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Short communication

Inhibition of intraerythrocytic proteasome retards the generation of hemorphins

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ABSTRACT

Hemorphins are a set of hemoglobin-derived opioid peptides. The production mechanism of these structural overlap peptides remains unclear. Based on the sequences of hemorphins, it could be inferred that hemorphins are probably generated by cleavage of hemoglobin β chain at sites favored by the chymotrypsin-like protease. 20S proteasome possesses the chymotrypsin-like activity and still persists in mature erythrocytes. This study attempts to clarify whether the intraerythrocytic proteasome involves in the formation of hemorphins. Hemorphins containing hemorphin-7 and V-hemorphin-7 are isolated by immunoprecipitation from culture supernatant of human erythrocytes. Bortezomib inhibits the chymotrypsin-like activity of intraerythrocytic proteasome and prevents the yield of hemorphins in a dose-dependent manner. The present study suggests that intraerythrocytic proteasome contributes to the generation of hemorphins.

1. Introduction

Hemorphin family consists of a series of endogenous morphinomimetic peptides. They derive from enzymatic hydrolysis of the β, γ, δ or ε chain of hemoglobin [4,11]. The spectrum of hemorphin differs from tripeptide to nonapeptide. A limited degradation of hemoglobin generates a set of sequentially overlapping peptides with N- and/or C-terminal extensions of Tyr-Pro-Trp core sequence. Hemorphin-3 to -7 represent the C-terminal truncations. The hemorphin sub-families are relevant to N-terminal sequences and categorize as hemorphins, V-hemorphins, VV-hemorphins and LVV-hemorphins [3,14]. Hemorphins function as ligands of different opioid receptors.

Erythrocyte harbors a large amount of hemoglobin which is known as the precursor of hemorphins [10]. The generation mechanism of hemorphins is not fully understood. Erythrocytes discard nuclei and get rid of organelles such as lysosome during their maturation. However, the recent studies indicate that functional 20S proteasome and its subunits still exist in mature erythrocytes [12,15,18]. The 20S proteasome, the main extralysosomal proteinase, degrades proteins into peptides with length range from 3- to 22-residues [16,20]. V-hemorphin-3, V-hemorphin-5, V-hemorphin-7, and hemorphin-7 are preferentially formed and excreted by human erythrocytes [19]. The productions of above mentioned hemorphins are independent of the age, sex, or blood type of the healthy donors [13]. According to the sequences of hemorphins, it is reasonable to infer that hemorphins are probably generated by cleavage of hemoglobin β chain at sites favored by the chymotrypsin-like protease. The present study attempts to clarify whether the intraerythrocyte proteasome involves in the origin of hemorphins. Bortezomib, a cell-permeable dipeptide boronate molecule, selectively inhibits chymotrypsin-like activity of 20S proteasome [2]. As a therapeutic agent, bortezomib is the first proteasome inhibitor approved by FDA for treatment of multiple myeloma. The pharmacokinetic study indicates that bortezomib is preferentially taken by erythrocytes from the plasma [22]. Therefore, we employ bortezomib to uncover the function of intraerythrocytic proteasome in the generation of hemorphins.

2. Materials and methods

2.1. Preparation of hemorphin-7 IgG antibody

Hemorphin-7 IgG antibody was achieved by affinity purification of immune rabbit sera. Briefly, synthetic YPWTQRF peptide coupled with keyhole limpet hemocyanin (KLH) was used as the antigen. Each of the New Zealand rabbits received 500 μg peptide-KLH emulsified with complete Freund's adjuvant. Booster immunization was given monthly until an appropriate response occurred. Hemorphin-7 IgG antibody was purified from rabbit antisera on protein A-coupled agarose beads.

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2.2. Cultivation of erythrocyte

Fresh venous blood was collected from healthy volunteers. The whole blood with 0.25% citrate was centrifuged at 10 °C for 20 min at 350 × g. The leukocyte- and reticulocyte-free erythrocytes were prepared by percoll density procedure. Erythrocyte pellets were resuspended to 2×10^7 cells/ml in Hank's buffered salt solution (HBSS) (pH 7.2). The cell suspension was dispensed on 6-well culture plates. The erythrocytes incubated in the absence or presence of various concentrations of bortezomib for different time periods at 37°C. Supernatants were collected by centrifugation.

2.3. Chymotrypsin-like activity assay

2×10^6 erythrocytes per well in 24-well plates were treated with bortezomib for 50 min and then incubated with 20 μM proteasomal substrate succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin for 1 h. The fluorophore aminomethylcoumarin in culture supernatant was detected by spectrofluorometer [17].

2.4. Isolation of hemorphins

The culture supernatants of erythrocytes were loaded into hemorphin-7 IgG antibody-conjugated agarose beads and incubated overnight at 4 °C. The unbound or nonspecific components were washed away with 0.05 M sodium phosphate buffer (pH 7.2). The hemorphins were eluted with sterile water at room temperature. The aliquots of dialysis were lyophilized for analysis.

