0.01$ vs. control). Preload with $ZnCl_2$ resulted in substantial inhibition of carbachol-induced ion secretion (*$P<0.01$ carbachol plus Zn^{2+} vs. carbachol alone), which is consistent with inhibition of Ca^{2+}-mediated ion secretion. The upper right side of the figure shows the maximal Isc modifications observed after the addition of carbachol (10^{-6}M) and $ZnCl_2$ (35 μM), alone or in combination. Preload with $ZnCl_2$ resulted in significant inhibition of maximal Isc modification induced by carbachol. Each experiment was repeated at least 3 times. Results are expressed as means ± S.D.

4. Discussion

Here we report evidence that, by directly interacting with enterocytes, Zn^{2+} reduces intestinal Cl^- secretion elicited by the Ca^{2+}- and NO-mediated intracellular pathways. Zinc significantly inhibited carbachol-induced ion secretion, and the effect on intestinal ion transport was paralleled by $[Ca^{2+}]_i$ modifications. In fact, pre-incubation with Zn^{2+} significantly inhibited the increase of $[Ca^{2+}]_i$ induced by carbachol in Caco2 cells. Carbachol, by activating M-type receptors, induces hydrolysis of polyphosphoinositides and mobilization of $[Ca^{2+}]_i$ stores as a consequence of activation of the G protein-phospholipase-C pathway. This in turn elicits activation of capacitative Ca^{2+} entry through the store-operated-Ca^{2+}-channels (Carlson et al., 2006). Our data suggest that in enterocytes, both these mechanisms contribute to $[Ca^{2+}]_i$ modifications in response to carbachol. The former produces a rapid but transient response and the latter induces a long-lasting event. HIV-1 Tat protein induced similar effects in Caco2 cells (Berni Canani et al., 2003). As observed in other extraintestinal cell types (Kresse et al., 2005; Priel and Hershfinkel, 2006), we found that Zn^{2+} inhibits both mechanisms in Caco2 cells.

Our results are consistent with the finding that Zn^{2+} reduces Ca^{2+}-dependent ion secretion in piglet small intestinal epithelium (Carlson et al., 2006), but contrast with the finding that Zn^{2+} does not inhibit Ca^{2+}-mediated secretion in isolated rat ileum (Hoque et al., 2005). In addition to factors related to the type of experimental model, these discrepancies may be related to the different Zn^{2+} doses used in these studies. In fact, we previously reported that in basal condition the effects of Zn^{2+} on intestinal ion transport are strictly dose-related. Within a normal plasma concentration (i.e., <40 μM), Zn^{2+} induces ion absorption. In contrast, at higher concentrations (i.e., >100 μM), Zn^{2+} induces ion secretion as demonstrated by an increase in Isc observed in human enterocytes (Berni Canani et al., 2005).

Nitric oxide is the most recently discovered pathway involved in intestinal ion (Fasano, 2002). We show that Zn^{2+} significantly inhibited intestinal Cl^- secretion induced by IFN-γ or by the NO donor sodium nitroprusside and that the addition of Zn^{2+} to Caco2 cells was associated with a decrease in NO production. These results confirm previous observations, obtained in *in vivo* animal models, showing that Zn^{2+} inhibits lipopolysaccharides and IL-1-induced diarrhea by inducing a reduction of NO production, and by removing NO chemically generated in the gut (Wingertzahn et al., 2003). There is compelling evidence that Zn^{2+} and NO play regulatory roles in intestinal ion transport. A low NO concentration sustained by constitutive NO synthase maintains the physiological intestinal ion pro-absorptive tone (Berni Canani et al., 2003a,b). On the contrary, an excess of NO production stimulated by pro-inflammatory cytokines or microbial toxins induces intestinal fluid secretion through up-regulation of iNOS (Wingertzahn et al., 2003). This mechanism may be activated in intestinal infections and inflammation (Chavez et al., 1999) and over-expression of the iNOS gene has been demonstrated in a Zn^{2+}-deficient animal model (Cui et al., 1997). The results of our study suggest that Zn^{2+} is part of this network in the enterocyte.

The antisecretory effects of Zn are particularly relevant to childhood diarrhea. In fact, infectious diarrhea is one of the leading causes of pediatric morbidity and mortality worldwide (Lazzerini and Ronfani, 2008) and *Rotavirus*, the major agent of acute diarrhea, stimulates Cl^- secretion through a phospholipase C-dependent Ca^{2+} signaling pathway induced by NSP4 (Lorrot and Vasseur, 2007). Our experimental data showing that Zn^{2+} exerts a direct effect on Ca^{2+}-induced intestinal ion secretion support the use of Zn^{2+} in the treatment of childhood diarrhea due to *Rotavirus*.

