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Fetuin-A and Cystatin C Are Endogenous Inhibitors of Human Meprin Metalloproteases†

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ABSTRACT: Meprin α and β, zinc metalloproteinases, play significant roles in inflammation, including inflammatory bowel disease (IBD), possibly by activating cytokines, like interleukin 1β, interleukin 18, or tumor growth factor α. Although a number of potential activators for meprins are known, no endogenous inhibitors have been identified. In this work, we analyzed the inhibitory potential of human plasma and identified bovine fetuin-A as an endogenous meprin inhibitor with a K_i (inhibition constant) of 4.2×10^{-5} M for meprin α and a K_i of 1.1×10^{-6} M meprin β. This correlated with data obtained for a fetuin-A homologue from carp (nephrosin inhibitor) that revealed a potent meprin α and β inhibition (residual activities of 27 and 22%, respectively) at a carp fetuin concentration of 1.5×10^{-6} M. Human fetuin-A is a negative acute phase protein involved in inflammatory diseases, thus being a potential physiological regulator of meprin activity. We report kinetic studies of fetuin-A with the proteolytic enzymes astacin, LAST, LAST_MAM, trypsin, and chymotrypsin, indeed demonstrating that fetuin-A is a broad-range protease inhibitor. Fetuin-A inhibition of meprin α activity was 40 times weaker than that of meprin β activity. Therefore, we tested cystatin C, a protein structurally closely related to fetuin-A. Indeed, cystatin C was an inhibitor for human meprin α ($K_i = 8.5 \times 10^{-6}$ M) but, interestingly, not for meprin β. Thus, the identification of fetuin-A and cystatin C as endogenous proteolytic regulators of meprin activity broadens our understanding of the proteolytic network in plasma.

Human meprin α and β are zinc metalloendopeptidases of the astacin family and metzincin superfamily (*1*, *2*). Although meprin α and β have 44% identical amino acid sequences, they exhibit striking differences in activation, substrate specificity, and quaternary structure (*3*). Meprin α forms chain and ring superstructures up to the megadalton range, and therefore, it is the largest known secreted protease (*4*, *5*). In contrast, meprin β is dimeric and remains predominantly membrane-bound due to a C-terminal transmembrane region. However, proteolytic shedding may release active meprin β from the cell surface (*6*).

Meprins are abundantly expressed in several tissues, including kidney, intestinal epithelial cells, skin, and certain populations of leukocytes (*7*−*11*). Because of their ability to cleave a wide range of biologically active proteins, as well as proteins of the extracellular matrix [such as collagen type IV, laminin-1, nidogen-1, and fibronectin (*3*)], they are thought to be involved in cell migration, differentiation, and proliferation. The cell-adhesion molecule E-cadherin, for example, is processed by meprin β, resulting in the loss of cell−cell contacts (*12*). Similarly, incubation of cultured keratinocytes with recombinant human meprin β induced dramatic changes in cell morphology and viability (*9*). Recently, the activation of tumor growth factor α (TGF-α) (*13*), interleukin 18 (IL-18) (*7*), interleukin 1β (IL-1β) (*14*), and the vascular endothelial growth factor (VEGF-A) (*15*) could be attributed to meprin activity, thereby revealing functions in immunology and angiogenesis. With regard to pathological conditions, it was shown that meprins are involved in inflammatory disorders like morbus Crohn or inflammatory bowel disease (*16*). Moreover, in colon cancer, meprin α is expressed not only apically but also basolaterally (*17*). Thus, the protease is facing the basement membrane and may therefore promote cancer cell evasion through this barrier by virtue of its proteolytic activity. Some of these proteins are grossly degraded by meprins, while others are specifically processed. Meprin α and β differ in their substrate specificity (*3*) largely because of binding preferences around the active site. Meprin α prefers neutral aliphatic and aromatic side chains in the P1′ position, while meprin β prefers negatively charged amino acid residues (*18*, *19*). Despite the fact that a considerable number of potential substrates and molecular features are known, many questions regarding the (patho)physiological roles of meprins remain open.

To understand the proteolytic network, it is especially important to understand the regulatory interaction among enzymes, substrates, and inhibitors. In skin, we could identify human tissue kallikrein-related peptidase 4 (klk4), klk5, and klk8 as activators for meprins, whereas meprin α is activated by klk 5 only (*9*, *20*). In contrast, meprin α is specifically transformed to its mature form by plasmin (*5*, *21*). Once proteases are proteolytically active, inhibitors are necessary for their physiological regulation. In the

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case of human meprins, no endogenous inhibitors have been identified. Although the mannan binding protein was found to inhibit rat meprins (*22*), such evidence is lacking for the human enzymes. Several synthetic inhibitors developed as therapeutics targeting other metalloproteases exhibited inhibitory capacity against meprins (*19*). To date, the most effective compound in this respect is the naturally occurring hydroxamate actinonin, which inhibits meprin α 100 times better than meprin β (*19*). However, the physiological relevance of this molecule is doubtful.

In this work, we provide evidence of the inhibition of meprins by extracellular members of the cystatin superfamily of protease inhibitors. The cystatins are subdivided into three groups by structural similarity, with type I cystatins being intracellular proteins. The extracellular type II cystatins comprise, for example, cystatin C (*23*), which consists of a single 120-amino acid chain, known to inhibit papain-like proteases, such as cathepsin B, H, and L, and papain (*24*, *25*). Cystatin C is expressed in many human tissues such as epididymis, vas deferens, brain, thymus, and ovary but can also be found in various body fluids such as plasma and cerebrospinal fluid (*26*, *27*). Fetuin-A (α-2HS-glycoprotein) and its homologue from carp, the nephrosin inhibitor, are members of the fetuin family, consisting of type 3 cystatins. Fetuins are plasma proteins with three characteristic domains: two N-terminal cystatin-like domains (D1 and D2) and an unrelated C-terminal domain (D3). Fetuins undergo proteolytic processing. The processing was studied in detail in human fetuin-A, which is cleaved from a single-chain precursor into a mature circulating two-chain form connected by a disulfide bond (*28*−*30*).

Fetuin-A is predominantly expressed in the liver and constitutively secreted into the blood throughout life (*29*). During development, fetuin-A expression in various tissues has also been reported (*31*, *32*). Fetuin-A serum levels drop after trauma and infection, and therefore, fetuin-A is a negative acute phase plasma protein (*33*).

