Induction of cytolytic activity and interferon-gamma production in murine natural killer cells by polymyxins B and E

Ming Zhong*   Yusuke Kadota[†]

Yoshio Shimizu[‡]   Eiichi Gohda**

*Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences

[†]Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences

[‡]Bizen Chemical Co., Ltd

**Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, gohda@pheasant.pharm.okayama-u.ac.jp

Induction of cytolytic activity and interferon-γ production in murine natural killer cells by polymyxins B and E

Ming Zhong [a,b], Yusuke Kadota [a], Yoshio Shimizu [b], Eiichi Gohda [a]*

[a] *Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima-naka, Okayama 700-8530, Japan*

[b] *Bizen Chemical Co., Ltd., 363 Tokutomi, Akaiwa City, Okayama 709-0716, Japan*

*Corresponding author:

    Eiichi Gohda, Ph.D.

    Professor

    Department of Immunochemistry

    Division of Pharmaceutical Sciences

    Okayama University Graduate School of Medicine,

    Dentistry and Pharmaceutical Sciences

    Tsushima-naka, Okayama 700-8530, Japan

    Tel.: +81-86-251-7960

    Fax: +81-86-251-7926

    E-mail: gohda@pheasant.pharm.okayama-u.ac.jp

Abstract

Natural killer (NK) cells are the primary effector cells of the innate immune system and have well-established roles in tumor rejection and resistance to viruses, bacteria and certain parasites. There is a need for more specific immune modulators of NK cell activity that lack the wide-ranging side effects of NK cell-stimulatory interleukins. The polycationic antibiotic polymyxin B (PMB) has been shown to have a unique ability to enhance activities of some immune cells, independent of its antibiotic properties. Here we report that both PMB and its analog polymyxin E (PME) markedly enhanced the activity of NK cells enriched from the murine spleen. Maximal activation of NK cell activity was obtained after 24 h of incubation with PMB at a dose of 300 µg/ml. PMB nonapeptide, one of the two PMB domains, and PME methanesulfonate, the negatively charged derivative of PME, had little effect on NK cell activity. PMB induced interferon (IFN)-γ and tumor necrosis factor-α production in NK cells. Proliferation of NK cells *in vitro* was significantly stimulated by being incubated with PMB. Administration of PMB to mice for 7 consecutive days stimulated splenic NK cell acitivity and increased NK cell populations in the spleen. These results suggest that the polycationc antibiotics PMB and PME may up-regulate innate and adaptive immune responses by induction of NK cell activity and IFN-γ production.

Keywords: Polymyxin B (PMB); Polymyxin E (PME); NK cells; IFN-γ

1. Introduction

Natural killer (NK) cells are the primary effector cells of the innate immune system and have well-established roles in tumor rejection in a variety of spontaneous and induced cancer models and resistance to viruses, bacteria and certain parasites [1-5]. The functions of NK cells are regulated by a complex balance of inhibitory and activating signals that allow them to selectively target and kill cells that display an abnormal pattern of cell surface molecules while leaving normal healthy cells unharmed. These signals are transmitted by inhibitory receptors that bind class I major histocompatibility complex (MHC) molecules and by activating receptors that bind ligands on tumors and pathogen-infected cells. Other than surface receptors, cytokines, such as interleukin (IL)-2, IL-12, IL-15, IL-18 and interferon (IFN)-α/β, have been shown to promote NK cell priming [6,7]. Clinical trials to enhance the anti-tumor potential of NK cells and the innate immune system have been performed over the last decade [8]. However, systemic administration of cytokines that regulate NK cell proliferation and/or activation perturb complex regulatory pathways, and serious side effects, including hypertension, cachexia, fever, chills, vomiting and diarrhea, have been observed [9-11]. There is an obvious need for more specific immune modulators that lack the wide-ranging side effects of the cytokines currently found in the clinical trials.

