Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 630-635

UNGLYCOSYLATED RAT α_1-PROTEINASE INHIBITOR HAS A SIX-FOLD SHORTER PLASMA HALF-LIFE THAN THE MATURE GLYCOPROTEIN

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Received December 6, 1984

Summary: The plasma half-lives of glycosylated and unglycosylated α_1-proteinase inhibitor - radioactively labeled with $|^{35}S|$methionine in rat hepatocyte primary cultures - were determined in the rat. Unglycosylated α_1-proteinase inhibitor was synthesized by hepatocytes in the presence of tunicamycin. Media from hepatocytes containing ^{35}S-labeled glycosylated or unglycosylated α_1-proteinase inhibitor were injected into the tail veins of rats. At different times after injection α_1-proteinase inhibitor was isolated from plasma by affinity chromatography with anti-α_1-proteinase inhibitor Sepharose. Radioactivity measurements revealed a plasma half-life of 170 min for glycosylated α_1-proteinase inhibitor and of 30 min for the unglycosylated form of the inhibitor.

Introduction: Many proteolytic reactions occur in blood and require control. One way of regulating proteolytic activities in blood is by specific proteinase inhibitors (for reviews see 1-9). They control the proteolytic cascades responsible for immune reactions, for coagulation and other responses to inflammation. Among the anti-proteases α_1-proteinase inhibitor (α_1PI)[1] plays an important role by protecting tissues against proteolytic attack by leukocyte elastase (10). Deficiency of α_1PI is associated with pulmonary emphysema (11). Recently, it has been reported that the development of emphysema can be limited by intravenous injection of α_1PI partially purified from human serum (12). For a large scale production α_1PI has been synthesized in E. coli (13,14) and yeast (15) by recombinant DNA technology. As recently emphasized (15,16) a major question with respect to the therapeutic use of the recombinant α_1PI is, whether the unglycosylated α_1PI has a plasma half-life comparable to one of the glycosylated inhibitor.

[1] Abbreviations: α_1PI, α_1-proteinase inhibitor, also known as α_1-antitrypsin, α_1-antiproteinase, and α_1-trypsin inhibitor; SDS, sodium dodecyl sulfate.

0006-291X/85 $1.50

Here we report that unglycosylated α_1PI, synthesized in rat hepatocyte primary cultures in the presence of tunicamycin, has a 6-fold shorter plasma half-life than the glycosylated α_1PI.

Materials and Methods: L-[^{35}S]Methionine (>600 Ci/mmol) was purchased from the Radiochemical Center, Amersham. Protein A-Sepharose and CNBr-activated Sepharose 4B were from Pharmacia, Freiburg. Tunicamycin was from Calbiochem-Behring, Giessen.

Male Wistar rats of 220 g body weight were generously supplied by Prof. Dr. H. Ueberberg, Thomae GmbH, Biberach. The animals had free access to water and a carbohydrate-rich 20% protein diet (Altromin, Lage).

Rat hepatocyte primary cultures were prepared as previously described (17,18).

Preparation of immobilized anti-α_1PI: The purification of α_1PI from rat serum and the preparation of a specific anti-serum has been previously described (19). For the preparation of the immobilized anti-α_1PI, anti-α_1PI (rabbit) dialyzed against 10 mM phosphate buffer, pH 7.5, 0.15 M NaCl was added onto a protein A-Sepharose column. The column was washed with the same buffer and the immunoglobulins were eluted with 3 M NaSCN. The anti-α_1PI IgGs were then coupled to CNBr-activated Sepharose essentially as described by the manufacturer (Pharmacia, Freiburg).

Immunoprecipitation: The immunoprecipitations of α_1PI from the hepatocyte media were carried out as previously described (17,18). The eluted protein was directly subjected to SDS[1]polyacrylamide slab gel electrophoresis (20) and fluorography (21).

