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Toll-like Receptor 3 Activation Affects Serotonin Transporter Activity and Expression in Human Enterocyte-like Caco-2 Cells

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Key Words

TLR3 • 5-HT • Serotonin transporter • Intestine • Caco-2 cells

Abstract

Serotonin, a neurotransmitter/autocrine agent mainly synthesized by intestinal enterochromaffin cells, regulates the whole intestinal physiology. Toll-like receptor 3 (TLR3) also contributes to the intestinal physiology by modulating intestinal innate immunity responses. Both serotonin and TLR3 are involved in intestinal inflammatory processes; however, the role of TLR3 in the regulation of intestinal 5-HT availability remains unexplored. The present study analyzes the effect of TLR3 activation on serotonin transporter (SERT) activity in Caco-2 cells. Treatment with poly(I:C), dsRNA synthetic analogue and TLR3 ligand, was assayed and SERT activity determined by 5-HT uptake and transepithelial flux. SERT expression was analyzed by qRT-PCR and western blotting. Poly(I:C) short-term treatment inhibited SERT activity in the apical and basal membrane of epithelial cells and diminished SERT protein content in the membrane. SERT total protein and mRNA levels were not affected by poly(I:C), suggesting a post-translational alteration of SERT. The poly(I:C) effect on SERT activity did not appear to be mediated by PKC, cAMP, PKR or JNK signaling pathways; however, the p38 MAPK pathway seemed to be involved. Our results demonstrate that TLR3 inhibits SERT activity, which may increase 5-HT extracellular levels and contribute to the inflammatory response; however, 5-HT treatment did not affect TLR3 expression.

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Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an important intestinal neurotransmitter/autocrine agent synthesized and released from enterochromaffin cells. This molecule has been shown to regulate the complete intestinal physiology by modulating the absorption of nutrients [1], intestinal motility [2] and intestinal secretion of ions and water [3]. The effects of 5-HT depend on its extracellular availability, which is, in part, determined by the serotonin transporter (SERT), a membrane protein expressed in the apical and basal membranes of the enterocytes, which reuptakes 5-HT with high affinity ($K_t = 0.2\text{--}0.6\ \mu\text{M}$).

It has also been suggested that 5-HT is involved in the pathogenesis of chronic gastrointestinal pathologies such as IBD (Inflammatory Bowel Disease) [4–6] and intestinal alterations such as Irritable Bowel Syndrome (IBS) [7, 8]. Recent results have demonstrated that SERT activity is impaired in intestinal epithelial cells treated with inflammatory factors [9–11] and in animal models and human forms of intestinal inflammation [4, 12–15]. Moreover, several studies have demonstrated that 5-HT activates the immune cells, producing pro-inflammatory mediators [16], and recent results support the concept that 5-HT is a pro-inflammatory mediator in the gut [17].

Toll-like receptors (TLRs) are innate receptors that recognize and induce inflammatory responses to bacterial products. TLRs are mainly expressed in the intestinal mucosa, in underlying hematopoietic and epithelial cells [18, 19], and they contribute to intestinal homeostasis by promoting mucosal integrity and tissue repair [20, 21].

Virally derived nucleic acids are also recognized by TLRs, and they act as potent modulators of the intestinal innate immunity. Viral-derived double-stranded RNA (dsRNA) induces interferon (IFN) and inflammatory responses by activating TLR3 [22, 23]. However, TLR3 activation has also been shown to protect against colitis in animal models [24, 25]. TLR3 seems to be abundantly expressed in the normal human small intestine and colon [19] and, although numerous studies have already elucidated the signal transduction of virus-sensing TLRs and how they regulate the antiviral inflammatory response, fewer studies have focused on their effects on intestinal epithelial cell activity.

TLR3 activity has been shown to be involved in the inflammatory responses in the intestine, and 5-HT has been described as playing an important role in intestinal physiology and/or inflammatory responses. However, the interaction between TLR3 and SERT activity is unknown. The aim of this work was to determine whether TLR3 activation, mediated by the synthetic dsRNA polyinosinic-polycytidylic acid [poly(I:C)], affects the activity and expression of SERT in human intestinal epithelial cells. The Caco-2/TC7 cell line has been used as it has been described as constitutively expressing SERT [26], and results obtained in our laboratory have shown that these cells are an excellent model for the study of intestinal SERT regulation [27,28].

