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INHIBITION OF ACTIVE OXYGEN GENERATION IN GUINEA-PIG NEUTROPHILS BY BISCOCLAURINE ALKALOIDS

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Abstract—Effect of biscoclaurine alkaloids, such as cepharanthine, on active oxygen production of neutrophils was investigated. Cepharanthine inhibited both superoxide generation and luminol-dependent chemiluminescence (CL) induced by either formylmethionyl-leucyl-phenylalanine, opsonized zymosan, arachidonic acid or by phorbol myristate acetate. Ca^{2+}- and phospholipid-dependent protein kinase (PKC) activity and the phosphorylation of cytoplasmic protein including 47 kDa proteins of neutrophils were also inhibited by cepharanthine; dose dependent inhibition of CL was quite similar to that of PKC. Among various biscoclaurines tested, the inhibitory effect of cepharanthine, tetrandrine and isotetrandrine was strong, but that of berbamine and cycreanine was weak; the inhibitory action of the former on lipid peroxidation and platelet aggregation were also stronger than those of the latter. These and other observations indicated that these alkaloids inhibited the active oxygen generation by way of stabilizing plasma membrane and inhibiting PKC and NADPH oxidase activation.

When challenged with various stimuli, neutrophils show metabolic responses [1–4]. Superoxide (O_2^-) generated by NADPH oxidase is the major source for other reactive oxygens, such as H_2O_2, $^1O_2$ and $^{\cdot}OH$ [5]. Although these active oxygens occur both intra and extracellularly, the former compartment is highly enriched with superoxide dismutase (SOD**) [6], catalase, glutathione peroxidase [7], and low molecular weight antioxidants, such as glutathione [8]. Thus, intracellular reactive oxygens are effectively scavenged *in situ*. In contrast, the levels of these enzymes and scavengers in extracellular space are extremely low [9], and, hence, the active oxygens in this compartment cannot be scavenged efficiently. Thus, oxidative injury of tissues might be induced when a large amount of active oxygens is generated in an extracellular space.

Previous studies in this laboratory revealed that various types of bisoclaurine alkaloids, such as cepharanthine, have a potent inhibitory action on O_2^- generation by neutrophils [10]. However, the mechanism for inhibition and the effect of such alkaloids on the formation of reactive species other than O_2^- remain to be studied. The present work demonstrates the effect of various alkaloids on the formation of reactive oxygen species. Kinetic analysis of luminol-dependent CL revealed that biscoclaurine type alkaloids also inhibited the generation of various types of reactive oxygens by guinea-pig neutrophils.

MATERIALS AND METHODS

Chemicals. Aprotinin, arachidonic acid (AA), calf thymus H_1 histone (type IIIs), cytochrome *c*, egg phosphatidylcholine (PC), FMLP, 2-mercaptoethanol, PMA, phenylmethylsulfonyl fluoride (PMSF), *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (Hepes) and zymosan were purchased from the Sigma Chemical Co. (St Louis, MO). Phosphatidylserine (PS) was obtained from Serdary Research Lab. (Canada). Sephadex G-25 and Superose 12 were from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE-cellulose (DE-52) and GF/C glass filters were from Whatman (Maidstone, U.K.) and TSK-G3000 SW from Toso (Tokyo, Japan). Nutrose was from Kodak Laboratory Chemicals (Rochester, NY). [γ-^{32}P]ATP was obtained from ICN Radiochemicals (Irvine, CA). 5-Amino-2,3-dihydro-1,4-phthalzinedione (luminol), Leupeptin and other chemicals used were of analytical grade from nacalai tesque (Kyoto, Japan). Biscoclaurine alkaloids were kindly donated by Kaken Pharmaceutical Co. (Tokyo, Japan) and used as ethanol or dimethylsulfoxide (DMSO) solution. Final concentrations of these solvents were lower than 1%. Luminol was dissolved in 0.1% triethylamine (2 mg/mL).

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** Abbreviations used: AA, arachidonic acid; CBB, Coomassie brilliant blue; CL, chemiluminescence; PC, phosphatidylcholine; DMSO, dimethylsulfoxide; EGTA, ethyleneglycol bis (β-amino-ethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; FMLP, formylmethionyl-leucyl-phenylalanine; Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid; KRP, Krebs–Ringer–phosphate buffer solution; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; MPO, myeloperoxidase; OZ, opsonized zymosan; PS, phosphatidylserine; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase.

