Biochemical Pharmacology, Vol 46, No 8, pp. 1487–1490, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

INHIBITION BY THE PROTEIN KINASE INHIBITOR HA1077 OF THE ACTIVATION OF NADPH OXIDASE IN HUMAN NEUTROPHILS

MICHIKO ARAI, YASUHARU SASAKI* and RYUSHI NOZAWA†

Laboratory of Microbiology and Host Defenses, University of Shizuoka School of Food and Nutritional Sciences, Shizuoka 422; and *Life Science Center, Asahi Chemical Industry Co. Ltd., Nobeoka 882, Japan

(*Received* 22 *March* 1993; *accepted* 4 *June* 1993)

Abstract—The effect of an inhibitor of protein kinase, HA1077 [1-(5-isoquinolinesulfonyl)-homopiperazine HCl], and its hydroxylated metabolite, HA1100, on the activation of NADPH oxidase in human neutrophils were studied. Cells were preincubated with each drug for 10 min and then activated by treatment with phorbol myristate acetate (PMA) or formylmethionyl leucyl phenylalanine (FMLP). After activation, the rate of superoxide dismutase-inhibitable reduction of cytochrome *c* was estimated. HA1077 and HA1100 inhibited the PMA-induced production of O_2^- by neutrophil NADPH oxidase in a concentration-dependent manner (IC_{50} = 15 and 24 μM, respectively). The sensitivity of the FMLP-induced production of O_2^- to these drugs was similar. The production of O_2^- in 1,25-dihydroxyvitamin D_3-treated HL-60 cells, which differentiated to macrophage-like cells, was also inhibited by the drugs. The extent of inhibition by HA1077 was almost the same as that by a calmodulin inhibitor (W-7) and by inhibitors of protein kinase (H-7 and H-8). In a cell-free lysate of neutrophils, the NADPH-dependent production of O_2^- can be induced by sodium dodecyl sulfate (SDS). HA1077 at 100 μM had only a weak inhibitory effect on the cell-free, SDS-induced production of O_2^-, an indication that HA1077 inhibits the activation of NADPH oxidase, not the actual activity. The effects of H-7 and H-8 were similar to that of HA1077, whereas W-7 inhibited the production of O_2^- by the cell-free extract of HL-60 cells. This action of HA1077 could explain, in part, its ability to protect neuronal cells from death after ischemia.

A novel antivasospasm drug, 1-(5-isoquinolinesulfonyl)homopiperazine, known as HA1077, belongs to a class of calcium antagonists that differ from the calcium entry blockers [1]. The drug also protects neuronal cells in the hippocampus of the Mongolian gerbil brain from death after ischemia [2]. The role of oxygen free radicals in brain ischemia has been discussed by many investigators [3–5]. Oxygen radicals are produced during specific biochemical reactions in a variety of tissues and cells [6]. Phagocytes are well known producers of oxygen radicals and generate O_2^- via the action of NADPH oxidase in the respiratory burst. Components of NADPH oxidase are segregated to the membrane and the cytosol in inert phagocytes [7]. Upon activation by phagocytic and inflammatory stimuli, and by a tumor promotor, a cytosolic component known as p47 is phosphorylated. It is transported with another cytosolic component(s) to the membrane and forms an active NADPH oxidase complex with heterodimeric cytochrome b_{558} [8–10]. A tumor promotor, phorbol myristate acetate (PMA‡), activates phagocytes by directly stimulating protein kinase C [11], and a chemotactic peptide, formylmethionyl leucyl phenylalanine (FMLP), activates phagocytes by binding to the G-protein-coupled receptor with subsequent release of second messengers, such as inositol phosphates and diacylglycerol [12]. HA1077 has been shown recently to be an inhibitor of cyclic nucleotide-dependent protein kinases and protein kinase C [13]. Therefore, we studied the effects of HA1077 and HA1100 [1-(5-(1-hydroxy)isoquinolinesulfonyl)-homopiperazine] on the activity of NADPH oxidase in human phagocytes that had been activated by treatment with PMA or FMLP. Since cell-free components of NADPH oxidase can be activated by sodium dodecyl sulfate (SDS) [14], the effect of HA1077 on a cell-free oxidase assay was tested to determine whether the drug affects the steps in the activation of NADPH oxidase. Also, the effects of known inhibitors of calmodulin (W-7) [15] and of kinases (H-7 and H-8) [16, 17] on these assays were compared. HA1077 as well as HA1100 inhibited the production of O_2^- in human neutrophils and macrophage-like cells, probably by blocking the activation of NADPH oxidase.

