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Increased expression of mature cathepsin B in aging rat liver

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Abstract Senescence has been proposed as an important safeguard against neoplasia. One of the hallmarks of cellular senescence in vitro as well as human aging in vivo is a reduced intracellular protein catabolism. The pathways affected and the mechanisms responsible for the decrease in overall protein turnover in aging cells are not well understood. Our aim was to determine whether or not expression of one of the major hepatic lysosomal cysteine peptidases, cathepsin B, changes during aging of Sprague-Dawley rats. Cathepsin B activity was assessed in whole rat liver homogenates, and was found to be increased fourfold ($P \leq 0.001$) in aged livers compared with younger counterparts. This was paralleled by an at least a twofold increase in mature cathepsin B protein. Nonetheless, Northern blot analysis of total liver RNA revealed no change in steady-state levels of cathepsin B mRNAs. These findings seem to contradict the present dogma according to which aging tissues have a reduced intracellular capacity to catabolise proteins. We propose that our earlier observation of the accumulation of T-kininogen, a potent but reversible cysteine peptidase inhibitor, in aging rat liver may provide a plausible explanation for this discrepancy.

Keywords Cysteine peptidases · Lysosomes · Aging · Protein turnover · Proteolysis · Rat (Sprague Dawley)

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Introduction

Aging is characterized by a decline in several physiological functions, as well as by a loss in responsiveness to external stimuli. The molecular mechanisms leading to and responsible for the maintenance of the senescent phenotype in vivo comprise both systemic and cellular components (for a recent review, see Johnson et al. 1999). Many features characterize senescence at the cellular level, and these include the decrease in stress resistance and repair mechanisms as well as the accumulation of modified and/or damaged proteins (for a review, see Dice 1993).

The accumulation of ubiquitinated proteins (Ivy et al. 1989; Pan et al. 1993) as well as damaged proteins (Reznick et al. 1981; Dovrat and Gershon 1983; Lavie et al. 1982) has been observed in aged animals. Part of this accumulation process appears to be due to the loss of defense and repair mechanisms in aged cells (Takahashi and Goto 1990; Agarwal and Sohal 1996), but an equally important mechanism is the lower rate of removal of such proteins (Reznick et al. 1981; Agarwal and Sohal 1994). The rate of intracellular protein degradation decreases with aging in mouse (Reznick et al. 1981; Lavie et al. 1982) and rat (Ward 1988) liver, in rat lens (Dovrat and Gershon 1983), and in nematodes (Reznick and Gershon 1979). Hepatocytes from aged mice have a lower rate of protein turnover (Ishigami and Goto 1988) as well as a lower rate of removal of microinjected oxidized proteins than do cells from younger counterparts (Takahashi and Goto 1987). Similar observations are seen in senescent human lung fibroblasts (Dice 1982). Removal of microinjected damaged proteins is dependent on the activity of the proteasome, which has been shown to be significantly impaired during aging (Agarwal and Sohal 1994).

Another major pathway of intracellular protein degradation is represented by the lysosomal proteolytic system (Barrett and Kirschke 1981). However, only a few studies have analyzed the changes that occur in the degradation machinery of lysosomes. The lysosomal aspartic

peptidase cathepsin D accumulates as an inactive form in many tissues of aged rats (Wiederanders and Oelke 1984). In contrast, others have found that the activity of this peptidase significantly increases with aging of rat brains (Matus and Green 1987). Both the cathepsin D protein level and enzyme activity increase in aging rat thymus and spleen but decrease in aging stomach (Nishishita et al. 1996). In many tissues such as the kidney, liver, and brain, cathepsin B is one of the most abundant lysosomal cysteine peptidases (Kominami et al. 1985). Most studies on this papain-like cysteine peptidase, however, have been done on either tissue specimens from aged patients with brain (Cataldo and Nixon 1990; Bernstein et al. 1990; Nakamura et al. 1991) or malignant disease (for a recent review and references, see Lah and Kos 1998). While one report showed overexpression of mature cathepsin B during replicative senescence of human lung fibroblasts in vitro (DiPaolo et al. 1992), to our knowledge no studies have analyzed expression of cathepsin B during normal aging in vivo.