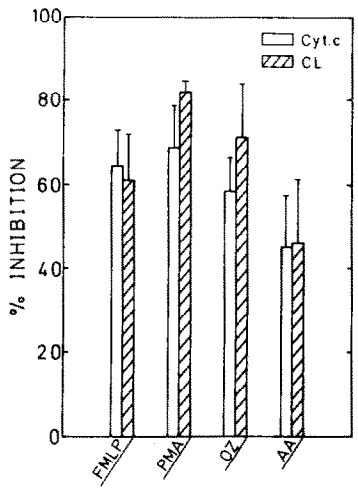

Fig. 1. Inhibitory effect of cepharanthine on stimulus responsive O_2^- generation and luminol CL in neutrophils. Guinea-pig neutrophils were suspended in KRP (5×10^5 cells/mL) containing 10 mM glucose and 1 mM $CaCl_2$. Concentrations of various stimuli were 1.25×10^{-8}M FMLP, 3×10^{-9}M PMA, 7.5×10^{-6}M AA and 125 μg/mL OZ. Active oxygen generation was measured by CL on 100 μM luminol at 37°. For the measurement of O_2^- generation, 0.025% cytochrome *c* was added to the medium and cytochrome *c* reduction was recorded at 550–540 nm with 5 mM NaN_3 at 37°. Cepharanthine (20 μm) dissolved in DMSO was added to the reaction medium before the start of experiment. The same volume of DMSO as solvent control showed no inhibitory effect on O_2^- generation but showed a slight inhibitory effect on CL (approx. 5% inhibition). O_2^- generation per unit period was compared with the luminol CL intensity as voluntary unit under the same condition. O_2^- and active oxygen production by FMLP for 3 min, those by AA for 5 min and those by PMA and OZ for 6 min are shown, respectively. The average and the SD of four measurements are shown. Cytochrome *c*, per cent inhibition of O_2^- generation; CL, per cent inhibition of chemiluminescence production.

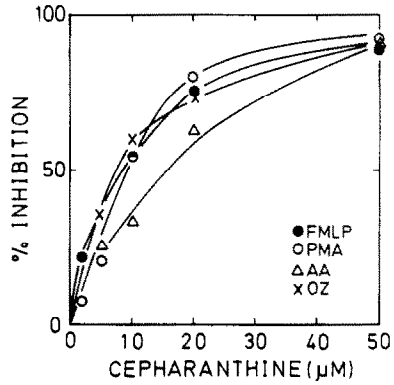

Fig. 2. Inhibitory effect of cepharanthine on the luminol CL by various stimuli in neutrophils and its dose dependency. Experimental conditions were the same as described in Fig. 1.

Stimulation of neutrophils. Four different ligands were employed for the stimulation of neutrophils. FMLP (1.25×10^{-8}M) was used for receptor-mediated activation of neutrophils while OZ (125 μg/mL) was used as a phagocytic ligand. AA (7.5×10^{-6}M) and PMA (3.0×10^{-9}M) were used as membrane perturber and activator for PKC, respectively.

Preparation of neutrophils and cytoplasmic proteins. Peritoneal neutrophils were prepared from guinea-pigs. One tenth body weight of 2% nutrose (casein) containing saline solution was injected intraperitoneally as described previously [10]. Sixteen hours after injection, cells were collected and washed three times with Krebs–Ringer–phosphate buffer solution (KRP) by centrifugation; more than 96% of the cells were neutrophils as confirmed by Giemsa staining.

To prepare cytoplasmic proteins, cells were suspended in a medium containing 0.15 M KCl, 20 mM Hepes-K buffer (pH 7.4), 4 mM iodoacetate, 1 mM Na-O-vanadate, 10 mM β-glycerophosphate, 1 mM *p*-nitophenylphosphate, 1 mM PMSF, 1 μg/mL aprotinin, 0.01% leupeptin, and 1 mM EGTA at 4° and homogenized by a glass–Teflon homogenizer with 50 strokes up and down at 2000 rpm. The homogenate was centrifuged for 60 min at 100,000 *g* at 4°. The supernatant fraction was subjected to gel-filtration chromatography on a Sephadex G-25 column (0.5 × 10 cm) to separate proteins from low molecular weight compounds [11]. The supernatant fraction was incubated at 55° for 3 min to inactivate endogenous protein kinases.

Preparation of PKC. PKC was partially purified from the soluble fraction of rat brain according to the method of Kikkawa *et al.* [12].

Measurement of O_2^- generation. Neutrophils (5×10^5 cells/mL) were suspended in KRP solution containing 1 mM cytochrome *c*, 1 mM $CaCl_2$ and 10 mM glucose with or without 1 mM NaN_3, and incubated at 37°. The SOD inhibitable O_2^- generation was measured by cytochrome *c* reduction method [13]. Measurement of the cytochrome *c* reduction was carried out continuously at 550 nm using a dual beam spectrophotometer (Shimadzu UV-300) as described previously [10].

