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Effect of Agroclavine on NK Activity in Vivo under Normal and Stress Conditions in Rats

M. STAREC, A. FIŠEROVÁ[3], J. ROSINA[1], J. MÁLEK[2], M. KRŠIAK

Department of Pharmacology, [1]Department of Biophysics, [2]Department of Anesthesiology, Third Faculty of Medicine, Charles University, and [3]Institute of Microbiology, Academy of Sciences, Prague, Czech Republic

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Summary

Agroclavine is a natural, clavine type of ergot alkaloid with D_1 dopamine and α-adrenoceptor agonistic properties. We showed previously that *in vitro* agroclavine enhances natural killer (NK) cell activity, increases interleukin-2 and interferon-gamma production and prolongs the survival time of tumor-bearing mice. The aim of this study was 1) to test the effect of agroclavine on NK activity *in vivo*, and 2) to assess the potential toxicity of high doses of agroclavine on cardiac and liver functions using creatine kinase MB (CKMB) and alanine aminotransferase (ALT) as biochemical markers in normal and stressed animals. The effect of stress was studied because we examined promising anticancer properties of agroclavine and malignant diseases are supposed to be a potent stressful event for patients. In our experiments 3-month-old male rats of the Wistar-Kyoto strain were used. Agroclavine was injected intraperitoneally (0.5 mg/kg or 0.05 mg/kg) 30 min before stress (four hours' restraint and immersion in 23 °C water). The animals were killed 30 min after stress, blood was collected and the spleen was removed. Non-stressed animals treated with agroclavine were killed 5 h after the drug administration. The results confirmed our previous *in vitro* results and showed that also *in vivo* agroclavine increases NK cell activity under non-stress conditions. Agroclavine only slightly increased CKMB and had no influence on ALT in non-stressed animals. These promising results are limited by the fact that agroclavine (0.5 mg/kg) diminished NK cell activity and significantly increased ALT and CKMB under stress conditions.

Key words

Agroclavine • Ergot alkaloid • Stress • Rat • NK cell activity • Toxicity

Introduction

Ergot alkaloids and their derivatives are known to exert diverse pharmacological effects. They influence the neuroendocrine system as many biogenic amines by interacting with various neurotransmitter receptors. Agroclavine is a clavine-type alkaloid, one of the natural products of *Claviceps spp*. and it has partial D_1-dopamine and α_1-adrenoceptor agonistic properties (Berde and Schild 1978, Berde 1984). Antineoplastic activity of

Fax+4202 24920590
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

agroclavine (and festuclavine) was described (Eich *et al.* 1984, 1986) on the L5178y mouse lymphoma cell system. The EC_{50} of agroclavine is 6.3 μM; this is comparable in potency with the therapeutic dose of cytostatic alkaloid camptothecin. Our previous *in vitro* experiments indicated that agroclavine enhances natural killer (NK) cell activity, production of IL-2 and interferon gamma in concentrations 10^{-7}-10^{-8} M (Fišerová *et al.* 1997).

NK cell activity is the most sensitive screening marker of immune responses during stress. Its major role is immunosurveillance against invading pathogens, control of tumor growth and regulation of hematopoiesis *in vivo* (Trinchieri 1989, Phillips *et al.* 1992). High susceptibility to pituitary hormones and neurotransmitters makes them a suitable target for the stress immunomodulatory reaction.

Acute exposures to a stressor usually result in the suppression of splenic NK cell activity, for example swimming stress (Ben Eliyahu *et al.* 1999), restraint stress (Okimura *et al.* 1986), or stress due to acute inescapable footshock (Saperstein *et al.* 1992). Other results are more conflicting. There have been reports of stressors enhancing NK activity (Fiatarone *et al.* 1988, Hoffman-Goetz *et al.* 1992. Millar *et al.* 1993), stressor indifferent response (Mizobe *et al.* 1997) or changed NK activity limited only to a specific group of animals, e.g. forced water-immersion stress decreased NK activity in virgin female rats, but had no effect in the pregnant rats (Nakamura *et al.* 1997).

In our experimental procedures, we used restraint and water immersion stress. These stressors induce heart tissue damage measured by mercury incorporation into the damaged tissue or with creatine kinase MB (CKMB) (Starec *et al.* 1994). The increase of plasma creatine phosphokinase, lactic dehydrogenase, and alanine aminotransferase (ALT), urea and glucose levels has also been described (Arakawa *et al.* 1997).

