Direct Effect of Human Immunodeficiency Virus Protease Inhibitors on Neutrophil Function and Apoptosis via Calpain Inhibition

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Impairment of neutrophil functions and high levels of apoptotic neutrophils have been reported in human immunodeficiency virus (HIV) patients. The aim of the present study was to investigate the direct in vitro effects of the different HIV protease inhibitors (PIs) on neutrophil functions and apoptosis and to explore their mechanisms of action. The effects of nelfinavir (NFV), saquinavir (SQV), lopinavir (LPV), ritonavir (RTV), and amprenavir (APV) in the range of 5 to 100 µg/ml on neutrophil function, apoptosis, and µ-calpain activity were studied. The neutrophil functions studied included superoxide production stimulated by 5 ng/ml phorbol myristate acetate, 5 × 10−7 M N-formyl-methionyl-leucyl-phenylalanine, and 1 mg/ml opsonized zymosan; specific chemotaxis; random migration; and phagocytosis. Apoptosis was determined by DNA fragmentation, fluorescein isothiocyanate-annexin V binding, and nuclear morphology. All three neutrophil functions, as well as apoptosis, were similarly affected by the PIs. SQV and NFV caused marked inhibition and LPV and RTV caused moderate inhibition, while APV had a minor effect. µ-Calpain activity was not affected by the PIs in neutrophil lysate but was inhibited after its translocation to the membranes after cell stimulation. SQV, which was the most potent inhibitor of neutrophil functions and apoptosis, caused significant inhibition of calpain activity, while APV had no effect. The similar patterns of inhibition of neutrophil functions and apoptosis by the PIs, which coincided with inhibition of calpain activity, suggest the involvement of calpain activity in the regulation of these processes.

Neutrophils are the front line of defense against microbial infection. Neutrophil functions, which include chemotaxis, oxidative respiratory burst, phagocytosis, and killing activity against bacteria and fungi, have been reported to be impaired during human immunodeficiency virus (HIV) infection (10). In patients with advanced HIV infection, neutrophils exhibited decreased chemotaxis (11, 13) due to reduced expression of chemotactic receptors (37). This reduced expression can result from continued exposure to HIV or viral products, such as Tat protein, which can desensitize phagocytes to chemotactic stimuli (40). The defective activity of neutrophils may also contribute to the development of secondary infections in AIDS patients, such as Pneumocystis carinii, Mycobacterium avium-M. intracellulare complex, Histoplasma capsulatum, Toxoplasma gondii, Rhodococcus equi, Cryptococcus neoformans, and Candida albicans infections (38). In addition, it has been shown that neutrophils from AIDS patients exhibit accelerated apoptosis ex vivo (42). Apoptotic neutrophils display morphological and biochemical characteristics of apoptotic cells, including cell shrinkage, compaction of chromatin, and loss of the multilobed shape of the nucleus (1), and are nonfunctional (9, 53). Thus, apoptosis might be one of the mechanisms responsible for the loss of function and reduction in neutrophils in AIDS patients (41). Treatment of AIDS patients with highly active antiretroviral therapy cocktails of compounds, including drugs designed to inhibit the HIV protease (12), leads to a reduction of inflammatory parameters and of neurological problems (28, 39). This treatment induced significant improvement in neutrophil chemotactic and fungicidal activities and enhancement of the oxidative burst, although there was no full recovery of these functions (35, 36). It has been shown that the protease inhibitors (PIs) counteract T-cell depletion (23, 29) and reduce apoptosis of T cells and neutrophils (36) in AIDS patients, even in the absence of inhibition of viral spread. Furthermore, PIs have been shown to increase in vitro cell viability by inhibiting apoptosis of infected and uninfected T cells (48, 52).

