Tumor necrosis factor-alpha impairs intestinal phosphate absorption in colitis

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Chen H, Xu H, Dong J, Li J, Ghishan FK. Tumor necrosis factor-α impairs intestinal phosphate absorption in colitis. Am J Physiol Gastrointest Liver Physiol 296: G775–G781, 2009. First published February 5, 2009; doi:10.1152/ajpgi.90722.2008.—Phosphate homeostasis is critical for many physiological functions. Up to 85% of phosphate is stored in bone and teeth. The remaining 15% is distributed in cells. Phosphate absorption across the brush-border membrane transporter (BBM) of enterocytes occurs mainly via a sodium-dependent pathway, which is mediated by type IIb sodium-phosphate cotransporters (NaPi-IIb). Patients of inflammatory bowel diseases (IBDs) suffer not only from diarrhea and nutrient malabsorption but also from bone loss. About 31–59% of patients with IBD develop bone disorders. Since the intestine is a primary location for dietary phosphate absorption, it is logical to postulate that there is an inverse relationship between gastrointestinal disorders and phosphate transport, which, in turn, contributes to bone disorders observed in patients with IBD. Phosphate absorption and NaPi-IIb expression were studied with BBM vesicles isolated from trinitrobenzene sulphonic acid (TNBS) animals as well as in Caco-2 cells. The mechanism of TNF-α downregulation of NaPi-IIb expression was investigated by luciferase assay, gel mobility shift assay (GMSA), and coimmunoprecipitation. Intestinal phosphate absorption mediated by NaPi-IIb was reduced both in TNBS colitis and in TNF-α-treated cells. Transient transfection indicated that TNF-α inhibits NaPi-IIb expression by reducing NaPi-IIb basal promoter activity. GMSAs identified NFκB protein as an important factor in TNF-α-mediated NaPi-IIb downregulation. Signaling transduction study and coimmunoprecipitation suggested that TNF-α interacts with EGF receptor to activate ERK1/2 pathway. Intestinal phosphate absorption mediated by NaPi-IIb protein is reduced in colitis. This inhibition is mediated by the proinflammatory cytokine TNF-α through a novel molecular mechanism involving TNF-α/EGF receptor interaction.

trinitrobenzene sulphonic acid colitis; Caco-2 cells; type IIb sodium-phosphate cotransporters; EGF receptor

Inflammatory bowel diseases (IBDs) are one of the most prevalent gastrointestinal disorders in the United States with its treatment costs of more than $1.7 billion (18). The two major categories of IBD are Crohn’s disease and ulcerative colitis. Bone loss is a common outcome in patients of both subtypes of IBD (5, 13). The observed bone loss in patients with IBD is from either osteopenia or osteoporosis. It is estimated that 31–59% of adult patients with IBD are classified as osteopenic, whereas 18–24% are diagnosed with osteoporosis (1, 4, 13). The pathogenesis of IBDs is not fully understood, but the cytokine profiles of the patients hint at the possibility of cytokines playing a prominent role since these patients have an imbalance of proinflammatory cytokines (12, 26). Patients with IBD have increased levels in number of cytokines including IL-1, IL-6, interferon (IFN-γ), and tumor necrosis factor-α (TNF-α) (17). TNF-α has been named the mastermind of the inflammatory response since treatment of anti-TNF-α antibodies controls the progression of IBD and also increases bone mineral density (6, 16).

