Effect of catecholamines on IL-2 production and NK cytotoxicity of rats *in vitro*[1]

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ABSTRACT

AIM: To explore effects of exogenous and endogenous catecholamines on function of lymphocytes and primary mechanisms mediating the effects. **METHODS:** Splenocytes of rats were exposed to norepinephrine (NE), α- or β-adrenoceptor antagonists plus NE, or α-methyl-p-tyrosine (α-MT), and then concanavalin A (Con A)-induced interleukin-2 (IL-2) production and natural killer (NK) cell cytotoxicity were determined by MTT assay and LDH assay, respectively. **RESULTS:** Optical density (*OD*) values of NE-treated groups, which reflected IL-2 production, were 0.63, 0.61, and 0.60, respectively for 1×10^{-10}, 1×10^{-9}, and 1×10^{-8} mol/L NE. They were all significantly reduced in comparison with control value of 0.68 ($P<0.01$). The effect of NE was blocked by either phentolamine (an α-adrenoceptor antagonist) or propanolol (a β-adrenoceptor antagonist). *OD* values of α-MT, an inhibitor of tyrosine hydroxylase, at doses of 1×10^{-10}, 1×10^{-9}, and 1×10^{-8} mol/L respectively were 0.71, 0.71, and 0.69, which were all notably higher than that of control (0.65, $P<0.01$). NK cytotoxicity was markedly attenuated by both NE and α-MT at the three doses mentioned above (17.69 %, 17.06 %, and 16.89 % versus 25.18 % for NE; 18.85 %, 18.44 %, and 17.04 % versus 23.22 % for α-MT; all $P<0.01$). The suppression of NK cytotoxicity by NE was prevented by propranolol but not by phentolamine. **CONCLUSION:** Exogenous NE exerts a suppressive action in modulating functions of T and NK cells, with the former via both α- and β-adrenoceptor mediated mechanisms and the later mainly through β-adrenoceptors. Endogenous catecholamines synthesized by lymphocytes have also an autoregulatory effect on the lymphocytes themselves.

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INTRODUCTION

The central and peripheral lymphoid organs are innervated by the sympathetic fibers with nerve terminals forming synapsislike contacts to immune cells[1-4] and adrenergic receptors exist on immune cells[5-7], which provides potent evidence for direct modulation of immune cell functions by adrenergic nervous system and endocrine system. Therefore, catecholamines, including norepinephrine (NE), dopamine and epinephrine,

classically from the adrenergic nervous system and the endocrine system, have been largely reported to regulate immune functions at systematic, cellular, and molecular level[8-12]. Our previous studies found that catecholamines were closely correlated to humoral and cellular immunity[13-15]. It is clear that β_2-adrenoceptors exit on T lymphocytes and mediate the modulation of T cell functions by catecholamines[16]. However, whether α-adrenoceptors are present on lymphocytes is still controversial[17]. Interleukin-2 (IL-2) produced by concanavalin A (Con A)-activated lymphocytes and cytotoxicity of natural killer (NK) cells are major indexes for assessing functions of T cells and NK cells, respectively. Thus, in this study, we explored the functional presence of α- and β-adrenoceptors on lymphocytes and their roles in mediating the effect of catecholamines on lymphocytes by using α- and β-adrenoceptor antagonists and by testing the Con A-induced IL-2 production and the NK cell cytotoxicity.

Since catecholamines are predominantly synthesized and secreted by neurons and endocrine cells, the modulation of immune function by the catecholamines is generally regarded as the regulation by the nervous and endocrine systems. However, recent findings that immune cells were also able to synthesize and secrete catecholamines suggest that a third novel catecholaminergic system, apart from the nervous and endocrine systems, may exist in the body[18-21], but functional significance of the endogenous catecholamines synthesized by immune cells is less clear. We considered that if the endogenous catecholamines could also regulate function of lymphocytes as the exogenous ones do, the immune system would benefit a more direct and quicker modulation from the autoregulation and the immune homeostasis would be better maintained. Therefore, in the current study, we used α-methyl-p-tyrosine (α-MT), which can be taken into cells and inhibit activity of tyrosine hydroxylase, an initial rate-limiting enzyme in synthesis of catecholamines, to block the synthesis of catecholamines in lymphocytes, and observed changes of the IL-2 production by the Con A-activated lymphocytes and the NK cell cytotoxicity so as to comprehend functional significance of the endogenous catecholamines synthesized by lymphocytes.

