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EFFECT OF NPC 15669, AN INHIBITOR OF NEUTROPHIL RECRUITMENT AND NEUTROPHIL-MEDIATED INFLAMMATION, ON NEUTROPHIL FUNCTION *IN VITRO**

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Abstract—The new anti-inflammatory agent *N*-[9*H*-(2,7-dimethylfluorenyl-9-methoxy)carbonyl]-L-leucine (NPC 15669) inhibits inflammation in several animal models dependent upon neutrophil activation and recruitment into the inflammatory lesion. NPC 15669 appears to elicit its pharmacological action by inhibiting the cell surface expression of CD11b/CD18 (Mac-1) on the neutrophil and subsequent adhesion of the neutrophil to the vascular endothelium. The current study sought to further characterize the action of NPC 15669 on neutrophil function. In the range of 1–100 μM, this fluorene enhanced superoxide production in a concentration-dependent fashion. Using spin trapping/ESR spectroscopy, NPC 15669 was found to inhibit myeloperoxidase (MPO)-dependent hydroxyl radical primarily by scavenging hypochlorous acid, and secondarily by inhibiting agonist-stimulated degranulation as assessed by MPO and elastase release. These studies demonstrated that NPC 15669, in addition to inhibiting adhesion, alters other neutrophil functions. Whether the pharmacological activities described for NPC 15669 resulted directly from changes in Mac-1 expression or through some other mechanism is currently under investigation.

N - [9*H* - (2,7 - Dimethylfluorenyl - 9 - methoxy)-carbonyl]-L-leucine (NPC 15669**) is a member of a novel class of anti-inflammatory agents collectively known as "leumedin" [1]. The structure of NPC 15669 consists of a dimethyl-substituted fluorene ring linked through a carbamate to a leucine group (Fig. 1). These compounds were found to inhibit inflammation in several models characterized by activation and recruitment of neutrophils at the site of inflammation. NPC 15669, in particular, has been found to effectively inhibit (a) arachidonic acid- and phorbol ester-induced ear edema in mice [1], (b) reversed passive Arthus reaction in rats [2], (c) leukopenia and mortality in endotoxic shock in mice [3], (d) mortality in gram-negative sepsis in rats [4], (e) neutrophil recruitment and epithelial and vascular damage in acetic acid colitis [5], and (f) neutrophil activation and pulmonary damage in the post-perfusion syndrome following cardiopulmonary bypass [6].

Studies of mediator production and receptor binding have suggested that NPC 15669 does not act at the level of ligand–receptor interaction [1, 2]. Examination of the effects of NPC 15669 on neutrophil adhesion *in vitro* led to the demonstration that NPC 15669 prevents adhesion of activated neutrophils to either cultured vascular endothelium or artificial protein [7]. Furthermore, the compound was found to inhibit the upregulation of the CD11b/CD18 (Mac-1) adhesion receptor complex on the neutrophil cell surface [7].

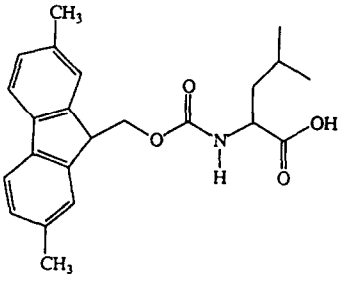

Fig. 1. Structure of *N*-[9*H*-(2,7-dimethylfluorenyl-9-methyoxy)carbonyl]-L-leucine (NPC 15669).

* Parts of this work were presented at the meeting of the American Federation for Clinical Research, Baltimore, MD, May, 1992.

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** Abbreviations: NPC 15669, *N*-[9*H*-(2,7-dimethylfluorenyl-9-methoxy)carbonyl]-L-leucine; fMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; DTPA, diethylenetriaminepentaacetic acid; MCD, monochlorodimedon; NaOCl, sodium hypochlorite; MPO, myeloperoxidase; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBSS, Hanks' balanced salt solution; and 4-POBN, α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone.

The purpose of the present study was to further explore the effects of NPC 15669 on neutrophil function to better understand the mechanism by which NPC 15669 mediates its pharmacological activity.

