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TNF-α down-regulates the Na^+-K^+ ATPase and the $Na^+-K^+-2Cl^-$cotransporter in the rat colon via PGE_2

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Abstract

TNF-α is believed to play a pivotal role in the pathogenesis of inflammatory bowel diseases which have diarrhea as one of their symptoms. This work studies the effect of the cytokine on electrolyte and water movements in the rat distal colon using an intestinal perfusion technique and attempts to determine its underlying mechanism of action. TNF-α inhibited net water and chloride absorption, down-regulated in both surface and crypt colonocytes the $Na^+-K^+-2Cl^-$ cotransporter, and reduced the protein expression and activity of the Na^+-K^+ ATPase. Indomethacin up-regulated the pump and the cotransporter in surface cells but not in crypt cells, and in its presence, TNF-α could not exert its effect, suggesting an involvement of PGE_2 in the cytokine action. The effect of TNF-α on the pump and symporter was studied also in cultured Caco-2 cells in isolation of the effect of other cells and tissues, to test whether the cytokine acts directly on intestinal cells. In these cells, TNF-α and PGE_2 had a similar effect on the pump expression and activity as that observed in crypt cells but were without any effect on the $Na^+-K^+-2Cl^-$ cotransporter. It was concluded that the effect of the cytokine on colonocytes is mediated via PGE_2. By inhibiting the Na^+-K^+ ATPase, it reduces the Na^+ gradient needed for NaCl absorption, and by down-regulating the expression of the $Na^+-K^+-2Cl^-$ symporter, it reduces basolateral Cl^- entry and luminal Cl^- secretion. The inhibitory effect on absorption is more significant than the inhibitory effect on secretion resulting in a decrease in net electrolyte uptake and consequently in more water retention in the lumen.

Keywords: TNF-α; Na^+-K^+ ATPase; $Na^+-K^+-2Cl^-$; PGE_2; Colon

1. Introduction

There is increasing evidence nowadays that multiple and complex interactions exist between inflammatory mediators and disturbances in ion transport processes that occur in bacterial, parasitic and autoimmune inflammatory diseases of the large intestine [1–3]. Today, three cytokines are known to be implicated in bowel inflammation namely interleukin-1 (IL-1), tumor necrosis factor (TNF) and IL-6 [4]. Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory conditions of unknown etiology whose common symptoms are abdominal pain and diarrhea. They include two main entities: Crohn's disease (CD) and ulcerative colitis (UC), and are characterized by chronic inflammation of the digestive tract with a prominent infiltration of affected tissues by lymphocytes, macrophages, plasma cells, and mast cells [4]. Increased levels of the pro-inflammatory cytokines IL-1, IL-6, IL-8, and TNF-α have been detected in IBD, and TNF-α is believed to play a pivotal role in its pathogenesis. The cytokine's level was elevated in mononuclear cells of the lamina propria isolated from patients inflicted with the disease and its administration for 10 days produced chronic inflammation of the intestinal lamina propria and submucosa [5]. TNF-α caused also a 640-fold increase in the median

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serum concentration in patients with UC as compared to healthy donors [6].

Although the increase in the level of pro-inflammatory cytokines has been well established, its correlation with the disease symptoms, mainly diarrhea and disturbed electrolyte and water movements, has not been well documented.

Diarrhea results from a decrease in net water absorption. Changes in water transport across the colon epithelium follow changes in net Na movements [7]. The mammalian colon absorbs sodium and chloride, and secretes potassium, bicarbonate, and chloride [8,9]. Under control conditions net absorption prevails [10], while secretory activities become apparent only upon stimulation by secretagogues [11].

Na^+ absorption can be electrogenic via Na^+ channels ENaC and is confined to the surface epithelium and upper part of the crypt [12] or electroneutral via parallel Na^+/H^+ and Cl^-/HCO_3^- exchange and is present in both crypts and surface epithelium [13,14]. In the rat and mouse colon, neutral NaCl absorption is dominant and is driven by the Na^+ gradient established by the Na^+-K^+ pump.

Secretion of Cl^- and K^+ occurs through their respective channels present on the luminal side and is also geared indirectly by the Na^+-K^+ pump, since it is dependent on the activity of the $Na^+-K^+-2Cl^-$ symporter which is itself energized by the Na^+ gradient established by the ATPase.

Although secretion is commonly believed to be restricted to crypt colonocytes, recent studies have shown that electrolyte secretion can occur in both surface and crypt epithelia [9,12].

Since the Na^+-K^+ ATPase is a major regulator of the transporters responsible for absorptive and secretory processes, any change in its activity is expected to lead to changes in net Na^+ and consequently water movements.

An increase in electrolyte secretion and a decrease in electrolyte absorption have been reported in inflammatory bowel diseases [15,16] together with elevated levels of TNF-α. Whether the cytokine is responsible for the observed diarrhea and targets the Na^+-K^+ pump has not been clearly elucidated. The aim of this work is to study the effect of TNF-α on net water and chloride movements in the rat colon, and determine its mechanism of action by studying its effects on the Na^+-K^+ pump and the $Na^+-K^+-2Cl^-$ cotransporter.

2. Methods

Male Sprague–Dawley rats (*Rattus norvegicus*) weighing 150–200 g were used and handled all through in accordance with the *Guide for Laboratory Animal Facilities and Care, US Department of Health, Education and Welfare*.