In this study, we compared expression of cathepsin B in the livers of young and old Sprague-Dawley rats. We show that cathepsin B activity was significantly increased in liver homogenates of old animals as compared with those of young animals. This increase was paralleled by an increased level of mature protein as determined by immunoblotting. However, Northern blot analysis of total RNA showed that the levels of cathepsin B transcripts remained unchanged. These findings, as well as previous observations (Sierra et al. 1989; Keppler et al. 1997) on the intrahepatic accumulation of active T-kininogen, a potent but reversible inhibitor of lysosomal cysteine peptidases, have led us to propose a novel model that may account for the overall decreased rate of intracellular protein degradation in senescent cells or aging tissues.

Materials and methods

Animals. Sprague-Dawley male rats were used in all experiments according to the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985). They were housed individually in a pathogen-free facility, and were fed laboratory chow ad libitum as previously described (Sierra et al. 1992). These rats have a median life span of about 24 months, and were sacrificed at the age of 15 months (young) or at the age of 25 months (old).

Preparation of liver homogenates. Rats were sacrificed under CO_2. Blood was taken by aortic puncture. Livers were perfused with physiological saline and cut into pieces for extraction of RNA (see below) and protein. The tissue was weighed and homogenized with a Polytron in four volumes of a 10 m*M* sodium phosphate buffer, pH 6.0, containing 0.4 *M* NaCl, 2 m*M* EDTA, and 0.2% (v/v) Triton X-100. The homogenates were spun at $10{,}000 \times g$ for 10 min at 4°C, and the supernatants were aliquoted, frozen in dry ice, and stored at –70°C until use. For the experiments described, both individual homogenates and pools of homogenates derived from five individual animals were used. The serum and liver from each animal used was individually tested for the absence of expression of α_1-acid glycoprotein, a marker of inflammation, as previously described (Sierra et al. 1989; Sierra et al. 1992). Proteins in liver homogenates were quantitated using a protein assay kit manufactured by Bio-Rad.

Assay of cathepsin B activity. In contrast to other lysosomal cysteine peptidases such as cathepsin L (Mason et al. 1985), cathepsin B readily dissociates from enzyme-inhibitor complexes upon dilution of samples (Assfalg-Machleidt et al. 1990). In order to be able to assay cathepsin B in liver homogenates, first an optimal dilution of samples has to be found (Higashi et al. 1986). Different amounts (0.5–100 µl) of pooled rat liver homogenates were preincubated for 30 min at 20°C in 490 µl (constant volume) of 50 m*M* sodium phosphate buffer, pH 6.0, containing 0.05% (v/v) Triton X-100, 2 m*M* EDTA, and 2.5 m*M* DTT (referred to below as assay buffer). Then, 10 µl of 1 m*M* benzyloxycarbonyl-L-arginyl-L-arginine-4-methyl-7-coumaryl-amide (Z-Arg-Arg-NHMec) or benzyloxycarbonyl-L-phenylalanyl-L-arginine-4-methyl-7-coumaryl-amide (Z-Phe-Arg-NHMec) was added and incubation continued for 10 min at 30°C. The enzymatic reaction was terminated by the addition of 500 µl of 0.1 *M* sodium monochloroacetate buffer, pH 4.3 (Barrett 1980), and fluorescence intensities were read on a calibrated Fluoroskan II microplate reader from Labsystems fitted with 355-nm excitation and 460-nm emission filters. Results are expressed as specific activities in units/mg protein, where one unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol 4-methyl-7-coumarylamine (NH_2Mec)/min.

