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Direct lysosomal uptake of α_2-microglobulin contributes to chemically induced nephropathy

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Background. An abnormal accumulation of α_2-microglobulin ($\alpha 2\mu$) in kidney lysosomes of male rats has been described in the nephropathy resulting from exposure to a variety of chemicals. The increment in lysosomal levels of $\alpha 2\mu$ cannot be explained by a decrease in its proteolytic susceptibility. Because a portion of $\alpha 2\mu$ resides in the cytosol of kidney cells, we decided to analyze whether this cytosolic form also contributes to the abnormal lysosomal accumulation of $\alpha 2\mu$ after exposure to chemicals.

Methods. Intact kidney lysosomes were isolated from untreated or 2,2,4-trimethylpentane (TMP) treated rats, and their ability to take up $\alpha 2\mu$ was compared.

Results. $\alpha 2\mu$ can be directly transported into isolated lysosomes in the presence of the heat shock cognate protein of 73 kDa (hsc73). $\alpha 2\mu$ specifically binds to a lysosomal membrane glycoprotein of 96 kDa, previously identified as the receptor for the hsc73-mediated lysosomal pathway of protein degradation. In rats exposed to TMP, the specific lysosomal transport of $\alpha 2\mu$ increases, as well as the ability of lysosomes to directly transport other substrates for this pathway. The increased lysosomal transport is mainly due to an increase in the levels of the receptor protein in the lysosomal membrane.

Conclusions. The hsc73-mediated lysosomal pathway contributes to the normal degradation of $\alpha 2\mu$ in rat kidney and liver, and the activity of this pathway is increased after exposure to TMP. Our results suggest that the chemically induced accumulation of cytosolic $\alpha 2\mu$ in lysosomes is mediated by an increased rate of direct uptake into lysosomes.

Exposure of male rats to a number of environmental chemicals, including gasoline derivatives (that is, aliphatic, alicyclic, and aromatic compounds), has been shown to cause a syndrome referred to as hyaline droplet nephropathy or α_2-microglobulin ($\alpha 2\mu$) nephropathy [1–7]. The major characteristic of the affected kidneys is the presence in the proximal tubules of hyaline droplets [8, 9], which are mainly composed of a single protein, $\alpha 2\mu$ [10, 11]. Previous studies have described the presence of lysosomal markers in these droplets, confirming that the storage of $\alpha 2\mu$ takes place inside secondary lysosomes [12, 13]. The abnormal accumulation of $\alpha 2\mu$ in kidney proximal tubular cells does not cause significant changes in urinary chemistry or glomerular function [14], but this lysosomal overload results in severe cellular damage and proximal tubule necrosis [15]. The cellular death stimulates a compensatory increase in cell proliferation (restorative hyperplasia) and carcinogenesis [14, 16, 17]. There is extensive evidence that the $\alpha 2\mu$ accumulation in this chemically induced nephropathy causes the cell damage: (*a*) After exposure to the chemicals, kidney proximal tubule cells show an increased $\alpha 2\mu$ immunoreactivity confined to protein droplets. (*b*) There is a dose-dependent increase in $\alpha 2\mu$ concentrations in kidney and in severity of the nephropathy. (*c*) There is a good correlation between levels of $\alpha 2\mu$ and cell proliferation [18]. One of the most potent hydrocarbons causing this nephropathy is 2,2,4-trimethylpentane (TMP), a major component in unleaded gasoline [19–21]. Chronic exposure to TMP and numerous other gasoline chemicals results in a progression of the kidney lesions and in an increase in the incidence of renal tumors in rats [6, 17, 18, 22].

α_2-Microglobulin is a secretory protein that belongs to the lipocalin family, proteins that bind small hydrophobic ligands [23]. $\alpha 2\mu$ is synthesized in the liver, and its rate of synthesis is controlled by multiple hormones [24]. $\alpha 2\mu$ has been proposed to participate in the transport of fatty acids to renal epithelial cells, where they are an important energy source [25]. The binding of $\alpha 2\mu$ to a chemotactic peptide has also lead to a suggestion of an immunomodulatory role for this protein [26]. $\alpha 2\mu$ also participates in the transfer of odorants (lipid pheromones) to the male urine [23]. Together with other low molecular weight proteins, $\alpha 2\mu$ is reabsorbed from the glomerular filtrate in proximal kidney tubules [27, 28], and after interaction with the brush border membranes, $\alpha 2\mu$ is internalized by endocytosis. This protein reaches the lysosomal matrix by fusion of endocytic vesicles with lysosomes, where

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it is completely degraded [27]. During endocytosis, part of α2μ can be processed to the kidney fatty acid-binding protein (k-FABP) by proteolytic attack by a protease at the tubular cell brush border membrane that cleaves four to six amino acids from its amino- and carboxyl-terminal regions [25]. α2μ and k-FABP have been found in only male rats, but proteins structurally similar to α2μ have been described in males of other species, including humans [29, 30]. Both α2μ and its derivative k-FABP have been also localized in the cytosol of tubular cells [25, 31, 32], and a role in the transport and metabolism of fatty acids for these cytosolic forms has been proposed [33]. The mechanisms by which α2μ and its derivative reach the cytosol remain unclear. The proposed endosomal leakage of α2μ prior to lysosomal fusion and a possible direct α2μ-specific reabsorption from the tubular lumen by a mechanism other than the endocytic-lysosomal pathway need to be proved [34]. In addition, nothing is known about how these cytosolic forms of α2μ are degraded.

The lysosomal accumulation of α2μ after exposure to gasoline derivatives seems to be selective for this protein, as no other major protein components have been identified in the hyaline droplets. No changes in liver concentration of α2μ mRNA [35, 36], rate of α2μ hepatic synthesis [37, 38], passage of serum proteins by the glomerulus [14], or in protein reabsorptive capacity of renal proximal tubular cells [37] have been found after exposure to those chemicals. In addition, none of those derivatives impair the lysosomal proteolytic activity itself, and an increase, not a decrease, in cathepsin activity has been detected [39]. Lehman-McKeeman et al have shown that lysosomal cysteine and aspartate proteases contribute to the degradation of α2μ in lysosomes [39]. α2μ is relatively long-lived in serum, with a half-life of six hours, and it shows high resistance to proteolysis when directly incubated with several proteases [40]. Specific changes in the structure of α2μ by several chemicals or their metabolites result in decreased proteolytic susceptibility of this protein to lysosomal proteases *in vitro* [39]. Under those conditions, the half-life of the protein changes from 6 hours to 10 hours. However, the magnitude and the speed of the chemically induced α2μ lysosomal accumulation is difficult to explain by the decrease in its lysosomal degradation alone. If only the described changes in the proteolytic susceptibility of α2μ after chemical exposure are considered, the total content of α2μ in kidney would double in one day, but most of the studies previously performed and our own observations reveal that after one day of exposure to most of the chemical derivatives analyzed, the levels of α2μ in kidney are nearly six times the initial values [41, 42].

A direct transport of certain cytosolic proteins into lysosomes has been described in human fibroblasts in culture [43] and in rat liver [44, 45]. This transport is mediated by a cytosolic heat shock protein of 73 kDa (hsc73) and requires adenosine triphosphate (ATP) [46–48]. The selective lysosomal uptake is activated by serum deprivation in cultured cells [43] or after prolonged starvation in rat liver [49, 50]. Substrate proteins bind to a lysosomal membrane glycoprotein of 96 kDa (lgp96) [51] and are subsequently transported into the lysosomal matrix and degraded there. A modified form of the cytosolic chaperone of 73 kDa, located in the lysosomal lumen, is necessary to achieve the complete transport of the substrate proteins [52, 53]. Levels of lgp96 at the lysosomal membrane and of hsc73 in the lysosomal matrix determine the activity of lysosomes for the selective protein uptake [51, 53]. This transport of cytosolic proteins to lysosomes has been reconstituted *in vitro* for human fibroblasts [47] and for rat liver [44, 48]. The first identified substrate for the selective lysosomal degradation was ribonuclease A (RNase A) [54]. Other cytosolic proteins also characterized as substrates for this pathway are glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [44], aldolase, and phosphoglyceromutase (A.M. Cuervo and E. Knecht, unpublished results), transcription factors such as c-Fos [55], and certain subunits of the proteasome [56].

A common target signal for all those proteins, biochemically related to the pentapeptide KFERQ, has been described, and this signal is recognized by the cytosolic hsc73 [57, 58]. The analysis of α2μ amino acid sequence revealed the presence of three potential targeting signals with conservative expansions of the proposed motif [57]: (*a*) amino acids 62–66, QHIDV, where H in addition to K and R is allowed as a basic amino acid; (*b*) amino acids 31–35, VDKLN, and (*c*) 76–80, RIKEN, where N is allowed to replace Q. Here we show that the cytosolic form of α2μ is, indeed, a substrate for selective lysosomal uptake, and we suggest that this pathway, in addition to stabilization of the α2μ-chemical complex, may contribute to the abnormal increase of lysosomal levels of α2μ after exposure to several chemicals.

METHODS

Animals

Adult male Wistar rats weighing 200 to 250 g and fasted for 20 hours before sacrifice were used. TMP, dissolved 1:1 in corn oil, was administered to the animals by gavage (1 g/kg of body weight) during seven consecutive days.

Chemicals

Sources of chemicals and antibodies were as described previously [42, 44, 46, 51]. Anti-α2μ monoclonal antibody was prepared as described earlier [42]. Other reagents were of the best analytical quality available.

Purification of α2μ

α_2-Microglobulin was purified from urine of rats as previously described [42]. Briefly, urine from adult male

Wistar rats was subjected to ammonium sulfate precipitation at 0°C (100% ammonium sulfate saturation). The precipitate was collected by centrifugation (3,500 × g for 30 min), dissolved in phosphate buffer (10 mM Na_2HPO_4, pH 7.4, and 150 mM sodium chloride), and loaded onto an Ultrogel AcA44 (Serva, Heidelberg, Germany) column. Relevant fractions were analyzed by PhastGel electrophoresis (Pharmacia, Freiburg, Germany).

Immunostaining of kidney sections

Paraffin wax sections (5 μm thick) of rat kidneys fixed by perfusion fixation with paraformaldehyde were deparaffinized according to standard procedures and were treated with methanol/hydrogen peroxide to destroy residual endogenous peroxidase [11]. Some samples were partially digested with protease type XIV from *Streptomyces griseus.* After blocking with nonfat dry milk (3% wt/vol in phosphate buffer), slides were incubated with 50 μg/ml of purified anti-α2μ antibody, followed by washing and incubation with a rabbit-antimouse streptavidin conjugate. After a final incubation with a biotin-labeled horseradish peroxidase conjugate, staining was performed by the addition of Hanker-Yates reagent [59]. Quantitation of α2μ-positive immunostaining was performed on micrographs using an Image Analyzer System (Inotech S-100; Sunnyvale, CA, USA). The areas of 8 to 10 proximal kidney tubules per micrograph were profiled, and the density (black spots) of those areas was automatically integrated. Densitometric values were corrected per total tubular areas, and mean values ± SD were calculated in control and TMP-treated rat samples.