All chemicals were purchased from Sigma (Sigma Chemical Co., St Louis, MP, USA). Recombinant Human TNF alpha was purchased from Endogen, MA, USA.

Rabbit anti-Na^+-K^+ ATPase α1 polyclonal antibody and anti-rabbit IgG HPR conjugated antibody were purchased from Upstate Biotechnology, NY, USA. The expression of the α1 catalytic subunit was studied because it is the main isoform present in the intestine [17]. Rabbit anti-rat $Na^+-K^+-2Cl^-$ cotransporter antibody was obtained from Alpha Diagnostic International, TX, USA.

The human colon carcinoma cell line (Caco-2) was a generous gift from Dr. Mark Donowitz from Johns Hopkins University, USA.

2.1. Effect of TNF-α on net water and chloride movements in the rat colon

Rats were injected i.p. with different doses of TNF-α (100, 170 or 200 ng/100 g BW) 4 h prior to the beginning of the experiment. The doses and time point have been selected based on a previous work with IL-1β [18]. When the effect of indomethacin was studied, it was injected i.p. (1 mg/100 g BW) 10 min before TNF-α. TNF-α was administered in physiological saline while indomethacin was dissolved in DMSO (3 mg/100 μl) and then added to phosphate buffer (0.088 M NaCl; 0.01 M Na_2HPO_4; 8 mM KH_2PO_4 pH 7.3) to a final concentration of 4 mg/ml. Control rats received a similar volume of the different vehicles.

At the time of the experiment rats were anesthetized by i.p. injection of pentobarbital (5 mg/100 g BW) and their abdomen opened through a midline incision. A segment of the distal colon was isolated, making sure to keep the mesenteries and vasculature intact, and was perfused for 15 min with Krebs-improved Ringer buffer (KIRB) (123.3 mM NaCl; 6.17 mM KCl; 3.29 mM $CaCl_2 \cdot 7H_2O$; 0.78 mM $MgSO_4 \cdot 7H_2O$; 32.14 mM $NaHCO_3$; 1.54 mM KH_2PO_4; 6.4 mM sodium pyruvate; 6.4 mM sodium glutamate; 7 mM sodium fumarate; 11.1 mM glucose) at a rate of 0.7 ml/min to clear it from wastes. In a second step the colon was perfused with the same buffer and at the same rate for 30 min, but the effluent was collected and its volume measured. Buffers were oxygenated (95% O_2, 5% CO_2) at 37 °C and then their pH adjusted to 7.4 with HCl or NaOH.

At the end of the second perfusion, the perfused segment was excised, cut longitudinally, its width and length measured and its surface area determined. Water movements during the second 30-min perfusion step were calculated as the difference in volume between the infused and collected buffers divided by the surface area of the perfused colon.

Chloride was measured by titration according to Mohr's method [19] and net chloride absorption was

calculated as the difference between the total number of moles present in the infused and collected buffers, divided by the area of the colon perfused.

2.2. PGE_2 involvement

To study the involvement of PGE_2 in the response to TNF-α, indomethacin was used as an inhibitor of COX-enzymes which inhibits both COX-1, the constitutive enzyme and COX-2 the cytokine-inducible isozyme, and is not specific to any one of them. Indomethacin (10 mg/kg BW) was injected i.p. 10 min before the injection of TNF-α.

2.3. Preparation of crude membranes from colonic surface and crypt cells

Surface and crypt cells were isolated from the colon and characterized as described by Homaidan et al. [20]. The isolated cells were re-suspended in a Tris buffer (buffer A) (200 mM NaCl; 5 mM $MgCl_2 \cdot 6H_2O$; 2 mM EGTA; 5 mM KCl; 200 mM Tris–HCl, pH 7.4), and homogenized in a glass–Teflon homogenizer with 20 strokes at 2100 rpm (Arthur Thomas Scientific Apparatus, Philadelphia, PA, USA) and then spun for 10 min at 1000g and 4 °C. A crude membrane fraction was then prepared by centrifuging the supernatant collected for 10 min at 33,000g, and was saved after the addition of protease inhibitors to assay later on for the Na^+-K^+ ATPase activity and determine the protein expression of the pump and the $Na^+-K^+-2Cl^-$ cotransporter as described below. All steps in the preparation of the homogenate were conducted at 4 °C. The percent recovery of the Na^+-K^+ ATPase activity in the crude membrane homogenate for both surface and crypt ranged between 84 and 92% while the percent recovery of the cotransporter, determined by western blot analysis ranged between 79 and 88%.

2.4. Culture and treatment of Caco-2 cells

Caco-2 cells were grown in Dulbecco's Minimal Essential Medium (DMEM) containing 4500 mg/L Glucose and supplemented with 15% fetal bovine serum, Penicillin (100 μg/ml) and Streptomycin (100 μg/ml), in a humidified incubator (95% air, 5% CO_2) at 37 °C.