MATERIALS AND METHODS

Reagents. Diethylenetriaminepentaacetic acid (DTPA), xanthine, dextran with an average molecular weight of 74,200, ferricytochrome *c* (type VI), monochlorodimedon (MCD), sodium hypochlorite (NaOCl), 5-amino-2,3-dihydro-1,4-pthalazinedione (luminol), human myeloperoxidase (MPO), phorbol 12-myristate 13-acetate (PMA), platelet-activating factor (PAF), formyl-methionyl-leucyl-phenylalanine (fMLP), dimethyl sulfoxide (DMSO) and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from the Sigma Chemical Co. (St. Louis, MO). Catalase, superoxide dismutase and xanthine oxidase were obtained from Boehringer-Mannheim (Indianapolis, IN). α-(4-Pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and was used without further purification. Hanks' balanced salt solution (HBSS) and bovine calf serum were obtained from Gibco Laboratories (Grand Island, NY). NPC 15669 was from the Scios-Nova Pharmaceutical Corp. (Baltimore, MD). Lymphoprep was from Nycomed (Oslo, Norway).

The buffer system used for the spin-trapping experiment was HBSS, pH 7.4, containing DTPA (0.1 mM), without phenol red and in the absence of magnesium and calcium salts.

Neutrophil isolation. Neutrophils were isolated from whole blood of normal human volunteers as previously described [8], with minor modification. Briefly, neutrophils and red blood cells were separated from other cellular components by centrifugation with Lymphoprep solution at 327 *g* for 20 min at room temperature. The resulting leukocyte suspension was sedimented over a 6% dextran solution followed by osmotic lysis of contaminating erythrocytes. Neutrophils were then suspended in HBSS buffer and stored on ice, and the concentration was adjusted accordingly. Giemsa stain revealed that more than 98% of the cells were neutrophils, and the viability was more than 95% as determined by exclusion of Trypan Blue dye.

Superoxide detection. Superoxide was measured at 37° as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* and monitored at 550 nm, as previously reported [9]. The rate of superoxide generation was calculated using an extinction coefficient of 21 $mM^{-1}\,cm^{-1}$. To stimulate superoxide production by neutrophils, PMA (100 ng/mL in DMSO) was added to a mixture of cells ($2-3 \times 10^6$/mL), various concentrations of NPC 15669 dissolved in DMSO, and ferricytochrome *c* (80 μM). The highest final concentration of DMSO used was 1.1% of the total volume (1 mL). Results are expressed as a percentage of the maximum control response produced in the absence of NPC 15669.

ESR/spin trapping of hydroxyl radical. Spin trapping of hydroxyl radical was performed as described previously [10] by mixing neutrophils ($10-20 \times 10^6$/mL), 4-POBN (10 mM), ethanol (170 mM), with and without NPC 15669 (100 μM), PMA (100 ng/mL in ethanol) and sufficient HBSS for a final volume of 0.5 mL. Reaction mixtures were transferred to a quartz ESR flat cell open to air, fitted into the cavity of the spectrometer (Century Line model E109, Varian Associates, Palo Alto, CA) and the spectra were recorded at 25°. Instrument settings were microwave power, 20 mW; modulation amplitude, 1.0 G; sweep time, 12.5 G/min; and response time, 1 sec. The receiver gain is given in the figure legends.

Decomposition of NaOCl. Sodium hypochlorite concentrations were measured using the ability of NaOCl to chlorinate MCD. Changes in MCD concentration following the addition of NaOCl were quantitated via the change in absorbance at 290 nm using an extinction coefficient of 19,000 $M^{-1}\,cm^{-1}$ for MCD [11].

Myeloperoxidase and elastase release. Human neutrophils were suspended to 1×10^7/mL in HBSS containing HEPES (10 mM), pH 7.4, without calcium or magnesium ion. The suspension was treated for 5 min with cytochalasin B (5 μg/mL); then $CaCl_2$ was added to 1 mM and $MgCl_2$ to 0.5 mM and the neutrophils were stimulated immediately with fMLP (10 μM), PAF (1 μM), or PMA (10 nM in ethanol) for 20 min at 37°. Cells were pelleted in a microfuge for 20 sec, and the supernatant was recovered for assay of released MPO or elastase. When NPC 15669 was present, it was added just after cytochalasin B. MPO was assayed spectrophotometrically using the *O*-dianisidine method of Krawiscz *et al.* [12], while elastase was determined by the method of Heubner [13]. The EC_{50} was determined using the Sigma plot software.

