Biochemical Pharmacology, Vol. 45, No. 3, pp. 766–768, 1993.
Printed in Great Britain.

0006-2952/93 $6.00+0.00
Pergamon Press Ltd

Polyene antibiotics inhibit superoxide-producing NADPH oxidase in a polymorphonuclear cell-free system

(*Received* 21 *July* 1992; *accepted* 23 *November* 1992)

Abstract—We studied the effect of polyene macrolide antibiotics on the NADPH-dependent superoxide production induced by arachidonic acid in a cell-free system consisting of the membrane and cytosolic fractions obtained from bovine polymorphonuclear leukocytes. Preincubation of the membrane fraction with polyenes before addition of the soluble components of the reaction mixture resulted in a dose-dependent inhibition of superoxide production.

Polymorphonuclear leukocytes respond to a variety of stimulating agents by a sharp increase in activity of a superoxide generating system that consists of both membrane and cytosolic components. Oxygen uptake corresponding to the respiratory burst is catalysed by the membrane-bound NADPH oxidase (EC 1.6.99.6), which is dormant in the resting state [1]. Inhibitors of the activation process can be broadly divided into two groups, those which inhibit the respiratory burst in intact cells by interfering with some of the stages of the activation process and those which exert a direct effect on the membrane-bound oxidase activity. The mechanism of action of these inhibitors and their role in regulating the superoxide generating system have been the subject of recent reviews [2, 3].

Polyene macrolide antibiotics have been reported to modify many properties of activated polymorphonuclear leukocytes by inhibiting chemotaxis, phagocytosis and killing of opsonized bacteria, and adherence and aggregation on polymeric fibers, as well as production of oxygen radicals. The alteration of the respiratory burst has been proved recently to be dissociable from the impairment of other leukocyte functions and to occur at relatively low drug concentrations [4, 5].

In this paper, we report experiments performed in a cell-free system by reconstituting membrane-bound NADPH oxidase activity with cytosolic components of bovine polymorphonuclear leukocytes [6]. Evidence is presented that polyenes inhibit the membrane-bound NADPH oxidase in a dose-dependent manner. The inhibition seems to be due either to interaction of polyenes with enzymatic protein(s) or to disorganization of lipid components of the membrane.

Materials and Methods

Amphotericin B was a kind gift from Squibb & Sons Inc. (Princeton, NJ, U.S.A.). Lucensomycin and *N*-acetyl lucensomycin were obtained from Farmitalia Inc. (Milan, Italy). The polyenes (10 mM) were dissolved in dimethyl sulfoxide and diluted in buffer. All other reagents were high purity commercial samples from the Sigma Chemical Co. (St Louis, MO, U.S.A.), Fluka A.G. (Buchs, Switzerland) and Boehringer A.G. (Mannheim, Germany).

Resting neutrophils were isolated from bovine blood according to the procedure described in Ref. 6. The membrane and cytosolic fractions were prepared by sequential centrifugation after ultrasonic cellular disruption [7]. Membranes were frozen at −80° and/or resuspended in buffer containing the polyene antibiotics (0–3 mM). The mixture was used for determining superoxide production.

Superoxide production was evaluated by determining the rate of superoxide dismutase-inhibitable cytochrome c reduction at 550 nm [7]. The reaction mixture contained a reconstituted system for activation of the superoxide-generating oxidase; namely, the membrane fraction (200–250 μg membrane protein/mL), the cytosolic fraction (400–450 μg protein/mL), arachidonic acid (250 μM), guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γS, 15 μM), ferricytochrome c (100 μM) and $MgCl_2$ (1 mM), unless otherwise stated. The reaction was initiated by addition of 250 μM NADPH. The difference between the rate without and with 100 μg/mL bovine superoxide dismutase (EC 1.15.1.1) was regarded as the rate of superoxide production. The rate of superoxide production was dependent on the concentration of the membrane fraction as well as on the preparation yield. In each group of experiments, comparisons between data were made by using the same membrane preparation. NADPH-dependent oxidase activity was no appreciably affected by dimethyl sulfoxide (up to 0.1 M).

