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Mouse α_1 -Protease Inhibitor Is Not an Acute Phase Reactant¹

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Abstract

Mouse plasma contains two major protease inhibitors, α_1 -protease inhibitor (α_1 -PI) and contrapsin, which have high affinity for bovine trypsin. Systemic injury, such as turpentine-induced inflammation, did not change the plasma concentration of α_1 -PI, but increased that of contrapsin by 50%. The concentration of hepatic α_1 -PI mRNA was determined by Northern blot hybridization and was not significantly affected by the acute phase reaction. J. M. Frazer, S. A. Nathoo, J. Katz, T. L. Genetta, and T. H. Finley ((1985) *Arch. Biochem. Biophys.* **239**, 112–119) have reported a threefold increase of mRNA for the elastase specific α_1 -PI but this increase was not demonstrated by the present study. The mRNAs for known mouse acute phase plasma proteins were, however, stimulated severalfold by the same treatment. These results indicate that in the mouse, as opposed to human, α_1 -PI is not an acute phase reactant.

Protease inhibitors in plasma play an essential role during the acute phase reaction of mammals by neutralizing extracellular proteolytic activity. Within hours following systemic tissue injury, the synthesis of a subset of plasma proteins, the acute phase reactants, is increased in the liver. These acute phase proteins include protease inhibitors. The increased production of the acute phase proteins results in a corresponding elevation in the plasma concentration of these proteins. It has been shown that in man, α_1 -antichymotrypsin and, to a lesser degree, α_1 -protease inhibitor (α_1 -antitrypsin; α_1 -PI³) are induced by injury (7, 8). Although mammals share a common set of acute phase reactants, one cannot assume that the homologous protease inhibitors are similarly regulated in different mammalian species (9, 10).

Characterization of the acute phase response in mouse liver has indicated that the cellular synthesis of α_1 -PI was not significantly affected (11, 12). However, contrapsin, the second major protease inhibitor specific for trypsin, but not elastase, appears to be a *bona fide* acute phase reactant (13–15).

Recently, Frazer *et al.* (10) have questioned whether the plasma protein identified by us as mouse α_1 -PI (11) was indeed the major protease inhibitor homologous to the human α_1 -PI. In the meantime, specific cDNA to mouse α_1 -PI has been isolated (16) and characterized as authentic α_1 -PI by sequence analysis and comparison to the human equivalent (17). In

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³Abbreviation used: α_1 -PI, α_1 -protease inhibitor.

addition, a mouse cDNA encoding contrapsin has been obtained. The nucleotide sequence has been shown to be homologous to human α_1 -antichymotrypsin (17). The sequence data also indicate that the active center of contrapsin has diverged considerably from the human α_1 -antichymotrypsin. This divergence presumably explains the different protease specificity of the two inhibitors (13, 14).

The cDNA to mouse α_1 -PI has provided the means for verifying whether its encoded protein was identical to the protein identified earlier by immunoprecipitation as α_1 -PI (18). Specific α_1 -PI mRNA was selected from total liver RNA by hybridization to immobilized cDNA and then translated in a cell-free system. The synthesized protein product was found by electrophoresis, immunoprecipitation, and partial proteolytic mapping to be indistinguishable from the α_1 -PI produced by liver cells.

Using the specific probes for α_1 -PI protein and its mRNA, the effect of the acute phase reaction upon the expression of that inhibitor has now been determined. We report in this communication that mouse α_1 -PI is not significantly affected by an inflammatory reaction both at the mRNA and plasma protein level. Together with our earlier result regarding cellular synthesis, it is evident that mouse α_1 -PI should not be included in the group of acute phase reactants.

Experimental Procedures

Animals

For all experiments 3-month-old males of the inbred mouse strain C57BL/6J were used. All animals were caged individually. Acute inflammation was induced either by two subcutaneous injections of 25 μ l turpentine in the lumbar region or by a single intraperitoneal injection of 200 μ l phosphate-buffered saline containing 10 μ g lipopolysaccharides (*Escherichia coli*; serotype 0127:B8).

