

## IFN- $\gamma$ and TNF- $\alpha$ decrease serotonin transporter function and expression in Caco2 cells

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**Foley KF, Pantano C, Ciolino A, Mawe GM.** IFN- $\gamma$  and TNF- $\alpha$  decrease serotonin transporter function and expression in Caco2 cells. *Am J Physiol Gastrointest Liver Physiol* 292: G779–G784, 2007. First published December 14, 2006; doi:10.1152/ajpgi.00470.2006.—Recent studies have shown that mucosal serotonin (5-HT) transporter (SERT) expression is decreased in animal models of colitis, as well as in the colonic mucosa of humans with ulcerative colitis and irritable bowel syndrome. Altered SERT function or expression may underlie the altered motility, secretion, and sensation seen in these inflammatory gut disorders. In an effort to elucidate possible mediators of SERT downregulation, we treated cultured colonic epithelial cells (Caco2) with conditioned medium from activated human lymphocytes. Application of the conditioned medium caused a decrease in fluoxetine-sensitive [<sup>3</sup>H]5-HT uptake. Individual proinflammatory agents were then tested for their ability to affect uptake. Cells were treated for 48 or 72 h with PGE<sub>2</sub> (10  $\mu$ M), IFN- $\gamma$  (500 ng/ml), TNF- $\alpha$  (50 ng/ml), IL-12 (50 ng/ml), or the nitric oxide-releasing agent S-nitrosoglutathione (GSNO; 100  $\mu$ M). [<sup>3</sup>H]5-HT uptake was then measured. Neither PGE nor IL-12 had any effect on [<sup>3</sup>H]5-HT uptake, and GSNO increased uptake. However, after 3-day incubation, both TNF- $\alpha$  and IFN- $\gamma$  elicited significant decreases in SERT function. Neither TNF- $\alpha$  nor IFN- $\gamma$  were cytotoxic when used for this period of time and at these concentrations. These two cytokines also induced decreases in SERT mRNA and protein levels. By altering SERT expression, TNF- $\alpha$  and IFN- $\gamma$  could contribute to the altered motility and expression seen in vivo in ulcerative colitis or irritable bowel syndrome.

SERT; tumor necrosis factor; interferon; colitis; inflammatory bowel disease; inflammation

SEROTONIN (5-hydroxytryptamine; 5-HT) is the main monoamine signaling molecule in the gut, where it acts as a paracrine neurotransmitter (9, 25). Specialized enteroendocrine cells, enterochromaffin cells, release 5-HT into the lamina propria of the intestines, where it activates receptors located on processes of intrinsic and extrinsic primary afferent neurons. This leads to activation of local motor and secretory reflexes and the transduction of sensory signals to the brain stem and spinal cord. A critical aspect of paracrine and neurotransmitter signaling is the termination of transmission by degradation or removal of the signaling molecule. In the case of 5-HT, this involves rapid clearance of 5-HT from the intercellular space by the 5-HT reuptake transporter (SERT), which is expressed by mucosal epithelial cells (7, 21, 36).

SERT is a member of the highly conserved Na<sup>+</sup>/Cl<sup>-</sup>-dependent transporter gene family containing 12 transmembrane domains (2). Transporters for 5-HT and other mono-

amines are of particular interest because they are sites of action for most clinically efficacious antidepressants, as well as for psychoactive drugs of abuse such as amphetamines, cocaine, and MDMA (ecstasy). Furthermore, abnormal expression of SERT in the central nervous system may contribute to a number of complex behavioral traits and disorders (1, 35, 38).

5-HT released by enterochromaffin cells is sequestered into mucosal epithelial cells (7, 36) and changes in SERT function can lead to changes in gastrointestinal (GI) function as evidenced by the GI side effects associated with 5-HT-selective reuptake inhibitors (10, 11, 32). Also, transgenic mice lacking SERT frequently exhibit diarrhea associated with watery stools interspersed with periods of constipation (5). Additionally, SERT inhibitors decrease guinea pig colonic motility (21, 36) as well as mouse intestinal transit time and fecal output (6).

We have recently demonstrated that SERT expression is downregulated in a number of animal and human forms of intestinal inflammation. These include guinea pig trinitrobenzene sulfonic acid (TNBS) colitis (21), guinea pig TNBS ileitis (26), mouse TNBS colitis (22), murine *T. Spiralis* ileitis (39), and humans with ulcerative colitis (UC) (7). We have also found that SERT expression is lower in the rectal mucosa of individuals with inflammatory bowel syndrome (IBS) than in healthy control samples (7).

