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TNF-α MEDIATED SECRETORY COMPONENT IS POSITIVELY REGULATED BY PI3K/AKT/MTOR IN CACO-2 CELLS

PI3K/AKT/MTOR U CACO-2 ĆELIJAMA POZITIVNO REGULIŠU SEKRETORNU KOMPONENTU ČIJI JE POSREDNIK TNF-α

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Summary

Background: Secretory component (SC) is the extracellular component of the polymeric immunoglobulin receptor (pIgR) that functions as a receptor for externally secreted polymeric immunoglobulins such as secretory IgA. SC expression is regulated by a signaling pathway involving TNF-α.
Methods: Caco-2 cells were cotreated with TNF-α plus either Wortmannin, which inhibits PI3K, or Rapamycin, which inhibits mTOR. The expression of SC and pIgR mRNA were assessed by immunocytochemistry, Western blotting, and Quantitative real-time PCR.
Results: Wortmannin and Rapamycin decreased the fraction of cells expressing SC, as well as the total expression of SC protein and pIgR mRNA.
Conclusions: TNF-α regulation of SC expression is mediated through a PI3K/AKT/mTOR signaling pathway in Caco-2 cells.

Keywords: secretory component, polymeric Ig receptor, tumour necrosis factor-alpha, phosphoinositide 3-kinase, mammalian target of rapamycin

Kratak sadržaj

Uvod: Sekretorna komponenta (SK) predstavlja izvanćelijsku komponentu polimeričnog imunoglobulinskog receptora (pIgR) koja funkcioniše kao receptor za polimerične imunoglobuline koji se izlučuju napolje poput sekretornog IgA. Ekspresiju SK reguliše signalna putanja u koju je uključen TNF-α.
Metode: Caco-2 ćelije kotretirane su sa TNF-α uz vortmanin, koji inhibira PI3K, ili rapamicin, koji inhibira mTOR. Ekspresija mRNK SK i pIgR određena je imunocitohemijski, pomoću vestern blota i kvantitativne PCR.
Rezultati: Vortmanin i rapamicin smanjili su udeo ćelija sa ekspresijom SK kao i ukupnu ekspresiju proteina SK i mRNK pIgR.
Zaključak: Regulacija ekspresije SK od strane TNF-α u Caco-2 ćelijama odvija se uz posredstvom signalne putanje PI3K/AKT/mTOR.

Ključne reči: sekretorna komponenta, polimerički Ig receptor, faktor nekroze tumora alfa, fosfoinozitid 3-kinaza, mTOR

Introduction

Secretory component (SC) is the extracellular component of the polymeric Ig receptor (pIgR) which functions as a receptor for polymeric immunoglobulin (pIg) that is secreted into external fluids as secretory immunoglobulin A (SIgA). SIgA is the most abundant intestinal immunoglobulin, as well as the first line of specific immunological defense against environmental antigens. SC is a nonspecific scavenger of microorganisms and plays a key role in protecting and balancing the intestinal mucous membrane in limiting the inflammation process (1). SC is a key defense to bacteria (2–5) and parasites (6). Moreover, SC can play a critical role in the immune neutralization of the Cholera toxin (7). Tumour necrosis factor alpha (TNF-α) is a well-known member of the TNF superfamily consisting of at least 18 ligands and 29 different receptors involved in numerous cellular processes. TNF signals through two distinct receptors, TNFR1

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and TNFR2, thereby controlling the expression of cytokines, immune receptors, proteases, growth factors and cell cycle genes which in turn regulate inflammation, survival, apoptosis, cell migration, proliferation and differentiation (8). TNF-α is also a key regulator of intestinal immunity, including roles in organogenesis of peripheral lymphoid structures, activation of innate antiviral and antibacterial responses, and transmission of signals to initiate the adaptive immune response (9).

SC expression in Caco-2 increases in response to proinflammatory cytokines – TNF-α (10). SC expression in HT-29 cells is regulated by TNF-α involved in the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide 3′-kinases (PI3K)/protein kinase B (AKT) activation (11). Our data suggest that the production of SC in Caco-2 cells is not only regulated by PI3K/AKT, but is also regulated by the mammalian target of rapamycin (mTOR).