The cells were treated when 80% confluent for different time periods with different concentrations of TNF-α to determine the optimal dose and incubation period. When the effect of indomethacin or PGE_2 was studied they were added at a respective concentration of 100 μM and 1 nM (chosen based on a previous work conducted on Caco-2 cells and LLC-PK1 [21,22] and were dissolved, respectively, in DMSO and ethanol. Indomethacin was added 15 min prior to TNF-α. The same volume of the vehicle(s) was added to control

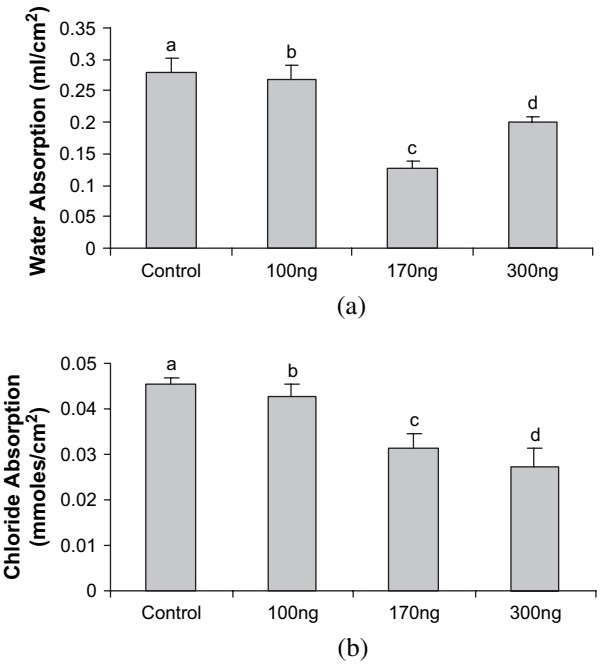

Fig. 1. a. Effect of different doses of TNF-α (ng/100 g BW) on net water absorption from the colon. For each group $n = 6$. $P < 0.01$: (a,c); (b,c). $P < 0.05$: (a,d); (c,d). b. Dose dependent effect of TNF-α (ng/100 g BW) on net absorption of chloride from the colon ($n = 5$). $P < 0.01$: (a,c); (a,d); (b,c) (b,d).

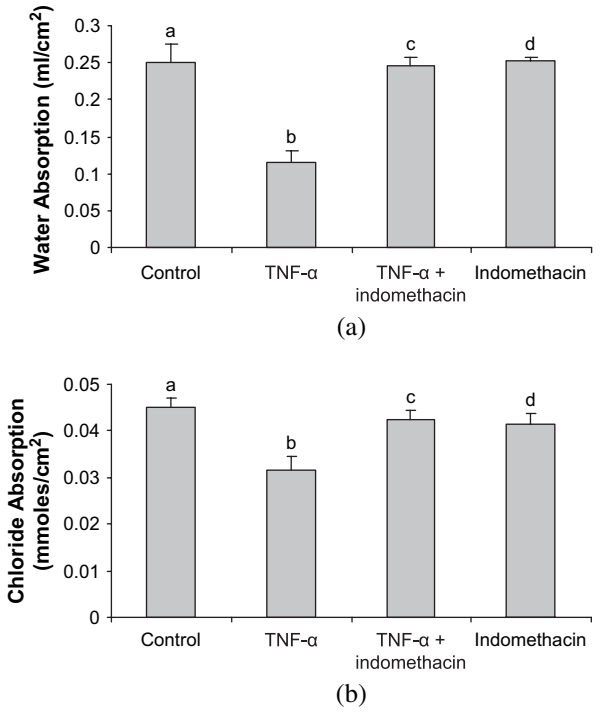

Fig. 2. a. Effect of TNF-α (170 ng/100 g BW) in presence or absence of indomethacin on net water absorption from the colon. For each group $n = 6$. Letters above the bars indicate significant differences. $P < 0.001$: (a,b); (b,c); (b,d). b. Effect of TNF-α in presence or absence of indomethacin on net absorption of chloride from the colon. ($n = 5$). Letters above the bars indicate significant differences. $P < 0.05$: (a,b); (b,c); (b,d).

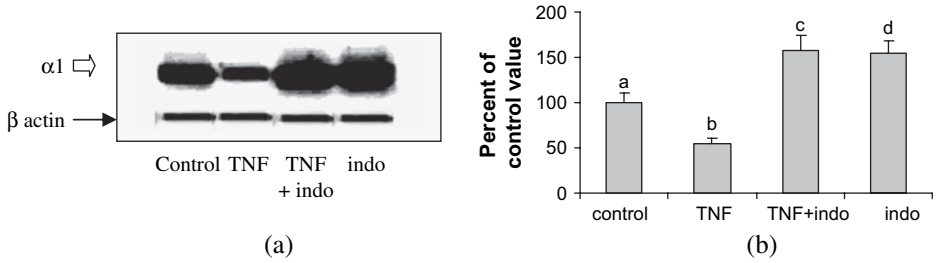

Fig. 3. (a) Effect of TNF-α in presence or absence of indomethacin on the protein expression of Na^+-K^+ ATPase in isolated colon surface cells (protein loaded: 40 μg). Results are representative of an experiment repeated three times. (b) Quantification of the results using a gel-pro analyzer (2) software. Results are reported as a percentage of the control value. $P < 0.05$: (a,b); (a,c); (a,d). $P < 0.001$: (b,c); (b,d).

plates. At the end of the incubation period, cells were washed, then lysed and homogenized in a polytron homogenizer at 20,000–22,000 rpm and 4 °C, and spun successively for 10 min at 1000g and 33,000g at 4 °C to remove nuclear and mitochondrial fraction and improve specific activity and yield.