The primary aim of our recent work was to confirm the possible positive effect of agroclavine on NK cell activity also *in vivo* under normal and stress conditions. Stress conditions were chosen as to approach a clinical situation. Malignant disease is supposed to be a potent stressful event for patients, so we decided to test the effects of agroclavine also under stress conditions.

The second aim of our study was to assess the potential toxicity of high doses of agroclavine on immune, cardiac, and liver functions. The level of creatine kinase MB and alanine aminotransferase as biochemical markers, and NK cell-mediated cytotoxicity have been investigated for this purpose.

Methods

Animals

We used 3-month-old male rats of the Wistar-Kyoto strain weighing 230-280 g. The rats were fed *ad libitum* on a commercial pelleted diet (Velaz Altromin 1310). They were housed under natural day/night conditions for at least two weeks before the experiment. The experimental protocol was approved by the Ethics Committee of the Third Faculty of Medicine of Charles University.

Drug

Agroclavine used in this study (kindly donated by Galena Pharmaceuticals Ltd., Opava, Czech Republic) was diluted in saline and injected intraperitoneally (i.p.) in the dose 0.05 mg/kg (low dose), or 0.5 mg/kg (high dose) 30 min before stress. Saline was administered to control stressed or non-stressed animals instead of agroclavine (0.2 ml/100 g of body weight i.p.).

Stress procedure

We used immobilization immersion stress (Klenerová and Šída 1994). Rats were immobilized in a wire mesh restrainer and vertically immersed up to the xiphoid processus in water at 23 °C for four hours. Thirty minutes after removal from the bath and restrainer the animals were killed, blood was collected and their spleen was excised.

Biochemical analysis

Serum ALT and CK-MB assays were performed using the corresponding Ektachem clinical chemistry slides methods (multiple-point rate test). The ALT and CK-MB Ektachem clinical chemistry slides are dry multilayer analytical elements coated on a polyester support. An 11 μl drop of a sample is deposited on the slide. The product of the final reaction is monitored by reflectance spectrophotometry (340 nm in the ALT method, 670 nm in the CK-MB method, respectively).

NK activity assay

Spleens were homogenized in glass Potter-Elvehjem homogenizer and separated on Ficoll-Hypaque density gradient (1.091 g/ml) to obtain mononuclear cells.

After repeated washing, the cells were used immediately for *in vitro* assays.

Long-term cultures (YAC-1 NK-sensitive - MLV induced mouse T lymphoma derived from A/Sn mice) were carried out in a RPMI-1640 medium enriched with L-glutamine (2 mM), antibiotics (penicillin 100 U/ml and streptomycin sulfate 100 μg/ml) and supplemented with 10 % fetal calf serum. Incubations was carried out at 37 °C in a humidified atmosphere containing 5 % CO_2 (IR 1500 - Flow Laboratories).

Effector cells were incubated with the target cells (YAC-1) labeled with ^{51}Cr (60 min) at 37 °C in round-bottomed 96-well microtiter plates (NUNC). After 4 h incubation 0.1 ml of the cell-free supernatant was taken and the radioactivity of released ^{51}Cr was measured in a gamma scintillation counter. All samples were tested in triplicates. The percentage of specific lysis was calculated according to the formula:

$$\% \text{ cytotoxicity} = \frac{\text{exp. cpm} - \text{spont. cpm}}{\text{max.cpm} - \text{spont. cpm}} \times 100$$

where exp. (experimental) cpm is the mean ^{51}Cr released in the presence of effector and target cells, spont. (spontaneous) cpm is the mean ^{51}Cr released by target cells incubated alone, and max. (maximal) cpm is the maximal amount of ^{51}Cr released by target cells after addition of 10 % Triton X-100.

The results are expressed as means ± S.E.M. Comparisons between the groups were made using one-way analysis of variance (ANOVA test).