The aim of the present study was to determine the direct effects of the various HIV PIs on neutrophil functions and on apoptosis. Since the involvement of the neutrophil cysteine protease calpain has been reported in spontaneous apoptosis (2, 25, 26, 49) and in neutrophil functions (8, 17, 25, 34), we studied whether calpain is affected by the PIs. The present study demonstrates that chemotaxis, phagocytosis, and superoxide production, as well as apoptosis, are inhibited by the PIs. The pattern of inhibition of neutrophil functions and apoptosis by the PIs coincided with inhibition of calpain activity.

MATERIALS AND METHODS

Neutrophil purification. Neutrophils were isolated from heparinized blood of healthy donors (within 3 hours after the collection of blood) at 95% purity by Ficoll/Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes, and their viability was determined by trypan blue exclusion (5). This study was approved by the Helsinki Committee of the Soroka University Medical Center.

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PIs. Nelfinavir (NEF) and saquinavir (SQV) were provided by Roche Pharmaceuticals, Tel Aviv, Israel. Lopinavir (LPV), ritonavir (RTV), and amprenavir (APV) were provided by Abbott Laboratories, Illinois. Apart from SQV, which was dissolved in dimethyl sulfoxide (DMSO), all the protease inhibitors were dissolved in ethanol (final solvent concentration, 0.025% [vol/vol]).

Superoxide anion measurements. The production of superoxide anion \((O_2^-)\) by neutrophils was measured as the superoxide dismutase-inhibitable reduction of acetyl ferricytochrome \(c\) by the microtiter plate technique, as previously described (7), with modifications. Cells (\(5 \times 10^7\) well) were suspended in 100 \(\muL\) Hanks' balanced salt solution (HBSS) containing acetyl ferricytochrome \(c\) (150 mM), with or without PIs, and stimulated by the addition of 5 ng/ml phorbol myristate acetate (PMA), 1 mg/ml oposazin zymosan (OZ), or \(10^{-7} M\) N-formyl-methionyl-leucyl-phenylalanine (fMLP). In addition, superoxide production in nonstimulated cells was determined. The reduction of acetyl ferricytochrome \(c\) was followed by a change of absorbance at 550 nm at 2- to 5-min intervals on a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA). The maximal rates of superoxide generation were determined and expressed as nanomoles \(O_2^-/10^6\) cells/10 min using an extinction coefficient \((E_{550})\) of 21 \(\text{mM} \cdot \text{cm}^{-1}\). OZ was measured as follows: 20 mg OZ was incubated with 1 ml of pooled human serum (lipopolysaccharide free) for 1 h at 37°C and washed three times with HBSS buffer.

Chemotaxis. Chemotaxis was assessed as previously described (33). Agarose was dissolved in sterile, distilled boiling water for 10 min. After being cooled to 48°C in a water bath, the agarose was mixed with an equal volume of prewarmed \(2 \times\) minimal essential medium with \(10\%\) heat-inactivated fetal calf serum and 7.5% (wt/vol) sodium bicarbonate. Five milliliters of the agarose medium was delivered to 60- by 15-mm tissue culture dishes and allowed to harden. A series of agarose dishes, 2.4 mm in diameter and spaced 2.4 mm apart, were formed. In the first well, 10 \(\muL\) of fMLP (\(10^{-7} M\)) was placed; in the center well, a 10- \(\muL\) aliquot of cell suspension (\(5 \times 10^6\) in HBSS, with or without PIs, was placed; and in the third well, 10 \(\muL\) of HBSS was placed. The dishes were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO\(_2\) in air for 2 h. The plates were fixed by the addition of 3 ml methanol at 4°C overnight. After the methanol was poured off, the plates were placed in glutaraldehyde (2.5%) for 30 min at room temperature. The agarose gel was removed intact after fixation, and the plates were stained with Giemsa stain and air dried. The random migration and the linear migration toward the chemoattractant (fMLP) were measured under a light microscope. Chemotaxis was defined as the ratio between the chemotactic and random migrations.