Human fetuin-A has many purported biological functions. Wang et al. (*34*) reported that fetuin-A may play a role in epithelial differentiation, because it is strongly expressed in the developing epithelial cell layer and can promote scar-free wound healing. Fetuin-A is foremost known to inhibit pathological calcification and is highly enriched in the mineralized bone matrix (*35*, *36*). Another major role is its involvement in the immune response (*37*). Fetuin-A was shown to be an inhibitor of the insulin receptor tyrosine kinase (*38*) and trypsin (*39*, *40*).

The nephrosin inhibitor from carp is likewise expressed in liver tissue and circulates in inhibition complexes with the astacin protease nephrosin (*28*). Zebrafish nehprosin is specifically expressed in granulocytes (*41*), suggesting a role in the inflammatory response like that of fetuin-A.

Thus, the goal of this work was the identification of an endogenous inhibitor of meprin α and β. Because meprins are expressed in circulating leukocytes, we analyzed human plasma, which is rich in protease inhibitors.

MATERIALS AND METHODS

Human Plasma Samples. The plasma samples used in this study were provided by the University Hospital of Mainz (Mainz, Germany). Control samples were voluntary blood donations from healthy persons. The Declaration of Helsinki Principles was followed, and all patients gave their written, informed consent.

Chemicals and Enzymes. All chemicals were of analytical grade and, if not stated otherwise, were obtained from Amersham Bioscience (Freiburg, Germany), Applichem (Darmstadt, Germany), Bio-Rad (Munich, Germany), Sigma/Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), or R&D Systems (Wiesbaden, Germany). Bovine fetuin-A isolated from bovine serum (catalog no. F-2379), human fetuin-A isolated from human plasma (catalog no. G0516), and fetuin-A antibody (rabbit, polyclonal; catalog no. HPA001524) were purchased from Sigma/Aldrich. Recombinant human fetuin-A was obtained from R&D Systems. Recombinantly expressed cystatin C (*Escherichia coli*; catalog no. RD172009100-H) was obtained from BioVendor (Heidelberg, Germany) and the penta-His antibody from Qiagen (Hilden, Germany).

Recombinant Protein Expression, Purification, and Activation. Recombinant meprin α (Q16819), recombinant meprin β (Q16820), recombinant LAST (B4F319), and recombinant LAST_MAM (B4F320) were expressed, purified, and activated as described previously (*5*, *9*, *42*). Astacin was isolated from the digestive tract of the crayfish *Astacus astacus* (*43*).

The cDNA for the recombinant nephrosin inhibitor was provided by F. Xavier Gomis-Rüth (Barcelona, Spain).

Inhibition Assay. The activity assay using azocasein as the substrate was used for inhibition studies. Enzymes at concentrations at least 10-fold below inhibitor concentrations were incubated with plasma [fetuin concentration in plasma of $1.02-2.54 \times 10^{-5}$ M (*29*, *44*)], FCS[1] (fetal calf serum; fetuin concentration in FCS of 1.1×10^{-4} M) (*36*), or potential inhibitors at 37 °C for 10 min. Afterward, the enzymes were incubated with 11 mg/mL azocasein in 20 mM ethanolamine (pH 9.5) at 37 °C overnight. The protein was precipitated via addition of TCA (trichloroacetic acid) to a final concentration of 3.5% (w/v) and centrifuged at 13000*g* for 10 min. Concentrations of cleaved azo dye in the supernatant were determined photometrically at 340 nm, which correlates to the proteolytic activity. The exact enzyme concentrations used in different inhibition assays are given in the figure legends.

The proteolytic activity and inhibition by bovine and human fetuin-A and cystatin C were also tested with the fluorogenic peptide substrate Mca-YVADAPK(Dnp)-OH (R&D Systems). The enzymatic activity was recorded with a Varioskan Flash fluorescence spectrophotometer (Thermo Scientific). Data were analyzed using SkanIt version 2.4 for Varioskan Flash. Meprin α [5 nM in 50 mM HEPES (pH 7.5)] and meprin β [1 nM in 50 mM HEPES (pH 7.5)] were incubated with an inhibitor at 37 °C for 10 min. After the assay had been started via addition of the fluorogenic peptide substrate [final concentration of 10 μM in 50 mM HEPES (pH 7.5)], upon excitation at 320 nm the fluorescence emission at 405 nm was monitored for 10−60 min. The proteolytic activity was related to the emission at 405 nm. The activity was determined by the slope of the initial linear range of the curve.

Fetuin Depletion. All incubations were conducted in 1.5 mL microtubes at 4 °C on a rotating wheel; 600 μg of immunoglobulin of a rabbit anti-bovine fetuin antiserum and a control normal rabbit serum (Dako Schweiz AG, Baar, Switzerland) were coupled with 100 μL of protein A Sepharose CL-4B beads (GE Healthcare Life Sciences, Glattbrugg, Switzerland) in phosphate-buffered saline for 3 h and subsequently washed three times with phosphate-buffered saline. Two hundred microliters of 1% inactivated FCS (57 °C for 20 min) in phosphate-buffered saline was precleared with 100 μL of protein A Sepharose beads

[1]Abbreviations: FCS, fetal calf serum; K_i, inhibition constant.

for 2 h and subsequently co-incubated overnight with the protein A-coupled immunoglobulins of the anti-bovine fetuin antiserum and control normal rabbit serum. The inhibitory capacity of the depleted FCS supernatants was assessed using recombinant human meprin β as outlined above.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Protein samples were separated under reducing conditions via SDS−PAGE (10%). Proteins were visualized with Coomassie staining [0.02% (w/v) Coomassie Brilliant Blue G-250, 5% (w/v) aluminum sulfate hydrate, 10% ethanol (99%), 2% *o*-phosphoric acid, and 83% Milli-Q water]. Prestained protein marker (ColorPlus P7-ladder prestained protein parker, Broad Range; New England Bio Laboratories, Frankfurt, Germany) was used to determine the molecular mass.

