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PHARMACODYNAMICS

E. Ueta · T. Osaki · N. Kawasaki · Y. Nomura

Suppression of respiratory burst of polymorphonuclear leukocytes by Azelastine hydrochloride (Azeptin)

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Abstract The inhibitory action of azelastine hydrochloride (Azeptin) on the respiratory burst in peripheral polymorphonuclear leukocytes (PMN) and pulmonary alveolar macrophages (PAM) has been studied. Azeptin in vitro suppressed chemiluminescence and superoxide (O_2^-) generation by human PMN in a dose- and time-dependent manner. Phorbol myristyl acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (FMLP)-induced O_2^- generation were strongly suppressed by 10^{-6}M and 10^{-5}M Azeptin, respectively. PMN and PAM from rabbits injected with Azeptin 0.2 mg · kg^{-1} for 5 days showed lower chemiluminescence and O_2^- generation than cells from untreated rabbits. Nitroblue tetrazolium reduction activity in human PMN was suppressed by treatment of PMN with 10^{-6}M Azeptin for 6 h. Inositol trisphosphate, intracellular free calcium, and protein kinase C activity were decreased by 10^{-6}M to 10^{-5}M Azeptin. The tyrosine phosphorylation of many proteins, especially a 115 kDa protein, was suppressed by 10^{-5}M Azeptin. However, superoxide dismutase activity in PMN, PAM, and lung tissue samples was only slightly decreased, even when the rabbits were treated with 1.0 mg · kg^{-1} Azeptin for 5 days. The results suggest that Azeptin suppresses multiple signal transduction steps in the respiratory burst of PMN. This suppressive action should be very useful in the prevention and treatment of reactive oxygen-associated disorders.

Key words Azelastine, Polymorphonuclear leukocytes, Respiratory burst oxidase; alveolar macrophage, superoxide, superoxide dismutase

Abbreviations Azeptin, azelastine hydrochloride; PMN, polymorphonuclear leukocytes; PAM, pulmonary alveolar macrophages; PMA, phorbol myristyl acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; PKC, protein kinase C; ROS, reactive oxygen species; O_2^-, superoxide; IP_3, inositol 1,4,5-trisphosphate; DG, diacylglycerol; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-alpha

E. Ueta (✉) · T. Osaki · N. Kawasaki · Y. Nomura
Department of Oral Surgery, Kochi Medical School, Kohasu, Okocho, Nankoku-city, Kochi 783, Japan

Azeptin is used in the treatment of many disorders, including allergic dermatitis, asthma, and Behcet's disease [1, 2]. The possible inhibitory action of this drug on leukocytes, for example suppression of the release of allergy-provoking chemical mediators, has been investigated [3, 4]. Azeptin also protects also protects cell membranes against hypotonic shock [5]. However, our understanding of its pharmacological actions remains incomplete.

The role of reactive oxygen species (ROS) in inflammation and in the impairment of tumour and normal cells has been investigated clinically and in the laboratory [6, 7]. ROS are essential for protecting the body against microbial invasion, and the bacterio- and fungicidal activity of phagocytic cells largely depends on ROS production [8, 9]. However, without exception ROS also impair cells if the ROS-scavenging capacity of the cells is insufficient. ROS occasionally become the initiators of pathogenic events as well as regulating the pathophysiology of many diseases.

Signal transduction in the induction and activation of cytotoxic T cells and in ROS generation of PMN has been investigated, and the importance of protein tyrosine phosphorylation has been shown in many types of cells [10–16]. There are two pathways of signal transduction for ROS generation, direct PKC (protein kinase C) activation and indirect activation of PKC via diacylglycerol (DG). PMA stimulates PKC directly, and FMLP induces phospholipase C activation and inositol phosphorylation, resulting in DG generation. Like T cell activation, the respiratory burst of PMN is regulated by protein tyrosine phosphorylation [13–16]. However, the details of signal transduction in ROS generation have yet to be clarified.

In the present study the effects of a suppressive agent, azelastine (Azeptin), on ROS and signal transduction, have been examined in human and rabbit cells.