† Corresponding author: Dr. Ryushi Nozawa, Laboratory of Microbiology and Host Defenses, University of Shizuoka School of Food and Nutritional Sciences, 52-1 Yada, Shizuoka-shi 422, Japan. Tel. 81-54-264-5553; FAX 81-54-264-5553.

‡ Abbreviations: PMA, phorbol myristate acetate; FMLP, formylmethionyl leucyl phenylalanine; VD_3, 1,25-dihydroxyvitamin D_3; SOD, superoxide dismutase; and SDS, sodium dodecyl sulfate.

MATERIALS AND METHODS

Materials. W-7, H-7 and H-8 were obtained from the Seikagaku Kogyo Co. (Tokyo, Japan). 1,25-Dihydroxyvitamin D_3 (VD_3) was from the Roussel Japan Co. (Tokyo); NADPH was from the Boehringer-Mannheim-Yamanouchi Co. (Tokyo);

cytochrome *c*, FMLP, PMA, phenylmethylsulfonyl fluoride (PMSF) and superoxide dismutase (SOD) were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); Dextran T500 was from Pharmacia (Tokyo); fetal bovine serum was from Bioserum (Victoria, Australia); Lymphoprep was from Nycomed Pharma As (Oslo, Norway); and leupeptin and pepstatin were from the Institute for Microbial Chemistry (Tokyo).

Cells and culture. Human promyelocytic leukemia HL-60 cells were inoculated at 2×10^5 cells/mL into F12 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 μg/mL kanamycin sulfate and 100 μg/mL aminobenzyl penicillin), and treated with 10^{-7} M VD_3 for 3 days at 37° in a 5% CO_2 incubator [17].

Preparation of neutrophils. Heparinized human peripheral blood was mixed with an equal volume of 3% Dextran T500 in phosphate-buffered saline and allowed to stand at room temperature for 15 min. The buffy coat fraction was layered on Lymphoprep solution and centrifuged at 480 *g* for 40 min at room temperature. The neutrophil-rich sediment was treated with H_2O, and contaminating red blood cells were disrupted [17].

Preparation of neutrophil lysate. Neutrophils (10^7 cells) were suspended in 3 mL of 6% sucrose in relaxation buffer [18] that contained 1 mM PMSF, 10 μg/mL leupeptin and 10 μg/mL pepstatin and sonicated at 4° as described elsewhere [19]. The disrupted cells were centrifuged at 800 *g* for 20 min, and the supernatant was used for the assay of cell-free NADPH oxidase activity.

Assay of production of O_2^- by intact cells. Cells were treated with each drug at room temperature for 10 min in Hanks' balanced saline solution. The assay mixture (0.5 mL of Hanks' solution in a cuvette) contained 80 μM cytochrome *c*, and 2×10^5 neutrophils or 5×10^5 VD_3-treated HL-60 cells. PMA or FMLP was added to the mixture to a final concentration of 10 ng/mL or 1 μM, respectively, and monitoring of the mixture at 550 nm was initiated immediately in a spectrophotometer (U3210; Hitachi, Tokyo). A cuvette with 150 U of SOD in the identical mixture was monitored as a reference. Assays were performed at room temperature. In some experiments, the assay mixture in an Eppendorf-type tube was incubated at 37° for 20 min and then rapidly cooled in iced water. The cells were pelleted by centrifugation, and the absorbance of the supernatant was measured at 550 nm.

Assay of NADPH oxidase in a cell-free system. The NADPH-dependent production of O_2^- in a cell-free lysate of neutrophils was measured in the presence of SDS by the method of Bromberg and Pick [14].

RESULTS

Inhibition by HA1077 of the production of O_2^- in neutrophils. The effects of HA1077 on the activity of PMA-stimulated NADPH oxidase in neutrophils were examined (Fig. 1B). Neutrophils were incubated with the indicated concentrations of HA1077 for 10 min, stimulated by treatment with 10 ng/mL of PMA, and the SOD-inhibitable reduction of cytochrome *c* was monitored at room temperature. HA1077 at 1–100 μM inhibited the activity of NADPH oxidase in a concentration-dependent manner. The concentration for 50% inhibition (IC_{50}) was 15 μM. HA1100 inhibited the reaction to a lesser extent (IC_{50} = 24 μM) (Fig. 1A). Neutrophils were then stimulated with 1 μM FMLP, and the effects of HA1077 and HA1100 were monitored (Fig. 2). HA1077 and HA1100 at 30 μM inhibited NADPH oxidase activity by 67 and 29%, respectively.

