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HUMAN PLASMA α_1- AND α_2-THIOL PROTEINASE INHIBITORS STRONGLY INHIBIT Ca-ACTIVATED NEUTRAL PROTEASE FROM MUSCLE

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Human plasma α_1- and α_2-thiol proteinase inhibitors ($\alpha_{1,2}$TPIs) inhibited purified Ca-activated neutral protease (CANP) most strongly among a number of thiol proteinases tested. When CANP was added to plasma, it was also inhibited by α_2-macroglobulin (α_2M). At low CANP concentrations, CANP was bound mainly to $\alpha_{1,2}$TPIs; and after saturation of $\alpha_{1,2}$TPIs the additional CANP was bound to α_2M. These data suggested that a probable role of $\alpha_{1,2}$TPIs is to neutralize the proteolytic activity of the CANP derived from the tissues in collaboration with α_2M.

The inhibitory effects of human plasma α_1- and α_2-thiol proteinase inhibitors ($\alpha_{1,2}$TPIs) on several thiol proteinases have been investigated mainly with proteinases of plant origin (1-4). Among thiol proteinases of animal origin, cathepsin B_1 has been tested, but since it was not so strongly inhibited as papain and ficin (1,2), the physiological role of $\alpha_{1,2}$TPIs has remained unclear.

Recent studies have revealed that several tissues and cells such as brain (5,6), muscle (7-10), liver (11), kidney (12), platelets (13), and erythrocytes (14) contain Ca-activated neutral protease(s) (CANP). Since this enzyme appears to be a thiol proteinase, we examined the interaction of $\alpha_{1,2}$TPIs with CANP from porcine muscle, and found that CANP was very strongly inhibited by $\alpha_{1,2}$TPIs. Human plasma, however, contains another proteinase inhibitor, α_2-macroglobulin (α_2M), which is capable of inhibiting thiol proteinases. In the

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Abbreviations used: $\alpha_{1,2}$TPIs, α_1- and α_2-thiol proteinase inhibitors; CANP, Ca-activated neutral protease; α_2M, α_2-macroglobulin; and TCA, trichloroacetic acid.

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present study the interaction of α_2M with CANP was also investigated, and the physiological role of these inhibitors toward tissue-derived thiol proteinases is discussed.

MATERIALS AND METHODS

α_1- and α_2-Thiol proteinase inhibitors ($\alpha_{1,2}$TPIs) were isolated from human plasma as previously described (4). α_1TPI had a molecular weight of 176,000, and α_2TPI used in this experiment was α_2TPI$_2$ with a molecular weight of 90,000 (4). α_2-Macroglobulin (α_2M) was purified by gel filtration on a Sephadex G-200 column, starch block electrophoresis, ion-exchange chromatography on DEAE Sephacel, and immuno-affinity chromatography on a column packed with several anti-plasma protein antibodies immobilized on Sepharose 4B. Ca-activated neutral protease (CANP) was isolated from porcine muscle by the method (9) developed for chicken muscle CANP. Ficin was purified by the method of Englund et al. (15). Papain was purchased from Worthington Biochemical Corp. Casein was a product of Difco Laboratories.

Anti-α_1TPI and anti-α_2M antisera were prepared by immunizing rabbit. The IgG fractions were separated by ammonium sulfate precipitation, followed by DEAE cellulose column chromatography. Anti-rabbit IgG antiserum was prepared by immunizing a goat with purified rabbit IgG, and the IgG fraction was separated as above. Separate agar plates containing anti-α_1TPI antibody and anti-α_2M antibody, respectively, were prepared for quantitative estimation of $\alpha_{1,2}$TPIs and α_2M in the plasma fractions separated by Sephadex G-200 chromatography. Purified α_1TPI and α_2M were used as standards.

To quantitate the binding capacity of $\alpha_{1,2}$TPIs and α_2M, purified CANP was labeled with ^{125}I by the Chloramin T method (16). Radioactivity of [^{125}I]CANP was measured by a well-type scintillation counter, Aloka JDC 755.

CANP activity was assayed by the method previously described (9) with an appropriate modification. A typical assay procedure was as follows: CANP solution (30 µl), 0.05 M Tris-HCl buffer, pH 7.5 (1.1 ml) and 0.4 M $CaCl_2$ (20 µl) were sequentially added, and preincubated for 1 min at low temperature (0-2°C). The reaction was started by the addition of freshly prepared 3% casein in 0.05 M Tris-HCl buffer, pH 7.5 (100 µl) containing 0.3 M β-mercaptoethanol. After a 20-min incubation at 30°C, the reaction was terminated by adding 10% TCA (1.25 ml). The mixture was allowed to stand for 60 min, filtered and the UV absorption of the filtrate was measured with a Hitachi-Perkin Elmer spectrophotometer at 275 nm.