Phagocytosis. Cells (\(5 \times 10^6/ml\)) suspended in HBSS were preincubated with the PIs and incubated at 37°C for 15 min with 5 \(\muL\) of OZ (1 mg/ml). Subsequently, the cells were smeared and stained with differential Wright-Giemsa stain. The maximal rates of superoxide generation were determined and expressed as nanomoles \(O_2^-/10^6\) cells\(/10^6\) cells/10 min using an extinction coefficient \((E_{550})\) of 21 \(\text{mM} \cdot \text{cm}^{-1}\). OZ was measured as follows: 20 mg OZ was incubated with 1 ml of pooled human serum (lipopolysaccharide free) for 1 h at 37°C and washed three times with HBSS buffer.

Immunoblot analysis. Membrane fractions (\(2 \times 10^6\) cell equivalents) were isolated from SDS sample buffer and electrophoresed on a 7% or 15% SDS-polyacrylamide gel (27). The resolved proteins were electrophoretically transferred to nitrocellulose membranes, and the detection of \(\mu\)-calpain protein was done using monoclonal antibodies that recognized the 80-kDa large subunit (kindly provided by Nechama S. Kosower, Tel Aviv University, Tel Aviv, Israel), followed by reaction with horse serum conjugated donkey anti-mouse antibody, which served as a secondary antibody, according to established procedure (31). Detection of the NADPH oxidase membrane subunit \(p22^{\text{phox}}\) was performed using goat antibodies against \(p22^{\text{phox}}\) (31). The relative amounts of calpain and \(p22^{\text{phox}}\) in the membrane fractions were quantitated using densitometry in ImageJ processing and analysis.

Statistical analysis. Statistical evaluation of the differences from the control was carried out by an unpaired Student’s \(t\) test with a 95% confidence interval. The differences between the various PI treatments were analyzed by analysis of variance.

RESULTS

The effects of SQV, NEF, LPV, RTV, and APV on neutrophil functions were studied in vitro. Figure 1 shows the dose-response effects of PIs on superoxide production stimulated by fMLP. The presence of these PIs during the superoxide production assay caused significant inhibition in a rank order of \(\text{SQV} = \text{NFV} > \text{LPV} = \text{RTV} > \text{APV}\). While SQV and NFV at 100 \(\muM\) caused almost complete inhibition, APV caused only slight and insignificant inhibition. The effects of the PIs were immediate, since preincubation for up to 60 min prior to stimulation did not change the effects (data not shown). The inhibitory effects of the PIs on superoxide production were not restricted to stimulation with fMLP but were also detected when neutrophils were stimulated with PMA or OZ, although to a lesser but significant extent \((P < 0.001)\), as shown in the dose-response inhibition of SQV on superoxide production stimulated with the three stimuli (Fig. 2). The effects of the PIs on neutrophil chemotaxis and on phagocytosis were similar to those on superoxide production. The chemotactic responsive-ness of neutrophils was inhibited in a dose-dependent manner by the presence of the PIs during the assay, as shown in Fig. 3A. SQV at 100 \(\muM\) caused total inhibition of chemotaxis, while APV caused only slight inhibition. Likewise, phagocytosis of OZ particles by neutrophils was inhibited by the PIs in a similar dose-dependent manner (Fig. 3B). Thus, there were similar rank order effects of the PIs on the three differ-
ent functions: superoxide production, chemotaxis, and phagocytosis.

The in vitro effects of the PIs on spontaneous neutrophil apoptosis were studied by three different methods. Spontaneous neutrophil apoptosis was induced by overnight culture in the presence of 25 g/ml of either the PIs or their solvents. Figure 4A presents the effects of the PIs as determined by DNA fragmentation. While cultured neutrophils exhibited significant DNA fragmentation, the presence of the different PIs during culture attenuated this process. The results, expressed as percentages of the control, are means ± standard errors of the mean from three experiments. There were significant inhibition compared to the control and significant differences between the effects of the three stimuli (P < 0.001).