Measurement of CL. Neutrophils were suspended in 1 mL KRP containing 100 μM of luminol and incubated at 37° [5]. CL was measured by an ATP photometer (ALL Co. Monolight 401) or a calcium analyser (Jasco, CAF-100, mode for chemiluminescence); the CL intensity was recorded for a period of 8–10 min. Tracings were evaluated by calculating the area under CL (cpm) curve (integral chemiluminescence).

Assay of PKC activity. PKC activity was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into calf thymus H_1-histone (Type IIIs) or cytoplasmic proteins of neutrophils as described previously [14].

RESULTS AND DISCUSSION

Effect of cepharanthine on O_2^- generation and CL production by neutrophils

We previously reported that cepharanthine markedly inhibited the SOD-sensitive, and CN^--insensitive, O_2 generation by neutrophils [10]. Superoxide

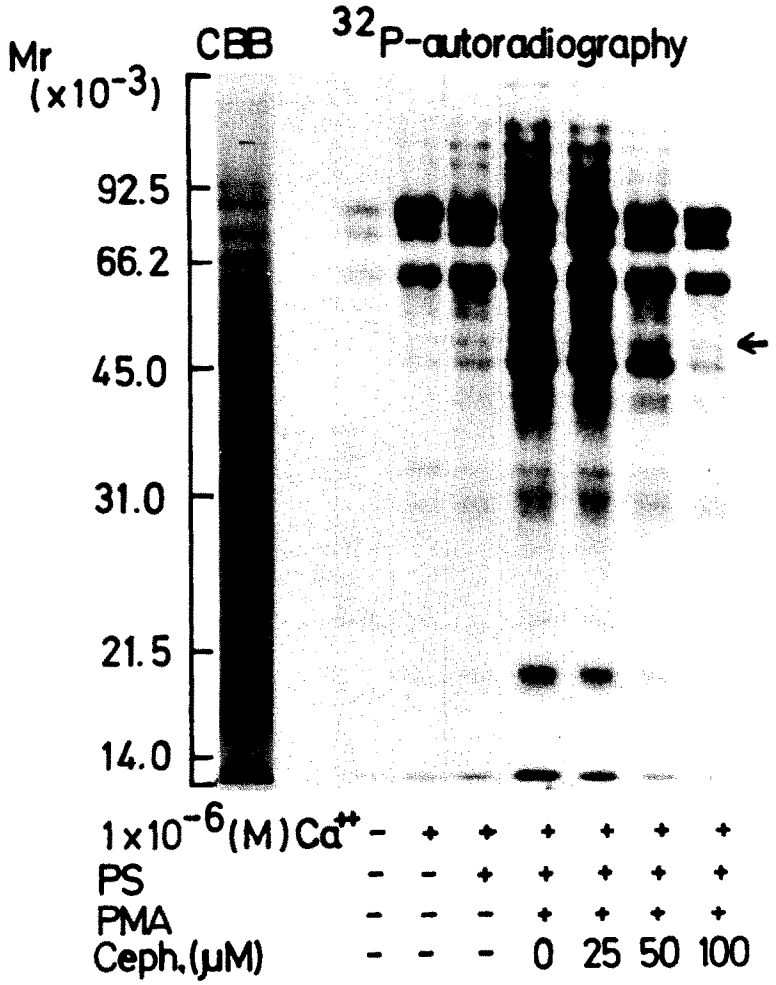

Fig. 3. Autoradiogram of endogenous proteins of guinea-pig neutrophils phosphorylated by partially purified rat brain PKC. Supernatant proteins were treated for 3 min at 55° and were phosphorylated in the medium of 1 μM $CaCl_2$, 0.1 mM phospholipid liposomes (egg PC/PS, 4/1 in molar ratio), 100 nM PMA, and 10 μM [γ-^{32}P]ATP and in the presence or absence of 25–100 μM cepharanthine. CBB, Coomassie blue staining; ^{32}P-autoradiography, autoradiography of phosphorylated proteins.

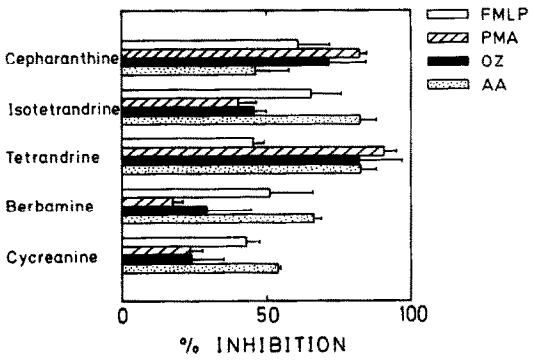

Fig. 4. Inhibitory effect of biscoclaurine alkaloids on the stimulus dependent luminol CL of neutrophils. Experimental conditions were the same as described in Fig. 1. Results indicate the percent inhibition by 20 μM alkaloids against solvent control (same volume of non-alkaloid containing DMSO). The average and the SD of four measurements are shown.

