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INHIBITORY EFFECT OF BIFEMELANE ON SUPEROXIDE GENERATION BY ACTIVATED NEUTROPHILS MEASURED USING A SIMPLE CHEMILUMINESCENCE METHOD

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We evaluated the effect of 4-(2-benzylphenoxy)-N-methylbutylamine hydrochloride (bifemelane hydrochloride) on superoxide production by human neutrophils using an MCLA-dependent chemiluminescence assay. Bifemelane hydrochloride dose-dependently inhibited superoxide production by neutrophils stimulated with phorbol myristate acetate, opsonized zymosan, or N-formyl-methionyl-leucyl-phenylalanine, while it had no effect on superoxide production by a hypoxanthine-xanthine oxidase system. These results indicate that bifemelane hydrochloride does not have a scavenging effect, but has an inhibitory effect on superoxide generation by neutrophils. Although this drug is commonly used for treating chronic cerebral infarction, it may also have a protective effect on acute ischemia/reperfusion injury.

KEY WORDS: Bifemelane hydrochloride, superoxide, MCLA-dependent chemiluminescence assay, activated neutrophils.

INTRODUCTION

Neutrophils play a major role in the body's defenses against bacterial infection and in the modulation of inflammation. They also have a significant influence on the development of inflammatory edema by increasing vascular permeability.[1] Recent reports[2] have suggested that cerebral ischemic damage is closely related to an acute inflammatory response characterized by neutrophil infiltration and edema formation. Although the mechanism by which neutrophils cause cerebral damage appears to be very complicated, the generation of superoxide and other active oxygen species by activated neutrophils may play a key role.[2,3] 4-(2-Benzylphenoxy)-N-methylbutylamine hydrochloride (bifemelane hydrochloride) is a cerebral metabolic activator, which has been shown experimentally and clinically to have a favorable effect on neuronal inactivity, amnesia, and emotional disturbances associated with cerebrovascular disease or aging.[4] Several previous studies[5] have suggested that bifemelane hydrochloride may act on active oxygen species. In the present study, we evaluated the effect of this drug on superoxide production by neutrophils.

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MATERIALS AND METHODS

Bifemelane hydrochloride was a kind gift of Eisai (Japan), and was used after being dissolved in Hanks' balanced salt solution without phenol red (HBSS, at pH 7.4). Phorbol myristate acetate (PMA), N-formyl-methionyl-leucyl-phenylalanine (FMLP), and zymosan A were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). PMA and FMLP were dissolved in 50% DMSO and 50% HBSS, as described previously,[6,7] and were stored at −80°C. Opsonized zymosan (OZ) was prepared from zymosan A using pooled human serum,[6,7] and was stored at −80°C until use after no longer than three weeks. Leukocytes were isolated from the peripheral blood of healthy volunteers by the method of Nishida *et al.*[6] The leukocytes thus obtained contained 65–91% neutrophils, and were suspended in HBSS at pH 7.4. and kept at 4°C for no longer than 3 h prior to use. Hypoxanthine (Wako, Japan) was dissolved in HBSS. Xanthine oxidase (from butter milk, grade III, Sigma) was dialysed overnight against HBSS and its activity was determined as described previously.[7] 2-Methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (MCLA) was purchased from Tokyo Kasei (Japan) and was prepared by the method described previously.[6,7]

Superoxide generation was determined by the MCLA-dependent chemiluminescence method using a BLR 301 Luminescence Reader (Aloka, Japan).[8] All experiments were carried out in the incubation chamber of the Luminescence Reader at 37°C in a total volume of 2 ml. The reaction mixture for the basal system consisted of neutrophils (5×10^4 cells/ml) and 1.0 μM MCLA in HBSS (pH 7.4) with preincubation for 5 min at 37°C. The reaction was started by the addition of each of the stimulants. To test the effect of bifemelane hydrochloride on superoxide generation by neutrophils, the basal system was preincubated for 5 min with this drug at various concentrations from 0 to 200 μM and then stimulated by the reagents. Cu-Zn superoxide dismutase (SOD; Sigma) derived from bovine erythrocytes or heat-treated SOD (autoclave-treated enzyme, 40 min at 120°C) was added to the basal system in some experiments. Superoxide production was expressed as photon counts per min. The maximum photon count for each reaction and the integrated photon count for the whole incubation period were defined as the peak count (PC) and the total photon count (TPC),[8] respectively. The incubation period was 10 min for the PMA- or OZ-stimulated systems and 5 min for the FMLP-stimulated system.