Western Blot Analysis. For Western blot analysis, proteins were subjected to SDS−PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF) (Immobilon P; Millipore, Eschborn, Germany). For specific protein detection with the anti-human fetuin-A antibody, the membrane was blocked with 5% (w/v) dry milk in a TBS buffer for 1 h at room temperature and incubated with the first antibody [polyclonal, rabbit human fetuin-A antibody, 1:1000 in 5% (w/v) dry milk Tris saline] for 1 h and afterward for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG [HRP; 1:10000 in 10% (w/v) dry milk saline]. For protein detection via His tag by penta-His antibodies (Qiagen), the membrane was blocked with a 3% (w/v) BSA/TBS solution for 1 h. The penta-His antibody [1:1000 in a 3% (w/v) BSA/TBS solution] was incubated for 1 h followed by HRP-conjugated anti-mouse IgG [1:10000 in 10% (w/v) dry milk TBS]. Detection on X-ray film was achieved using the ECL Plus substrate (GE Healthcare, Freiburg, Germany) or Rothi-Lumin substrate (Roth, Karlsruhe, Germany), following the manufacturer's instructions (Hyperfilm ECL, Amersham Pharmacia Biotech, Freiburg, Germany).

Inhibition Kinetics. Determination of the inhibition constant K_i was performed by nonlinear regression analysis using GraFit version 4.0 (Erithacus Software) by plotting the ratio of the inhibited and uninhibited enzyme activities against the inhibitor concentration and fitting the data to the following equation (*45*):

$$\frac{v_i}{v_o} = 1 - \left[E_o + l_o + K_i - \sqrt{(E_o + l_o + K_i)^2 - 4E_o l_o}\right]/(2E_o)$$

Inhibition kinetics were performed using the fluorogenic peptide substrate Mca-YVADAPK(Dnp)-OH (R&D Systems). Bovine fetuin was used at concentrations ranging from 1.9×10^{-7} to 3.4×10^{-4} M and cystatin C at concentrations ranging from 4.1×10^{-9} to 4.1×10^{-5} M. The final concentration of meprin α was 5 nM and that of meprin β 1 nM.

Proteolytic Processing of Bovine and Human Fetuin. Recombinant meprin α and meprin β at a final concentration of 9.4×10^{-8} M were incubated overnight with bovine or recombinant human fetuin-A at a final concentration of 1.07×10^{-5} M at 37 °C in a total volume of 20 μL. Afterward, samples were subjected to 10% SDS−PAGE. Proteins were visualized by Coomassie staining or blotted to polyvinylidene fluoride membranes. Blots were probed with the rabbit anti-human fetuin-A antibody. Signals could be detected because of the horseradish peroxidase-conjugated secondary antibodies.

Meprin Affinity Chromatography. Meprin affinity chromatography was performed to isolate specific ligands from human plasma. For that purpose, we expressed, purified, and activated human meprin α and β. Immobilization of the active enzymes on CH Sepharose 4BTM-Matrix was conducted using the manufacturer's instructions. Residual active groups were blocked with a 0.1 M Tris-HCl buffer (pH 8.0) for 1 h. To ensure that no free ligands remained bound electrostatically, the material was washed with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl. Sepharose prepared for affinity chromatography was packed into Bio-Rad columns, and 2.5 mL of plasma was applied to 1.5−3.0 mL of Sepharose matrix. Unbound substances were removed when the column was washed with HEPES/NaOH buffer (50 mM, pH 7.5). Eventually, meprin ligands were identified by SDS−PAGE and Western blot analysis; 500 μL of Sepharose matrix with a coupled ligand was boiled for 10 min under reducing and denaturing conditions and centrifuged (1 min at 15000*g*), and the supernatant was loaded on an SDS gel.

Plasma Preparation. Control plasma was obtained from healthy volunteers. Blood taken from Vena mediana cubiti was caught in serum tubes [(Z) Gel 7.5 mL; KABE Labortechnik, Nümbrecht-Elsenroth, Germany], centrifuged for 2 h (4000*g* and 4 °C), and stored at −20 °C.

Amino Acid Sequence Alignment. Sequences were aligned using ClustalX version 2.0 (*46*) via adjustment of opening GAP penalties to 5.0; other settings were default settings. Sequence alignments were edited with GeneDoc version 2.6.0241 and comprised complete sequences or single domains.

RESULTS

Cell Culture Medium Inhibits Meprin Activity. The presence of potential inhibitors in plasma was inferred from the use of FCS-containing medium in cell culture experiments, revealing reduced meprin activity (data not shown).

We first tested if α2-macroglobulin, a broad-spectrum protease inhibitor abundant in plasma, would inhibit meprin activity. Despite the fact that many astacin proteases are effectively inhibited by α2-macroglobulin (*43*, *47*−*49*), meprins were not (*19*). One reason might be that meprin dimers and oligomers are too bulky for caging inhibition by α2-macroglobulin because of the cage mechanism.

Human Plasma Inhibits the Proteolytic Activity of Meprin α and β. Inhibition assays with four different plasma samples from two female and two male adults revealed strong inhibition of human meprins (Figure 1A). Using azocasein as a substrate, recombinant meprin α exhibited a residual activity of 52−66% after plasma incubation, whereas recombinant meprin β was more strongly inhibited, with 2−15% remaining activity, by a final plasma concentration of 18%. Obviously, the individual plasma samples differed in their inhibitory capacity, indicating varying levels of endogenous inhibitors. Moreover, separation of human plasma proteins by gel filtration revealed molecular masses between 30 and 60 kDa for the main inhibiting molecules (data not shown).

Isolation and Identification of Human Fetuin-A as an Inhibitor of Meprin α and β. A recently described endogenous inhibitor of nephrosin, another astacin metalloprotease from the carp *Cyprinus carpio*, had been identified previously as a homologue of human fetuin-A (*28*). Prompted by the fact that the molecular mass of the meprin inhibitor detected by analytical gel filtration was in the fetuin-A range of 50−60 kDa, we performed immunoassays with a fetuin-A specific antibody, as well as inhibition assays with purified fetuin-A. To isolate binding

