Benzodiazepine Receptor Agonists Modulate Thymocyte Apoptosis Through Reduction of the Mitochondrial Transmembrane Potential

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ABSTRACT—Peripheral-type benzodiazepines have been shown to exert immunological effects. In this study, we examined the effects of the peripheral-type benzodiazepines on murine thymocytes. Murine thymocytes that were incubated with the peripheral-type benzodiazepines underwent apoptosis associated with the collapse of mitochondrial transmembrane potential ($\Delta \phi_m$). The drugs stimulated dexamethasone- and etoposide-induced apoptosis with the enhanced collapse of $\Delta \phi_m$. The central-type benzodiazepines had no effect on either the $\Delta \phi_m$ or apoptosis. The reduction of $\Delta \phi_m$ depended on protein synthesis and protein phosphorylation. These results suggest that the immunomodulating effect of benzodiazepines is in part due to the modulation of thymocyte apoptosis associated with the collapse of $\Delta \phi_m$.

Keywords: Benzodiazepine, Thymocyte, Mitochondrial permeability transition, Proteasome, Apoptosis

Benzodiazepines are a class of frequently prescribed anxiolytic, anticonvulsant and sedative drugs whose pharmacological effects are mediated through the central-type receptor associated with the GABA$_A$ receptor (1). A second class of benzodiazepine binding sites, peripheral-type benzodiazepine receptor (PBR), is found in many peripheral tissues such as heart, adrenal, testis, liver, and hemopietic and lymphatic cells (2, 3). The physiological and pharmacological roles of these binding sites have not yet been established. The two classes of the receptors differ in their specificity for ligands such as benzodiazepines (1) and an isoquinoline carboxamide derivative, PK 11195 (4). Specific effects of peripheral-type benzodiazepines on immune cells such as macrophages and lymphocytes have been reported (2). The peripheral-type benzodiazepines inhibit the proliferation of lymphoid cells, modulate the oxidative bursts of neutrophils and macrophages, and inhibit the macrophage secretion of cytokines (2).

The mitochondrial transmembrane potential ($\Delta \phi_m$) results from the asymmetric distribution of protons and other ions on both sides of the inner mitochondrial membrane, and it maintains mitochondrial functions (5). The mitochondrial permeability transition (MPT) is the regulatable opening of a large, nonspecific pore in the inner mitochondrial membrane, and its sudden disruption collapses the $\Delta \phi_m$, resulting in disorders of many mitochondrial functions. Although the molecular elements that form this pore have not been definitively established, they are presumed to derive from well-known inner- and outermembrane constituents, including adenosine nucleotide translocator, porin molecules which interact with hexokinase and glycero kinase, and the complex forming PBR and its endogenous ligand endozepin (5). The MPT has been shown to be a critical event in cell death, both necrosis and apoptosis, in various cells including hepatocytes (6), fibroblasts (7) and thymocytes (8).

Because the PBR is an important component of the MPT pore, we examined here the effects of benzodiazepine-receptor agonists on thymocyte MPT and apoptosis to document their roles in immunomodulation.

MATERIALS AND METHODS

Chemicals

The benzodiazepines used were diazepam, 4'-chlorodiazepam and clonazepam. The benzodiazepines and 3,3'-dihexyloxacarbocyanide iodide (DiOC$_6$(3)) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-(2-Chlorophenyl)-N-(1-methylpropyl)-3-isoquinoline-carboxamide (PK 11195) was obtained from Research Biochemicals (Natick, MA, USA). 1-(5-Isoquinolinesulfonyl)-2-methylpirazin hydrochloride (H-7) was purchased from Seikagaku Kogyo Co. (Tokyo). Culture materials were purchased from Immuno-Biological
Laboratories (Fujioka). Other reagents were obtained from Nacalai Tesque Co. (Kyoto). The protease inhibitor peptides benzoyloxy carbonyl-Ile-Glu(O-t-Bu)-Ala-leucinal (PSI) and acetyl-Tyr-Val-Ala-Asp-chloromethylketone (AcYVADcmk) were purchased from Peptide Institute, Inc. (Osaka).