Although it has been established that inflammation coincides with decreased expression of SERT in intestinal tissue, mediators that could evoke this change have not yet been identified. The goal of this study was to identify potential proinflammatory mediators that can decrease SERT function, as seen in animal models of intestinal inflammation as well as in human patients with IBS. For these studies we have used an immortalized human intestinal epithelial cell line (Caco2 cells), which natively express SERT (24). Originally derived from a human carcinoma of the colon, these cells form a polarized monolayer when grown in vitro (12, 13, 17). They possess a mucosal membrane that models intestinal transport and metabolic parameters of the human gut (see Refs. 4, 15, 30 for examples). Using conditioned medium from activated human lymphocytes and treatment with individual cytokines, we investigated the effects on the SERT protein in Caco2 cells.

Inflammation involves a myriad of interdependent responses in a given tissue, including infiltration of leukocytes and macrophages, release of proinflammatory mediators, and changes in the synthetic machinery of resident cells. To assess whether inflammatory mediators directly affect SERT 5-HT uptake, Caco2 cells were treated with the following inflamma-

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tory mediators: IFN- $\gamma$ , TNF- $\alpha$ , IL-12, PGE<sub>2</sub>, and nitric oxide [NO; from the NO donor *S*-nitrosoglutathione (GSNO)]. These agents have all been shown to be associated with intestinal inflammation. IFN- $\gamma$ , a well-known type 1 helper T (Th1) cytokine, was chosen on the basis of studies showing that UC and TNBS-colitis in animals are associated with a cell-mediated immune response involving the activation of Th1 lymphocytes and subsequent secretion of IFN- $\gamma$  (3, 33). IFN- $\gamma$  levels are elevated in UC and TNBS-colitis (23, 33); additionally, TNF- $\alpha$  as well as mRNA for TNF receptors (TNF-RI and TNF-RII) are significantly increased in the colon of experimental animals (29). Furthermore, TNF- $\alpha$  is the target for the drug infliximab, which is used in inflammatory bowel disease (IBD) treatment (34). IL-12 was investigated as it too has been implicated in the pathogenesis of IBD (27). Other inflammatory mediators investigated included PGE<sub>2</sub> and NO. These were considered on the basis of the increases in COX-2 and inducible NO synthase often observed during inflammation (14, 16).

The results of this study have demonstrated that TNF- $\alpha$  and IFN- $\gamma$  either alone or in combination decrease SERT function, protein, and mRNA levels. The identification of cytokines that lead to altered SERT function is important in understanding the functional changes that occur in the inflamed gut. Since altered 5-HT signaling can exacerbate, if not induce, the symptoms of IBD, understanding the mediators that trigger these changes is paramount. Therapeutic strategies for the treatment of IBD could result from a better understanding of the cytokine-induced changes that affect neurotransmitter signaling in the gut.

## MATERIALS AND METHODS

**Caco2 cell culture.** Caco2 cells were obtained from American Type Culture Collection (ATCC), Manassas, VA. Cells were grown in modified Eagle's medium (GIBCO) with the addition of 15% heat-inactivated FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin (also glutamate and sodium pyruvate). Cells were incubated at 37°C with 5.5% CO<sub>2</sub>. Cells were grown at least 5 days to allow for confluence and maturation (as described by Singh et al., Ref. 31). After the fifth day, the media were changed and cytokines were added. Cytokines were added daily and culture media was replaced concurrently. The concentrations of cytokines and inflammatory mediators were based on effective, published concentrations previously described in the literature. Concentrations used were PGE<sub>2</sub>, 10  $\mu$ M; IFN- $\gamma$ , 500 ng/ml; TNF- $\alpha$ , 50 ng/ml; IL-12, 50 ng/ml; the NO-releasing agent GSNO was used at 100  $\mu$ M. All agents were purchased from Sigma-Aldrich, Milwaukee, WI.

