Norepinephrine inhibits the cytotoxicity of NK92-MI cells via the β2-adrenoceptor/cAMP/PKA/p-CREB signaling pathway

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Abstract. Norepinephrine (NE) can regulate natural killer (NK) cell activity, but the mechanism remains unclear. In the present study the roles of adrenergic receptors (ARs) in inhibiting NK92-MI cells-mediated cytotoxicity by NE were investigated. To examine the effect of NE on NK92-MI cytotoxicity, a lactate dehydrogenase-release cytotoxicity assay was used to determine the cytotoxicity of NK92-MI cells against K562 cells. To evaluate the possible function of the α, β1 and β2 AR in mediating NE-induced effects, NK92-MI cells were pre-incubated with phenol-amine, CGP20712A and ICI118551 prior to stimulation by NE. To evaluate the role of cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling pathway in the inhibitory effect on cytotoxicity of NK92-MI cell by NE, NK92-MI cells were pre-incubated with PKA inhibitor Rp-8-Br-cAMP prior to stimulation by NE. It was demonstrated that NE decreased cytotoxicity and downregulated the expression of perforin, granzyme B and interferon (IFN)-γ of NK92-MI cells in a dose-dependent manner. Blocking NE functional receptors by ARs antagonists, particularly of β2 AR antagonist, suppressed the inhibitory effect of NE on cytotoxicity and expression of perforin, granzyme B, IFN-γ of NK92-MI cells significantly. Blockade of β2 AR in NE treated NK92-MI cells resulted in a reduction of the expression of phosphorylated (p)-cAMP-responsive element-binding protein (CREB) and intracellular cAMP concentration. Inhibiting the activity of PKA by Rp-8-Br-cAMP in NE treated NK92-MI cells resulted in increased cytotoxicity. The results of the present study suggest that NE can inhibit cytotoxicity and expression of perforin, granzyme B, IFN-γ of NK92-MI cell mainly via the β2-AR/cAMP/PKA/p-CREB signaling pathway.

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Abbreviations: NK, natural killer; NE, norepinephrine; AR, adrenergic receptor

Key words: norepinephrine, adrenergic receptor, NK92-MI cells, cytotoxicity

Introduction

Cross-talk between the sympathetic nervous system and immune system is necessary for health and well-being (1,2). Communication with immune cells occurs directly by neurotransmitter release from sympathetic nerves that bind to receptors expressed on immune cells (3). Norepinephrine (NE) is a member of the catecholamines family and is widely located in the central nervous system as well as peripheral tissues (4,5). NE modulates immune responses by binding to adrenergic receptors (ARs) expressed on immune cells (6).

Natural killer (NK) cells are large granular lymphocytes, which serve a role in the innate immune response (7). NK cells kill tumor cells and virally infected cells by direct cell-mediated cytotoxicity in a non-major histocompatibility complex restricted manner (8). Certain studies have demonstrated that NE has a regulatory effect on NK cell activation (9-11). However, the underlying mechanisms are not clear. NK92-MI cells established from patients with NK malignancies, serve an important role in the study of NK cell biology (12). In the present study, NK92-MI cells were used to investigate the mechanism by which NE regulates NK cell function.

NE exerts its effects by binding to α- and β-ARs (13). β2-AR is associated with G proteins via a stimulatory G protein subunit, which positively regulates the adenylate cyclase-cyclic adenosine monophosphate (cAMP) signal transduction pathway and cAMP response element-binding protein (CREB) activation. CREB (14,15), a transcription factor that has previously been revealed to be activated by protein kinase A (PKA), has important roles in regulating the activity of NK cells (16,17). However, whether the β2-AR/cAMP/PKA/phosphorylated (p)-CREB signaling pathway is involved in regulation of NK cells remains to be determined.

The results of the present study suggested that NE may inhibit cytotoxicity and expression levels of perforin, granzyme B and interferon (IFN)-γ in NK92-MI cells, predominantly

via the β2-adrenergic receptor/cAMP/PKA/p-CREB signal pathway. The present study may further the present understanding regarding the role of neuropeptides in the regulation of NK cell-associated innate immunity.