The supernatant was collected, and after addition of protease inhibitors (Protease inhibitors, Complete™, R) were used for western blot analysis or to assay for the Na^+-K^+ ATPase activity. The Bio-Rad assay was used for protein determination.

2.5. Na^+-K^+ ATPase assay

Membrane homogenates were diluted in Tris buffer (buffer A) to a final concentration of 2 mg protein/ml and incubated for 30-min with saponin (0.02% final concentration) at room temperature. Samples (50 μl) were then pre-incubated at 37 °C for 10 min in presence or absence of ouabain (4 mM final concentration). The reaction was later on initiated by addition of ATP to a final concentration of 1.25 mM and terminated after 1 h of incubation at 37 °C by addition of trichloroacetic acid (200 μl, 11%). The amount of inorganic phosphate liberated was measured colorimetrically according to the method of Taussky and Shorr [23], and the activity of the enzyme was determined by measuring the ouabain-inhibitable inorganic phosphate released.

The percent inhibition of the enzyme activity was calculated as follows:

$$1-\frac{\mathrm{Pi(treatment)}-\mathrm{Pi(treatment+ouabain)}}{\mathrm{Pi(control)}-\mathrm{Pi(control+ouabain)}}\times 100$$

2.6. Western blot analysis

Membrane proteins were equally loaded and resolved on 8% polyacrylamide gels, then transferred to a PVDF membrane (Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA). Protein expression of β actin was used to check for equal loading. The PVDF membrane was then washed, blocked and incubated overnight at 4 °C with a rabbit polyclonal anti-Na^+-K^+ ATPase α1 IgG (Upstate biotechnology, Lake Placid, NY 12946, USA), or with a rabbit anti-rat polyclonal sodium potassium chloride cotransporter antibody (Alpha Diagnostic International, TX, USA). Detection of the signal was enhanced by using chemiluminescence luminol reagent (Santa Cruiz Biotechnology Inc., CA, USA).

2.7. Statistical analysis

Results are reported as means ± SEM. Statistical significance was tested by a one-way analysis of variance

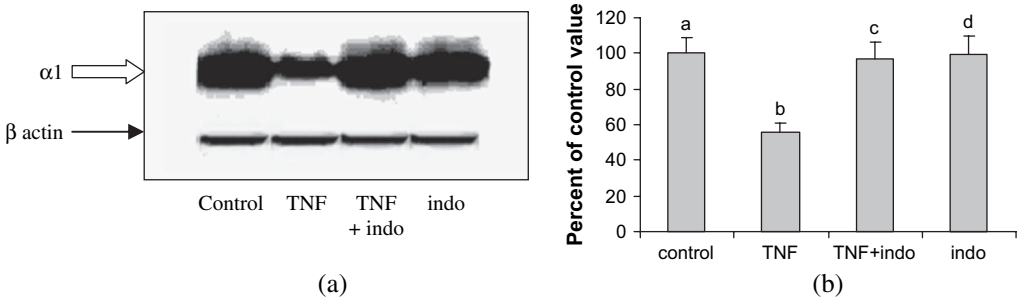

Fig. 4. (a) Effect of TNF-α in presence or absence of indomethacin on the protein expression of Na^+-K^+ ATPase in isolated colon crypt cells (protein loaded: 40 μg). Results are representative of an experiment repeated three times. (b) quantification of the results using a gel-pro analyzer (2) software. Results are reported as a percentage of the control value. $P < 0.05$: (a,b); (b,d).

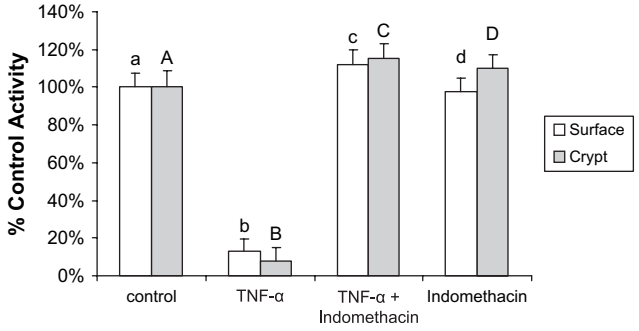

Fig. 5. Effect of TNF-α in presence or absence of indomethacin on the activity of the Na^+-K^+ ATPase in colon surface and crypt cells ($n = 3$). Letters above the bars indicate significant differences. $P < 0.01$: (a,b); (b,c); (b,d); (A,B); (B,C); (B,D).

followed by a Tukey–Kramer multiple comparisons test.

3. Results

3.1. Dose response study on the effect of TNF-α on net water and chloride absorption from the colon

A dose response study was conducted by assessing net water and chloride absorption at different doses of TNF-α (100, 170 or 300 ng/100 g BW) injected i.p., 4 h prior to the intestinal perfusion.

The cytokine reduced significantly net water absorption from the colon at 170 and 300 ng/100 g BW (Fig. 1a). The most prominent effect was observed at 170 ng/100 g BW. Net chloride absorption was also decreased at 170 and 300 ng/100 g BW (Fig. 1b).

Since the maximal inhibitory effects of the cytokine appeared at 170 ng/100 g BW, this dose was considered optimal and was used later on in all subsequent experiments.