**Conditioned medium experiments.** Human lymphocytes were isolated from 10 ml whole blood by using lymphocyte separation medium (MP Biomedicals, Irvine, CA). Cells were isolated by centrifuging at 350 g for 8 min. The lymphocyte layer was removed and added to 15 ml or RPMI medium containing 10% FBS. Cells were then centrifuged again to pellet lymphocytes. Cells were placed in 10 ml of RPMI + 10% FBS. Three ml of cell suspension was then added to culture tubes. To activate lymphocytes LPS (Sigma, Milwaukee, WI) was added to one tube at a final concentration of 200  $\mu$ g/ml. Another tube was treated with 25  $\mu$ l of a turbid solution of *Escherichia coli* grown in Luria broth base (ATCC no. 25922). A control tube of lymphocytes was also cultured that contained no LPS or bacteria. After 24 h tubes were centrifuged at 1,500 g and supernatants were removed, sterile filtered, and stored at -70°C. Aliquots were then thawed, and 75  $\mu$ l of this conditioned medium were added to the 500  $\mu$ l of culture medium of Caco2 cells growing in 24-well plates. Conditioned medium was added daily for 3 days

(media were changed daily). Preparation of conditioned medium (lymphocyte culture) was performed twice. Application of conditioned media to Caco2 cells was performed three times, in duplicate. For the measurement of TNF- $\alpha$  and IFN- $\gamma$  in conditioned medium, ELISA assay kits were purchased from Biosource (Camarillo, CA) and assays were performed according to the manufacturer's instructions.

**[<sup>3</sup>H]5-HT uptake experiments.** After incubation with various proinflammatory agents or control (PBS), Caco2 cells grown on 12-well plates were washed three times with cold Krebs-Ringer-HEPES (KRH). The KRH buffer contained the following ingredients: 124.0 mM NaCl, 2.9 mM KCl, 1.3 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.4 mM CaCl<sub>2</sub>, 5.2 mM D-glucose, 25.0 mM HEPES, 0.1 mM sodium ascorbate, 0.1 mM pargyline. After washing, cells were incubated in 1 ml KRH buffer containing 50 nM [<sup>3</sup>H]5-HT. Wells used to define nonspecific uptake also had 10  $\mu$ M fluoxetine present. All radiochemicals were purchased from PerkinElmer, Boston, MA. After the addition of [<sup>3</sup>H]5-HT, cells were transferred to a 37°C incubator for 10 min. After incubation at 37°C, cells were washed three times with ice-cold KRH to remove free [<sup>3</sup>H]5-HT. KRH was then aspirated and 600  $\mu$ l of 0.1% SDS were added to solubilize the cells. After the addition of detergent, 500  $\mu$ l of solubilized cells were transferred to scintillation vials and radioactivity in each vial was measured by using a Packard tri-carb 2800TR scintillation counter. Nonspecific uptake was defined as that which occurred in the presence of 10  $\mu$ M fluoxetine (a 5-HT-selective reuptake inhibitor). Each plate was normalized to itself. Nonspecific uptake was subtracted from all wells. All wells were then normalized to wells containing no fluoxetine and expressed as a percent of these control wells. All experiments were performed at least three times, in duplicate or triplicate. Uptake in the presence of fluoxetine gave dpm between 2,000 and 8,000 whereas total uptake typically ranged from 125,000 to 180,000 dpm. Results were compared using ANOVA followed by a Dunnett's posttest comparing all columns to control. Data were analyzed by using Graphpad Prism 4.01.

**Cell viability assays.** Caco2 cells were treated with TNF- $\alpha$  and IFN- $\gamma$  as described above, after which cells were incubated with 10  $\mu$ M of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reagent (Molecular Probes) to assess overall viability by monitoring the production of formazan at 540 nm. Results were expressed as percent survival compared with controls and represent an *n* of 3. Error bars represent SE.

**Quantitative RT-PCR.** Quantitative real-time PCR determination of SERT mRNA was performed. Caco2 cells were treated with 50 ng of TNF- $\alpha$  and 500 ng of IFN- $\gamma$  over a time course of 72 h. cDNA was synthesized as described above. SERT expression after cytokine treatment was determined by using SYBR GreenER qPCR SuperMix SYBR GreenER qPCR SuperMix (Invitrogen) with the following primers: SERT mRNA expression was normalized to the housekeeping gene  $\beta$ -actin. As an internal control, relative expression of the chloride channel CLCN3 was determined and normalized to  $\beta$ -actin using the following primers:  $\beta$ -actin, forward 5'-catgttgagacctcaacac-3'; reverse 5'-ccaggaaggaaggctggaa-3'. CLCN3: forward 5'-tgcttagtgctgctattg-3'; reverse 5'-ccagaacgggatacttcca-3'. SERT: forward 5'-gccgtgtcatcactgctt-3'; reverse 5'-cgtagtgtgctggcggctcat-3'.

