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Cytokine Regulation of OCTN2 Expression and Activity in Small and Large Intestine

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Running title: IFN-γ and TNF-α regulate intestinal OCTN2 expression

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Abstract (262 words 06.24.10, 250 limit)

Background: The organic cation transporter OCTN2 is located on the IBD5 risk allele and has been implicated in the pathogenesis of inflammatory bowel diseases (IBD). It expressed in the apical membrane of gut epithelium and transports many solutes including bacteria-derived mediators which appear to be involved in host-microbial interactions. To explore its role further, we examined its expression and potential regulatory factors in human IBD and in experimental models.

Methods: Human colonic epithelial cells (Caco2BBE) were used to investigate effects of inflammatory mediators on OCTN2 activity and expression. Apical membrane expression of OCTN2 was assessed by surface biotinylation. Rag-$1^{-/-}$deficient mice were used to determine the potential role of adaptive immune cells in the regulation of intestinal OCTN2 expression. C57Bl/6 mice were treated with the pro-inflammatory cytokines, IFN-γ and TNF-α, to determine expression and activity of OCTN2. OCTN2 expression in human IBD specimens was assessed by Western blotting and immunohistochemistry.

Results: OCTN2 activity and expression are regulated by the state of intestinal inflammation. OCTN2 expression in colonic tissues of Rag-$1^{-/-}$deficient mice was significantly reduced. Treatment with IFN-γ and TNF- α increased intestinal OCTN2 expression, particularly in the colon. IFN-γ increased both total and apical membrane expression of OCTN2, whereas TNF-α stimulated apical expression. Similar results were observed in Caco2BBE cells. Colonic epithelial OCTN2 expression was increased in actively inflamed areas of both Crohn's disease and ulcerative colitis.

Conclusions: Intestinal epithelial OCTN2 expression is increased by intestinal

inflammation, most likely through increased levels of proinflammatory cytokines. These findings suggest that OCTN2 may be involved in the restoration of intestinal homeostasis under conditions of inflammation-associated stress.

Introduction

OCTN1 and OCTN2 are members of the organic anion/cation transporter family SLC22 which are expressed in human tissues including kidney, heart, prostate, skeletal muscle as well as small and large intestine[1-4]. OCTN1 and OCTN2 were originally cloned from a rat kidney cDNA library, and have been shown to transport a number of endogenous substrates (carnitine, choline) as well as xenobiotics (tetraethyl ammonium, quinidine, cimetidine) in either Na^+-dependent manner (carnitine) or pH-dependent manner (tetraethyl ammonium)[2-4]. A naturally-occurring mutation of the OCTN2 gene in mice resulted in an early lethality attributed to carnitine-deficient metabolic disturbance and cardiac or skeletal muscle abnormalities[5-8]. Carnitine is required for mitochondrial β-oxidation of fatty acids[4,5].

Intestinal inflammation may alter the characteristics of epithelial cells through the secretions of pro-inflammatory cytokines such as IFN-γ and TNF-α known to be increased in the tissues of Crohn's disease patients[9, 10]. IFN-γ alters intestinal epithelial cell expression of a number of proteins such as adhesion molecules[11] , MHC molecules[12, 13], tight-junction related proteins[9], and a number of transport proteins including sodium/hydrogen exchangers[14], sodium-potassium-chloride cotransporter[15], CFTR[16], NKCCl[17], sodium-dependent glucose trasporter-1[18] and MDR-1[19-21]. Some of these

proteins are down-regulated, particularly those that are involved in maintaining the barrier function and electrolyte/nutrient transport properties of epithelial cells[22-26]. Their downregulation may be part of an autophagic process to reduce the metabolic requirements of these cells during stress, but they also contribute to the extent and severity of fluid, electrolyte, and nutrient malabsorption associated with inflamed mucosa[22,23]. In contrast, certain proteins such as PepT1 appear to be upregulated in IBD and experimental colitis, possibly as a response to restore intestinal homeostasis (VAVRICKA 2006, NGUYEN 2009). PepT1 is di/tripeptidyl transporter involved in nutrient absorption in the small bowel, but, in colon, may mediate the uptake of bioactive bacterial mediators such as chemotactic peptide (MERLIN 1998, BUYSE 2002) and muramyl dipeptide (VAVRICKA 2004).

In the present study, we investigate the expression of an organic cation transporter, OCTN2, in human IBD and its regulation by pro- and anti-inflammatory cytokines. Its encoding gene, SLC22A5, is located within the IBD5 susceptibility locus of Crohn's disease (PELTEKOVA et al., 2004, Vermeire et al., 2005, WALLER et al., 2006 and RIOUX et al., 2006), although it remains unclear whether the polymorphisms of OCTN2 have disease-causing or –modifying roles. In the colon, OCTN2 is primarily expressed by surface epithelial cells that are in direct contact with the luminal contents and microbes. Previously, we had demonstrated that it played an important role in mediating the uptake of bioactive bacterial mediators, including the quorum-sensing molecule CSF-1, which activated a number of cytoprotective and cell survival pathways (FUJIYA). These findings led to the proposal that OCTN2 is a host mechanism to sample the luminal content for

certain microbial constituents within the enteric microbiome, allowing the host to adjust to perturbations or changes that might otherwise affect intestinal homeostasis. The present study extend these observations and show that colonic epithelial OCTN2 is upregulated in response to immune- and microbial-mediated stress, possibly as a mechanism to restore intestinal homeostasis.

Materials and Methods

Mice

These studies were approved by the Institutional Animal Care and Use Committee. Rag-$1^{-/-}$ mice on a C57B16 background and age-matched controls were purchased from Jackson Laboratories (Bar Harbor, ME). Small and large intestine of mice that had been treated with IFN-γ and/or TNF-α or vehicle were removed and mucosa gently sheared off with microscope glass slides for protein or mRNA determination. To investigate the expression and localization of OCTN2, formalin fixed specimens of small and large intestine were analyzed by immunohistochemistry.

Cell culture

Human colonic epithelial Caco2BBE cells were grown as previously described on collagen-coated polycarbonate permeable filter supports (Transwell, 0.4 μm pore size, 24.5 mm diameter, 4.7 cm^2 growth surface, Costar 3412, Cambridge, MA) seeded at a density of 10^5 cells/ cm^2 and were allowed to differentiate for 10-14 days before experiments.

Human intestinal tissues

Approval for these studies was obtained from the University of Chicago Institutional Review Board. Informed consent was obtained from all subjects. Colonic pinch biopsy specimens were obtained from 11 patients with Crohn's colitis, 8 patients with left-sided ulcerative colitis (UC), and 6 healthy volunteers. Biopsies were either fixed in formalin for immunohistochemical OCTN2 staining or prepared for Western blot analysis. Samples were obtained from both inflamed and non-inflamed areas of the colon of IBD patients.