RESULTS AND DISCUSSION

The reaction mixture of neutrophils and MCLA in HBSS produced marked luminescence in response to PMA (0.1 μg/ml), OZ (0.5 mg/ml) or FMLP (1.0 μM). This response was completely eliminated by the addition of 0.5 μM SOD, but was not influenced by the addition of heat-treated SOD (Figure 1). When neutrophils or stimulating agents were omitted, only the basal emission from MCLA was detected, while reaction mixtures without MCLA produced no detectable emission. These findings indicate that MCLA-dependent chemiluminescence above the basal level was derived from superoxide produced by neutrophils.

Bifemelane hydrochloride suppressed the chemiluminescence of neutrophils stimulated by PMA, OZ, or FMLP (Figure 1). And the drug reduced both PC and TPC in a dose-dependent manner (Figures 2–4). Complete inhibition of the response of the basal system to any of the stimulants was achieved by addition of 200 μM bifemelane hydrochloride. The concentration required for 50% inhibition of the PC

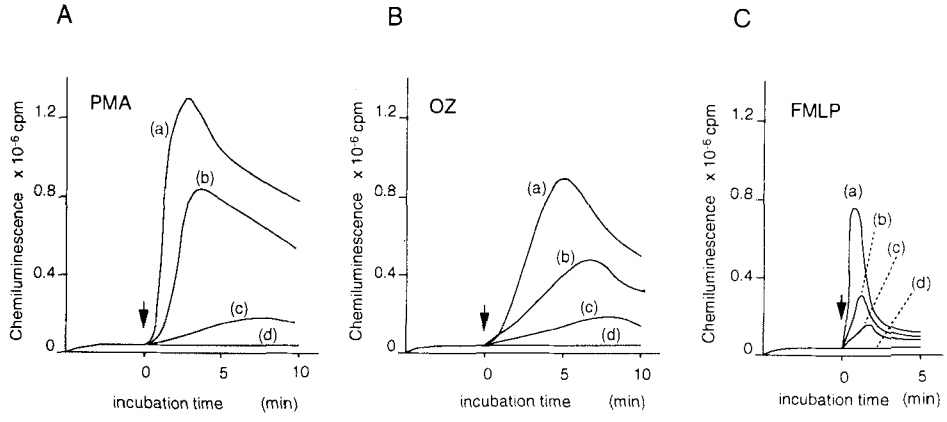

FIGURE 1 MCLA-dependent chemiluminescence response of stimulated neutrophils and the effect of bifemelane hydrochloride. The incubation mixtures contained 5×10^4 neutrophils and 1.0 μM MCLA with various concentrations of bifemelane hydrochloride in HBSS (pH 7.4), and were stimulated using 0.1 μg/ml PMA (Figure 1A), 0.5 mg/ml OZ (Figure 1B), or 1.0 μM FMLP (Figure 1C). The stimulant was added at time 0 (arrow). Systems (a), (b), (c), and (d) contained bifemelane hydrochloride at 0, 40, 80 and 200 μM, respectively. Addition of 0.5 μM SOD completely inhibited the chemiluminescence promoted by any of the stimulants, giving essentially the same result as shown for system (d). In contrast, heat-treated SOD had no effect on the chemiluminescence. The incubation mixture without stimulants or neutrophils produced only basal emission by MCLA identical to that in system (d).