MATERIALS AND METHODS

Cell culture medium Roswell park memoral institute (RPMI) 1640 medium (Gibco) supplemented with 10 % fetal calf serum, 2.5×10^{-2} mol/L *N*-2-hydroxyethyl-piperazine-*N*´-2-ethanesulfonic acid (HEPES, Sigma), 5×10^{-5} mol/L 2-mercaptoethanol, 1×10^{-3} mol/L sodium pyruvate and antibiotics (100 kU/L penicillin, 100 U/mL streptomycin) was used as a complete culture medium.

Cell suspensions Single lymphocyte suspensions were prepared from the spleens of Sprague-Dawley rats (from the Center of Experimental Animals, Nantong Medical College, China) weighing 200-250 g. Erythrocytes were lysed by sterilized distilled water. A final concentration of the lymphocytes in the complete culture medium was 2×10^6 or 5×10^6 cells/mL depending on different experimental aims.

MTT colorimetric assay for Con A-induced IL-2 production IL-2 production by Con A-activated T cells was quantitatively measured using MTT assay as described previously[22]. Briefly, single splenocytes suspended in the complete culture medium at a concentration of 5×10^6 cells/mL were incubated with 5 mg/L Con A (Sigma) in an incubator (ESPEC BNA-311, Japan) with 5 % CO_2 at 37 ºC for 24 h. Then they were centrifugalized at 1000×*g* for 25 min. The supernatants were added to another splenocyte cultures of 2×10^6 cells/mL that had been incubated with 2 mg/L Con A for 48 h and washed with methyl-α-*D*-mannopyranoside (Fluka). And then, the mixtures together with methyl-α-*D*-mannopyranoside (10 g/L) were incubated in 5 % CO_2 at 37 ºC for 48 h. The MTT (Fluka) solution of 5 mg/mL was added to the cultures containing IL-2 (10 μL MTT solution per 100 μL medium), followed by the incubation with 5 % CO_2 at 37 ºC for 4 h. Sodium dodecyl sulfate (20 %) containing 50 % *N*´,*N*-dimethylformamide was added to the cultures and mixed thoroughly to dissolve the dark blue crystals, and the cultures were incubated for 20 h at 37 ºC in 5 % CO_2. Lastly, optical density (*OD*) of each culture was read on a Universal Microplate Reader (Elx800, Bio-TEK instruments, Inc, USA) using a test wavelength of 570 nm.

The drugs used in this study, ie NE, phentolamine, propranolol, and α-MT (all from Sigma), were first added to the splenocyte suspensions of 5×10^6 cells/mL respectively according to the different experimental aims, and 15 min later, Con A was added to the suspensions, which were cultured for 24 h. The subsequent processes were the same as described above. The control groups were conducted simultaneously with the experimental groups and were similarly to the

experimental groups but without those drugs.

Lactate dehydrogenase (LDH) release assay for evaluation of NK cell cytotoxicity Yac-1 cell line (from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences), a Moloney leukemia virus induced mouse lymphoma, with noted sensitivity to NK cells was used as a target cell in this study. The target cells were maintained in continuous suspension culture at 37 ºC in a 5 % CO_2 incubator. A final concentration of the target cells in the complete culture medium was 5×10^5 cells/mL. Effector cells (NK cells) from the spleens of rats anaesthetized with urethane (1 g/kg, ip) were isolated as described by Konjević *et al*[23]. Briefly, the cell suspensions were incubated in culture flask at 37 ºC in 5 % CO_2 for 2 h. Non-adherent cells were collected and resuspended in the complete culture medium at a concentration of 5×10^6 cells/mL.