has been assumed to be a primary source for other reactive oxygens such as O_2^- H_2O_2, $^{\cdot}OH$, $^1O_2$, MPO-dependent OCl^-, etc. [5]. Thus, the effect of cepharanthine on luminol-dependent CL of neutrophils was tested. Various stimuli were used at concentrations required for the induction of half maximal response (ED_{50}) of neutrophils, such as 10^{-9}M PMA, 10^{-8}M FMLP, 10^{-5}M AA and 125 μg/mL OZ. Under these conditions, cepharanthine strongly inhibited O_2^- generations, especially when cells were stimulated by PMA and FMLP in a dose-dependent manner; more than 90% PMA-induced CL was inhibited by 50 μM cepharanthine (Fig. 1).

Similar inhibitory profiles were also seen in the cases of FMLP- and OZ-stimulated CL. However, the inhibitory activity against AA-stimulated CL was weak, particularly at its low concentration (Fig. 2). Although SOD has been reported to decrease FMLP-induced CL [15], azide increased the light emission response (data are not shown). However, OZ-induced CL intensity was inhibited partially by SOD, and remarkably by azide [5].

Under such conditions, OCL^- produced by MPO is released from azurophilic granules and the generated OCl^- produce $^1O_2$ [5]. These observations suggested that the FMLP-dependent CL might reflect the activation of NADPH oxidase while OZ-stimulated CL predominantly reflects MPO activity [16, 17]. Since cepharanthine inhibited CL induced either FMLP or OZ, the drug would have inhibited both NADPH oxidase and the release of MPO via its membrane stabilizing effect [18].

Effect of cepharanthine on PKC activity

In fact, when neutrophils were stimulated by various stimuli, PKC-dependent phosphorylation of cytosolic factors including 47 kDa protein and the association of these proteins with membranous cytochrome b_{-245} occurred with concomitant production of superoxide [19–21]. Thus, PKC [22] has been considered to play an essential role in transmembrane signal of transduction by way of phosphorylating 47 kDa protein. This hypothesis was further supported by the experiments of Lomax *et al.* [23] using recombinant 47 kDa cytosol factor. Thus, PKC might trigger the respiratory burst by way of phosphorylating of 47 kDa protein. To elucidate the inhibitory mechanism of cepharanthine on respiratory burst, we examined the effect of the alkaloid on PKC activity. Cepharanthine inhibited the phosphorylation of H_1 histone by rat brain PKC (data not shown). The concentration required for the inhibition of the enzyme was slightly higher than that required for the inhibition of PMA-dependent CL. Phosphorylation of cytosolic proteins of neutrophils was also inhibited by cepharanthine in a concentration dependent manner (Fig. 3).

Mori *et al.* [24] reported that various amphipathic drugs that interact with phospholipids, such as chlorpromazine, phentolamine, dibucaine, verapamil and tetracaine, competitively inhibited the interaction between PKC and phospholipid rather than directly inhibiting catalytic activity of the enzyme. Since cepharanthine also interacts predominantly with phospholipids, actions of this alkaloid are expected to be similar to those of these amphipathic drugs.

Effect of various biscoclaurine alkaloids on CL of neutrophils.

To test whether the inhibitory action of cepharanthine is a common property of biscoclaurine type alkaloids, the effect of other alkaloids on CL was examined. CL intensity of neutrophils was determined after stimulation by either OZ, FMLP, PMA or AA in the presence or absence of 20 μM of various alkaloids. Figure 4 shows the percent inhibitory effect by biscoclaurine alkaloids. The inhibitory effects of isotetrandrine and tetrandrine were stronger than those of berbamine and were similar to those of cepharanthine. The inhibitory action of these biscoclaurine alkaloids seems to coincide with their inhibitory effects on lipid peroxidation [25] and on platelet aggregation [26]. The inhibitory effects of berbamine and cycreanine varied depending on the type of stimuli. The mechanism for the difference in the inhibitory action of various alkaloids remains to be studied.

For the inhibitory mechanism of cepharanthine on reactive oxygen generation, the following three mechanisms may be proposed; inhibition of NADPH oxidase by interacting with the lipid binding domain of the enzyme; inhibition of PKC and phosphorylation of cytoplasmic proteins including 47 kDa protein; or inhibition of enzyme release by stabilizing plasma membrane lipid bilayers. The precise mechanism for the inhibitory actions of cepharanthine is under investigation in our laboratory.

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