**Western blotting.** Caco2 cells were treated as previously described with cytokines. After treatment, samples were lysed in HEPES buffer, pH 7.4, 0.44% SDS, 1% Triton, 111 mM NaCl, 2.2 mM EDTA, and protease inhibitor cocktail (Sigma). After lysis, samples are centrifuged at 14,000 rpm for 20 min followed by a brief sonication of the resulting pellet. Fifty micrograms of protein were then loaded on a 10% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad), and membranes were subsequently blocked in 5% milk in Tris-buffered saline (TBS). Membranes blocked overnight in TBS/milk were washed two times for 15 min in TBS containing 0.05% Tween 20 and incubated with primary antibody directed against SERT for 16 h at 4°C. The SERT antiserum was

a generous gift from Dr. Randy Blakely, Vanderbilt University. Membranes were washed three times for 20 min in TBS-Tween 20 and incubated with a peroxidase-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After a 30-min wash with TBS-Tween 20, conjugated peroxidase was detected by ECL according to the manufacturer's instructions (Amersham Biosciences). Densitometry was performed with an FX scanner. Mean densities were calculated and compared by ANOVA followed by Bonferroni's multiple-comparison test. The gel showed statistically significant differences at the 72-h time point ( $P < 0.05$ ).

## RESULTS

**SERT function experiments.** As previously described, intestinal inflammation has been associated with decreases in SERT function. To elucidate whether naturally produced inflammatory mediators alter SERT function in Caco2 cells, human lymphocytes were isolated and activated with either the bacterial endotoxin (LPS) or *E. coli*. Wild-type Caco2 cells take up radiolabeled 5-HT in a fluoxetine-sensitive manner (24). We have found this uptake to be between 90 and 95% specific and temperature sensitive (data not shown). At confluence, differentiated Caco2 cells were treated with medium conditioned by coculturing the isolated lymphocytes with *E. coli* or the addition of 200  $\mu\text{g/ml}$  LPS. As seen in Fig. 1, incubation with conditioned medium for 48–72 h resulted in a significant reduction in [ $^3\text{H}$ ]5-HT uptake compared with medium from nonactivated lymphocytes.

Experiments were then conducted to elucidate possible cytokines or agents responsible for this decrease in uptake. Figure 2 shows the effects of various proinflammatory cytokines on 5-HT uptake. After 48 h of incubation, only TNF- $\alpha$  and the combination of TNF- $\alpha$  plus IFN- $\gamma$  caused significant reductions in uptake. When the incubation period was extended to 72 h, both IFN- $\gamma$  and TNF- $\alpha$  each produced reductions in 5-HT uptake (Fig. 2). Transcripts for IFN- $\gamma$  and TNF- $\alpha$  (TNF-RI, TNF-RII, and IFNgR) were detected in RNA from Caco2 cells (data not shown). In time course studies, it was determined that both IFN- $\gamma$  and TNF- $\alpha$  caused significant inhibition of uptake when incubated for 72 h but in most cases did not cause

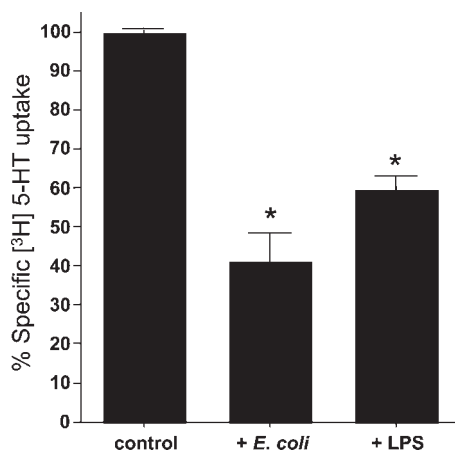


Fig. 1. Conditioned medium from activated lymphocytes decreases [ $^3\text{H}$ ]5-HT uptake in Caco-2 cells. Cells were incubated for 72 h with 75  $\mu\text{l}$  of conditioned media from human lymphocytes cultured in the presence of *Escherichia coli* or 200  $\mu\text{g/ml}$  LPS. Nonspecific uptake was defined using 10  $\mu\text{M}$  fluoxetine. Data were normalized to controls (wells containing media from nonactivated lymphocytes). The experiment was performed 3 times in duplicate.