Phosphate is an important element for the body because it is essential for ATP synthesis, acid/base regulation, and the formation of nucleotides. Most importantly, it is a key component of bone. In fact, the bone matrix stores 85% of the body phosphate. Mammalian phosphate homeostasis is tightly regulated by controlling intestinal and renal epithelial transport mechanisms. In the renal proximal tubules, phosphate reabsorption is mediated by type IIa sodium-phosphate cotransporters (NaPi-IIa). Intestinal absorption of dietary phosphate is mediated by another subtype of type II NaPi cotransporters expressed on the apical membranes of enterocytes named NaPi-IIb (14, 19, 37). Enterocytes also express another NaPi cotransport protein on the basolateral membranes called NaPi-III, and this isoform most likely plays a role in cellular phosphate homeostasis (3, 9). For intestinal phosphate absorption, however, NaPi-IIb is the most important of these cotransporters to study. Phosphate transport in the intestine has already been shown to be regulated by age (2, 36, 38), vitamin D3 (8, 11, 36), and hormones such as glucocorticoid (2) and estrogen (42). Since metabolic bone diseases like osteopenia and osteoporosis are associated with IBD, it is necessary to understand the molecular mechanisms of aberrant phosphate homeostasis in patients with IBD. Although TNF-α has been found to affect bone density by inducing osteoclasts to erode the bone and by inhibiting osteoblasts to lay new bone matrix (35), how the state of inflammation affects the intestinal phosphate absorption is unknown. Because of the fact that phosphate absorption is segment specific (ileum in mouse and jejunum in rat), we chose both of the animals as our in vivo model in our present studies. We aim to study the effects of inflammation on intestinal phosphate transport and ultimately bone health.

Materials and Methods

Animals. Six-week-old male Balb/C mice or 3-wk-old male Sprague Dawley rats were administered trinitrobenzene sulphonic acid (TNBS) in 50% ethanol (2 mg/mouse or 1 mg/rat) by an enema into the colonic lumen. Six days after TNBS administration, animals were euthanized, and the small intestinal mucosa was harvested for brush-border membrane vesicle (BBMV) isolation and RNA purification. All animal works have been approved by the University of Arizona Institutional Animal Care and Use Committee. All experiments were repeated at least three times with different groups of animals (3–4 animals per group).

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Cell culture. Human intestinal epithelial (Caco-2) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to ATCC guidelines. In some experiments, cells were incubated with human recombinant TNF-α (Peprotech, Rock Hill, NJ) for 40 h.

RNA purification and PCR analysis to detect NaPi-IIb expression. RNA was purified from Caco-2 cells or small intestinal mucosa. Real-time PCR was performed to detect NaPi-IIb expression. TATA binding protein (TBP) expression was used as an internal control to calculate the expression levels of NaPi-IIb. The primers used to detect NaPi-IIb and TBP were purchased from Applied Biosystems (Foster City, CA). Resulting data were analyzed by the comparative cycle threshold (Ct) method as a means of relative quantitation of gene expression, normalized to an endogenous reference (TBP) and relative to a calibrator (normalized Ct value obtained from control groups) and expressed as 2^-ΔCt (Applied Biosystems User Bulletin no. 2: Rev B “Relative Quantitation of Gene Expression”).

Phosphate uptake analysis in BBMV protein and in Caco-2 cells. Phosphate uptake with BBMV protein and in Caco-2 cells were performed as previously described methods (7, 40). The contribution of sodium-dependent uptake was calculated by subtracting the sodium-independent uptake values observed in the absence of sodium from the uptake values in the presence of sodium. The experiments were repeated in three to four different groups of animals or cells.

Protein purification and Western blot analysis. BBMVs were prepared from intestinal mucosa as previously described (36). Total crude membrane protein was isolated from Caco-2 cells with RIPA buffer method (10). A 1:4,000 dilution of mouse NaPi-IIb antibody (42) or 1:1,000 dilution of human NaPi-IIb antibody (Alpha Diagnostic International, San Antonio, TX) was used to detect NaPi-IIb protein. A 1:5,000 dilution of the β-actin antiserum (Sigma Aldrich, St. Louis, MO) was used to detect β-actin protein. Western detection was performed with the BM Chemiluminescence Western Blotting Kit (Roche Diagnostics, Basel, Switzerland). For protein expression quantitation, a ratio of NaPi-IIb protein intensity over β-actin protein intensity was used. Western blotting experiments were done with proteins isolated from three different groups of animals or Caco-2 cells.