Materials and methods

Cell culture. NK92-MI cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in a modification of Eagle's minimum essential medium (α-MEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 0.01 mM 2-mercaptoethanol, 0.02 mM folic acid, 2 mM L-glutamine, 12.5% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) 0.2 mM inositol and 12.5% horse serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C in a humidified 5% CO_2 incubator. K562 cells were also obtained from ATCC and cultured in RPMI-1640 (Hyclone; GE Healthcare Life Sciences) medium with 10% FBS at 37˚C in a humidified 5% CO_2 incubator.

Cytotoxicity assay. NK92-MI cell cytotoxicity was determined using a colorimetric, non-radioactive, assay that quantitatively measures the release of lactate dehydrogenase (LDH) following cell lysis. NK92-MI (effector) cells were incubated with a series of NE (Sigma-Aldrich; Merck KGaA) concentrations 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M at 37˚C for 48 h and then co-cultured with K562 (target) cells at an effector-to-target (E:T) ratio of 4:1 at 37˚C for 4 h. Samples were centrifuged at 1,500 x g at 4˚C for 5 min. The supernatants were collected and LDH release in the supernatants was evaluated using a colorimetric reaction (absorbance at 490 nm). The spontaneous and maximum LDH release was measured by adding 100 µl α-MEM or 1% NP-40 (Sigma-Aldrich, Merck KGaA) to the effector cells or target cells. NE demonstrated no direct cytotoxic effect on K562 cells or NK92-MI cells alone (data not shown). The percentage specific lysis was calculated as follows:

$$\text{Specific lysis (\%)} = [\text{optical density (OD)}_{\text{experimental group}} - \text{OD}_{\text{target cell natural release control}}] / (\text{OD}_{\text{target cell maximum release control}} - \text{OD}_{\text{target cell natural release control}}) \times 100\%.$$

To evaluate the possible function of the AR α, β1 and β2 in mediating NE-induced effects, NK92-MI cells were pre-incubated with specific α receptor antagonist phentol-amine (PH), β1 receptor antagonist CGP20712A (CGP) or β2 receptor antagonist ICI118551 (ICI; all were provided by Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min prior to stimulation by NE. The concentration of antagonists were 0.1, 0.3 and 1.0 µM. The above-described procedures were used to detect NK92-MI cell cytotoxic activity against K562 cells.

The present study also aimed to evaluate the role of cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) signaling pathway in inhibitory effect on cytotoxicity of NK92-MI cell by NE. NK92-MI cells were pre-incubated with PKA inhibitor (Rp-8-Br-cAMP) 10 or 50 µM at 37˚C for 30 min prior to stimulation by NE. The above-described procedures were used to detect NK92-MI cell cytotoxic activity against K562 cells.

Western blotting. NK92-MI cells exposed to NE (10^{-8} M) at 37˚C for 24 h were harvested and washed with ice-cold PBS. Cells were resuspended in SDS-protein lysis buffer [0.1% SDS, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40 and 1% phenylmethylsulfonyl fluoride], solubilized for 30 min on ice and then centrifuged at 4˚C at 12,000 x g for 5 min. Protein concentrations were measured using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For western blotting, equivalent amounts (40 µg) total protein per sample were separated by SDS-PAGE on a 12.5% gel. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane by semi-dry blotting. The membrane was blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) overnight at 4˚C and then incubated with primary antibodies at room temperature for 2.5 h against the following proteins: Perforin (cat. no. ab97305; 1:1,000; Abcam, Cambridge, UK), granzyme B (cat. no. ab53097; 1:1,000; Abcam) and β-actin (cat. no. ab8277; 1:2,000; Abcam). Following washing, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (cat. no. ab107866; 1:10,000; Abcam) at room temperature for 1 h. The bands were then visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific Inc.) at 0.5-10 min post-washing. The protein expression was analyzed by Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), which was represented as the density ratio vs. β-actin.

To investigate the possible function of the β2 AR in mediating NE-induced effects, NK92-MI cells were pre-incubated with ICI for at 37˚C 30 min prior to stimulation by NE. The concentrations of antagonists used were 0.1, 0.3 and 1.0 µM. The above-described procedures were used to detect the expression of CREB and p-CREB using the following primary antibodies: Anti-CREB (cat. no. ab31387; 1:1,000; Abcam) and anti-p-CREB (cat. no. ab10564; 1:1,000; Abcam). Following this, the membrane was incubated with HRP-conjugated anti-rabbit IgG, (cat no. ab107866; 1:10,000; Abcam).

