·Original Article·

Neuropeptide Y promotes TGF-β1 production in RAW264.7 cells by activating PI3K pathway via Y1 receptor

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Abstract: Objective To examine the effect of neuropeptide Y (NPY) on TGF-β1 production in RAW264.7 macrophages. **Methods** Enzyme linked immunosorbent assay (ELISA) was used to detect TGF-β1 production. Cell counting kit 8 (CCK-8) was used to assay the viability of RAW264.7 cells. Western blot was used to detect the phosphorylation of PI3K p85. **Results** NPY treatment could promote TGF-β1 production and rapid phosphorylation of PI3K p85 in RAW264.7 cells via Y1 receptor. The elevated TGF-β1 production induced by NPY could be abolished by wortmannin pretreatment. **Conclusion** NPY may elicit TGF-β1 production in RAW264.7 cells via Y1 receptor, and the activated PI3K pathway may account for this effect.

Keywords: neuropeptide Y; TGF-β1; phosphoinositide-3 kinase; RAW264.7 cells

1 Introduction

Neuropeptide Y (NPY), a 36-amino acid peptide, is widely distributed in the central and peripheral nervous systems serving as neuromodulator and neurohormone[1]. It plays an important role in regulation of food intake, memory retention, cardiovascular functions and anxiety[2-5]. NPY exerts its pleiotropic functions through activation of several G-protein-coupled NPY receptors, including Y1, Y2, Y3, Y4, Y5 and Y6 receptors[6]. NPY and Y1 receptor are expressed by many kinds of hemopoietic and immune cells such as T cells, B cells, monocytes and macrophages[7]. Recent findings point to a role of this neuropeptide in adaptive immunity: expression of Y1 receptor in dendritic cells is essential for antigen presenting cells (APC) function; and Y1 signaling plays a regulatory role in T cells, which will be hyper-responsive without Y1[8]. Elevated serum level of NPY has also been detected in the patients with asthma and systemic lupus erythematosus[9]. Thus NPY is regarded as an important factor involved in immune responses and pathogenesis of autoimmune diseases.

A principle source of TGF-β1 during inflammation is the macrophages[10,11]. Stimulating macrophages with priming and/or triggering agents, known to be present during inflammation, can alter their proteolytic profile, respiratory burst, and cytokine expression[12]. It has been reported that lipopolysaccharide (LPS) stimulated human peripheral blood monocytes can induce secretion of TGF-β1[13]. Further, LPS-stimulated murine peritoneal macrophage cells can activate endogenous latent TGF-β1[14]. These results indicate that LPS-stimulated macrophage cells produce TGF-β1. In the present study, we demonstrated that NPY could promote the TGF-β1 production by RAW264.7 macrophages. Phosphatidylinositol 3-kinase (PI3K) pathway was also found activated following the NPY stimulus via Y1 receptor. The effect of elevated TGF-β1 production by NPY could be abolished by wortmannin pretreatment. On the basis of these findings, we proposed that NPY interacted with macrophages through Y1 receptors, leading to the increase of TGF-β1 production via the PI3K pathway.

2 Materials and methods

2.1 Reagents ELISA kit for mouse TGF-β1 was from R and D Systems. Anti-phospho-p85 antibody was purchased from

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Cell Signal Technology. Antibodies against β-actin and anti-rabbit IgG labeled with horseradish peroxidase were purchased from Santa Cruz Biotechnology. PI3K inhibitor wortmannin was from Calbiochem. Y1 receptor inhibitor PD160170 was from Tocris. Cell Counting Kits-8 was from Beyotime.

2.2 Cell culture The murine macrophage-like cell line RAW264.7 was cultured in RPMI 1640 (PAA Laboratories) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2.

2.3 Assays for cytokines The culture supernatants of RAW264.7 cells were collected, centrifuged at 500 *g* for 5 min, and frozen at –20 °C until used for detection of TGF-β1 by ELISA.

2.4 Western blotting After various treatments, proteins in the whole cell lysate were resolved on 10% SDS–PAGE and then transferred onto nitrocellulose membrane (Schleicher and Schuell). The membranes were blocked overnight in phosphate-buffered saline containing 10% nonfat dry milk and 0.5% Tween-20, and incubated with primary antibodies for 2 h. Horseradish peroxidase-conjugated anti-rabbit IgG was used as the secondary antibody. Target proteins were imaged with the ECL system (Pierce).

2.5 Statistical analysis Data are expressed as mean±SEM. Each experiment was performed at least three times, and statistical analysis was performed using Student's *t* test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Promotion effect of NPY on TGF-β1 production in RAW264.7 macrophages LPS-stimulated macrophage cells can produce TGF-β1, but whether NPY can affect the TGF-β1 production in unstimulated macrophages remains unknown. We thus examined the effect of NPY on the expression of TGF-β1 in RAW264.7 macrophages. As shown in Fig. 1, the production of TGF-β1 in the RAW264.7 cells treated with NPY (10 nmol/L) was up-regulated 600%, significantly higher than that in the control group, which only showed low level of TGF-β1 after 24 h. If the Y1 receptor specific inhibitor PD160170 (1 μmol/L) was added before NPY treatment, the elevated TGF-β1 production could not be observed. These results indicated that NPY could increase TGF-β1 production in RAW cells via Y1 receptor.