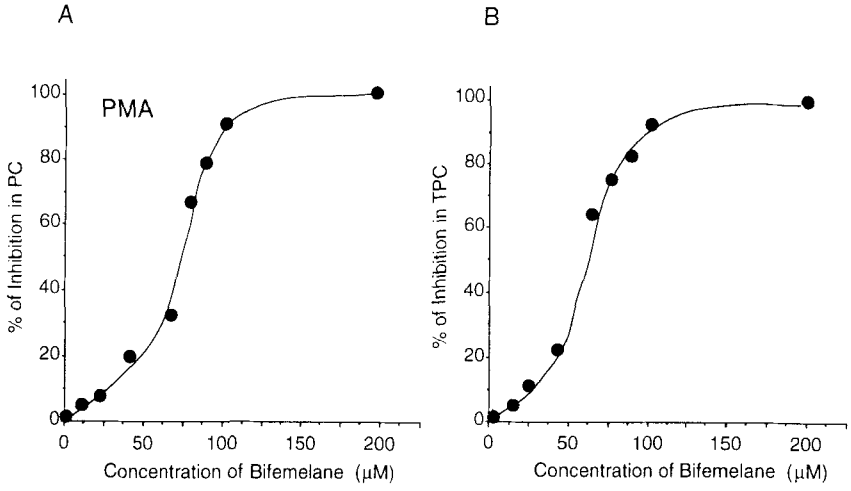

FIGURE 2 Relationship between the percent inhibition of PC (A) or TPC (B) for PAM-stimulated neutrophils and the concentration of bifemelane hydrochloride. The incubation mixture contained 5×10^4 neutrophils, 1.0 μM MCLA, and 0.1 μg/ml PMA with various concentrations of bifemelane hydrochloride in 2 ml of HBSS.

and TPC responses to PMA was 60–70 μM, while it was about 30 μM in the OZ-stimulated system and 40–50 μM in the FMLP-stimulated system. The viability of the neutrophils determined by 0.2% trypan blue staining was equally 95% before and after preincubation with bifemelane hydrochloride. To test a scavenging effect on superoxides and a possible interaction with MCLA, 200 μM bifemelane hydro-

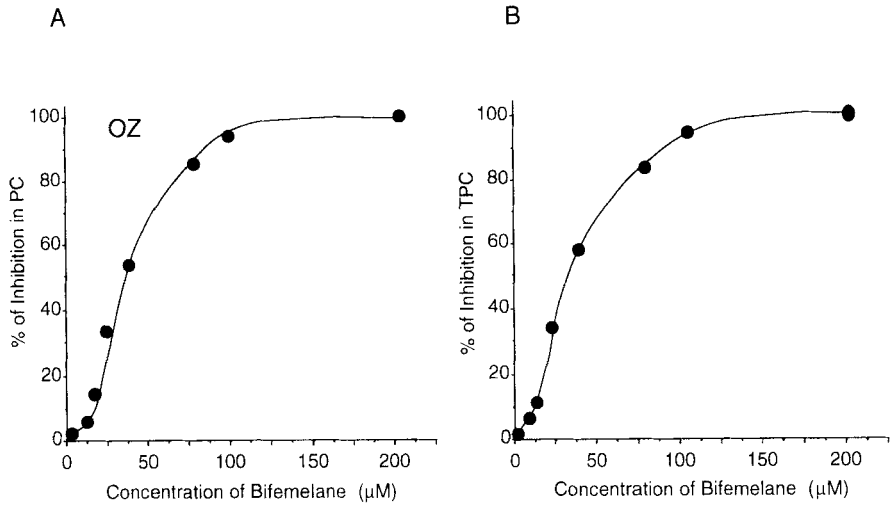

FIGURE 3 Relationship between the percent inhibition of PC (A) or TPC (B) for OZ-stimulated neutrophils and the concentration of bifemelane hydrochloride. The incubation mixture contained 5×10^4 neutrophils, 1.0 μM MCLA, and 0.5 mg/ml OZ with various concentrations of bifemelane hydrochloride in 2 ml of HBSS.

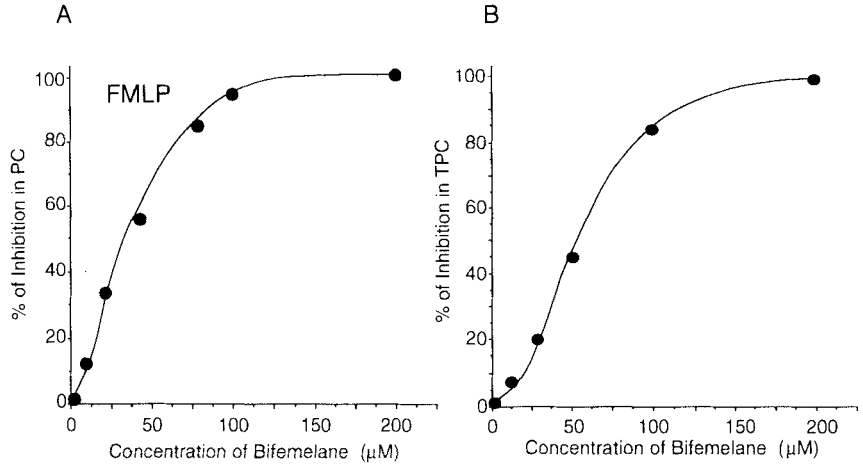

FIGURE 4 Relationship between the percent inhibition of PC (A) or TPC (B) for FMLP-stimulated neutrophils and the concentration of bifemelane hydrochloride. The incubation mixture contained 5×10^4 neutrophils, 1.0 μM MCLA, and 1.0 μM FMLP with various concentrations of bifemelane hydrochloride in 2 ml of HBSS.