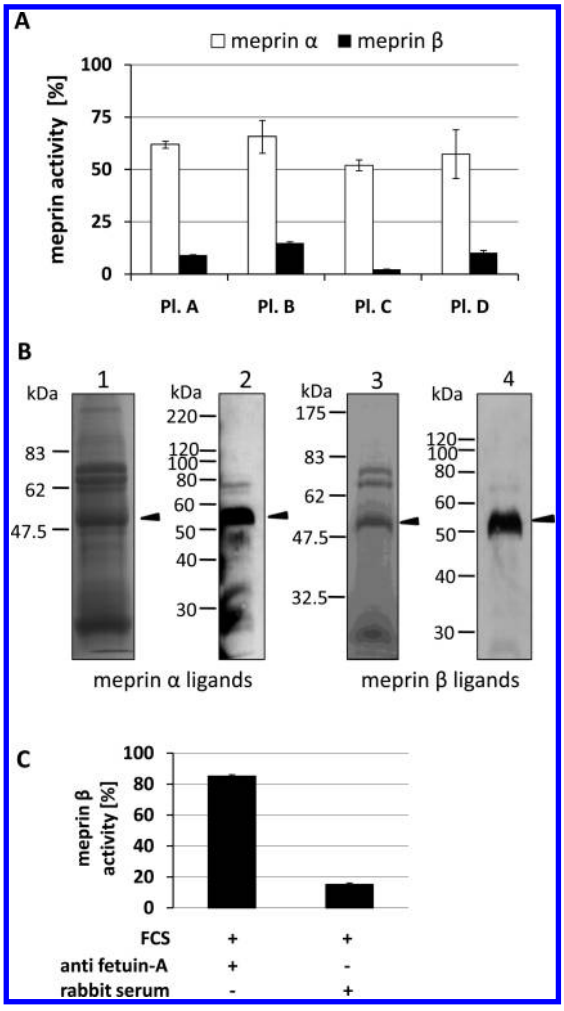

FIGURE 1: Inhibition of recombinant human meprin α and β by human plasma and identification of fetuin-A as a meprin binding molecule. (A) Recombinant active meprin α or β (each at 9.4×10^{-8} M) was incubated with four different human blood plasma samples from healthy subjects for 10 min (18% plasma) and afterward tested for azocasein cleaving activity in an end point (24 h) assay. The optical density at 340 nm correlates to proteolytic activity. The data represent averages of two independent activity assays (±maximum/minimum). The full enzyme activity without plasma incubation is 100%. Pl. denotes plasma samples. (B) Recombinantly expressed and activated meprin α or β was coupled to an activated Sepharose matrix. Plasma from healthy subjects was applied to the affinity column, washed with 50 mM HEPES/NaOH buffer (pH 7.5). Because of the strong interaction of the ligands withstanding different pH values and ionic strengths, part of the column was heat denatured under reducing conditions. The supernatant from meprin α (lanes 1 and 2) and meprin β affinity columns (lanes 3 and 4) was separated by SDS−PAGE (10%). Proteins were visualized either by Coomassie blue staining (lanes 1 and 3) or by immunodetection (lanes 2 and 4). Western blots were probed with the rabbit anti-human fetuin-A antibody. (C) Recombinant active meprin β was incubated with 1% FCS preincubated with specific fetuin-A antibodies or rabbit serum. Afterward, samples were tested for azocasein cleaving activity corresponding to the optical density at 340 nm. The data represent averages of two independent activity assays (±maximum/minimum). The enzyme activity is 100% without FCS.

partners of human meprin α and β, we coupled both enzymes covalently to a Sepharose matrix, loaded plasma samples, and analyzed the bound proteins. In Coomassie-stained gels, we observed several bands isolated from meprin α and β columns (Figure 1B, lanes 1 and 3). In both cases, Western blot assays with an anti fetuin-A antibody revealed a strong band at ~55 kDa, corresponding to proteolytically processed fetuin (*30*) and weaker bands in the range of 70−75 kDa (Figure 1B, lanes 2 and 4). Additionally, fetuin-A depletion of FCS samples with a specific fetuin-A antibody resulted in a strongly decreased level of inhibition of meprin β activity (Figure 1C).

Kinetic Characterization of Fetuin-A Inhibition. For obtaining kinetic parameters, a fluorogenic meprin substrate was used in assays with purified bovine fetuin-A, as well as with purified and recombinant human fetuin-A. The strongest inhibition of meprin β (12% residual activity) could be observed with bovine fetuin-A (final concentration of 1×10^{-4} M) (Figure 2A), which is 62% identical in amino acid sequence with the human homologue (Figure 4A). Recombinant meprin α was slightly less inhibited by the bovine fetuin-A (25% residual activity), but interestingly, at a final concentration of 1×10^{-4} M, purified human fetuin-A exhibited less residual activity (41%) than meprin β (62%). Surprisingly, a 3×10^{-6} M solution of recombinant human fetuin-A revealed an increased activity of meprin α (141%), while meprin β was still inhibited, with 70% residual activity (Figure 2A). Likewise, bovine fetuin-A at the lower concentration of 3×10^{-6} M increased the activity of meprin α to 115% but strongly inhibited recombinant meprin β, with 16% activity. Data from inhibition kinetics with bovine fetuin-A at a range of concentrations from 1×10^{-7} to 4×10^{-4} M allowed the calculation of the inhibition constant (K_i) for recombinant meprin α (4.2×10^{-5} M) and meprin β (1.1×10^{-6} M) (Figure 2B). To demonstrate that the inhibition was not merely due to substrate effects, we incubated recombinant meprin α and β with gelatin, which is a known substrate of these enzymes (*5*). During a 32 h digestion, no inhibitory effect was detected (data not shown).

Proteolytic Processing of Fetuin-A. To investigate if proteolytic cleavage of the inhibitor by the target protease occurs or if it is even required for the reactivity of fetuin-A against meprins, the interaction between the two proteins was further analyzed by SDS−PAGE and immunoblotting.

Digests of bovine fetuin-A or recombinant human fetuin-A and recombinant meprins suggested limited proteolysis of fetuin-A in that cleavage was complete after incubation for 24 h (Figure 2C,D). Bovine fetuin-A incubated with meprin α resulted in a major band at 60 kDa and secondary bands at 55 and 50 kDa. These signals could also be observed in the fetuin-A control, albeit at a lower intensity. Upon incubation with meprin β, cleavage products at 50, 45, 40, and 30 kDa were generated in addition to the major band at 60 kDa (Figure 2C,D).

Inhibitory Capacity in Patient Plasma. The analysis of plasma from four randomly selected patients suffering from various diseases showed strong differences. While the inhibitory effect of samples from patients with cephalaea myalgica and psoriasis showed inhibition of meprin α and β, inhibition was weak or absent in plasma samples from two multimorbid patients (patient 3, alcoholic cirrhosis, renal failure, diabetes mellitus type 2, esophagus varices bleeding, hepatic encephalopathy, and atrial fibrillation; patient 4, Crohn's disease, pulmonary embolism, intestinal inflammatory disease, gastrointestinal bleeding, and hypertension) (Figure 3A). Moreover, immunoblot detection of fetuin-A (Figure 3B) in plasma samples from healthy controls (lanes 1 and 2) compared to those from diseased patients (lanes 3 and 4) revealed slightly higher serum levels as well as an additionally contained proteolytic fragment of ~25 kDa (lanes 1 and 2, arrowhead).