3.2. Involvement of PGE_2

To see whether the effect of TNF-α is mediated via PGE_2 production, the cyclooxygenase (COX) enzymes were inhibited with indomethacin (1 mg/100 g BW), injected 10 min before TNF-α. Indomethacin alone did not have any effect on net water or chloride absorption but abrogated completely that of TNF-α (Fig. 2a and b).

3.3. Effect of TNF-α on the protein expression of the Na^+-K^+ ATPase in colon surface and crypt cells isolated from treated rats

TNF-α down-regulated the protein expression of the alpha-1 subunit of the Na^+-K^+ pump in colonic surface and crypt cells isolated from animals treated with the cytokine (170 ng TNF-α/100 g BW for 4 h), as illustrated in Figs. 3 and 4. Indomethacin alone up-regulated the pump in surface cells but did not have any effect in crypt colonocytes. In presence of indomethacin, the down-regulatory effect of TNF-α did not appear in both types of cells.

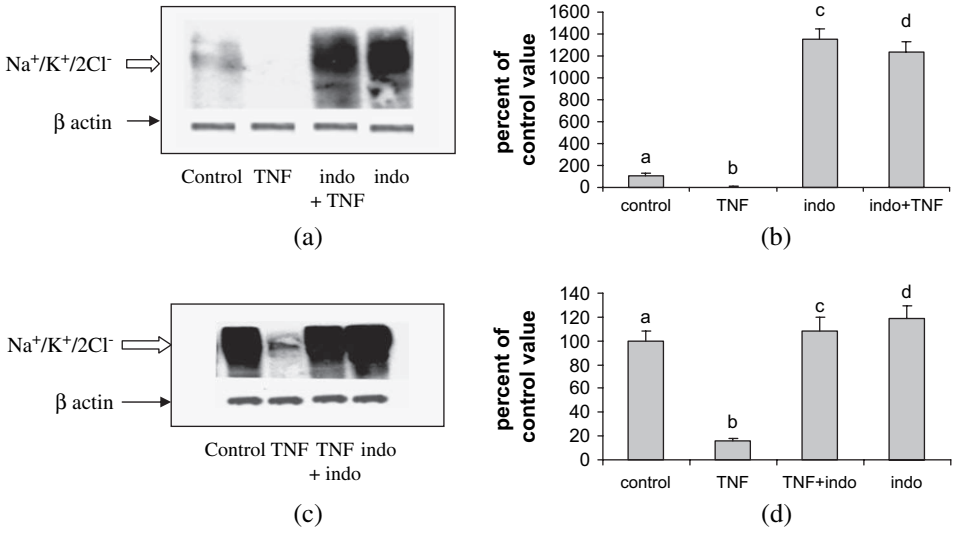

Fig. 6. (a) Effect of TNF-α in presence or absence of indomethacin on the protein expression of the $Na^+-K^+-2Cl^-$ cotransporter in colon surface cells. Results are representative of an experiment three times (protein loaded: 35 μg). (b) Quantification of the results using a gel-pro analyzer (2) software. Results are reported as a percentage of the control value. $P < 0.05$: (a,b). $P < 0.01$: (a,c); (a,d); (b,c); (b,d). (c) Effect of TNF-α in presence or absence of indomethacin on the protein expression of the $Na^+-K^+-2Cl^-$ cotransporter in colon crypt cells. Results are representative of an experiment three times (protein loaded: 35 μg). (d) Quantification of the results using a gel-pro analyzer (2) software. Results are reported as a percentage of the control value. $P < 0.001$: (a,b); (b,c); (b,d).

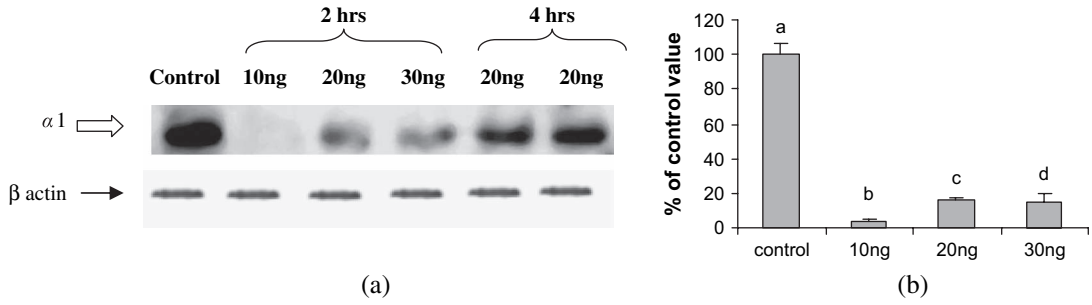

Fig. 7. (a) Time (2, 3, and 4 h) and dose (10, 20, and 30 ng/ml) dependent effect of TNF-α on the protein expression of α1 subunit of the Na^+-K^+ ATPase in Caco-2 cells. Results are representative of an experiment three times (protein load: 30 μg). (b) Quantification of the results obtained for a 2 h incuabtion using a gel-pro analyzer (2) software. $P < 0.001$: (a,b); (a,c) (a,d); (b,c); (b,d).

3.4. Effect of TNF-α on the activity of the Na^+-K^+ ATPase in colon surface and crypt cells isolated from treated rats

In parallel to the protein expression, TNF-α (170 ng/100 g BW) inhibited significantly the activity of the Na^+-K^+ATPase in surface and crypt cells isolated from treated animals, by 87 and 92%, respectively (Fig. 5). Pre-treatment with indomethacin, led to 100% recovery of the enzymatic activity in both types of cells, abrogating thus the effect of TNF-α (Fig. 5) and suggesting that the cytokine exerts its effect through PGE_2.