Apical membrane uptake studies

Apical membrane carnitine transport was performed as previously described (FUJIYA CH&M). All transport experiments utilized confluent monolayers of Caco2BBE cells grown on Transwell permeable supports for 14 days to maximize differentiation and OCTN2 expression. Apical uptake studies were performed in an acidic (pH 5.5) apical medium where OCTN2-mediated uptake represents a majority of carnitine uptake in the presence of Na^+. OCTN2-mediated flux was calculated by subtracting Na^+-independent uptake from the total flux.

Surface biotinylation and Western blotting

Apical surface proteins were biotinylated by previously published protocols (ROCHA, VAVRICKA, FUJIYA). For all analyses, total as well as apical surface OCTN2 were quantified by densitometry of Western blots. For mouse intestinal samples, light mucosal scrapings were made by gently shearing with glass slides and then processed for

protein analysis. For human biopsies, the entire biopsy was used for homogenate preparation. Rabbit polyclonal anti-mouse OCTN2 antiserum (Alpha Diagnostic International, San Antonio, TX) was used for blots overnight at 4°C and signals were detected using Super-Signal West Pico enhanced chemiluminescence (Pierce Chemical Company).

Real-time PCR

RNA was harvested from differentiated Caco2BBE monolayers using Trizol after treatment with cytokines when appropriate. RNA was reverse transcribed using random primers and Superscript II RT (Invitrogen). OCTN2 was amplified using the iCycler iQ real-time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA) using specific primers (sense, 5'-TTGACCCGAGTGAGTTACAAGACC-3', anti-sense, 5'-alphaGCGAAAGCCCAAAATAGCC-3' for the analysis of Caco2BBE cells and sense, 5'-TGGATTGGGGCATCTGTCC-3', anti-sense, 5'-GAGAGGGAAAAAGACCT-3' for the analysis of mouse intestine) in triplicate. OCTN2 mRNA expression levels were normalized to GAPDH expression (GAPDH specific primers; sense, 5'-TCATCTCTGCCCCCTCTGCT-3', anti-sense, 5'-CGACGCCTGCTTCACCACCT-3' for the analysis of human Caco2BBE cells and sense, 5'-GCAAATTCAAGGGCACAGT-3', anti-sense, 5'-GATGGTGATGGGCTTCCC-3' for the analysis of mouse intestine) and calculated using the comparative threshold cycle method (SCHMITTGEN).

Studies to silence OCTN2 using siRNA

To specifically inhibit expression and function of OCTN2, the BLOCK-iT RNAi designer program (Invitrogen, CA) was used to select primers (human OCTN2 bp 1331-1355). The corresponding double stranded Stealth oligo was introduced into Caco2BBE monolayers as previously described (FUJIYA).

Immunohistochemistry

The avidin-biotin indirect immunoperoxidase method was used for immunohistochemistry of paraffin-embedded sections, as previously described[28]. The Vectastain Elite ABC kit was used according to the manufacturer's instruction. Sections were counterstained with hematoxylin. Parallel sections were incubated in PBS as a primary antibody for negative controls.

Intraperitoneal injection of cytokines

Concentrations of cytokines were selected based on previous studies[14, 23, 26]. The study treatments included IFN-γ (100 ng), TNF-α (2 μg), anti TNF-α monoclonal antibody (XT22, 500 μg) or isotype-matched rat control monoclonal antibody (GL113, 500 μg), which were all administered by single intraperitoneal injection. This protocol had been previously used to investigate the effects of these agents on rat intestinal apical sodium/hydrogen exchange[14], mouse-sodium/potassium-αTPase[23] and mouse-PepT1[26] where alterations of activity and/or function of these proteins were observed. Intestinal sections were harvested 48 hours later and analyzed by Western blotting and for

Na-dependent ^{3}H-carnitine uptake.

Mouse intestinal uptake study

Two days after the initiation of cytokine treatments, intestinal segments were opened along the mesenteric border and kept in warmed (37°C) saline. The segments were minced to small pieces of approximately 0.2 cm and placed into 5 ml pre-warmed and oxygenated apical flux buffers (used for Caco2BBE ^{3}H-carnitine uptake). Pieces were incubated in the flux buffer for 10 minutes, then rapidly (less than 30 seconds) washed 3 times with ice cold flux buffer with only unlabeled carnitine to reduce extracellular trapped [^{3}H] carnitine. Trapped extracellular space was estimated using [^{3}H]-mannitol (100µmoles/l, 4400 cpm/nmole), which resulted in 317±48 cpm/mg protein in jejunum (1.8 % of control ^{3}H-carnitine counts), 261±51 in ileum (1.8 % of control ^{3}H-carnitine counts), 107±28 in proximal colon (0.9 % of control ^{3}H-carnitine counts), and 84±29 in distal colon (0.6 % of control ^{3}H-carnitine counts). The tissues were placed into 10% (wt/vol) trichloroacetic acid, homogenized with a Teflon pestle homogenizer, and allowed to sit on ice for 30 min. Precipitated proteins were pelleted (14,000 x g for 10 min at 4°C) and the supernatant removed and counted for tissue accumulated uptake of ^{3}H-carnitine. The precipitated proteins were then solubilized with 1N NaOH and quantified using the bicinchoninic acid procedure[29]. The uptakes were expressed as pmoles carnitine/10 min-mg protein.

Results

OCTN2 expression is decreased in Rag-1-deficient mouse intestine

To assess intestinal OCTN2 expression and if immune cells affect expression, immunohistochemical staining was performed on intestinal tissues of control and recombinase activating gene-1 (Rag-$1^{-/-}$)-deficient mice that lack mature T and B cells. In control mice, OCTN2 was highly expressed in the brush border (apical membrane) and subapical region of epithelial cells nearest the intestinal lumen in both colon and small intestine (Figure 1A). OCTN2 appeared to be more strongly expressed in colon rather than small intestine. Decreased OCTN2 staining was found in both small and large intestine of Rag-1-deficient mice (Figure 1B). As immunohistochemistry is not quantitative (but allows cellular location), intestinal epithelia homogenates were analyzed by Western blotting. OCTN2 expression is significantly less in the large intestine of Rag-1-deficient mice (Figure 1C).

IFN-γ and TNF-α, but not IL-1β, IL-2, IL-4 and IL-10, increase Na^+-dependent carnitine uptake in Caco2BBE cells

To confirm that the Na^+-dependent carnitine uptake is mainly due to OCTN2, Caco2BBE cells were treated with OCTN2 siRNA 24 hours before the uptake measurements were made. The Na^+-dependent ^{3}H-carnitine uptake in cells treated with OCTN2 siRNA was reduced from 0.88 ± 0.10 to 0.16 ± 0.06 pmol/mg protein-3 minutes, demonstrating that more than 80% of Na^+-dependent carnitine uptake is due to OCTN2 in Caco2BBE cells (Figure 2A).