The subsequent processes of LDH release assay for evaluation of the NK cell cytotoxicity were conducted according to the following formula:

$$\frac{OD_{\text{experimental}}-OD_{\text{spontaneous}}}{OD_{\text{maximal}}-OD_{\text{spontaneous}}}\times100\ \%$$

Where $OD_{\text{experimental}}$ stands for LDH release activity resulting from cocultures of effector cells and target cells at 10:1 ratio, ie 100 μL effector cell (5×10^6 cells/mL) and 100 μL target cells (5×10^5 cells/mL); $OD_{\text{spontaneous}}$ stands for the activity released from separate cultures of Yac-1 cells, ie 100 μL target cells and 100 μL complete medium; and OD_{maximal} stands for LDH activity released from Yac-1 cells after lysed by nonidet P 40 (NP 40, Fluka), ie 100 μL target cells and 100 μL 1 % NP 40. Each group was placed into the well of a 96-well flat-bottom plate and incubated in 5 % CO_2 at 37 ºC for 2 h, followed by the centrifugation at 500×g for 5 min. The supernatants were read as *OD* on the Universal Microplate Reader using the test wavelength of 570 nm. Lastly, NK cell cytotoxicity was calculated as percentage in accordance with the formula described above.

In the $OD_{\text{experimental}}$, the drugs used in this study were first added to the NK cell suspensions and incubated for 1 h, then the target cells (Yac-1 cells) were added to the cultures and cultured for 2 h. The other processes were the same as those described above. The control groups were conducted simultaneously with the experimental groups and were similarly to the experimental groups but without the drugs.

Statistical analysis The data were expressed as the mean±SD. Statistical analysis was carried out with the Stat software (Computer Resource Center 7.0, USA). The data were submitted to the two-way analysis of variance. The Student-Newman-Keul's test was also used to compare the data of all groups between each other. Differences were considered statistically significant at $P<0.05$.

RESULTS

Inhibitory effect of NE on Con A-induced IL-2 production NE exerted an inhibitory effect on the IL-2 production by the Con A-activated lymphocytes. The IL-2 production by T lymphocytes that were stimulated by Con A was remarkably decreased after NE treatment (1×10^{-10}, 1×10^{-9}, or 1×10^{-8} mol/L) when compared with the control group without NE (Fig 1).

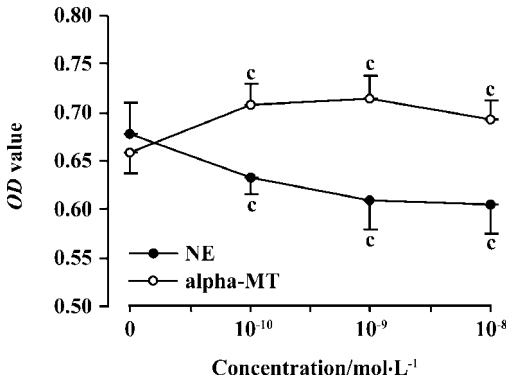

Fig 1. Effect of NE and α-MT on the Con A-induced IL-2 production. NE or α-MT was added to the splenocyte suspensions and the suspensions were incubated with Con A. *n*=8. $^cP<0.01$ *vs* control (0 mol/L NE or α-MT).

Blocking effect of α- and β-adrenoceptor antagonists on the NE inhibition of IL-2 production The splenocytes were exposed to NE or NE plus phentolamine, an α-adrenoceptor antagonist, with the concentration of 1×10^{-9} or 1×10^{-8} mol/L of each drug. The *OD* values of both the doses of NE were significantly lower than that of the control group (Fig 2). However, after NE together with phentolamine acted on the lymphocytes, the IL-2 production increased to the level near the control with a marked difference from the NE group, at the two used doses (Fig 2).