Long-term inflammation (5 days) was achieved by injection of turpentine every second day or by daily injection of lipopolysaccharides. Chronically inflamed animals were analyzed 24 h after the last injection. None of the animals in this study were subjected to periods of food or water deprivation, and were killed in the morning between 9 and 10 o'clock. Blood (100 μ l) was collected by retroorbital puncture into heparinized tubes and freed of cells by centrifugation. Plasma was frozen until use.

Analysis of plasma proteins

Total plasma proteins were separated by crossed immunoelectrophoresis as outlined by Weeke (19). The first dimension represents electrophoresis in 1% agarose gel at 5 V/cm for 3.25 h at 15°C. The second dimension was performed in agarose containing antiserum raised against a preparation of the two major mouse inhibitors for trypsin, α_1 -PI and contrapsin (generous gift of Dr. J. Gauldie, Department of Pathology, McMaster University, Hamilton, Canada). The original antigen preparation contained a trace amount of albumin resulting in the presence of immunoglobulin against that protein. The immunoprecipitation of albumin in the crossed immunoelectrophoresis experiment served as a convenient endogenous marker for electrophoresis.

In order to obtain metabolically labeled plasma proteins, hepatocytes were prepared by collagenase perfusion of adult mouse liver (20), freed of nonparenchymal cells by differential centrifugation, and placed in collagen-coated tissue culture plates (8×10^5 cells/10 cm²) (11). After 45 min, the adherent liver cells were washed two times and then labeled for 6 h with [³⁵S]methionine (1000 Ci/mmol; 100 μ Ci/ml) in serum-free Dulbecco's modified Eagle's medium (11). The secreted proteins present in 50 μ l cell-free culture medium supernatant were subjected to crossed immunoelectrophoresis using nonradioactive plasma as carrier. The radioactivity associated with precipitin lines was visualized by autoradiography. The labeled antigens were cut out of the crossed immunoelectrophoresis plate, recovered by boiling in a buffer containing 10 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate, and 5% 2-mercaptoethanol, and separated again by two-dimensional polyacrylamide gel electrophoresis (21). These gels were processed for fluorography (22).

Analysis of RNA

Total RNA was extracted from liver by the guanidine-HCl procedure (23, 24). Isolation of polyadenylated mRNA was not performed in order to avoid biased selection of mRNA. For Northern blot analysis, 15 μ g of RNA was fractionated on a 1.5% agarose gel containing 2.2 M formaldehyde (25) transferred to nitrocellulose (26) and hybridized to ³²P-labeled cDNA probes (27). The following labeled cDNA were utilized: p1796 encoding the carboxy terminal half of mouse α_1 -PI and, therefore, not cross-hybridizing with contrapsin (16, 17); pSAA-1, human SAA, (28) (generously provided by Dr. J. Sipe, Boston University School of Medicine, Boston, Mass.); pIRL10, rat α_1 -acid glycoprotein (29); and pIRL-9, rat haptoglobin (30). All cDNAs encoding nonmurine proteins were found to hybridize specifically to the corresponding mouse mRNAs at normal stringency, which was 0.6 M sodium chloride at 62°C. Quantitation of the hybridization was achieved by densitometric scanning of the Northern blots. The linear relationship of signal to the amount of RNA analyzed was established using serial dilutions. The hybridization intensity was expressed in standardized densitometric units per microgram total RNA.

Results

The Effect of Inflammation on the Plasma Level of α_1 -PI and Contrapsin

In rodents, most if not all, major acute phase plasma proteins attain their maximal plasma concentration 24 h after initiation of inflammation (4, 6). In view of this observation, we determined the corresponding changes that can be observed for the two major trypsin inhibitors (Fig. 1). Individual variation in plasma concentration was randomized by analyzing a pool of plasma collected from the same 10 animals prior to, and 24 h after, turpentine-induced inflammation. The identities of the immunoprecipitated antigens were further verified by separation on two-dimensional polyacrylamide gels using metabolically labeled proteins obtained from primary cultures of adult mouse hepatocytes (Fig. 2). α_1 -PI shows an apparent molecular weight of 55,000 and pI of 4.5–5.0 and contrapsin of 65,000 and 4.1–4.5, respectively. These molecular properties are in agreement with those reported earlier (11, 13, 14, 31). Furthermore, Fig. 2 illustrates that the identified α_1 -PI and contrapsin are major plasma proteins because a significant amount of the total radioactivity in the medium is confined to these proteins (see Refs. (11, 12) for quantitation).