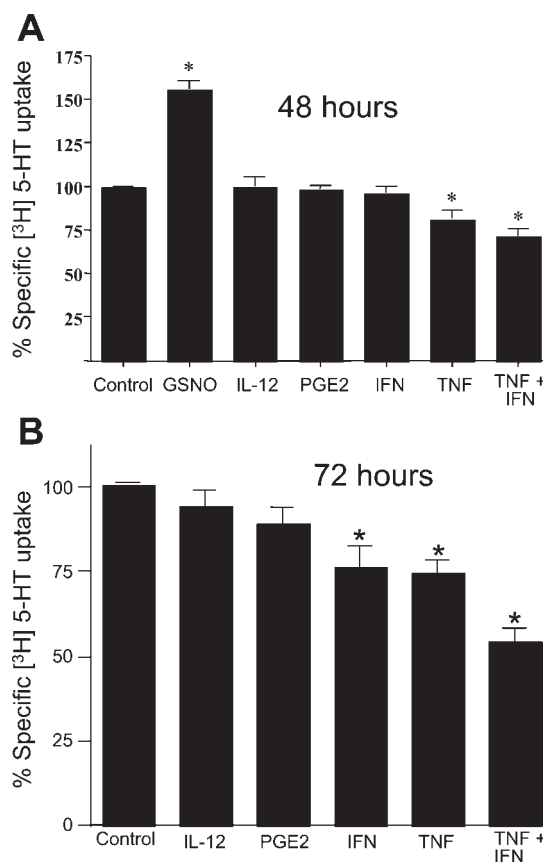


Fig. 2. Screening various proinflammatory agents for effects on specific 5-HT transporter (SERT) uptake. A: after 48 h only TNF- $\alpha$  and a combination of TNF- $\alpha$  and IFN- $\gamma$  caused significant decreases in specific [ $^3\text{H}$ ]5-HT uptake. B: at 72 h TNF- $\alpha$  and IFN- $\gamma$  alone could cause significant decreases in uptake. PGE<sub>2</sub>: 10  $\mu\text{M}$ , IFN- $\gamma$ : 500 ng/ml, TNF- $\alpha$  50 ng/ml, IL-12 50 ng/ml, S-nitrosoglutathione (GSNO): 100  $\mu\text{M}$ . \* $P < 0.05$ .

significant changes at 48 h unless both cytokines were used simultaneously (Fig. 3).

Conditioned media from both LPS-treated lymphocytes and those cocultured with *E. coli* contained IFN- $\gamma$  (nonactivated lymphocyte medium, 3 pg/ml; LPS-treated lymphocyte medium, 80 pg/ml; *E. coli*-cocultured lymphocyte medium, 220 pg/ml). Conditioned media also contained TNF- $\alpha$ . Media from LPS-treated cells contained ~2,300 pg/ml whereas *E. coli*-conditioned medium contained 5,702 pg/ml of TNF- $\alpha$ . After dilution for coculturing with Caco-2 cells, the concentrations of IFN- $\gamma$  and TNF- $\alpha$  in wells treated with *E. coli*-conditioned medium were 15 and 397 pg/ml, respectively.

**Viability experiments.** MTT cytotoxicity assays were used to ensure that TNF- $\alpha$  and IFN- $\gamma$  treatments were not associated with a loss of cells, which could contribute to the decrease in 5-HT uptake described above. As can be seen in Fig. 4, exposure to IFN- $\gamma$  plus TNF- $\alpha$  for 72 h, which is the treatment that caused the most significant reductions in 5-HT uptake, did not affect the survivability of the cells. No significant differences were found between groups ( $P > 0.05$ ; ANOVA). Trypan blue staining was also performed and yielded findings similar to the MTT results. Taken together, data from these experiments indicate that TNF- $\alpha$  and IFN- $\gamma$  do not affect Caco2 cell viability.