To determine if immune cell-derived cytokines affect activity, cells were treated with

50 ng/ml of pro-inflammatory (IFN-γ, TNF-α, IL-1β, IL-2, IL-4) or anti-inflammatory cytokines (IL-10) for 48 hours before apical membrane carnitine uptake. These cytokine concentrations were selected based on our previous work[10, 14, 30]. Both IFN-γ and TNF-α significantly increased the Na^+-dependent carnitine uptake, but the other cytokines had no effect (Figure 2B). IFN-γ increased the Na^+-dependent carnitine uptake at 24 hours or greater (Figure 3), while TNF-α increased Na^+-dependent carnitine uptake within 30 minutes and significantly after 24 hours (Figure 2C).

IFN-γ and TNF-α increase OCTN2 expression in Caco2BBE cells.

We next determined the time- and dose-dependent effects of the IFN-γ or TNF-α effects on OCTN2 expression in Caco2BBE cells. IFN-γ increased both total and apical membrane OCTN2 expression 24 hours after addition of cytokines, at concentrations as low as 10 ng/ml (Figure 3A and B). In contrast, TNF-α increased apical membrane abundance as early as 30 min that was not associated with any corresponding increases in total expression (Figure 3C and D).

To determine if IFN-γ and TNF-α stimulate OCTN2 mRNA expression, quantitative PCR analysis was performed. IFN-γ increased OCTN2 mRNA expression in a concentration-dependent manner at 24 hours (Figure 3E), consistent with increased OCTN2 gene transcription or mRNA stability. In contrast, TNF-α had no effects on the OCTN2 mRNA expression (Figure 3E)

IFN-γ and TNF-α stimulate the uptake of Na^+-dependent ^3H-carnitine in mouse intestine

To determine if the in vitro effects of IFN-γ and TNF-α on intestinal epithelial OCTN2 and Na^+-dependent carnitine uptake reflected their physiological actions *in vivo*, mice were treated with IFN-γ (100ng/mouse), TNF-α (2μg/mouse) and TNF-neutralizing antibody XT22 (500μg/mouse) 48 hrs before initiation of the flux studies. IFN-γ treatment significantly increased Na^+-dependent carnitine uptake in the proximal colon, but not in the jejunum, ileum and distal colon (Figure 4). In contrast, TNF-α increased Na^+-dependent carnitine uptake in the jejunum as well as ileum, but not in the colon (Figure 4). XT22 neutralizing antibody decreased Na^+-dependent carnitine uptake in all parts of intestine, suggesting that TNF-α has a role in maintaining basal OCTN2 expression in the intestine. XT22 also inhibited the effect of IFN-γ on Na^+-dependent carnitine uptake in proximal colon, suggesting that TNF-α is essential for IFN-γ mediated OCTN2 increases.

OCTN2 protein expression was measured by Western blot in mice treated with IFN-γ, TNF-α, and XT22. IFN-γ only increased colonic OCTN2 expression without changing expression in the jejunum or ileum (Figure 5A). In contrast, TNF-α treatment had no effect on the protein expression of OCTN2 in either small or large intestine of mice (Figure 5A).

To determine if IFN-α or TNF-α increased the OCTN2 gene transcription, we quantified OCTN2 mRNA expression by real-time PCR (Figure 5B). The control C_T

values for OCTN2 were 22.71 ± 0.59 for jejunum, 22.28 ± 1.13 for ileum, 24.16 ± 0.28 for proximal colon, and 24.80 ± 0.87 for distal colon (n=4). IFN-γ increased the OCTN2 mRNA expression in proximal colon, but not TNF-α (Figure 5B). These data are consistent with in vitro data, suggesting that OCTN2 function is increased by IFN-γ and TNF-α by different mechanisms. IFN-γ increases OCTN2 by stimulating gene expression whereas TNF-α primarily regulates OCTN2 through changes in OCTN2 cellular distribution.

OCTN2 expression in human IBD patients

OCTN2 protein expression was analyzed in colonic biopsies from Crohn's disease (CD) and ulcerative colitis (UC). Biopsies were obtained from endoscopically-determined areas of inflamed and non-inflamed mucosa of patients with Crohn's colitis and left-sided ulcerative colitis which were compared with samples obtained from healthy human subjects. There was no change of colonic OCTN2 protein expression between in the patients with inactive IBD and healthy volunteers (Figure 6A), determined by Western blot analysis. In contrast, inflamed regions of both CD and UC all exhibited increased OCTN2 expression. Immunohistochemistry showed that OCTN2 was strongly expressed in both apical membrane and cytosol of the colonocytes in upper half crypt in active Crohn's disease patients, while OCTN2 expression appeared predominantly in the apical membrane of the surface colonocytes in non-inflamed mucosa (Figure 6B).

Discussion

Several important cellular processes have now been identified that play a key role in mediating host-microbial interactions. Toll like receptors, for instance, are activated by a broad range of microbe-derived molecules that are collectively referred to as pathogen-associated molecular patterns (PAMPs) and play a large role in innate immunity (MORGENSEN). This study provides evidence for another unique form of host-microbial interaction that may have relevance to maintaining intestinal homeostasis. Like the di/tripeptidyl transporter, PepT1, OCTN2 is expressed throughout the small and large intestine where its expression is greatest in the apical membrane and subapical regions of intestinal epithelial cells. Both transporters have been shown to transport and mediate microbe-derived bioactive agents that activate many types of cellular and tissue processes (MERLIN, BUYSE, VAVRICKA -2, NGUYEN, FUJIYA). Both are upregulated in inflamed intestinal mucosa and by pro-inflammatory cytokines. For PepT1, uptake of chemotactic peptides (e.g. fMLP) and muramyl dipeptide may promote a pro-inflammatory defensive response. For OCTN2, the uptake of small bacterial peptides like the quorum sensing CSF1 (competence and sporulation factor 1) results in the induction of cytoprotective heat shock proteins and activation of the Akt and MAPK kinase pathways (FUJIYA). These events confer protection to intestinal epithelial cells and intestinal mucosa to the injurious effects of reactive oxygen species. From these findings, we proposed that epithelial transporters like OCTN2 and PepT1 (and possibly OCTN1) mediate host responses to a variety of bacteria-derived signals that can either have beneficial or potentially disease-promoting actions. Thus, certain membrane transporters may facilitate host-microbe/pathogen interactions by providing selective portals for

transmission of bioactive agents.

