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Suppressive effect of diazepam on IFN-γ production by human T cells

Min Wei [a,b], Li Li [a], Rui Meng [a], Yanying Fan [a], Yun Liu [a], Liang Tao [c], Xianguo Liu [d,*], Changyou Wu [a,*]

[a] Department of Immunology, Zhongshan School of Medicine; Sun Yat-Sen University, Guangzhou 510080, PR China
[b] Department of Pharmacy, Nursing School of Guangzhou Medical University, Guangzhou 510180, PR China
[c] Department of Pharmacology, Zhongshan School of Medicine; Sun Yat-Sen University, Guangzhou 510080, PR China
[d] Department of Physiology, Zhongshan School of Medicine; Sun Yat-Sen University, Guangzhou 510080, PR China

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ABSTRACT

Many studies showed that benzodiazepines could modulate immune responses through interaction with peripheral benzodiazepine receptors (PBRs) in immune cells but most of the studies were focused on monocytes and macrophages. In the present study, we revealed that diazepam, a mixed-type benzodiazepine, inhibited IFN-γ production by human peripheral blood mononuclear cells (PBMCs) induced by anti-CD3 in dose-dependent manner. Flow cytometry analysis demonstrated that diazepam could inhibit the frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells. The inhibitory effect of diazepam on IFN-γ production is similar to that of $R_0$5-4864, a selective PBRs ligand. However, D8555, a selective ligand for PBRs in microglia in the central nervous system, is a much weak inhibitor compared with $R_0$5-4864 or diazepam. The inhibitory effect of $R_0$5-4864 could be antagonized by PK11195, which is recognized as selective PBRs antagonist, and suppressive effect of diazepam on T cells is partially antagonized by PK11195. Collectively, these results suggested that diazepam suppressed human T cell function through PBRs.

1. Introduction

Diazepam is one of the classic benzodiazepines, which are widely used due to their central actions, including anxiolytic, sedative, hypnotic, anticonvulsant and muscle-relaxant properties. The special benzodiazepine receptors (BDZ-Rs) in cortex are responsible for the central actions of benzodiazepines. BDZ-Rs are a component of $GABA_A$ receptor chloride ion channel complex in the cell membrane. Binding of benzodiazepine to BDZ-Rs causes a conformational change of the $GABA_A$ receptors and the exposure of high affinity sites to $GABA_A$, thus intensifying the binding of GABA with the receptors, resulting in anxiolytic, sedative, hypnotic, anticonvulsant and muscle-relaxant effects of the drug [1,2].

Two major classes of BDZ-Rs have been identified as central BDZ-Rs and peripheral BDZ-Rs. The central benzodiazepine receptors (CBRs are one component of $GABA_A$ receptor in brain cortex. The PBRs are first discovered in peripheral organs and later in the CNS, where they are associated with glial cells [3]. PBRs are expressed in immune cells abundantly and there are reports that benzodiazepines could modulate immune responses through interaction with PBRs in immune cells [4–8]. However, most of the studies are focused on monocytes and macrophages. The modulatory effect of benzodiazepines on T cells is limited and the underlying mechanism is poorly defined [9–11].

Our study found that diazepam, which is a mixed-type benzodiazepine and can act on both CBRs and PBRs, had inhibitory effect on human T cell function. To further explore the property, several selective BDZ-Rs ligands, $R_0$5-4864, PK11195 and D8555 were used in vitro on human peripheral blood cells. $R_0$5-4864 and PK11195 are selective PBRs ligands, whose affinity for PBRs is 1000 times as that for CBRs [12] while D8555 is a selective ligand for PBRs in microglia in the central nervous system. The results indicated that diazepam inhibited human T cell function through PBRs in T cells.

2. Materials and methods

2.1. Peripheral blood mononuclear cells (PBMCs) preparation

Peripheral blood mononuclear cells were obtained from 15 healthy volunteer donors (13 female and 2 male, aged from 20 to 35 years) without autoimmune or infectious disease. Adequate informed consent was obtained from all individuals involved in this study. The study was approved by the Medical School Review Board at Sun Yat-sen University.

2.2. Reagents and mAbs

Diazepam, $R_0$5-4864, D8555, PK11195 and Brefeldin A were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-CD3 antibodies used for cell culture were purchased from BD Bioscience Pharmingen (San Jose, CA, USA). RPMI 1640 medium were purchased

* Corresponding authors.
E-mail addresses: liuxg@mail.sysu.edu.cn (X. Liu), changyou_wu@yahoo.com (C. Wu).

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from Invitrogen (Grand Island, NY). Fetal calf serum (FCS) was purchased from HyClone (Logan, UT). Ficoll-Paque was purchased from Tianjin HaoYang Biological Manufacture (Tianjin, China). The following monoclonal antibodies (mAbs) were used for intracellular cytokine analysis: phycoerythrin (PE)-labeled monoclonal anti-CD4, fluourescein isothiocyanate (FITC)-labeled monoclonal anti-CD8 and allophycocyanin (APC)-labeled IFN-γ was purchased from BD Bioscience Pharmingen (San Jose, CA, USA).

2.3. Cell preparation

Heparinized venous blood was obtained from healthy donors. PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation. Cells were washed twice in Hank's balanced salt solution. The cells were finally adjusted to a final concentration of 2×10^6 cells/mL in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U penicillin/ml, 100 μg streptomycin/ml, 2 mM L-glutamine and 50 μM 2-mercaptoethanol.

2.4. Cell culture conditions and IFN-γ detection by ELISA

PBMCs were seeded into the wells of 96-well culture plates (Becton Dickinson) (2×10^6 cells/mL) in triplicate. Anti-CD_3 (0.2 μg/mL) was added with or without diazepam or different PBR_S ligands to the wells. The plates were incubated for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Supernatants were collected and the level of IFN-γ was measured by ELISA kit (BD Bioscience Pharmingen) according to the manufacturer's instructions.