Isolation of liver and kidney subcellular fractions

Rat liver and kidney lysosomes were isolated from a light mitochondrial-lysosomal fraction in a discontinuous metrizamide density gradient [60] by the shorter method reported previously [44]. After isolation lysosomes were resuspended in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (0.3 M sucrose/10 mM MOPS, pH 7.2). In some experiments, two separate lysosomal fractions with different activities for the selective proteolytic pathway were isolated as described [53]. Levels of contaminant organelles or cytosolic proteins in the lysosomal fractions were determined based on the activities or levels of different enzymatic markers (succinate dehydrogenase for mitochondria, cation-independent mannose 6-phosphate receptor for late endosomes, catalase for peroxisomes, and lactate dehydrogenase for cytosol) as previously described [44, 53]. Cytosolic fractions were obtained by centrifugation of the supernatant of the light mitochondrial-lysosomal fraction at 150,000 × g for one hour at 4°C.

Lysosomal membranes were obtained as Ohsumi et al described [61]. Briefly, lysosomal pellets were resuspended in 0.025 M sucrose and were incubated for 30 minutes at 0°C. After hypotonic shock lysosomes were centrifuged for 30 minutes at 105,000 × g using a 1002 Ti rotor (Beckman Instruments Inc., Palo Alto, CA, USA), and after removing supernatants (lysosomal matrix), the pelleted membranes were washed once with 0.5 M NaCl and were resuspended in 0.3 M sucrose (lysosomal membranes).

Proteolysis measurements

Freshly isolated rat liver or kidney lysosomes (25 μg of protein) were incubated in a final volume of 60 μl with 260 nM GAPDH, 260 nM α2μ, or 260 nM 2,2,4-trimethyl pentane-α_2-microglobulin conjugate (TMP-α2μ), radioactively labeled by reductive methylation ([^{14}C]GAPDH, [^{14}C]α2μ, [^{14}C]TMP-α2μ) [62]. Incubations were carried out at 25°C for 90 minutes in MOPS buffer, 1 mM dithiothreitol, and 5.4 μM cysteine, in the presence or absence of ATP and hsc73, and were stopped by the addition of trichloroacetic acid to a final concentration of 10%. Other proteins added (nonradioactive α2μ and ovalbumin) were dissolved in the same incubation buffer. Acid-soluble material (amino acids and small peptides) was collected by filtration through a Millipore Multiscreen Assay System (Millipore, Bedford, MA, USA) using a 0.45 μm pore filter, and the acid-precipitable material (protein) was collected on the filter. Radioactivity in the samples was converted to disintegrations per minute in a P2100TR Packard liquid scintillation analyzer (Packard Instruments, Meriden, CT, USA) by correcting for quenching using an external standard. Proteolysis was expressed as a percentage of the initial acid-insoluble radioactivity converted to acid-soluble radioactivity at the end of the incubation. For freshly isolated lysosomes, the integrity of the lysosomal membranes during incubations was evaluated by measuring the leakage of cathepsins as described [44].

In vitro uptake of proteins by lysosomes

Freshly isolated lysosomes (100 μg of protein) previously treated with 100 μM chymostatin for 10 minutes at 0°C were incubated with the protein substrate RNase A (25 μg) or [^{14}C]α2μ (10 μg) at 37°C for 20 minutes in 0.3 M sucrose, 10 mM MOPS, pH 7.2 (final volume 30 μl) [44, 48]. At the end of the incubation, samples were treated with proteinase K and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with a specific antibody against RNase A or exposed to a phosphorimager screen. Other proteins added to the medium were dissolved in the incubation buffer before adding to lysosomes. The efficiency of the exogenous protease in removing all of the substrate present in the medium at the end of the incubation was analyzed by subjecting lysosomal supernatants to SDS-PAGE and immunoblotting for the substrate protein.

Binding of proteins to lgp96

Specific binding of cytosolic proteins to the receptor for the selective lysosomal pathway of proteolysis was analyzed as described [51]. Briefly, lysosomal membranes were subjected to SDS-PAGE and were electrotransferred to a nitrocellulose membrane. After blockage with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.1% Tween-20, membranes were incubated with [^{14}C]α2μ (250 nM) in a renaturation buffer (50 mM Tris-HCl, 0.1 M potassium acetate, 0.15 M NaCl, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, and 0.3% Tween-20) for 12 hours at 4°C. Specifically bound protein was detected after extensive washing by exposure to a phosphorimager screen.

Immunoblotting

Cellular fractions and isolated proteins were subjected to SDS-PAGE (10 or 16%) in slab gels [63] or to isoelectric focusing in a pH range of 3 to 10 [64] and were electrotransferred to nitrocellulose membranes using a Mini-Trans Blot SD wet transfer cell (Bio-Rad, Richmond, VA, USA). Membranes were incubated with specific antibodies followed by peroxidase conjugated antimouse IgG (for α2μ) or antirabbit IgG (for RNase A). Recognized proteins were revealed by standard chemiluminescence methods. Densitometric quantitation of the immunoblotted membranes was performed using an Image Analyzer System (Inotech S-100).

General methods

Protein was determined by the Lowry method [65] using bovine serum albumin as a standard. Standard procedures were used for the determination of enzymatic activities as reported previously [44, 46]. Hsc73 was purified from rat liver cytosol by ATP-agarose affinity chromatography [66]. Statistical analyses were carried out by the Student's *t*-test.

RESULTS

Subcellular distribution of α2μ in liver and kidney of male rats

α_2-Microglobulin has been described as mainly located in secretory vesicles in rat liver or in the endosome-lysosome compartment in rat kidney [6, 11]. The presence of this protein in the kidney cytosolic fraction has also been reported [25, 31, 32], but until now, quantitative studies of this cytosolic form have not been published. In order to determine the percentage of total α2μ located in the cytosolic fraction, we used immunohistochemical staining of fixed and paraffin-embedded sections of male rat kidneys with a specific antibody against α2μ. Figure 1 shows a representative section containing mainly kidney tubules. As shown in Figure 1A, generalized staining for α2μ was observed in the epithelial cells of the tubular segments (notice that the unstained tubules correspond to kidney distal tubules). The staining of the tubular lumen may be due to the presence of α2μ not yet endocytosed by the epithelial cells. When kidney sections were previously subjected to a controlled protease treatment (**Methods** section), a typical vesicular pattern was revealed (Fig. 1B). These vesicular structures have been previously identified by others as components of the endosomal-lysosomal system [6, 11].

Quantitative analysis of untreated and protease-treated sections allowed us to determine that the protease-sensitive α2μ (cytosolic form) represents 24.5 ± 2.5% of the total cellular α2μ in rat kidney. Similar values (23 ± 4.3% of the total in homogenate) were obtained after SDS-PAGE and immunoblotting of the cytosolic fraction from rat kidney with a specific antibody against α2μ (Fig. 2A; the upper and lower panels correspond to two different protein amounts for the same samples). In rat liver, the immunoblot analysis revealed that 19.4 ± 1.6% of the total α2μ in the homogenate was located in cytosol (Fig. 2B). For comparison, only 1% of cathepsin B was found in the cytosolic fraction, so the presence of α2μ cannot be due to lysosomal breakage. For the same amount of total protein, the content of α2μ was approximately 4.5 times higher in kidney than in liver (compare lanes 1 and 2 in Fig. 2A upper panel with lanes 1 and 2 in Fig. 2B). A low molecular weight protein (approximately 17.5 kDa) also recognized by the antibody was detected in the cytosolic fractions of both kidney and liver tissues. In order to separate both proteins in kidney better, the lower panel in Figure 2A contains half of the amount of protein used in the upper panel for each fraction. This protein corresponds in size to k-FABP, the previously described α2μ proteolytic derivative [25]. In addition, isoelectric focusing of the kidney fractions followed by immunoblotting with the specific antibody for α2μ (Fig. 2C) revealed the presence of at least four isoforms, two of which (pI 5.3 and pI 6.26) correspond to the isoelectric points described for k-FABP [32, 67]. The other isoforms correspond to two of the different isoforms described for intact α2μ [67].

As shown in Figure 2 A and B, α2μ was also found associated with lysosomes from rat kidney (Fig. 2A) and liver (Fig. 2B). Quantitative analysis of the distribution of α2μ between lysosomal matrix and membrane in both tissues is shown in Table 1. In spite of the higher levels of α2μ in the lysosomal membrane in liver when compared with kidney, treatment of those lysosomes with proteinase K revealed that only 40% of the membrane-associated α2μ is susceptible to protease treatment (data not shown). This result suggests that part of the α2μ associated with the lysosomal membrane is located at its