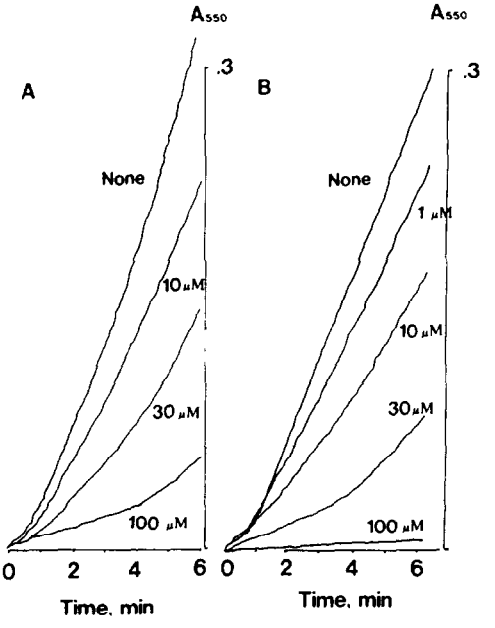

Fig. 1. Inhibition by HA compounds of the production of O_2^- in PMA-stimulated neutrophils. Neutrophils were preincubated at room temperature for 10 min with HA1100 (A) and HA1077 (B) at indicated concentrations, and then cells were activated by the addition of PMA at 10 ng/mL at time 0. The production of O_2^- was monitored at room temperature.

Inhibition by HA1077 of the production of O_2^- in VD_3-treated HL-60 cells. HL-60 cells were treated with 10^{-7} M VD_3 for 3 days and, as a result, they were induced to differentiate into monocyte-lineage cells [17]. These cells were preincubated with indicated concentrations of HA1077, HA1100, W-7, H-7, and H-8 for 10 min, and the PMA-stimulated activity of NADPH oxidase was estimated at 37° (Fig. 3 and Table 1). The IC_{50} values for these drugs ranged from 10 to 21 μM. The inhibitory effect of HA compounds was not due to their cytotoxicity since neither drug at 100 μM had any great effect on the growth of HL-60 cells.

Effects of HA1077 on the cell-free production of O_2^-. A cell-free lysate of neutrophils was prepared and incubated with 0.003% SDS in the presence of 50 μM NADPH. O_2^- was produced by the SDS-activated NADPH oxidase (Fig. 4). HA1077 and HA1100 at 100 μM only inhibited the production of O_2^- by 20%. The effects of W-7, H-7 and H-8 on the cell-free production of O_2^- were also examined (Table 1). W-7 inhibited the SDS-activated NADPH oxidase, whereas H-7 and H-8 had no inhibitory effect under these conditions.

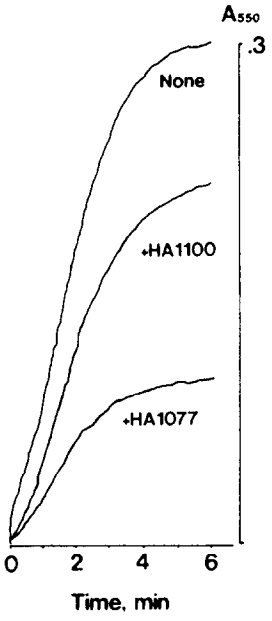

Fig. 2. Inhibition by HA compounds of the production of O_2^- in FMLP-stimulated neutrophils. Neutrophils were preincubated at room temperature for 10 min with HA compounds at 0 or 30 μM, and then they were activated by the addition of FMLP at 1 μM at time 0. The production of O_2^- was monitored at room temperature.

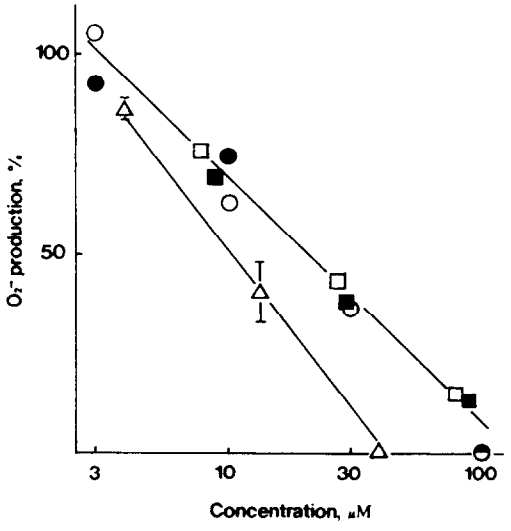

Fig. 3. Effects of HA compounds and of inhibitors of calmodulin and protein kinases on the production of O_2^- in VD_3-treated HL-60 cells. The HL-60 cells were preincubated at room temperature for 10 min with HA-1077 (○), HA1100 (●), W-7 (△), H-7 (□), and H-8 (■) at the indicated concentrations, and then cells were activated by the addition of PMA at 10 ng/mL. Assay tubes were incubated at 37° for 20 min as described in Materials and Methods. Production of O_2^- by drug-treated cells is expressed as a percentage of that by untreated cells. Experiments were performed in duplicate (HA compounds) or in triplicate. Bars represent standard deviations. Absence of bars indicates that standard deviations fell within the symbols.