To demonstrate the specificity of our assay, enzyme activities of liver homogenates were titrated in parallel with *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl-4-guanidino-butylamide (E-64) and its derivative *N*-(L-3-*trans*-propyl-carbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074). E-64 is a stoichiometric and irreversible inhibitor of most papain-like cysteine peptidases (Barrett et al. 1982) whereas CA-074 is selective for lysosomal cathepsin B (Murata et al. 1991). The two pools of liver homogenates, the inhibitors, and the substrate were all diluted in assay buffer. Diluted samples (50 µl; 1/125) were preincubated for 30 min at 20°C with various concentrations of inhibitor (50 µl; 0.0, 0.3, 1.6, 8, 40, 200, 1000 and 5000 n*M*). After addition of 100 µl of 200 µ*M* Z-Arg-Arg-NHMec, the initial velocity of product formation at 37°C in the presence of inhibitor (v_i), was recorded. Results were expressed as residual enzyme activity ($v_i/v_o \times 100\%$) after normalization for the initial velocity of product formation in the absence of inhibitor (v_o) (Keppler et al. 1996a).

Each of five individual liver homogenates, rather than pools, from both young and old rats was analyzed in triplicate for cathepsin B activity using optimal conditions established as described above. For this, 2 µl of liver homogenates was preincubated for 30 min at 20°C in 498 µl assay buffer. After this preincubation period, 500 µl of fresh assay buffer containing 200 µ*M* Z-Arg-Arg-NHMec was added and incubation continued for 20 min at 37°C. Reactions were stopped and samples processed as before. Controls were made up for each sample with 5 µ*M* (final concentration) CA-074. After subtraction of control values, results were expressed as specific activities as described above. The means ± SEM (n=5) were calculated for both age classes and statistically compared using Student's *t* test.

Western blot analysis. Fifty micrograms of proteins from individual or pooled rat liver homogenates containing 5 µ*M* E-64 were boiled for 5 min in Laemmli buffer containing 100 m*M* DTT (final concentration), and samples were loaded onto a 16% (w/v) SDS-polyacrylamide gel. Two identical gels were prepared and electrophoresis was carried out for 90 min at 120 V. One gel was fixed and resolved proteins visualized with 0.1% (w/v) Coomassie Brilliant Blue G-250. Separated proteins in the second gel were electrophoretically transferred (200 mA for 45 min) to nitrocellulose membranes. Immunoblotting was performed with rabbit anti-human liver cathepsin B antibodies, kindly prepared and donated by Mrs. M. Sameni and Dr. B.F. Sloane (Department of Pharmacology, Wayne State University). These antibodies were used at a final concentration of 3 µg/ml as previously described (Keppler et al. 1996b), followed by incubation with peroxidase-conjugated

secondary antibodies. Detection was performed using the enhanced chemiluminescent (ECL) system from Amersham according to the instructions supplied by the manufacturer. An Ambis v.4.01 image analysis system was used to scan the Coomassie-stained gel and the ECL film and evaluate relative amounts of immunoreactive cathepsin B versus albumin (used as loading control).

RNA isolation and Northern blot analysis. Total liver RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method as modified by Puissant and Houdebine (1990) to eliminate glycogen contamination. Total RNA was then electrophoretically fractionated on glyoxal gels (McMaster and Carmichael 1977), transferred to GeneScreen membranes (DuPont-New England Nuclear), fixed by exposure to UV light, and hybridized to random primed ^{32}P-labeled probes in 50% formamide at 42°C overnight. Human cathepsin B cDNA was a generous gift of Dr. Shu Jin Chan, HHMI Research Laboratories, University of Chicago, Chicago, USA (Chan et al. 1986). The rat β-actin cDNA probe was a kind gift of Dr. Alphonse LeCam (INSERM-CNRS, Montpellier, France).

