An Antagonistic Activity of Etizolam on Platelet-Activating Factor (PAF)
In Vitro Effects on Platelet Aggregation and PAF Receptor Binding

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Abstract—The antagonistic effect of etizolam, an anti-anxiety drug, on platelet-activating factor (PAF) was investigated in rabbit platelets in vitro. Etizolam inhibited PAF-induced aggregation in a dose-dependent manner, with an IC50 of 3.8 \(\mu\)M, about one tenth that of triazolam (IC50=30 \(\mu\)M). At 300 \(\mu\)M, it inhibited both ADP and arachidonic acid-induced aggregation only slightly, while the other anti-anxiety drugs tested had no effect on PAF-induced aggregation even at this concentration. Etizolam and triazolam inhibited the specific binding of 3H-PAF to PAF receptor sites on washed rabbit platelets with IC50 values of 22 nM and 320 nM, respectively. Diazepam and estazolam were inactive even at 1 \(\mu\)M. These results indicate that etizolam is a specific antagonist of PAF.

The psychotropic benzodiazepine drugs alprazolam and triazolam have been reported to inhibit specifically platelet-activating factor (PAF)-induced platelet aggregation (1). Etizolam (Depas®), a thienodiazepine drug used as an anti-anxiety agent (2, 3), has a chemical structure similar to that of triazolam. Its inhibitory effects on PAF-induced bronchoconstriction, PAF-induced hypotension and PAF-induced mortality in vivo were reported in a previous paper (4). These results indicate that it may be a PAF receptor antagonist. In this report, we demonstrate that etizolam is a specific PAF receptor antagonist by examining its effect on platelet aggregation and on PAF receptor binding in vitro.

Materials and Methods

1. Animals: Male Japanese albino rabbits (Seiwa Institute of Experimental Animals, Fukuoka Japan) were used. Animals were housed at constant temperature (23±2°C) and relative humidity (55±5%) and were allowed free access to food and water.

2. Materials: Etizolam, triazolam, diazepam, estazolam, lorazepam and chlordiazepoxide were synthesized by the chemical division of our laboratory and were dissolved in dimethyl sulfoxide. Platelet-activating factor (PAF, 1-o-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) was purchased from Serdary Research Lab. 3H-PAF (1-o-3H-octadecyl, 80 Ci/mmol) was obtained from Amersham (England). Stock solutions were made in ethanol and stored at −20°C. These solutions were diluted in 0.9% saline and placed on ice for use.

3. Preparation of platelet rich plasma (PRP), platelet poor plasma (PPP) and washed platelets: Blood samples were taken from the carotid arteries of rabbits with siliconized syringes containing 0.1 volumes of 3.8% sodium citrate and 0.1 volumes of ACD solution (38 mM citric acid, 38 mM sodium citrate and 136 mM glucose, pH 6.4) for platelet aggregation and PAF receptor binding assay, respectively. PRP was made
by centrifuging the blood at 200 g for 10 min, and PPP was made by centrifuging at 1400 g for 10 min. Washed platelet suspensions were prepared from PRP. PRP was sedimented with equal volumes of standard platelet buffer (3.2 mM KH₂PO₄, 4.8 mM Na₂HPO₄, 80 mM citric acid, 4.4 mM glucose, pH 6.5) at 1000 g for 10 min at 4°C. The pellets were resuspended in the same buffer as above and centrifuged at 1400 g for 5 min at 4°C. This procedure was repeated 3 times. The platelet pellets were finally resuspended in standard platelet buffer containing 0.9 mM CaCl₂ and 0.1% bovine serum albumin (BSA) at a concentration of 1.1×10⁸ platelets/ml.

**4. Assay for platelet aggregation:** Aggregation was measured at 37°C with a turbidimetric device (NKK Hematracer 1, model PAT-6A, Niko Bioscience, Tokyo, Japan) by a previously described method (5). Inhibition of platelet aggregation was assessed by comparing the maximal change in transmitted light in drug-treated PRP with that in vehicle treated PRP. The aggregometer was adjusted in sensitivity to give light transmission values of 0 and 100% for PRP and PPP, respectively. The aggregating agents were used at the minimal concentration which induces maximal aggregation.

**5. Assay for receptor binding:** The experiments were done according to the method of Valone (6) with a slight modification. A 920 µl aliquot of buffer containing 10⁸ platelets were added to siliconized tubes containing 40 µl of test compound solution and preincubated for 5 min at 25°C. The binding reaction was begun by adding 40 µl of [³H]-PAF (8 nM) to the tubes at 25°C and stopped by adding 4 ml of ice-cold saline containing 0.1% BSA (washing solution). Platelets were isolated by vacuum filtration through glass filters (Whatman GF/F filter, England). The filters were rapidly washed 4 times with 4 ml of ice-cold washing solution, and the radioactivity was measured in 4 ml of ACS-II scintillator (Amersham, England) with a scintillation counter (model 3380, Packard, U.S.A.). The experiments were done in triplicate. The inhibition percentage by the test compounds was calculated as:

\[
\text{Inhibition percentage} = \frac{\text{total binding} - \text{total binding with test compound}}{\text{total binding}} \times 100\%
\]

Results

1. **Platelet aggregation:** Incubation of rabbit PRP with etizolam for 2 min before administration of aggregating agents inhibited aggregation induced by PAF (190 nM) with an IC₅₀ of 3.8 µM. Etizolam at a higher concentration (300 µM) only slightly inhibited ADP (15 µM) and arachidonic acid (AA, 0.25 mM)-induced aggregation (Fig. 1). Among the compounds tested, it was the most potent inhibitor of PAF-induced aggregation and about 10 times more potent than triazolam (IC₅₀=30.0 µM). Other anti-anxiety drugs tested had no effect on PAF-induced aggregation (Fig. 2, Table 1).