All the experiments were performed in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4 at 25°. The protease inhibitor mixture, if present, contained 10 μM 4-amidinophenylmethanesulphonyl fluoride, 10 μM pepstatin, 20 μM leupeptin and 1 mM EDTA. Protein content was determined as described in Ref. 7.

Results

Activity of membrane-bound NADPH oxidase was dependent on a number of factors, including NADPH, GTP-γS and arachidonic acid concentrations as well as cytosolic and membrane components [7]. The optimal concentration of GTP-γS was found to be around 15 μM. The amount of arachidonic acid was properly adjusted with respect to the amount of membrane and cytosolic components. Under our experimental conditions, maximal oxidase activity occurred in the presence of 250–350 μM arachidonic acid, higher concentrations being inhibitory.

Freshly prepared membranes generally exhibited the highest oxidase activity. The activity slowly decreased with time upon incubation at 25°. Inactivation behaved as an exponential process (relaxation time of 15–20 min) that asymptotically resulted in the loss of about half of the initial activity. Inactivation was at least in part prevented by storing membrane preparations at −80°. After the freezing and thawing cycle, however, membrane inactivation at 25° was more rapid and pronounced than the inactivation of freshly prepared membranes. Several consecutive freezing cycles of the membranes led to enzymatic preparations, which retained the original activity just after thawing but lost up to 90% of the initial activity after about 1 hr at 25° (Table 1).

Addition of the polyene antibiotic lucensomycin (1–800 μM) to membrane suspensions at 25° increased the rate of oxidase inactivation (Fig. 1). This effect appeared to be a biphasic process characterized by an initial rapid drop in the enzyme activity, followed by monoexponential inactivation. The activity of freshly prepared polyene-treated membranes approached that of membrane preparations subjected to several freezing and thawing

Table 1. Effect of repeated freeze/thaw cycles on NADPH oxidase inactivation at 25°

Preincubation at 25° (min)	Enzyme activity (nmol O_2^-/min/mg membrane proteins)		
	Fresh preparation	Freeze/thaw 1st cycle	2nd cycle
0	33	31	30
60	15	10	5

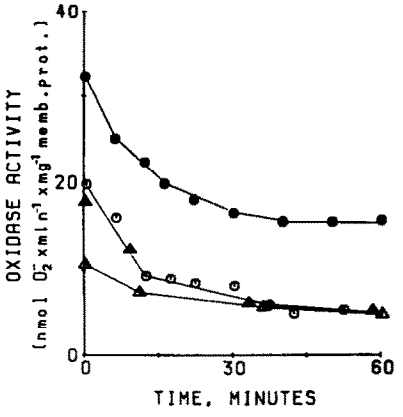

Fig. 1. Time course of polymorphonuclear leukocyte NADPH oxidase inactivation. (○, ●) Freshly prepared membranes; (△, ▲) membrane incubated for 1 hr at 25°, frozen at −80°, thawed and then assayed for the residual activity. Lucensomycin concentration was 0 (closed symbols) and 20 (open symbols) μM. Other experimental conditions were as described in Materials and Methods.

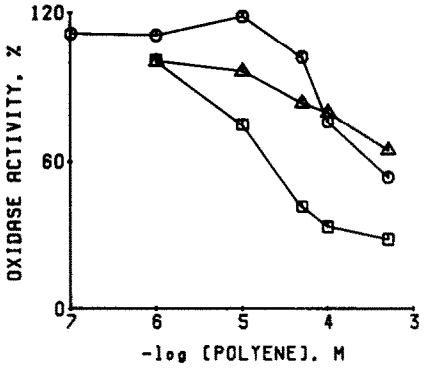

Fig. 2. NADPH oxidase activity as function of polyene concentration. Membranes were incubated for 2 min at 25° in the presence of lucensomycin (□), *N*-acetyl lucensomycin (△) and amphotericin B (○), and then used for the determination of oxidase activity. The reference activity was 25 nmol O_2^-/min/mg membrane proteins. Other conditions were as described in Fig. 1.