Table 1. Effects of HA compounds and of inhibitors of calmodulin and protein kinases on the production of O_2^- in intact cells and in a cell-free system

Drug	IC_{50} (μM)	
	Intact	Cell-free
W-7	10	30
H-7	21	300
H-8	18	300
HA1077	16	ND*
HA1100	21	ND

The cell-free lysate for the assay of SDS-dependent NADPH oxidase activity was prepared from VD_3-treated HL-60 cells as described in the text. The IC_{50} of each drug for intact cells was calculated from the results in Fig. 3.
* Not done.

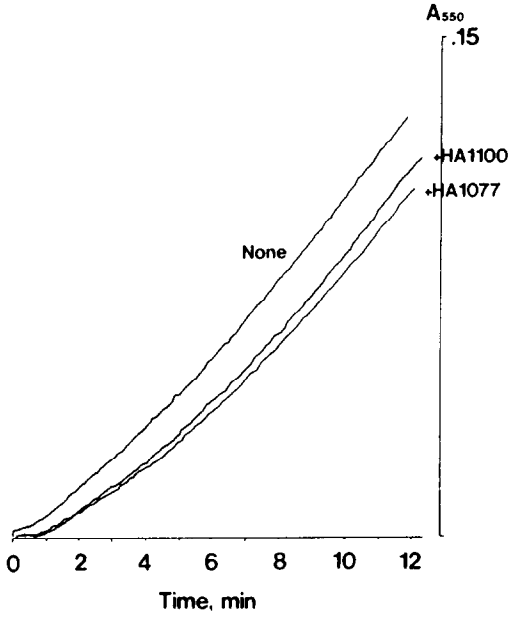

Fig. 4. Effects of HA compounds on the cell-free production of O_2^-. NADPH oxidase in a lysate of neutrophils was activated by SDS, and production of O_2^- was monitored at room temperature in the absence or presence of HA compounds at 100 μM.

DISCUSSION

Activated phagocytes develop the capacity for multiple functions, such as aggregation, adhesion, chemotaxis, phagocytosis, degranulation and the respiratory burst [20]. The respiratory burst is the most suitable of these functions for assays of the effects of drugs that might be expected to affect the activation of phagocytes, since the oxidase activity associated with the respiratory burst can be measured in a cell-free system as well as in intact cells. In a cell-free system, SDS, probably by acting as an anionic detergent, mimics the effects of the phosphorylation of components of NADPH oxidase with resultant formation of the activated cytochrome b_{558} complex and the subsequent production of O_2^-. HA1077 and HA1100 inhibited NADPH oxidase activity in intact cells but not in a cell-free system,

a result that indicates that the HA compounds affect steps in the process of activation of NADPH oxidase (formation of the activated cytochrome b_{558} complex) rather than the activated NADPH oxidase itself. The well known inhibitors of protein kinases, H-7 and H-8, had the same effect. Therefore, the effect of HA1077 appears to be due to its inhibitory effect on calcium-associated protein kinases [13]. Further experiments to examine the effects of HA1077 on the phosphorylation of components of NADPH oxidase may confirm this possibility.

It was demonstrated recently that lazaroids, which were developed for use in the treatment of trauma to the central nervous system and ischemia, can prevent ischemia–reperfusion injury in a variety of organs [21, 22]. Lazaroids inhibit the production of O_2^- by NADPH oxidase in human neutrophils, a result that suggests that oxygen free radicals produced by activated phagocytic cells are involved in postischemic cell death [23]. HA1077 had a protective effect on selectively vulnerable CA_1 pyramidal neurons in the hippocampus of the Mongolian gerbil brain after ischemia [2]. The inhibitory effect of HA1077 on the production of O_2^- in phagocytes may explain its protective effect against ischemic damage. HA1077 appears to inhibit the activation of NADPH oxidase, whereas lazaroids inhibit the activated enzyme itself [23]. The development of drugs with different types of inhibitory effect should increase the therapeutic strategies available to clinicians.