Determination of α_1PI clearance: Media from 7 dishes of hepatocytes treated without or with tunicamycin (legend to Fig. 1) were centrifuged at 10,000 g for 10 min, the resulting supernatants were concentrated by ultrafiltration (Amicon PM10). Ultrafiltration was repeated 4-5 times to remove free |^{35}S| methionine and tunicamycin. Samples of 400 µl containing ^{35}S-labeled proteins were injected rapidly into the tail vein of rats under light ether anesthesia. At various time intervals after injection, blood samples of about 200 µl were taken from the tail vein, transferred to citrate-coated plastic tubes and centrifuged at 10,000 g for 5 min. 100 µl aliquots of the plasma were mixed to 1 ml (bed volume) of anti-α_1PI Sepharose (see above), gently shaken at 4°C overnight, washed 3 times with 20 mM Tris/HCl, pH 7.5 containing 0.14 M NaCl, 5 mM EDTA, 2 mM methionine and 1% Triton X-100 and twice with 50 mM NaP_i buffer, pH 7.5. The radioactivity bound to IgG-Sepharose was measured.

Results: Studies on the plasma half-life of α_1PI have been carried out with α_1PI radioactively labeled by iodination (22-27), reductive methylation (28,29) or reductive amination (30,31). By all these labeling methods the chemically modified protein is altered in its physical and/or biological properties (29,32-41) possibly affecting the rate of removal from circulation. Therefore, in our studies we used α_1PI radioactively labeled with |^{35}S| methionine during its biosynthesis in rat hepatocyte primary cultures. To obtain unglycosylated α_1PI the hepatocytes were incubated with the glycosylation inhibitor tunicamycin (19,42-45). Fig. 1 shows fully glycosylated (lane 1) or unglycosylated α_1PI (lane 2) immunoprecipitated from the hepatocyte media by use of a specific antiserum to α_1PI.

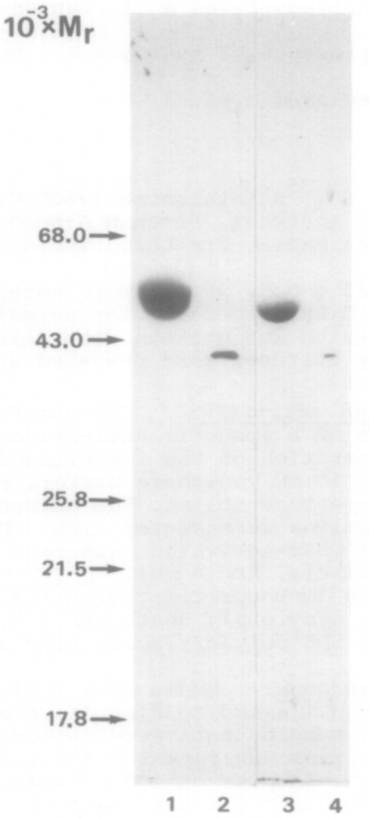

Figure 1: SDS polyacrylamide gel electrophoresis of unglycosylated and glycosylated α_1-proteinase inhibitor
Hepatocytes (8 x 10^6 cells/dish) were incubated at 37°C for 1 h without (lane 1) or with 2.5 µg/ml of tunicamycin (lane 2). The media were replaced by fresh media without and with tuncamycin at the same concentration and the hepatocytes were labeled with 100 µCi of [^{35}S]methionine for 4.5 h. α_1PI was immunoprecipitated from the media and subjected to SDS polyacrylamide gel electrophoresis and fluorography. Lanes 3 and 4 show glycosylated and unglycosylated α_1PI, respectively, purified from rat plasma by affinity column chromatography 3h after injection of the radioactively labeled hepatocyte media into rats as described in Materials and Methods. Bovine serum albumin (68,000), ovalbumin (43,000), elastase (25,800), soybean trypsin inhibitor (21,500) and myoglobin from equine skeletal muscle (17,800) were used as molecular weight standards.

For the half-life studies the concentrated media containing all the ^{35}S-labeled proteins synthesized and secreted by the hepatocytes were injected into the tail vein of adult Wistar rats. 200 µl aliquots of blood were taken at different times. α_1PI was isolated from the plasma by anti-α_1PI covalently linked to Sepharose 4B and its radioactivity was determined. Fig. 2A shows the decrease of radioactivity of labeled glycosylated (open circles) or unglycosylated α_1PI (closed circles) in the rat plasma. Unglycosylated α_1PI is removed at a much faster rate as compared to the native