cycles. The residual 10% of activity was resistant to inactivation. Proteases seemed to be unlikely to be involved in the inactivation process, since the loss of oxidase activity was not prevented by adding mixtures of protease inhibitors. We were unable to demonstrate the reversibility of the inactivation process, owing to the almost complete inactivation of the diluted plasma membranes after high-speed centrifugation even in the absence of polyenes. No appreciable effect was observed when polyenes (up to 0.1 mM) were added last to already activated membranes, i.e. to reaction mixtures containing membrane and cytosolic components as well as NADPH, arachidonic acid and GTP-γS. By contrast, the rapid drop in NADPH oxidase activity was not appreciably affected by preincubating the cytosolic fraction with polyene (up to 0.1 mM) before the addition of the other reaction components (data not shown).

The amplitude of the initial drop of activity depended on the concentration of polyenes (50% inhibition was observed in the presence of about 20 μM lucensomycin). The effect was less evident in the case of amphotericin B. Lucensomycin acetylation strongly reduced the capacity of the drug to interfere with the enzymatic activity (Fig. 2). Arachidonic acid concentration required for maximal activation of membrane oxidase was not greatly affected by polyene addition (Fig. 3).

Discussion

It has already been reported [4, 5] that polyene antibiotics

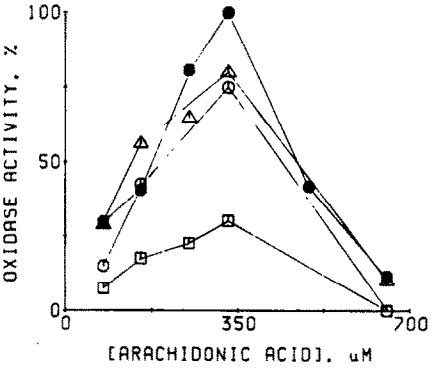

Fig. 3. NADPH oxidase activity as function of arachidonic acid concentration. Membrane were incubated for 2 min at 25° in the absence (●) or in the presence of 0.1 mM polyenes (□ lucensomycin; △ *N*-acetyl lucensomycin; ○ amphotericin B). Membrane suspensions were then used for the determination of oxidase activity in the presence of different arachidonic acid concentrations. The reference activity was 25 nmol O_2^-/min/mg membrane proteins. Other conditions were as described in Fig. 1.

interfere with oxygen radical production by activated polymorphonuclear leukocytes and that respiratory burst inhibition does not correlate with the impairment of other cellular functions (e.g. chemotaxis, phagocytosis, cell aggregation) as well as with membrane depolarization, which is observed in the presence of these drugs. Dissociation between permeability changes and impairment of cellular functions was also observed in mammalian cell culture by studying polyene effects that were attributed to modifications of the structure of the whole cell plasma membrane and/or of some of its components rather than to ionophoretic properties of the drugs [8].

Our results support the hypothesis that polyenes inhibit NADPH oxidase activity even in a cell-free system. The inhibition cannot be attributed to a scavenger effect of polyenes on oxygen-reactive species. Firstly, polyenes are unable to decrease the rate of cytochrome *c* reduction if added last to the reaction mixture. Moreover, the extent of inhibition increases with time upon incubating mixtures of membranes and polyenes in the absence of other reaction components. Finally, polyenes do not appreciably affect oxygen radical production in a xanthine–xanthine oxidase model system [4]. Competition between polyenes and arachidonic acid for membrane target(s) and/or the formation of ineffective aggregates seem to be also unlikely since the arachidonic acid concentration required for maximal activation of the membrane oxidase is not greatly affected by addition of polyenes.

