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Short communication

Cholinergic regulation on polymeric immunoglobulin receptor expression in Caco-2 cells

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ABSTRACT

The polymeric immunoglobulin receptor (pIgR) mediates transcytosis of IgA, a pivotal anti-inflammatory player of the mucosal immune system. Transcytosis mediated by pIgR entails protein effectors of vesicle-mediated transport involved in signal pathway activation that lead to the sorting of pIgR-IgA complexes from the basolateral to apical membrane. Each step of pIgR transport encompasses multiple targets for regulation, but the role of cholinergic system components, *i.e.* acetylcholine (ACh), the ligand of nicotinic (nAChR) and muscarinic (mAChR) receptors, is unclear. This study evaluated the effect of the cholinergic system on pIgR at transcriptional and protein levels. Accordingly, lipopolysaccharide (LPS)-primed Caco-2 cells were treated with nicotine (nAChR agonist) and/or mecamylamine (nAChR antagonist) or with muscarine (mAChR agonist) and/or atropine (mAChR antagonist), and then pIgR was analysed *in situ* by immunofluorescence and by RT-qPCR. In general terms, cholinergic antagonists counteracted the upmodulatory outcome of both cholinergic agonists on both pIgR cellular location and mRNA levels. These findings suggest that the cholinergic system plays a key role in the regulation of epithelial immunity by modulating pIgR expression. The study provides insights into the interaction between the cholinergic system and intestinal immune mechanisms for future research in mucosal immunity and possible therapeutic strategies.

Keywords: polymeric Ig receptor, nicotine/mecamylamine, nicotinic-receptors, muscarinic-receptors, Caco-2 cells

INTRODUCTION

Polymeric immunoglobulin receptor (pIgR) is a heavily-glycosylated type I transmembrane protein (~83 kDa) composed of an N-terminal extracellular domain and a C-terminal cytoplasmic tail connected by a single transmembrane helix (1). The prime function of pIgR is the transport of dimeric or polymeric immunoglobulin A (dIgA) from the basolateral to apical surface, known as transcytosis (2). The latter entails the i) endocytosis of the pIgR-dIgA complex through clathrin-dependent coated vesicles and their delivery in basolateral

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early endosomes, ii) translocation to a tubulo-vesicular compartment that underlies the centre of the apical membrane known as apical recycling endosome (ARE), and iii) their delivery to the apical plasma membrane and the proteolytic cleaving of pIgR joined to dIgA, resulting in a proteolytic product known as secretory component that is released along with dIgA in the luminal environment to generate the secretory IgA (sIgA) (3).

A presumable signal pathway that accounts for pIgR-IgA transcytosis assumes that pIgR-IgA interaction favours the activation of Yes, a SRC family tyrosine kinase, that in turn phosphorylates the epidermal growth factor receptor (EGFR), leading to phosphorylation of ERK kinase, and resulting in Yes→EGFR→ERK signalling. The latter drives FIP5 activation, a Rab-11-interacting protein located in ARE, resulting in a YES-EGFR-ERK-FIP5 signal pathway (4).

The role of the cholinergic system in pIgR-IgA transcytosis is not fully clear. The cholinergic system operates through the prime agonist acetylcholine (ACh), synthesised by choline acetyltransferase (ChAT), and by the seven transmembrane-muscarinic receptors and pentameric ligand-gated channel nicotinic receptors. Muscarinic receptors (mAChRs) are activated by the agonist muscarine and act as G-protein coupled receptors of metabotropic type with a single protein chain with N-extracellular- and C-intracellular domains that display seven transmembrane portions. The nicotinic receptors (nAChRs) are sensitive to their agonist nicotine and are ligand-gated ionotropic receptors arranged in five subunits encircled by a central ion-conducting protein pore (5).

Experimental settings showed that the non-selective cholinergic agonist carbachol upmodulated the pIgR mRNA levels in cell cultures of rat parotid acinar cells, human submandibular cells and human colorectal adenocarcinoma (HT-29) cell line (6). Assays conducted in perfused porcine ileum showed that the IgA secretion was increased in electrically stimulated conditions with atropine (a competitive mAChR antagonist) co-infused with phentolamine (a competitive non-selective α-adrenergic receptor antagonist); the stimulatory effect of both antagonists upon the IgA secretion was abrogated by hexamethonium (a non-selective nAChR antagonist) (7). In murine experimental settings in whole small intestine samples, pIgR mRNA expression was down-modulated by both GTS-21 (a selective α7nAChR agonist) and by mecamylamine (non-selective nAChR antagonist) (8).