Results

Increased specific activity of cathepsin B in aged rat liver

To evaluate total cathepsin B activity in whole tissue homogenates, the enzyme has first to be dissociated from its reversible complexes with inhibitors. This is generally achieved by dilution of the sample (Assfalg-Machleidt et al. 1990). The dilution-dependent release of Z-Phe-Arg-NHMec- and Z-Arg-Arg-NHMec-hydrolyzing activities from enzyme-inhibitor complexes is illustrated in Fig. 1A and B, respectively. The shapes of the dilution curves were similar for both substrates and fitted logarithmic curves. Specific enzyme activities were recovered in a saturable way, i.e., rapid recovery occurred up to a dilution of 1:200 and progressively plateaued thereafter. Although Z-Phe-Arg-NHMec is a better substrate for cathepsin B than is Z-Arg-Arg-NHMec (Barrett and Kirschke 1981), it is also cleaved by many other lysosomal cysteine peptidases (Wang et al. 1998). In contrast, Z-Arg-Arg-NH-Mec is a more selective substrate for cathepsin B (Wang et al. 1998). We plotted the ratios of the specific enzyme activities (Z-Phe-Arg-NHMec/Z-Arg-Arg-NHMec) as a function of the dilution factor for each pool of liver homogenate. In pools from both young and old rats, this ratio increased with increasing dilution and plateaued at a value between 6.0 and 7.0 (Fig. 1C). Since others have reported a ratio of 6.4 for purified rat liver cathepsin B and 7.2 for the recombinant unglycosylated enzyme (Hasnain et al. 1992), our dilution experiments strongly suggest that only cathepsin B dissociated from enzyme-inhibitor complexes and was assayed under our experimental conditions. This is substantiated by the fact that the corresponding ratios for purified cathepsin L and