Measurement of interferon (IFN)-γ production. IFN-γ levels in culture supernatants were measured using an IFN-γ ELISA kit (Cusabio Biotech, Co., Ltd., Wuhan, China; cat no. CSB-E04577h) following manufacturer's protocol.

Measurement of intracellular cAMP levels. Intracellular cAMP levels were measured using cAMP Enzyme immunoassay kit according to the manufacturer's protocol (Sigma-Aldrich; Merck KGaA; cat no. CA200).

Statistical analysis. The difference among groups was analyzed with SPSS statistical software (20.0; IBM, Corps., Armonk, NY, USA) using one-way analysis of variance with a post-hoc Fisher's least significant difference test. All values were expressed as mean ± standard deviation. All experiments were performed in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of NE on cytotoxicity of NK92-MI cells. An LDH-release cytotoxicity assay was used to determine the cytotoxicity of NK92-MI cells against K562 cells (Fig. 1). Compared with

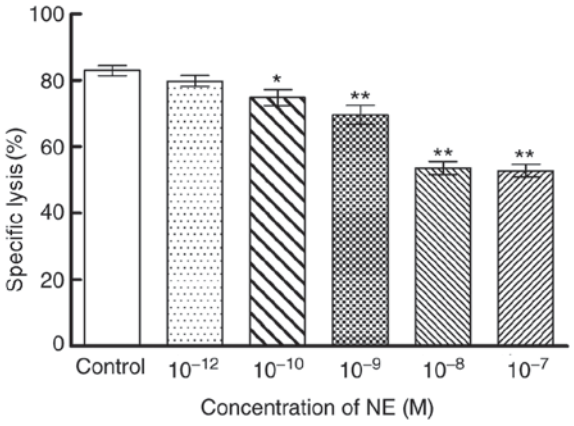

Figure 1. Effects of NE on NK92-MI cell-mediated cytotoxicity. NK92-MI cells were incubated with NE for 48 h, the lactate dehydrogenase-release cytotoxicity assay was used to determine cytotoxicity of NK92-MI cells against K562 cells. *P<0.05, **P<0.01 vs. control. NE, norepinephrine.

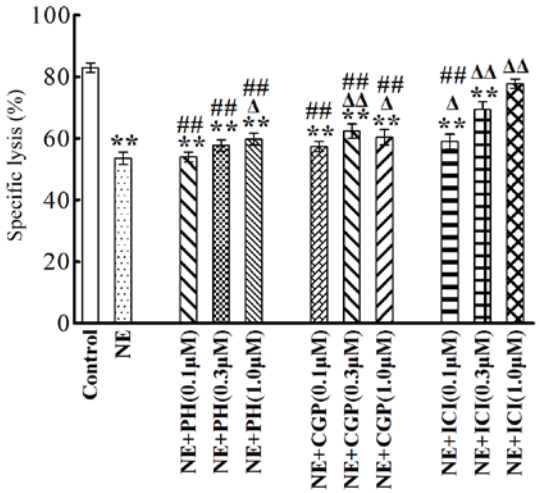

Figure 2. Effects of α, β1 and β2 AR antagonists (PH, CGP and ICI) on inhibition of NK92-MI cells cytotoxicity by NE. NK92-MI cells were pre-incubated with AR antagonists PH, CGP or ICI for 30 min prior to stimulation by NE (10^{-8} M). **P<0.01 vs. the control group; ΔP<0.05 and ΔΔP<0.01 vs. the NE group; ##P<0.01 vs. the NE+ICI (1.0 μM) group. AR, adrenergic receptor; NE, norepinephrine; PH, phentolamine; CGP, CGP20712A; ICI, ICI118551.

the control group, NE (10^{-10}-10^{-7} M) significantly inhibited NK92-MI cells cytotoxicity in a dose-dependent manner (P<0.05 and P<0.01).