**Thymocyte culture**

Thymocytes from 6- to 7-week-old male BALB/c mice were incubated at a cell density of $4 \times 10^6$ to $7 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 10 mM HEPES, 50 $\mu$M 2-mercaptoethanol, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin at 37°C under 5% CO$_2$ (9).

**DNA fragmentation**

DNA fragmentation was determined by a fluorometric assay as described previously (9). The percentage of fragmented DNA was defined as the ratio of DNA in the detergent-soluble supernatant (fragmented) to DNA in the lysate (total DNA).

**Fluorometric analysis of $\Delta\Psi_m$**

After incubation, thymocytes were collected, washed once with phosphate-buffered saline (PBS), and further incubated at a cell density of $5 \times 10^6$ cells/ml in PBS containing $\text{DiOC}_6(3)$ at 40 nM at 37°C for 15 min. The cells were then washed once with PBS and resuspended in PBS, followed by analysis on a flow cytometer, FAC-SCalib (Becton Dickinson, San Jose, CA, USA).

**PCR analysis of PBR**

Total RNA from the thymocytes was prepared by the method of acid guanidin thiocyanate phenol chloroform. The first-strand cDNA was synthesized using 5 $\mu$g RNA as described previously (10). An aliquot of 2 $\mu$l of first-strand cDNA was used for the PCR. Primers specific for the PBR were synthesized using the sequence of mouse Leydig cell PBR cDNA (11). The 5' primer was 5'GCAATCACCATGCTGAATCCG3', and the 3' primer was 5'CATTCCTGCAAATGGCCTTCG3'. The G3PDH primer was purchased from Clonetech (Palo Alto, CA, USA). The PCR amplification was done using a cycler set for 30 cycles. The temperatures used for the PCR were as follows: denaturing at 94°C for 1 min; primer annealing at 55°C for 1 min; primer extension at 72°C for 2 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel, and DNA was visualized using ethidium bromide staining.

**RESULTS**

**Benzodiazepine receptor agonists induced apoptosis in thymocytes**

To examine whether benzodiazepine-receptor agonists modulate apoptosis, thymocytes were incubated with various compounds that bind to the peripheral- or central-type benzodiazepine receptors for 24 hr, and DNA fragmentation was determined. All of the benzodiazepines used except clonazepam induced DNA fragmentation dose-dependently (Fig. 1). Clonazepam, which is a central-type receptor agonist, did not induce significant DNA fragmentation (Fig. 1), although it induced a slight increase in DNA fragmentation at 50 $\mu$M. PK 11195, which is a derivative of isoquinoline carboxamide, also induced DNA fragmentation in a dose-dependent manner. Electrophoresis on agarose gels showed an internucleosomal ladder of DNA cleavage (data not shown), which is a characteristic of thymocyte apoptosis.

The DNA fragmentation induced by chlorodiazepam and PK 11195 was inhibited by actinomycin D, cycloheximide and H-7 (Fig. 2). The concentration of the inhibitors used was the optimum obtained in a previous study to inhibit thymocyte apoptosis (9). The inhibition of DNA fragmentation was accompanied by the reduction of lactate dehydrogenase (LDH) release into the medium.

![Fig. 1. Benzodiazepine agonists-induced thymocyte apoptosis.](image-url) Thymocytes were incubated with each of the benzodiazepine-receptor agonists shown for 24 hr, and DNA fragmentation was determined. Chlorodiazepam (□), PK 11195 (●), diazepam (■) and clonazepam (▲) were used at the concentrations indicated. Data show means of three separate experiments. ** and *: Significantly different from the control at P<0.01 and P<0.05, respectively (Student’s t-test).
(data not shown). These results suggest that the drugs capable of binding to the PBR induce thymocyte apoptosis in a process dependent upon the synthesis of macromolecules and protein phosphorylation.