Polymyxins B and E, which consist of a polycationic decapeptide and a fatty acid residue linked by an amide bond, show antibiotic activity against a diverse array of organisms, including bacteria, yeasts, and protozoa [12,13]. Polymyxin B (PMB) is especially active in causing permeabilization and killing of Gram-negative bacteria and has therefore been used for treating certain bacterial infections. Membrane disruption of the bacteria appears to be induced by interaction between PMB and bacterial phospholipids and lipopolysaccharide (LPS) in the outer membrane [12]. The high affinity of PMB for LPS is currently exploited to remove contaminating LPS from solutions and reagents, thus avoiding endotoxin shock or excluding the presence of potential LPS contaminations in experimental systems [14]. In addition to its abilities to neutralize LPS and kill microbes, PMB has been shown to enhance

activities of some immune cells: it activated B cells [15], induced maturation of monocyte-derived dendritic cells [16], and enhanced immunostimulatory sequence-induced IFN-α production in plasmacytoid dendritic cells [17]. Prompted by these reports indicating direct activity of PMB on innate immune cells, we examined the effect of PMB on activity of NK cells, the primary effector cells of the innate immune system. We found in this study that PMB remarkably enhanced murine NK cell cytolytic activity both *in vitro* and *in vivo*.

2. Materials and methods

PMB sulfate salt, polymyxin E (PME, also known as colistin) sulfate salt, PME sodium methanesulfonate, and PMB nonapeptide hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IL-2 was purchased from Hoffmann-La Roche (Nutley, NJ). FITC-conjugated anti-mouse CD11c monoclonal antibody (mAb) was obtained from BD Biosciences Pharmingen (San Jose, CA). Anti-mouse IFN-γ mAb, biotinylated anti-mouse IFN-γ mAb, IFN-γ, TNF-α, and IL-12 were purchased from Endogen (Woburn, MA). Anti-mouse TNF-α antibody, biotinylated anti-mouse TNF-α antibody, anti-mouse IL-12 mAb, biotinylated anti-mouse IL-12 antibody, and a mouse IFN-α ELISA kit were obtained from R&D Systems (Minneapolis, MN).

Female C57BL/6 mice were purchased from Charles River Japan, Inc. (Yokohama, Japan) and maintained under specific pathogen-free conditions in the animal facility of Okayama University. All experiments were performed according to the guidelines for animal experiments of Okayama University. Murine spleen cells from 8- to 12-week-old mice depleted of erythrocytes were prepared by lysis of erythrocytes with ammonium chloride. The spleen NK cells were enriched from erythrocyte-depleted spleen cells by negative selection using a mouse NK cell enrichment set (BD Biosciences Pharmingen) according to the manufacturer's instructions, except that adherent cells were removed by incubating the cells on plastic dishes for 90 min at 37 °C before the procedure for negative selection. The purity of recovered viable NK cells ranged between 75 and 80% when the cells were stained with

PE-conjugated anti-mouse NK1.1 mAb (BD Biosciences Pharmingen), FITC-conjugated anti-mouse CD3 ε chain mAb (BD Biosciences Pharmingen) and propidium iodide after preincubation of the cells with anti-mouse CD16/CD32 mAb (BD Biosciences Pharmingen) and then analyzed by a flow cytometer (Epics XL, Beckman Coulter, Miami, FL). In some experiments, enriched NK cells were further purified by positive selection using a mouse NK cell separation set (BD Biosciences Pharmingen) according to the manufacturer's instructions. The purification step resulted in 96-97% NK1.1$^+$CD3$^-$ NK cells.