To estimate inhibitory activity, α_1 or α_2TPI was first added to the enzyme solution, and mixed. The following procedure was the same as for the enzyme assay. Assays of papain and ficin were carried out by the same procedure as for CANP, but adding only buffer instead of $CaCl_2$ solution.

RESULTS

Inhibition of CANP by $\alpha_{1,2}$TPIs. Inhibition of CANP activity by $\alpha_{1,2}$TPIs was compared with that of ficin and papain, which had proved to be most sensitive to $\alpha_{1,2}$TPIs. As shown in Fig. 1 (A and B), CANP was most strongly inhibited by both α_1TPI and α_2TPI. The approximate amounts of α_1TPI to give 50% inhibition of CANP, ficin, and papain activities were 7, 18, and 28 µg, respectively, and those of α_2TPI were 11, 20, and 30 µg.

Inhibitors in plasma. In order to identify the plasma components that combine with CANP, the inhibitor patterns in plasma fractions separated by Sephadex

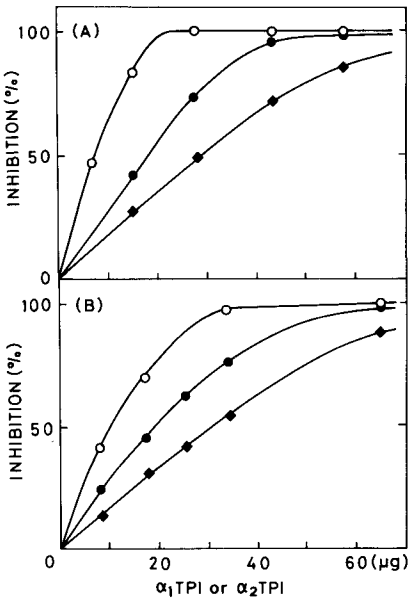
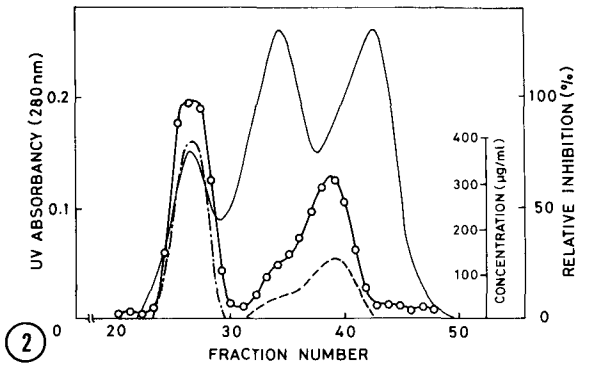


Figure 1. Inhibition patterns of CANP, ficin and papain by α_1TPI and α_2TPI. To each solution amount of CANP, ficin, and papain yielding the same caseinolytic activity in the standard assay (E=0.3 at 275 nm) was added an increasing amount of α_1TPI (A) or α_2TPI (B). For further experimental conditions, see Materials and Methods. Inhibition of CANP, —○—; ficin, —●—; and papain, —◆—.

G-200 chromatography were examined. As shown in Fig. 2, one inhibitor activity was found in the pass-through fraction, where α_2M antigen was positive, and $\alpha_{1,2}$TPIs antigens negative. The other inhibitor activity was eluted between the second and third protein peaks of the plasma fractions, with a shoulder under the second protein peak. Here only $\alpha_{1,2}$TPIs antigens and no α_2M antigen was detected. The former inhibitor activity disappeared on preincubation with anti-α_2M antibody and the latter on preincubation with anti-α_1TPI antibody. This indicates that the first inhibitor activity is due to α_2M and the second to $\alpha_{1,2}$TPIs.

Distribution ratio of CANP in plasma. To estimate the distribution of CANP binding to $\alpha_{1,2}$TPIs and α_2M in the plasma, purified CANP was labeled with ^{125}I, and an increasing amoung (5 - 300 μl) of [^{125}I]CANP was added to a constant amount (5 μl) of fresh human plasma. After a 30-min incubation at 30°C, anti-α_1TPI antibody and anti rabbit IgG antibody were sequentially added to precipitate the [^{125}I]CANP bound to $\alpha_{1,2}$TPIs. The precipitates thus