Materials and Methods

Reagents and antibodies

The following drugs and substances were used (abbreviations and suppliers in parentheses): Serotonin (5-HT), dibutyryl-cAMP (d-cAMP), (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT 5720), Bisindolylmaleimide IV (BIS IV), 5-(2-Aminopyrimidin-4-yl)-4-(4-fluorophenyl)-1-(4-piperidinyl) imidazole trihydrochloride (SB 220025) and specific primers from Sigma-Aldrich (St. Louis, MO). Polyinosinic-polycytidylic acid [poly(I:C)] and 2-aminopurine were supplied by InvivoGen (San Diego, CA). Anthra[1-9-cd]pyrazol-6(2H)-one (SP 600125), was procured from Tocris Bioscience (Bristol, UK). [^3H]-5-HT (specific activity 28 Ci/mmol) was from Perkin-Elmer (Boston, MA). Mouse monoclonal antibody anti-human TLR3 was supplied by AbD serotec (MCA2267, Dusseldorf, Germany), mouse monoclonal antibody anti-human SERT was supplied by Abcam (Ab1125, Cambridge, UK) and rabbit polyclonal anti-human actin antibody (SC-1616r) and secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA). All generic reagents were purchased from Sigma-Aldrich and

Roche Applied Sciences (Sant Cugat del Vallés, Barcelona, Spain).

Cell line and cell culture

Caco-2/TC7 cells [29] were cultured at 37°C in an atmosphere of 5% CO₂ and maintained in high glucose DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids and 20% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA). The cells were passaged enzymatically (0.25% trypsin – 1 mM EDTA) and sub-cultured in 25 or 75 cm² plastic culture flasks (Sarstedt, Nuembrecht, Germany). The medium was changed 48 h after seeding and daily thereafter. The cells were always used between passages 19 and 35. For 5-HT uptake assays, cells were seeded in 24-well plates or in 12-well permeable polyester (PET) membranes (0.4 µm porous size and 1 cm² growth area; Millipore, Billerica, MA), at a density of 4x10⁴ cells per well. Uptake measurements were carried out 14 days after seeding (9 days after confluence). Previous results have shown that Caco-2/TC7 cells express SERT and its activity reaches a plateau on the fifth day after confluence [26]. In the experiments, the cell medium was free of FBS 24 h before using the cells. The double-stranded RNA synthetic analogue poly(I:C) and the different modifiers were added to the culture medium at different concentrations and periods. Preliminary assessment of the epithelium morphology was carried out in the cell culture under the different experimental conditions. The results showed that none of the assayed treatments seemed to affect cell culture morphology, proliferation and monolayer integrity (data not shown).

5-HT uptake studies

Uptake measurements were performed on cells attached to 24-well plates, as previously described [26], either under control or after different experimental conditions. The transport medium composition in mM was as follows: 137 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 10 HEPES pH 7.4, 4 Glutamine, 0.1% BSA, and both 0.2 µM 5-HT and [³H]-5-HT (1.5 µCi/ml). Before measuring uptake, cells were pre-incubated at 37°C in an atmosphere of 5% CO₂ with a substrate-free transport medium for 30 min. The cells were immediately washed with a substrate-free transport medium at 37°C and then incubated with a transport medium at 37°C for 6 min. Transport was stopped by removing the transport medium and washing the cells twice with an ice-cold substrate-free transport medium containing 20 µM 5-HT. The cells were solubilized in 0.1 M NaOH, and samples were taken for radioactivity counting (Wallac Liquid Scintillation Counter, Perkin-Elmer). Protein was measured using the Bradford method (Bio-Rad, Hercules, CA), with BSA as standard. Results were calculated in pmol 5-HT/mg protein and were expressed as a percentage of the control value (100%). In the experiments in which 5-HT fluxes were measured, Caco-2/TC7 cells were seeded in 12-well permeable polyester (PET) membranes. These inserts establish apical (A) and basal (B) compartments. Bidirectional fluxes, apical to basal (A–B) and basal to apical (B–A), were measured at intervals of 10 min during 1 h, after adding 0.1 µM 5-HT plus [³H]-5-HT (2.5 µCi/ml) either to the apical or basal compartment, respectively. The results were calculated in pmol 5-HT/10 min and were expressed as a percentage of the control (100%). Cell monolayer integrity and confluence were checked by measuring transepithelial resistance (TER) with an Epithelial VoltOhmmeter (Millicell Electrical resistance system, Millipore) before the start of each experiment.