**Benzodiazepine receptor agonists stimulated apoptosis induced by dexamethasone and etoposide**

Dexamethasone and etoposide are known to induce apoptosis associated with the Δψₘ reduction (12). To elucidate the effects of benzodiazepine-receptor agonists on the dexamethasone- and etoposide-induced apoptosis, thymocytes were treated for 6 hr together with the agonists, and DNA fragmentation was determined. The peripheral-type agonists did not induce DNA fragmentation above the control level at 6 hr of incubation as shown in Fig. 3, but they stimulated DNA fragmentation which was induced by dexamethasone or etoposide, whereas clonazepam, a central-type agonist, had no effect.

**Benzodiazepine-receptor agonists reduced the Δψₘ**

To address whether the induction of MPT by the benzodiazepine-receptor agonists relates to apoptosis and stimulates the dexamethasone-induced apoptosis, thymocytes were incubated with chlorodiazepam or PK 11195 in the presence or absence of dexamethasone, and we analyzed the Δψₘ with a flowcytometer using DiOC₆(3).

Chlorodiazepam and PK 11195 both provoked a gradual reduction in the Δψₘ (Fig. 4A). Chlorodiazepam induced DNA fragmentation of about 14% and 31% at 6 and 12 hr, respectively, increasing progressively to about 53% at 16 hr. The DNA fragmentation by PK 11195 was about 26% and 35% at 12 and 16 hr of incubation, respectively. Dexamethasone induced a profound reduction of the Δψₘ, which was enhanced by the addition of chlorodiazepam (Fig. 4B).

The addition of cycloheximide or H-7 did not affect the Δψₘ of the control thymocytes, but it inhibited the Δψₘ reduction induced by chlorodiazepam or PK 11195 (data not shown), followed by the inhibition of DNA fragmentation as seen in Fig. 2. Dexamethasone induced DNA fragmentation of about 39% (Fig. 3) and the low Δψₘ fraction was about 51% at 6 hr of incubation (Fig. 4B). The DNA fragmentation induced by dexamethasone was inhibited by cycloheximide or H-7 (9) and was associated with the suppression of the Δψₘ reduction (data not shown). The dexamethasone-induced collapse of the Δψₘ was enhanced by chlorodiazepam (Fig. 4B, Table 1). The enhanced reduction of the Δψₘ and DNA fragmentation were inhibited by cycloheximide and H-7 (Table 1). The synthesis and phosphorylation of protein(s) are required for both the Δψₘ reduction and DNA fragmen-

**Fig. 2.** Effects of inhibitors of macromolecular synthesis and protein phosphorylation. Thymocytes were incubated without or with chlorodiazepam (100 μM) or PK 11195 (100 μM) in the absence (open column) or presence of actinomycin D (closed column, 500 ng/ml), cycloheximide (dotted column, 10 μg/ml) or H-7 (hatched, 50 μM) for 24 hr, and DNA fragmentation was determined. Vertical bars represent the S.D. of six independent experiments. a: Significantly different from the values of each corresponding control at P < 0.05 (Tukey's t-test). b: Significantly different from the value in the presence of either chlorodiazepam or PK 11195 at P < 0.05.

**Fig. 3.** Effects of benzodiazepine agonists on dexamethasone- and etoposide-induced DNA fragmentation. Thymocytes were incubated with or without dexamethasone (1 μM) or etoposide (10 μM) in the absence (open column) or presence of chlorodiazepam (dotted column), diazepam (hatched column), PK 11195 (closed column) or clonazepam (checkered column), each at 50 μM, for 6 hr, and DNA fragmentation was determined. Data show means ± S.D. of three separate experiments. a: Significantly different from the values of each corresponding control at P < 0.01 (Tukey's test). b: Significantly different from the values in the presence of either dexamethasone or etoposide at P < 0.05.
tation, and the reduction of the $\Delta \phi_m$ is associated with the induction of DNA fragmentation.