Transient transfection and functional promoter analysis. Caco-2 cells were transfected with human NaPi-IIb (hNaPi-IIb) promoter constructs and control plasmids by Effectene-mediated transfection (Qiagen, Valencia, CA). Promoter reporter assays were performed using the dual luciferase Assay kit (Promega, Madison, WI). Luciferase activities were measured with a luminometer (Femtoscanter FB 12; Berthold Detection System, Pforzheim, Germany). Renilla luciferase activity driven by pRL-CMV (Promega, Madison, WI) was used as an internal control to calculate the relative luciferase activity. For TNF-α treatment, 20 ng/ml human recombinant TNF-α was used. To determine the involvement of EGF receptor (EGFR) pathways, cells were treated with monocolonal anti-EGFR antibodies (50 ng/ml), AG1478 (1 μM, an inhibitor of ERK/MAPK) or PD98059 (25 μM, an inhibitor of PKC), or PD98059 (25 μM, an inhibitor of PKC), or PD98059 (25 μM, an inhibitor of PKC), or PD98059 (25 μM, an inhibitor of PKC), or PD98059 (25 μM, an inhibitor of PKC), or PD98059 (25 μM, an inhibitor of PKC). The contribution of sodium-dependent uptake was calculated by subtracting the sodium-independent uptake values observed in the absence of sodium from the uptake values in the presence of sodium. The experiments were repeated in three to four different groups of animals or cells.

Preparation of nuclear extracts for GMSA. Nuclear extracts were prepared from Caco-2 cells and gel mobility shift assays (GMSAs) were performed by a previously described method (41). Synthetic DNA oligonucleotides covering NaPi-IIb promoter region –37 bp to –13 bp were end labeled with [32P]ATP, and 5 μg of nuclear extract was incubated with 1 ng of labeled probe in GMSA binding buffer [10 mM HEPES, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM diithiothreitol, and 50 μg/ml poly(dI-dC)]. After incubation at room temperature for 20–30 min, the mixture was electrophoresed on a 6% polyacrylamide gel. For competition experiments, 100- to 500-fold molar excess of unlabeled oligos was added to the reaction mixture before adding labeled oligo probes. For supershift assays, 4 μg of anti-human NF1 antibody, rabbit IgG, or anti-human ELK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixtures. The resulting products were separated on 6% polyacrylamide gel and exposed to X-ray film.

Coominoprecipitation. Caco-2 cells were cultured in 100-mm plates and treated with normal or TNF-α-containing medium. Cells were then lysed in 0.5 ml RIPA buffer. Coominoprecipitation was performed according the protocol provided by the antibody manufacturer. Anti-EGFR mouse monoclonal antibody (Calbiochem) or anti-TNF-α goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used for coominoprecipitation. Rabbit anti-EGFR antibody and anti-TNF-α antibody (Santa Cruz Biotechnology) were used for Western detection.

Statistical analysis. ANOVA post hoc tests (StatView 5.0.1; SAS Institute, Cary, NC) were used to compare values of the experimental data. P values <0.05 were considered significant.

RESULTS

Effect of TNBS colitis on phosphate absorption and NaPi-Ib expression in mouse small intestine. Male mice received TNBS (2 mg/mouse in 50% ethanol) or PBS buffer in a total volume of 100 μl by enema into the colonic lumen. Six days after TNBS administration mice were killed, ileal mucosa was harvested and used for BBMV purification. Phosphate uptake and Western blot were then performed with purified BBMV proteins. Our data showed that TNBS administration significantly reduced intestinal sodium-dependent phosphate absorption in mice, from 26.3 ± 1.2 nmol/mg protein per 10 s in control mice to 17.3 ± 2.8 nmol/mg protein per 10 s in TNBS mice (n = 3; P = 0.028) (Fig. 1A). Western blot with BBMV protein showed that TNBS administration also decreased NaPi-IIb immunoreactive protein abundance (indicated by the ratio of optical densities of the NaPi-IIb band to that of the β-actin band) from 1.97 ± 0.45 in control mice to 0.73 ± 0.18 in TNBS mice (n = 3; P = 0.017) (Fig. 1B).