Similarly, after NE together with propranolol acted on lymphocytes at the dose of 1×10^{-9} or 1×10^{-8} mol/L of each drug, the IL-2 production of the each group nota-

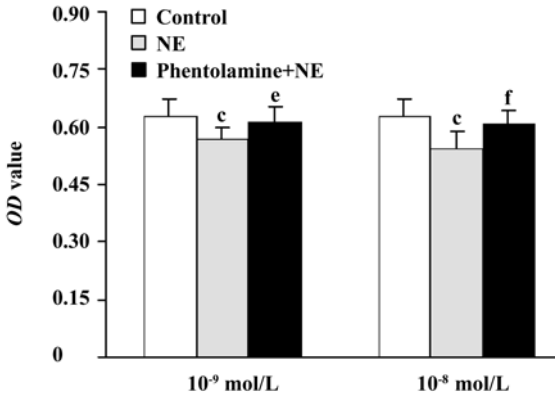

Fig 2. Blocking effect of α-adrenoceptor antagonist phentolamine on the NE inhibition of the IL-2 production. The lymphocytes from the spleens were exposed to NE plus phentolamine. *n*=8. $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* NE group.

bly increased in comparison with the reduced IL-2 production induced by the alone NE treatment at the same dose, and had no striking difference from the control (Fig 3).

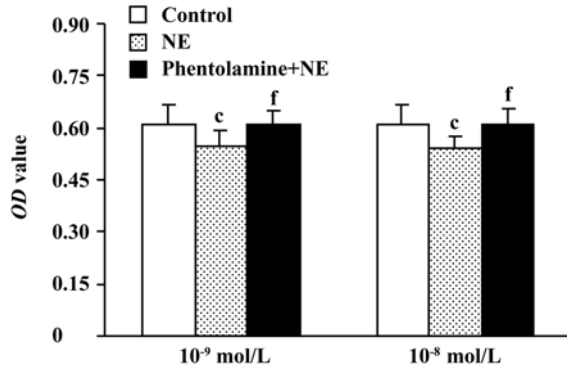

Fig 3. Blocking effect of α-adrenoceptor antagonist propranolol on the NE inhibition of the IL-2 production. The lymphocytes from the spleens were exposed to NE plus propranolol. *n*=7. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* NE group.

Enhancing action of α-MT on the Con A-induced IL-2 production α-MT had a facilitating effect on the Con A-induced IL-2 production. α-MT of 1×10^{-10}, 1×10^{-9}, or 1×10^{-8} mol/L was used in the experiment. The IL-2 production of each dose group was dramatically higher than that of the control group without α-MT, and no significant difference between the various concentrations of α-MT groups was found (Fig 1).

Suppressive effect of NE on the NK cell cytotoxicity The cytotoxicity of NK cells that were pretreated with 1×10^{-10}, 1×10^{-9}, or 1×10^{-8} mol/L NE was markedly reduced at the three used NE doses when compared with the control group without NE (Fig 4). The information demonstrated that NE could attenuate the cytotoxicity of NK cells.

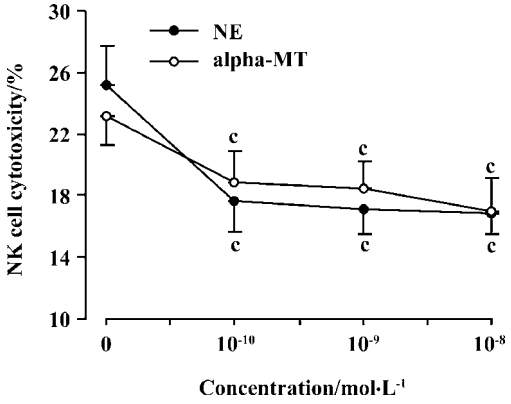

Fig 4. Inhibitory action of NE and α-MT on the NK cell cytotoxicity. The effector cells from the spleens of rats were pretreated with NE or α-MT, and then were cultured with the target cells (Yac-1 cell line) for 2 h. *n*=6. $^{c}P<0.01$ *vs* control (0 mol/L NE or α-MT).

