Effect of NCO-700, an inhibitor of thiol protease, on reactive oxygen production by chemotactic peptide-stimulated rabbit peripheral granulocytes

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Summary. The specific thiol protease inhibitor, NCO-700, which is related to L-trans-epoxysuccinylpeptides, inhibited oxidant production by chemoattractant-stimulated rabbit polymorphonuclear leukocytes. NCO-700 could also scavenge active oxygen generated from sodium hypochlorite-hydrogen peroxide and hypoxanthine-xanthine oxidase systems.
Key words. Active oxygen; rabbit polymorphonuclear leukocyte; thiol protease inhibitor; chemiluminescence.

It has been postulated that protease activity is essential for the production of oxidants by polymorphonuclear leukocytes (PMNs), since serine protease inhibitors such as chlormethyl ketone derivatives inhibit oxidant production by PMNs[1,2]. However, these inhibitors have non-specific effects, and it has been shown that inhibition of oxidant production by chlormethyl ketones is largely due to their inhibition of sulfhydryl groups[3]. A new class of compounds that show promise that they might act as class-specific inhibitors of the thiol (cysteine) proteases are the L-trans-epoxysuccinylpeptides, related to the compound bis[ethyl (2R, 3R)-3-[(S)-3-methyl-1-[4-(2,3,4-tri-methoxyphenylmethyl) piperazin-1-yl-carbonyl] butyl-carbamoyl] oxiran-2-carboxylate] sulfate (NCO-700). NCO-700 was shown to inhibit papain, calcium-activated neutral protease (CANP) and cathepsin B[4,5]. In contrast, the serine proteases trypsin, plasmin and pancreatic elastase, the aspartic protease pepsin, and the metalloprotease thermolysin were unaffected. NCO-700 did not inactivate lactic dehydrogenase, a non-proteolytic thiol-dependent enzyme (unpublished observation). All of these characteristics suggested that NCO-700 might be a valuable inhibitor in the study of a possible role of cysteine proteases in the generation of oxidants by activated PMNs.

We therefore studied the effect of NCO-700 on the oxidant production of chemoattractant-stimulated rabbit PMNs.

Materials and methods. Formyl-methionyl-leucyl-phenylalanine (FMLP), luminol, xanthine oxidase (XOD) and catalase were obtained from Sigma Chemical Co. Hypoxanthine, hydrogen peroxide (H_2O_2), sodium hypochlorite (NaOCl) and taurine were purchased from Wako Pure Chemical Co. NCO-700 was donated by Nippon Chemipher Co., Tokyo. Heparinized blood was obtained from male albino rabbits (2.2–3.0 kg). PMNs were isolated by a modified method of Böyum[6], then washed twice and resuspended in HEPES-saline (5 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, 137 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, pH 7.4). Twelve male albino rabbits weighing 2.0 to 2.3 kg were randomly assigned to either a NCO-700 treatment or a control (normal saline) group. NCO-700 (20 mg · kg^{-1}) was administered i.v. over a 5-min period. Heparinized blood was obtained 5 min after infusion. PMNs were prepared as described above. In the present study, we monitored the generation of reactive oxygen species by using luminol-dependent chemiluminescence (CL)[7,8]. CL was measured with a lumiphotometer (model TD 4000; LABO SCIENCE, Tokyo, Japan) at 37 °C and recorded in millivolts. Compounds were also tested for their ability to inhibit luminol-dependent CL, generated by NaO-Cl-H_2O_2 and hypoxanthine-XOD systems. For statistical analysis of CL measurements, light production was expressed as the peak height and as the area under the CL curve, and these were compared with control studies in which the CL response was set at 100%.

Results and discussion. Table 1 shows that NCO-700, at concentrations between 1 μM and 1 mM, produced a progressive decrease in CL production when compared to production from the control cells. Studies were performed to determine whether inhibition by NCO-700 required its presence at the time of assay. As shown in table 1, the extent of inhibition of CL production in FMLP-stimulated PMNs was almost consistent with that in washed cells. The 30-min incubation in NCO-700 did not result in either microscopic cramping of the PMNs, or a change in their viability, as measured by trypan blue exclusion.

We tested the effect of NCO-700 on the co-oxidation of luminol by hypochlorite plus hydrogen peroxide[8]. NCO-700, and its dehydrated active metabolite, quenched CL production in a dose-dependent manner (data not shown). We further examined the effect of NCO-700 on CL production by the hypoxanthine-XOD system which has often been employed as an indirect index of the presence of scavenger. Table 2 indicates that high concentrations of the drug (> 0.1 mM) inhibited CL production. Since low concentrations of superoxide dismutase (1–10 μg · ml^{-1}) completely

Table 1. Effects of NCO-700 on luminol-dependent CL production. PMNs (1×10^8 cells · ml^{-1}) were incubated with varying concentrations of NCO-700 for 30 min at 37 °C. The cells were then centrifuged, washed twice with HEPES-saline at 4 °C to remove the drug and subsequently assayed for FMLP (3×10^{-8} M)-stimulated CL production after 10-min preincubation at 37 °C. The activity of these cells (Washed) was compared with that of unwashed cells (Direct) assayed for CL production in the presence of the same concentrations of NCO-700. Numbers in parentheses are the number of animals in each group. Values are the means ± standard deviation of the mean.