Effect of TNBS colitis on NaPi-IIb expression in rat intestine. Male rats received TNBS (1 mg/rat in 50% ethanol) or PBS buffer in a total volume of 250 μl by enema into the colonic lumen. Six days after TNBS administration, rats were killed and jejunal mucosa was harvested for BBMV and RNA purification. Western blot was used to determine NaPi-IIb protein abundance from BBMV protein. Real-time PCR was used to determine NaPi-IIb mRNA expression. Western blot results showed that TNBS administration significantly reduced NaPi-IIb mRNA expression in TNBS colitis rats compared with control rats, from 1.09 ± 0.02 in control rats to 0.63 ± 0.07 in TNBS rats (n = 3; P = 0.004) (Fig. 2B).

Effect of TNF-α on phosphate uptake and NaPi-Ib expression in Caco-2 cells. The endogenous NaPi-Ib expression in Caco-2 cells has been shown in our previous work (38). To study the effect of TNF-α on cellular phosphate absorption, Caco-2 cells were grown in 24-well plates and treated with normal or TNF-α-containing medium (20 ng/ml) for 40 h. Cellular phosphate uptake assays were performed to measure the rate of phosphate uptake in Caco-2 cells. Western blot detection was used to assess NaPi-IIb protein levels in Caco-2 cells. Real-time PCR was conducted to determine the expression of NaPi-IIb mRNA in Caco-2 cells.
Effect of TNF-α treatment on hNaPi-IIb gene promoter activity. To explore whether the inhibition of TNF-α on human NaPi-IIb (hNaPi-IIb) gene expression is mediated by transcriptional mechanism, hNaPi-IIb promoter activity assays were conducted. Caco-2 cells were first transfected with hNaPi-IIb promoter constructs and then treated with TNF-α (20 ng/ml, 40 h) before analyzing promoter activity. As shown in Fig. 4A, hNaPi-IIb promoter activity was significantly decreased in TNF-α-treated Caco-2 cells (n = 7; P < 0.01). To locate the TNF-α responsive region, series of 5’ deletion constructs within the 2783 bp of the hNHE8 gene promoter region were used. Promoter reporter gene assays showed that promoter constructs pGL3b/2783, pGL3b/1103, pGL3b/380, and pGL3b/58 were all responsive to TNF-α treatment, suggesting that the TNF-α response element is located in the minimal promoter region of the hNaPi-IIb gene.

GMSA identification of DNA sequences involved in the TNF-α response of the hNaPi-IIb promoter. Previous studies have identified that NF1 transcriptional factor is involved in activating hNaPi-IIb gene expression in Caco-2 cells (41). To identify whether the basal promoter activation region is also shown in Fig. 3, sodium-dependent phosphate absorption was significantly decreased in Caco-2 cells after TNF-α treatment, from 2.51 ± 0.27 nmol/mg protein per 15 min in control cells to 1.14 ± 0.37 nmol/mg protein per 15 min in TNF-α-treated cells (n = 3; P = 0.013) (Fig. 3A). Western blot detection using human NaPi-IIb antibody indicated that NaPi-IIb immunoreactive protein abundance was reduced from 0.31 ± 0.03 in control cells to 0.18 ± 0.01 in TNF-α-treated cells (n = 3; P = 0.006) (Fig. 3B). Real-time PCR using human NaPi-IIb and TBP primers showed that TNF-α treatment inhibited NaPi-IIb gene expression by ~44%, from 1.002 ± 0.016 in control cells to 0.56 ± 0.065 in TNF-α-treated cells (n = 4; P < 0.001) (Fig. 3C).
involved in the TNF-α response, DNA oligos homologous to the hNaPi-IIb promoter region from −37 bp to −13 bp were used as the probe for GMSAs. Nuclear protein was isolated from Caco-2 cells treated with normal or TNF-α-containing medium. As shown in Fig. 4, a strong DNA-protein interaction was detected with radiolabeled oligos probes, which could be inhibited in the presence of NF1 consensus oligos (TTTGATGTAAGCCAATATGATA); underline indicates core sequences for NF1 protein binding). NF1 antiserum (a blocking antibody that recognizes all NF1 family members) blocked the DNA-protein interaction, whereas control IgG or unrelated supershift antibody (Elk antibody) had no effect on the DNA-protein interaction (Fig. 4B). Furthermore, TNF-α treatment reduced NF1 binding at the hNaPi-IIb basal promoter region (Fig. 4C).