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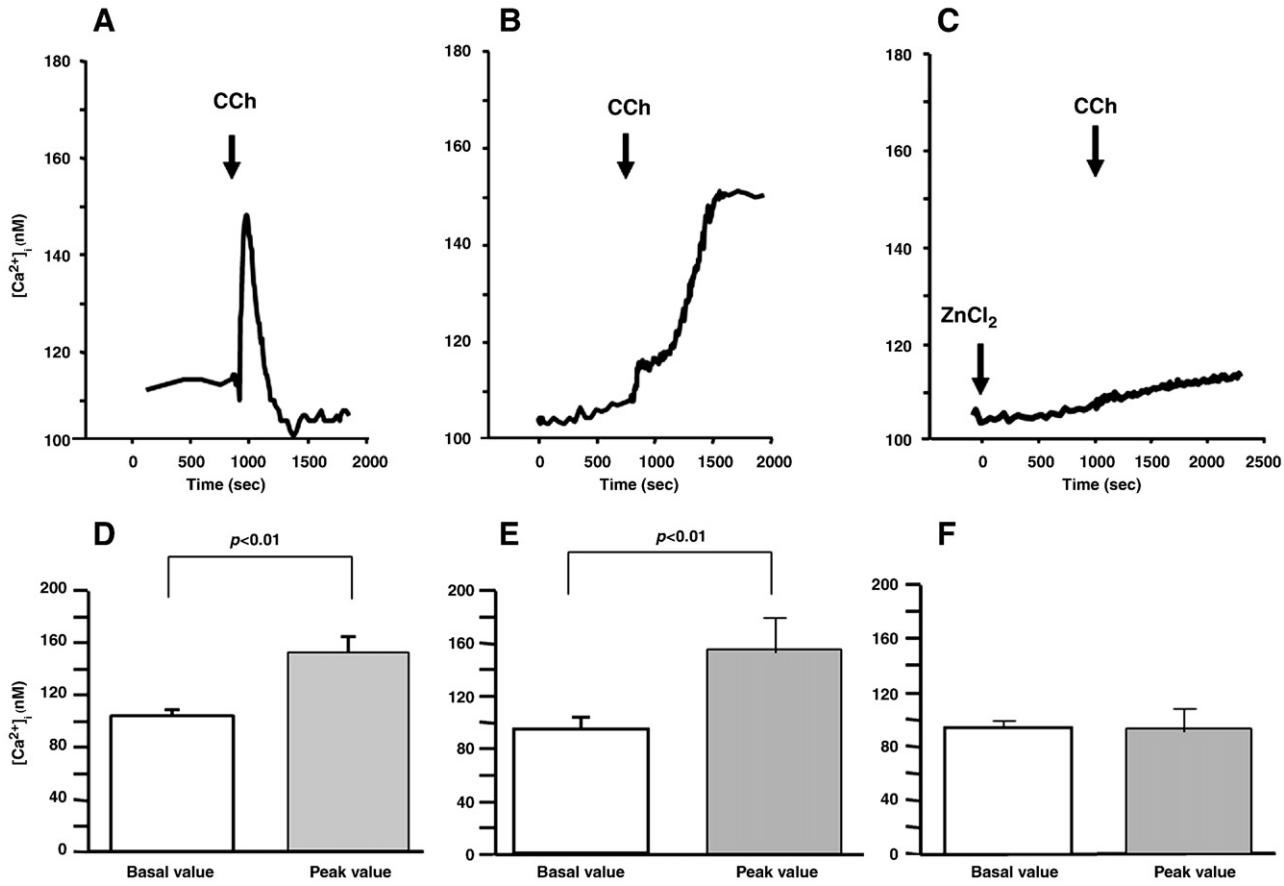

Fig. 2. Effect of Zn^{2+} on $[Ca^{2+}]_i$ increase induced by carbachol. Panels A and B report the effect of carbachol (10^{-6}M) on $[Ca^{2+}]_i$ in two single Caco2 cells loaded with Fura-2 A (see Section 2). These traces are representative of 150 cells recorded in 3 different experiments. The arrows show when the drug was put in the perfusion chamber. Panel C is a representative trace of the effect exerted by Zn^{2+} (35 μM) on $[Ca^{2+}]_i$ increase induced by carbachol (10^{-6}M). This trace is representative of 100 cells recorded in 3 different experiments. The arrows show when the drug was put in the perfusion chamber. Panels D, E and F show the maximal $[Ca^{2+}]_i$ modifications observed. The experiments were performed with 1.2 mM extracellular Ca^{2+}. Results are expressed as means ± S.D. * $P<0.01$ peak vs. basal value.