Very little is known about the role or regulation of intestinal epithelial OCTN2 in health or disease. Polymorphisms of the encoding genes for OCTN1 and OCTN2, (SLC22A4 and SLC22A5, respectively) are found in the IBD5 risk allele and have been implicated in increasing risk for the development of inflammatory bowel diseases. However, these genes are one of several in a region of high linkage disequilibrium (ONNIE), making it difficult to pinpoint the causative factor. Our approach has been to look at the physiological role of OCTN2 and its regulation. While these studies do not shed light on the controversy surrounding OCTN2 in IBD, they do implicate it as an important mediator of host-microbe interaction (FUJIYA). The studies reported here extend these initial observations by showing that OCTN2 expression is increased in inflamed regions in both Crohn's disease as well as ulcerative colitis. This is in contrast to many other proteins involved in fluid, nutrient and electrolyte transport that are selectively down-regulated, including NHE2, NHE3, DRA, SGLT1 and Na-K-ATPase. Their down-regulation may be part of an autophagic response to reduce the metabolic requirements of the cell. We believe that the up-regulation of colonic OCTN2 during active mucosal inflammation must be relevant to host responses to injury and stress. It should be noted that our findings of inflammation-induced increases in OCTN2 conflict with a report in TNBS-induced colitis where decreased OCTN2 expression was observed (D'ARGENIO). This difference could be related to two possibilities: (1) differences in the severity of mucosal inflammation which, at the extreme, can overwhelm any process that might be involved in restoring intestinal homeostasis and promote necrosis and apoptosis, and (2) subtle differences in the

animal model versus human IBD.

This study also defined mechanisms for increased OCTN2 expression and activity in inflamed mucosa. IFN-γ stimulates OCTN2 mRNA and protein expression along with increased total protein expression in proximal colon and also increases apical OCTN2 abundance. TNF-α does not alter colonic OCTN2 expression or activity, but increases apical abundance and OCTN2 activity in small intestine. In addition to these mechanisms, it appears that the distribution of OCTN2 between the apical membrane and subapical compartment is regulated by pro-inflammatory mediators, particularly TNF-α. In this regard, OCTN2 is not the only transporter that undergoes cellular redistribution after TNF-α stimulation. TNF-α, for example, also stimulates the redistribution of the iron transporter IREG-1 from basolateral to apical membranes over a 24 hour period[24].

In summary, we find that OCTN2 is well expressed throughout the GI tract, and prominently by surface intestinal epithelial cells of the normal colon. The colonic epithelial expression of OCTN2 is highly dependent on signals received from adaptive immune cells. In human IBD, OCTN2 expression is further stimulated, probably because of the increased levels of pro-inflammatory cytokines such as TNF-alpha and IFN-gamma. These agents increase intestinal epithelial expression and/or activity of OCTN2 through activation of gene expression and redistribution of cellular OCTN2. These findings support the notion that OCTN2 is involved in the restoration of intestinal homeostasis under conditions of inflammation-associated stress.

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Figure Legends

Figure 1. OCTN2 is expressed at the brush border of the small and large intestine in control mice (A). OCTN2 expression in these regions is decreased in Rag-$1^{-/-}$-deficient mice (B). Segments of jejunum or proximal colon were fixed for immunohistochemical analyses (A and B) and mucosa scrapings analyzed by Western blotting (C) using rabbit polyclonal anti-OCTN2 antiserum or mouse monoclonal anti-villin as a constitutively-expressed, epithelial-specific control protein. Images shown are representative of 3 separate experiments, each in a different mouse. Densitometry was performed using NIH Image J, setting the control sample at 100% for each segment. * $p < 0.05$ compared with control at the same time point by analysis of variance.

Figure 2. OCTN2 mediates most of the apical carnitine uptake in Caco2BBE cells (A) that is increased by treatment with IFN-γ or TNF-α (B) in a time-dependent manner (C). For OCTN2 silencing (A), Caco2BBE monolayers were treated with silencing, double-stranded RNA oligonucleotide to OCTN2 or a control for 24 hours and Na^+-dependent ^{3}H-carnitine measured. For cytokine treatment (B), Caco2BBE monolayers were treated with the designated cytokines at 50ng/ml for 24 hours and Na^+-dependent ^{3}H-carnitine uptake measured. Data are means ± SE for 4 experiments. * $p < 0.05$ compared with control in each group by analysis of variance using a Bonferroni correction.

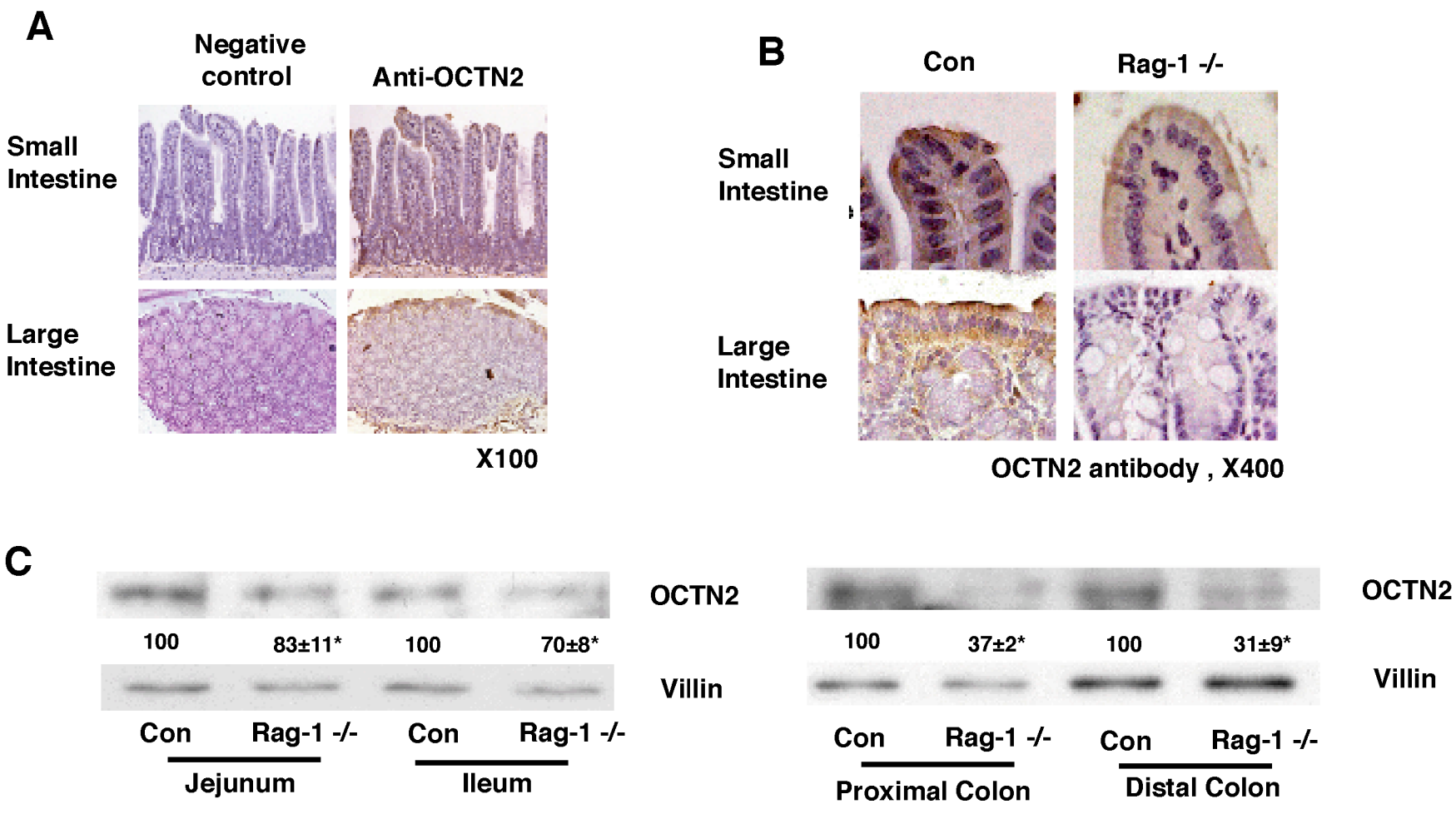
Figure 3. IFN-γ increases total and apical OCTN2 expression in time- and concentration-dependent manner (A) and increases OCTN2 mRNA (C). TNF-α increased apical OCTN2 expression without changes in total expression (B) or changes in mRNA (C). Caco2BBE monolayers were treated as designated with 50ng/ml IFN-γamma or TNF-α for varying times or varying concentrations for 24 hours and apical proteins biotinylated. Biotinylated (apical) and total OCTN2 were analyzed by Western blots and quantified using Image J software. The densitometry values for samples obtained from untreated cells were set to 100% for each analysis. Villin expression was used as a constitutively-expressed control protein. * $p< 0.05$ compared with control at the same time point of analysis of variance.