2.5. Reversed-phase HPLC analysis

The lyophilized samples were dissolved in 10 mM ammonium acetate buffer (pH 6). The reversed-phase HPLC (RP-HPLC) was performed on a Waters Spherisorb C18 column (4.6 mm × 250 mm, 5 μm) with a linear gradient of 0–39% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 0.85 ml/min. The UV-absorbance was detected at 226 nm.

2.6. Enzyme linked immunosorbent assay (ELISA)

Immunoreactive hemorphins were analyzed with ELISA method. 96 well microtiter plates were coated overnight at 4 °C with 100 μl/well sample in carbonate buffer (50 mM sodium carbonate, pH 9.6). Each well was treated with 0.5% bovine serum albumin in phosphate-buffered saline (PBS) to block unoccupied protein-binding sites. Hemorphin-7 IgG antibody was incubated at 37 °C for 2 h and alkaline phosphatase-conjugated goat anti-rabbit antibody was incubated for 1 h at 37 °C. Between each of the incubation steps, plates were washed with pH 7.4 PBS containing 0.05% Tween-20. 100 μl of p-nitrophenyl phosphate (1 mg/ml) substrate in pH 9.6 carbonate buffer containing 0.5 mM $MgCl_2$ were added. Reaction was terminated by the addition of 1 M NaOH (50 μl/well). The absorbance was measured at 405 nm on spectrophotometer.

3. Results

Currently no commercial antibodies for hemorphin-7 are yet available. We prepared hemorphin-7 IgG antibody by affinity purification of immune rabbit sera. Hemophins in culture supernatant of erythrocytes were isolated by immunoprecipitation and detected by RP-HPLC. The chemically synthetic hemorphin-7 and V-hemorphin-7 were used as standards. As seen in Fig. 1D, hemorphin-7 and V-hemorphin-7 in the immuneprecipitated products were eluted at the retention time of 23.7 min and 25.5 min, respectively.

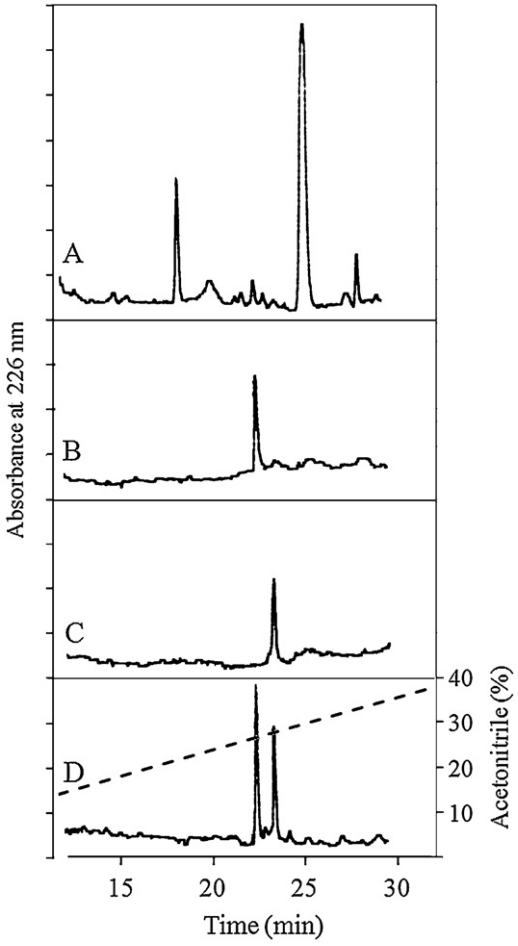

Fig. 1. RP-HPLC analysis of hemorphins. (A) Supernatant of human erythrocytes incubated in HBSS (pH 7.2) at 37°C for 50 min. (B) The synthetic hemorphin-7. (C) The synthetic V-hemorphin-7. (D) Immunoprecipitated hemophins. Peaks 1 and 2 corresponded to hemorphin-7 and V-hemorphin-7, respectively.

To directly assess the inhibitory effect of bortezomib on intraerythrocytic proteasome, we evaluated the chymotrypsin-like activity of proteasome in intact living erythrocytes. The fluorogenic peptide substrate was added to culture media following the treatment of erythrocytes with different concentrations of bortezomib. Proteasome efficiency was determined by measuring the appearance of fluorescence in culture supernatant. As shown in Fig. 2A, bortezomib exhibited a dose dependent inhibition of the chymotrypsin-like activity of intraerythrocytic proteasome.

ELISA analysis showed a low yield of immunoreactive hemorphins following the treatment of erythrocytes with proteasome inhibitor (Fig. 2B). 4 nM bortezomib was sufficient to reduce the production of hemorphins. The effect of bortezomib reached a plateau at 8 nM. In addition, we examined the time course for the influence of bortezomib on the formation of hemorphins. After 30 to 60 min of incubation, both 5 nM and 7 nM bortezomib showed significant inhibition of hemorphin generation compared to 3 nM bortezomib (Fig. 2C). In comparison with the control, bortezomib significantly decreased the formation of hemorphins 30 min after the onset of incubation. The maximal inhibition of hemorphin yield occurred at 50 min.

4. Discussion

Although some studies have reported that hemorphins released by erythrocytes, little is known about the production mechanism.