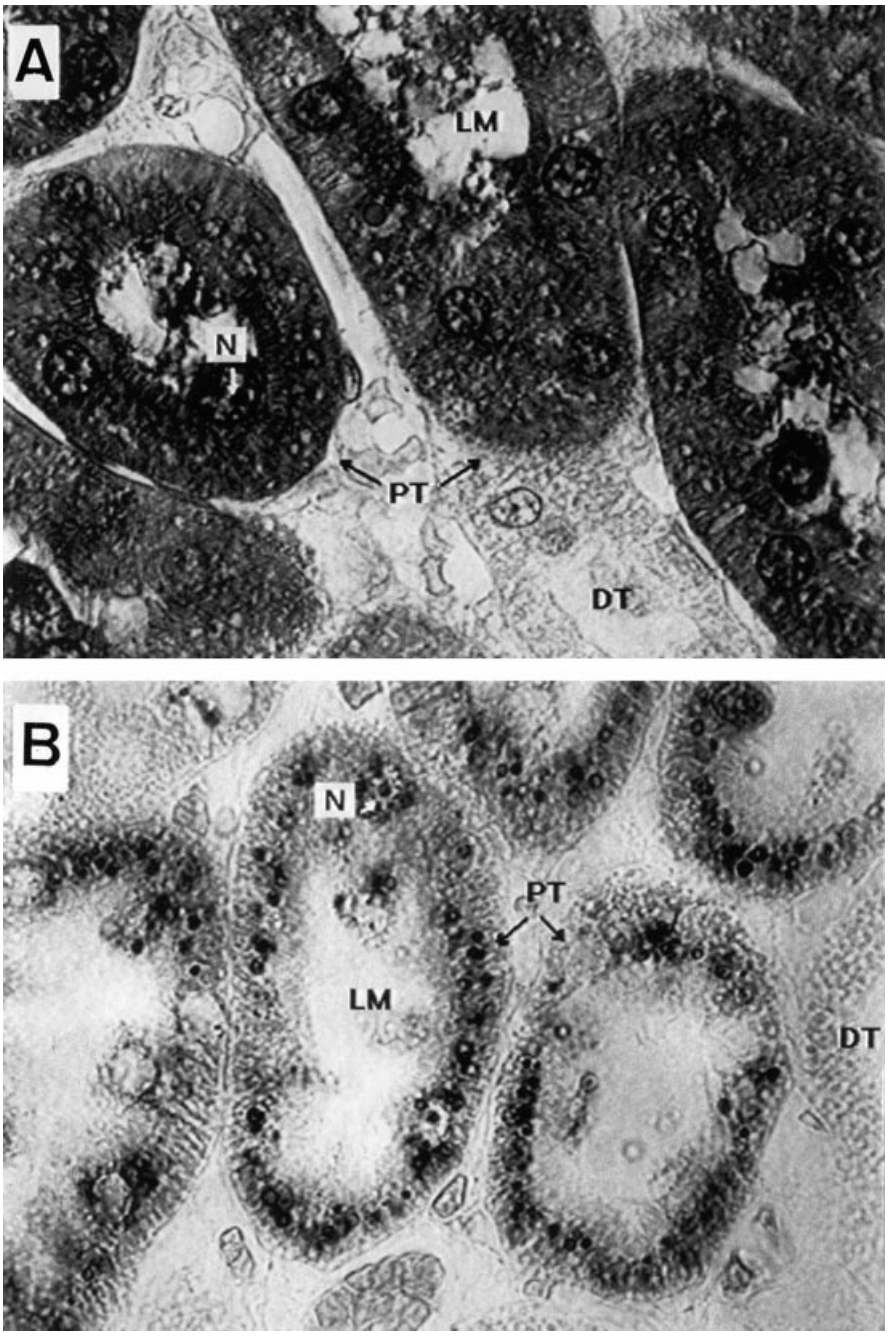


Fig. 1. Immunostaining of kidney sections for alpha-2-microglobulin (α2μ). Rat kidney sections processed as described in the **Methods** section were stained with a specific antibody for α2μ directly (*A*) or after treatment with an exogenous protease (*B*) (magnification ×1,000). Abbreviations are: LM, tubular lumen; N, nucleus; PT, proximal tubules; DT, distal tubules.

lumenal side. k-FABP was also detected in lysosomes from both tissues, and it was mainly located in the lysosomal matrix (Fig. 2 A, B, lane 5; Table 1).

To determine if the presence of α2μ in lysosomes, in addition to the previously described endocytic pathway, could also be the result of a direct transport of the cytosolic α2μ through the lysosomal membrane, we analyzed the content of α2μ in different lysosomal populations. We have previously characterized two groups of lysosomes with very different activity for the selective uptake of cytosolic proteins [53]. The main difference between these groups of lysosomes is the higher levels of hsc73, the chaperone involved in the direct transport, in the matrix of the active population. As shown in Figure 2D, the content of α2μ was significantly higher in the lysosomes enriched in hsc73 (HSC+ lysosomes) when compared with the lysosomes less active for this selective uptake (HSC−). In liver, 78% of the total α2μ associated with lysosomes was found in the HSC+ lysosomal population (Fig. 2D; compare lanes 1 and 2). In kidney, HSC+

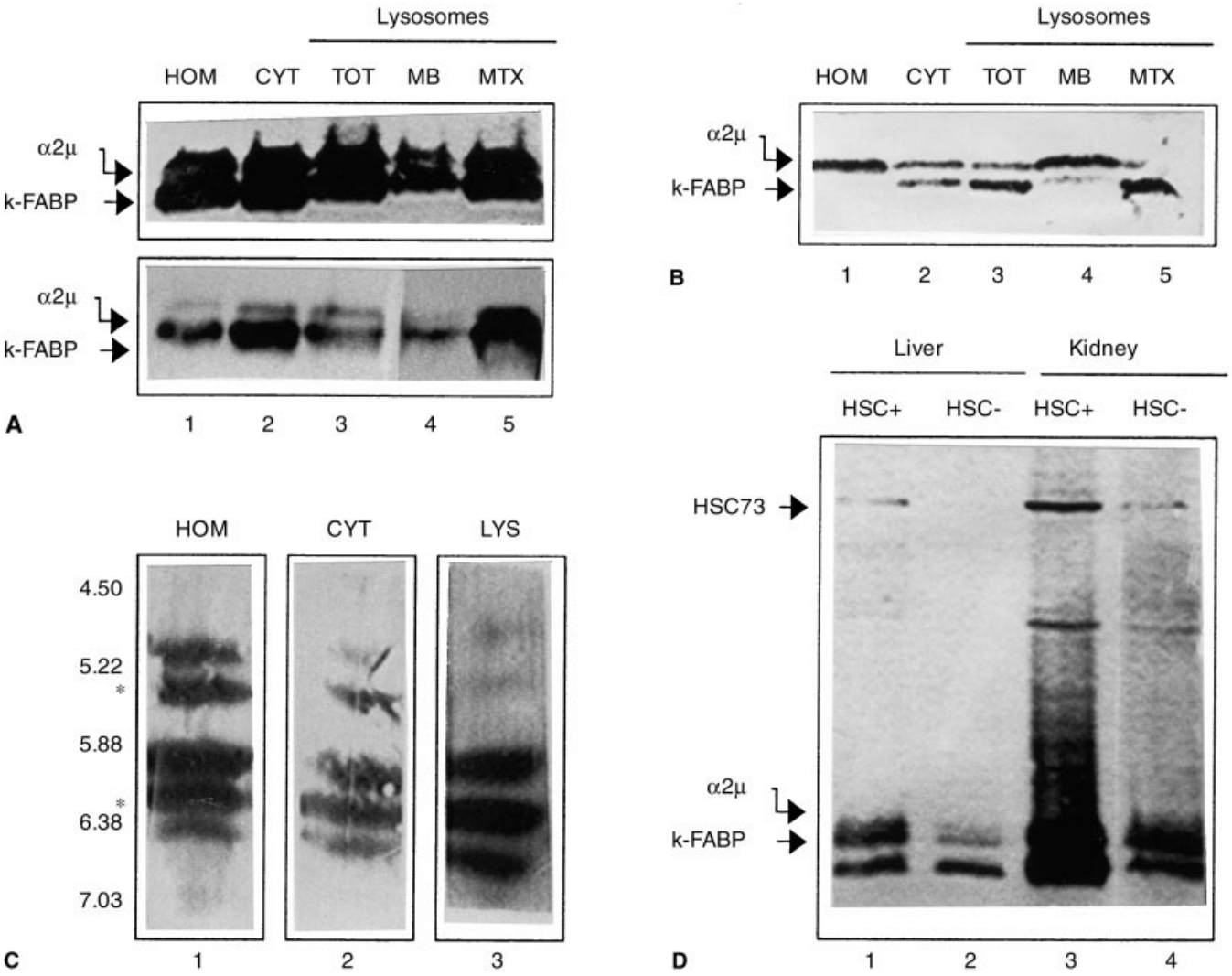


Fig. 2. Immunolocalization of α2μ in lysosomes. Different subcellular fractions [homogenate (HOM) cytosol (CYT) and lysosomes (TOT)] were isolated from rat kidney (*A* and *C*) or liver (*B*) as described under the **Methods** section. For each fraction, 100 μg of protein (*A* upper panel, *B* and *C*) or 50 μg of protein (*A* lower panel) were subjected to SDS-PAGE (*A* and *B*) or isoelectrofocusing (*C*) and immunoblotting with a specific antibody against α2μ. In *A* and *B*, lysosomes were also separated into membrane (MB) and matrix (MTX) fractions and were subjected to the same procedure (lanes 4 and 5). In (*D*), lysosomes were separated in two groups (HSC+ and HSC−) with different activity for the selective lysosomal pathway and subjected to the same procedure as mentioned earlier here. An additional incubation with a specific antibody against hsc73 was also performed to analyze the purity of both lysosomal subpopulations.

Table 1. Distribution of alpha-2-microglobulin (α2μ) in lysosomes from rat kidney and liver

	Membrane	Matrix
	% of total	
Kidney		
α2μ	25.2	74.8
k-FABP	21.5	78.5
Liver		
α2μ	54.3	45.7
k-FABP	10.6	89.4

Rat kidney and liver lysosomes were separated in membrane and matrix (**Methods** section) and 100 μg of protein from each fraction were subjected to SDS-PAGE and immunoblot with a specific antibody against α2μ. Immunoblots from four independent experiments were densitometrically analyzed. Values are expressed as an average percentage of the total density (calculated as the sum of the reflectance values of membrane and matrix for each tissue). Variations between the experiments were less than 10%.

lysosomes contain 66% of the total lysosomal α2μ (Fig. 2D; compare lanes 3 and 4). The higher content of α2μ in kidney HSC− lysosomes (lane 4) when compared with liver HSC− lysosomes (lane 2) could be related with a higher content in hsc73 in the kidney lysosomes (compare levels of hsc73 in lane 2 and 4). No differences were observed in the content of k-FABP between both lysosomal populations in liver (Fig. 2D; lanes 1 and 2), but in kidney, lower levels of k-FABP were detected in HSC− lysosomes when compared with HSC+ lysosomes.