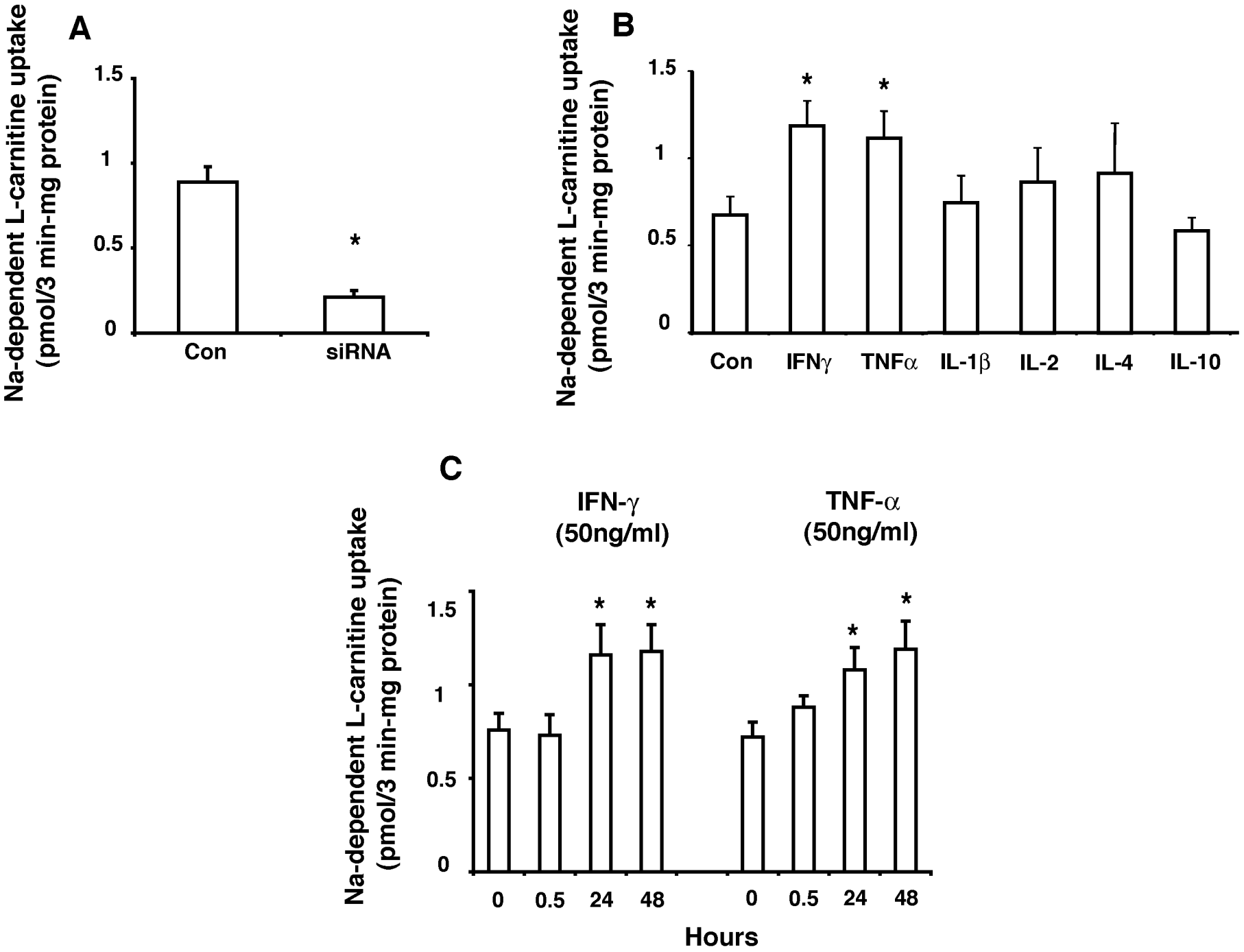
Figure 4. IFN-γ increases mouse proximal colon Na^+-dependent carnitine uptake while TNF-α increases jejunal and ileal uptake. Mice were treated with cytokines (IFN-γ 100ng; TNF- α, 2μg) with or without simultaneous treatment with anti-TNF- α antibody XT 22 (2μg). Mouse intestines were harvested 2 days later and Na^+-dependent ^3H-carnitine uptake measured as detailed in Methods. Neutralization of TFN- α with XT 22 antibody decreased carnitine uptake in all parts of intestine, even when treated with IFN-γ or TNF-α . Data are means ± SE for 4 experiments. * $p < 0.05$ compared with control at the same time point by analysis of variance.