RNA extraction, RT-PCR and real-time quantitative RT-PCR

RNA extractions were carried out with the RNeasy mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions and as previously described [27]. Total RNA was extracted from cells cultured in 25 cm² flasks, 14 days after seeding and under different experimental conditions. The extracted RNA (1 µg) was used as a template for first-strand cDNA synthesis using Oligo(dT) primers and a modified M-MLV reverse transcriptase (Life Technologies). Negative amplification control and lack of DNA contamination in the RNA preparation were verified by PCR amplification in the absence of reverse transcriptase. One tenth of the resultant cDNA was used for PCR amplification of human TLR3, using the follow specific primers [30]: TLR3 sense (5'-CGGGCCAGCTTTCAGGAACCTG-3') and TLR3 antisense (5'-GGCATGAATTATATATGCTGC-3'). The PCR amplification was carried out after 40 cycles under the following conditions: 94°C for 2 min; 55°C for 30 s; 72°C for 1 min, being the amplicon size 400 bp.

The relative abundance of SERT mRNA in control and poly(I:C)-treated cells was quantitated by real-time RT-PCR. cDNAs obtained by reverse transcription were used to determine SERT mRNA expression levels. Reactions were run using the StepOne Plus Real-Time PCR System (Life Technologies) and hSERT Gene Expression Assay from Applied Biosystems (Life Technologies, Assay number Hs00169010_m1

[SLC6A4]) following the manufacturer's conditions. Each sample was run in triplicate, and the mean Ct was determined. Relative SERT mRNA expression under each experimental condition (control or treatment) was expressed as $\Delta Ct = Ct_{\text{SERT}} - Ct_{\text{calibrator}}$, being hGAPDH (Assay number Hs99999905_m1) and hHPRT1 (Assay number Hs99999909_m1) housekeeping genes expression used as calibrators. Then relative SERT mRNA expression was calculated as $\Delta\Delta Ct = \Delta Ct_{\text{control}} - \Delta Ct_{\text{treatment}}$. Finally, the relative gene expression levels were converted and expressed as fold difference ($2^{-\Delta\Delta Ct}$).

Preparation of the brush border enriched fraction and western blotting

Caco-2/TC7 cells were cultured in 75 cm² flasks and used 14 days after seeding. The preparation of the intestinal brush border membrane enriched fraction followed the procedure previously described [28]. The cells were washed twice with PBS and immediately resuspended with a cold Tris-manitol buffer (Tris 2 mM, Manitol 50 mM, pH 7.1) containing protease inhibitors and 0.02% sodium azide. The suspension was homogenized, and the cells were disrupted by sonication (fifteen 1-s bursts, 60W). One sample was taken from the lysate for total protein analysis and protein quantification. CaCl₂ (final concentration 20 mM) was added to the cell lysate and, after standing for 10 min in ice, the mixture was centrifuged for 10 min at 950 g. The supernatant was taken and centrifuged at 33500 g for 30 min. The pellet (brush border-enriched fraction) was resuspended in phosphate buffer (KH₂PO₄ / K₂HPO₄ 10 mM pH 6.8) and a sample was taken for protein analysis. Protein was measured using the Bradford method (Bio-Rad).

Brush border-enriched samples and cell lysate (total protein 60 µg) from Caco-2/TC7 cells were electrophoresed in 9% SDS-PAGE gels and then transferred to PVDF membranes (Millipore) by electroblotting. The membranes were blocked with 5% non-fat dried milk plus 1% BSA and probed with mouse monoclonal anti-human TLR3 1:250 or mouse monoclonal anti-human SERT 1:500. The primary antibody was detected using a secondary goat anti-mouse Ig coupled to horseradish peroxidase and the ECL Plus detection kit (GE Healthcare, Buckinghamshire, UK) and was visualized with X-ray film (Hyperfilm MP, GE Healthcare). The blots were reprobed, after stripping, with a rabbit polyclonal anti-human actin antibody to determine differences in the sample load. The SERT/β-actin protein ratio was calculated in densitometric units from the film with Quantity One Analysis Software (Bio-Rad) and the results were expressed as a percentage of the control values (100%).