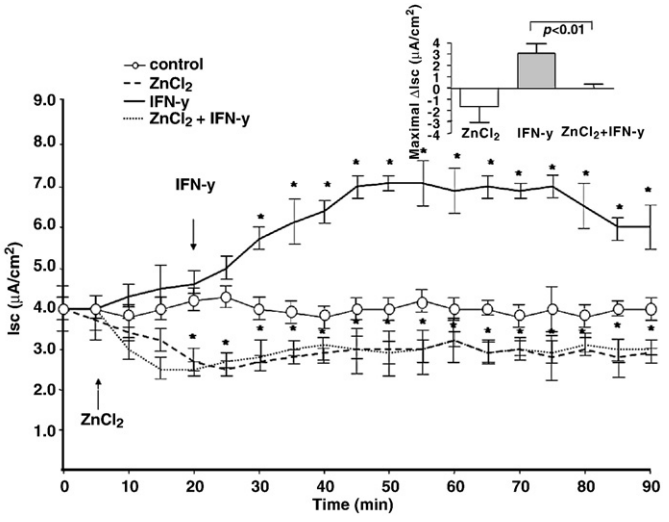

Fig. 3. Effects of Zn^{2+} on interferon-γ-induced ion secretion. Time course effects of IFN-γ (300 UI/ml) and $ZnCl_2$ (35 μM), alone or in combination, on short-circuit current (Isc) in Caco2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent. IFN-γ induced a significant increase in Isc, consistent with Cl− secretion (* $P<0.01$ vs. control). $ZnCl_2$ induced a significant decrease in Isc, consistent with chloride absorption. Preload with $ZnCl_2$ resulted in substantial inhibition of IFN-γ-induced ion secretion (*$P<0.01$ IFN-γ plus Zn^{2+} vs. IFN-γ alone), consistent with inhibition of NO-mediated ion secretion. The upper right side of the figure shows the maximal Isc modifications observed after the addition of IFN-γ (300 UI/ml) and $ZnCl_2$ (35 μM) alone or in combination. Preload with $ZnCl_2$ resulted in significant inhibition of maximal Isc modification induced by IFN-γ. Each experiment was repeated at least 3 times. Results are expressed as means ± S.D.

The number of intestinal pathogens that induce diarrhea via NO is limited, but not negligible, *Shigella* enterotoxin 1 exerts an irreversible, dose-dependent enterotoxic effect via an increased NO intestinal concentration that is partially blocked by iNOS inhibitors (Fasano, 2002). However, high NO production can also contribute to diarrhea by acting as a secretagogue, particularly in inflammatory diarrhea. In this

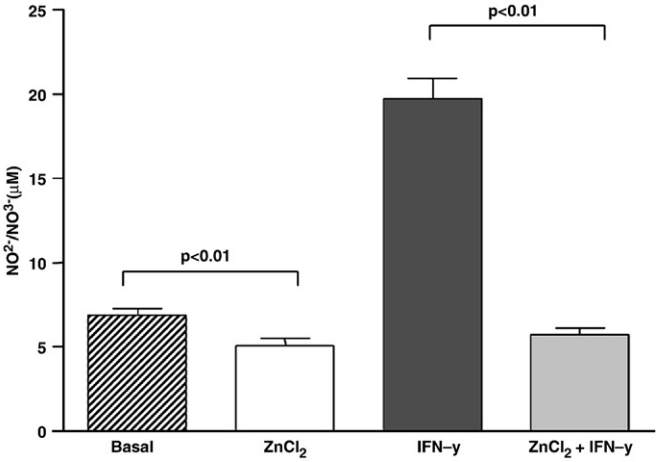

Fig. 4. Effects of Zn^{2+} on the end stable degradation products of NO (NO_2^-/NO_3^-). Total NO_2^-/NO_3^- production in Caco2 cells determined in basal condition, in the presence of $ZnCl_2$ (35 μM) and IFN-γ (300 UI/ml), alone and in combination. Data are means ± S.D. of 3 different observations. *$P<0.01$ IFN-γ plus Zn^{2+} vs. IFN-γ alone.

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context, the effects of Zn^{2+} on the NO pathway open new perspectives in the treatment of diarrhea induced by intestinal inflammatory diseases. In addition to its effects on ion transport, Zn^{2+} stimulates enterocyte growth and differentiation, reduces intestinal permeability and positively regulates oxidative stress and inflammation (Hoque and Binder, 2006). Few other drugs exert such a variety of beneficial effects in the intestine. As an adjunct to ORS, Zn^{2+} has the potential to improve the management of diarrhea at affordable costs and without the need for major equipment or medical facilities. Recently, it has been reported that Zn^{2+} enhanced clinical recovery and reduced stool output in severe diarrhea caused by *Vibrio cholerae* in children (Roy et al., 2008). It has been estimated that implementation of the WHO/UNICEF recommendations on the use of Zn^{2+} in the treatment of diarrhea could save nearly 400,000 lives annually (WHO/UNICEF, 2004), and that Zn^{2+} supplementation together with ORS is less expensive and more effective than ORS alone in the treatment of acute diarrhea (Lazzerini and Ronfani, 2008). The composition of ORS has undergone many changes in the last 30 years (Guarino et al., 2001), our results support the use of a new super-ORS containing Zn^{2+}, but further clinical studies are needed to explore the selective effects of Zn^{2+} against specific pathogens responsible for diarrhea.

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