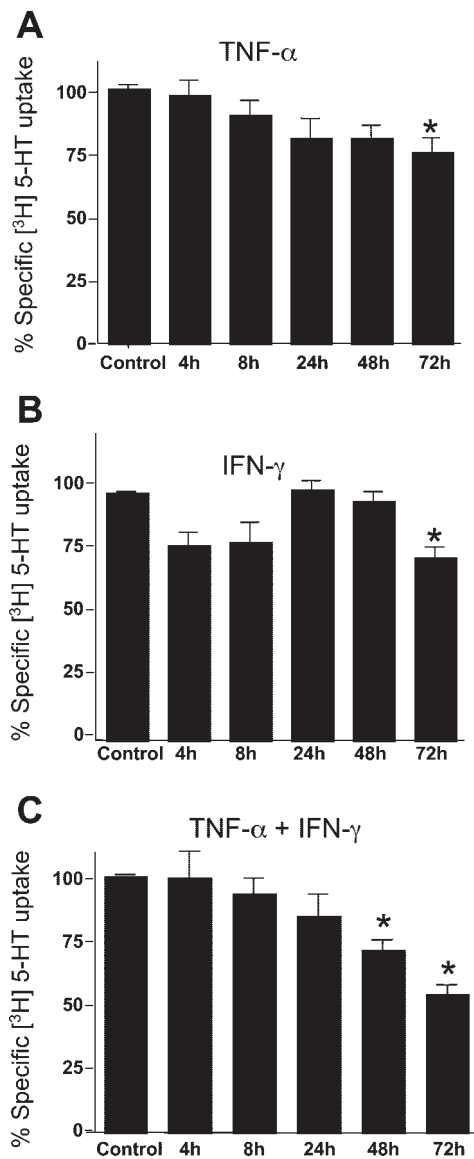


Fig. 3. TNF- $\alpha$  and IFN- $\gamma$  time course experiments. Individually both cytokines caused significant decreases in uptake after 72 h incubation (A and B); when they were combined, significant decreases in uptake were observed after 48 or 72 h (C). Bars represent the mean of at least 3 experiments each performed in triplicate. \* $P < 0.05$

**Changes in SERT mRNA and protein levels.** As described above, treatment of Caco2 cells with the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  resulted in decreased SERT activity (Fig. 2). Additionally, we have previously shown that SERT mRNA is decreased in the colonic mucosa of animal models of intestinal inflammation and of individuals with IBS (7, 22). Therefore, we tested whether treatment of Caco2 cells with these cytokines led to a decrease in SERT mRNA. Using real-time quantitative PCR, we determined the relative expression of SERT mRNA, compared with the housekeeping gene  $\beta$ -actin, after 72 h of cytokine treatment. As can be seen in Fig. 5A, cytokine treatment significantly decreased SERT mRNA levels by  $\sim 40\%$ , compared with transcript levels in untreated cells. To determine whether this cytokine treatment indiscriminately decreased the ex-

pression of other membrane proteins, the mRNA expression of the chloride channel CNCL3 was evaluated. Cytokine treatment did not decrease CNCL3 mRNA levels, demonstrating some degree of specificity for this treatment in reduction of SERT mRNA (Fig. 5B).

Using an antibody for SERT, we investigated the effects of TNF- $\alpha$  and IFN- $\gamma$  on SERT protein expression in Caco2 cells. As can be seen in Fig. 6, incubation of Caco2 cells with a combination of TNF- $\alpha$  and IFN- $\gamma$  caused a visible decrease in SERT protein expression at 48 and 72 h. Densitometric evaluation of immunoblots revealed a  $23.5 \pm 1.8\%$  reduction in SERT at the 72 h time point ( $P < 0.05$ ), with actin serving as a loading control.

## DISCUSSION

The significant morbidity and public health significance of IBD are greatly appreciated; however, the cellular events that lead to the altered motility, secretion, and sensation seen in IBD have not been resolved. Recent findings suggest that serotonergic signaling is altered in IBD, and these alterations in 5-HT release or reuptake could have significant effects on GI function. Animal studies as well as studies in humans have revealed that SERT expression is decreased in the mucosa of inflamed colon (7, 21, 22). Because of its physiological role in modulating peristalsis and visceral sensation in the gut, loss of the SERT could contribute to the altered motility and sensation seen in IBD.