2.5. Cell culture conditions, flow cytometry staining and analysis

For cell surface staining of detection for CD4 and CD8 cells, PBMCs were cultured in tubes with anti-CD_3 (0.2 μg/mL) in the presence or absence of diazepam 10^{-5} M or $R_0$5-4864 (10^{-4} M). The tubes were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. For intracellular staining of IFN-γ, brefeldin A was added to cells at a final concentration of 10 μg/mL 6 h before the culture was completed to prevent secretion of IFN-γ. PBMCs were washed twice with phosphate-buffered saline (PBS) containing 0.1% BSA and 0.05% sodium azide, and the cells were incubated with PE-labeled anti-CD4 and FITC-labeled anti-CD8 at 4 °C in the dark for 30 min. The cells were thereafter washed twice and fixed in 0.5% paraformaldehyde before analysis using a FACS Calibur ™ (Becton Dickinson, San Jose, CA). Staining was performed after fixation in 4% paraformaldehyde, followed by permeabilization and staining in buffer containing 0.1% saponin. Analysis of flow data was performed using FlowJo software (Treestar, San Carlos, CA).

2.6. Statistical analysis

Unpaired student's *t*-tests were used to compare two groups. A value of $P<0.05$ was considered significant.

3. Results

3.1. Diazepam inhibited IFN-γ production by PBMCs

To determine whether diazepam affects IFN-γ production by PBMCs, PBMCs were cultured with anti-CD3 in the presence or absence of various concentrations of diazepam. After stimulation of 72 h, the level of IFN-γ in cell-free culture supernatants was assessed by ELISA. The results revealed that no IFN-γ was detected when PBMCs were cultured with medium alone and significant high levels of IFN-γ were detected with the stimulation of anti-CD3. Also detected was that diazepam inhibited IFN-γ production by PBMCs which was induced by anti-CD3 in dose-dependent manner ($rs=-0.952$, $P<0.001$) (Fig. 1). The inhibition rate at 10^{-5} M reached 81.5 ± 24.9%. Of note, the fact that the dead cells observed under microscope with Trypan blue dye exclusion assay in each group was below 5% showed that this inhibitory effect was not produced due to the death of cells caused by diazepam.

3.2. Diazepam inhibited IFN-γ production by $CD4^+$ and $CD8^+$ T cells

To further determine which subset of T cells was inhibited for the production of IFN-γ, intracellular cytokine staining was performed. After stimulation with anti-CD3 alone, about 5.24% of $CD4^+$ and 15.33% of $CD8^+$ T cells produced IFN-γ. However, the percentages were dropped to 2.74% for $CD4^+$ and 6.73% for $CD8^+$ T cells respectively in the presence of diazepam (10^{-5} M). These results indicated that diazepam could inhibit the rate of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells after stimulation with anti-CD3 (Fig. 2).

3.3. $R_0$5-4864 and D8555 inhibited IFN-γ production by PBMCs

To explore the mechanism of inhibitory effect of diazepam on PBMCs, the action of two selective ligands, $R_0$5-4864 and D8555 was compared. $R_0$5-4864 is a selective ligand for PBRs, whose affinity for PBRs is 1000 times as that for CBRs [12]. D8555 is a selective ligand for PBRs in microglia in the central nervous system. The results showed that $R_0$5-4864(10^{-4} M–10^{-6} M) inhibited IFN-γ production by PBMCs ($P<0.001$), whereas D8555 only showed inhibitory effect at relatively higher doses (10^{-4} M and 10^{-5} M) and was a weak inhibitor compared with $R_0$5-4864. Significant difference was detected between them (Fig. 3).

3.4. $R_0$5-4864 inhibited IFN-γ production by $CD4^+$ and $CD8^+$ T cells

To further determine which subset of T cells was inhibited for the production of IFN-γ by $R_0$5-4864, intracellular staining was performed. After stimulation with anti-CD3 alone, about 0.67% $CD4^+$ and 17.01% of $CD8^+$ T cells produced IFN-γ. However, the percentages were dropped to 0.26% for $CD4^+$ and 7.64% for $CD8^+$ T cells respectively in the presence of R_o 5-4864 (10^{-4} M). The results demonstrated that $R_0$5-4864 could inhibit the rate of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells (Fig. 4).

3.5. PK11195 antagonized inhibitory effect of diazepam on IFN-γ production by PBMCs

The inhibitory effect of diazepam on T cells is similar to that of $R_0$5-4864 and $R_0$5-4864 is a selective ligand for PBRs on T cells. To determine whether diazepam inhibited T cells through PBRs on T cells,

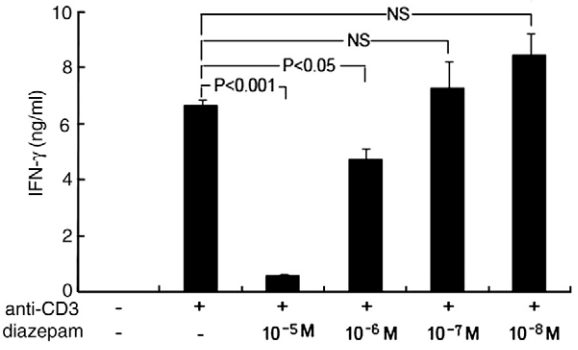

Fig. 1. Suppressive effects of diazepam on the production of IFN-γ by PBMCs. PBMCs were stimulated with anti-CD3 in the presence or absence of diazepam at different concentrations. After incubation for 72 h, culture supernatants were harvested and the levels of IFN-γ were measured by ELISA. The data shown are representative of results obtained in 3 independent experiments.