The in vitro effects of the PIs on chemotaxis and phagocytosis were studied by three different methods. Spontaneous neutrophil apoptosis was induced by overnight culture in the presence of 25 μg/ml of either the PIs or their solvents. Figure 4A presents the effects of the PIs as determined by DNA fragmentation. While cultured neutrophils exhibited significant DNA fragmentation, the presence of the different PIs during culture attenuated this process. The results, expressed as percentages of the control, are means ± standard errors of the mean from three experiments. There were significant inhibition compared to the control and significant differences between the effects of the three stimuli (P < 0.001).

FIG. 1. The dose-response effects of PIs on superoxide production stimulated by fMLP (5 × 10⁻⁷ M). PIs were added to the reaction mixture before stimulation. The results, expressed as percentages of the control, are means ± standard errors of the mean from three experiments. DMSO was added to the control of SQV, and ethanol was added to the control of the other PIs. ( ), APV; ( ), RTV; ( ), LPV; ( ), NFV; ( ), SQV. For all PIs, there was significant inhibition compared to the control (P < 0.001). a, significant differences compared to b and c (P < 0.001); b, significant differences compared to c (P < 0.001).
FIG. 4. The effects of HIV PIs on spontaneous apoptosis of peripheral blood neutrophils. Neutrophils were cultured for 16 h in the absence or presence of the different PIs (25 μg/ml) or their solvents. (A) DNA fragmentation analysis. Starting from the left side, lane 1, freshly isolated neutrophils; lane 2, cultured neutrophils (in the presence of DMSO, the solvent of SQV); lane 3, cultured neutrophils in the presence of SQV (S); lane 4, cultured neutrophils (in the presence of ethanol, the solvent of all other PIs); lanes 5 to 8, cultured neutrophils in the presence of NFV (N), RTV (R), LPV (L), and APV (A), respectively; lane 9, 1-kb ladder. (B) Bar graphs of the effects of the PIs on neutrophil apoptosis detected by the relative amounts of the fragmented DNAs, which were quantified by using densitometry in ImageJ processing and analysis. (C) Fluorescein isothiocyanate-annexin V binding assayed by flow cytometric analysis. Shown are representative results for cultured neutrophils in the presence of NFV (top) or its solvent (bottom). Early apoptotic cells with intact membranes were annexin V positive and propidium iodide negative (lower right quadrant). Late apoptotic or necrotic cells that lost cell membrane integrity were positive for both annexin V and propidium iodide (upper right quadrant). (D) Bar graphs of the effects of PIs on neutrophil apoptosis detected by labeled annexin V. (E) Giemsa staining of cultured neutrophils. The arrow shows the condensed nuclei in apoptotic neutrophils. A total of 40 to 65% of neutrophils cultured for 16 h were apoptotic (F) Bar graphs of the effects of the PIs on neutrophil apoptosis detected by changes in nuclear morphology after Giemsa staining. The results, expressed as percentages of the control, are means ± standard errors of the mean of four experiments. The significances of the results compared to the control were as follows: *, P < 0.05; **, P < 0.001. P values were as follows: a in comparison to b and c, <0.001 (for panels B, D, and F); b in comparison to c, 0.2, <0.001, and <0.01 (for panels B, D, and F, respectively). A P value of <0.05 was considered significant.
The effects of the PIs on neutrophil lysates and on recombinant calpain \( \mu \)-isoform were studied. Addition of 100 \( \mu \)g/ml of the two most effective PIs, SQV and NFV, to the lysates did not cause any inhibition of calpain activity, nor did higher concentrations up to 400 \( \mu \)g/ml (data not shown). Likewise, the most effective PI, SQV, had no effect on recombinant \( \mu \)-calpain activity (Fig. 5B). Calpain has been shown to translocate to the membranes after cell stimulation (43, 50); thus, its activity was assayed on the membrane fractions separated from stimulated neutrophils. The effects of the two most potent PIs, SQV and NFV, as well as that of APV, which had only slight effects on neutrophil functions and on apoptosis, were tested on calpain activity after its translocation to neutrophil membranes. As shown in Fig. 5C, membrane fractions of cells preincubated with either SQV or NFV exhibited significantly \( (P < 0.001) \) lower calpain activity than the membrane fractions of the untreated control cells. SQV was more potent in inhibiting calpain activity \( (P < 0.001) \), while APV did not cause any inhibition.