Integration of the areas under the precipitin lines of the protease inhibitors in Fig. 1 indicated a lack of inflammation-induced change in α_1 -PI concentration, while contrapsin was increased by 50%. This finding corroborates our earlier report (11) which demonstrated that there was no significant change in the hepatocellular synthesis of α_1 -PI, but a 50% increase of contrapsin.

The Effect of Inflammation of α_1 -PI mRNA in the Liver

At the maximal hepatic acute phase response, we were not able to detect any increase of α_1 -PI synthesis or plasma concentration. However, the possibility remained that inflammation could exercise a stimulatory effect at the pretranslational level (10). Therefore, we determined the effect of turpentine injection upon α_1 -PI mRNA concentration in the liver. Total liver RNA was separated by gel electrophoresis and probed for specific mRNA by hybridization with a labeled cDNA (Fig. 3). The achievement of a proper inflammatory reaction was illustrated using cDNAs that hybridized to the mRNAs for the known mouse acute phase plasma proteins, serum amyloid A, α_1 -acid glycoprotein, and haptoglobin.

Quantitation of the hybridization to α_1 -PI mRNA revealed a remarkably large variation among individual animals even within the same experimental group (Table I). A similar variation was also noticed when identically treated litter mates were analyzed (data not shown). The average hybridization value for α_1 -PI mRNA in control animals represented about 6000 mRNA copies per cell based on liquid hybridization studies reported previously (16). There is no statistically significant increase either after 9 h, when according to Frazer *et al.*, RNA for α_1 -PI(E) is increased threefold, or after 24 h. The quantitation of mRNA for haptoglobin demonstrates that a full scale inflammation reaction of the livers was achieved.

The mRNA concentration in animals which had been maintained for 5 days in an inflammatory state was measured (Table I) in order to test whether the proposed induction of α_1 -PI mRNA by inflammation was delayed relative to the standard response. The α_1 -PI mRNA concentration in chronically treated animals is not significantly different from that of the control as shown in acutely inflamed animals.

Discussion

The main purpose of this communication is to demonstrate that the major inhibitor for trypsin and elastase, α_1 -PI of the mouse, is not an acute phase reactant. In this study, cloned α_1 -PI cDNA was utilized for the identification and quantitation of α_1 -PI mRNA. Earlier reports from this laboratory (11, 18) identified the plasma form of α_1 -PI on the basis of immunoprecipitation with the aid of monospecific antibodies to purified mouse α_1 -PI (32). In this way, the identified protein was found to be a major component of the hepatic synthesized plasma proteins (Fig. 2). This protein corresponds in physicochemical and functional parameters to mouse α_1 -PI described in detail by several laboratories (10, 13, 14, 31, 32).

Nathoo *et al.* (33) and Frazer *et al.* (10) have reported the presence of two similar α_1 -PIs in mouse plasma. The inhibitors, termed α_1 -PI(T) and α_1 -PI(E), show preferential inhibition of trypsin and elastase, respectively. The information provided regarding protease specificity

and molecular weights of cell-free precursor and mature plasma forms of the two α_1 -PIs indicated an extremely high similarity to two protease inhibitors described previously by Takahara and Sinohara (13, 14). These protease inhibitors are contrapsin, or at least one possible form of it (15), and α_1 -PI. Frazer *et al.* also showed, however, that the plasma level of α_1 -PI(E) and not α_1 -PI(T) is increased following inflammation. Our conflicting report, which showed (11) that hepatic α_1 -PI synthesis is not changed during acute phase, was dismissed on the premise that the protein measured did not represent the major inhibitor for trypsin and elastase and also because it is distinct in size and charge from α_1 -PI(E). This alleged discrepancy in the electrophoretic properties was, however, not demonstrated, because minimal apparent molecular weight of 55,000 for α_1 -PI in our experiment coincided with that of α_1 -PI(E) of Frazer *et al.*