Statistical analysis

All results are expressed as means ± the standard error of the mean (SEM). Statistical comparisons were performed by one-way ANOVA followed by the Bonferroni post-test with a confidence interval of 95% (p<0.05). Statistical analysis was accomplished with the computer-assisted Prism GraphPad Program (Prism version 4.0, GraphPad Software, San Diego, CA).

Results

Expression of TLR3 in Caco-2/TC7 cells and the effect of poly(I:C) treatment on 5-HT uptake

The expression of TLR3 in Caco-2 cells has already been described [31, 32]. However, the present study used the Caco-2/TC7 clone and the demonstration of TLR3 expression in these cells was considered essential. We therefore determined both TLR3 mRNA and protein expression in these cells by RT-PCR and western blotting respectively, under conditions described in the "Materials and Methods" section. The results obtained show that a 400 bp sequence was amplified by PCR, and a 117 kDa band was immunodetected in Caco-2/TC7 cell homogenates, both corresponding with the expected TLR3 mRNA and protein sizes (Fig. 1A, B, respectively).

The effect of TLR3 activation by poly(I:C) on SERT activity in Caco-2/TC7 cells was analyzed. The cells were treated with two concentrations of poly(I:C) (1 or 5 µg/ml), according to previous studies [31, 33], for short (6 min and 30 min) or long (1 day) periods, and the 5-HT uptake was measured. The results showed that poly(I:C) significantly diminished 5-HT uptake with short-term treatment, although none of the poly(I:C) concentrations affected 5-HT uptake after 1 day of treatment (Fig. 2).

In addition, 5-HT fluxes were measured in Caco-2/TC7 cells treated with poly(I:C) 5

Fig. 1. TLR3 mRNA and protein expression in Caco-2/TC7 cells. (A) TLR3 mRNA expression was determined by RT-PCR. The PCR amplification product was obtained with primers and conditions as detailed in the “Materials and Methods” section. A band of 400 bp corresponding to TLR3 is shown. (B) TLR3 protein expression was assessed by western blotting in homogenate of Caco-2/TC7 cells, and rat intestinal mucosa was used as a control. The detection of a 117 kDa band is shown.

Fig. 2. Effect of the concentration and period of treatment of poly(I:C) on 5-HT uptake. Uptake was measured after 6 min incubation of 0.2 µM 5-HT. Two concentrations of poly(I:C) were assayed (1 and 5 µg/ml). The treatment periods were 6 or 30 min (short term) or 1 day (long term). The results are expressed as the percentage of the uptake control (100%), and are the mean ± SEM of 5 independent experiments. Absolute control values were 12.04 ± 0.60, 11.32 ± 0.17 and 10.87 ± 0.34 pmol 5-HT/mg prot at 6 min, 30 min or 1 day respectively. ***P<0.001 compared with the control (untreated cells). ###P<0.001 compared with poly(I:C) 1 µg/ml.

Fig. 3. Effect of poly(I:C) on 5-HT transepithelial fluxes. Poly(I:C) 5 µg/ml was added to the apical or basal side for 30 min and 5-HT apical-to-basal (A-B) or basal-to-apical (B-A) fluxes were measured, respectively. The control condition corresponds to untreated cells. 5-HT concentration was 0.1 µM, and samples were taken every 10 min. The results are expressed as a percentage of the control (100%) and are the mean ± SEM of 4 independent experiments. Absolute control values were 0.63 ± 0.03 and 0.15 ± 0.01 pmol 5-HT/10 min in A-B and B-A fluxes respectively. ***P<0.001 compared with the control value.