Accordingly, the impact of the cholinergic system on pIgR-IgA transport addressed in the aforementioned models is not fully clear. Therefore, *in vitro* culture assays in Caco-2 cells (derived from human colorectal adenocarcinoma cells) provide more controlled environmental conditions to address functional and molecular mechanisms underlying the pIgR-IgA transcytosis pathway (9–11). Thus, the study was focused on addressing the outcome of mAChR and nAChR receptors on pIgR protein localisation and its mRNA expression in *in vitro* cultures of Caco-2 cells.

EXPERIMENTAL

Reagents and dosages

Nicotine (nAChR agonist; 50 μmol L^{-1}; Sigma-Aldrich, N3876), mecamylamine (MLA, nAChR antagonist; 500 μmol L^{-1}; Sigma-Aldrich, M9020) (12), muscarine (mAChR agonist; 100 μmol L^{-1} ; Sigma-Aldrich, M6532) (13), and atropine (mAChR antagonist; 500 μmol L^{-1}; Sigma-Aldrich, A0132) (14, 15) were used for receptor modulation. Lipopolysaccharide (LPS)

from *Escherichia coli* O127:B8 (LPS; 1 μg mL^{-1}; Sigma-Aldrich, L3129) was applied 3 h prior to stimulation to mimic a pro-inflammatory environment. All reagents were diluted in Dulbecco's Modified Eagle's Medium (DMEM Gibco, 11995040), which served as the vehicle for drug dilutions.

Caco-2 Cell culture

Caco-2 cells were cultured in DMEM (Gibco, 11995040) supplemented with 10 % fetal bovine serum (FBS; Gibco, 26140079), 2 mmol L^{-1} L-glutamine (Gibco, 25030081), and 1 % penicillin-streptomycin (Sigma-Aldrich, A5955). Cells were maintained at 37 °C in a humidified 5 % CO_2 incubator. For experiments, cells were seeded at a density of 1×10^5 cells per well on 13 mm coverslips in 24-well plates. Culture medium was refreshed two to three times per week, and cells were used once they reached 80 % confluence.

Drug treatment protocol

The drug treatment protocol was conducted according to five consecutive steps: i) Caco-2 cells were seeded as above mentioned; ii) cells were incubated overnight in DMEM supplemented with 10 % FBS; iii) the medium was replaced with fresh DMEM with 2 % FBS and treated with the agonists/antagonists to the concentrations mentioned above; LPS was included as a positive control; iv) all treated cells were incubated 3 h at 37 °C in 5 % CO_2; v) cells were washed with PBS and fixed in cold methanol for 10 min. Untreated Caco-2 cells were included as a negative control.

Immunofluorescence staining

After treatment, cells were fixed in cold methanol (10 min), and then the methanol was removed, after which cells were permeabilised with 1 % Triton X-100 in PBS for 10 min at room temperature. Coverslips were incubated overnight at 4 °C with rabbit anti-pIgR (1:200 Abcam ab96196), washed, then incubated with goat anti-rabbit IgG FITC-conjugated (1:200 Abcam ab6717) for 2 h at room temperature in the dark. Nuclei were counterstained using Vectashield with DAPI (Vector Laboratories, H-1200). Images were acquired on a Nikon Eclipse E600 fluorescence microscope and processed with Image-Pro Plus software (version 5.1).

RNA extraction and Real Time quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (1 mL/sample: Invitrogen™ Thermo Fisher Scientific; 15596026). Chloroform (200 μL), isopropanol (500 μL), and 75 % ethanol (1 mL) were used for phase separation, precipitation, and washing steps, respectively. RNA concentration was assessed on a NanoDrop Lite spectrophotometer (Thermo Scientific) by the A260/A280 ratio. cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Table I) (Roche Diagnostics; 04379012001). Quantitative RT-qPCR was performed using LightCycler® Nano (Roche Diagnostics) and Universal ProbeLibrary system (Table I). pIgR relative expression was normalised to GAPDH using the ΔΔCq method (assuming 100 % efficiency). The nucleotide sequences of primers were designed using Probe Finder version 4.5 (Table II).