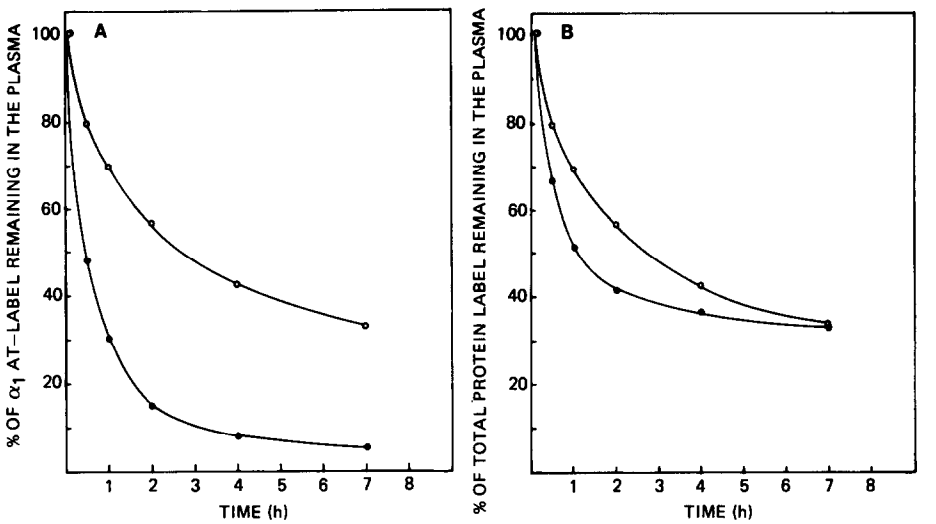

Figure 2: Rates of disappearance from rat plasma of unglycosylated and glycosylated α_1-proteinase inhibitor
Hepatocytes were prepared and radioactively labeled as described in Materials and Methods and in the legend to Fig. 1. Media from 7 dishes of hepatocytes treated without or with tunicamycin were concentrated and injected into the tail vein of rats as detailed in Materials and Methods. At 5 min, 30 min, 1 h, 4 h and 7 h after injection, blood samples of about 200 µl were taken and used for the determination of α_1PI (A) and total TCA-precipitable radioactivity (B). Radioactivity of glycosylated (o) and unglycosylated (●) α_1PI (A) or total TCA-precipitable proteins (B) is expressed as percent of the value obtained 5 min after injection.

protein. From a semilogarithmic plot of the data of Fig. 2 complex kinetics consisting of at least 3 phases were obtained. Half-lives of 170 min and of 30 min were estimated for the overall decrease of glycosylated and unglycosylated forms of α_1PI, respectively. To confirm that the radioactivity eluted from the affinity column represented α_1PI, the material was analyzed by SDS polyacrylamide gel electrophoresis (Fig. 1, lanes 3 and 4). For comparison we have determined the decrease of radioactivity of total proteins in rat plasma (Fig. 2B). Within the first 2 h the rate of disappearance of radioactivity from total proteins obtained from tunicamycin-treated hepatocytes was faster than that of controls. Thereafter both curves converge. This can be explained by the faster initial disappearance of the unglycosylated forms of glycoproteins, for example α_1PI, while the plasma half-lives of the other proteins remain unchanged after inhibition of glycosylation.

Discussion: The half-life of the glycosylated form of α_1PI of 170 min reported in this paper is in the same range as the half-lives of human α_1PI

in the rat circulation of 150 and 120 min, respectively, obtained by Yu and and Gan (46) and Omichi et al. (31). In contrast to the glycosylated α_1PI the unglycosylated protein disappears from rat plasma 6 times faster. The underlying mechanisms involved in the removal of α_1PI from the rat circulation are presently unknown. Entrance into extravascular space or the action of scavanger systems (47,48) could be responsible for the rapid clearance of α_1PI, particularly of the unglycosylated form. It can be concluded from this study that unglycosylated human α_1PI obtained by recombinant DNA technology and used for supplementation therapy is very likely to be eliminated from the circulation at a much faster rate than the glycosylated proteinase inhibitor. Therefore, an in vitro glycosylation system seems desirable.

Acknowledgements: The authors should like to thank E. Koetter for helpful discussions, K. Gyufko for excellent technical assistance and H. Gottschalk for her help with the preparation of this manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Bonn, the Fonds der Chemischen Industrie, Frankfurt, and the Müller-Fahnenberg-Stiftung, Freiburg. W. Weber is a recipient of the Fritz-Thyssen-Stiftung, Köln.

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