Effects of AR antagonists on inhibition of NK92-MI cell cytotoxicity by NE. AR antagonists were adopted to assess the roles of ARs in mediating the inhibition of NK92-MI cell cytotoxicity by NE (Fig. 2). Results demonstrated that, compared with the NK92-MI cells stimulated by NE alone, α, β1 and β2 AR antagonists PH (1.0 μM), CGP (0.3 and 1.0 μM) and ICI (0.1, 0.3 and 1.0 μM) could all partly block the inhibitory effect on cytotoxicity of NK92-MI cells by NE (at optimal concentration 10^{-8} M; P<0.05). Among them, the effect of β2 AR antagonists ICI was the most marked, the cytotoxicity of NK92-MI cells stimulated by NE+ICI (1.0 μM) was significantly increased compared with both PH and CGP (0.1, 0.3 and 1.0 μM; P<0.05).

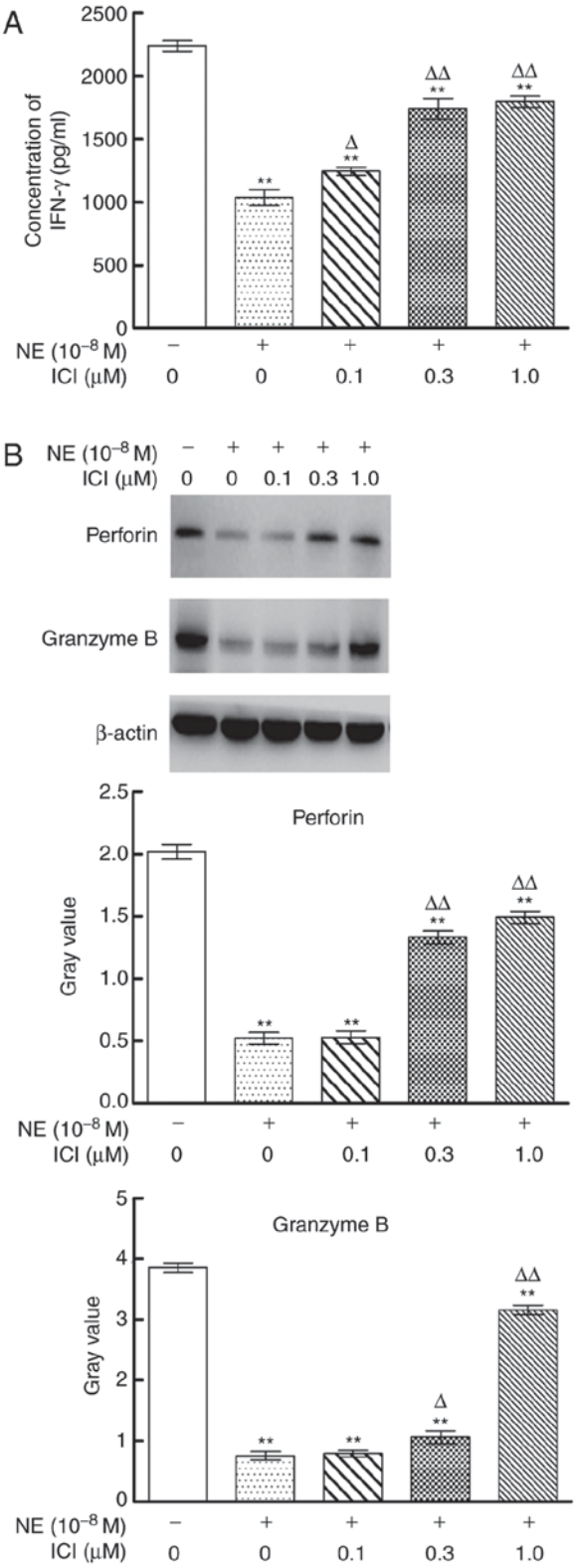

Figure 3. Effects of β2 AR antagonist on expression of (A) IFN-γ (B) perforin and granzyme B of NK92-MI cell treated by NE. NK92-MI cells were pre-incubated with ICI for 30 min prior to stimulation by NE (10^{-8} M). **P<0.01 vs. the control group; ΔP<0.05 and ΔΔP<0.01 vs. the NE group. NE, norepinephrine; ICI, ICI118551; INF, interferon.