chloride was incubated with a system containing 1.0 μM MCLA, 0.18 mg/ml of bovine serum albumin and 45 μM hypoxanthine in HBSS at pH 7.4 and 37°C. By the addition of xanthine oxidase (28 unit/ml), this system yielded luminescence as shown in Figure 5, which was completely eliminated by the addition of 0.5 μM SOD. The intensity and pattern of the luminescence were essentially the same as those found

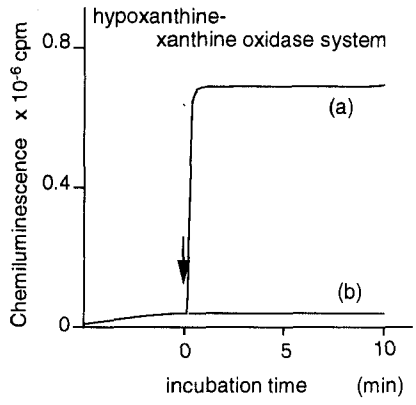

FIGURE 5 MCLA-dependent chemiluminescence by hypoxanthine-xanthine oxidase system and the effect of SOD or bifemelane hydrochloride on the chemiluminescence. System (a) contained 45 μM hypoxanthine, 0.18 mg/ml of bovine serum albumin and 1.0 μM MCLA in 2 ml of HBSS at pH 7.4. Xanthine oxidase (28 units/ml) was added at time 0 (arrow). Chemiluminescence produced in the presence of 200 μM bifemelane hydrochloride was essentially the same as shown for system (a). The addition of 0.5 μM SOD completely inhibited the chemiluminescence as indicated by system (b).

in a system without bifemelane hydrochloride (Figure 5), indicating that the drug did not interact with MCLA and had no detectable scavenging effect, but had an inhibitory effect on the superoxide-generating system of neutrophils.

The MCLA-dependent chemiluminescence assay that we employed in the present study is a simple and sensitive method of determining superoxide and singlet oxygen specifically and quantitatively. The major source of superoxide was considered to be neutrophils in this study. Neutrophils and non-phagocytosing lymphocytes are the major types of leukocytes. Although monocytes also secrete superoxide and eosinophils secrete a small amount of singlet oxygen when stimulated,[9] these two cell types amount to less than 10% of the peripheral leukocytes under normal circumstances. In addition, the complete inhibition of luminescence by SOD indicates that singlet oxygen was negligible under our experimental conditions.

Liu *et al.*[5] examined the scavenging effects of bifemelane hydrochloride and its two major metabolites on 1,1-diphenyl-2-picryl hydrazyl radicals, superoxide, and hydroxyl radicals using electron spin resonance spectrometry. They reported that 250 μM bifemelane hydrochloride showed little scavenging effect on hydroxyl radicals and no effect on superoxide. At higher levels than 2.5 mM, however, bifemelane hydrochrolide scavenged hydroxyl radicals significantly. In the present study, we also demonstrated that bifemelane hydrochloride did not scavenge superoxide, but instead acted on the superoxide-generating system. Since the superoxide is a potential precursor of hydroxyl radical which seems to be a more hazardous free radical, bifemelane hydrochloride may prevent hydroxyl radical formation indirectly by means of inhibition of superoxide production from the neutrophils at low concentrations and may quench hydroxyl radical directly at high concentrations. Bifemelane hydrochloride is promptly incorporated by cell membranes and may then alter membrane function.[10] This may be responsible for its inhibitory effect on superoxide generation system, because the common signal pathway transmitting PMA, FMLP, or OZ stimulation is present in the cell membrane and its related structures.[11]

Recently, neutrophils have suggested to have a role in ischemia/reperfusion injury. Shiga *et al.*[2] reported that the removal of circulating neutrophils reduced ischemic brain edema. The superoxide produced by neutrophils is one of the critical chemical mediators which alter vascular permeability and accelerate the development of cerebral edema.[2,12] Although bifemelane hydrochloride is commonly used for the treatment of patients with chronic cerebral infarction,[4] our findings suggest that it may also have a protective effect during the acute phase of ischemia/reperfusion injury.

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