3.2 NPY having no effect on the viability of RAW264.7 macrophages Apoptotic cells recognition and clearance could induce TGF-β1 secretion[15]. To determine whether the up-regulated TGF-β1 production was elicited by apoptotic RAW cells, we investigated the viability of RAW cells after NPY treatment. As shown in Fig. 2, the viability of NPY-treated RAW264.7 cells was similar to that of control group ($P > 0.05$). This result indicated that NPY could not affect the viability of RAW cells.

3.3 NPY activating PI3K through Y1 receptor in RAW264.7 macrophages Activated PI3K pathway may account for the elevated TGF-β1 production in macrophage, and NPY could activate PI3K pathway in different cells[16,17]. Therefore we

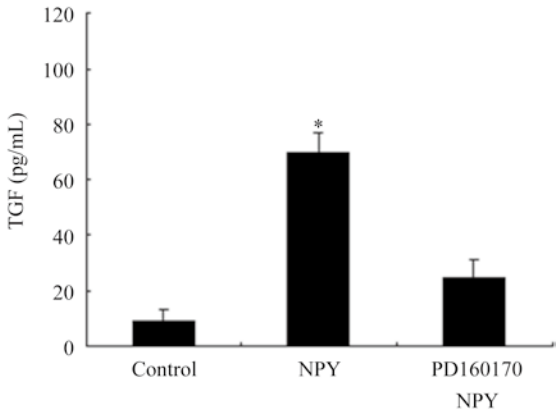

Fig. 1 Promotion effect of NPY on TGF-β1 production in RAW264.7 macrophages via Y1 receptor. RAW264.7 cells were treated with 10 nmol/L NPY or 1 μmol/L PD160170 for 2 h. Expression levels of TGF-β1 were measured 24 h later by ELISA. NPY promoted TGF-β1 production and this effect was abolished by Y1 receptor antagonist PD160170. The relative values of all results were determined by three independent experiments and expressed as mean± SEM. *$P < 0.05$ *vs* control group.

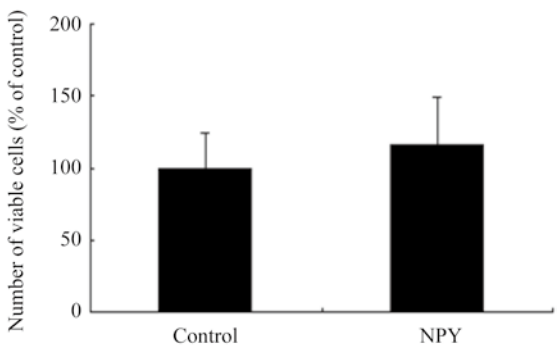

Fig. 2 Effect of NPY on the viability of RAW264.7 macrophages. RAW cells were treated with 10 nmol/L NPY for 2 h; the viability of RAW cells was detected 24 h later by CCK-8 test. NPY had no effect on the viability of RAW264.7 cells.

test the phosphorylation of PI3K p85 trying to determine whether the PI3K pathway was activated in the NPY-treated RAW cells. As shown in Fig. 3, after RAW cells were treated with 10 nmol/L NPY, PI3K p85 was phosphorylated obviously in less than 10 min and the effects could be blocked by PD160170. These findings indicated that NPY could rapidly activate intracellular PI3K pathway via Y1 receptor.

3.4 Blockage of PI3K inhibitor on NPY-induced TGF-β1 elevation in RAW macrophages To determine whether NPY up-regulated the production of TGF-β1 through activation of PI3K pathway, we incubated cells with wortmannin before adding NPY. The TGF-β1 production was detected by ELISA. As shown in Fig. 4, pre-incubation of cells with 100 nmol/L PI3K inhibitor wortmannin did abolish the promotion effect of NPY on TGF-β1 production. This result indicated that PI3K was involved in the up-regulation of NPY on TGF-β1 production.

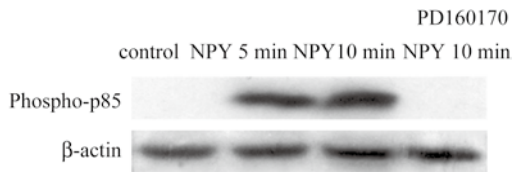

Fig. 3 NPY activated PI3K in RAW cells via Y1 receptor. RAW cells were treated with or without NPY (10 nmol/L) for 5 or 10 min. Phosphorylation of PI3K p85 was detected by Western blot. NPY rapidly activated PI3K p85 and this effect was blocked when Y1 receptor antagonist PD160170 was added before NPY treatment.