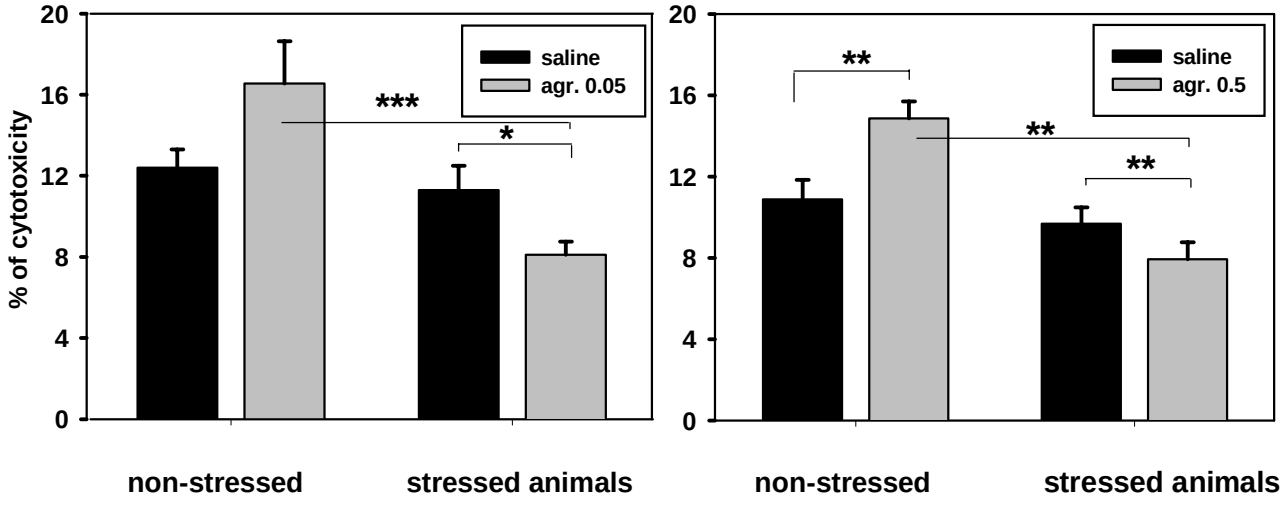

Fig. 1. *Effect of agroclavine (0.05 or 0.5 mg/kg) and water immersion-immobilization stress on natural killer cells activity. Saline = control (stressed or non-stressed) animals treated with saline in a volume of 0.2 ml/100 g of body weight (black columns); agr. 0.05 = animals treated with agroclavine (0.05 mg/kg); agr. 0.5 = animals treated with agroclavine (0.5 mg/kg); * p<0.05, ** p<0.01, *** p<0.001. The results are means of % of cytotoxicity ± S.E.M., 18 animals were in groups of both stressed animals treated with agroclavine 0.5 mg/kg or saline, 12 animals were in all other groups.*

Results

Effect of agroclavine on NK cell activity (Fig. 1)

Non-stressed animals treated with the higher dose of agroclavine (0.5 mg/kg) exhibited a significant increase in spleen NK cell activity ($p<0.01$). The lower dose (0.05 mg/kg) only caused a moderate increase in spleen NK cell activity, which was not significant compared to non-stressed rats treated with saline.

Water immersion and restraint stress for 4 h did not change NK cell activity significantly in control animals (treated with saline only). We observed only a slight non-significant decrease.

NK activity in stressed animals treated with agroclavine was lower ($p<0.05$ for the low dose and $p<0.01$ for the high dose) compared to stressed saline-treated rats.

Effect of agroclavine on creatine kinase MB (Fig. 2)

In non-stressed animals the CKMB value was not influenced significantly after the low dose of agroclavine but was increased after the higher dose ($p<0.05$).