Broad-Range Inhibition of Metalloprotease and Serine Proteases by Fetuin-A. To investigate whether fetuin-A acts like a broad-range inhibitor in plasma or is specific for meprins,

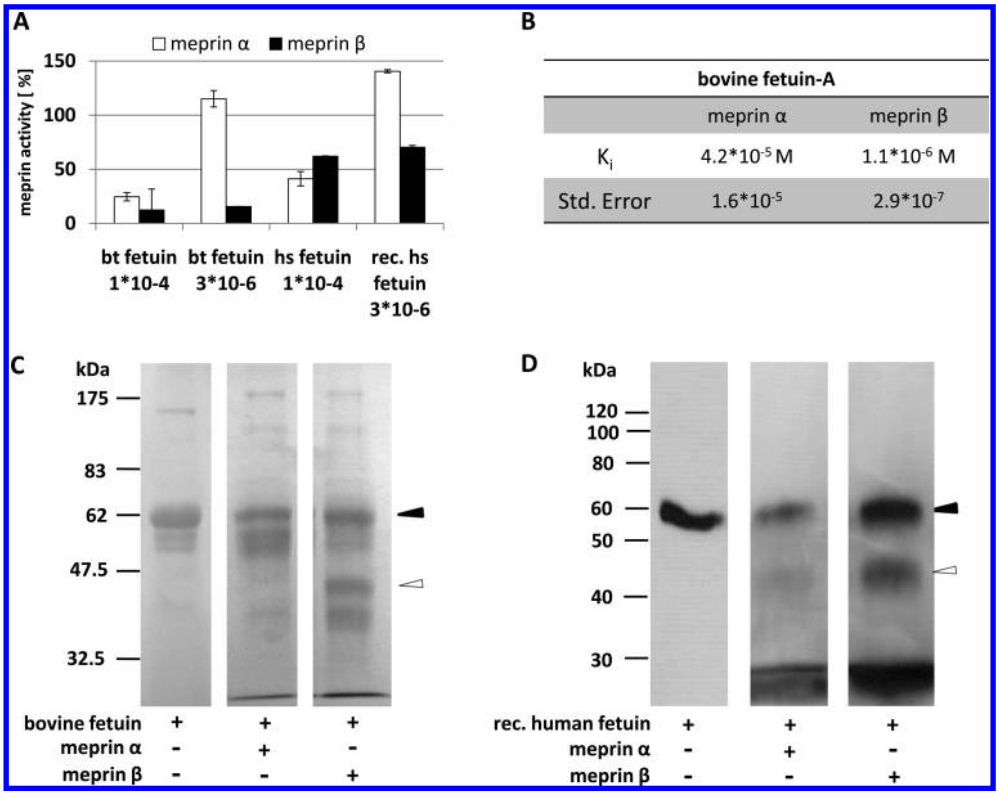

FIGURE 2: Inhibition of recombinant human meprin α and β by fetuin-A. (A) Recombinant activated meprin α or β (meprin α at 5×10^{-9} M or meprin β at 1×10^{-9} M) was incubated with different fetuin-A samples for 10 min and later tested for proteolytic activity with a meprin specific fluorogenic peptide substrate. Concentrations: purified bovine fetuin (bt fetuin), 1.0×10^{-4} or 3×10^{-6} M; purified human fetuin-A (hs fetuin), 1×10^{-4} M; recombinantly expressed human fetuin-A (rec. hs Fetuin), 3×10^{-6} M. Emission at 405 nm correlates with proteolytic activity. The enzyme activity without an inhibitor is 100%. The data represent averages of two independent activity assays (±maximum/minimum). (B) K_i values of bovine fetuin. Inhibitor profiles with bovine fetuin (concentration of 1×10^{-7} to 3.4×10^{-4} M) were assessed in determining inhibition constants (K_i). The K_i observed for meprin α was 4.2×10^{-5} M and for meprin β 1.1×10^{-6} M. (C and D) Proteolytic processing of bovine and human fetuin by meprin α and β. Bovine (C) or recombinant human fetuin-A (D) was incubated with active recombinant meprin α or β overnight at 37 °C. Proteins were separated by SDS−PAGE (10%). Bovine fetuin was stained with Coomassie brilliant blue, and human fetuin was visualized by Western blotting using the mouse anti-His antibody. The filled arrowheads denote the fetuin-A full-length protein, while the empty arrowheads denote the main processed fragment.

we performed activity assays with astacin metalloproteases from crayfish (*A. astacus*) and recombinant LAST and LAST_MAM from the horseshoe crab (*Limulus polyphemus*) (Figure 4B). At a fetuin-A concentration of 2×10^{-5} M, LAST and LAST_MAM exhibited 16 and 11% residual activity, respectively, while astacin exhibited a remaining proteolytic activity of 28%. In addition, we tested the inhibitory potential of bovine fetuin-A against trypsin and chymotrypsin, resulting in residual activities of 32 and 45%, respectively, as described previously (*40*). Both serine proteases are thought to be responsible for the processing of human fetuin-A to the mature form (*30*), a fact we could not observe for recombinant human fetuin-A (data not shown).

Fetuin from Carp (*nephrosin inhibitor*) *Inhibits Astacin Metalloproteases*. Recombinant nephrosin inhibitor from carp (1.5×10^{-6} M) revealed strong inhibitory potency against recombinant human meprin α and β (residual activities of 27 and 22%, respectively), much weaker inhibition of recombinant LAST and LAST_LAM (residual activities of 89 and 64%, respectively), but no inhibitory effect against crayfish astacin (residual activity of 112%) (Figure 4C). Comparable to that of fetuin-A, incubation of the recombinant nephrosin inhibitor (42 kDa) with recombinant meprin α and β revealed a truncated form of ~37 kDa, whereas astacin had almost no effect (Figure 4D). The nephrosin inhibitor precursor is expressed in the liver and is proteolytically processed in head kidney, kidney, and spleen to become an active inhibitor, comparable to the human protein. The sequence of recombinant carp fetuin-A contains amino acids 17−282, representing the active form of carp fetuin described by Tsai (*28*).