The inflammation-mediated increase of mRNA for α_1 -PI(E) and α_1 -PI(T) reported by Frazer *et al.* (10) differed remarkably from that of other *bona fide* acute phase plasma proteins. Functional mRNA for both α_1 -PIs reached a maximal concentration (threefold above control) 9 h after initiation of inflammation and returned within 24 h to basal level. Unfortunately, the quantitation of the mRNAs, which was dependent on *in vitro* translation and immunoprecipitation of α_1 -PI precursors, was performed without the use of internal standards which would have corrected for potential variation in efficiency of translation, and recovery of the synthesized protein. Considering this omission and the fact that there is high individual variation of α_1 -PI mRNA concentration, the statement that mouse α_1 -PI is an acute phase reactant is unwarranted. Our present and previous results strongly indicate that the expression of the mRNA for the major α_1 -PI in the mouse is not significantly affected by inflammation.

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Fig. 1.

Crossed immunoelectrophoresis of α_1 -PI and contrapsin. Plasma was collected from 10 male mice prior to (Control) and 24 h after injection of turpentine (Inflamed). Fifty microliters of plasma derived from each animal and time point was pooled. From each pool, 0.1 μl was separated by crossed immunoelectrophoresis (the first dimension is horizontal from left to right, the second dimension is vertical from bottom to top). The second dimension agarose contained 0.3% rabbit antiserum against mouse α_1 -PI and contrapsin (CT). The antiserum also contained low titer antibodies against mouse albumin (ALB) that was present in trace amounts in the original antigen preparation. The precipitin bands were visualized by Coomassie blue staining.

Fig. 2.

Separation of α_1 -PI and contrapsin by two-dimensional polyacrylamide gel electrophoresis. In order to localize α_1 -PI and contrapsin on the two-dimensional polyacrylamide gel pattern, radiolabeled mouse plasma proteins were used. A freshly prepared primary culture of hepatocytes from a control male mouse was labeled for 6 h with [35 S]methionine. A 50- μ l aliquot of the labeled culture medium was combined with 0.1 μ l control mouse plasma and separated by crossed Immunelectrophoresis as shown in Fig. 1. The radioactive bands corresponding to α_1 -PI (B) and contrapsin (C) were cut out and separated by two-dimensional polyacrylamide gel electrophoresis. For comparison 25 μ l of the nonfractionated hepatocyte medium was similarly separated by two-dimensional gel electrophoresis (A). The position of molecular weight markers and the tracking dye, bromophenol blue (BPB), are shown on the left and the pH gradient achieved in the first dimension is indicated at the top. The following proteins are indicated: ALB, albumin; AT-III, antithrombin III; CT, contrapsin; β -HP, β -haptoglobin; HPX, hemopexin; MUP, major urinary proteins; α_1 -PI, α_1 -protease inhibitor.

Fig. 3.

Northern blot analysis of mouse liver RNA. Total liver RNA (15 μ g) from individual animals, which were treated as indicated, was separated by electrophoresis, transferred to nitrocellulose, and hybridized simultaneously with two different 32 P-labeled cDNAs. Panel A shows hybridization to mRNA for α_1 -PI and α_1 -acid glycoprotein and panel B to mRNA for haptoglobin and serum amyloid A (SAA). The control lanes in A represent threefold serial dilutions of RNA from a nontreated animal. Autoradiographs were exposed for 24 h.

Table I
Quantitation of mRNA for α_1 -PI and Haptoglobin

Treatment	N	Hybridization (units/ μ g RNA)	
		α_1 -PI	Haptoglobin
Control	17	10.3 $\pm$ 4.5	2.3 $\pm$ 0.3
9 h inflamed	4	14.3 $\pm$ 3.1	9.3 $\pm$ 2.3
24 h inflamed	12	12.5 $\pm$ 5.5	16.9 $\pm$ 1.9
5 days inflamed	6	11.7 $\pm$ 4.2	9.5 $\pm$ 3.7

Note. Total liver RNA from control animals and animals after injection of turpentine was analyzed by Northern blot hybridization as shown in Fig. 3. Hybridization was quantitated by densitometry of the bands on autoradiographs under conditions such that a linear densitometric signal to the serially diluted amounts of RNA was obtained. In all Northern blot separations a standard RNA preparation was included in order to allow cross comparison. The data are expressed in densitometric units per μ g RNA. The values shown represent means and standard deviations. *N* indicates the number of independent RNA preparations and Northern blot analyses.