For *in vitro* NK activity and cytokine production assays, NK cells (5×10^4 cells/200 μl/well) were incubated for 24 h, unless otherwise specified, with or without PMB and other agents in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml of penicillin G and 100 μg/ml streptomycin (complete medium), at 37℃ in an atmosphere containing 5% CO_2 in 96-well round-bottom plates (Nunc, Roskilde, Denmark). NK cell activity was then determined by target cell retention of the fluorescent dye Calcein acetoxymethyl ester (Calcein-AM; Molecular Probes, Eugene, OR) [18-20]. Briefly, YAC-1 cells (10^6/ml of complete medium), obtained from Riken BRC Cell Bank (Tsukuba, Japan), were pre-incubated with 15 μM Calcein-AM for 30 min at 37℃ with occasional shaking and washed twice with the complete medium. The pre-cultured NK cells (effector cells) in the plate were washed once with the complete medium and incubated for 4 h with the YAC-1 cells (target cells, 10^4) at an E:T ratio of 5:1 in 200 μl/well of complete medium. Following the incubation, the plate containing cells was centrifuged at $420 \times g$ for 10 min, and the resulting supernatant was removed from the cellular fraction by rapidly inverting the plate and flicking the supernatant out. The plate was blotted dry, and 200 μl of 2% Triton X-100 in 0.05 M borate buffer (pH 9.0) was added to each well. After incubation for 10 min at room temperature to solubilize remaining cells, individual wells were assayed for fluorescence with a microplate fluorometer (Fluoroskan). The wavelengths of the filters used were excitation at 485 nm and emission at 527 nm. The percentage of cytotoxic activity was calculated as: Cytotoxic activity (%) = (average fluorescence in wells incubated with target cells alone − fluorescence in the well of the

experimental group)/(average fluorescence in wells incubated with target cells alone − average fluorescence in wells incubated with effector cells alone) × 100. The IFN-γ level in each cultured supernatant was measured by an ELISA as described previously [20]. Levels of TNF-α, IL-12, and IFN-α in each cultured supernatant were measured by an ELISA according to the manufacturer's instructions.

For determination of DNA synthesis, NK cells (5×10^4 cells/200 μl/well) were incubated for 24 h with or without PMB. The cells were then pulse-labeled with [^{3}H]thymidine (0.5 μCi/well, 2.5 Ci/mmol) for 18 h and harvested on glass fiber filters using a cell harvester. The amount of [^{3}H]thymidine incorporated was measured in a liquid scintillation counter.

For determination of the *in vivo* effect of PMB on NK activity, erythrocyte-depleted spleen cells (1×10^6 cells/200 μl/well) from mice administered PMB instead of enriched NK cells were incubated with the target cells at E:T ratios of 25:1, 50:1 and 100:1 for the NK cytotoxicity assay described above.

3. Results and discussion

NK cells enriched by negative selection were incubated with PMB for 24 h, and their cytotoxic acitivity against YAC-1 target cells was assayed. NK cell activity was enhanced in a dose-dependent manner by 30−300 μg/ml of PMB (Fig. 1A). The effective concentrations are comparable to those previously reported for B cells and dendritic cells [15-17]. Maximal effect of PMB was observed at the concentration of 300 μg/ml, inducing a 7-fold increase in NK cell activity (Fig. 1A) and as marked as the effect of 5 U/ml of IL-2 (Fig. 1C). The activity of NK cells purified by negative plus positive selection was similarly enhanced by PMB (Fig. 1A). Incubation for 24 h or more was necessary for the full enhancing effect (Fig. 1B). PME, in which D-phenylalanine of PMB cyclic peptide is replaced by D-leucine, was also effective (Fig. 1C). Both PMB and PME have two major domains: a polycationic decapeptide domain containing a heptapeptide ring and a fatty acid residue linked by an amide bond. In order to determine whether one or both domains are required to stimulate NK

cell activity, PMB nonapeptide and PME methanesulfonate were employed. PMB nonapeptide lacks the hydrophobic tail but retains the positively charged cyclic peptide domain. PME methanesulfonate is negatively charged instead of being positively charged, but the hydrophobic tail is intact. Neither PMB nonapeptide nor PME methanesulfonate had a notable effect on NK activity (Fig. 1C).

It is known that IFN-γ is a key cytokine during innate and adaptive immune responses, and activated NK cells and T cells, which are the major types of innate and adaptive immune cells, respectively, produce IFN-γ [21,22]. We examined whether IFN-γ production is up-regulated in NK cells cultured with PMB and PME. As shown in Fig. 2A, both PMB and PME enhanced IFN-γ production as markedly as did 5 U/ml of IL-2. NK cells can produce TNF-α, and production of this cytokine is increased by activation with IL-2 [23,24]. PMB also enhanced TNF-α production as potently as did 5 U/ml of IL-2 in NK cells enriched by negative selection (Fig. 2B) and in NK cells purified by negative plus positive selection (data not shown). There were a very few dendritic cells in the both NK cell preparations purified by negative selection only and negative plus positive selection (less than 0.5% and 0.1%, respectively). Neither IFN-α nor IL-12 was detected in the culture supernatants of NK cells incubated with or without PMB (data not shown).