Role of α- and β-adrenoceptor antagonists in the NE depression of the NK cell cytotoxicity After the NK cells were pretreated with α-adrenoceptor antagonist phentolamine (1×10^{-9} or 1×10^{-8} mol/L) plus NE (1×10^{-9} mol/L), their cytotoxicity was still significantly lower than that of the control group without any treatment and had no notable difference from the alone NE-treated group (Fig 5). However, after the NK cells were pretreated with β-adrenoceptor antagonist propanolol (1×10^{-9} or 1×10^{-8} mol/L) plus NE (1×10^{-9} mol/L), their cytotoxicity mostly recovered from the NE suppression ($P<0.01$), although there was a mild reduction of the cytotoxicity in comparison with the control group without any drug ($P<0.05$, Fig 5). These findings revealed that propanolol, but not phentolamine, could basically prevent the NK cell cytotoxicity from the suppressive effect of NE.

Attenuating action of α-MT on the NK cell cytotoxicity α-MT used concentrations of 1×10^{-10}, 1×10^{-9}, and 1×10^{-8} mol/L all notably diminished the NK cell cytotoxicity as NE did (Fig 4), showing that α-MT played an attenuating role in the influence of the NK cell cytotoxicity.

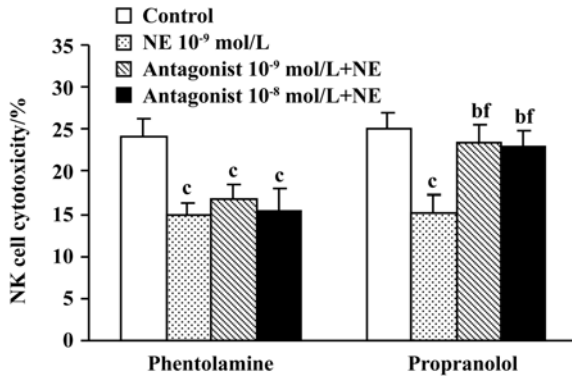

Fig 5. Effect of α- and β-adrenoceptor antagonists on the NE attenuation of the NK cell cytotoxicity. NK cells were pretreated with NE plus α-adrenoceptor antagonist phentolamine, or with NE plus β-adrenoceptor antagonist propranolol. *n*=6 or 8. $^{b}P<0.05$ *vs* control. $^{f}P<0.01$ *vs* NE group.

DISCUSSION

β-Adrenoceptors have been clarified to exist on almost all immune cells and they principally play an inhibitory role in the influence of T cell proliferation, differentiation and cytokine production when stimulated[17]. For example, NE down-regulates IL-2 production by binding specifically to the β-adrenergic receptors[24]; NE and terbutaline stimulate the β_2-adrenoceptors to decrease the level of IL-2 produced by freshly isolated murine splenic naive $CD4^+$ T cells[25]. However, few study has reported the presence of the α-adrenergic receptors on T cells[16], and the existence of α-adrenergic receptors on lymphocytes and monocytes is controversial[17]. Our present study showed that NE reduced the IL-2 production by the Con A-activated lymphocytes and that both of phentolamine and propanolol blocked the effect of NE. These findings suggest that apart from α-adrenoceptors, β-adrenoceptors may also exist on lymphocytes and participate in the modulation of NE on T cell function as well. Our previous study by using another functional index, the T cell proliferation, indicated that NE exerted an inhibitory effect on the T cell proliferation through both α- and β-adrenergic receptors[15]. The current results consistent with our previous observations further support and approve the hypothesis of the presence of α-adrenergic receptors on lymphocytes and their involvement in neuroimmunomodulation. Recently, Kavelaars reported that lymphocytes could express α_1-adrenergic receptors[27]. However, the functional significance of α-adrenergic receptors on lymphocytes is studied much less than that of β-adrenoceptors and is primarily unknown. Our study, from the functional profile, provided more evidence for the presence and the role of α-adrenergic receptors in the immune system. Probably, in the intact body, the α- and β-adrenergic receptors and the relevant receptor-linked mechanisms are respectively responsible for their own distinct roles.