Effects of β2 AR antagonist on expression of perforin, granzyme B and IFN-γ by NK92-MI cells treated by NE. The effects of ICI on the expression of perforin, granzyme B and

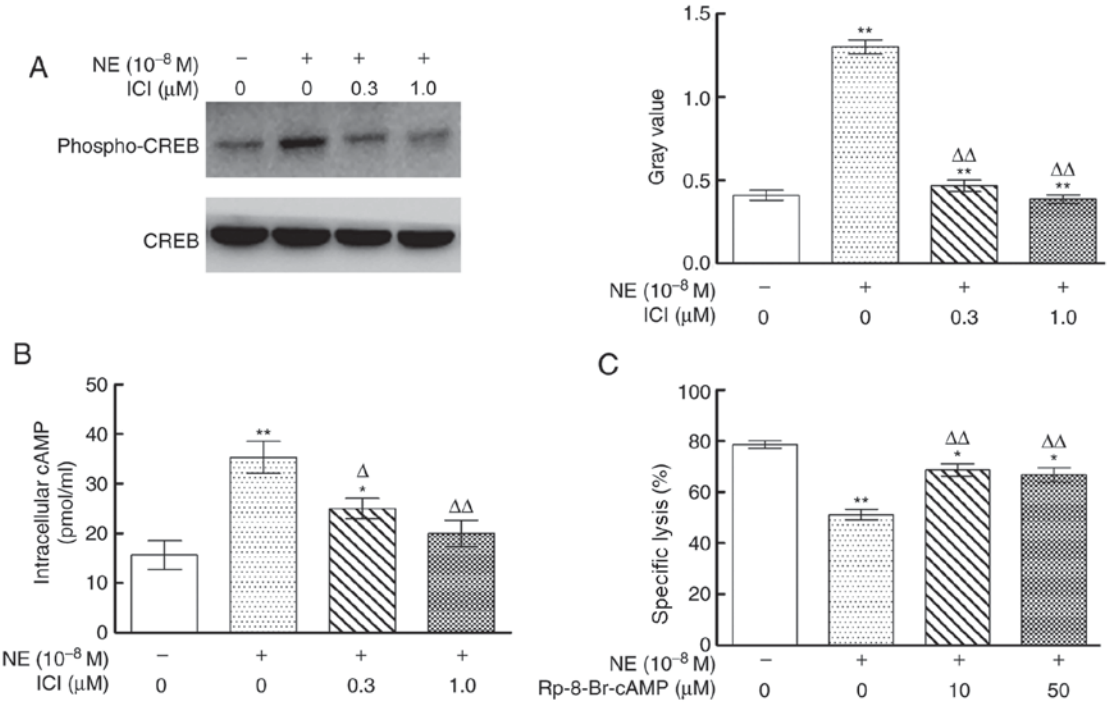

Figure 4. Roles of cAMP-PKA signaling pathway in inhibitory effect on cytotoxicity of NK92-MI cell by NE. NK92-MI cells were pre-incubated with ICI for 30 min prior to stimulation by NE, (A) expression of CREB, phospho-CREB and (B) intracellular cAMP concentration were detected. (C) NK92-MI cells were pre-incubated with PKA inhibitor (Rp-8-Br-cAMP) for 30 min prior to stimulation by NE, cytotoxicity of NK92-MI cells was detected. *P<0.05 vs. the control group, **P<0.01 vs. the control group; $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 vs. the NE group. cAMP, cyclic adenosine monophosphate; CREB, cAMP response element binding protein; NE, norepinephrine; PKA, protein kinase A.

IFN-γ of NK92-MI cells treated by NE were assessed (Fig. 3). Compared with NE alone treated NK92-MI cells, blockade of β2 AR by ICI (0.1, 0.3 and 1.0 μM) in NE treated NK92-MI cells resulted in significantly increased expression of IFN-γ (P<0.05), ICI (0.3 and 1.0 μM) could upregulate the expression of both perforin and granzyme B in NE treated NK92-MI cells (P<0.05).