REFERENCES

1. Asano T, Ikegaki I, Satoh S, Suzuki Y, Shibuya M, Takayasu M and Hidaka H, Mechanism of action of a novel antivasospasm drug, HA1077. *J Pharmacol Exp Ther* **241**: 1033–1040, 1987.
2. Asano T, Ikegaki I, Satoh S, Mochizuki D, Hidaka H, Suzuki Y, Shibuya M and Sugita K, Blockade of intracellular actions of calcium may protect against ischaemic damage to the gerbil brain. *Br J Pharmacol* **103**: 1935–1938, 1991.
3. Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, McCord JE and Harman D, Oxygen radicals and human disease. *Ann Intern Med* **107**: 526–545, 1987.
4. Halliwell B, Oxidants and human disease: Some new concepts. *FASEB J* **1**: 358–364, 1987.
5. Floyd RA, Role of oxygen free radicals in carcinogenesis and brain ischemia. *FASEB J* **4**: 2587–2597, 1990.
6. Fridovich I, The biology of oxygen radicals. *Science* **201**: 875–880, 1978.
7. McPhail LC, Shirley PS, Clayton CC and Snyderman R, Activation of the respiratory burst enzyme from human neutrophils in a cell-free system. Evidence for a soluble factor. *J Clin Invest* **75**: 1735–1739, 1985.
8. Segal AW, Heyworth PG, Cockcroft S and Barrowman MM, Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a M_r-44,000 protein. *Nature* **316**: 547–549, 1985.
9. Okamura N, Malawista SE, Roberts RL, Rosen H, Ochs HD, Babior BM and Curnutte JT, Phosphorylation of the oxidase-related 48K phosphoprotein family in the unusual autosomal cytochrome-negative and X-linked cytochrome-positive types of chronic granulomatous disease. *Blood* **72**: 811–816, 1988.
10. Rotrosen D, and Leto TL, Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor: Translocation to membrane is associated with distinct phosphorylation events. *J Biol Chem* **265**: 19910–19915, 1990.
11. Nishizuka Y, The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308**: 693–698, 1984.
12. Boulay F, Tardif M, Brouchon L and Vignais P, The human *N*-formylpeptide receptor. Characterization of two cDNA isolates and evidence for a new subfamily of G-protein-coupled receptors. *Biochemistry* **29**: 11123–11133, 1990.
13. Asano T, Suzuki T, Tsuchiya M, Satoh S, Ikegaki I, Shibuya M, Suzuki Y and Hidaka H, Vasodilator actions of HA1077 *in vitro* and *in vivo* putatively mediated by the inhibition of protein kinase. *Br J Pharmacol* **98**: 1091–1100, 1989.
14. Bromberg Y and Pick E, Activation of NADPH-dependent superoxide production in a cell-free system by sodium dodecyl sulfate *J Biol Chem* **260**: 13539–13545, 1985.
15. Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fujii Y and Nagata T, *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc Natl Acad Sci USA* **78**: 4354–4357, 1981.
16. Hidaka H, Inagaki M, Kawamoto S and Sasaki Y, Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**: 5036–5041, 1984.
17. Nozawa R, Kato H, Ito T and Yokota T, Identification and characterization of a differentiation antigen in human neutrophils and monocytes. *Blood* **71**: 1288–1294, 1988.
18. Borregaard N, Heiple JM, Simons ER and Clark RA, Subcellular localization of the *b*-cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. *J Cell Biol* **97**: 52–61, 1983.
19. Nozawa R, Kato H and Yokota T, Induction of cytosolic activation factor for NADPH oxidase in differentiated HL-60 leukemia cells. *J Biochem (Tokyo)* **103**: 43–47, 1988.
20. Tauber AI, Protein kinase C and the activation of the human neutrophil NADPH-oxidase *Blood* **69**: 711–720, 1987.
21. Braughler JM, Pregenzer JF, Chase RL, Duncan LA, Jacobsen EJ and McCall JM, Novel 21-amino steroids as potent inhibitors of iron-dependent lipid peroxidation. *J Biol Chem* **262**: 10438–10440, 1987.
22. Jacobsen EJ, McCall JM, Ayer DE, VanDoornik FJ, Palmer JR, Belonga KL, Braughler JM, Hall ED, Houser DJ, Krook MA and Runge TA, Novel 21-aminosteroids that inhibit iron-dependent lipid peroxidation and protect against central nervous system trauma. *J Med Chem* **33**: 1145–1151, 1990.
23. Thomas PD, Mao GD, Rabinovitch A and Poznansky MJ, Inhibition of superoxide-generating NADPH oxidase of human neutrophils by lazaroids (21-aminosteroids and 2-methylaminochromans). *Biochem Pharmacol* **45**: 241–251, 1993.