Drug conc. (M)	% Inhibition (data)	
	Direct (5)	Washed (3)
1×10^{-6}	10.1 ± 3.7	13.6 ± 5.1
3×10^{-6}	17.3 ± 4.8	23.1 ± 3.9
1×10^{-5}	30.3 ± 5.3	32.7 ± 3.1
3×10^{-5}	50.3 ± 7.4	90.4 ± 3.8
1×10^{-4}	70.5 ± 5.9	93.4 ± 2.3
3×10^{-4}	92.0 ± 2.3	98.5 ± 1.2
1×10^{-3}	99.5 ± 1.8	–

Table 2. Effects of NCO-700 on luminol-dependent CL of the hypoxanthine-xanthine oxidase system. A 20 μl aliquot of XOD (4 U · ml^{-1}) was added to a cuvette containing hypoxanthine (1 mM), luminol (50 μM), and varying concentrations of NCO-700. The chemiluminescence was expressed as the peak height (peak) and the area under the CL curve (data) and then compared with control studies in which the CL response was set at 100%. Results are the average of three separate incubations and are presented as means ± standard errors of the means.

Drug conc. (M)	% Inhibition Data	Peak
Buffer	0.00	0.00
3×10^{-5}	4.63 ± 0.93	3.25 ± 2.05
1×10^{-4}	18.90 ± 2.19	18.05 ± 2.43
3×10^{-4}	47.21 ± 1.03	45.46 ± 1.37
1×10^{-3}	85.00 ± 0.43	84.31 ± 0.43
3×10^{-3}	98.88 ± 0.11	96.92 ± 1.23

Table 3. Effects of intravenously administered NCO-700 (20 mg · kg^{-1}) on FMLP-induced CL in rabbit PMNs.

	Chemiluminescence (mV)			
	Data control	Data drug	Peak control	Peak drug
Means (6)	11.23	6.23*	15.79	7.75*
SD	1.91	3.13	4.99	4.57

* $p < 0.05$ (Mann-Whitney U test).

inhibited this CL production, NCO-700 may act by scavenging superoxide anions. The high concentrations employed in this study did not affect urate formation by XOD (data not shown).

The effect of NCO-700 on FMLP-stimulated CL production was further examined in PMNs in vivo. Table 3 shows that PMNs from NCO-700 pre-treated rabbits exhibited a decreased CL production response to FMLP compared to cells prepared from control rabbits. Although we have demonstrated that NCO-700 can quench luminol-dependent CL generated in both NaOCl-H_2O_2 and hypoxanthine-XOD systems, the scavenging effect of the free NCO-700 does not explain its ability to interfere with drug-treated and washed PMNs or PMNs treated in vivo. Indeed, the ability of NCO-700 to interfere with PMNs under these conditions suggests that its primary effects are independent of scavenging and due to a direct, but undetermined, effect on PMNs.

While the present results indicate that FMLP-triggered active oxygen production is prevented by NCO-700, they do not identify the mechanism through which the inhibition is achieved. Although luminol-amplified CL in phagocytosing PMNs depends largely on the presence of myeloperoxidase (MPO) released from the cells, it has been shown that FMLP-triggered CL production was independent of the presence of MPO [9]. Moreover, NCO-700 did not affect MPO activity or FMLP-stimulated MPO release (unpublished observation) in spite of the significant inhibitory effect of NCO-700 on CL production.

Although the evidence in this study is not wholly conclusive, it suggests that NCO-700 may inhibit the production of reactive oxygen. A plausible explanation is that the thiol proteases, presumably CANP, may play important roles in activating NADPH-oxidase, responsible for superoxide anion production, and/or hydrogen peroxide generation. Other factors which could alter the sensitivity of PMNs to FMLP include changes in FMLP-binding to specific receptors on the cell surface and changes in post-receptor activation.

Weiss and others suggested that the critical PMN oxidants are hypochlorous acid or its amino adducts, and/or the N-chloramines, which is consistent with his and other previous studies [10–13]. The MPO-derived hypochlorous acids are probably the most potent oxidants generated by stimulated PMNs. Since NCO-700 scavenges several reactive oxygen species generated in both NaOCl-H_2O_2 and hypoxanthine-XOD systems and inhibits reactive oxygen production, NCO-700 may have some beneficial effects on oxidant-induced tissue injury triggered by activated PMNs.

Interestingly, NCO-700 has recently been described as a protective agent against cardiac muscle degradation induced by coronary artery occlusion in rabbits [14]. There is experimental evidence that drugs which impair PMNs function may reduce the size of acute myocardial infarction [15]. Since NCO-700 inhibits oxidant formation by PMNs, its cardioprotective effect in animals may partly be due to the decrease in cytotoxic oxygen radicals produced by PMNs which have migrated into the ischemic area.

It is tempting to assume that thiol protease, but not serine protease, has some role in generating active oxygen production in FMLP-triggered rabbit PMNs. Although further studies are required to support this hypothesis, the present data indicate that the direct action of NCO-700 is distinct from its scavenging effects.

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Specific radioimmunoprecipitation of histone H2A antigens by protein A conjugated sepharose

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Summary. A modified radioimmunoprecipitation technique is described which allows the specific detection of histone H2A antigens. The technique circumvents unspecific binding of histones to the bacterial adsorbent.
Key words. Protein A; radioimmunoprecipitation; histone H2A.

Protein A, a cell wall constituent of certain *Staphylococcus aureus* strains, binds specifically to the Fc regions of many IgG molecules [1, 2]. Because of its high adsorption capacity, rapid binding and advantageous sedimentation properties, fixed cells of the Cowan strain of the bacterium *Staphylococcus aureus* are widely used as an adsorbent for antigen-antibody-complexes and for rapid isolation and characterization of labeled antigens in radioimmunoassays [3, 4]. The use of this precipitation technique is limited by the fact that fixed *St. aureus* cells bind radiolabeled proteins non-specifically in the