Degradation of α2μ by lysosomes

To determine if the presence of α2μ in the lysosomal population active for the direct protein transport was related to its degradation, we measured levels of α2μ

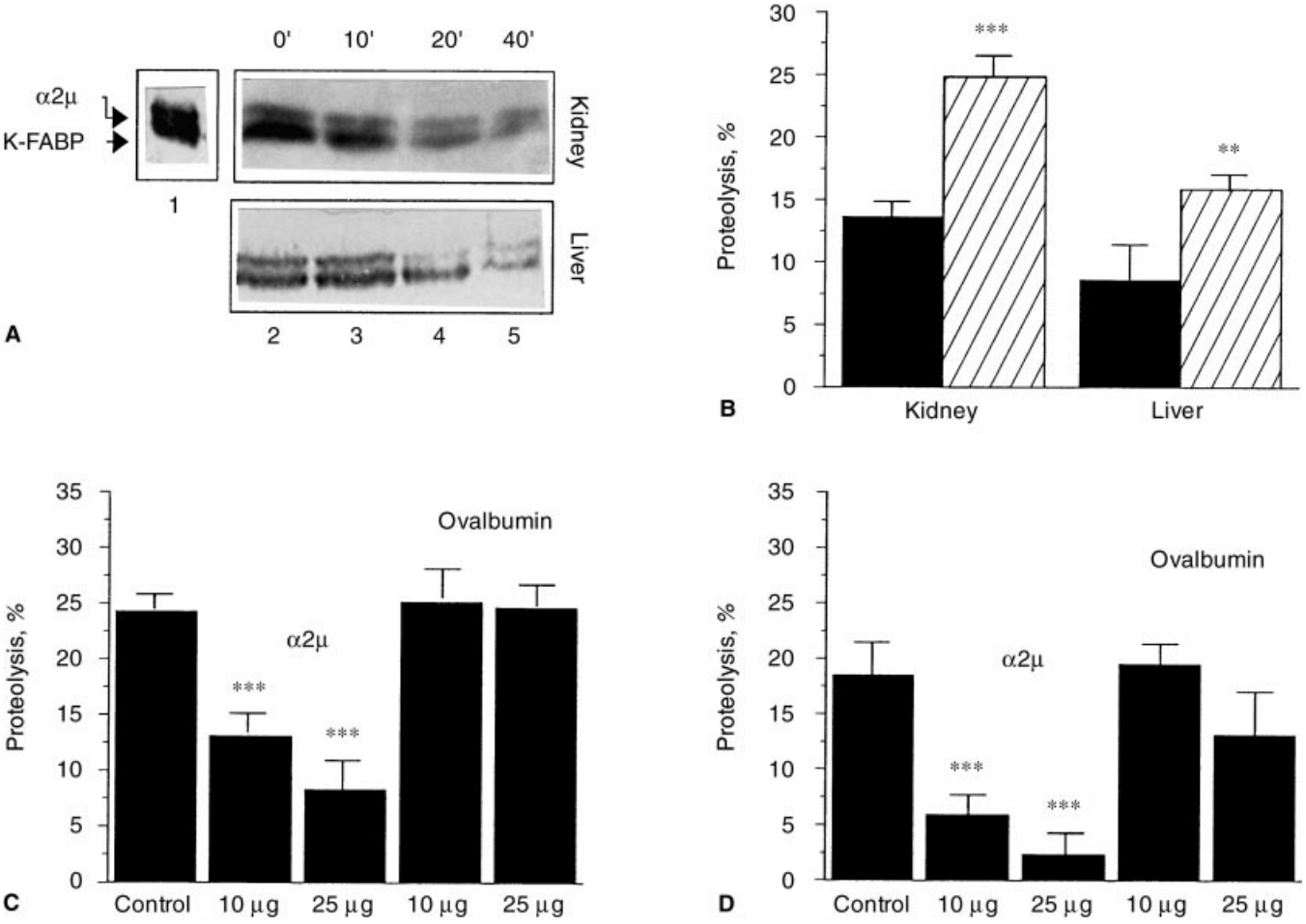


Fig. 3. Degradation of α2μ by intact lysosomes. (*A*) Intact rat kidney (upper panel) and liver (lower panel) lysosomes were incubated in an isotonic medium at 37°C. At the indicated times, remaining lysosomal levels of α2μ were analyzed by immunoblotting with a specific antibody against α2μ. (*B*) [^{14}C]α2μ was incubated with intact rat kidney and liver lysosomes under standard conditions with the indicated additions. Symbols are: (■) control; (▨) ATP + HSC. At the end of the incubation, proteolysis was measured as described in the **Methods** section. (*C* and *D*) Proteolysis of [^{14}C]GAPDH by liver (*C*) and kidney (*D*) lysosomes was analyzed as in (*B*). In the indicated samples, cold α2μ or ovalbumin (as labeled) was added to the incubation medium. In (*B*) and (*C*), results are mean ± SD of four and three different experiments, respectively. Differences with respect to control values are significant for $^{***}P < 0.001$ and $^{**}P < 0.01$.

in intact rat kidney and liver lysosomes after different incubation times. Incubations were performed in an isotonic medium to maintain the lysosomal integrity. As shown in Figure 3A, the amount of α2μ in the lysosomal fraction decreased with the incubation time in both tissues. Interestingly, the levels of the proteolytic derivative of α2μ, k-FAPB, decreased more slowly than α2μ levels. These differences could be due to a higher resistance to degradation of k-FAPB when compared with α2μ or with processing of α2μ to k-FAPB during the incubation. By densitometric quantitation of three experiments similar to the one shown here, we determined that the half-life of α2μ once inside lysosomes is 52 minutes in kidney and 165 minutes in liver (degradation rates of 0.84 and 0.25 μg/hr).

Since one of the main characteristics of the group of lysosomes analyzed here is its ability to take up certain proteins directly, we further analyzed possible direct transport of α2μ.

Specific uptake of α2μ by lysosomes

To check the possible direct transport of α2μ into lysosomes by the hsc73-mediated pathway, we incubated [^{14}C]α2μ with isolated lysosomes under previously described conditions (**Methods** section) [46]. As shown in Figure 3B, a percentage of the added α2μ was directly transported through the lysosomal membrane and degraded once in the lysosomal matrix. Degradation rates significantly increased in the presence of hsc73 and ATP. In addition, α2μ was able to compete with the uptake and degradation of GAPDH, a well-characterized substrate of the hsc73-mediated lysosomal pathway [44], by liver (Fig. 3C) and kidney (Fig. 3D) lysosomes. Degradation of GAPDH was not significantly modified by oval-

bumin, a nonsubstrate protein of this pathway (Fig. 3 C, D). The competition between α2μ and GAPDH in its lysosomal degradation pathway seems to be related to their binding or transport through the lysosomal membrane because no differences were found in the degradation of GAPDH by broken lysosomes in the presence of α2μ (data not shown). These results suggest that the transport of α2μ to lysosomes requires some of the components involved in the transport of cytosolic proteins by the hsc73-mediated pathway.

To directly analyze the α2μ uptake by lysosomes, we used a variant of the previously mentioned system [44, 48], in which the proteolytic activity of lysosomes was previously inhibited by chymostatin, an inhibitor of cathepsins B, H, and L [44, 48]. After incubation of the substrate protein with intact lysosomes treated with chymostatin, the transported protein can be detected by its resistance to proteolysis by an exogenously added protease. In these studies, we used radiolabeled [^{14}C]α2μ, because the high endogenous lysosomal levels of this protein masked the α2μ uptake. As shown in Figure 4A, upper panel, after incubation with liver or kidney lysosomes, [^{14}C]α2μ is found associated with lysosomes from both tissues (lanes 2 and 4). In addition, a portion of this lysosome-associated [^{14}C]α2μ was resistant to proteinase K treatment (lanes 3 and 5) and, consequently, was located at the lysosomal matrix. In contrast, after incubation of rat kidney and liver lysosomes under the same conditions with RNase S-protein (residues 21–124 of RNase A), not a substrate protein for this selective lysosomal pathway, only a small percentage of the added RNase S-protein could be detected associated with the lysosomal membrane, and none was detected in the lysosomal matrix (Fig. 4A, lower panel). To verify that this transport took place following the direct hsc73-mediated pathway, we incubated RNase A, another substrate described for this pathway, with isolated lysosomes in the presence of α2μ or ovalbumin (Fig. 4B). A decrease in the amount of RNase A transported into lysosomes was detected when α2μ was added to the incubation medium, but no significant effect was found in the presence of ovalbumin. The decrease in RNase A uptake in the presence of α2μ seems to result from α2μ binding and uptake by lysosomes because, under those circumstances, we found an increase in the levels of α2μ in the lysosomal matrix (data not shown).

To determine if α2μ bound to the lysosomal receptor involved in RNase A and GAPDH transport, we analyzed direct binding of α2μ to immobilized lysosomal membranes. As shown in Figure 5, left panel (lanes 2 and 3), α2μ preferentially associated with a lysosomal membrane protein of 96 kDa, which is the same molecular weight as lgp96, the receptor for the hsc73-mediated lysosomal pathway [51]. An antibody to lgp96 was used to identify this protein in the nitrocellulose membrane

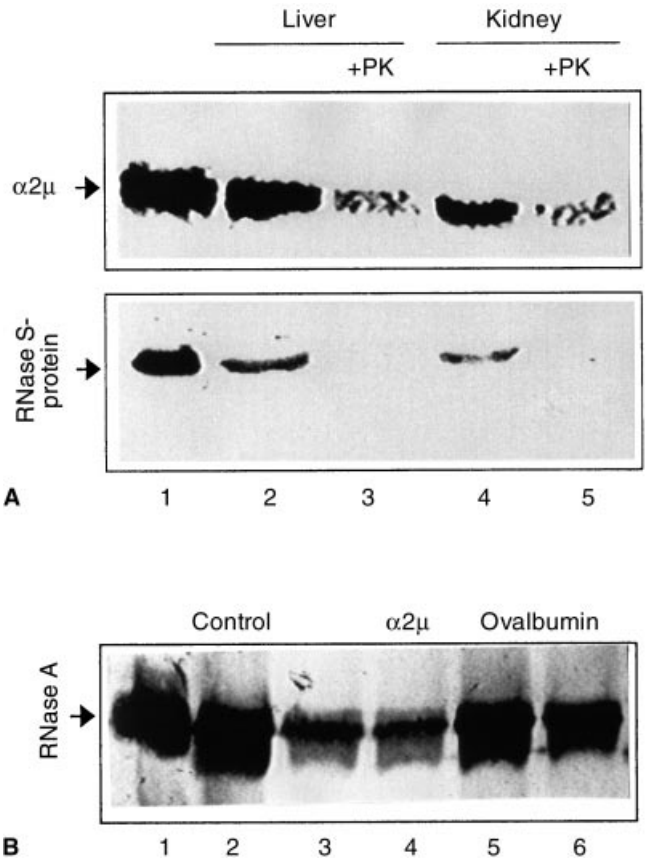


Fig. 4. Direct uptake of α2μ by lysosomes. (*A*) Isolated rat kidney and liver lysosomes were incubated with [^{14}C]α2μ (5 μg; upper panel) or RNase S-protein (10 μg; lower panel) under standard conditions. Where indicated, proteinase K was added, and all samples were subjected to SDS-PAGE and autofluorography (upper panel) or immunoblotting with a specific antibody against RNase A (lower panel). [^{14}C]α2μ (0.5 μg) and RNase S-protein (1 μg) are in lane 1. (*B*) RNase A (25 μg) was incubated with freshly isolated lysosomes alone (CTR; lane 2) or in the presence of α2μ (25 μg; lanes 3 and 4) or ovalbumin (25 μg; lanes 5 and 6). At the end of the incubation, lysosomes were treated with proteinase K, and samples were processed as in (*A*; lower panel). RNase A (1 μg) is in lane 1. The low molecular band recognized by the antibody against RNase A corresponds to an intermediate transport form previously described [48].

in these experiments (Fig. 5, middle panel, lanes 5 and 6). The lysosomal membrane protein pattern after electrophoresis and Coomassie blue staining for kidney and liver is shown in the right panel of Figure 5 (lanes 7 and 8). Notice that a minor amount of α2μ also associated with three low molecular weight lysosomal membrane proteins (of approximately 30 and 42 kDa in kidney and 32 kDa in liver). The specificity of this binding to lower molecular weight proteins remains to be determined.