The inhibition of hNaPi-IIb expression by TNF-α is mediated through EGFR signaling transduction pathway. To further elucidate the signaling pathways involved in TNF-α-mediated hNaPi-IIb gene expression downregulation, various inhibitors were selected in our study. Caco-2 cells were transfected with promoter construct pGL3/−58 and pretreated with various inhibitors for 2 h before TNF-α was added. As shown in Fig. 5, hNaPi-IIb gene promoter activity was reduced 40% by TNF-α in Caco-2 cells. Administration of 50 nM mouse monoclonal anti-EGFR antibody blocked the response of hNaPi-IIb promoter to TNF-α. AG1478 (1 μM), a specific inhibitor of EGFR tyrosine kinase activity, also abolished the TNF-α effect on the hNaPi-IIb promoter activity. Furthermore, administration of ERK1/2 inhibitor PD98059 (25 μM) fully restored the hNaPi-IIb promoter activity, whereas administration of PKC inhibitor H7 (10 μM) had no effect on restoring hNaPi-IIb promoter activity. The inhibitors themselves used in this study had no effect on the hNaPi-IIb promoter activity.

Complex formation between TNF-α and EGFR in Caco-2 cells. Since EGFR activation pathway is involved in the regulation of TNF-α on hNaPi-IIb expression, we thought to explore the interaction between TNF-α and EGFR in Caco-2 cells. To assess the physical interaction between TNF-α and EGFR, immunoprecipitation was performed. Immunoprecipitation results indicated that TNF-α/EGFR complexes were formed after TNF-α treatment in Caco-2 cells. TNF-α was detected by Western blot from samples coimmunoprecipitated with a mouse monoclonal anti-EGFR antibody, and EGFR was detected by Western blot from samples coimmunoprecipitated with a goat anti-TNF-α antibody. As control experiments, neither EGFR nor TNF-α could be detected from samples coimmunoprecipitated with mouse or goat IgG (Fig. 6).

**DISCUSSION**

Phosphate homeostasis is critical for many physiological functions. In bone and teeth, calcium apatite \([\text{Ca}_{10}(\text{PO}_4)_{6}](\text{OH})_2]\) serves as the inorganic filling in the organic network of collagen. Up to 85% of phosphate is stored in bone and teeth; the remaining 15% is distributed in cells as a key component for nucleic acids (DNA and RNA), energy molecules (e.g., ATP), and metabolic mediators. Phosphate absorption across the BBM of enterocytes occurs mainly via the sodium-dependent pathway, which is mediated by NaPi-IIb (14, 19, 37). Under normal physiological conditions, NaPi-IIb transporter is regulated by a number of factors, including dietary phosphate intake, vitamin D, estrogen, EGFR, and glucocorticoids (2, 8, 27, 30, 36, 39, 42). However, essentially nothing is known regarding the regulation of phosphate transport in inflammatory pathophysiological conditions.