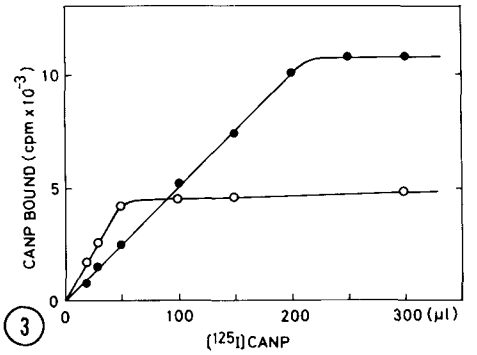


Figure 2. Inhibition profiles of CANP by plasma components fractionated on Sephadex G-200 column.
Fresh human plasma (10 ml) was filtered on a Sephadex G-200 column (2.0 x 140 cm) at a flow rate of 6 ml/hr. Each fraction volume was 10 ml. UV absorption (——) was estimated at 280 nm. Inhibition of CANP (—○—) was assayed as described in Materials and Methods. Concentrations of $\alpha_{1,2}$TPIs (– – –) and α_2M (— · —) were estimated on the agar plates containing anti-α_1TPI antibody or anti-α_2M antibody, respectively, using purified α_1TPI and α_2M as standard materials.

Figure 3. CANP binding capacities of $\alpha_{1,2}$TPIs and α_2M.
To the fresh human plasma (5 μl) [^{125}I]CANP solution (20 - 300 μl) was added, and the distribution of [^{125}I]CANP to $\alpha_{1,2}$TPIs and α_2M was determined. For further procedures, see text. —○—, [^{125}I]CANP bound to $\alpha_{1,2}$TPIs; —●—, [^{125}I]CANP bound to α_2M.

obtained were washed 3 times with chilled saline, and the radioactivity was estimated. The amount of [^{125}I]CANP bound to α_2M was also estimated by the same procedure, using anti-α_2M antibody in place of anti-α_1TPI antibody. Figure 3 shows a typical pattern of the [^{125}I]CANP in the plasma. When a small amount of [^{125}I]CANP was added, it was bound mainly to $\alpha_{1,2}$TPIs (ratio of [^{125}I]CANP binding to $\alpha_{1,2}$TPIs and α_2M, 3.5 : 2); after these were saturated, the additional [^{125}I]CANP was bound to α_2M, indicating that the overall binding capacity of α_2M was larger than that of $\alpha_{1,2}$TPIs. The final ratio of [^{125}I]CANP binding to $\alpha_{1,2}$TPIs and α_2M was approximately 2 : 5.

DISCUSSION

Thiol proteinases effectively inhibited by $\alpha_{1,2}$TPIs have not been identified in animal tissues, although Pagano and Engler (17) have very recently reported that human lysosomal enzyme, cathepsin L, is strongly inhibited by $\alpha_{1,2}$TPIs, and suggested that one of the physiological functions of $\alpha_{1,2}$TPIs is to inhibit cathepsin L. On the other hand, Iwata et al. (18) reported that, among cathepsin type thiol proteinases from rat liver,

cathepsin H was the most susceptible to rat serum low molecular weight TPI, followed by cathepsin C. However, inhibition of cathepsin H by the TPI did not exceed that of papain. In addition, since the optimum pH of the cathepsin type thiol proteinases is generally considerably lower than the blood pH, the proteolytic activity of these proteinases is more or less reduced when they enter the blood circulation. Accordingly, another type of thiol proteinase has been considered to exist in animal tissues. CANP appears to possess the properties required for this proteinase: The optimum pH of the CANP (6,7,9) is very close to the blood pH, and the inhibition of CANP by $\alpha_{1,2}$TPIs was far stronger than that of papain and ficin. This is the first evidence for a proteinase from animal tissues with a much higher susceptibility to $\alpha_{1,2}$TPIs than papain and ficin. Moreover, several tissues and cells including muscle, kidney, liver, and especially erythrocytes and platelets have the potential to release CANP into the blood in the event of inflammation and tissue damage or even under physiological conditions. Thus, at present CANP would be the most probable thiol proteinase target for $\alpha_{1,2}$TPIs.

The present experiments further demonstrated that CANP is also inhibited by α_2M with greater capacity than that of $\alpha_{1,2}$TPIs. However, since α_2M also inhibits other proteinases, it is likely that in case of tissue damage a large part of its binding capacity would be consumed for binding to other classes of released proteinases such as serine, metallo-, and carboxyl proteinases (19); and since $\alpha_{1,2}$TPIs do not bind to other classes of proteinases, their binding capacity is conserved for thiol proteinases. The relationship between $\alpha_{1,2}$TPIs and α_2M can be reviewed as follows: $\alpha_{1,2}$TPIs are the primary inhibitors specific for the thiol class of proteinases, and α_2M is present to compensate for the limited capacity of $\alpha_{1,2}$TPIs.

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