3.5. Effect of TNF-α on the protein expression of the $Na^+-K^+-2Cl^-$ cotransporter in surface and crypt colonocytes isolated from treated rats

Because the diarrhea that accompanies elevated levels of TNF-α in IBD may result from increased water secretion, and since the $Na^+-K^+-2Cl^-$ symporter is involved in secretory processes, we opted to study the effect of TNF-α on the protein expression of the cotransporter. In both surface and crypt cells, the cytokine down-regulated the cotransporter (Fig. 6). Indomethacin, on the other hand caused an up-regulation in surface cells (around 12-folds), and in its presence the effect of TNF-α did not appear neither in surface nor in crypt cells.

3.6. Time and dose dependent effect of TNF-α on the expression of α1 subunit of the Na^+-K^+ ATPase in Caco-2 cells

TNF-α reduced in a dose and time dependent manner, the protein expression of the alpha-1 subunit of the Na^+-K^+ ATPase in Caco-2 cells (Fig. 7). The inhibition was more prominent at 2 h than 3 or 4 h. At 10 ng/ml and 2 h the expression of the Na^+ pump was almost totally inhibited (Fig. 7).

3.7. Time and dose dependent effect of TNF-α on the activity of the Na^+-K^+ ATPase in Caco-2 cells

TNF-α caused also a dose and time dependent inhibition of the activity of the Na^+-K^+ ATPase in Caco-2 cells. The most significant effect was observed again at 10 ng/ml ($P < 0.01$) (Fig. 8a) and 2 h ($P < 0.05$) (Fig. 8b).

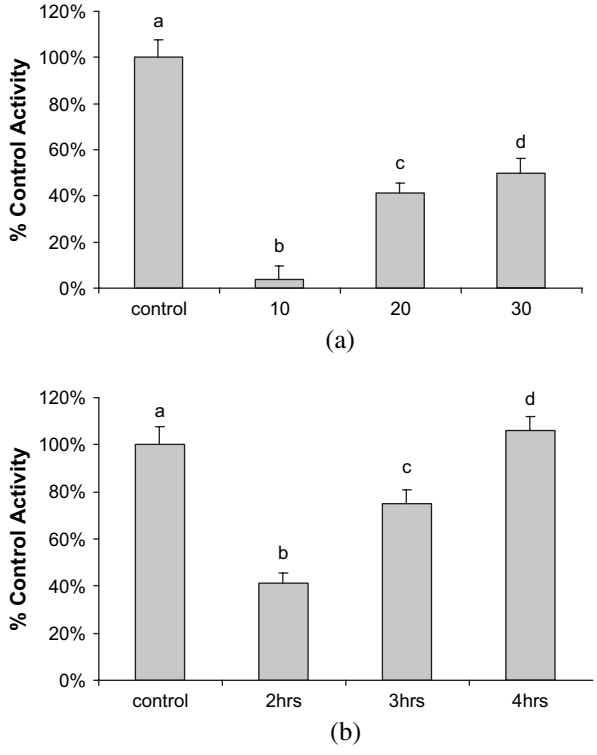

Fig. 8. (a) Dose dependent effect of TNF-α (10, 20, and 30 ng/ml) at 2 h on the Na^+-K^+ ATPase activity ($n = 3$) in Caco-2 cells. Letters above the bars indicate significant differences. $P < 0.01$: (a,b); (a,c); (a,d); (b,c); (b,d). (b) Time dependent effect of TNF-α (2, 3, and 4 h) at a concentration of 20 ng/ml on the Na^+-K^+ ATPase activity ($n = 3$) in Caco-2 cells. Letters above the bars indicate significant differences. $P < 0.05$: (a,b); (b,c); (b,d).

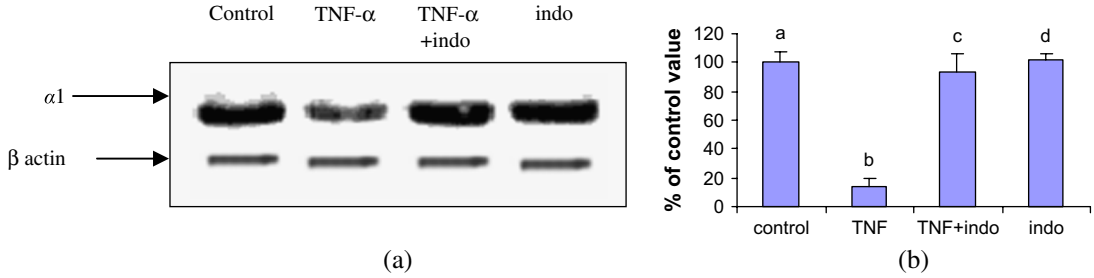

Fig. 9. (a) Effect of (TNF-α 10 ng/ml for 2 h) in presence or absence of indomethacin (100 μM pre-incubated for 15 min) on the expression of the α1 subunit of the Na^+ pump in Caco-2 cells. Results are representative of an experiment three times. (b) Quantification of the results using a gel-pro analyzer (2) software. $P < 0.001$: (a,b); (b,c); (b,d).