Materials and Methods

Cell culture

Caco-2 cells were derived from the Cell Biological Institute of Shanghai in China. Caco-2 cells were grown on 25 mm plastic petri dishes at 37 °C in air and 5% CO_2 in 1640 (GiBCO, Invitrogen Co, Carlsbad, CA, USA), supplemented with 20% FCS, 100 μg/mL streptomycin, 100 IU/mL penicillin, 3.7 g/L $NaHCO_3$. We selected the Caco-2 cell line because this cell line has often been used to examine the mechanisms of SC production by TNF-α and we considered that Caco-2 a very good SC expression model (10, 12). Prior to treatment, cells (1×10^6 cells/mL) were plated with fresh medium and cultured. On the second day after plating, 50 ng/mL of human recombinant TNF-α, with or without Wortmannin/Rapamycin, was added to the medium of the serum-free condition and cultivation continued for 24 h. Then cultured cells were harvested, the RNA and protein of Caco-2 cells were extracted. All experiments were repeated five times.

Immunocytochemistry

Caco-2 cells on coverglasses were fixed with 4% paraformaldehyde for 30 min and incubated with 3% H_2O_2. For SC staining, sections were reacted with 10% goat serum for 30 min, then with mouse anti-human SC at 4 °C overnight. The sections were washed with PBS and incubated with biotin-goat F(ab′)2 anti-mouse IgG for 30 min and with avidin-peroxidase for 30 min. The sections were rinsed three times with PBS between each incubation, and counterstained with hematoxylin. Sections from the same cells processed without the primary antibody were determined using the procedure detailed above as a control for nonspecific binding of the secondary antibody.

Western blot analysis of SC protein in Caco-2 cells

Extracted proteins of Caco-2 cells were separated by SDS-PAGE and transferred to polyvinylidine fluoride membranes. The membranes were blocked with Tris-buffer containing 50 ng/L skim milk and probed with polyclonal mouse anti-human SC antibodies or β-actin followed by peroxidase-conjugated secondary antibody. They were then incubated with an enhanced chemiluminescent substrate and exposed to X-OMAT film.

Quantitative real-time PCR

Total cellular RNA was measured as described earlier (13–14). Briefly, total cellular RNA was extracted from Caco-2 cells using the RNeas Mini kit from Takara. The quality of extracted RNA was determined by agarose gel electrophoresis. cDNA was synthesized using 100 μg of RNA. The levels of individual RNA transcripts were quantified by quantitative real-time PCR.

The primers of SC were:
PIgR–F: 5′-TGTTGCCACCACTGAGAGCAC-3′,
PIgR-R: 5′-CTTTGTAGGCCATCTCGGCTTC-3′,
GAPDH–F: 5′-GCACCGTCAAGGCTGAGAAC-3′,
GAPDH–R: 5′-ATGGTGGTGAAGACGCCAGT-3′.

Primers and fluorescent probes for SC were purchased from Takara. The PCR conditions comprised a preliminary cycle of 95 °C for 10 sec, followed by 45 cycles of 95 °C for 5 s and 60 °C for 20 s, followed by 60 for 1 min and 95 °C for 5 s. We also confirmed that the efficiency of amplification of each target gene was 100% in the exponential phase of the PCR. The housekeeping gene GAPDH (Takara, Dalian, China) was used as an endogenous control. The relative level of SC gene expression for each sample was calculated according to the following formula: $2^{-\Delta\Delta ct}$, $\Delta\Delta ct$= test(CT_{SC}-CT_{GADPH})-control(CT_{SC}-CT_{GADPH}).

Statistical analysis

Statistical differences among the treatment groups were determined by t test with SPSS 13.0.

Results

The effect of TNF-α with or without PI3K inhibit Wortmannin/mTOR inhibit Rapamycin on SC-positive cells

Immunocytochemistry demonstrated that the SC-positive cells accounted for about 0.5–0.6% of Caco-2 cells cultured in the absence of TNF-α (*Figure 1A*). After treatment of Caco-2 cells with only TNF-α 50 ng/mL for 24 h, the proportions of SC-positive cells were approximately 1.2–1.4% (*Figure 1B*). After treatment of Caco-2 cells with TNF-α 50 ng/mL and Wort-