In order to examine the *in vivo* effect of PMB on NK activity, mice were intraperitoneally administered PMB for 7 consecutive days, and NK activities of their splenocytes were determined. The splenic NK activity in mice treated with PMB significantly increased at all of the three different effector:target ratios (Fig. 3). PMB had no significant effect on body weight gain of mice during the experimental periods (data not shown). NK cell populations also significantly increased in the mice treated with PMB, as determined by flow cytometry (Fig. 4A and 4B). Proliferation of NK cells *in vitro* was measured by being labeled with [3H]thymidine for the last 18 h during 42-h incubation with or without PMB. The antibiotic caused a significant increase in [3H]thymidine uptake by NK cells (Fig. 4C).

PMB has been used due to its high affinity for LPS and being devoid of pleiotropic effects to exclude the presence of potential contaminations of LPS, a potent activator of innate

immune cells, in experimental systems. However, recent studies have shown that PMB is capable of directly activating some functions of dendritic cells [16,17]. We demonstrated in this study that PMB activated murine NK cells, the primary effector cells of the innate immune system, to kill tumor cells and produce IFN-γ and TNF-α at doses comparable to those effective on dendritic cells. To the best of our knowledge, this is the first report on the regulation of NK cell function by PMB. We also demonstrated stimulation of NK cell activity and proliferation by PMB administration *in vivo*.

The mechanism responsible for PMB-induced NK cell activation has not been elucidated, but our studies using derivatives of PMB and PME suggested that both polycationic peptide and hydrophobic tail domains of the polymyxins are required for promoting cytolytic activity of NK cells. The two domains of PMB confer on the antibiotic the amphipathic property of solubilizing in water or lipid membranes, which is necessary for optimal membrane disruption of Gram-negative bacteria induced by the antibiotic. In contrast, it has been reported that polyanions such as heparin and dextran sulfate inhibit NK activity [25]. PMB is known as an inhibitor of protein kinase C (PKC) [26]. This activity of PMB, however, may not be involved in the activation of NK cells because PKC has been shown to play an important role in activating NK cell cytotoxicity and because the PKC inhibitor H-7 inhibited NK cell activity [27,28]. Valentinis et al. recently reported that PMB activates IκB-α/NF-κB and extracellular signal-regulated kinase (ERK)1/2 signal transduction pathways in human dendritic cells [16]. A recent study by Ortaldo et al. showed that both ERK and p38 mitogen-activated protein kinase (MAPK) pathways are required for the synergistic production of IFN-γ by cross-linking of activating Ly49D or NKG2D murine NK cell receptor combined with IL-12 or IL-18 [29]. Thus, the ERK pathway is one of the candidates involved in PMB-induced activation of NK cells and IFN-γ production. In fact, we have obtained the results that the PMB-induced cytolytic activity of NK cells was inhibited by either the ERK kinase inhibitor PD98059 or the p38 MAPK inhibitor SB203580 (our unpublished data). Detailed biochemical and molecular analyses of PMB activity may provide new insights into the mechanism of NK cell activation.

Since NK cells are critical players during tumor growth control through a cytotoxic response and since IFN-γ plays a significant role in preventing development of primary and transplanted tumors [1,2,22], our experiments suggest that an anti-tumor effect of PMB should be investigated. Moreover, NK cells are capable of mediating antibody-dependent cellular cytotoxicity (ADCC) against an antibody-coated target via their expression of a low-affinity receptor for IgG (FcγRIII or CD16) [4]. Thus, our observations also suggest that PMB will become an effective adjuvant to administer in combination with anti-tumor antibodies.

References

[1] Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. Nat Rev Immunol 2001;1:41-9.

[2] Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer 2002;2:850-61.