µg/ml for 30 min. Poly(I:C) was added to the apical (A) or basal (B) side, and apical to basal (A-B) or basal to apical (B-A) 5-HT fluxes were measured. The results showed that both A-B and B-A fluxes were significantly diminished by poly(I:C) treatment (Fig. 3). Transepithelial resistance (TER) was also determined after TLR3 activation and the results showed that TER was not significantly affected by apical or basal poly(I:C) (in Ω/cm²; control 396.50 ± 8.50; apical poly(I:C) 391.67 ± 7.17; basal poly(I:C) 377.50 ± 4.50. The results are the mean ± SEM of 4 independent experiments).

The effect of poly(I:C) on the SERT mRNA and protein expression levels

The above results show that poly(I:C) treatment inhibits SERT activity. To gain an in-depth knowledge of the molecular effects of poly(I:C) on SERT, mRNA and protein levels of SERT were measured in Caco-2/TC7 cells treated for 30 min with poly(I:C) 1 or 5 µg/ml. The mRNA level was determined by real-time quantitative RT-PCR and the results showed that

Fig. 4. Effect of poly(I:C) on SERT mRNA and protein expression levels. (A) Real-time quantitative RT-PCR analysis of SERT mRNA expression level in cells treated for 30 min with poly(I:C) 1 or 5 $\mu\text{g}/\text{ml}$. Relative quantification was performed using comparative Ct ($2^{-\Delta\Delta\text{Ct}}$). (B) Immunodetection of SERT by western blot in cell lysate and apical membrane from Caco-2 cells treated with poly(I:C) 1 or 5 $\mu\text{g}/\text{ml}$ for 30 min. (C) Quantitation of SERT protein in both cell lysate and apical membrane using β -actin as an internal control of the protein load (SERT/ β -actin ratio). The results are expressed as a percentage of the control value and are the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$ compared with the control value.

poly(I:C) treatment did not significantly alter the level of mRNA (Fig. 4A). SERT protein was also measured in cells under poly(I:C) treatment by western blotting and the results showed that the SERT protein level in cell homogenate was not affected by poly(I:C) treatment; however, the expression of SERT protein in the apical membrane of the cells was significantly reduced (Fig. 4B, C).

Analysis of signaling pathways involved in the poly(I:C) effect on SERT

In order to identify the pathways which may mediate the poly(I:C) effects on SERT, the role of the signaling pathways mediated by cAMP-protein kinase A (PKA), protein kinase C (PKC), the double-stranded RNA(dsRNA)-dependent protein kinase (PKR), c-Jun N-terminal kinase (JNK) and p38 MAPK were assessed in cells treated with poly(I:C) (5 $\mu\text{g}/\text{ml}$).

First, the role of cAMP/PKA and PKC, which have been described to be involved in the regulation of SERT activity and expression [26], was considered. Uptake of 5-HT was measured in Caco-2/TC7 cells treated with poly(I:C) for 30 min and either d-cAMP (1 mM), a nonmetabolizable analogue of cAMP, or PMA (1 μM), an activator of PKC. Cells were also treated with either KT 5720 (1 μM) or BIS IV (2 μM), inhibitors of PKA and PKC respectively; this treatment began 30 min before the addition of poly(I:C). The results showed that the poly(I:C) inhibitory effect was additive to the inhibition yielded by cAMP or PKC on SERT activity, and that KT 5720 and BIS IV did not reverse the poly(I:C) effect (Fig. 5A, B, respectively).

Since PKR has been described as a protein with ds-RNA-dependent kinase activity, which plays a critical role in the antiviral defense mechanism of the host [34], the mediation of PKR on poly(I:C) effects on SERT was determined. The cells were treated with poly(I:C) and/or 2-aminopurine, an inhibitor of PKR, at two concentrations (0.1 and 1 μM). The results showed that the effect of poly(I:C) was not reversed by 2-aminopurine. In addition, 2-aminopurine 0.1 μM did not affect SERT activity, but at 1 μM it was shown to significantly