Statistical analysis

All experiments were performed in triplicate using at least three independent biological replicates ($n = 3$). Data are presented as mean ± SEM. Statistical significance was

Table I. cDNA synthesis and RT-qPCR components and conditions

	Component	Conditions
cDNA Synthesis	Transcriptor First Strand cDNA Synthesis Kit	
	Total RNA	1 μg per 20 μL reaction
	Random primers	1 μL
	RNase-free water	To adjust volume to 11 μL
	Final reaction volume	20 μL
RT-qPCR	LightCycler® TaqMan® Master Mix	1 × (Roche Diagnostics, USA)
	LightCycler Uracil-DNA Glycosylase	0.5 U
	Template cDNA	2 μL
	Final reaction volume	20 μL

Table II. Primer sequences

Gene	Forward (5′→3′)	Reverse (5′→3′)
pIgR	CTTCGCTAGGCTGGCTGACATCG	ACTGCAGCCGTCTATGTGG
gapdh	TTTGATGTTAGTGGGTCTCG	AGCTTGTCATCAACGGGAAG

assessed by one-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* test using GraphPad Prism software (version 9.0; GraphPad Software). A $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Caco-2 cells confluence

Caco-2 cells showed progressive growth and monolayer formation during the 14-day culture period. On day 3 (Fig. 1, panel A), cell density was low (30 % confluence). By day 7 (Fig. 1, panel B), cell growth was observed without reaching a confluent monolayer, while on day 14 (Fig. 1, panel C), confluence reached approximately 80 % with no signs of morphological deterioration.

Nicotinic and muscarinic cholinergic agonist receptor stimulation induces the tissular presence of pIgR in Caco-2 cells

For the characterisation of pIgR in Caco-2 cells, immunofluorescence staining was performed using an anti-pIgR antibody (green) and DAPI (blue) for nuclear visualisation. We observed at 3 hours of Caco-2 cells incubation with the cholinergic agonists nicotine (Fig. 2A, panel n, white arrows) and muscarinic (Fig. 2A, panel r, white arrows) an intense

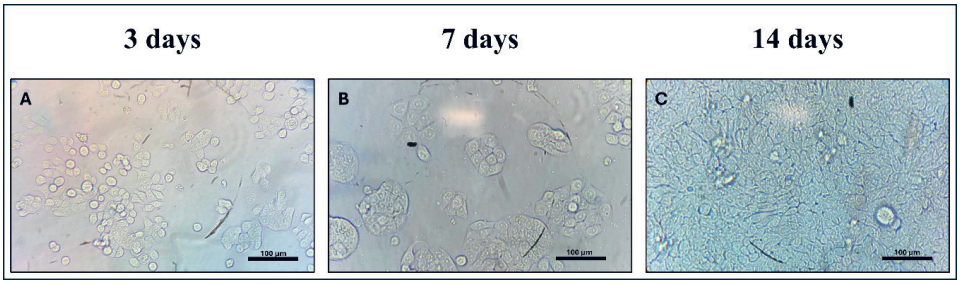

Fig. 1. Cell confluence. Brightfield microscopy images showing Caco-2 cell morphology and confluence at different time points. Images correspond to: A) day 3 (30 % confluence, low density); B) day 7 (60 % confluence, near confluence); C) day 14 (80 % confluence), showing uniform morphology and no signs of deterioration. Scale bar: 100 µm.

positive fluorescence for pIgR. This effect was mimicked by the group incubated with LPS (Fig. 2A, panel j, white arrows). Fluorescent counterstaining with DAPI evidenced the presence of cell nuclei in both nicotine (Fig. 2A, panel o, yellow arrows) and muscarinic (Fig. 2A, panel s, yellow arrows) treated cells, as well as in the control groups, LPS (Fig. 2A, panel k, yellow arrows), control without agonist drug treatment (Fig. 2A, panel g, yellow arrows) and control without anti-pIgR antibody showed no positive fluorescence (Fig. 2A, panel c, yellow arrows). Marked fluorescence is seen in the groups treated with cholinergic agonists, in contrast to the group not treated with cholinergic agonists, in which no positive fluorescent signal for pIgR was detected (Fig. 2A, panels b and f).

Stimulation with cholinergic antagonist drugs decreases the presence of pIgR in Caco-2 cells

Immunofluorescent assays indicated that incubation for 3 hours with the nicotinic cholinergic antagonist (nicotine + MLA) (Fig. 2B, panel n, white arrow) and with the muscarinic cholinergic antagonist (muscarine + atropine) (Fig. 2B, panel r) decreased the fluorescence positive for pIgR. Caco-2 cells treated with cholinergic antagonists showed little or no detectable positive label to pIgR, in contrast to cells incubated with cholinergic agonists. Caco-2 cells incubated with LPS (Fig. 2B, panel j, white arrows) showed positive pIgR label; control without drug treatment and control without anti-pIgR antibody showed no positive fluorescence (Fig. 2B, panels b, f). Counterstaining with DAPI evidenced the presence of cell nuclei in all groups (Fig. 2B, panels c, g, k, o and s; yellow arrows).