Materials and methods

Cell preparation

PMN were separated from heparinised peripheral blood obtained from rabbits and healthy persons. After centrifugation at 400 g for 10 min, the buffy coat layer was collected, diluted in 3 vol PBS, and centrifuged on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) gradients, according to Böyum's method [17]. The PMN pellets were resuspended in PBS containg 3 % (w/v) dextran to remove any contaminating erythrocytes. After centrifugation, the PMN were resuspended in a hypotonic buffer solution, and any residual erythrocytes were removed. PMN puritiy of more than 95 % and cell viability of more than 98 % were microscopically confirmed by Giemsa staining and trypan blue exclusion, respectively. Rabbit PAM were obtained from bronchial lavage fluid by the method of Reynolds et al. [18]. Their purity and viability were also examined microscopically.

Chemiluminescence

Chemiluminescence was measured with a calcium analyser (CAF-100, JASCO Co., Ltd., Tokyo, Japan). A cell suspension (5×10^5 cells $\cdot$ ml^{-1}) in HBSS containing 100 μM luminol was incubated for 1 min, and 50 ng $\cdot$ ml^{-1} PMA (Sigma Chemical Co., St. Louis, MO) or 10^{-7}M FMLP (Sigma) were added. The mixture was kept at 37 °C during the experiment. Activity was expressed as peak chemiluminescence intensity (mV).

O_2^- generation

O_2^- was assayed spectrophotometrically by a cytochrome C reduction method, using a Shimadzu UV-300 spectrophotometer (Shimadzu Ltd., Kyoto, Japan). Cells 1×10^7/ml suspended in HBSS, and 100 μM cytochrome C (Sigma, type VI) was poured into each cuvette, followed by adjustment to a final cell concentration of 1×10^6 cells $\cdot$ ml^{-1}. The reaction mixtures in the cuvettes were preincubated at 37 °C for 1 min, and a stimulating agent [50 ng $\cdot$ ml^{-1} PMA or 10^{-7}M FMLP] was added. The kinetics of cytochrome C reduction were measured as the change in absorbance at 540–550 nm. O_2^- concentration was calculated from the linear portion of the cytochrome C reduction curve.

Assay of IP_3, DG, and PKC

IP_3, DG, and PKC were assayed using commercial kits from Amersham (UK). The IP_3 assay was based on competition between unlabelled IP_3 and a fixed quantity of tritium-labelled IP_3 for a limited number of binding sites on a bovine adrenal binding protein preparation [19]. For the assay of DG, enzyme-catalyzed phosphorylation of DG was performed and the [^{32}P] phosphatidic acid reaction product was extracted and separated by TLC. The radioactivity attributable to [^{32}P] phosphatidic acid was determined by liquid scintillation counting. PKC was measured by a modification of a mixed micelle assay [20].

Assay of [Ca^{++}]i

PMN were loaded with 0.1 nmol Fura 2 AM (Dojin Chemical Co., Osaka, Japan) for 30 min at 37 °C, in medium containng 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM $NaHPO_4$, 5.5 mM glucose, and 20 mM HEPES (pH 7.4). Cells were washed twice and resuspended in HBSS, and the intracellular ionized calcium ([Ca^{++}]i) level was measured periodically after FMLP (10^{-7}M) stimulation, using a CAF-100 Ca analyzer.

NBT reduction assay

The capacity of PMN to reduce NBT was determined by a microtitre plate assay method [21]. Briefly, 100,000 viable PMN in HBSS were plated into flat-bottomed microwells, and the plates were incubated in a humidified, 5 % CO_2 atmosphere, for 1 h at 37 °C. The wells were then washed twice with warm HBSS to yield a monolayer of adherent PMN that were more than 98 % viable by trypan blue exclusion. The PMN were incubated for 1 h or 6 h in the presence or absence of 10^{-6} to 10^{-4}M Azeptin. Each vertical row of eight wells contained sufficient NBT solution to yield a final dilution of 1 mg $\cdot$ ml^{-1}, with or without 10 ng $\cdot$ ml^{-1} PMA. The incubations were done for 1 h, at 37 °C, in a 5 % CO_2 atmosphere. The optical density in each well was examined at 550 nm, using a MTP-32 microplate reader (Corona Electric Co., Ltd., Tokyo, Japan).