Human Cystatin C Inhibits Recombinant Meprin α but Not Meprin β. We reasoned that the fetuin-A-related protein cystatin C, which is present at high concentrations in plasma, might be another potential endogenous meprin inhibitor. Indeed, an activity assay revealed cystatin C to be a potent meprin α inhibitor (Figure 5A) whereas meprin β was not inhibited. The K_i of 8.5×10^{-6} M for the inhibition of recombinant human meprin α by recombinant human cystatin C was calculated from kinetic data yielding cystatin C concentrations ranging from 4.1×10^{-9} to 4.1×10^{-5} M (Figure 5A,B). As a control experiment, the known inhibitory activity of recombinant cystatin C toward papain was verified (data not shown).

DISCUSSION

In this study, we identified and characterized fetuin-A and cystatin C from human plasma as endogenous inhibitors of meprin metalloproteinases.

Isolation of Fetuin-A from Meprin Affinity Columns. Fetuin-A reversibly bound to affinity columns with immobilized recombinant human meprins. Fetuin-A antibodies detected a major proteolytically processed fetuin-A fragment, at 55 kDa, which corresponds to the processed fetuin-A (chain A). It is

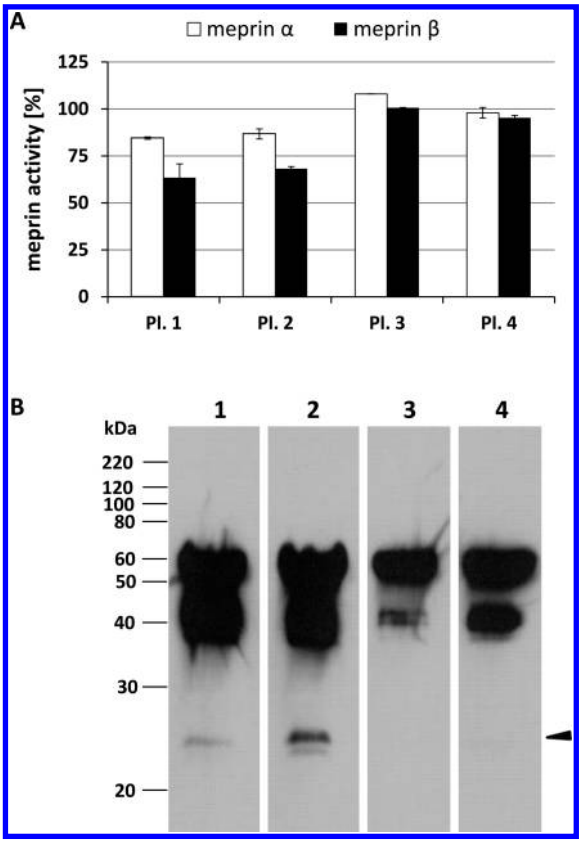

FIGURE 3: Plasma samples from patients exhibited strong differences in meprin inhibition and fetuin processing. (A) Recombinant active meprin α or β (each at 9.4×10^{-8} M) was incubated for 10 min with four different human plasma samples from patients suffering from various diseases (18% plasma). Afterward, samples were tested for azocasein cleaving activity corresponding to the optical density at 340 nm. The data represent averages of two independent activity assays (±maximum/minimum). The enzyme activity without plasma incubation is 100%. Diseases: plasma 1 (Pl. 1), cephalaea myalgica; plasma 2 (Pl. 2), psoriasis; plasma 3 (Pl. 3), alcoholic cirrhosis, renal failure, diabetes mellitus type 2, esophagusvarices bleeding, hepatic encephalopathy, and atrial fibrillation; plasma 4 (Pl. 4), morbus Crohn, pulmonary embolism, acute intestinal inflammatory disease, gastrointestinal bleeding, and hypertension. (B) Detection of fetuin-A in plasma samples. Plasma proteins from two healthy and two diseased persons, corresponding to panel A, were separated by SDS−PAGE (10%) and transferred to a PVDF membrane. Blots were probed with the rabbit anti-human fetuin-A antibody. Lane 1 contained plasma from a healthy subject (Figure 1A, plasma C). Lane 2 contained plasma from a healthy subject (Figure 1A, plasma A). Lane 3 contained plasma from a diseased subject (plasma 3). Lane 4 contained plasma from a diseased subject (plasma 4).

known that human fetuin-A with a theoretical mass of 39 kDa appears in a range of 60 kDa upon reduced SDS−PAGE, due to glycosylation. Subsequently, inhibition assays with fetuin-A from different sources (Figure 2A) and fetuin-A depletion (Figure 1C) confirmed the inhibitory potential against meprin α and β.

Inhibition Constants of Fetuin-A. Fetuin-A purified from bovine plasma, which is 62% identical to the human homologue, appeared to be a stronger inhibitor for recombinant meprin β ($K_i = 1.1 \times 10^{-6}$ M) than for recombinant meprin α ($K_i = 4.2 \times 10^{-5}$ M). Purified human fetuin-A revealed a reduced inhibitory efficiency for both meprins, while recombinant human fetuin-A (unprocessed one-chain form), at micromolar concentrations, exhibited an enhancing effect of meprin α activity. The reason might be due to the lack of processing of the recombinant fetuin.

The calculated K_i values for recombinant meprin α and meprin β with bovine fetuin-A were 4.2×10^{-5} and 1.1×10^{-6} M, respectively (Figure 2B). The K_i of meprin α is higher than that of meprin β, and this correlates with observations that fetuin-A at low concentrations leads to slightly increased meprin α activity. At an inhibitor concentration of 9×10^{-6} M, meprin α activity is 85% while it is at least 47% at a bovine fetuin-A concentration of 1.8×10^{-5} M. Although the inhibition capacity of human fetuin-A is weaker than that of the bovine homologue, the concentration in human adult plasma ($1.02-2.54 \times 10^{-5}$ M) (*29*) is striking in its physiological relevance for meprin inhibition.

Processing of Fetuin-A by Meprin. The observed difference in recombinant meprin α or β inhibition by plasma proteins is most likely due to the molecular variations within their active site clefts. Previously, we demonstrated the inhibitory capacity of several synthetic components with striking differences against human meprins, probably based on the accessibility of the active site (*19*).