The $\Delta \phi_m$ reduction was inhibited by a proteasome inhibitor but not by an inhibitor of caspases

We and others have shown that proteasome and AcYVADcmk-inhibitable caspases regulate apoptosis in various cells including thymocytes (13–15). We analyzed the effect of inhibitors of proteasome and caspases on the $\Delta \phi_m$. PSI, a proteasome inhibitor peptide, inhibited the thymocyte apoptosis induced by dexamethasone and etoposide (14, 15). PSI did not affect the $\Delta \phi_m$ and DNA fragmentation at 6 hr of incubation. However, PSI itself induced apoptosis (13, 14) associated with the reduction of $\Delta \phi_m$ after a 12-hr incubation (data not shown). Therefore, we could not identify the direct effect of PSI on the chlorodiazepam-induced $\Delta \phi_m$ reduction and DNA fragmentation at an early time of incubation. Dexamethasone induced DNA fragmentation of about 39% with the reduction of $\Delta \phi_m$ at 6 hr of incubation (Figs. 3 and 4B). PSI inhibited both the DNA fragmentation and the $\Delta \phi_m$ collapse induced by dexamethasone (Table 1). PSI also reduced the enhanced collapse of $\Delta \phi_m$ by chlorodiazepam plus dexamethasone and suppressed the DNA fragmentation (Fig. 5, Table 1), although the suppression was not complete. AcYVADcmk, which is an inhibitor of caspases, did not affect the $\Delta \phi_m$ in the cells treated with dexamethasone and with dexamethasone plus chlorodiaz-

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**Fig. 4.** Benzodiazepine-induced $\Delta \phi_m$ reduction. A: Thymocytes were incubated with chlorodiazepam or PK 11195, each at 50 $\mu$M, for 6 and 12 hr, and the $\Delta \phi_m$ was determined by DiOC$_6$(3) labeling. B: Thymocytes were incubated with 1 $\mu$M dexamethasone (a) or with 1 $\mu$M dexamethasone plus 50 $\mu$M chlorodiazepam (b) for 6 hr, followed by DiOC$_6$(3) labeling. Results are typical of three independent experiments.
Table 1. Effects of cycloheximide, H-7, PSI and AcYVADcmk on the reduction of the mitochondrial transmembrane potential ($\Delta \phi_m$) and DNA fragmentation induced by dexamethasone plus chlorodiazepam

<table>
<thead>
<tr>
<th>Conditions</th>
<th>$\Delta \phi_m$ (%)</th>
<th>DNA fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.2 ± 0.9a</td>
<td>10.5 ± 1.7</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>51.5 ± 1.9a</td>
<td>39.4 ± 0.7a</td>
</tr>
<tr>
<td>+ PSI</td>
<td>22.9 ± 2.3ab</td>
<td>11.2 ± 0.8c</td>
</tr>
<tr>
<td>+ AcYVADcmk</td>
<td>52.7 ± 2.0a</td>
<td>12.1 ± 0.9b</td>
</tr>
<tr>
<td>Chlorodiazepam</td>
<td>16.8 ± 2.0</td>
<td>13.9 ± 1.5</td>
</tr>
<tr>
<td>+ Chlorodiazepam</td>
<td>71.3 ± 0.2ab</td>
<td>53.5 ± 2.1ab</td>
</tr>
<tr>
<td>+ Cycloheximide</td>
<td>13.1 ± 2.2c</td>
<td>7.6 ± 0.6c</td>
</tr>
<tr>
<td>+ H-7</td>
<td>13.8 ± 1.6c</td>
<td>8.0 ± 0.8c</td>
</tr>
<tr>
<td>+ PSI</td>
<td>27.3 ± 1.1c</td>
<td>14.6 ± 0.7c</td>
</tr>
<tr>
<td>+ AcYVADcmk</td>
<td>73.2 ± 1.0c</td>
<td>9.8 ± 0.4c</td>
</tr>
</tbody>
</table>

Thymocytes were incubated with or without dexamethasone (1 μM) plus chlorodiazepam (50 μM) in the presence or absence of cycloheximide (10 μg/ml), H-7 (50 μM), PSI (20 μM) or AcYVADcmk (200 μM) for 6 hr, and DNA fragmentation and $\Delta \phi_m$ were determined. Data show means ± S.D. of three separate experiments. The values were evaluated by Tukey's test. *: P < 0.05 vs control, #: P < 0.05 vs the values only in the presence of dexamethasone, #: P < 0.05 vs the values in the presence of dexamethasone plus chlorodiazepam.