The importance of cytokines in GI inflammation is well documented. However, there have been no studies that directly link cytokine levels to decreases in SERT. The results reported here demonstrate that conditioned medium from activated human lymphocytes contains agent(s) capable of decreasing SERT function and SERT mRNA levels. When incubated with conditioned medium from activated lymphocytes for 72 h, a 40–60% decrease in SERT uptake was observed. We also attempted to determine specifically which agent or agents are capable of decreasing SERT function. Six proinflammatory agents were investigated, and only TNF- $\alpha$  and IFN- $\gamma$  led to a decrease in SERT function. Both TNF- $\alpha$  and IFN- $\gamma$  decreased 5-HT uptake and SERT transcript levels in Caco2 cells. The presence and role of TNF- $\alpha$  in IBD are well known (37). However, less is known about the role IFN- $\gamma$  might play in the cellular mechanism of this disease. Interestingly, the NO donor

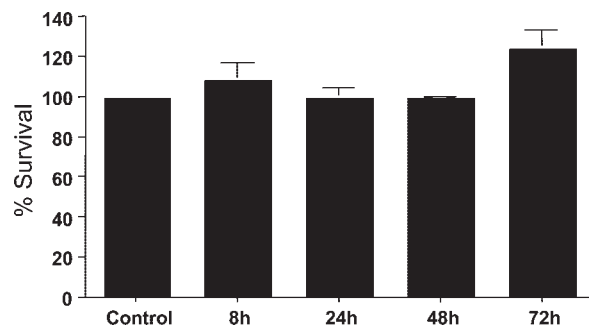


Fig. 4. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay. Combined treatment with IFN- $\gamma$  and TNF- $\alpha$  evoked the greatest change in uptake; this treatment was also investigated for possible cytotoxicity. An MTT viability assay showed no decreases in cell survival when these cytokines were used at the incubation times investigated (no significant differences between these groups).

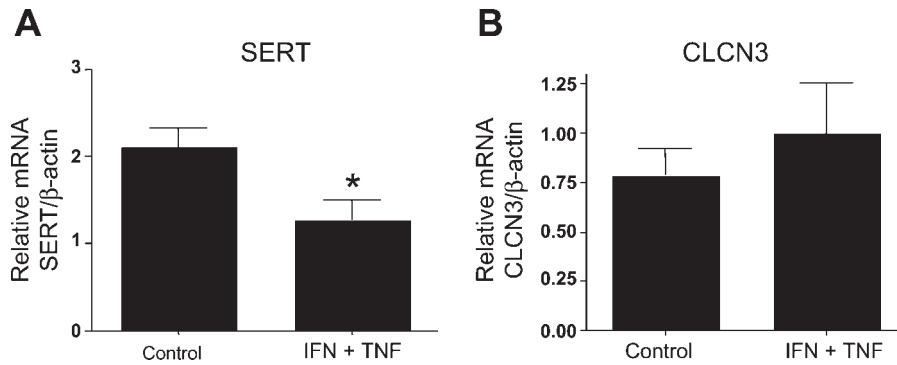


Fig. 5. RT-PCR for SERT. SERT transcript was measured in Caco-2 cells treated with IFN- $\gamma$  and TNF- $\alpha$  for 72 h. Relative quantity of SERT compared with  $\beta$ -actin is shown (A). Graph represents the mean from 8 experiments. \* $P < 0.02$ . For the sake of comparison, mRNA levels for another regulated membrane protein were also measured. RT-PCR for the chloride channel 3 (CLCN3) was performed ( $n = 3$ ). The cytokine incubation had no significant effect on the expression of this protein (B).

GSNO increased SERT uptake. These results are consistent with recent findings from Zhu et al. (41), who showed that another NO donor (hydroxylamine), increased SERT activity in rat basophilic leukemia 2H3 cells. The decrease in 5-HT uptake by Caco2 cells treated with conditioned medium demonstrates the presence of factors that can decrease SERT function. On the basis of the finding that TNF- $\alpha$  and IFN- $\gamma$  can decrease SERT function and mRNA, the conditioned medium was assayed for these two cytokines via an ELISA. Both of these cytokines were detected. The levels of TNF- $\alpha$  in wells treated with *E. coli*-conditioned medium was  $\sim 125$  times lower than the TNF- $\alpha$  concentration used in subsequent experiments. The concentration of IFN- $\gamma$  in the conditioned medium was over 30,000 times lower than the concentration used in subsequent experiments. Despite this, the reductions in uptake were greater after treatment with conditioned medium than after treatment with either cytokine alone or in combination. It is therefore likely that there are additional cytokines or inflammatory agents present in the conditioned medium that work in concert to bring about the greater reduction in SERT activity seen with application of the conditioned medium. However, despite the possible effects of other, unidentified, proinflammatory agents, data reported here indicate that IFN- $\gamma$  or TNF- $\alpha$  alone (72 h) or in combination (48 h) could decrease 5-HT uptake.