[3] Kirwan SE, Burshtyn DN. Regulation of natural killer cell activity. Curr Opin Immunol 2007;19:46-54.

[4] Sun PD. Structure and function of natural-killer-cell receptors. Immunol Res 2003;27:539-48.

[5] Vivier E, Nunes JA, Vely F. Natural killer cell signaling pathways. Science 2004;306:1517-9.

[6] Zwirner NW, Fuertes MB, Girart MV, Domaica CI, Rossi LE. Cytokine-driven regulation of NK cell functions in tumor immunity: role of the MICA-NKG2D system. Cytokine Growth Factor Rev 2007;18:159-70.

[7] Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP. Natural killer cells in antiviral defence: function and regulation by innate cytokines. Annu Rev Immunol 1999;17:189-220.

[8] Salgaller ML, Lodge PA. Use of cellular and cytokine adjuvants in the immunotherapy of cancer. J Surg Oncol 1998;68:122-38.

[9] Rodolfo M, Colombo MP. Interleukin-12 as an adjuvant for cancer immunotherapy. Methods 1999;19:114-20.

[10] Salem ML, Gillanders WE, Kadima AN, El-Naggar S, Rubinstein MP, Demcheva M, et al. Novel nonviral delivery approaches for interleukin-12 protein and gene system: curbing toxicity and enhancing adjuvant activity. J Interferon Cytokine Res 2006;26:593-608.

[11] Chada S, Ramesh R, Mhashilkar AM. Cytokine- and chemokine-based gene therapy for cancer. Curr Opin Mol Ther 2003;5:463-74.

[12] Storm DR, Rosenthal KS, Swanson PE. Polymyxin and related peptide antibiotics. Annu Rev Biochem 1977;46:723-63.

[13] Evans ME, Feola DJ, Rapp RP. Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant Gram-negative bacteria. Ann Pharmacother 1999;33:960-7.

[14] Bhor VM, Thomas CJ, Surolia N, Surolia A. Polymyxin B: an ode to an old antidote for endotoxic shock. Mol BioSyst 2005;1:213-22.

[15] Smith CIE, Hammarström L. B-cell activating properties of polymyxin B. Scand J Immunol 1978;7:67-72.

[16] Valentinis B, Bianchi A, Zhou D, Cipponi A, Catalanotti F, Russo V, et al. Direct effects of polymyxin B on human dendritic cells maturation. The role of IkB-α/NF-kB and ERK1/2 pathways and adhesion. J Biol Chem 2005;280:14264-71.

[17] Marshall JD, Higgins D, Abbate C, Yee P, Teshima G, Ott G, et al. Polymyxin B enhances ISS-mediated immune responses across multiple species. Cell Immunol 2004;229:93-105.

[18] Neri S, Mariani E, Meneghetti A, Cattini L, Facchini A. Calcein-acetoxymethyl cytotoxicity assay: standardization of a method allowing additional analyses on recovered effector cells and supernatants. Clin Diagn Lab Immunol 2001;8:1131-5.

[19] Kolber MA, Quinones RR, Gress RE, Henkart PA. Measurement of cytotoxicity by target cell release and retention of the fluorescent dye bis-carboxyethyl-carboxyfluorescein (BCECF). J Immunol Methods 1988;108:255-64.

[20] Zhong M, Tai A, Yamamoto I. *In vitro* augmentation of natural killer activity and interferon-γ production in murine spleen cells with *Agaricus blazei* fruiting body fractions. Biosci Biotechnol Biochem 2005;69:2466-69.

[21] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-γ. Annu Rev Immunol 1997;15:749-95.

[22] Ikeda H, Old LJ, Schreiber RD. The roles of IFNγ in protection against tumor development and cancer immunoediting. Cytokine Growth Factor Rev 2002;13:95-109.

[23]   Cuturi MC, Murphy M, Costa-Giomi MP, Weinmann R, Perussia B, Trinchieri G. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. J Exp Med 1987;165:1581-94.

[24]   Hunter CA, Gabriel KE, Radzanowski T, Neyer LE, Remington JS. Type I interferons enhance production of IFN-γ by NK cells. Immunol Lett 1997;59:1-5.