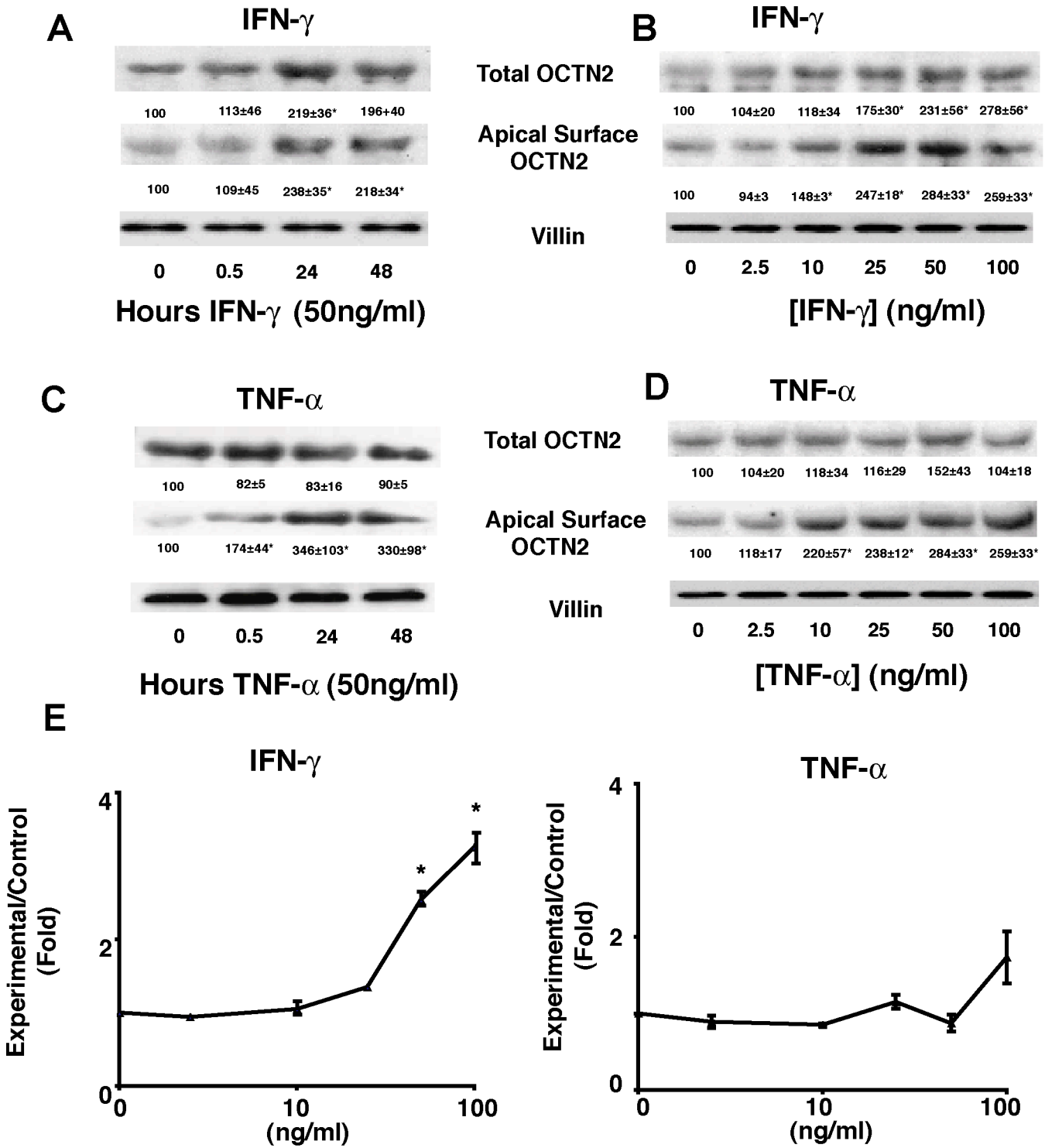
Figure 5. IFN-γ increases OCTN2 protein expression in mouse proximal and distal colon and increases OCTN2 mRNA in proximal colon (B), TNF-α did not increase OCTN2 protein or mRNA expression in any intestinal segment. Mice were treated with cytokines (IFN-γ 100ng; TNF-α, 2μg) for 2 days, mucosa scraped from each region and either analyzed for protein (OCTN2 and villin) expression by Western blots or OCTN2 mRNA by real time PCR (B). Images as well as real time data are representative of 4 separate experiments. Densitometry was performed using NIH Image J, setting the untreated control to 100% for each time point. * $p< 0.05$ compared with control at the same time point of analysis of variance.

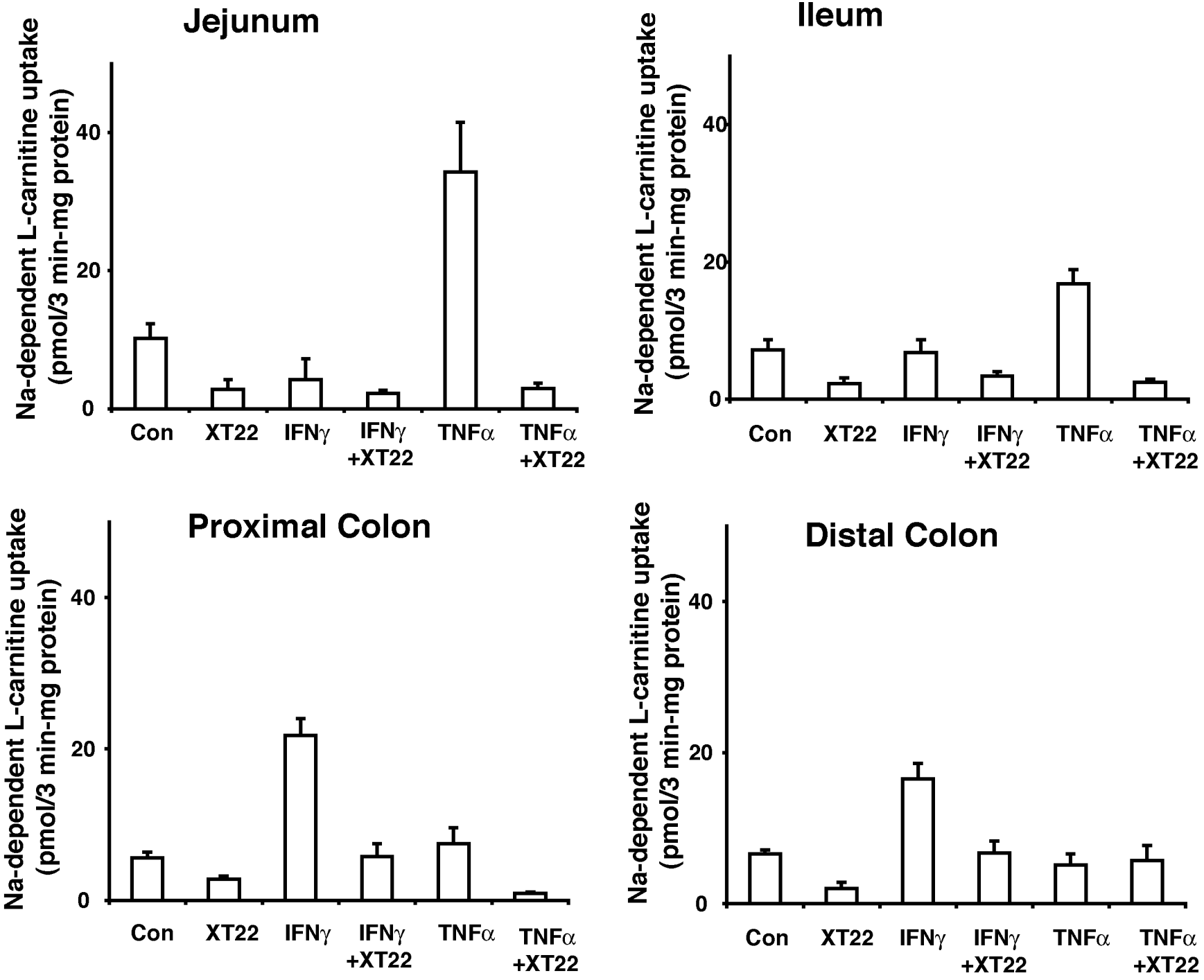
Figure 6. Colonic OCTN2 expression is increased in Crohn's disease and human ulcerative colitis. Colonic biopsies were obtained from 6 healthy volunteers, 6 Crohn's