To determine whether the inhibition of calpain activity by the PIs was due to a direct effect on the enzyme or to inhibition of its translocation to the membranes, their effects on calpain translocation were analyzed. As shown in Fig. 5D, stimulation of neutrophils with fMLP caused a marked translocation of calpain to the membrane fractions, as determined by immunoblotting. Preincubation of neutrophils with each of the PIs did not affect the levels of calpain in the membrane fractions of stimulated neutrophils.

**DISCUSSION**

The present study demonstrates that PIs have a direct effect on inhibiting neutrophil functions (superoxide production,}

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**FIG. 5.** The effects of HIV PIs on calpain activity. (A) Neutrophil lysates. Cell lysate equivalents \((1.5 \times 10^7)\) were pretreated (10 min) without (\( \times \)) or with NFV (\( \Delta \)), SQV (\( \square \)), or APV (\( \square \)) at 100 \( \mu \)g/ml before assay of calpain activity using 200 \( \mu \)M of the calpain substrate Suc-LLVY AMC. The results are presented as relative fluorescence units (RFU). (B) Purified \( \mu \)-calpain. Recombinant \( \mu \)-calpain (100 ng) was pretreated with solvent (\( \times \)) or 100 \( \mu \)g/ml SQV (\( \square \)) for 10 min before assay of activity using the synthetic substrate Suc-LLVY AMC. (C) Neutrophil membranes. Membrane fractions were prepared from neutrophils (10^6 cells/ml) preincubated without (\( \times \)) or with the PIs NFV (\( \Delta \)), SQV (\( \square \)), and APV (\( \square \)) at a concentration of 25 \( \mu \)g/ml for 10 min and stimulated for 3 min with fMLP, and 1 \( \times \) 10^7 cell membrane equivalents were analyzed for calpain activity using the synthetic substrate Suc-LLVY AMC. The significance of the effect was a \( P \) value of <0.001. (D) The effects of the PIs on calpain in membrane fractions of stimulated neutrophils. Membrane fractions from neutrophils preincubated without PIs or with PIs at a concentration of 25 \( \mu \)g/ml for 10 min and stimulated for 3 min with fMLP were separated on SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis against anti-\( \mu \)-calpain or anti-p22sphix antibodies to confirm equal loading of membrane fractions; 5 \( \times \) 10^5 cell membrane equivalents were applied per lane. Starting from the left side, lane 1, unstimulated neutrophils; lane 2, stimulated neutrophils; lanes 3 to 7, stimulated neutrophils in the presence of SQV (S), NFV (N), LPV (L), RTV (R), and APV (A), respectively. The relative amounts of the proteins were quantified using densitometry, and the ratios between \( \mu \)-calpain and p22sphix membrane proteins are expressed in the bar graph. The results are from one representative experiment out of three presenting identical results.
calpain, and phagocytosis) and on neutrophil apoptosis in similar rank orders. SQV and NFV, the most effective PIs in attenuating neutrophil functions, caused a significant inhibition of apoptosis, while APV had only small effects on neutrophil functions and on neutrophil apoptosis. The concentrations of the PIs used in this study are readily achievable with oral dosing regimens in humans (46, 51). The PIs act by blocking the HIV aspartyl protease, a viral enzyme that cleaves the HIV Gag and Gag-Pol polyprotein backbone, thereby interrupting the viral life cycle. These PIs have been reported to have different affinities, inhibition constants, physiochemical properties, and cell culture efficiencies (16, 47). The highest cell culture efficiencies were detected for SQV and NFV and the lowest for APV. In addition, in HIV pharmacotherapy, it is important to consider intracellular drug concentrations. The distribution of the PIs from plasma into cells and tissues is dependent on many factors, including uptake mechanisms and the relative affinities for cells and tissues versus plasma components (18). The PIs show differential accumulations within lymphoblastoid cell lines (21) and peripheral blood mononuclear cells of virologically suppressed patients in vivo, with NFV and SQV accumulations higher than those of LPV and RTV (24). Thus, the rank order effects of the different PIs on neutrophil functions and apoptosis shown in our study are similar to their reported cell culture efficiencies (which represent the concentrations of inhibitor that result in 50% inhibition of HIV type 1 replication [47]) and to their rank order for cellular accumulation (18, 24). The significant recovery of neutrophil functions reported after the treatment of HIV patients (35) raises the possibility that the main in vivo effect of the PIs on neutrophils is in reducing the number of functionally defective apoptotic cells (53). However, the fact that there was no full recovery in those patients may be explained by the inhibitory effects of the PIs on neutrophil function.