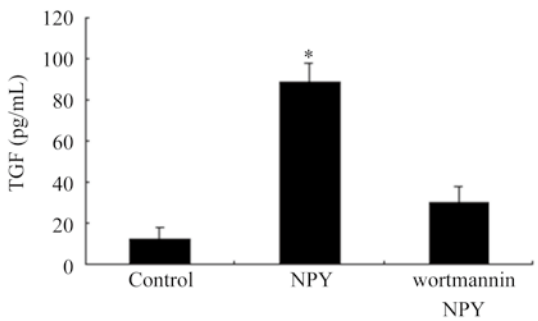

Fig. 4 Inhibition of wortmannin on NPY-induced TGF-β1 elevation in RAW264.7 cells. RAW cells were treated with or without NPY (10 nmol/L) for 2 h. Wortmannin was added 30 min before NPY treatment. TGF-β1 in the supernatant was detected by ELISA. Wortmannin could abolish the promotion effect of NPY on TGF-β1 production. The relative values of all results were determined by three repeated experiments and expressed as mean±SEM. *$P < 0.05$ *vs* control group.

4 Discussion

We have demonstrated that NPY could increase the production of TGF-β1 in RAW264.7 macrophages. Earlier studies indicated that $Y1^{-/-}$ macrophages produced significantly less TNF-α than $Y1^{+/+}$ peritoneal macrophages in response to LPS; Y1 receptor antagonist was capable of inhibiting TNF-α secretion by normal LPS-activated peritoneal macrophages[8]. These findings indicate that macrophage-derived NPY produced by activated macrophages are required for normal macrophage proinflammatory cytokines production. However, no study had been done to investigate the NPY's effect on anti-proinflammatory cytokines production in macrophages. We asked, in the present study, whether NPY could affect TGF-β1 production in macrophage cells, by using a macrophage cell line, RAW264.7 macophages. We have demonstrated that NPY could up-regulated the production of TGF-β1 by RAW264.7 macrophages.

A recent study revealed that liposomes composed of phosphatidylserine (PS-liposomes) induced TGF-β1 mRNA expression and protein production by activating PI3K pathway[15]. In keeping with these results, we found that NPY treatment activated PI3K pathway rapidly in RAW264.7 cells. Using highly specific PI3K inhibitor wortmannin, we were able to elucidate whether NPY regulates the TGF-β1 expression through the PI3K signaling pathway. We have shown that addition of PI3K inhibitors reduced significantly the increase of TGF-β1 levels in RAW cells treated with NPY. These findings are consistent with previous evidence that activation of PI3K/Akt pathway is important for TGF-β1 expression[16].

Monocytes/macrophages secrete TGF-β1 which, in turn, regulates numerous responses such as monocyte activation, cytokine production, host defense, and chemotaxis[12,14]. Beyond the accumulating evidence implicating TGF-β1 as a potent immunosuppressive agent[18], recent studies have highlighted the bidirectional effect of this cytokine on monocyte/macrophage function[11,19]. Specifically, the action of TGF-β1 on these cells is dependent upon the state of cellular differentiation, the cytokine milieu, and the concentration gradient of TGF-β1 itself[20]. *In vitro*, femtomolar concentrations of TGF-β1 induce the most potent chemoattractant response observed by human circulating blood monocytes[21,22]. This *in vitro* chemoattractant effect has been shown *in vivo*

to affect the recruitment of monocytes to the sites of inflammation, for example intradermal or intraarticular injection of TGF-β1 stimulates monocyte infiltration and matrix deposition[23,24]. Moreover, TGF-β1 mediates monocyte production of cytokines which act as additional mononuclear cell chemoattractants[12,25]. Thus, during the initial stages of inflammation, TGF-β1 locally acts as a proinflammatory agent by recruiting and activating resting monocytes.

Our study demonstrated that NPY can promote the unstimulated RAW264.7 macrophage production of TGF-β1. We postulated that NPY could act as a proinflammatory factor in the early stage of inflammation by up-regulating the production of TGF-β1 by macrophage itself.

Collectively, our study demonstrated that NPY could up-regulate the TGF-β1 production in RAW264.7 macrophages through the promoted PI3K activity via Y1 receptor. Thus, NPY and its receptors may become targets for novel therapies in the treatment of inflammatory diseases such as endoxin shock and sepsis.

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神经肽 Y 通过 Y1 受体激活 PI3K 通路促进 RAW264.7 细胞中 TGF-β1 的产生

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摘要：目的 探讨神经肽Y对巨噬细胞系RAW264.7分泌转化生长因子TGF-β1的影响。**方法** 酶联免疫吸附实验检测细胞培养上清液中的TGF-β1水平；细胞计数试剂盒CCK-8检测细胞活力；Western blot检测磷脂酰肌醇3激酶PI3K p85的磷酸化水平。**结果** 神经肽Y主要通过激活其Y1受体信号通路增加RAW264.7细胞中TGF-β1的产生，并可在10 min内快速激活PI3K通路。PI3K通路阻滞剂可消除NPY对TGF-β1产生的促进作用。**结论** 神经肽Y能够通过Y1受体促进RAW264.7细胞的TGF-β1的表达，此作用可能由PI3K介导。

关键词：神经肽 Y；转化生长因子 β1；磷脂酰肌醇 3 激酶；RAW264.7 细胞