Modifications in the direct transport of α2μ into lysosomes after TMP exposure

We also analyzed the effect of exposure to TMP on the direct transport of α2μ into lysosomes and its possible

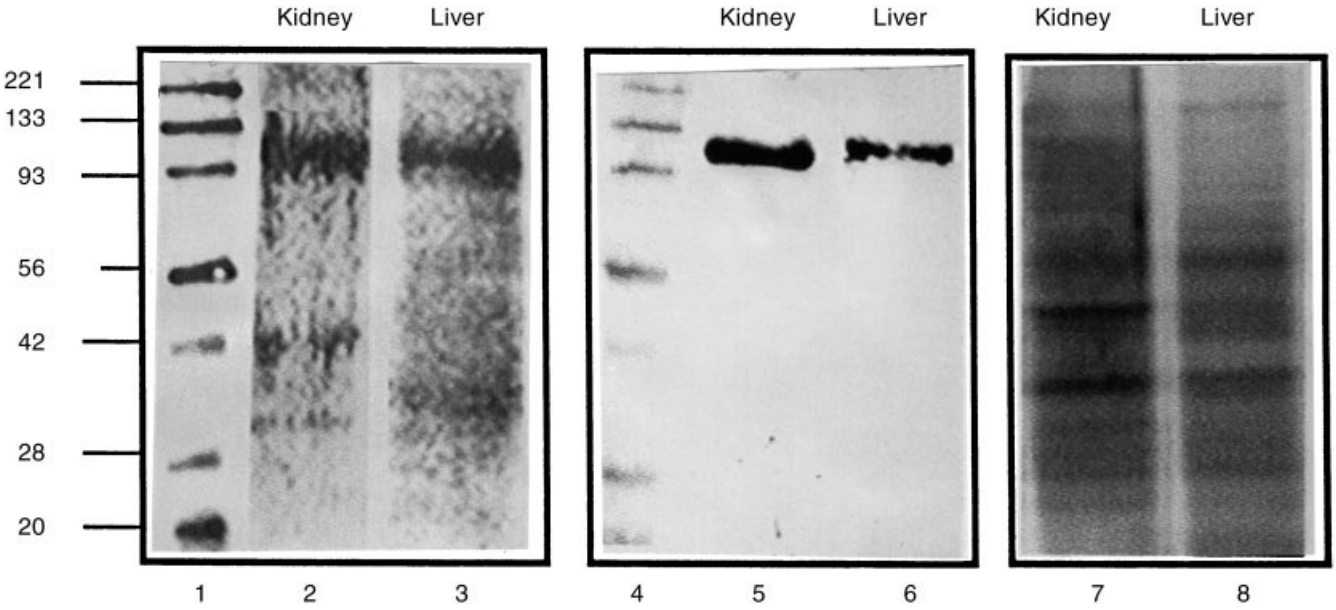


Fig. 5. Binding of α2μ to lysosomal membrane proteins. Rat kidney and liver lysosomal membranes were separated by SDS-PAGE and were electrotransferred to a nitrocellulose membrane (left and middle panels) or Coomassie blue stained (right panel). The nitrocellulose membranes were subjected to immunoblotting with a specific antibody against lgp96 (middle panel) or to a binding assay for [^{14}C]α2μ (left panel) as described in the **Methods** section. Lanes 1 and 4 show prestained molecular weight marker proteins.

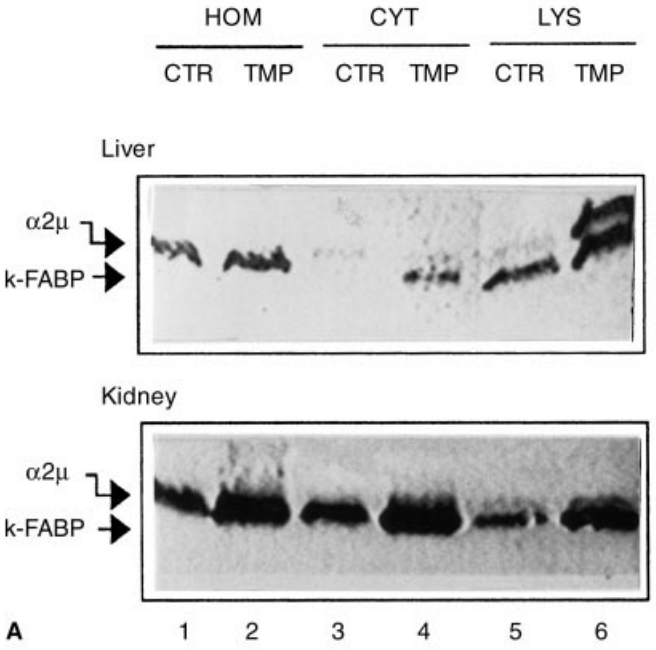
contribution to the hyaline droplet nephropathy. No major differences were found in the purity or activity of enzymatic markers between lysosomal fractions isolated from kidney of nontreated (control) or TMP-treated animals (TMP; Table 2). In liver, the increase in total protein in the homogenate after TMP treatment is higher than in kidney, and that makes the recovery of lysosomal protein (percent of protein in homogenate) lower than in untreated animals. However, the equivalent recovery for the lysosomal marker (hexosaminidase activity) in livers of treated and untreated rats indicates that the purity of the lysosomal preparations from both groups of animals was similar. The analysis of the leakage of lysosomal enzymes in the medium during the incubation was also unaffected by TMP treatment, suggesting that this compound does not modify lysosomal membrane stability (data not shown).

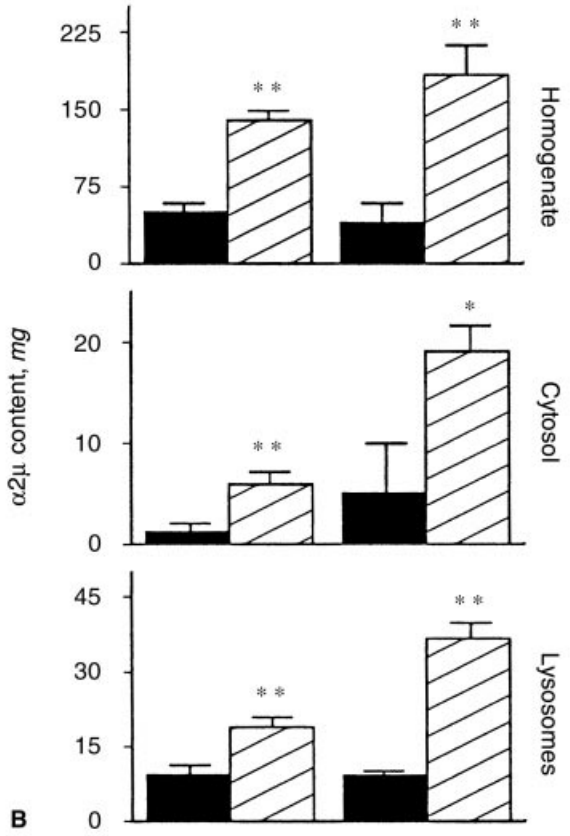
When levels and distribution of α2μ were analyzed in liver and kidney of untreated or TMP-treated rats, we found that the total content of α2μ for 100 μg of homogenate protein was similar or only slightly higher after TMP treatment (Fig. 6A, lanes 1, 2). However, when calculated for the total homogenate protein (Fig. 6B), levels of α2μ markedly increased after exposure to TMP (2.8 and 4.6 times for liver and kidney, respectively). We also found an increase in the cytosolic levels of k-FABP after TMP treatment, but no significant differences were observed in the cytosolic levels of α2μ (Fig. 6A, lanes 3 and 4, and Fig. 6B). A clear increase in α2μ content was observed in lysosomes from liver and kidney of treated animals (Fig. 6A, lanes 5 and 6). When corrected for total lysosomal protein (Fig. 6B), the increase after TMP treatment was 5.0 and 3.8 times for liver and kidney,

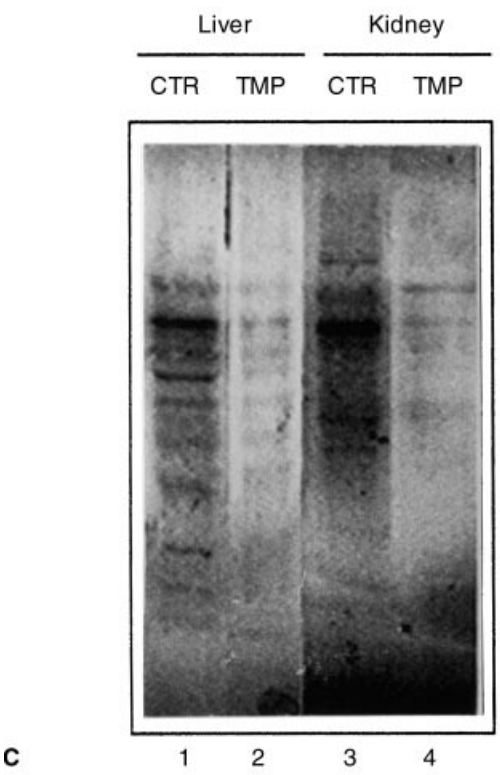
Table 2. Biochemical characterization of the lysosomal fractions isolated from untreated (Control) or 2,2,4-trimethylpentane-treated (TMP) rats