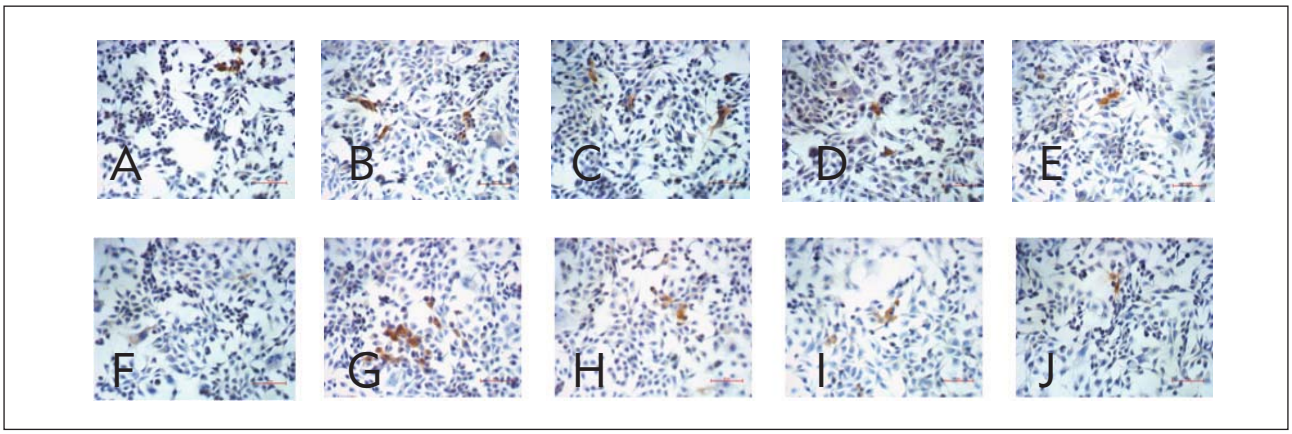

Figure 1 The effect of TNF-α with or without Wortmannin/Rapamycin on SC-positive cells 400x.

A) The percentage of SC-positive cells of the Caco-2 cells untreated with TNF-α is about 0.5–0.6%.
B) Caco-2 cells treated with TNF-α; the proportion of SC-positive cells is 1.2–1.4%.
C–F) Caco-2 cells treatmented by TNF-α and Wortmannin (50 nmol/L, 100 nmol/L, 500 nmol/L or 1000 nmol/L); the proportions of SC-positive cells were approximately 0.60–0.62%, 0.51–0.55%, 0.42–0.50% and 0.24–0.30%, respectively.
G-J) Caco-2 cells treatment with TNF-α and Rapamycin (1 nmol/L, 10 nmol/L, 100 nmol/L or 200 nmol/L) led to SC-positive cells populating approximately 1.20–1.30%, 0.55–0.56%, 0.54–0.55% and 0.36–0.40%, respectively.

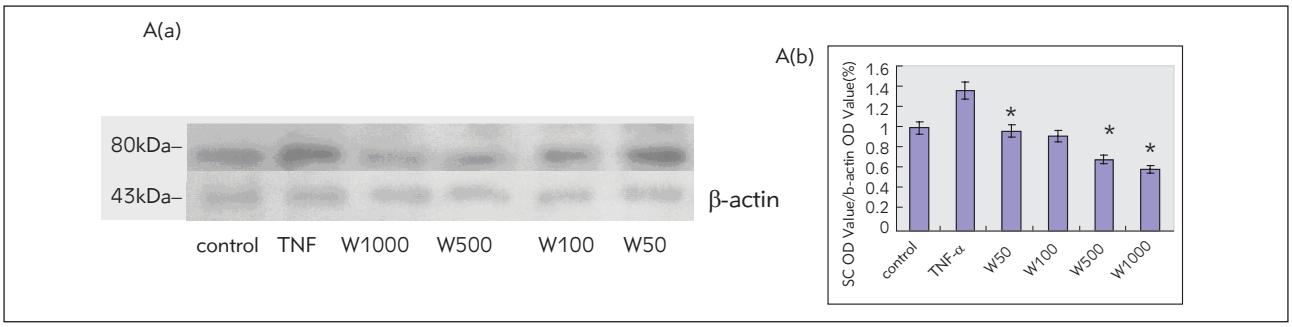

Figure 2A Effect of TNF-α/Wortmannin on the expression of SC in the Caco-2 cells. A(a): Specific bands for SC. A(b): Densitometric analysis. Compared with the relative value of Caco-2 cells treated with TNF-α, those of Caco-2 cells treated with TNF-α/Wortmannin were significantly decreased (n=5 in the group, *P<0.01).