Chemiluminescence. One milliliter of 10^{-4} M

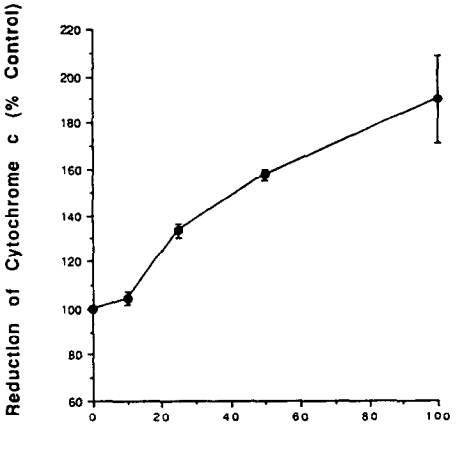

Fig. 2. Effect of increasing concentrations of NPC 15669 on superoxide-inhibitable ferricytochrome *c* reduction resulting from the addition of 100 ng/mL of PMA. Results are means ± SEM of at least three separate experiments. The rate of superoxide production in the absence of NPC 15669 was 3.38 ± 0.01 μM/min/10^6 cells.

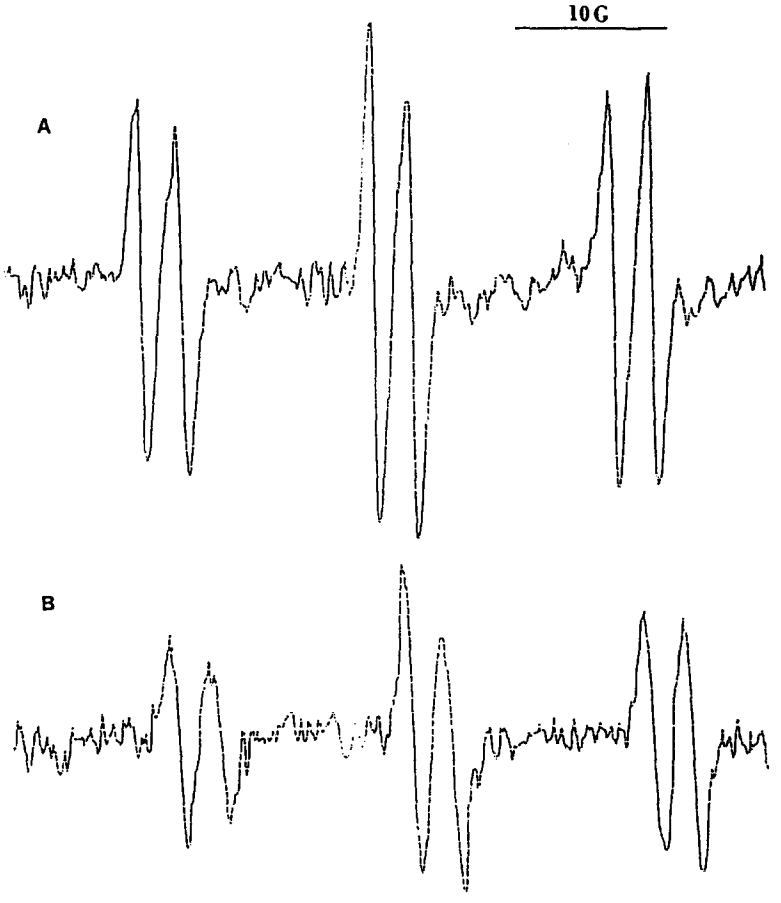

Fig. 3. ESR spectra obtained 18 min following the addition of PMA (100 ng/mL) to neutrophils (20×10^6/mL) (A) in the absence of NPC 15669, and (B) in the presence of NPC 15669 (100 μM). The final concentrations of ethanol and 4-POBN were 170 and 10 mM, respectively. The receiver gain was 6.3×10^4.

luminol and 1 mL of neutrophils (5×10^6) were added to the chamber of an integrating photometer (model 3000; SAI Technology, San Diego), and after 4 min of baseline recording, PMA (100 ng/mL) was added in the presence and absence of NPC 15669. Luminescence was then recorded as described previously [14].

RESULTS AND DISCUSSION

During inflammation, reduction products of oxygen, including superoxide, are generated by stimulated neutrophils [15]. These reactive species have been proposed to mediate tissue injury at the site of inflammation. Steroids and non-steroidal anti-inflammatory drugs have been shown to inhibit the NADPH oxidase of human neutrophils in whole-cell and cell-free systems [16, 17]. Because leumedins have exhibited remarkable anti-inflammatory activity [1], we examined the effects of these compounds on free radical formation by stimulated human neutrophils.