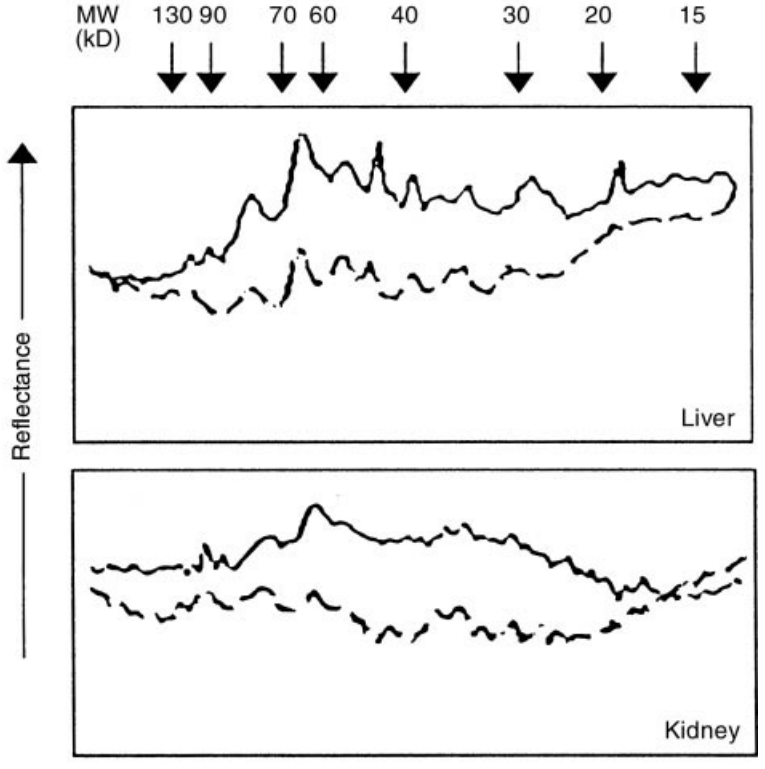
	Protein		Hexosaminidase			
	mg	Recov %	S.A. USA	T.A. UA	Enrich fold	Recov %
Kidney						
Control						
HOM	393.2		0.93	365		
CYT	114.9	29.23				
LYS	1.1	0.28	24.5	27	26	7
TMP						
HOM	524.7		1.31	385		
CYT	179.1	31.14				
LYS	1.3	0.24	29.6	38	23	5
Liver						
Control						
HOM	1969.4		0.24	481		
CYT	534.6	27.15				
LYS	4.2	0.21	14.45	60	59	12
TMP						
HOM	2921.2		0.31	910		
CYT	580.6	19.81				
LYS	3.5	0.12	12.75	44	41	5

Isolation of fractions, protein measurement, and hexosaminidase assay were performed as described in the **Methods** section. Recovery (Recov) was calculated as total activity (T.A.) in the fraction relative to total activity in the homogenate. Enrichment (Enrich) was calculated by dividing the specific activity (S.A.) in the fraction by the specific activity in the homogenate. Abbreviations are: HOM, homogenate; CYT, cytosol; LYS, lysosomes; USA, units of specific activity (UA/mg protein); UA, units of activity.


HOM
CYT
LYS
CTR TMP CTR TMP CTR TMP
Liver
α2μ
k-FABP
Kidney
α2μ
k-FABP
A
1 2 3 4 5 6


α2μ content, mg
225
150
75
0
Homogenate
20
10
0
Cytosol
45
30
15
0
Lysosomes
B


Liver
Kidney
CTR TMP CTR TMP
C
1 2 3 4


MW (kD)
130 90 70 60 40 30 20 15
Reflectance
Liver
Kidney

respectively. Interestingly, both α2μ and k-FABP levels increased in liver lysosomes, whereas in kidney lysosomes, the k-FABP increase was more evident than for α2μ. Lysosomal fractionation into matrix and membrane, as described earlier here, revealed that the increase in lysosomal α2μ after TMP treatment was mainly due to an increase in its matrix content (data not shown).

We also analyzed the effect of TMP on the cellular levels of other substrates for this selective lysosomal pathway. As shown in Figure 6C, the total content of most of the KFERQ-containing proteins in cytosol, determined by immunoblot of the cytosolic fractions with an antibody raised against KFERQ [43], was consistently lower in the liver and kidneys of TMP-treated rats. This result suggests that all substrates for this lysosomal degradative pathway are degraded more rapidly in response to TMP.

A decrease in the proteolytic susceptibility of α2μ to lysosomal proteases after exposure to TMP has been previously suggested as a possible cause for its lysosomal accumulation [39]. We compared the degradation of α2μ and TMP-modified α2μ isolated from urine of normal and TMP-treated rats by lysosomes isolated from untreated rats. When both proteins were incubated with intact lysosomes from kidney and liver of untreated rats, we found no significant differences (Fig. 7A). No significant differences in protein stability were observed when both proteins were incubated with broken lysosomes (Fig. 7B). In addition, when we measured direct uptake of radiolabeled TMP-α2μ by lysosomes isolated from untreated rats (as described in Fig. 4 for α2μ), we found that the percentage of transported TMP-α2μ and α2μ were similar (Fig. 7C). Finally, the capability of TMP-α2μ to bind to lgp96 at the lysosomal membrane was also preserved (data not shown). The small differences between degradative rates of α2μ and TMP-α2μ (Fig. 7 A, B) do not seem to explain the differences in lysosomal levels of α2μ after TMP treatment (Fig. 6A). These results suggest that the increase in lysosomal content of α2μ after exposure to TMP in the group of lysosomes analyzed in this study was not due to modifications in α2μ itself, but that it was due to changes in the lysosomal activity of the hsc73-mediated pathway.

When we analyzed the direct lysosomal uptake of α2μ after TMP treatment as described earlier here, we found a significant increase (20%) in α2μ transport into kidney lysosomes from TMP-treated rats (Fig. 8A). The *in vitro* uptake of other substrates for this pathway also increased; RNase A uptake increased 15% in rat kidney lysosomes (Fig. 8B). The inserts show two representative experiments. Interestingly, competition studies revealed a preference for lysosomal uptake of α2μ in kidney after TMP treatment when compared with other substrates. Thus, addition of the same amounts of α2μ during RNase A uptake by lysosomes from both groups of animals resulted in a higher inhibitory effect in lysosomes from treated rats; RNase A inhibition by α2μ was 10% in lysosomes from untreated rats and 67% in lysosomes from TMP-treated rats (Fig. 8B, compare lanes 2 and 4).

To determine if the increased lysosomal ability to take up specific cytosolic proteins after TMP treatment was also present *in vivo*, we separately analyzed the lysosomal levels of several cytosolic proteins. The *in vivo* lysosomal uptake of α2μ, GAPDH, and hexokinase was measured as the increase in the lysosomal levels of those proteins after a single injection of leupeptin, a protease inhibitor, to TMP-treated and untreated animals (Fig. 9A). Under those conditions, we found that the increase in levels of α2μ in lysosomes was markedly higher for the group of rats treated with TMP. Similar results were found when levels of GAPDH, another substrate for the hsc73-mediated lysosomal pathway, were measured. No significant differences between untreated and TMP-treated animals were found for hexokinase, a nonsubstrate for this pathway, after the treatment with leupeptin. A densitometric quantitation of three such experiments is shown in Figure 9B. As the inhibitory effect of leupeptin in broken lysosomes from both groups of rats was similar (data not shown), those results suggest that TMP induces an increase in the lysosomal uptake of some, but not all, cytosolic proteins.

Changes in the components of the hsc73-mediated lysosomal pathway after TMP exposure

The best identified components of the selective lysosomal pathway are the cytosolic and lysosomal hsc73 and lgp96, the receptor in the lysosomal membrane. Total and cytosolic levels of hsc73 in kidney were not significantly modified by TMP (Fig. 10A). In lysosomes, similar

Fig. 6. Levels of different cytosolic proteins in rat liver and kidney after treatment with TMP. (*A*) Different subcellular fractions [cytosol (CYT), lysosomes (LYS), and homogenates (HOM)] from liver (100 μg protein) and kidneys (50 μg protein) of untreated rats (control; CTR) or rats treated with TMP (treated; TMP) as described in the **Methods** section were subjected to SDS-PAGE and immunoblotting with a specific antibody against α2μ. (*B*) Total content of α2μ in different cellular fractions in kidney and liver was calculated after densitometric quantitation of immunoblots similar to the ones shown in (*A*). Symbols are: (■) control; (▨) TMP. Values were corrected for total protein content in each of the fractions and are the means ± SD of three different experiments. Notice that values from both α2μ + k-FABP have been added together. Differences with respect to control values are significant for $^*P < 0.1$ and $^{**}P < 0.01$. In (*C*), the left panel shows the immunoblot of the cytosolic fractions from the same group of rats with a specific antibody against KFERQ, and the right panel corresponds to the densitometric profile of KFERQ-containing proteins in the cytosol of liver and kidneys of untreated (——) and TMP-treated (- - -) rats.

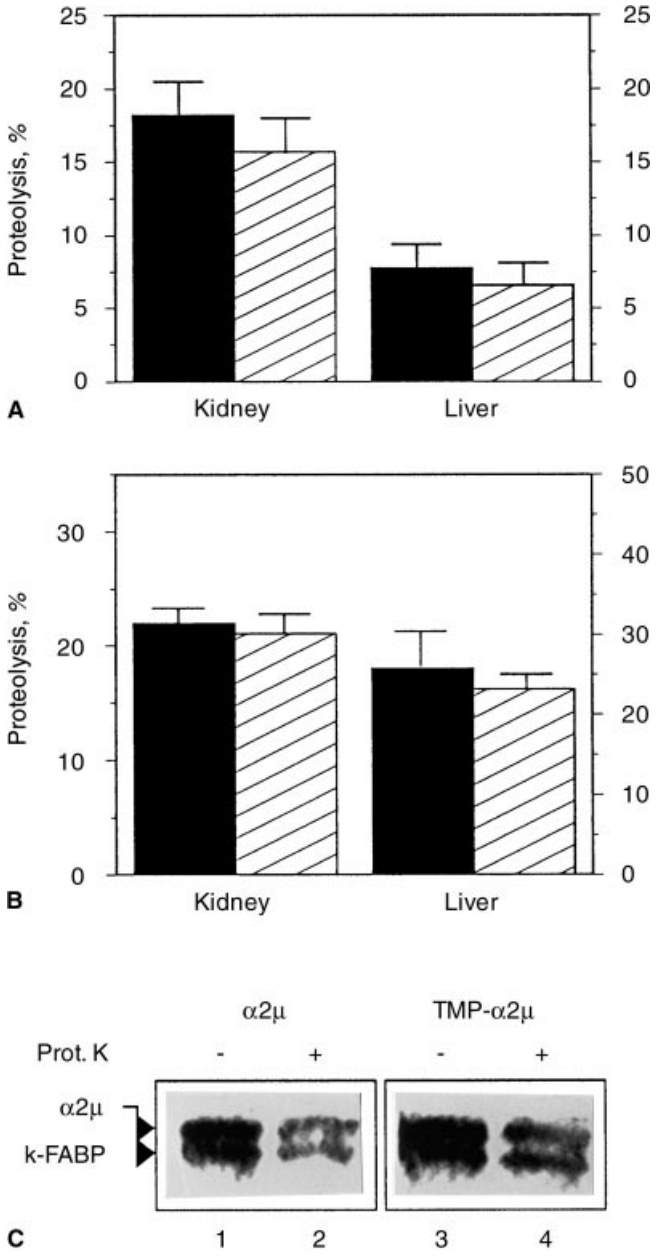


Fig. 7. Specific uptake and degradation of TMP-α2μ by lysosomes. [^{14}C]α2μ (■) and [^{14}C]TMP-α2μ (▨) were incubated with intact (*A*) or broken (*B*) lysosomes from untreated rats in standard conditions. At the end of the incubation, proteolysis was calculated as described in the **Methods** section. Results are means ± SD of 4 different experiments. (*C*) [^{14}C]α2μ (lanes 1 and 2) and [^{14}C]TMP-α2μ (lanes 3 and 4) were incubated with intact lysosomes previously treated with chymostatin under standard conditions. Uptake of both proteins was determined as described in Figure 4.

amounts of hsc73 were also detected in control and TMP-treated rats (Fig. 10B). However, the total amount of lgp96 in the lysosomal fraction after TMP treatment was significantly higher (2.6 times) than in control rats (Fig. 10B). This difference was even more evident when distribution of lgp96 between matrix and membranes of lysosomes was analyzed (Fig. 10C). The increase in the amount of lgp96 in lysosomal membranes after TMP treatment was approximately 3.6 times (Fig. 10C, left panel, compare lanes 1 and 3). This increase correlates well with an increase in the binding of α2μ to those lysosomal membranes (Fig. 10C, right panel).