A conceivable inhibition mechanism could be based on proteolytic cleavage of the inhibitor as a substrate with slow dissociation due to strong enzyme−product interaction. Indeed, incubation of fetuin-A with meprin β revealed proteolytic fragmentation (Figures 1B and 2C,D). Most likely, the protein fragment of 55 kDa of fetuin-A is the inhibiting protein, while the cleavage products observed after meprin incubation are not. It is remarkable that bovine as well as human recombinant fetuin-A exhibited strictly limited proteolysis even after 24 h, still exhibiting the 55 kDa fragment. Additionally, we observed proteolytic cleavage of the recombinant nephrosin inhibitor by recombinant meprin α and β, but not by crayfish astacin. Although only astacin was not affected by the inhibitor, it is still unclear whether proteolytic processing is essential for inhibition. Inhibiting cleavage and proteolytic cleavage by the free enzyme in parallel might be a result of reversible inhibition. Hence, several questions arise. (i) Is proteolytic cleavage of the inhibitor (Figures 1B and 2C,D) involved in the inhibition of meprin by fetuin? (ii) Is this processing due to the action of meprin itself or to other proteases (*30*)? (iii) Does it exhibit only the virgin inhibitor, only the processed inhibitor, or both inhibitory capacities? Further investigations are necessary, and the most promising approach would be the crystallization of an enzyme−inhibitor complex.

To determine if the reduced activity of meprins against azocasein in the presence of fetuin-A was caused by the presence of another substrate and not by inhibition, we analyzed gelatin, a known substrate (*5*), under the same conditions over a range of 32 h. Such an effect could be excluded (data not shown), which is further supported by the depletion of fetuin-A, which resulted in the loss of inhibitory capacity (Figure 1C).

Clinical Data. Plasma samples from four healthy subjects (two female and two male, 25−32 years old) compared to those from four patients revealed significant differences in inhibitory potency, which suggest complex and highly coordinated regulatory mechanisms, adapted to physiological and pathophysiological conditions.

It is known that meprins are involved in inflammatory diseases, like inflammatory bowel disease or Crohn's disease (*16*, *50*), and in cancer progression (*17*). These proteases are directly in contact with plasma, because they are expressed in leukocytes and secreted by metastasizing cells. Hence, meprin inhibition by fetuin-A seems to be pathophysiologicallt relevant. Results with fetuin-A deficient mice showed strongly increased or diminished tumor incidence and progression depending on the

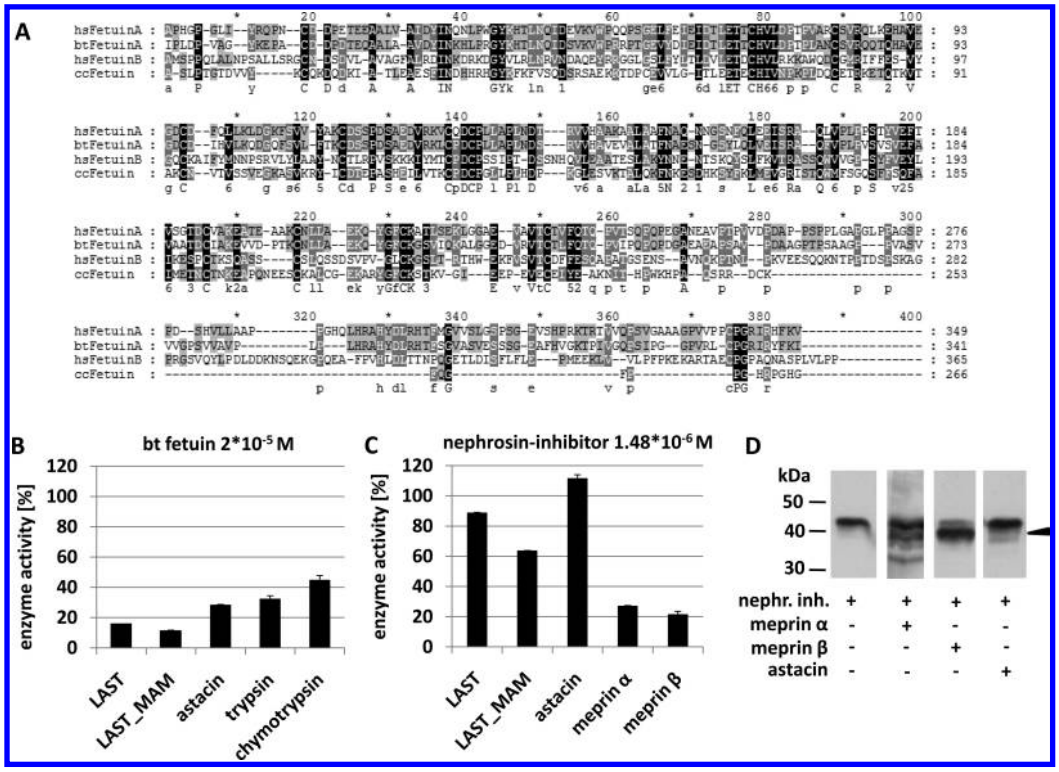

FIGURE 4: Multiple-amino acid sequence alignment of human, bovine, and carp fetuin-A and human fetuin-B. Protease inhibition by bovine and carp fetuin. (A) The sequence alignment was generated with ClustalX version 2.0 and edited with GeneDoc version 2.6.02. Species are noted at the left. Identical or similar residues in all sequences have a black background. A truncated sequence of carp fetuin-A is displayed, which was used for recombinant expression. Bovine fetuin-A shows a level of sequence identity with the human homologue of 62%, and levels of 24 and 23% with carp fetuin-A and human fetuin-B, respectively. (B) Astacin proteases (9.4×10^{-8} M) were incubated with purified bovine fetuin-A (2×10^{-5} M) for 10 min and were then tested for azocasein cleaving activity. Abbreviations: LAST, limulus astacin (B4F319); LAST_MAM, limulus astacin with the MAM domain (B4F320); astacin, crayfish astacin (P07584). Trypsin (P00761) and chymotrypsin (Q7M325) were from porcine pancreas. (C) Inhibition of astacin metalloproteases (each at 9.4×10^{-8} M) by the recombinant carp fetuin (1.5×10^{-6} M). Enzymes were incubated for 10 min with carp fetuin and then tested for azocasein degrading activity. Absorption of the azo dye at 340 nm correlates with proteolytic activity. All data represent averages of two independent activity assays (±maximum/minimum). The full enzyme activity without inhibitor is 100%. (D) Processing of the recombinant nephrosin inhibitor by astacin metalloproteases. The inhibitor (1.5 μM) was incubated with 13 nM enzyme at 37 °C for 6 h. Proteins were separated by SDS−PAGE, transferred to a PVDF membrane, and detected with an anti-strep tag antibody. The arrowhead denotes proteolytic fragments.

tumor model (*51*, *52*). These results were partially attributed to different protease activities in the tumor stroma. In view of the novel findings presented here, the mouse models should be revisited with a strong emphasis on meprin activity. Intriguingly, the weakened inhibition capacity of plasma was present in two multimorbid patients (patients 3 and 4).