Initial experiments were conducted to determine

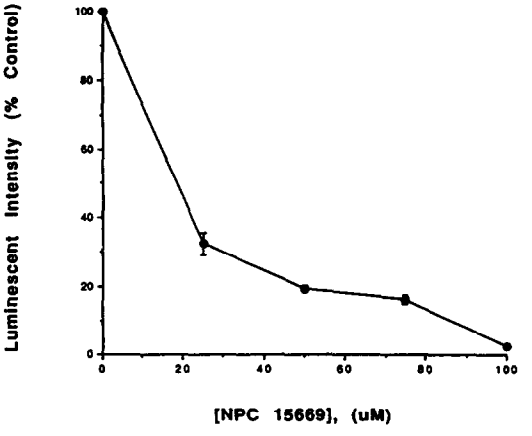

Fig. 4. Effect of increasing concentrations of NPC 15669 on chemiluminescence resulting from the addition of PMA (100 ng/mL) to neutrophils (5×10^6/mL). Results are means ± SEM of at least three separate experiments. Luminescence in the absence of NPC 15669 was 109 ± 7 mV.

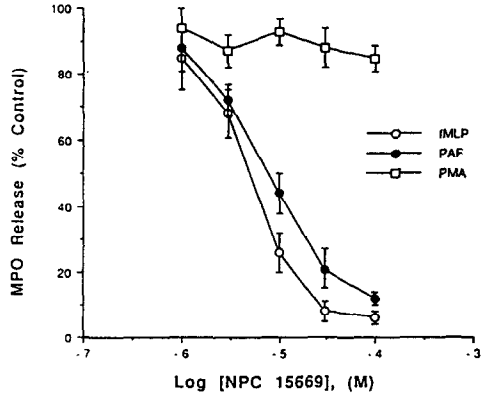

Fig. 5. Effect of increasing concentrations of NPC 15669 on MPO release resulting from the addition of 10 μM fMLP, 1 μM PAF and 10 nM PMA. Results are means ± SEM of at least three separate experiments. MPO released from unstimulated cells was 0.2 ± 0.03 units, and was increased to 0.7 ± 0.1 units by fMLP, 2.4 ± 0.3 units by PAF, and 1.6 ± 0.3 units by PMA.

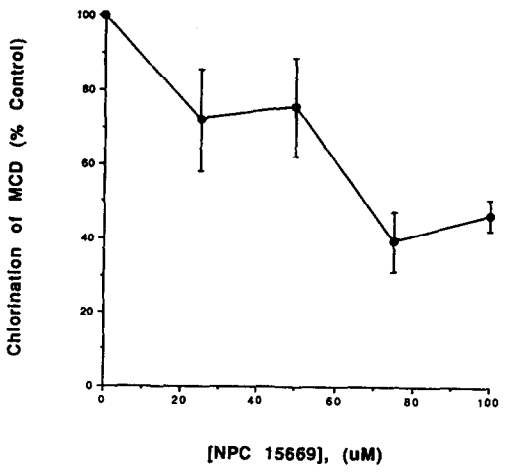

Fig. 7. Effect of increasing concentrations of NPC 15669 on the oxidation of MCD by NaOCl. Results are means ± SEM of at least three separate experiments. The concentration of MCD oxidized per 100 nmol NaOCl added in the absence of NPC 15669 was 62.2 ± 18.6 nmol.

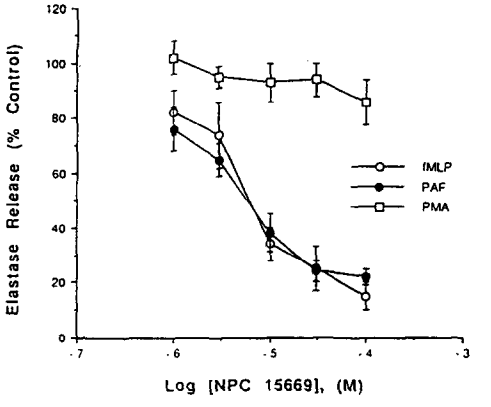

Fig. 6. Effect of increasing concentrations of NPC 15669 on elastase release resulting from the addition of 10 μM fMLP, 1 μM PAF and 10 nM PMA. Results are means ± SEM of at least three separate experiments. Elastase release from unstimulated cells was 0.08 ± 0.01, and was increased to 0.19 ± 0.02 by fMLP, 0.56 ± 0.11 by PAF, and 0.36 ± 0.05 by PMA.