For patients of IBDs, decreased bone density is a common outcome of their disease (4). How IBD results in bone loss is not completely understood since many factors, like disease state, calcium and vitamin D3 deficiency, glucocorticoid treat-
ment, estrogen levels, and overall nutrition, could result in unhealthy bones. Humans with inflammatory processes such as IBD result in elevated proinflammatory cytokine levels including IL-1β, TNF-α, and IL-6 (20, 24, 25, 31, 33). In TNBS colitis animal model, the levels of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1β are also increased (23). In particular, TNF-α is a key cytokine responsible for many of the symptoms of IBD, and anti-TNF-α antibodies reduce the severity of established colitis (29, 34). To explore whether intestinal phosphate absorption is impaired in IBDs, we used TNBS mouse and TNBS rats as our in vivo colitis models. The reason for utilizing the mouse and rat relates to the observations that phosphate absorption occurs in the ileum in the mouse, whereas the jejunum is the site of phosphate absorption in the rat. We found that the sodium-dependent phosphate absorption in the ileum was significantly reduced in mouse colitis. The reduction in intestinal phosphate absorption is correlated with the decrease of the intestinal NaPi-IIb expression. The similar results were also seen in colitis rats. These observations suggest that the intestinal phosphate absorption is impaired in colitis, and the involved protein is most likely the intestinal sodium-phosphate cotransporter (NaPi-IIb).

Since TNF-α is the main culprit in pathogenesis of colitis, we tested whether TNF-α is the main player in the reduction of the intestinal phosphate absorption in colitis. We treated Caco-2 cells with TNF-α (20 ng/ml for 40 h) and analyzed phosphate absorption and NaPi-IIb gene expression in these cells. Our data showed that TNF-α treatment not only reduced the phosphate transport rate in Caco-2 cells, but also reduced NaPi-IIb protein and mRNA expression. All these reductions in phosphate absorption and NaPi-IIb expression in Caco-2 cells are similar to what was observed in TNBS colitis animals. These results suggest that TNF-α is indeed an important factor that contributes to the abnormal phosphate metabolism in patients with IBD.

To understand the mechanism of TNF-α regulation on intestinal NaPi-IIb expression, we transfected Caco-2 cells with hNaPi-IIb promoter constructs and exposed these cells to TNF-α. Our results showed that TNF-α treatment (20 ng/ml, 40 h) reduced hNaPi-IIb gene promoter activity by ~40%, a level that agrees with the observed NaPi-IIb mRNA reduction. This observation suggests that a transcriptional inhibition mechanism is likely involved in TNF-α-mediated NaPi-IIb downregulation. Further transfection with shortened hNaPi-IIb promoter constructs (pGL3/-2783, pGL3/-1103, pGL3/-380, pGL3/-58) and pRL-CMV. TNF-α (20 ng/ml) was applied 40 h before measuring promoter activities. The degree of inhibition is shown as the ratio of luciferase activity in TNF-α-treated cells over luciferase activity in vehicle-treated cells. Results are means ± SE from 6 separate experiments. *P < 0.01 for control vs. TNF-α treatment.