The mechanism by which polyenes inhibit superoxide production is not clear. It has been suggested already that polyenes could interact with membrane proteins. This hypothesis is in line with circular dichroism studies showing that spectra of amphotericin B bound to cell membranes look similar to those of amphotericin B bound to isolated proteins [9]. Interactions of antibiotics with membrane proteins have also been proposed by studying the kinetics of binding of lucensomycin to erythrocyte ghosts [10]. Another hypothesis is that polyenes act by perturbing lipid–protein interactions that are expected to play a crucial role in the enzyme activation and/or in the catalytic cycle. In fact, other ionic detergents such as cetyltrimethylammonium bromide [2] are very powerful inhibitors of the isolated oxidase. Inhibition by high concentrations of arachidonic acid [7] is possibly due to a similar detergent-like effect. Inhibition of oxidase activity may be also due to lipid bilayer disorganization, which results from the formation of sterol–polyene adducts [8]. Lucensomycin, which shows the highest affinity for membrane cholesterol, is more effective in inhibiting oxidase activity than the other tested drugs.

Inhibition by polyenes seems to be not additive with respect to oxidase inactivation induced by freezing and thawing cycles. It is conceivable that membrane freezing induces irreversible disorganization of membrane structure eventually leading to loss of activity. Disorganization of the membrane and/or loss of some of its components have already been taken into account to explain the instability of freshly prepared NADPH oxidase with time [3].

Departments of Biochemical Sciences and Human Biopathology
University of -Rome La Sapienza, and
Centre of Molecular Biology
CNR, Rome, Italy

ELISABETTA CAPUOZZO
CARLO CRIFÒ
COSTANTINO SALERNO*

* Corresponding author: C. Salerno, Department of Human Biopathology, University of Rome la Sapienza, V. le Regina Elena, 324 (Policlinico), 00185 Rome, Italy.

REFERENCES

1. Rossi F, The O_2^--forming NADPH oxidase of the phagocytes: nature, mechanisms of activation and function. *Biochim Biophys Acta* **853**: 65–89, 1986.
2. Cross AR, Inhibitors of the leukocyte superoxide generating oxidase: mechanisms of action and methods for their elucidation. *Free Radic Biol Med* **8**: 71–93, 1990.
3. Seifert R and Schultz G, Reconstitution and regulation of NADPH oxidase activity in cell-free systems. *Rev Physiol Biochem Pharmacol* **117**: 177–202, 1991.
4. Jullien S, Capuozzo E, Salerno C and Crifò C, Effects of polyene antibiotics on the activation of human polymorphonuclear leukocytes. *Biochem Pharmacol* **41**: 2037–2040, 1991.
5. Jullien S, Capuozzo E, Salerno C and Crifò C, Effects of polyene macrolides on the membrane potential of resting and activated human leukocytes. *Biochem Int* **24**: 307–319, 1991.
6. Doussière J, Pilloud MC and Vignais PV, Cytosolic factors in bovine neutrophil oxidase activation. Partial purification and demonstration of translocation to a membrane fraction. *Biochemistry* **29**: 2225–2232, 1990.
7. Ligeti E, Doussière J and Vignais PV, Activation of O_2^--generating oxidase in plasma membranes from bovine polymorphonuclear neutrophils by arachidonic acid, a cytosolic factor of protein nature, and nonhydrolyzable analogues of GTP. *Biochemistry* **27**: 193–200, 1988.
8. Bolard J, How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim Biophys Acta* **864**: 257–304, 1986.
9. Szponarski W and Bolard J, Temperature-dependent modes for the binding of the polyene antibiotic amphotericin B to human erythrocyte membranes. A circular dichroism study. *Biochim Biophys Acta* **897**: 229–237, 1987.
10. Capuozzo E, Salerno C, Strom R and Crifò C, Kinetics of binding of lucensomycin to natural and artificial membranes. *Eur J Biochem* **167**: 59–64, 1987.