In animal models of intestinal inflammation as well as in human patients with IBD, SERT mRNA levels are decreased (7, 21, 22). Treatment of Caco2 cells caused a significant decrease in SERT function, message, and protein levels after 72 h incubation with TNF- $\alpha$  and IFN- $\gamma$ , suggesting that inflammatory cytokines can directly regulate transcription of the SERT gene. To rule out whether this decrease in SERT was the result of general transcriptional decreases in the cell, we also measured transcript for the CLCN3, a highly regulated chloride

transporter protein (8, 20). Transcript levels for CLCN3 were not altered with TNF- $\alpha$  and IFN- $\gamma$  treatment, suggesting the transcriptional regulation of SERT is somewhat specific. Experiments to confirm decreased expression of SERT at the membrane were conducted and a decrease in SERT was seen at 48 and 72 h by Western blotting. The decreases in SERT mRNA likely lead to decreased expression of SERT and subsequent decreases in uptake function. Previous studies using immunohistochemistry to assess SERT expression also showed measurable decreases with inflammation (7, 22, 26, 39).

TNF- $\alpha$  and IFN- $\gamma$  may decrease SERT uptake simply by decreasing SERT mRNA (as our RT-PCR data demonstrate), which leads to less translation of the SERT protein (as seen in Western blots). However, it is also possible that SERT function is modulated via phosphorylation by protein kinase C, as has been shown in other systems (19). The finding that the decrease in SERT function occurred over a period of days, rather than minutes or hours, suggests that transcriptional changes rather than the more acute phosphorylation-induced changes are responsible for decreased SERT function.

This is not the first report that cytokines can regulate SERT function. Zhu et al. (40) recently found that TNF- $\alpha$ -stimulated 5-HT uptake in rat embryonic raphe cells and in mouse synaptosomes. This interesting finding is in contrast to the inhibition we observed in Caco2 cells. The fact that Zhu et al. used cells of neuronal origin and much shorter incubation times ( $\leq 60$  min) may explain this difference. These results suggest that regulation of neuronal SERT expression and function differs from regulation of SERT in intestinal epithelium.

In conclusion, the findings reported here support the concept that inflammation can lead to decreases in mucosal SERT expression and function. Furthermore, the data reported here demonstrate that TNF- $\alpha$  and IFN- $\gamma$  are primary candidates for mediating this effect. The ability of both TNF- $\alpha$  and IFN- $\gamma$  to decrease 5-HT uptake capacity could have profound implications on GI function. Loss of SERT would be expected to cause increases in 5-HT in the lamina propria, leading to enhanced stimulation, and possibly desensitization, of neuronal 5-HT receptors located on the processes of intrinsic and extrinsic primary afferent neurons. These related changes in 5-HT signaling changes could alter gut sensation, secretion, and motility. Restoring SERT function in the inflamed gut could have therapeutic potential for colitis patients if secretion, motility, and sensation could be normalized. Understanding which inflammatory mediators can cause lowered SERT function and expression would be a necessary step for developing novel

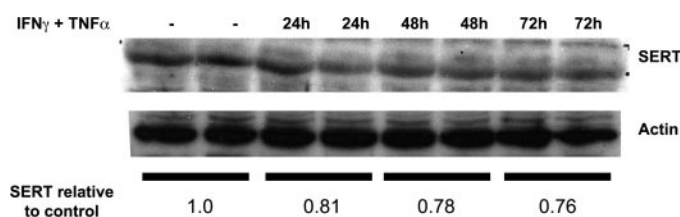


Fig. 6. SERT protein expression. SERT expression was evaluated by Western blotting. Caco-2 cells were treated with IFN- $\gamma$  and TNF- $\alpha$ . Expression of  $\beta$ -actin was used as a loading control. Western blots were performed 3 times; the blot shown is representative. Densitometric evaluation was used to calculate fold changes relative to control values.

approaches aimed at restoring normal 5-HT signaling in the inflamed gut.

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