Fig. 5. Intracellular mechanisms involved in poly(I:C) effect on SERT activity. Cells were treated for 30 min with poly(I:C) 5 μ g/ml and/or the different modifiers, and compared with untreated cells (control). Uptake of 5-HT was measured after 6 min of incubation and 5-HT concentration was 0.2 μ M. (A) Effect of cAMP/PKA pathway. Cells were treated with poly(I:C), d-cAMP 1 mM and/or KT 5720 1 μ M. Control absolute value was 10.26 ± 0.47 pmol 5-HT/mg prot. (B) Effect of PKC pathway. Cells were treated with poly(I:C), PMA 1 μ M and/or BIS IV 2 μ M. Absolute control value was 9.17 ± 0.08 pmol 5-HT/mg prot. (C) Effect of PKR. Cells were treated with poly(I:C) and/or 2-aminopurine 0.1 or 1 μ M. Absolute control value was 9.57 ± 0.31 pmol 5-HT/mg prot. (D) Effect of JNK pathway. Cells were treated with poly(I:C) and/or SP 600125 1 μ M. Absolute control value was 12.55 ± 1.58 pmol 5-HT/mg prot. (E) Effect of p38 MAPK pathway. Cells were treated with poly(I:C) and/or SB 220025 0.1 or 1 μ M. Absolute control value was 11.13 ± 1.08 pmol 5-HT/mg prot. The results are expressed as the percentage of the uptake control, and are the mean $\pm$ SEM of 5 independent experiments. *** $P < 0.001$ and ** $P < 0.01$ compared with the control; ### $P < 0.001$ and ## $P < 0.01$ compared with poly(I:C) treatment.

inhibit 5-HT uptake (Fig. 5C).

The involvement of mitogen-activated protein kinases (MAPKs) in TLR3 signaling pathways is well known. JNK and p38 MAPK have been described as mediators of TLR3 activity [23], and the role of both of these was considered. In the case of JNK mediation, the cells were treated with poly(I:C) and/or SP 600125 (1 μ M), a selective inhibitor of JNK. The

Fig. 6. Effect of 5-HT on TLR3 protein expression. Experimental conditions were: C, untreated cells; 5-HT, cells treated with 5-HT 0.1 mM for four days. (A) Expression of TLR3 by electrophoresis and immunoblotting. (B) Quantitation of TLR3 protein in cell lysate using β -actin as an internal control of protein load (TLR3/ β -actin ratio). The results are expressed as a percentage of the control value and are the mean $\pm$ SEM of 3 independent experiments.

results showed that SP 600125 did not reverse the effect of poly(I:C); moreover, it inhibited SERT activity on its own, and this effect was additive to the poly(I:C) inhibition (Fig. 5D). In order to assess the role of p38 MAPK in the poly(I:C) effect on SERT, the cells were treated with poly(I:C) and/or SB 220025, a specific p38 MAPK inhibitor. The results showed that poly(I:C) inhibition of SERT activity was reversed by SB 220025 either at 0.1 or 1 μ M, and SB 220025 alone did not affect SERT activity (Fig. 5E).

5-HT feedback regulation of TLR3 expression

From the results obtained in this study, it can be concluded that TLR3 activation by poly(I:C) inhibits SERT activity, and, as a consequence, it may induce an increase of 5-HT availability in the intestinal epithelium. A number of studies have demonstrated that 5-HT availability is increased in chronic intestinal inflammatory processes [4, 5], and 5-HT has been described as modulating the intestinal physiology, but the question of whether TLR3 expression is regulated by a chronic increase of 5-HT has not, until now, been answered. Caco-2/TC7 cells were treated with 5-HT at a high concentration (0.1 mM) for 4 days and the TLR3 protein expression was measured by western blotting. The results showed that 5-HT treatment did not affect TLR3 expression (Fig. 6).

Discussion

In the present study, TLR3 mRNA and protein expression in Caco-2/TC7 cells has been demonstrated. Analysis of the TLR3 effects on SERT was carried out by poly(I:C) treatment of the cells, and the results showed that short-term TLR3 activation reduced SERT activity in both apical and basal membranes. TLR3 activation with a high concentration of poly(I:C) (100 and 25 μ g/ml) has been reported to induce apoptosis and cytotoxicity [32, 35]; however, in the present study, the Caco-2 cell culture maintained the monolayer morphology (data not shown) and epithelial integrity (TER measurement) after poly(I:C) treatment, indicating that the inhibitory effect is not due to cellular alteration.

The effect of long-term treatment of the cells with poly(I:C) was also assessed, and, under these conditions, SERT activity did not show to be affected. This result might be due to the activation of mechanisms that are involved in the control of TLRs overactivation after long-term treatment with poly(I:C) [36].