Detection of tyrosine phosphorylation

PMN (2×10^6 cells $\cdot$ ml^{-1}) suspended in HBSS containing various concentrations of Azeptin were incubated at 37 °C in the presence or absence of 50 U $\cdot$ ml^{-1} TNF-α. After 1 h incubation, the reaction was terminated by adding an ice-cold 15 % trichloroacetic acid solution containing 2 mM phenylmethylsulphonyl fluoride and 1 mM sodium vanadate. After centrifugation, the precipitate was washed with ice-cold ether : ethanol (1 : 1), dissolved in sodium dodecyl sulphate (SDS) sample buffer, and subjected to SDS-PAGE. After electrophoresis (30 mA, 3 h), proteins were transferred to an Immobilon-P filter (Millipore) using the Sartorius semidry blotting apparatus. After 60 min incubation in 5 % powdered skimmed milk solution at room temperature, the filters were incubated with a phosphotyrosine-specific monoclonal antibody for 40 min. The monoclonal antibody was then detected with peroxidase-conjugated rabbit anti-mouse IgG. Peroxidase-positive bands were detected using an ECL Western blotting detection system (Amersham). After staining with Coomassie brilliant blue, the molecular weights of the proteins were determined using Daiichi-kagaku standards.

SOD activity

Japanese white rabbits were treated with intramuscular injections of saline or Azeptin 0.04 to 1.0 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} for 5 days. Five hours after the final injection, peripheral PMN, PAM, and lung tissue samples were obtained, and their total SOD activity was measured by Õyanagi's method [22]. The lung tissue samples were obtained by homogenising 1.0 g (wet weight) specimens in 1 ml HBSS, and protein concentration was determined by Lowry's method [23]. Briefly, the sample (0.1 ml), Reagent A (0.2 ml pH 7.0, 0.2 mM hydroxylamine plus 0.2 mM hypoxanthine 1.77 mM hydroxylamine O-sulfonic acid), 0.1 ml distilled water, and Reagent B (0.2 ml: 1.25 U $\cdot$ ml^{-1} xanthine oxidase and 10^{-4}M EDTA-2Na) were mixed. The mixture was incubated for 30 min at 37 °C, and Reagent C (2.0 ml: 30 μg $\cdot$ ml^{-1} sulphanilic acid, 5 μg $\cdot$ ml^{-1} N-1-naphthylethylenediamine, and 16.7 % acetic acid) was added. The final mixture was allowed to stand for 20 min at room temperature and optical absorption was measured at 550 nm.

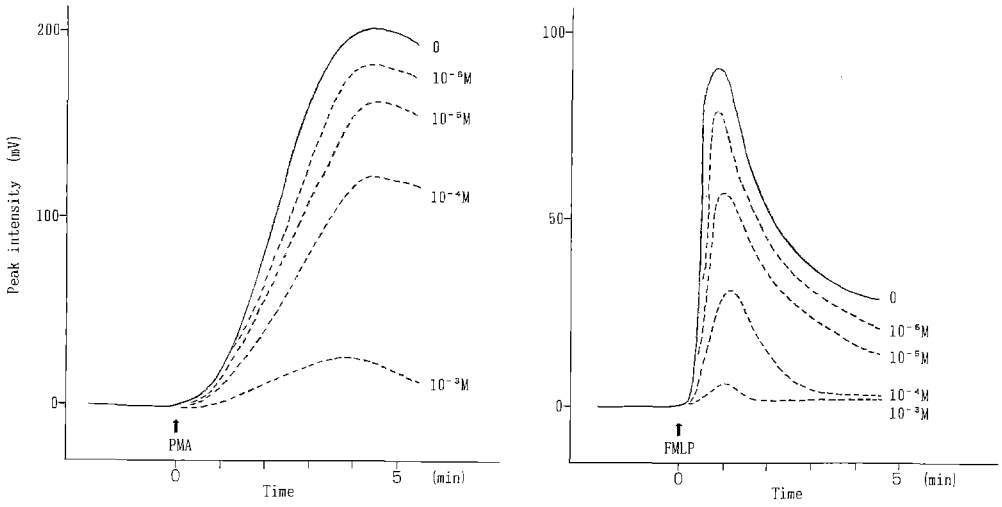

Fig. 1. Suppression of chemiluminescence in human PMN by in vitro Azeptin. PMN from 2 healthy individuals were cultured in the presence of the indicated doses of Azeptin for 1 h. The cells were then stimulated with 50 ng/ml PMA or 10^{-7} M FMLP, and chemiluminescence was measured