3.8. Effect of indomethacin and TNF-α on the Na^+-K^+ pump in Caco-2 cells

Pre-incubation with indomethacin (100 μM for 15 min) prior to TNF-α treatment (10 ng/ml for 2 h) eliminated completely the inhibitory effect of the cytokine on protein expression (Fig. 9) and pump activity (Fig. 10) in Caco-2 cells. Indomethacin alone had no effect.

3.9. Effect of TNF-α on the expression of $Na^+-K^+-2Cl^-$ cotransporter in Caco-2 cells

TNF-α (10 ng/ml for 2 h) had no effect on the protein expression of the $Na^+-K^+-2Cl^-$ cotransporter in presence or absence of indomethacin (100 μM; added 15 min before TNF-α). Indomethacin alone also showed no effect compared with the control (Fig. 11).

3.10. Effect of PGE_2 on the Na^+-K^+ ATPase and the $Na^+-K^+-2Cl^-$ cotransporter in Caco-2 cells

Caco-2 cells incubated for 30 min with PGE_2 showed a reduced Na^+-K^+ ATPase activity and expression (Fig. 12). The expression of the $Na^+-K^+-2Cl^-$ was, however, not affected (Fig. 13).

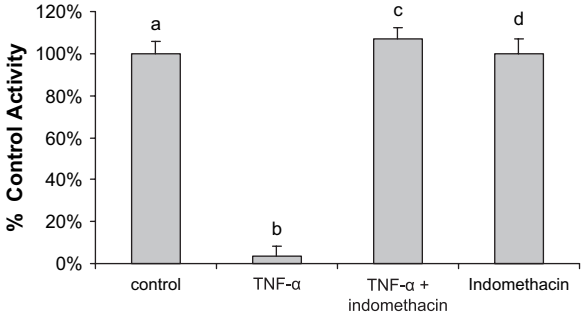

Fig. 10. Effect of TNF-α (10 ng/ml for 2 h) in presence or absence of indomethacin (100 μM; pre-incubated for 15 min) on the activity of the Na^+ pump in Caco-2 cells. Letters above the bars indicate significant differences. Results are representative of an experiment repeated three times. $P < 0.05$: (a,b); (b,c); (b,d).

4. Discussion

This work demonstrated a decrease in net water and chloride absorption from the colon of rats injected with TNF-α (Fig. 2a and b) which could be due to either a decrease in absorption or an increase in secretion [11,24] or both. Water movements are geared by the osmotic gradient that results from the different distribution of electrolytes across the plasma membrane, mainly Na^+.

Absorption of NaCl in the rat distal colon is mainly electroneutral [25] via parallel Na^+/H^+ and Cl^-/HCO_3^- exchange. The driving force for this transport process derives from the Na^+ gradient established by the Na^+-K^+ pump. Since the activity and protein expression of the Na^+-K^+ ATPase were reduced in both surface and crypt colonocytes isolated from rats treated with TNF-α (Figs. 3–5), the pump seems to be a direct site of action for the cytokine, causing a decrease in the Na^+ gradient and net NaCl absorption, and consequently, a decrease in water uptake which usually follows electrolyte movements by osmosis.

It has been recently demonstrated that secretion can occur in both surface and crypt epithelia [26] and involves the $Na^+-K^+-2Cl^-$ cotransporter and the Na^+-K^+ ATPase situated both on the basolateral side. Chloride, sodium and potassium ions are taken up by the cotransporter from the serosal fluid using the Na^+ gradient established by the pump. Sodium is pumped out back by the Na^+-K^+ ATPase while K^+ ions are

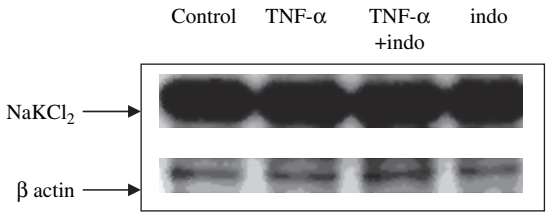

Fig. 11. Effect of TNF-α (10 ng/ml for 2 h) in presence or absence of Indomethacin (100 μM; pre-incubated for 15 min) on the expression of $Na^+-K^+-2Cl^-$ cotransporter in Caco-2 cells. Results are representative of an experiment three times.

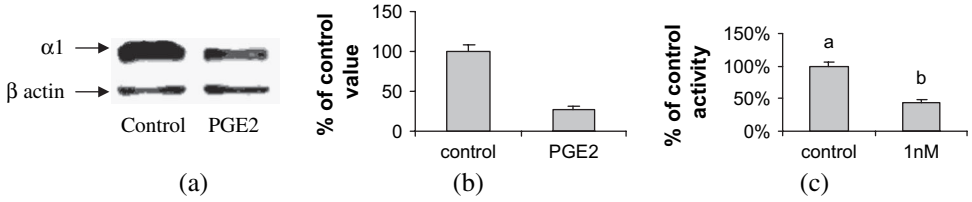

Fig. 12. (a) Effect of 1 nM PGE_2 on the protein expression of the Na^+-K^+ ATPase in Caco-2 cells. Results are representative of an experiment repeated three times. (b) Quantification of the results using a gel-pro analyzer (2) software. Values are significantly different from each other ($P < 0.001$) as shown by Student's t test. (c) Effect of PGE on the Na^+-K^+ ATPase activity in Caco-2 cells. Values are significantly different from each other ($P < 0.001$) as shown by Student's t test.

recycled via K^+ channels. This leads to hyperpolarization of the epithelial cells and an electrochemical gradient that favors Cl^- secretion into the lumen, and water follows osmotically [27]. This work has demonstrated a decrease in the protein expression of the $Na^+-K^+-2Cl^-$ symporter in both surface and crypt cells (Fig. 6). Down-regulation of the cotransporter is expected to decrease basolateral Cl^- entry and Cl^- secretion.