Role of cAMP-PKA signal pathway in the inhibitory effect on the cytotoxicity of NK92-MI cell by NE. NK92-MI cells were pre-incubated with ICI for 30 min prior to stimulation by NE, expression of CREB, p-CREB and intracellular cAMP concentration were detected (Fig. 4A and B). NE could significantly increase the expression of p-CREB and intracellular cAMP concentration (P<0.01). Compared with NE alone treated NK92-MI cells, blockade of β2 AR by ICI (0.3 and 1.0 μM) in NE treated NK92-MI cells resulted in a significant reduction of the expression of p-CREB and intracellular cAMP concentration (P<0.05).

NK92-MI cells were pre-incubated with PKA inhibitor (Rp-8-Br-cAMP) for 30 min prior to stimulation by NE, cytotoxicity of NK92-MI cells was detected (Fig. 4C). Compared with NE alone treated NK92-MI cells, inhibiting the activity of PKA by Rp-8-Br-cAMP (10 and 50 μM) in NE treated NK92-MI cells resulted in significantly increased cytotoxicity (P<0.01).

Discussion

In mammals under stress, an increased level of stress-associated hormone can be induced by the activation of the hypothalamic-pituitary-adrenal and the sympathetic-adrenal medullary axes, such as corticotropin releasing hormone, adrenocorticotropic hormone and NE (18,19). Activation by stress of the sympathetic nervous system results in the release of catecholamines from the adrenal medulla and sympathetic nerve terminals (20,21). Catecholamines consist of several types of substances including NE, dopamine, histamine, serotonin and epinephrine. NE is one of the primary catecholamines of the sympathetic nervous system released during a stress response and serves an important role in modulating immune function (22).

In the present study, the effects of NE on NK92-MI cells and associated mechanisms *in vitro* were investigated. The major findings from the present study are that NE reduced NK92-MI cell cytotoxicity, downregulated the expression of perforin, granzyme B and IFN-γ in a dose-dependent manner. Granzyme B, an essential cytotoxic effector, is the main NK cell granule serine protease, and is produced by activated NK cells and initially stored in cytoplasmic granules with other cytolytic proteins including perforin (23). Perforin not only induces necrotic death but also apoptosis independently of granzyme B and facilitates entry of granzyme B into the target cells (24). NK cells are a major source of IFN-γ (25). NE exerts its effects through binding to α- and β-ARs (13). Blockade of NK α-, β1- and β2-ARs could suppress the inhibitory cytotoxicity and expression of perforin, granzyme B and IFN-γ of NK cells by NE. The results indicated that NK ARs (α, β1 and β2) are all involved in the reduction of NK cells cytotoxicity and perforin, granzyme B, IFN-γ expression by NE. Among ARs (α, β1 and β2) antagonists, the effect of β2 AR antagonist

(ICI) was the most marked. The results of the present study indicated that β2 AR served a more dominant role in this process than the two others.

β2 AR, a G-protein linked receptor that classically leads to intracellular accumulation of cAMP and activation of PKA upon stimulation (14,15). The second messenger cAMP regulates a number of cellular processes (26,27). With the exception of certain ion channels, all known effects of cAMP are mediated via the activation of PKA (28). It has demonstrated that increased cAMP levels could inhibit the killing effect of NK-cell on tumor cells (16,29). Blockade of β2 AR by ICI in NE treated NK92-MI cells resulted in a reduction of the expression of p-CREB and intracellular cAMP concentration. Inhibition of the activity of PKA by Rp-8-Br-cAMP in NE treated NK92-MI cells resulted in increased cytotoxicity. Results demonstrated that NE inhibited NK92-MI cells through the β2-AR/cAMP/PKA/p-CREB signaling pathway.

In conclusion, the results of the present study suggest that NE can inhibit cytotoxicity and expression of perforin, granzyme B and IFN-γ of NK92-MI cell mainly through the β2-AR/cAMP/PKA/p-CREB signaling pathway. This novel study should aid understanding of the role of neuropeptides in the regulation of NK cell-associated innate immunity.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ZS and ZL conceived and designed the experiments; ZS, DH, SL and WF performed the experiments; JW analyzed the data; and ZS wrote the manuscript.

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the guidelines provided by the Ethical committee of Experimental Animal Care at China Medical University (Shenyang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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