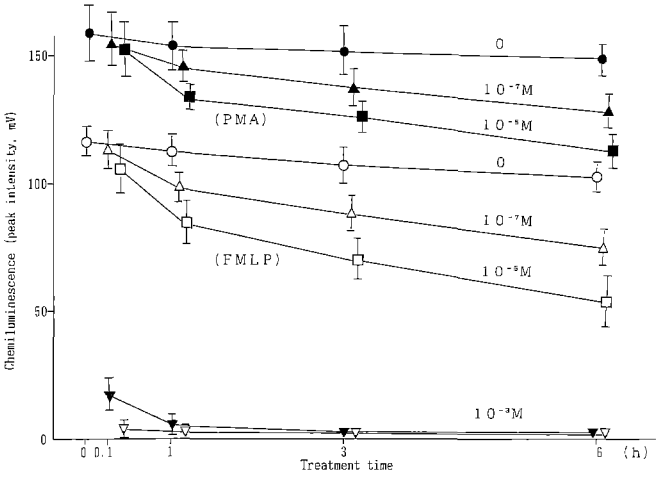

Fig. 2. Influence of in vitro Azeptin on chemiluminescence in PMN. PMN from 4 healthy donors were pretreated with each indicated dose of Azeptin for 0.1 h to 6 h. The cells were then stimulated with 50 ng/ml^{-1} PMA (closed symbols) or 10^{-7}M FMLP (open symbols). Luminol-dependent chemiluminescence was measured with a Ca^{2+} analyzer. Each experiment was triplicated. Each bar indicates the mean with SD

Results

Chemiluminescence was dose-dependently reduced by the short term in vitro treatment of human PMN with Azeptin (Fig. 1). When PMN were pretreated with 10^{-6}M, 10^{-5}, and 10^{-4}M Azeptin for 1 h, the peak intensity induced by PMA was lowered from the control level (203 mV) to 180, 162, and 126 mV, respectively. By pretreatment with 10^{-3}M Azeptin, chemiluminescence was suppressed to an extremely low level (26 mV). The suppression of FMLP-induced chemiluminescence was similar to that of PMA. Further, Azeptin time-dependently suppressed the respiratory burst of PMN (Fig. 2). The peak intensity decreased in parallel with the duration of the Azeptin pretreatment of PMN, although the graph was not so steep. The time-dependent suppression was more prominent when FMLP was used as the stimulant.

The generation of O_2^- from PMN was virtually not suppressed by low concentrations of Azeptin, but it was markedly suppressed by concentrations of 10^{-6}M or more (Fig. 3). Following pretreatment with 10^{-6}M, 10^{-5}, and 10^{-4}M Azeptin for 5 min, PMN generated 58 (8), 23 (4), and 9 (3) pmol of O_2^-, respectively, on PMA stimulation. FMLP-induced O_2^- was not as sharply reduced by Azeptin O_2^-; there was a significant decrease in O_2^- generation after 10^{-5}M Azeptin or higher concentrations, while PMA-induced O_2^- generation was significantly reduced by Azeptin 10^{-6}M.

Suppression of reactive oxygen generation was also observed in Azeptin-treated rabbits (Table 1). PAM and PMN from rabbits treated with 0.04 mg Azeptin · kg^{-1} for 5 days exhibited only a slightly decreased respiratory burst. However, PAM and PMN from rabbits treated with 0.2 mg · kg^{-1} exhibited significantly decreased chemiluminescence and O_2^- generation (13.5 (2.4) and 30.1 (2.2) pmol : 10^4 cells of O_2^-, respectively; these values were significantly lower than the control level). The peak intensity was decreased regardless of the inducer of the respiratory burst. Even when the Azeptin dose was raised to 1.0 mg · kg^{-1}, further suppression of the respiratory burst was negligible.

NBT reduction by human PMN was suppressed by pretreatment with 10^{-5}M Azeptin, with significantly more suppression after 10^{-4}M Azeptin (Table 2). This suppression was observed in the presence of PMA, but not in the absence of the stimulant.

Azeptin had a negligible effect on SOD activity (Table 3). Even when a large dose of Azeptin was injected (1.0 mg · kg^{-1} for 5 days), peripheral PMN and alveolar macrophages, as well as pulmonary tissue, showed sustained SOD activity. SOD activity in PAM from control rabbits was 9.60 (0.82) U · mg^{-1} protein, while that in alveolar macrophages from rabbits treated with 1.0 mg · kg^{-1} · day^{-1} of Azeptin was 9.38 (0.93) U · mg^{-1} protein.