the effects of this leumedin on neutrophil viability. NPC 15669 up to 100 μM had a minimal effect on neutrophil viability for at least 20 min at 25° as assessed by the exclusion of Trypan Blue dye. Next, experiments were performed to determine the effects of NPC 15669 on the production of superoxide by stimulated neutrophils. For these studies, the initial rate of superoxide production by PMA-activated human neutrophils was measured as a function of NPC 15669 concentration. For simplicity, we represent the change in the rate of superoxide generation in the presence and in the absence of NPC 15669. As shown in Fig. 2, NPC 15669 actually led to increased formation of superoxide. The rate of production increased in a stepwise fashion at concentrations of the leumedin from 10 to 100 μM. It is interesting to note that the effective concentration of NPC 15669 in mediating a variety of anti-inflammatory actions and in inhibiting adhesion of neutrophils to the endothelium is 30–100 μM [2–8], the concentration which maximally enhanced superoxide production. Thus, in contrast to steroid and non-steroidal anti-inflammatory drugs, the effective concentration of NPC 15669 necessary to elicit this pharmacological activity did not inhibit neutrophil-generated superoxide.

Our next series of experiments were designed to determine the effect of NPC 15669 on hydroxyl radical production by stimulated human neutrophils, using spin trapping combined with ESR spectroscopy [18]. This technique consists of using a nitrone or nitroso compound to trap the initial unstable radical to give a long lived spin-trapped adduct, a nitroxide which can be observed by conventional ESR at room temperature [19]. In a previous study [10], we demonstrated that using the spin-trapping system of 4-POBN/ethanol, PMA-stimulated neutrophils produce hydroxyl radicals through an MPO-dependent pathway as shown:

$$O_2^{-\cdot} + O_2^{-\cdot} \rightarrow H_2O_2$$

$$H_2O_2 + NaCl + MPO \rightarrow HOCl$$

$$HOCl + O_2^{-\cdot} \rightarrow HO^{\cdot} + Cl^- + O_2.$$

Based on the observation that NPC 15669 increased superoxide flux, we expected an enhancement in the hydroxyl radical production when neutrophils were treated with leumedins. However, when PMA-stimulated neutrophils were treated with NPC 15669 (100 μM), we found a significant decrease in spin-trapped hydroxyl radical (Fig. 3). This inhibition was not due to the direct scavenging of hydroxyl

radical by NPC 15669, since NPC 15669 had no effect on the ESR signal generated by the action of xanthine oxidase on xanthine in the presence of the spin trap 4-POBN, ethanol and ferrous ion (data not shown). Alternatively, this leumedin might inhibit some aspect of the MPO-dependent reaction. To test this hypothesis we monitored luminol-dependent luminescence generated by PMA-stimulated neutrophils. A variety of reactive oxygen species, most reliably hypochlorous acid, elicit this luminescence [14]. Light detection was inhibited by NPC 15669 in a concentration-dependent manner (Fig. 4). This decrease in luminescence could result from inhibition of appropriate mobilization or MPO activity. Alternatively, it could result from scavenging of hypochlorous acid by leumedins. All of these possibilities were examined. MPO activity was monitored by measuring oxidation of 4-aminopyrine. In this case, NPC 15669 (100 μM) did not inhibit the activity of this enzyme (data not shown). The release of MPO was measured in response to fMLP, PAF and PMA. As shown in Fig. 5, NPC 15669, while exhibiting remarkable inhibition of MPO release with an EC_{50} of less than 10 μM for fMLP and PAF, had no effect on PMA-induced release of MPO. Similar results were observed for another granular enzyme, elastase (Fig. 6). Finally, this leumedin was found to inhibit the oxidation of MCD by hypochlorous acid (Fig. 7). Thus, the hypochlorous acid scavenging effect of NPC 15669 may be responsible for decreased hydroxyl radical generation as detected by spin-trapping experiments.

In this paper, we report on the effects of a new class of anti-inflammatory agents, the leumedins, on important physiologic functions of the neutrophil. Surprisingly, NPC 15669 increased superoxide production, while inhibiting hydroxyl radical formation. The latter radical is believed to be a more significant inflammatory oxidant. We have demonstrated previously MPO-dependent hydroxyl radical formation by neutrophils in the absence of redox active metals [10]. It appears that NPC 15669 inhibited the formation of this free radical by primarily scavenging hypochlorous acid. These studies suggest another mechanism by which NPC 15669 and other leumedins might mediate their anti-inflammatory activity.

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