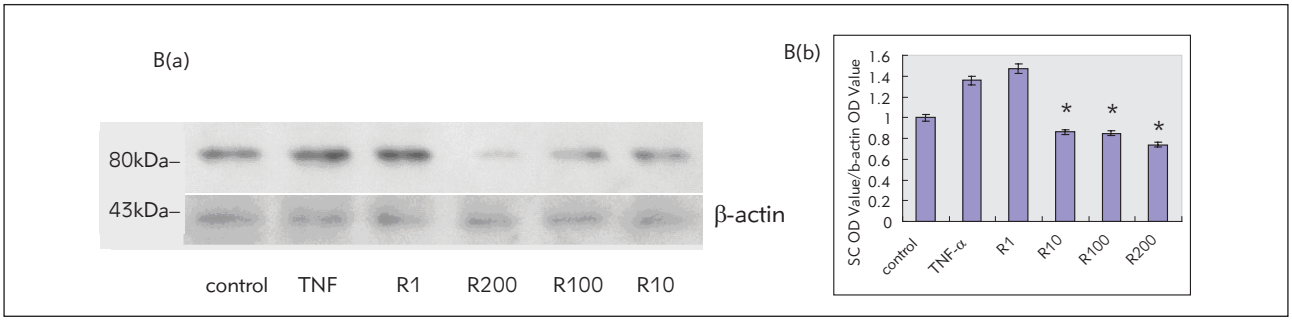

Figure 2B Effect of TNF-α/Rapamycin on the expression of SC in the Caco-2 cells. B(a): Specific bands for SC. B(b): Densitometric analysis (n=5 in the group, *P<0.01).

mannin (50 nmol/L, 100 nmol/L, 500 nmol/L, 1000 nmol/L) for 24 h, the proportions of SC-positive cells were approximately 0.60–0.62% (*Figure 1C*), 0.51–0.55% (*Figure 1D*), 0.42–0.50% (*Figure 1E*) and 0.24–0.30% (*Figure 1F*), respectively. After treatment of Caco-2 cells with TNF-α 50 ng/mL and Rapamycin (1 nmol/L, 10 nmol/L, 100 nmol/L, 200 nmol/L) for 24 h, the proportions of SC-positive cells were approximately 1.20–1.30% (*Figure 1G*), 0.55–0.56% (*Figure 1H*), 0.54–0.55% (*Figure 1I*), 0.36–0.40% (*Figure 1J*), respectively. The SC was localized mainly in the cytoplasm and cell membranes of Caco-2 cells.

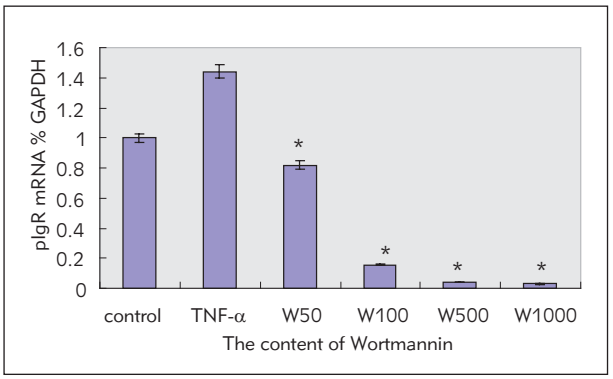

Figure 3 Dose-dependent decrease of SC mRNA expression in the Caco-2 cells after treatment with TNF-α/Wortmannin compared with TNF-α (*P<0.01).

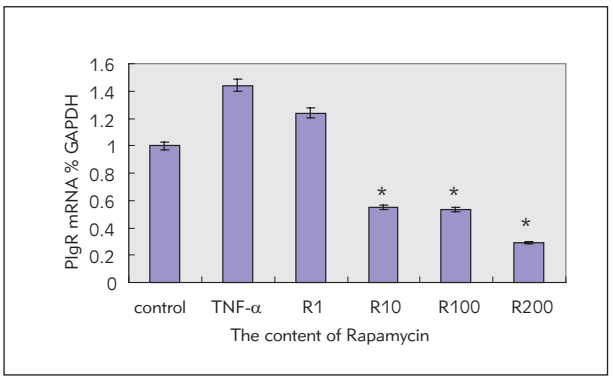

Figure 4 Dose-dependent decrease of SC mRNA expression in the Caco-2 cells after treatment with TNF-α/Rapamycin compared with TNF-α (*P<0.01).