It has been shown in our previous and other authors' studies that lymphocytes could synthesize catecholamines[18-21]. Therefore, further research into whether the endogenous catecholamines synthesized by lymphocytes could also regulate function of lymphocytes as the exogenous catecholamines did was one of our present concerns. We found that when the synthesis of the catecholamines in the lymphocytes was blocked by α-MT, the IL-2 production by the Con A-activated lymphocytes increased. The information suggests that the endogenous catecholamines synthesized by the lymphocytes may also exert a negative influence on the cytokine production as the exogenous NE does. Although we have still not known the exact action pathways and mechanisms of the endogenous catecholamines, we infer, on the basis of the similar action of the endogenous catecholamines to the exogenous NE, that the endogenous catecholamines may use a paracrine or autocrine pathway and a receptor-mediated mechanism to affect functions of the lymphocytes themselves.

NK cells are another type of lymphocytes and they can kill target cells, such as tumor cells, as well as virus, which are named as the cytotoxicity of NK cells. NE was found to attenuate the cytotoxicity of NK cells in the present study, and this depressive effect was abolished by propranolol but not by phentolamine. These results demonstrated that the inhibition of the NK cell cytotoxicity by NE was mediated only by β-adrenoceptors but not by α-adrenoceptors. The data, on the one hand, revealed extensive inhibitory actions of NE on the lymphocyte functions (including T and NK cells), on the other hand, implied different receptor mechanisms mediating the actions of NE on the T and NK cells. Whalen and Bankhurst[26] reported that application of epinephrine and isoproterenol *in vitro* elevated cAMP about 2.5-fold and induced an inhibition of NK cell activity, and that these effects were blocked by propranolol and mimicked by terbutaline, a β_2-adrenoceptor agonist, indicating the involvement of β_2-adrenoceptors in the process. Our present results concerning NE were similar to Whalen and Bankhurst's findings regarding epinphrine, suggesting that both NE

and epinephrine can diminish NK cell function and the effects may be mediated by β-adrenoceptors. Moreover, our current facts implied the possibility of no involvement of α-adrenoceptors in the NE modulation of the NK cell function. Similarly, Jetschmann *et al*[7] reported that infusion of NE did not appreciably alter α-adrenoceptor numbers on peripheral human NK cells. Thus, the functional significance of the presence of α-adrenoceptors on the NK cells will need to be further explored.

Unlike the IL-2 production, the cytotoxicity of NK cells was still decreased after these cells were treated with α-MT, showing that the reduction of the catecholamines in NK cells resulted in an attenuation of the NK cell function. It appeared that the endogenous catecholamines had a positive effect on the NK cell activity, which may be contrary to the negative influence of the exogenous NE. The information proposes that the endogenous catecholamines may employ some other intracellular mechanisms that are different from the exogenous ones to affect the function of the NK cell themselves. Furthermore, since α-MT can block the synthesis of catecholamines that include NE, dopamine, and epinephrine, the endogenous dopamine or epinephrine that is likely to play a distinct effect from NE may also contribute to the difference in the modulation of NK cell activity between the endogenous catecholamines and exogenous NE.

Although we have known that catecholamines can modulate function of lymphocytes, the modulations were generally attributed to the exhibition of the nervous and endocrine systems. Our present findings suggest that apart from the nervous and endocrine systems, the third novel catecholaminergic system, ie the endogenous catecholamines synthesized by lymphocytes, may also participate in the regulation of lymphocyte function. This kind of dual modulation of lymphocyte function by the exogenous and endogenous catecholamines may have more important significance than that of only exogenous catecholamines in keeping homeostasis of immune and other functions of the body, and potentially, the autoregulation of the endogenous catecholamines may play a greater role owing to its quicker and more direct action.

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