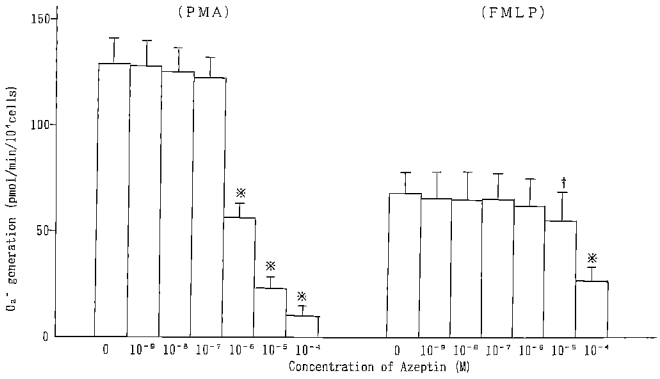

Fig. 3 In vitro effects of Azeptin on O_2^- generation by PMN. PMN from 5 healthy persons were cultured in the presence of the indicated doses of Azeptin for 5 min, and PMN were stimulated with 50 ng · ml^{-1} PMA or with 10^{-7} M FMLP. Columns and bars indicate the mean and SD, respectively, of duplicated experiments for the 5 donors. * $P < 0.001$, † $P < 0.05$ by t-test (vs control = without Azeptin)

Azeptin dose-dependently suppressed IP_3 and DG levels (Figs. 4, 5). When PMN were not treated with Azeptin, IP_3 and DG increased from control levels of less than 5 and 6 pmol : 6×10^6 cells, respectively, to more than 12 and 8 pmol : 6×10^6 cells 15 s after addition of FMLP. About 30 s after the stimulation of PMN with FMLP, both IP_3 and DG decreased, and rapid increases in these second messengers were observed 45 s after the addition of FMLP. The increases in the first peak in IP_3 and the second peak in DG were suppressed by Azeptin in a dose-dependent manner.

Azeptin also suppressed [Ca^{++}]i level and PKC activity in human PMN in vitro (Figs. 6, 7). After the addition of FMLP, [Ca^{++}]i in PMN first increased rapidly and subsequently decreased slowly. When PMN were pretreated with 10^{-5}M Azeptin, the maximum [Ca^{++}]i was reduced to about 165 nM, compared to a maximum of more than 200 nM in PMN not so pretreated (Fig. 6). PKC activity in the cytosol fraction was reduced from 972 pmol in the control to 954 pmol, 932 pmol, 891 pmol, and 867 pmol, respectively, by treating PMN for 1 h with 10^{-6}M, 10^{-5}M, 10^{-4}M, and 10^{-3}M Azeptin. Similarly, PKC activity in the membrane fraction was also suppressed by Azeptin in a dose-dependent manner. The duration of treatment had very little influence, as 6 h treatment produced very little more suppression of PKC activity than 1 h treatment.

Western blots demonstrated the suppression of protein tyrosine phosphorylation by Azeptin (Fig. 8), especially of a 115 kDa protein by 10^{-4}M Azeptin.

Discussion

ROS are implicated in many disorders. Many animal tissues are damaged not only by free radicals but also by other ROS, such as hydrogen peroxide and lipid perox-

Table 1 Influence of in vivo Azeptin on reactive oxygen generation

Cells and inducers	Saline (Control)	Dose of Azeptin		
		(0.04 mg · kg^{-1})	(0.2 mg · kg^{-1})	(1.0 mg · kg^{-1})
Alveolar macrophages				
FMLP-induced CL	22.0 (2.0)[a]	19.7 (0.6)	18.5 (0.7)*	16.7 (1.4)*
PMA-induced CL	26.0 (3.6)	20.0 (1.7)*	18.9 (1.1)*	16.5 (1.5)*
PMA-induced O_2^-	19.0 (2.6)[b]	15.0 (5.6)	13.5 (2.4)*	12.4 (2.6)*
Peripheral PMN				
FMLP-induced CL	45.7 (7.1)	35.7 (6.5)	31.6 (4.9)*	28.0 (3.7)*
PMA-induced CL	45.0 (6.6)	36.0 (4.0)	33.2 (3.3)*	32.8 (2.6)*
PMA-induced O_2^-	39.3 (4.0)	33.3 (2.5)	30.1 (2.2)*	27.4 (3.1)*