Fig. 4. TNF-α response region on human NaPi-IIb gene promoter. A: Caco-2 cells were cotransfected with human NaPi-IIb promoter constructs (pGL3/-2783, pGL3/-1103, pGL3/-380, pGL3/-58) and pRL-CMV. TNF-α (20 ng/ml) was applied 40 h before measuring promoter activities. The degree of inhibition is shown as the ratio of luciferase activity in TNF-α-treated cells over luciferase activity in vehicle-treated cells. Results are means ± SE from 6 separate experiments. *P < 0.01 for control vs. TNF-α treatment. B: identification of nuclear protein bound on promoter region (~37 bp~13 bp) by gel mobility shift assays (GM-SAs). A 32P-labeled double-stranded oligonucleotide probe covering the proximal promoter region (~37 bp~13 bp) was incubated with 5 μg of Caco-2 cell nuclear extract in the presence or absence of unlabeled 100 × excess NF1 consensus oligos (NF1 oligos), 4 μg rabbit IgG or anti-Elk antibody (αELK) or anti-NF1 antibody (αNF1). Image is representative of 3 independent experiments. C: identification of DNA region involving TNF-α regulation. Nuclear proteins were isolated from Caco-2 cells treated with normal medium or TNF-α medium. GM-SAs were performed with DNA probe covering the basal promoter region (~37 bp~13 bp). Results shown are representative of 4 separate experiments.
EGFR activation is required in TNF-α-mediated NaPi-IIb downregulation. Our earlier study showed that EGF could inhibit NaPi-IIb expression in rat intestine and in Caco-2 cells (38, 39). Therefore, we considered the possibility of TNF-α utilizing EGFR activation pathway to downregulate NaPi-IIb gene expression. Caco-2 cells were transfected with NaPi-IIb promoter construct pGL3b/58 and were treated with various inhibitors 2 h before 40 h of TNF-α treatment. Administration of PKC inhibitor H7 (10 μM) had no effect on restoring NaPi-IIb promoter activity, suggesting that PKC pathway is not involved in TNF-α-mediated NaPi-IIb downregulation. Pretreatment with ERK1/2 inhibitor PD098059 (25 μM) completely restored the NaPi-IIb promoter activity, implying the involvement of MAPK activation by TNF-α. Our data also showed that a specific EGFR tyrosine kinase inhibitor, AG 1478 (1 μM), blocked the inhibitory effect of TNF-α on NaPi-IIb promoter activity, which suggests the involvement of EGFR tyrosine kinase activation after TNF-α administration. Furthermore, a mouse monoclonal anti-EGFR antibody abolished the effect of TNF-α on NaPi-IIb promoter activity, indicating the participation of EGFR in this TNF-α-mediated NaPi-IIb downregulation. All these observations imply that a ligand-mediated EGFR activation is required in TNF-α-induced NaPi-IIb expression downregulation.

TNF-α has been shown to transactivate EGFR in human pancreatic cancer cells (32). In Caco-2 cells, TNF-α was also found to activate EGFR by nonligand-mediated means (28). Furthermore, EGFR transactivation by TNF-α happens as a result of TNF-α converting enzyme cleaving the membrane-bound form of the cytokine (15). Our data indicated that TNF-α affects NaPi-IIb expression through EGFR-MAPK cascade in Caco-2 cells, and this effect requires ligand binding-induced EGFR activation. The requirement of both EGFR activation and the following MAPK activation indicates that TNF-α-mediated NaPi-IIb gene regulation is unlikely a result of the crosstalk between the TNFR pathway and the EGFR pathway (21, 22) because EGFR activation is not required for MAPK activation in this case. Moreover, TNF-α effect on NaPi-IIb gene expression requires prolonged treatment time (40 h). This excludes the possibility of EGFR transactivation since EGFR transactivation by TNF-α is usually transient (28) and requires TNF-α converting enzyme-cleaved membrane-bound TNF-α (15).

To test the possibility of a direct interaction between TNF-α and EGFR, we performed coimmunoprecipitation with different antibodies and followed by Western blot detection. Immunoprecipitation with a mouse monoclonal anti-EGFR antibody resulted in the detection of the soluble form of TNF-α at 17 kDa by Western blot with the use of a rabbit anti-TNF-α antibody, whereas immunoprecipitation with a goat anti-TNF-α antibody resulted in the detection of the EGFR at ~170 kDa by Western blot with the use of a rabbit anti-EGFR antibody. These observations suggest that there might be a direct interaction between soluble TNF-α and EGFR in TNF-α-mediated NaPi-IIb gene regulation in Caco-2 cells. Further studies will need to be conducted to identify the detail interaction between TNF-α and EGFR in regulating NaPi-IIb expression in Caco-2 cells.

In conclusion, we have shown that the intestinal phosphate absorption is decreased in TNBS colitis through reduced NaPi-IIb expression, and proinflammatory cytokine TNF-α is a main player in this regulation. TNF-α-mediated NaPi-IIb expression inhibition involves a novel pathway that requires direct TNF-α/EGFR interaction and EGFR/MAPK activation. With TNF-α considered the main perpetrator of inflammation in numerous inflammatory diseases and the fact that EGFR is expressed prominently in epithelial cells, the existence of this kind of interaction would significantly further our understanding of the pathogenesis and consequences of inflammatory disorders ranging from IBD to rheumatoid arthritis.

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