DISCUSSION

Together with the classic endocytic pathway of degradation for α2μ in male rat kidney, we describe here the participation of a selective lysosomal pathway in the normal turnover of a portion of this protein that resides in the cytosol. The direct transport of the cytosolic α2μ to lysosomes is active in liver and kidney. After exposure to TMP, the direct transport of α2μ significantly increases mainly due to an increase in the lysosomal levels of lgp96, the receptor protein for the selective pathway. α2μ stabilization does not seem to contribute to the accumulation of the directly transported protein from cytosol into lysosomes, as we could not detect significant changes in the degradation rates of the cytosolic α2μ once it reaches the lysosomal matrix. Instead, the 3.5-fold increase in the activity of the hsc73-mediated selective pathway appears to be the main reason for the accumulation of the cytosolic α2μ inside lysosomes after TMP exposure.

One of the main limitations in the study of the α2μ nephropathy is the inability to reproduce the α2μ lysosomal storage in kidney cells in culture. This fact clearly reduces the number of possible approaches for implication of the hsc73-mediated pathway in α2μ degradation. Thus, the typical measurement of protein degradation rates in the presence and in the absence of serum in cultured cells, which has been successfully applied for the characterization of other substrates of this pathway, cannot be used for α2μ. However, our studies with lysosomal inhibitors *in vivo* together with those involving a previously developed *in vitro* system for the direct lysosomal transport of proteins allow us to present consistent evidence in the current study for α2μ lysosomal degradation following the hsc73-mediated pathway.

The selective pathway of lysosomal degradation, first described in human fibroblasts [43] and later characterized in rat liver [44, 48], is also active in rat kidney. Previous studies have shown that cytosolic proteins containing the targeting sequence for this pathway (peptide sequences biochemically related to KFERQ) selectively decreased in the kidney cytosol during starvation [43, 49]. Using isolated kidney lysosomes, we also found that there is a direct transport of GAPDH and RNase A into kidney lysosomes, similar to that described for liver. This uptake is stimulated by ATP and hsc73, involves the binding of substrates to lgp96, and increases after prolonged starvation (A.M. Cuervo and J.F. Dice, unpublished results). The facts that (*a*) α2μ was more abundant in the lyso-

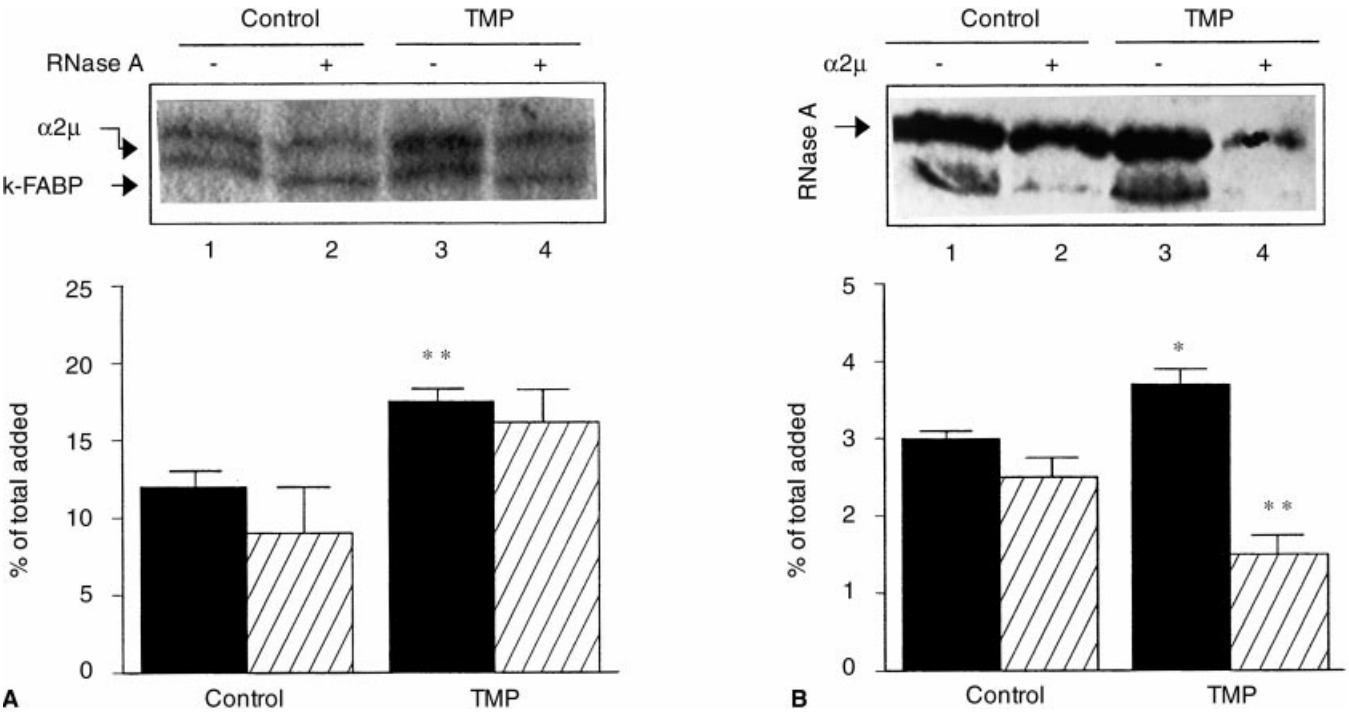


Fig. 8. Effect of TMP on the direct uptake of α2μ and RNase A by lysosomes. Freshly isolated lysosomes from kidney of untreated (Control) or TMP-treated rats (TMP) were incubated with [^{14}C]α2μ (5 μg) (*A*) or RNase A (25 μg) (*B*) under standard conditions, alone (■) or in the presence of RNase A (1 μg) (*A*) or α2μ (5 μg) (*B*) (▨), as labeled. At the end of the incubation, lysosomes were treated with proteinase K, collected by centrifugation, and were subjected to SDS-PAGE and autofluorography (*A*) or immunoblotting with a specific antibody against RNase A (*B*). Values were obtained by densitometric quantitation of five immunoblots similar to the ones shown in the insets. Values (means ± SD) are expressed as the percentage of the total substrate protein added to the incubation medium detected at the lysosomal matrix. Differences with respect to control values are significant for $^*P < 0.1$ and $^{**}P < 0.01$.

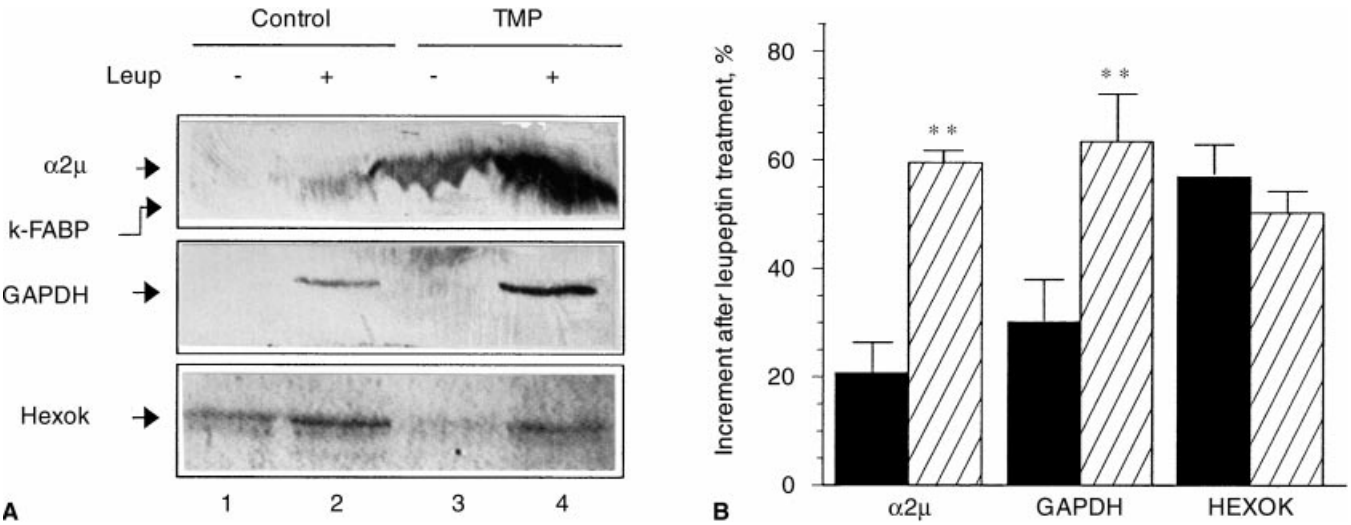


Fig. 9. Effect of TMP on the activity of the hsc73-mediated lysosomal pathway *in vivo*. (*A*) Lysosomes (100 μg of protein) isolated from control and TMP-treated rats injected (lanes 2 and 4) or not (lanes 1 and 3) with leupeptin before sacrifice (**Methods**) were subjected to SDS-PAGE and immunoblot with specific antibodies against several cytosolic proteins. (*B*) Densitometric quantitation (means ± SD) of three experiments was as the one shown here. Symbols are (■) control; (▨) TMP. Differences with respect to control values are significant for $^{**}P < 0.01$.

