Effect of Etizolam (Depas®) on Production of Superoxide Anion by Platelet-Activating Factor and N-Formyl-Methionyl-Leucyl-Phenylalanine-Stimulated Guinea Pig Polymorphonuclear Leukocytes

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Accepted March 26, 1988

Abstract—Effect of etizolam on platelet activating factor (PAF) and N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced superoxide anion (O$_2^-$) production in guinea pig polymorphonuclear leukocytes (PMNL) was investigated. Etizolam showed the inhibitory effect on PAF-induced O$_2^-$ production concentration dependently, with an IC$_{50}$ value of 4.7 µM, but it had no inhibitory effect on FMLP-induced O$_2^-$ production at 100 µM. These results suggest that etizolam has a selectively strong inhibitory effect on PAF-induced O$_2^-$ production in guinea pig PMNL.

Platelet activating factor (PAF, 1-o-hexadecyl/octadecyl-2-acetyl-sn-3-phosphocholine) has been found to be present in many cells and tissues (1-4). PAF has the ability to activate platelets, constrict bronchiole and induce hypotension, indicating that PAF may act as an important mediator in pathological processes such as anaphylaxis and inflammation (5). Etizolam (6-(o-chlorophenyl)-8-ethyl-1-methyl-4H-s-triazolo[2,3-e][1,4]diazepine, Depas®, Y-7131) is one of the thienodiazepine derivatives containing a triazole ring (6). It is used clinically as a potent and characteristic anti-anxiety drug which is different from the benzodiazepines. Etizolam has been found to possess an anti-PAF activity in vivo and in vitro as previously described (7, 8). In response to the inflammatory response, polymorphonuclear leukocytes (PMNL) generate superoxide anion (O$_2^-$) and its metabolites. The active oxygen generated by PMNL have been implicated as putative mediators of the tissue injury associated with the inflammatory process. This paper demonstrates the effect of etizolam on PAF and FMLP-induced O$_2^-$ production in vitro.

Female Hartley guinea pigs were used and fed ad libitum. Etizolam (Depas®), indomethacin, BW-755c, FPL-55712 and MK-447 were synthesized by the chemical division of our laboratories. Nifedipine was purchased from Bayer. All drugs were dissolved in ethanol, and the final concentration of ethanol in the reaction mixture was 1%. The preparation of guinea pig PMNL was described in another paper (9). The cells were suspended in 10 mM phosphate buffer, pH 7.4, containing 138 mM NaCl, 2.7 mM KCl, 0.6 mM CaCl$_2$ and 1.0 mM MgCl$_2$; and the number of cells was adjusted to 6.3×10$^6$ cells/ml. The production of O$_2^-$ was measured by superoxide dismutase-inhibitable reduction of cytochrome c as previously described (9, 10). The effects of test drugs were represented as the concentration of 50% inhibition (IC$_{50}$).

The production of O$_2^-$ by both PAF and FMLP stimulated guinea pig PMNL was maximal within 5 min and at the concentration of 10$^{-6}$ M (Fig. 1). The inhibitory effect of etizolam on PAF-induced O$_2^-$ production was concentration-dependent, with an IC$_{50}$ value of 4.7 µM; and it had no in-
hibitory effect on FMLP-induced $O_2^-$ production at 100 nM (Table 1). The results of the test drugs are summarized in Table 1. Indomethacin, BW-755c, FPL-55712 and nifedipine had no inhibitory effect, but MK-447 showed an inhibitory effect on PAF and FMLP-induced $O_2^-$ production with an IC50 value of 460 and 290 nM, respectively.

The guinea pig PMNL produced $O_2^-$ in response to PAF and FMLP. It was reported that the production of $O_2^-$ is maximal at about 30 min in the case of insoluble stimulants such as opsonized zymosan and sodium urate crystals (9, 11). However, in the case of PAF and FMLP, the maximal production was observed within 5 min. This finding was identical with that using human PMNL reported by Smith et al. (10). The $O_2^-$ production induced by PAF and FMLP increased concentration dependently up to 1 nM, but

\[ \text{Table 1. Effect of etizolam and standard compounds on PAF and FMLP-induced } O_2^- \text{ production in guinea pig PMNL} \]

| Compounds     | IC50 (nM) PAF | IC50 (nM) FMLP |
|---------------|---------------|---------------|
| Etizolam      | 4.7 ±0.1*     | >100          |
| Indomethacin  | >100          | >100          |
| BW-755c       | >100          | >100          |
| FPL-55712     | >10*          | >10*          |
| MK-447        | 460±20*       | 290±6*        |
| Nifedipine    | >100          | >100          |

PMNLs were preincubated with cytochalasin B (5 μg/ml) and test drugs for 10 min at 37°C, followed by exposure to PAF and FMLP (1 μM) and incubated for 10 min. All drugs were dissolved in ethanol. The values represent the mean of 4 experiments. *Mean±S.E. †insoluble at 100 μM.
it decreased at 10 μM. Although the mechanism of this phenomenon is not clear, it may be due to a decrease in the function of PMNL aggregated by high concentration of PAF and FMLP (Fig. 1).

Etizolam concentration-dependently inhibited the PAF-induced \( \text{O}_2^- \) production, but failed to inhibit FMLP-induced \( \text{O}_2^- \) production (Table 1). This finding suggests that etizolam acts specifically on the reaction induced by PAF. Etizolam was found to have a potent anti-PAF activity in vivo and in vitro (7, 8). Moreover, it was reported that etizolam was a specific PAF receptor antagonist as determined by receptor binding assay (8). These results suggest that the inhibitory effect of etizolam on PAF-induced \( \text{O}_2^- \) production is mediated via PAF receptors. However, etizolam may not be a FMLP receptor antagonist, so it may fail to inhibit FMLP-induced \( \text{O}_2^- \) production.

The cyclooxygenase inhibitor, cyclooxygenase-lipoxygenase inhibitor and leukotriene antagonist had no inhibitory effect. The inactivity of these compounds suggests that cyclooxygenase and lipoxygenase products of arachidonic acid do not mediate this reaction.

\( \text{O}_2^- \) production by PAF in human PMNL was reported to increase in the presence of divalent cation (10), especially \( \text{Ca}^{2+} \). Even in the presence of the \( \text{Ca}^{2+} \) chelator EGTA, \( \text{O}_2^- \) production by PAF was not affected. The \( \text{Ca}^{2+} \) entry blocker verapamil was reported to have little effect on FMLP-induced \( \text{O}_2^- \) production at high concentration (12), so that extracellular \( \text{Ca}^{2+} \) is not considered to play a role in the regulation of \( \text{O}_2^- \) production. Therefore, nifedipine may have no effect in the present study.

It was reported that MK-447 could act as a radical scavenger (13). MK-447 had an inhibitory effect in the present study. However, this effect may be a non-specific effect because the concentration used was higher than that of PAF and FMLP (Table 1).

Neutrophils infiltrated into the inflammatory site are supposed to produce active oxygen (14). Active oxygen generated in the living body is supposed to cause tissue injury in the inflammatory process. Although it is not evident that PAF plays a role in the production of active oxygen, active oxygen which is generated by PAF is suggested to worsen the inflammation. Etizolam inhibited the PAF-induced \( \text{O}_2^- \) production as reported in this paper, so that it may prove to have an enhancing effect on any anti-inflammatory drug when it is combined with such a drug.

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