SC protein of Caco-2 cells detected by Western blot

The expression of SC protein was significantly decreased in Caco-2 incubated with TNF-α/Wortmannin or TNF-α/Rapamycin. The average OD levels of SC protein were compared with the average OD levels of β-actin. The ratio of untreated Caco-2 cells was supposed to be one; the others were compared with it. The relative value of Caco-2 cells treated with TNF-α was 1.36±0.2. The relative values were 0.97±0.1, 0.92±0.2, 0.69±0.1, 0.60±0.1 respectively when the doses of Wortmannin were 50 nmol/L, 100 nmol/L, 500 nmol/L, 1000 nmol/L. Compared with the relative value of Caco-2 cells treated with TNF-α, those of Caco-2 cells treated with TNF-α/Wortmannin were significantly decreased (*Figure 2 A*). The relative values were 1.47±0.1, 0.86±0.2, 0.85±0.2, 0.74±0.1 respectively when the doses of Rapamycin were 1 nmol/L, 10 nmol/L, 100 nmol/L, 200 nmol/L. Compared with the relative value of Caco-2 cells treated with TNF-α, those of Caco-2 cells treated with 10 nmol/L, 100 nmol/L, 200 nmol/L of Rapamycin were significantly decreased (P<0.01) (*Figure 2 B*).

Real-time PCR analysis of SC in Caco-2 cells

Caco-2 cells were cultured for 24 h with TNF-α/Wortmannin or TNF-α/Rapamycin treatment, and steady-state mRNA levels were determined by real-time PCR for SC. The mRNA levels of these target genes were normalized by the mRNA levels of the housekeeping gene GADPH. The SC mRNA level of Caco-2 cells treated with TNF-α was supposed to be one. The SC mRNA levels of Caco-2 cells treated with TNF-α/Wortmannin or TNF-α/Rapamycin were obtained by comparison with those of the cells treated with TNF-α. Expression of SC mRNA decreased significantly in response to various concentrations of Wortmannin (P<0.01) (*Figure 3*) or 10 nmol/L, 100 nmol/L, 200 nmol/L Rapamycin treatment (P<0.01) (*Figure 4*).

Discussion

PI3Ks are a family of lipid kinases that propagate intracellular signalling cascades regulating a wide range of cellular processes. PI3K phosphorylates the 3′-OH group on phosphatidylinositols in the plasma membrane. This leads to recruitment of the protein Ser/Thr-kinase and AKT to the cell membrane where it becomes activated. PI3K activation is essential for adherens junction integrity, as well as the functional and morphological differentiation of intestinal epithelial cells (15). The signalling mechanisms will enhance the expression level of pIgR by various cytokines. For example, when intestinal epithelial cells – HT-29 cells are treated with IFN-α and IL-4, the expression level of pIgR mRNA is upregulated, and the protein tyrosine kinase is involved in the upregulation of pIgR (16). In addition, the PI3K and MAPK/ERK signal pathway are involved in the expression of SC by TNF-α (11). Our data indicate that the production of SC by TNF-α was decreased by Wortmannin, suggesting that the PI3K/AKT pathway positively regulates the SC production at the transcriptional level. As it has been reported that PI3K is involved in the transcytosis of pIgR (17–18), inhibition of SC production by the PI3K inhibitor is controlled at the transcriptional level. These data suggest that PI3K is involved in the transcytosis of pIgR in the intestinal epithelial cells as previously indicated (17–19) and our findings confirm these results.

AKT may directly lead to phosphorylation of Ser2448 on mTOR and active mTOR and its downstream as well as control of cell growth and translation. mTOR is vitally important for protein biosynthesis. mTOR is a member of the phosphatidylinositol kinase-related kinase family and regulates protein translation, cell cycle progression and cell proliferation (20). mTOR activation is essential for cell proliferation, regulating the entry into the G1 phase of the cell cycle. mTOR plays an important role in the G1-S transition of the cell cycle and is regulated by PI3K activity. Rapamycin, a bacterially derived drug with potent antitumour properties, inhibits mTOR activity and leads to dephospho-

rylation of mTOR downstream kinases p70S6K and 4E-BPs and therefore blocks protein synthesis. In this study, our data showed that Rapamycin rapidly decreased the synthesis of SC protein and the expression of SC mRNA in a dose-dependent manner. This suggests that TNF-α-mediated SC expression is positively regulated by mTOR at the transcriptional level and the translation level.

In conclusion, TNF-α-mediated SC is positively regulated by PI3K/AKT/mTOR in Caco-2 cells. The complicated mechanisms of TNF-α-mediated SC regulated by PI3K/AKT/mTOR however need to be explained.

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Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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