[25]   Yamamoto H, Fujiya S, Arai S, Sendo F. Inhibition of mouse natural killer cytotoxicity by heparin. Cell Immunol 1985;96:409-17.

[26]   Mazzei GJ, Katoh N, Kuo JF. Polymyxin B is a more selective inhibitor for phospholipid-sensitive Ca^{2+}-dependent protein kinase than calmodulin-sensitive Ca^{2+}-dependent protein kinase. Biochem Biophys Res Commun 1982;109:1129-33.

[27]   Ting AT, Schoon RA, Abraham RT, Leibson PJ. Interaction between protein kinase C-dependent and G protein-dependent pathways in the regulation of natural killer cell granule exocytosis. J Biol Chem 1992;267:23957-62.

[28]   Itoh M, Tanabe F, Sato A, Takami Y, Shigeta S. A potent inhibitor of protein kinase C inhibits natural killer activity. Int J Immunopharmacol 1998;10:211-6.

[29]   Ortaldo JR, Winkler-Pickett R, Wigginton J, Hormer M, Bere EW, Mason AT, et al. Regulation of ITAM-positive receptors: role of IL-12 and IL-18. Blood 2006;107:1468-75.

Legends for the Figures

Fig. 1. Enhancement of murine NK cell cytotoxic activity *in vitro* by PMB. Murine NK cells enriched by negative selection were incubated for 24 h with the indicated doses of PMB (A), for the indicated periods with or without 300 μg/ml of PMB (B), and for 24 h with or without 5 U/ml of IL-2 or 300 μg/ml of each of PME, PME methanesulfonate, PMB nonapeptide, and PMB (C). NK cells purified by negative plus positive selection were also incubated for 24 h with or without 300 μg/ml of PMB (A). The NK cell cytotoxic activity against YAC-1 tumor cells was then determined. The data are means ± S.D. of three independent experiments (A and C) or representative of two independent experiments with similar results (B). * $p<0.05$, ** $p<0.01$, and *** $p<0.001$, as compared with the values of control cultures incubated in the medium alone (Dunnett's *t*-test).

Fig. 2. Induction of IFN-γ and TNF-α production in murine NK cells *in vitro* by PMB and PME. Murine NK cells enriched by negative selection were incubated for 24 h with or without 300 μg/ml of PMB, 300 μg/ml of PME or 5 U/ml of IL-2. The levels of IFN-γ (A) and TNF-α (B) in the culture supernatants were measured by an ELISA. The data are means ± S.D. of three independent experiments. ** $p<0.01$ and *** $p<0.001$, as compared with the values of control cultures incubated in the medium alone (Dunnett's *t*-test).

Fig. 3. Augmentation of splenic NK cell cytotoxic activity by intraperitoneal administration of PMB. Mice were intraperitoneally administered 5 mg/kg of PMB in 0.2 ml/head of saline or saline alone (control) for 7 consecutive days. NK cell cytotoxic activity of erythrocyte-depleted splenocytes against YAC-1 tumor cells was determined at the indicated E:T ratios. The data are means ± S.D. (n=4). * $p<0.05$ and ** $p<0.01$, as compared with the values of control mice (Student's *t*-test).

Fig. 4. Proliferation of splenic NK cells *in vivo* and *in vitro* induced by PMB. Mice were

intraperitoneally administered 5 mg/kg of PMB in 0.2 ml/head of saline or saline alone (control) for 7 consecutive days. Spleen cells were then stained with PE-conjugated NK1.1 mAb and FITC-conjugated CD3 mAb and analyzed by a flow cytometer. Profiles (A) are representative of three mice in each group. A percentage of NK1.1$^+$CD3$^-$ NK cell populations were shown in the profiles (A) and the graph (B). NK cells purified from spleen of untreated mice by negative plus positive selection were incubated with or without 300 μg/ml of PMB for 42 h, being pulse-labeled with [^{3}H]thymidine for the last 18 h (C). The data are means ± S.D. (n=3). ** $p<0.01$, as compared with the values of control (Student's t-test).