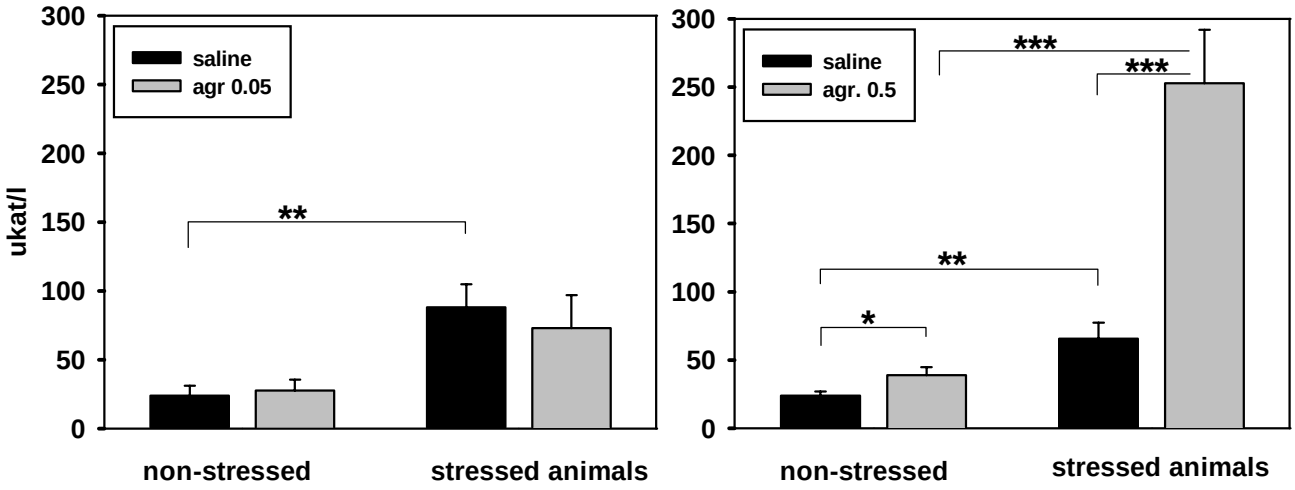

Fig. 2. *Effect of agroclavine (0.05 or 0.5 mg/kg) and water immersion-immobilization stress on CKMB values. Saline = control (stressed or non-stressed) animals treated with saline in a volume of 0.2 ml/100 g of body weight, agr. 0.05 = animals treated with agroclavine (0.05 mg/kg); agr. 0.5 = animals treated with agroclavine (0.5 mg/kg); * p<0.05, ** p<0.01, *** p<0.001. The results are means ± S.E.M. of CKMB values expressed in μkat/l, 15 animals were in groups of both stressed animals treated with agroclavine 0.5 mg/kg or saline, 6 animals were in all other groups*

Immobilization immersion stress significantly increased the CKMB value (stressed versus non-stressed control saline-treated animals, $p<0.01$). The combination of the high dose of agroclavine and stress caused more than a twofold increase of CKMB values compared with stressed saline-treated rats ($p<0.001$).

Agroclavine effect on alanine aminotransferase (Fig. 3)

Similar results as for CKMB were obtained. Agroclavine had no effect in non-stressed animals. However, stress significantly increased ALT values in saline-treated rats ($p<0.01$). The high dose of agroclavine in stressed rats substantially increased ALT values compared to stressed saline-treated controls ($p<0.01$).

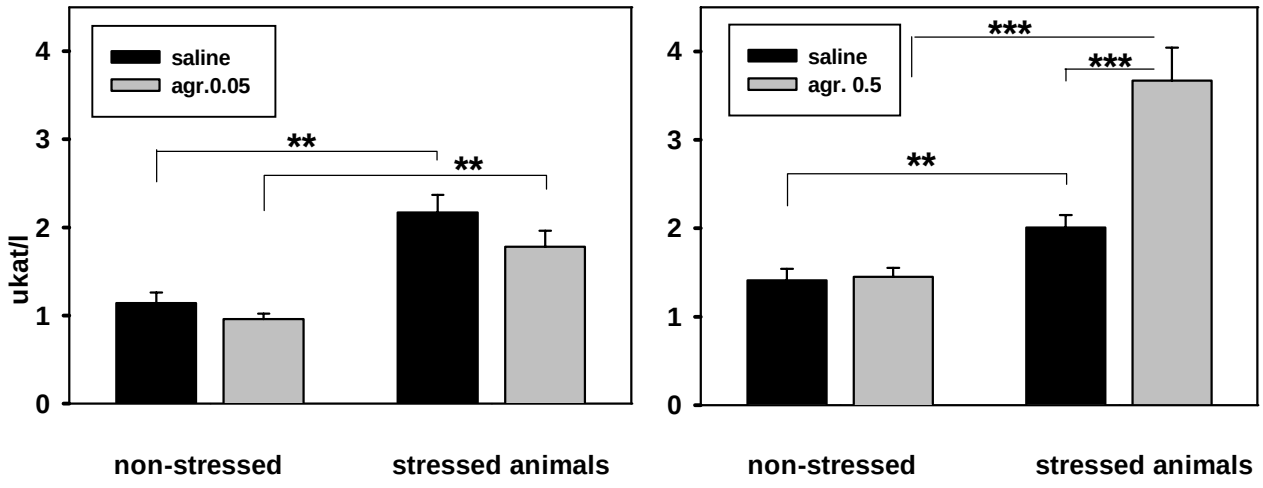

Fig. 3. *Effect of agroclavine (0.05 or 0.5 mg/kg) and water immersion-immobilization stress on ALT values. Saline = control (stressed or non-stressed) animals treated with saline in a volume of 0.2 ml/100 g of body weight (black columns), agr. 0.05 = animals treated with agroclavine (0.05 mg/kg); agr. 0.5 = animals treated with agroclavine (0.5 mg/kg); * p<0.05, ** p<0.01, *** p<0.001. The results are means ± S.E.M. of ALT values expressed in μkat/l, 15 animals were in groups of both stressed animals treated with agroclavine 0.5 mg/kg or saline, 6 animals were in all other groups.*