2. **PAF receptor binding:** The time course for [³H]-PAF binding to rabbit platelets showed an increase in radioactivity associated with the platelets. Specific binding reached half-maximal values in 5 min, and approximate steady-state binding was achieved after a 10 min incubation. Therefore, we used an incubation time of 10 min in subsequent studies. Unlabelled PAF (1 µM) reversed the specific binding of [³H]-PAF in a time-dependent manner. Three min after the addition of PAF, the specific binding of [³H]-PAF was almost completely displaced (Fig. 3). Figure 4 shows the inhibition percentages of the test compounds on the specific binding of [³H]-PAF to the PAF receptor sites in the platelets. Etizolam inhibited [³H]-PAF (0.32 nM) receptor binding with an IC₅₀ of 22 nM, about one fifteenth that of triazolam (IC₅₀=320 nM). Diazepam and estazolam were inactive even at 1 µM (Fig. 4, Table 2).

Discussion

Etizolam, an anti-anxiety drug, potently inhibited PAF-induced aggregation of rabbit platelets. The effect was specific to PAF.
Etizolam and PAF

Fig. 1. Inhibitory effect of etizolam on aggregation induced by PAF (190 nM), ADP (15 μM) or arachidonic acid (AA, 0.25 mM) in rabbit platelets. Each concentration of etizolam (▲) was incubated with platelet-rich plasma for 2 min at 37°C before the administration of aggregating agents (▲). Numbers in the figures are the concentration (μM) of etizolam.

Fig. 2. The effects of etizolam and triazolam on PAF-induced aggregation of rabbit platelets in vitro. Results are shown as the mean±S.E. from 3 separate experiments. —— etizolam, —— triazolam.

Table 1. Effects of anti-anxiety drugs on PAF-induced aggregation of rabbit platelets

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>Etizolam</td>
<td>3.8</td>
</tr>
<tr>
<td>Triazolam</td>
<td>30.0</td>
</tr>
<tr>
<td>Estazolam</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Diazepam</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Chlordiazepoxide</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Oxazolam</td>
<td>&gt;300</td>
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Fig. 3. Time course for total and nonspecific binding of $^3$H-PAF to washed rabbit platelets and reversibility of specific binding of $^3$H-PAF to platelets. The platelets were incubated with 0.32 nM $^3$H-PAF for 3 to 30 min at 25°C. Unlabelled PAF was added to the incubation medium 15 min after addition of $^3$H-PAF (†). Nonspecific binding was defined as the amount of binding not inhibited with 1 μM unlabelled PAF. Results are shown as the mean from triplicate assays performed in a single experiment.

Fig. 4. The inhibitory effects of anti-anxiety drugs on specific binding of $^3$H-PAF to washed platelets. The platelets were preincubated with test compounds for 5 min at 25°C and then incubated with 0.32 nM $^3$H-PAF for 10 min at 25°C. Results are shown as the mean±S.E. from 3 experiments. – – – : etizolam, – – – : triazolam, – – – – : diazepam, – – – : estazolam.

Table 2. Inhibition of $^3$H-PAF binding to washed rabbit platelets by test compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etizolam</td>
<td>22</td>
</tr>
<tr>
<td>Triazolam</td>
<td>320</td>
</tr>
<tr>
<td>Diazepam</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Estazolam</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

activation, since it did not affect the responses of ADP and AA. Triazolam, a PAF receptor antagonist (1), also inhibited PAF-induced aggregation, but was 10 times less potent than etizolam. Other anti-anxiety drugs tested had no effect on PAF-induced aggregation, indicating that the inhibitory activity of etizolam and triazolam on PAF-induced aggregation had no relation with anti-anxiety activity.

Benzodiazepine receptors have been found in platelets and their roles on platelet functions have been discussed (7). Diazepam and flurazepam were reported to inhibit human platelet aggregation induced by ADP, AA or epinephrine at high concentrations (8), suggesting that their anti-platelet activity was mediated by the benzodiazepine receptors. However, O'Beirne and Williams (9) indicated that human platelets do not possess detectable binding sites for peripheral type benzodiazepines such as Ro 5-4864. Furthermore, in this report, the anti-anxiety drugs tested which had a high affinity for benzodiazepine receptors (10) did not inhibit PAF-induced aggregation of rabbit platelets. These results suggest that the anti-platelet effects of benzodiazepine derivatives are not
mediated via benzodiazepine receptors, and the inhibitory effects of etizolam and triazolam on PAF-induced aggregation are mediated via PAF receptors.

A PAF receptor binding assay was made to clarify the relation between PAF receptors and anti-anxiety drugs. Etizolam inhibited 3H-PAF binding to the platelets with an IC50 of 22 nM, about one fifteenth that of triazolam, a potency ratio comparable to that for inhibition of PAF-induced platelet aggregation in PRP. Diazepam and estazolam were ineffective in this study as in the platelet aggregation study. Although it is difficult to compare the anti-PAF activity of etizolam with other PAF-antagonists which have already been reported because of differences in both PAF concentration and the platelet source used, the anti-PAF activity of etizolam may be about the same as those of BN-52021 (11), kadsurenone (12) and CV-3988 (13).

In conclusion, etizolam is a specific PAF receptor antagonist, specifically inhibiting PAF-induced aggregation of rabbit platelets. It may be useful in treating disorders caused by PAF and may be useful tool for studying the pathophysiological significance of PAF.

References