Cholinergic agonists significantly increase the number of cells fluorescent to pIgR in Caco-2 cells

Quantitative analysis of the number of fluorescent cells with positive fluorescence to pIgR is shown in Fig. 2C. Treatment with cholinergic agonists (nicotine and muscarine) significantly increased the number of pIgR-positive cells in Caco-2 monolayers compared to the untreated control (****$p < 0.0001$) (Fig. 2C). Co-treatment with their respective antagonists (MLA and atropine) significantly reduced the number of fluorescent cells compared

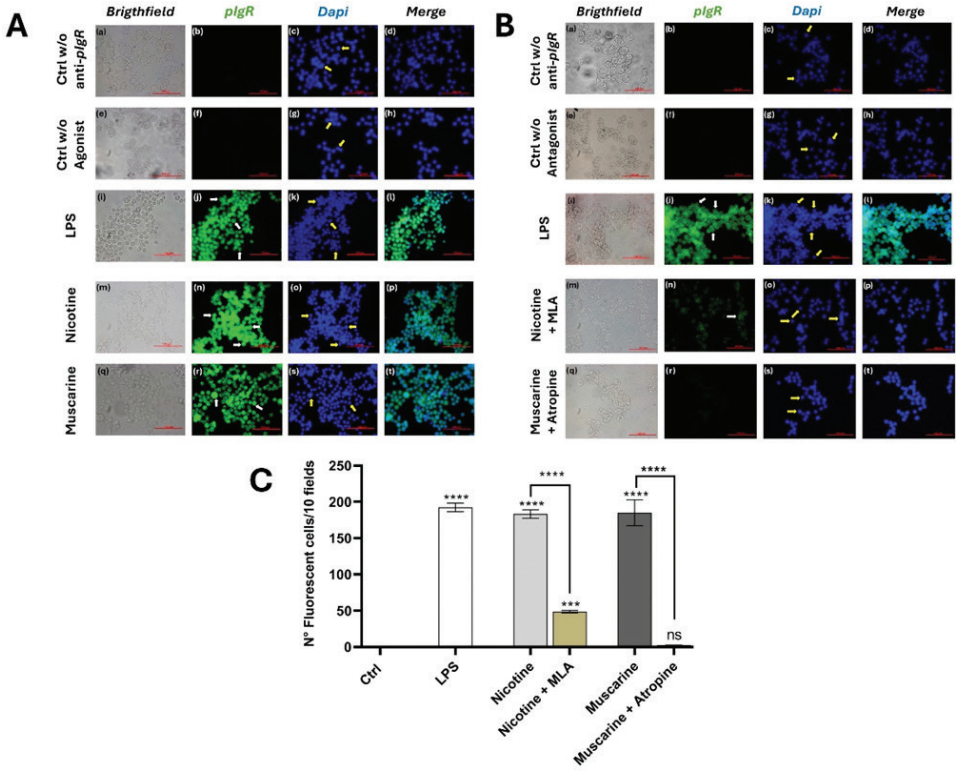

Fig. 2. Detection and quantification of pIgR fluorescence in Caco-2 cells after cholinergic stimulation and inhibition. Representative fluorescence images show pIgR (green) and nuclear (DAPI) labelling in Caco-2 cells after 3 h of treatment. A) cholinergic agonist stimulation; B) cells incubated with their respective antagonists (Nicotine + MLA and Muscarine + atropine); C) fluorescence analysis, cells treated with nicotine or muscarine or co-incubation with nicotine + MLA or muscarine + atropine group. One-way ANOVA: F = 205.7. Significant statistical differences ****$p < 0.0001$, ***$p < 0.001$. Data are presented as mean ± SEM ($n = 3$).

to the agonist alone (nicotine *vs.* nicotine + MLA, ****$p < 0.0001$; muscarine *vs.* muscarine + atropine ****$p < 0.0001$) (Fig. 2C). However, only the nicotine + MLA group maintained a significant difference versus the control (***$p < 0.001$), while muscarine + atropine did not show a statistical difference compared to the control (ns) (Fig. 2C). One-way ANOVA revealed F = 205.7, $p < 0.0001$.

pIgR gene expression is increased in Caco-2 cells incubated with a nicotinic cholinergic agonist but not with a muscarinic cholinergic agonist

Analysis of pIgR gene expression revealed that nicotine treatment in Caco-2 cells significantly increased mRNA levels, with a 3.94-fold increase compared to the untreated control (****$p < 0.0001$) (Fig. 3). This effect was counteracted by co-incubation with the