Benzodiazepine-induced thymocyte apoptosis

PBR mRNA was expressed in thymocytes

The above data indicate that benzodiazepines capable of binding to the peripheral-type binding sites, PBR, act on thymocytes, resulting in the modulation of apoptosis. To confirm the presence of PBR in thymocytes, the expression of PBR mRNA was analyzed. PBR mRNA was expressed in thymocytes (Fig. 6). The PCR product with a size of 549 bp was confirmed to be PBR by sequencing. It was also expressed in the mouse kidney, testis and liver, but at a lesser amount in the mouse brain.
DISCUSSION

The PBR was initially identified in many peripheral tissues and in some blood cells, including human lymphocytes and human and murine lymphoma cells. Drugs that bind with high affinity to the PBR have previously been described as having complex immunomodulating properties. The drugs inhibit the proliferation of mitogen-stimulated lymphocytes and the cellular immune responses (2, 3). In contrast, the in vivo administration of a lower dose of the drugs enhanced the T-cell-dependent antibody response (2). The drugs have also been reported to affect the functions of neutrophils and macrophages, resulting in the modulation of inflammation and immune responses (3).

The present study showed that the drugs which bind to the PBR induced apoptosis in the thymocytes (Figs. 1 and 2) and stimulated the dexamethasone- and etoposide-induced apoptosis (Fig. 3). The effect was accompanied by a reduction in the $\Delta \Phi_m$ (Fig. 4). The $\Delta \Phi_m$ is controlled by the mitochondrial permeability transition pore that contains the PBR as a component (16). The expression of PBR mRNA in thymocytes (Fig. 6) suggests that the peripheral-type benzodiazepines act on thymocytes through PBRs, although it cannot be concluded that the effect is due to the direct result of binding to the PBR. The enhanced $\Delta \Phi_m$ reduction by benzodiazepine plus dexamethasone is likely to depend on the synthesis of macromolecules and protein phosphorylation (Fig. 5) since it was inhibited by cycloheximide and H-7. The results suggest that the process is energy-dependent as is that by dexamethasone alone.

It has been shown that PBR agonists such as PK 11195 and diazepam potentiate cell killing by rotenone or tumor necrosis factor and are associated with the $\Delta \Phi_m$ reduction (7, 17). Protoporphyrin IX, which is an endogenous ligand of the mitochondrial benzodiazepine receptor in the kidney and liver, is well known for its MPT-triggering capacity (18), and it has been shown to induce apoptosis associated with the loss of $\Delta \Phi_m$ in thymocytes (8). However, protoporphyrin IX-induced thymocyte apoptosis does not occur in the absence of protein synthesis (8). PK 11195 and chlorodiazepam have no inhibitory effect on protein synthesis (19). The $\Delta \Phi_m$ reduction by benzodiazepines depended on the synthesis of macromolecules and protein phosphorylation, suggesting that the receptor-mediated signals evoke the $\Delta \Phi_m$ reduction and apoptosis. It has been reported that at least two types of PBR exist in human lymphocytes (20, 21); one is located in the plasma membrane and the other, in mitochondria. The plasma membrane PBR is recognized essentially only by an endogenous ligand, diazepam-binding inhibitor, whereas the mitochondrial type binds to both the diazepam-bind-
apoptotic cascade (16), the present study suggests that
the immunosuppressing effect of benzodiazepines may
in part be due to the thymocyte apoptosis induced by
the drugs in addition to the inhibition of lymphocyte
proliferation.

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