Differences in physiological fetuin-A processing were observed under pathological conditions (*30*). Western blot analysis of plasma samples from healthy controls and diseased patients confirmed a different concentration and band pattern of fetuin-A for the tested samples (Figure 3B). Different processing of fetuin-A might finally result in altered meprin inhibition. Deregulated meprin activity was observed in several inflammatory disorders, and further investigations are necessary to understand the correlation between fetuin-A processing and meprin regulation under pathological conditions.

Cystatin C Is an Inhibitor for Meprin α. Because fetuin-A was not efficient in inhibiting meprin α, in a physiological manner, further reactive components must exist in plasma. Therefore, cystatin C, another member of the cystatin superfamily of protease inhibitors (*53*), was positively identified as an additional inhibitor of meprin α ($K_i = 8.5 \times 10^{-6}$ M) but not for meprin β. These findings are further proof of variance in the inhibitor susceptibility of both meprins, subsequently revealing a different physiological regulation of both enzymes. Although the concentration of cystatin C in plasma (0.64−0.84 mg/L, $2.9-3.8 \times 10^{-8}$ M) and the concentration in cerebrospinal fluid 5 times higher (*27*, *54*) are not within the range of the inhibition constant, local aggregates may well influence meprin α activity under certain conditions (*55*).

The described inhibition mechanism of cystatin C depends on a tight complex in which a binding region of the inhibitor comprising three different regions of the polypeptide chain interacts with the proteinase active site cleft. Further studies showed that cystatin C binds to pig legumain without cleaving and even with a completely disrupted peptidase-binding site (*23*). Additional analysis of latexin, a structural homologue of cystatin and an endogenous inhibitor for metallocarboxypeptidases, also revealed that strictly conserved reactive sites are not essential for inhibition effects (*56*). A different situation has been observed for inhibition of cathepsin B by cystatin C, which proceeds in two steps, involving an initial weak interaction followed by a conformational change (*57*). Therefore, the N-terminus of cystatin C is important, but not in all cases, as shown for the inhibition of ficin (*58*). The involvement of the N-terminus of chicken egg white cystatin was also described by Bode et al. (*59*). There, the inhibitor's N-terminus enters the active site cleft without any covalent interactions or proteolysis and substrate cleaving, resulting in inhibition. These findings indicate that the cystatin C inhibition mechanism may vary depending on the target protease.

Fetuin-A Is a Broad-Range Protease Inhibitor. Analyzing further astacin metalloproteases and serine proteases (Figure 4B)

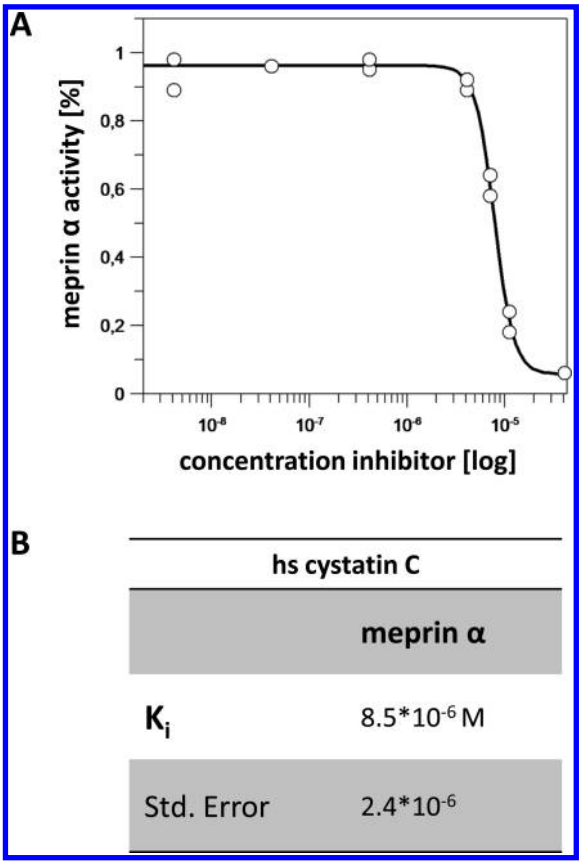

FIGURE 5: Inhibition of human meprin α by cystatin C. (A) Inhibitor profiles with recombinant human cystatin C using a meprin specific fluorogenic peptide substrate were performed. A meprin α inhibition kinetic with cystatin C concentrations from 4.1×10^{-9} to 4.1×10^{-5} M and a meprin α concentration of 5×10^{-9} M was calculated. Emission at 405 nm correlates to proteolytic activity. The enzyme activity without inhibitor is 100%. The log of inhibitor concentration is given on the *x*-axes. The data represent averages of two independent activity assays (±maximum/minimum). (B) The inhibitor constant ($9K_i$) for meprin α was calculated to be 8.5×10^{-6} M.

led to the assumption that fetuin-A might be a broad-range protease inhibitor. Trypsin and chymotrypsin are described in the literature as processors of fetuin-A in vitro (*30*), and fetuin-A is described as a trypsin inhibitor (*39*, *40*). We could demonstrate that the recombinantly expressed nephrosin inhibitor from carp also inhibits several astacin proteases (Figure 4C). Recombinant meprin α and β were especially effectively inhibited (27 and 22% residual activity, respectively), while astacin and recombinant LAST were almost unaffected. Thus, fetuin-A has the general regulatory function of controlling proteolytic activity in plasma. This is important because the known broad-spectrum inhibitor α2-macroglobulin does not inhibit human meprins. Because fetuin is known as a negative acute phase protein, involved in inflammatory diseases, further studies of fetuin-A regulation with regard to common protease activity are warranted.

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