disease patients and 5 patients with ulcerative colitis. Biopsies from IBD patients were obtained from the same patient from areas with and without inflammation. For Western analysis, homogenates were analyzed for OCTN2 and villin (A) and a small section was prepared for immunohistochemistry (B). Images shown are representative of #####. Densitometry was performed using NIH image, setting one healthy volunteer to 1 at each time point (B).


A
Negative control
Anti-OCTN2
Small Intestine
Large Intestine
X100
B
Con
Rag-1 -/-
Small Intestine
Large Intestine
OCTN2 antibody , X400
C
OCTN2
100
83±11*
100
70±8*
Villin
Con
Rag-1 -/-
Con
Rag-1 -/-
Jejunum
Ileum
OCTN2
100
37±2*
100
31±9*
Villin
Con
Rag-1 -/-
Con
Rag-1 -/-
Proximal Colon
Distal Colon

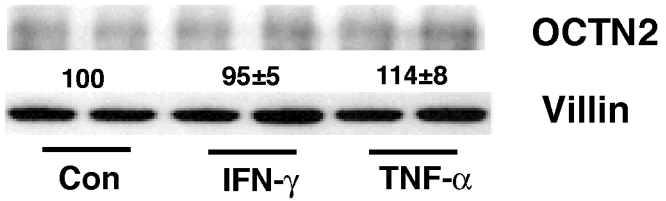

A
Na-dependent L-carnitine uptake
(pmol/3 min-mg protein)
1.5
1
0.5
0
*
Con
siRNA
B
Na-dependent L-carnitine uptake
(pmol/3 min-mg protein)
1.5
1
0.5
0
* *
Con
IFNγ
TNFα
IL-1β
IL-2
IL-4
IL-10
C
IFN-γ
(50ng/ml)
TNF-α
(50ng/ml)
Na-dependent L-carnitine uptake
(pmol/3 min-mg protein)
1.5
1
0.5
0
* * * *
0
0.5
24
48
0
0.5
24
48
Hours


A
IFN-γ
100
113±46
219±36*
196+40
100
109±45
238±35*
218±34*
0
0.5
24
48
Hours IFN-γ (50ng/ml)
B
IFN-γ
Total OCTN2
100
104±20
118±34
175±30*
231±56*
278±56*
Apical Surface OCTN2
100
94±3
148±3*
247±18*
284±33*
259±33*
Villin
0
2.5
10
25
50
100
[IFN-γ] (ng/ml)
C
TNF-α
100
82±5
83±16
90±5
100
174±44*
346±103*
330±98*
0
0.5
24
48
Hours TNF-α (50ng/ml)
D
TNF-α
Total OCTN2
100
104±20
118±34
116±29
152±43
104±18
Apical Surface OCTN2
100
118±17
220±57*
238±12*
284±33*
259±33*
Villin
0
2.5
10
25
50
100
[TNF-α] (ng/ml)
E
IFN-γ
Experimental/Control (Fold)
4
2
0
0
10
100
(ng/ml)
*
*
TNF-α
Experimental/Control (Fold)
4
2
0
0
10
100
(ng/ml)

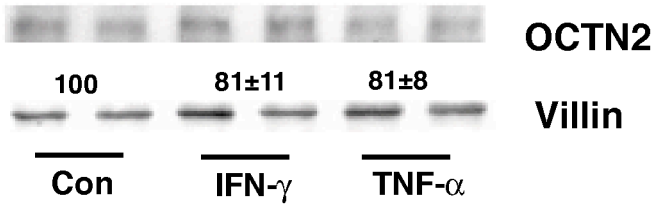

Jejunum
Ileum
Proximal Colon
Distal Colon
Na-dependent L-carnitine uptake
(pmol/3 min-mg protein)
0
20
40
Con
XT22
IFNγ
IFNγ +XT22
TNFα
TNFα +XT22