Three rabbits received subcutaneous injections of saline or the indicated doses of Azeptin for 5 days, and alveolar macrophages and peripheral PMN were obtained and separated. Stimulation of the cells with PMA and FMLP, as well as the assay methods for determining chemiluminescence and O_2^- were the same as in Figs. 1 and 2. Each value indicates the mean (SD) of duplicate in the 3 rabbits

[a] Peak intensity (mV); [b] pmol · min^{-1} : 10^4 cells; * $P < 0.05$, vs control, by U-test

Table 2 In vitro effects of Azeptin on NBT reduction

PMA (10 ng/ml)	Time of treatment with Azeptin	Concentration of Azeptin (M)			
		0	10^{-6}	10^{-5}	10^{-4}
−	1 h	15.4 (2.1)	15.7 (1.7)	16.0 (1.3)	15.2 (2.5)
−	6 h	14.3 (2.6)	14.4 (2.2)	14.1 (3.0)	13.5 (1.7)
+	1 h	59.2 (4.7)	58.6 (4.3)	57.4 (4.6)	16.7 (2.9)***
+	6 h	55.4 (3.6)	46.2 (2.9)*	40.1 (3.2)**	11.6 (3.4)***

PMN obtained from 4 healthy persons were pretreated with the indicated concentrations of Azeptin for 1 h or 6 h, and the reduction of 1 mg · ml^{-1} NBT was measured by a densitometer int he presence or absence of 10 ng · ml^{-1} PMA. This detection was duplicated. NBT reduction is expressed as an arbitrary unit (AU) of optical density, each value indicates the mean units ± standard deviation.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (vs control, by t-test)

Table 3 Influence of in vivo Azeptin on superoxide dismutase (SOD)

Cells and tissue	Saline (Control)	Azeptin ($mg \cdot kg^{-1} \cdot day^{-1}$)		
		(0.04 mg)	(0.2 mg)	(1.0 mg)
Peripheral PMN	3.97 (0.38)[a]	3.92 (0.51)	3.60 (0.42)	3.10 (0.45)
Alveolar M_φ	9.60 (0.82)	9.28 (0.82)	9.09 (0.76)	8.38 (0.93)
Lung tissue	9.02 (1.37)	8.43 (1.69)	8.30 (1.62)	8.16 (1.72)

Three rabbits received intramuscular injections of saline or the indicated doses of Azeptin for 5 days. Five hours after the last injection, peripheral PMN, alveolar macropahges (M_φ), and lung tissue specimens were obtained; these were assayed for total SOD activity. Each value shows the mean (SD) of duplicate experiments in 3 rabbits

[a] $U \cdot mg^{-1}$ protein

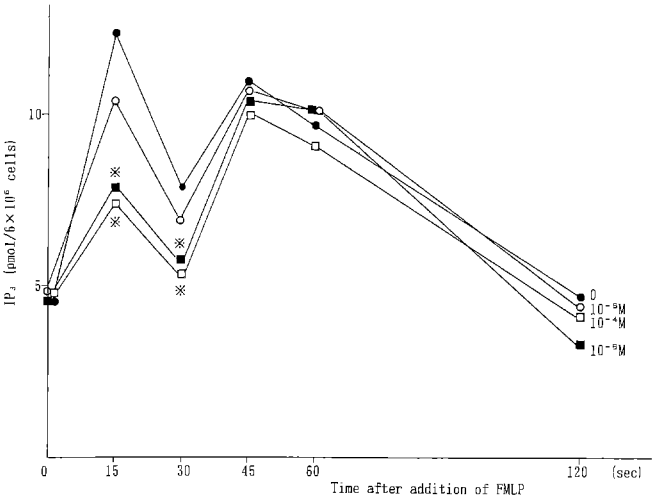

Fig. 4 Influence of Azeptin on IP_3 levels in human PMN. PMN from 3 healthy subjects were treated with the indicated concentrations of Azeptin for 1 h at 37 °C. After being washed, the cells were stimulated with 10^{-7} M FMLP, and the IP_3 level was assayed. Each point shows the mean IP_3 level of duplicated experiments for the 3 subjects. * $P < 0.05$, by U-test (vs control)