The similar patterns of inhibition of the three different neutrophil functions and neutrophil apoptosis raised the possibility that the different processes have a common key regulator. Several studies have reported the involvement of calpain in regulation of different neutrophil functions, as well as of apoptosis. It has been shown that inhibition of calpain activation prevents phagocytosis of iC3b-opsonized particles (8) and neutrophil transendothelial migration or adhesion to endothelial cells (3). Calpain activities are responsible for rearrangement of the plasmalemmal cytoskeleton, including dissociation of protein from actin and loss of immunodetectable α-actinin and ezrin, a process that is important for neutrophil chemotaxis and phagocytosis (17, 25), and may affect membrane NADPH oxidase activity. In addition, calpain has been implicated as a critical calcium-dependent regulator of the actin cytoskeleton and cell migration (20, 22). The role of calpain in the regulation of apoptosis, in addition to that of the caspases, has been recently reported (2, 6, 25). While constitutive calpain activity in neutrophils has been shown to mediate apoptosis by degradation of a caspase inhibitor, inhibition of calpain by calpeptin calpastatin delays the initiation of apoptosis (26, 49).

The present study demonstrates that the different PIs inhibited calpain activity in the membrane fractions of activated neutrophils in the same rank order of inhibition as for neutrophil functions and apoptosis. These results, together with the reported finding that calpain is involved in both neutrophil functions and apoptosis, raise the possibility that the effects of the PIs on both processes are mediated by calpain. In line with this suggestion, it was recently reported that elevated apoptosis and calpain activity of the neutrophils of AIDS patients decreased after PI treatment (32). HIV protease and calpain share similar secondary structures, in which the active site is flanked by hydrophobic regions (51). Although HIV protease is an aspartyl protease while calpain is a cysteine protease, peptid aldehyde inhibitors of calpain have been shown to inhibit HIV protease (44). The inhibition of μ-calpain in the membrane fraction by the PIs demonstrated in our study (Fig. 5C) is in line with the findings that μ-calpain, but not m-calpain, is the dominant isoform in neutrophils (50) and is activated in spontaneous and Fas receptor-mediated apoptosis of neutrophils (2). μ-Calpain activity was inhibited by the PIs after its translocation to the plasma membranes induced by neutrophil stimulation (Fig. 5C), but not in neutrophil lysates (Fig. 5A) or in its purified form (Fig. 5B), suggesting that its activated form is more susceptible to inhibition. Thus, it is possible that the site of the PI binding is exposed only when calpain is found on the membranes after its translocation.

In conclusion, the PIs tested in our study exhibited direct effects on neutrophil functions and on apoptosis, with similar rank orders. In addition, the PIs most effective in these processes were also potent in inhibiting neutrophil calpain activity in the membrane fractions, suggesting the involvement of calpain activity in the regulation of these processes. The treatment of HIV patients with PIs, which brings about improvement but not total recovery of neutrophil functions, probably reflects a balance between their effects in reducing the number of apoptotic cells that are functionally defective and their direct inhibition of neutrophil functions.

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