SERT expression analysis demonstrated that neither SERT mRNA nor protein levels in cell lysate were affected by poly(I:C) treatment. However, SERT protein content in the apical membrane was significantly reduced, suggesting a post-translational modification of SERT that might affect the intracellular trafficking of the transporter. This kind of inhibition has recently been described in relation to the effect of TLR4 activation on SERT in intestinal epithelial cells [37].

Different transduction modules have been identified in the TLR3 triggered signaling pathway. In order to determine the intracellular response that mediates the TLR3 effect on SERT, different signaling pathways were assessed. cAMP and PKC have been described as regulating SERT activity and expression [26, 27]; in addition, an IFN-independent pathway

involving PKC has been described as being responsible for poly(I:C) effects [35]. Therefore, PKC and cAMP involvement were analyzed, and the results showed that neither PKC nor cAMP mediated the TLR3 effect.

Another intermediary component in the dsRNA/TLR3 signaling cascade is PKR [38, 39], which has been described as acting upstream on several proteins such as TRAF6 and MAPKs [40]. Since PKR might be involved in the poly(I:C) effect on SERT, this possibility was considered. The results showed that PKR did not appear to be involved in the poly(I:C) inhibitory effect.

It has been demonstrated that one of the TLR3 signal transduction pathways leads to the transcription factor AP-1, which is controlled by MAPKs. Moreover, previous results have shown that poly(I:C) induces activation of JNK and p38 MAPK [23]. Therefore, the involvement of JNK and/or p38 MAPK in the poly(I:C) effect on SERT was assessed. With JNK, the results showed that the poly(I:C) effect was not reversed by SP 600125, a specific JNK inhibitor. SP 600125 alone significantly diminished SERT activity, being additive to the poly(I:C) inhibition. These results not only rule out the mediation of JNK in the poly(I:C) inhibitory effect, but also suggest a possible JNK stimulatory effect on SERT activity. With regard to the p38 MAPK mediation of poly(I:C) effects, the results showed that SB 220025, a specific inhibitor of p38 MAPK, reversed the poly(I:C) effect on SERT. Consequently, p38 MAPK appears to be involved in the inhibitory effect of poly(I:C) on SERT. Multiple pathways have been described as mediating viral dsRNA cellular responses [41, 42]; therefore the involvement of other recognition pathways in poly(I:C) effect on SERT cannot be discarded.

Several studies have analyzed the role of p38 MAPK in SERT regulation, and their conclusions have been inconsistent: a study carried out in platelets and in transfected cells showed that p38 MAPK agonists yielded an increase in SERT activity independent of SERT intracellular traffic [43]. These results contradict, in part, those of the present work, although it should be recognized that the cells studied and the mechanisms triggering the signaling pathway were different. Moreover, a different study concluded that p38 MAPK effects on SERT activity depend on cellular phenotypes and SERT coding variants [44].

Numerous investigations have demonstrated that pro-inflammatory factors induce SERT inhibition [9, 10]. Moreover, the results of this study show that TLR3 activation inhibits SERT activity. SERT removes 5-HT from the intestinal extracellular compartment and, as such, the inhibition of SERT activity yielded by intestinal inflammation and TLR3 may induce an increase of the 5-HT availability, which might alter the expression of TLR3 by feedback regulation. To clarify this point, TLR3 protein expression was measured in Caco-2/TC7 cells treated with 5-HT at high concentration and for a long-term period. The results indicated that 5-HT did not alter TLR3 expression; therefore, 5-HT did not seem to induce feedback regulation of TLR3 expression.

To summarize, our study provides, for the first time, evidence that TLR3 activation modulates SERT activity and expression in intestinal epithelial cells, and suggests p38 MAPK as the signaling pathway involved. Since 5-HT has been shown to regulate intestinal motility, secretion and immune responses, the transient increase of extracellular 5-HT induced by TLR3 activation might contribute to intestinal defense against viral damage by flushing viral particles from the gastrointestinal tract and by activating intestinal immune pathways.

The results of the current work may be useful for clarifying the serotonergic response in intestinal inflammation and may also contribute to our knowledge and understanding of the regulation of the intestinal serotonergic system under physiological and pathological conditions.

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