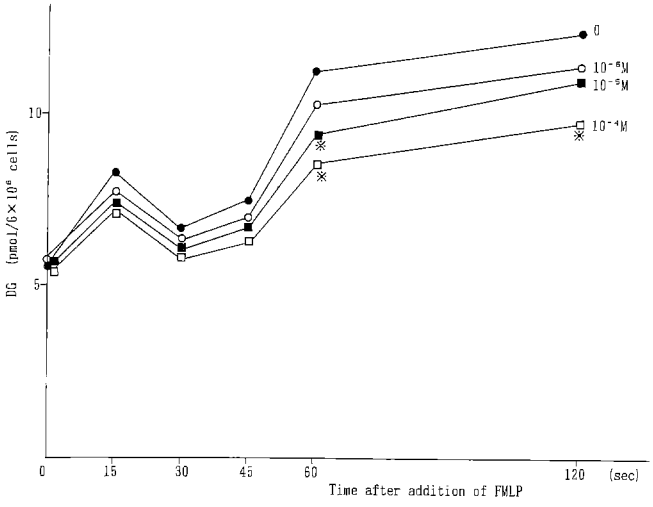

Fig. 5 Influence of Azeptin on DG level in human PMN. PMN from 3 healthy donors were treated with the indicated concentrations of Azeptin for 1 h at 37 °C. After being washed, the cells were stimulated with 10^{-7} M FMLP, and DG level was assayed. Each point shows the mean DG level of duplicate experiments for the 3 subjects. * $P < 0.05$, by U-test (vs control)

ides. ROS play an important pathological role in necrosis in transplanted organs [24], ulceration of the mucous membranes [25], pulmonary alveolar injury [26], and in impairment of the retina [27]. Agents that suppress ROS generation and increase ROS-scavenging activity should be of value in the treatment of ROS-associated disorders.

Azeptin is already being used in the treatment of many disorders, because of its anti-allergic and leukocyte function suppressing activity. So far, however, its influence on ROS generation has not been explored. In a previous study, Azeptin was administered to patients with oral cancer in an attempt to prevent chemoradiotherapy-induced erosive mucositis; it had a good prophylactic effect [28]. Our clinical experience led us to examine whether the drug would suppress reactive oxygen generation.

Here, Azeptin was shown dose-dependently to suppress the respiratory burst. Even at a low concentration (10^{-7}M), the respiratory burst of PMN was suppressed when they were treated for a long time (6 h). Both in vitro and using PAM and PMN from rabbits given a small dose ($0.2 mg \cdot kg^{-1}$) of Azeptin for 5 days there was suppression of chemiluminescence and O_2^- generation. Taking these findings and the tissue concentration in patients given Azeptin into consideration, ROS generation activity in these patients appeared to be suppressed.

With regard to O_2^- generation, the suppressive action of Azeptin was more prominent in PMN stimulated by PMA than by FMLP. As PMA directly stimulates PKC and induces O_2^- generation, the suppression of PKC activity by Azeptin was anticipated. In fact, Azeptin dose-dependently suppressed PKC activity both in the cytosol and membrane fractions. Apart from suppressing PKC, Azeptin also appeared to exhibit a further suppressant effect on the respiratory burst system because FMLP-induced chemiluminescence was also inhibited. To explore which step in signal transduction was suppressed by Azeptin, the levels of the second mesengers IP_3 and DG were examined in PMN. Treatment with Azeptin led to a dose-dependent suppression of both in human PMN. Thus Azeptin appeared to suppress PKC not only directly, but also via signal transduction. Exploration of any effect of Azeptin on phospholipase C gamma and GTP-binding protein would appear to be of interest.

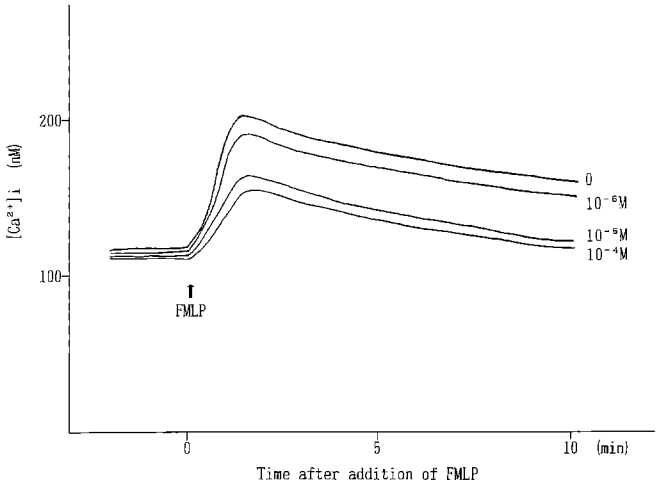

Fig. 6 Influence of Azeptin on [Ca $^{++}$]i level in human PMN. PMN were pretreated with Azeptin (10^{-6} to 10^{-4} M) for 1 h and loaded with Fura-2 AM during the last 30 min. After stimulation with 10^{-7} M FMLP, [Ca $^{++}$]i level was measured