Discussion

The main aim of the present study was to verify the immunostimulatory effect of agroclavine on NK cell activity *in vivo*, which had been determined previously *in vitro* (Fišerová *et al.* 1997). The *in vivo* administration of agroclavine under non-stressful conditions did increase NK cell activity. As agroclavine is known to be a partial dopaminergic agonist, we may speculate about the role of dopamine receptor involved in stimulatory effect of this drug. The dopamine receptor was demonstrated to be responsible for some of the direct effects of antipsychotics on lymphocytes (Boukhris *et al.* 1988, Won *et al.* 1995). This is in agreement with recent molecular biology studies performed in human peripheral blood lymphocytes. The demonstration of different subtypes of dopamine receptors in a primary immune organ such as the thymus and in circulating immune cells supports the hypothesis that dopamine is involved in the control of immune function (Deleplanque *et al.* 1994, Ricci *et al.* 1997, 1999). Nozaki *et al.* (1996) found that natural killer cell activity was markedly decreased after intraperitoneal administration of haloperidol (a dopamine receptor blocker) for 5 days. We thus can suggest that the stimulatory effect of dopamine agonist agroclavine on NK cell activity could be mediated through dopamine receptors. On the other hand, agroclavine caused a significant decrease of NK activity under stress conditions. This effect may be due to the interaction of various factors. We can hypothesize that a large number of mediators (cytokines, neurotransmitters) is released during stress *via* activation of the hypothalamic-pituitary-adrenal (HPA) axis. Especially catecholamines could have synergistic effects with the sympathomimetic activity of agroclavine.

Activation of HPA axis could also be mediated indirectly *via* the production of proinflammatory cytokines induced by agroclavine. Indeed, agroclavine was found to increase the production of IL-1α, IL-2 and INFγ (Fišerová *et al.* 1995). These cytokines are known to play an important role in the feedback effect on the HPA axis and brain monoamines (Shintani *et al.* 1995). The engagement of these receptors on lymphoid cell membranes is one of the most important mechanisms preferentially regulating the NK cell activity *in vivo*. High levels of catecholamines produced during stress enhance α-adrenoceptor expression on lymphocytes, and subsequently inhibit NK cell functions. The role of β_2-adrenoceptors was also demonstrated (Hellstrand and Hermodsson 1989).

In our previous experiments (unpublished data), an α-adrenoceptor antagonist phentolamine administered in mice 30 min prior to the stress conditioning reversed the inhibitory effect of agroclavine on NK cell activity. We can thus suggest that the inhibitory effect of agroclavine on NK cell activity under stress conditions is preferentially caused by stimulation of α-adrenoceptors on the cell membrane.

Hence, the *in vivo* use of α blockade seems to be promising for eliminating the stress-induced effect on NK cells and for restoration of the positive effects of agroclavine mediated by D_1 receptors. The possible role of an α-adrenergic blocker to reverse this effect needs further examination.

The increased toxicity of agroclavine during stress could be explained by altered pharmacodynamic and/or pharmacokinetic properties of this substance.

Changes of pharmacodynamic properties could be explained by sympathetic nerve activity during stress. It has been proved (Arakawa *et al.* 1997, Starec *et al.* 1994) that the effect of stress on plasma CKMB and ALT values could be prevented by a β-adrenergic blocker. We can hypothesize that agroclavine further potentiates the stress reaction effect by its agonist properties at D_1 dopamine and/or α-adrenoceptors. The toxicity of agroclavine is thus enhanced during stress conditions. Another explanation might be that the pharmacokinetic properties of agroclavine are influenced by stress (alteration of body temperature) affecting the metabolism, distribution, and elimination of this substance. Decreased metabolism of the drug caused by a lower body temperature after immersion in cold water may be the reason for increased plasma concentration and toxicity.

The presented data have demonstrated that agroclavine increases NK cell activity *in vivo* under normal conditions and has little or no toxicity according to the ALT and CKMB changes. These promising results are limited by the fact that agroclavine is toxic under stress condition according to ALT and CKMB elevation and diminishes NK cell activity. The possible role of α-adrenergic blockers to reverse these adverse effects is under investigation.

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Reprint requests

Dr. Miroslav Starec, Department of Pharmacology, Third Faculty of Medicine, Charles University, Ruská 87, 100 00 Praha 10, Czech Republic, fax. 0042 2 67102461, e-mail Miroslav.Starec@LF3.cuni.cz