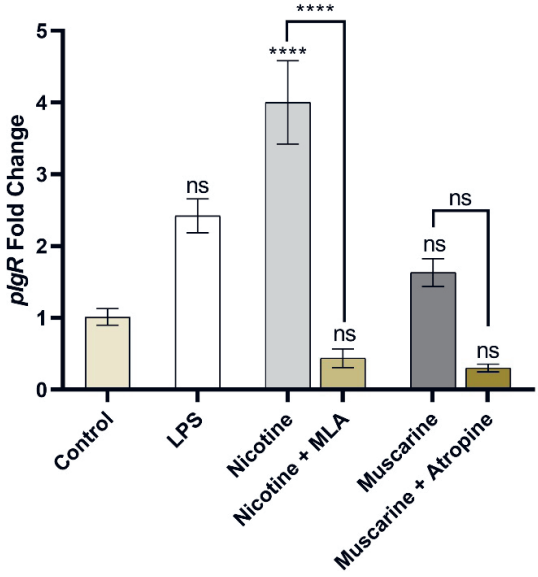

Fig. 3. Relative pIgR mRNA expression in the Caco-2 cell line after cholinergic stimulation. pIgR expression was quantified by RT-qPCR and calculated using the $2^{\wedge-\Delta\Delta Ct}$ method, normalised to GAPDH. Bars represent mean ± SEM ($n = 3$). Significant statistical differences ****$p < 0.0001$. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's *post hoc* test.

nicotinic antagonist MLA, which reduced expression by 9.10-fold relative to the nicotine-treated group (****$p < 0.0001$), showing no significant difference *versus* control (ns) (Fig. 3). In contrast, treatment with muscarine induced a moderate increase in pIgR expression, which was not statistically significant compared to the control, and no differences were observed when comparing this group with the group treated with muscarine + atropine (ns) (Fig. 3). Likewise, treatment with LPS did not generate significant changes under the conditions evaluated (Fig. 3). Statistical analysis by one-way ANOVA confirmed significant differences between groups (****$p < 0.0001$). These results suggest that the activation of nicotinic, but not muscarinic, cholinergic receptors promotes pIgR gene expression in Caco-2 cells, reinforcing the role of nicotinic signaling in the regulation of epithelial IgA transcytosis.

The current research conducted on LPS-primed Caco-2 cell cultures evidences the effect of cholinergic antagonists blocking the increase of pIgR protein induced by cholinergic agonists. This finding is in line with the up-modulatory effects of the cholinergic system on pIgR mRNA (6) and IgA secretion (7). The underlying mechanism accounting for the cholinergic modulation of pIgR is not fully clear, although there is evidence of the impact of mAChR on regulated secretory pathways (16). It is known that the regulated secretion relies on Rab3D proteins, which enable the sorting and storing of proteins into granules that, after degranulation, release their protein content. Instead, transcytosis entails the YES-EGFR-ERK-FIP5 signal pathway for the intracellular transport of endocytosed protein-receptor complexes for their extracellular release (4, 16, 17).

Cholinergic modulation of pIgR transport addressed in rabbit lacrimal gland acinar cells (LGACs) was evidenced by the upmodulating action of carbachol on the secretion of secretory component residing in mature secretory vesicles. Secretory component secretion displayed a pattern of secretion similar to the syncolin protein as a marker of secreted proteins via the regulated secretory system (16). Furthermore, pIgR was colocalised with the small GTPase Rab3D (a regulated secretory vesicle marker localised in zymogen granule membranes) within subapical membrane vesicles to be secreted by exocytosis. Thus, carbachol may modulate the pIgR-Rab3D interaction and ultimately the secretion through the regulated secretory pathway (16).

CONCLUSIONS

This work demonstrates that muscarinic and nicotinic receptor modulation alters pIgR-IgA trafficking in Caco-2 cells. Although the precise signaling pathways require further elucidation, these findings establish a functional link between cholinergic signaling and secretory IgA transcytosis. Future studies using pathway-specific inhibitors, receptor knockdown, and live-cell imaging are warranted to define underlying molecular mechanisms.

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Authors contributions. – Conceptualisation, G.H.-M., M.E.D.-S., and J.P.-Y.; methodology, G.H.-M., M.E.D.-S., A.A.R.-A., I.M.A.-M., D.L.-L., and M.G.-R.; formal analysis, G.H.-M., M.E.D.-S., A.A.R.-A., I.M.A.-M., and J.P.-Y.; data curation, G.H.-M. and M.E.D.-S.; writing – original draft preparation, G.H.-M., M.E.D.-S., and J.P.-Y.; writing – review and editing, G.H.-M., M.E.D.-S., A.A.R.-A., I.M.A.-M., D.L.-L., M.G.-R., and J.P.-Y.; supervision, M.E.D.-S. and J.P.-Y.; project administration, J.P.-Y.

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