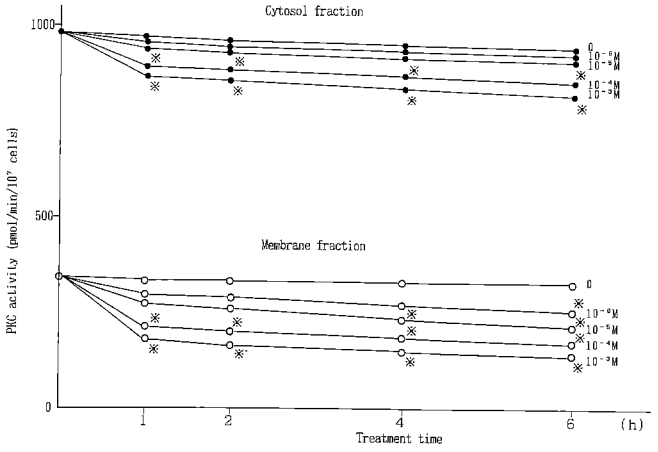

Fig. 7 Kinetics of PKC activity after the treatment of PMN with Azeptin. Following treatment with the indicated concentrations of Azeptin for the indicated time, the cells were fractionated into cytosol and membrane fractions, and PKC activity in both fractions was measured. The experiment was duplicated; each PKC level indicates the mean for 3 subjects

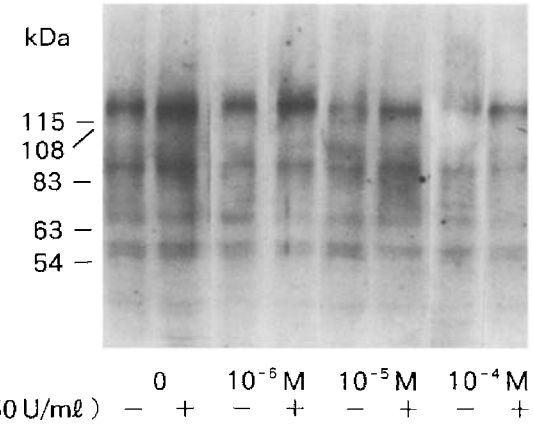

Fig. 8 Tyrosyine phosphorylation in PMN treated with Azeptin-PMN from healthy individuals were pretreated with Azeptin (0 to 10^{-4} M) for 1 h in the presence or absence of 50 U/ml TNF-α. The PMN proteins were subjected to SDS-PAGE followed by transfer to an Immobilon-P membrane. Tyrosine residues in the phosphorylated proteins were detected by immunoblotting with anti-phosphotyrosine antibody

The importance of protein tyrosine phosphorylation in cell activation has been reported [10–16]. In the O_2^- generation of PMN, tyrosine phosphorylation of a 115 kDa protein has been reported to play a critical role [15]. In the present study, reduced protein phosphorylation was observed in parallel with the Azeptin-induced inhibition of O_2^- generation. The block of signal transduction by Azeptin will be better understood once the position of the 115 kDa protein in the signal transduction system has been decided.

Many cytokines regulate ROS generation. Of these, TNF-α is the most potent stimulator of ROS generation, increasing cell membrane PKC and upregulating protein tyrosine kinase phosphorylation [15, 29–32]. Many other cytokines, such as interferon-gamma, granulocyte-macrophage colony-stimulating factor and interleukin-6, work in cooperation with each other and with TNF-α, and so enhance PMN function [33–38]. As a result of the cooperation, these cytokines, which are released from mononuclear leukocytes and PMN, indirectly upregulate ROS generation. Cytokine generation by mononuclear leukocytes and PMN is largely suppressed by Azeptin (data not shown). The inhibition of cytokine generation seems to contribute to the suppression of ROS generation by Azeptin.

Fortunately, Azeptin did not suppress SOD activity. This fact is very important in regard to clinical use of the drug to avoid ROS-induced damage. If scavenging activity is not decreased and only ROS generation is greatly suppressed, tissue damage by ROS will be avoided. Further investigations are required to elucidate the action of Azeptin, but it does appears to be of value in suppressing the respiratory burst.

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