A possible involvement of PGE_2 in the action of TNF-α was previously suspected in the colon [28]. To see whether the effect of TNF-α on water and chloride absorption is mediated via PGE_2, indomethacin was used as an inhibitor of PGE_2 synthesis.

In surface cells the protein expression of the Na^+-K^+ ATPase and the $Na^+-K^+-2Cl^-$ symporter was up-regulated by indomethacin (Figs. 3 and 6) and in its presence the effect of TNF-α on the two transporters did not appear. These results suggest that PGE_2 is involved in the regulation of the basal expression of both transporters and is indeed a mediator of TNF-α action. Up-regulation of the pump in presence of indomethacin was not accompanied by an increase in its activity (Figs. 3 and 5) suggesting that basal levels of PGE_2 increase the number of the ATPase molecules but reduce their activity. Changes in the activity of the pump may be due to its phosphorylation/dephosphorylation [29] by some TNF-α-activated kinases/phosphatases like p38 MAPK and Jun N-terminal kinase (JNK).

In crypt cells, indomethacin had no effect on the protein expression of both transporters but still abrogated the effect of TNF-α (Figs. 4 and 6c), inferring that PGE_2 is not involved in regulating the basal level of the two transporters but is mediating only the induced down-regulatory effect of TNF-α.

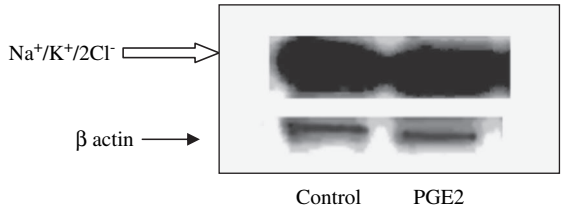

Fig. 13. Effect of PGE_2 on the protein expression of the Na^+-K^+ ATPase in Caco-2 cells. Results are representative of an experiment three times.

In both cells, the effect of TNF-α on Na^+-K^+ ATPase activity was more prominent than its effect on its protein expression and can be ascribed to a decrease in both the number and activity of pump molecules.

In crypt cells the cotransporter was expressed at higher density (Fig. 6a and c), probably due to the known greater role of crypt cells in secretion than surface cells [30].

It can be postulated that TNF-α decreases in vivo both Na^+ and Cl^- absorption resulting in a net decrease in electrolyte uptake that causes changes in the osmotic gradient favoring more water retention in the colon.

An inflammatory response is the result of a cytokine cascade, where one cytokine leads to the release of another until the final response is elicited. Thus TNF-α injected to animals may have exerted its effect on the colonocytes directly or indirectly by acting on other target cells and causing the release of other cytokines or inflammatory mediators that could act in an autocrine, parcrine or endocrine way. To clarify this point Caco-2 cells derived from a human colon carcinoma cell line were cultured and grown in isolation from any other tissues, and treated with TNF-α. These cells have been shown to retain cellular polarity, exhibit vectorial ion transport, form intercellular tight junction, and respond to hormones and neurotransmitters [31,32]. A Cl^- secretory response, although lower in intensity than the one observed in T84 or HT-19 cell lines, was elicited in these cells by many secretagogues (cAMP, VIP, epinephrine…) [32].

TNF-α reduced in Caco-2 cells also, the activity and protein expression of the Na^+-K^+ pump (Figs. 7 and 8), which is consistent with our previous in vivo results. Indomethacin alone had no effect on the ATPase but abrogated the effect of TNF-α (Figs. 9 and 10). Since Caco-2 cells are known to possess crypt cell properties [31], the effect of indomethacin on the pump was similar to that observed in crypt cells isolated from animals treated in vivo with the cytokine.

TNF-α had no effects on the protein expression of the $Na^+-K^+-2Cl^-$ cotransporter in Caco-2 cells in presence or absence of indomethacin (Fig. 11) while it reduced it significantly in crypt cells isolated from treated animals. Since the observed in vivo effect disappeared in presence of indomethacin it is speculated to be due to PGE_2 produced by neighboring cells and acting in a paracrine way.

The involvement of PGE_2 in TNF-α action was confirmed by the direct addition of exogenous PGE_2 to Caco-2 cells. PGE_2 caused as TNF-α, a decrease in the pump activity and expression (Fig. 12) but had no effect on the $Na^+-K^+-2Cl^-$ cotransporter (Fig. 13).

It can be concluded that TNF-α decreases electrolyte absorption by inhibiting the Na^+-K^+ pump, and reduces electrolyte secretion by down-regulating the $Na^+-K^+-2Cl^-$ cotranporter. The latter step may come as a regulatory measure to counteract the reduced absorption. The effect on absorption is probably more prominent than the effect on secretion resulting in the observed decrease in net Cl^- and water uptake. The effect on the two transporters is mediated through PGE_2.

Acknowledgements

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