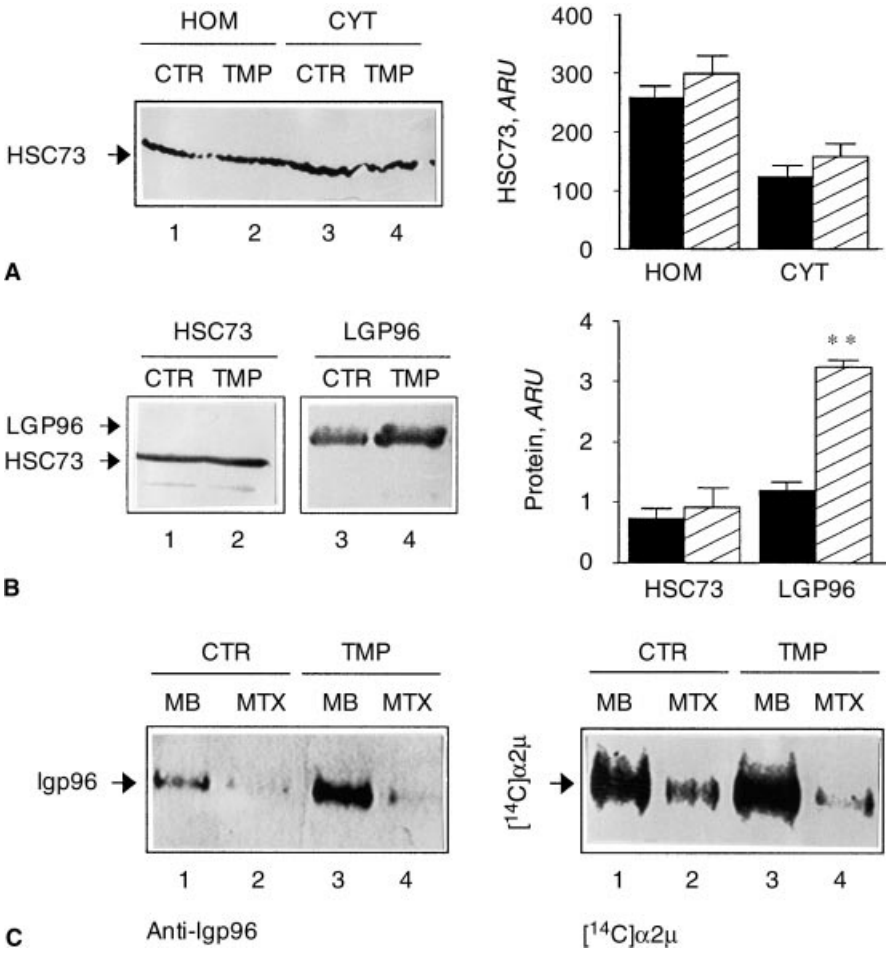


Fig. 10. Kidney lysosomal levels of different components of the hsc73-mediated lysosomal system after treatment with TMP. Homogenates (HOM), cytosol (CYT) (*A*) and lysosomes (*B*) prepared from kidney of untreated (■; CTR) or TMP-treated rats (▨; TMP) as described under **Methods** were subjected to SDS-PAGE and immunoblotting with specific antibodies against hsc73 (*A* and *B*) and lgp96 (*B*). Total hsc73 and lgp96 content in each fraction calculated by densitometric analysis as described in Figure 6B is shown in the right panels of (*A* and *B*). Values are expressed as arbitrary refractance units (ARU) and are means ± SD of four different experiments. Differences are significant for $^{**}P < 0.001$. (*C*) Lysosomal matrixes and membranes from untreated (CTR) or TMP-treated rat kidney (TMP) prepared as described in **Methods** were subjected to SDS-PAGE and immunoblotting with a specific antibody against lgp96 (left panel) or incubated with [^{14}C]α2μ and exposed to a phosphorimager screen (right panel).

somal population active for the hsc73-mediated transport (Fig. 2C), (*b*) α2μ could be directly transported into isolated lysosomes in an ATP/hsc73-stimulated manner (Figs. 3C and 4A), (*c*) α2μ was able to compete for direct transport of other substrates for this degradative pathway (Figs. 3C and 4B), and (*d*) α2μ binds to a lysosomal membrane protein of 96 kDa consistent with binding to lgp96, the receptor for the lysosomal pathway (Fig. 5), support the notion that the cytosolic form of α2μ is transported into lysosomes by the hsc73-mediated pathway. Interestingly, in spite of the comparable activity for this selective pathway in kidney and liver, there also seems to be some tissue-regulated substrate selectivity. Thus, we have shown that α2μ can be directly transported into lysosomes from both tissues (Figs. 3 and 4), but levels of α2μ are higher for kidney lysosomes (Fig. 2). A larger inhibitory effect of α2μ on GAPDH degradation (Fig. 3C) was also observed in kidney lysosomes when compared with liver lysosomes.

The origin of the cytosolic form of α2μ and its derivative k-FABP is unclear. Different mechanisms have been shown to result in the localization of a single gene product in more than one intracellular compartment [68]. Thus, cytosolic forms of other secretory proteins such as invertase, gelsolin, or plasminogen activator inhibitor 2 have been proven to be the result of alternative transcription initiation, alternative pre-RNA splicing, and inefficient or thwarted translocation, respectively. Future studies will be needed to determine which of those mechanisms applies for the cytosolic form of α2μ in liver. Cytosolic α2μ in kidney may be due to some molecules escaping from the endosome/lysosome system or to reabsorption mechanisms other than the endocytic pathway.

Regarding k-FABP, our results suggest that the role of lysosomes in processing of α2μ to this derivative might be different for kidney and liver. A protease in the brush border of tubular cells has been shown to participate in the α2μ processing in kidney during its endocytosis [33]. In liver, where reabsorption of α2μ is minor [69], a different protease should be involved in the production of k-FABP. The presence of k-FABP at the lysosomal matrix in rat liver (Fig. 2) along with its resistance to degradation (Fig. 3) might implicate lysosomes in the normal processing of α2μ to k-FABP in liver.

The lysosomal accumulation of the cytosolic α2μ after TMP treatment seems to be caused by increased uptake rates of this cytosolic protein by lysosomes (Fig. 8), more than to changes in its susceptibility to lysosomal proteases (Fig. 7). The direct measurements of the transport of α2μ into lysosomes *in vitro*, working with isolated lysosomes (Fig. 8), and also *in vivo* (Fig. 9), after injection of leupeptin, revealed a marked increase (3.5-fold) in the transport of this protein into lysosomes after TMP treatment. This increase in the direct uptake of α2μ seems to be related to changes in the lysosomal function rather than to changes in α2μ itself. Thus, there were no significant differences between the uptake of TMP-α2μ and that of the unmodified α2μ by lysosomes from nontreated rats (Fig. 7C). This increase in the activity of the selective lysosomal transport seems to be caused by an increase in the levels of lgp96 in the lysosomal membrane (Fig. 10C). Changes in lysosome membrane levels of this protein and a good correlation between levels of lgp96 and the activity of the hsc73-mediated lysosomal pathway have also been found in other cellular conditions (prolonged starvation, aging, etc.; A.M. Cuervo and J.F. Dice, unpublished results). The mechanisms involved in the lysosomal increase in lgp96 under those circumstances are not known.

The fact that none of the other substrates for the hsc73-mediated pathway accumulate inside kidney tubule lysosomes after exposure to TMP might be related to the specific characteristics of α2μ. As described previously, α2μ is very resistant to protease attack (compare its lysosomal half-life of 52 min with the lysosomal half-lives of 10 to 15 min for most of the other substrate proteins) [44, 48, 56]. Most of the cytosolic proteins that lysosomal uptake increases after TMP exposure would be rapidly degraded once inside the lysosomal matrix, but α2μ would remain longer inside lysosomes. A general TMP-related increase in the selective lysosomal uptake for all the substrates analyzed was evident not only when working with isolated lysosomes (Fig. 8), but also after injection of the rats with leupeptin (Fig. 9). However, the higher competition of RNase A uptake by α2μ after treatment with the drug suggests that the increased uptake could be somewhat preferential for α2μ. This selectivity for α2μ uptake under TMP exposure is clearly manifested *in vivo*, where the increase in lysosomal content of α2μ in treated rats was significantly higher than for other substrates of this pathway (data not shown). Whether this selectivity is just the result of the regular higher cytosolic levels of this protein in kidney compared with other proteins or of the reported α2μ, altered stability once inside lysosomes should be further analyzed.

Once again, the treatment with TMP also revealed a different processing mechanism for α2μ and its derivative k-FABP in kidney and liver. Thus, while TMP-treated liver lysosomes showed an increase in both α2μ and k-FABP, a preferential accumulation of k-FABP was found in kidney lysosomes (Fig. 6A, lane 6). A possible explanation for those differences would be that the TMP-induced increase in α2μ uptake could result in a saturation of the lysosomal processing system for α2μ in liver, and meanwhile in kidney, where processing takes place in the brush border, it would not be affected by the drug.

The increase in the direct transport of α2μ in kidney and liver after TMP exposure might be a compensatory mechanism activated by those cells in response to abnormal cytosolic levels of k-FABP (Fig. 6A, compare lanes 3 and 4). Even though the origin of this cytosolic form in both tissues is still unknown, it might be possible that the processing of α2μ to k-FABP or its complete lysosomal degradation are in a continuous balance. TMP-induced changes in this balance could result in an increase in the amount of k-FABP that is released into the cytosol. To eliminate these abnormally increased levels of k-FABP in the cytosol of the cell the hsc73-mediated pathway will be activated. The higher constitutive levels of α2μ in kidney compared with liver may explain why this defensive mechanism, which seems to be successful in eliminating the abnormal protein in liver, fails in kidney. The dual source of α2μ in kidney lysosomes, endocytosis, and direct lysosomal transport, when compared to liver lysosomes, could also explain the development of lysosomal deposits only in kidney.

We conclude that the chemically induced α2μ nephropathy is the result of an increase in the direct transport of the cytosolic form of α2μ into lysosomes. This hsc73-mediated uptake of the cytosolic α2μ by lysosomes contributes to its degradation in kidney and liver in normal cellular conditions. After TMP exposure, an increase in the lysosomal membrane levels of the receptor for this pathway accelerates the lysosomal uptake of specific cytosolic proteins and mostly of α2μ. This is the first evidence of a chemically mediated modification in the activity of the hsc73-mediated lysosomal pathway.

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APPENDIX

Abbreviations used in this study are: α2μ, α_2-microglobulin; ATP, adenosine triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsc73, heat shock cognate protein of 73 kDa; k-FABP, kidney fatty acid-binding protein; lgp96, lysosomal glycoprotein of 96 kDa; MOPS, 3-(N-morpholino) propanesulfonic acid; RNase A, ribonuclease A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMP, 2,2,4-trimethylpentane; TMP-α2μ, 2,2,4-trimethyl pentane-α_2-microglobulin conjugate.

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