A

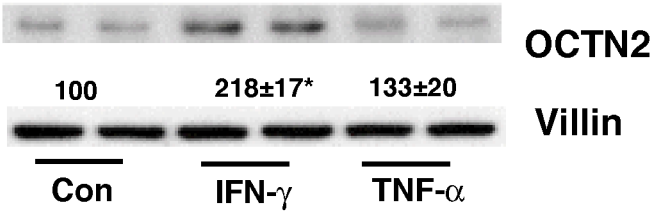
Jejunum



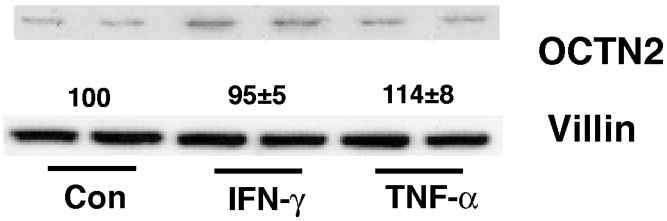
Ileum



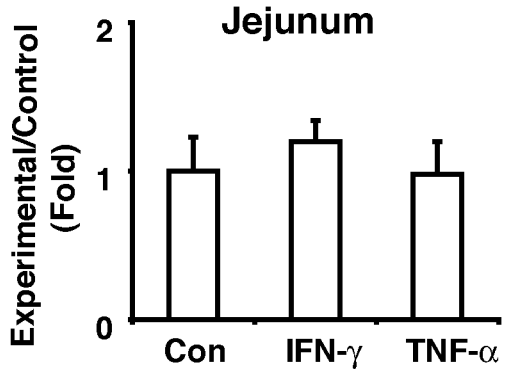
Proximal Colon

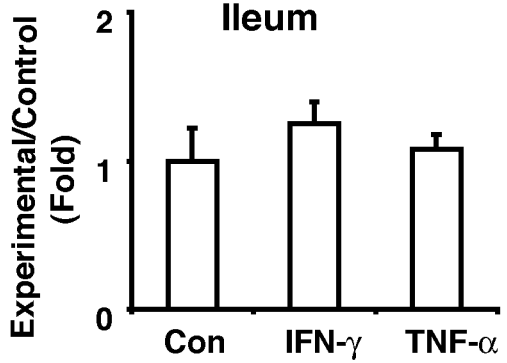


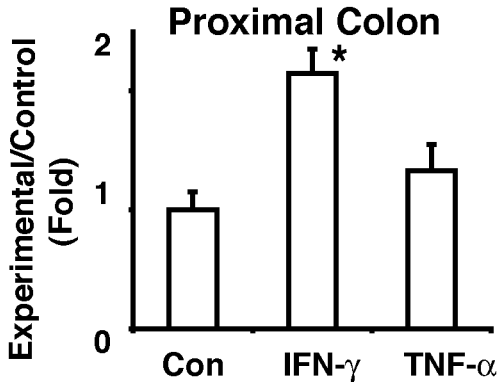
Distal Colon

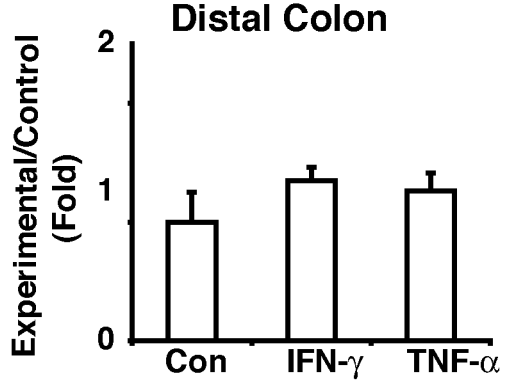


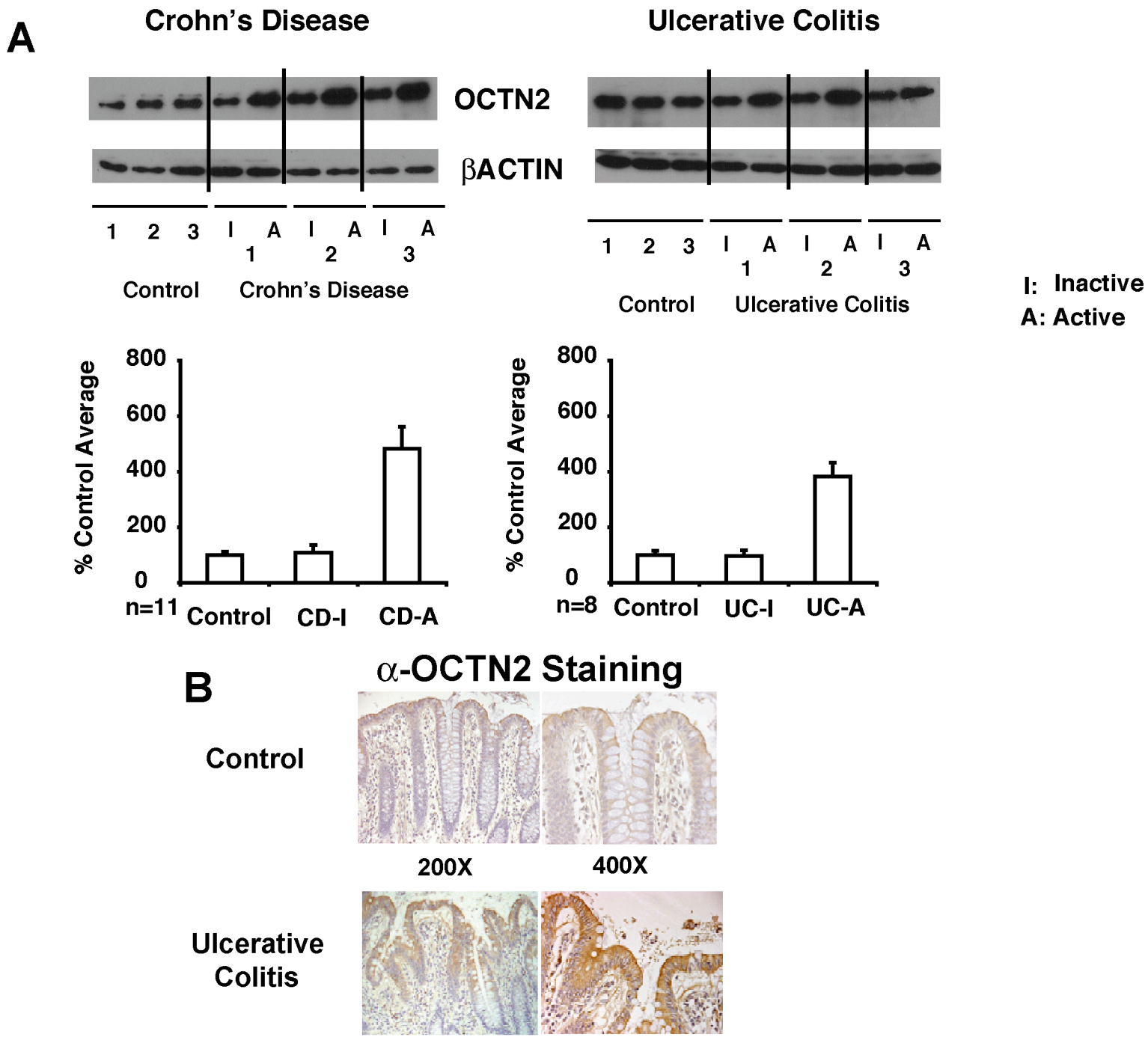
B










A
Crohn's Disease
Ulcerative Colitis
OCTN2
βACTIN
1 2 3 I A I A I A
1 2 3
Control
Crohn's Disease
Ulcerative Colitis
I: Inactive
A: Active
% Control Average
800
600
400
200
0
n=11
Control
CD-I
CD-A
n=8
UC-I
UC-A
B
α-OCTN2 Staining
Control
200X
400X
Ulcerative Colitis