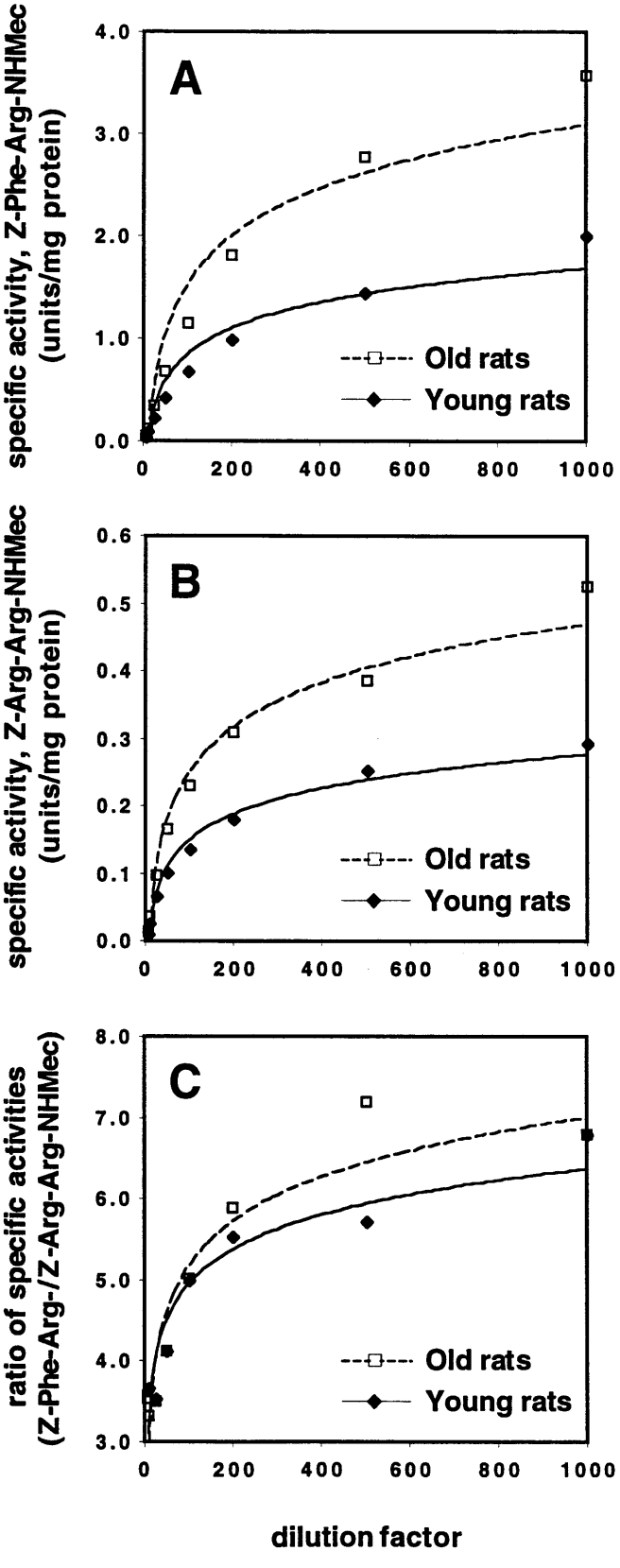

Fig. 1A–C Specific enzyme activity as a function of dilution of liver homogenates. Various amounts of pooled rat liver homogenates (0.5–100 µl) were preincubated for 30 min at 20°C in a constant volume (490 µl) of 50 m*M* sodium phosphate buffer, pH 6.0, containing 0.05% (v/v) Triton X-100, 2 m*M* EDTA, and 2.5 m*M* DTT (assay buffer). Then, 10 µl of 1 m*M* substrate solution was added, and incubation continued for 10 min at 30°C. The substrates used were Z-Phe-Arg-NHMec (**A**) and Z-Arg-Arg-NHMec (**B**). **C** The ratio of specific activities (Z-Phe-Arg-NHMec/Z-Arg-Arg-NHMec) was plotted as a function of the dilution factor

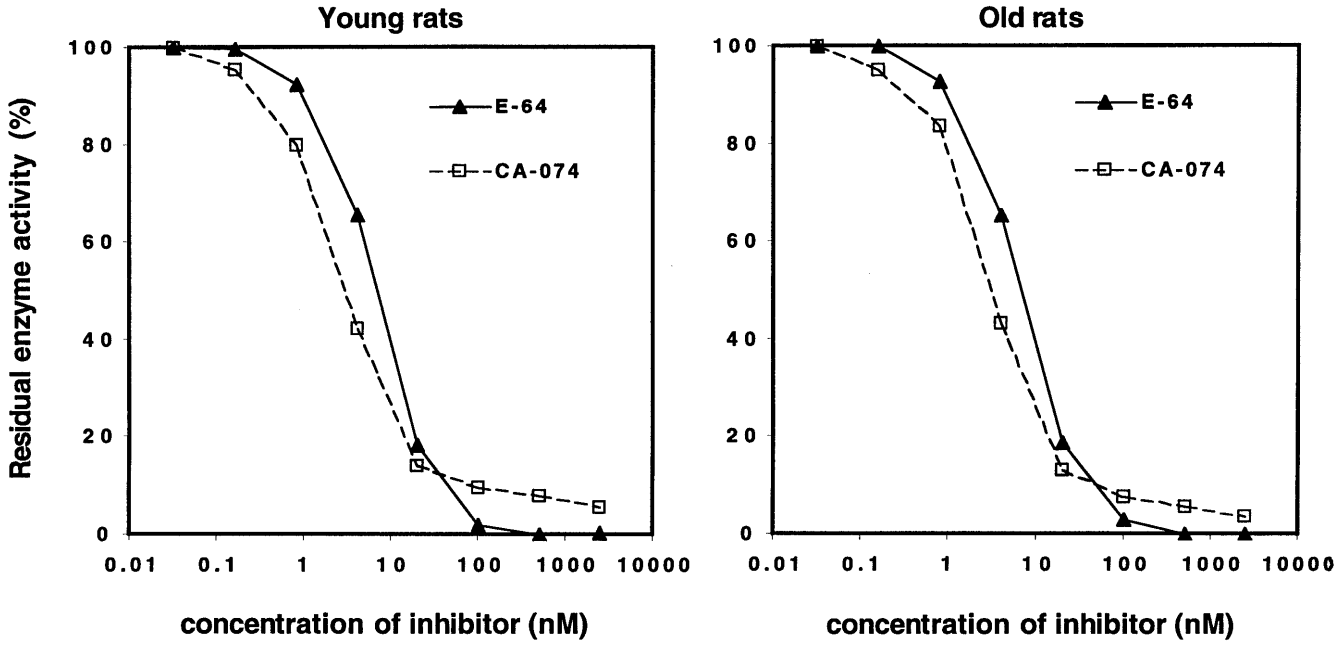

Fig. 2 Titration of cathepsin B activity in liver homogenates by E-64 and CA-074. The two pools of liver homogenates, the inhibitors, and the peptidase substrate were all diluted in assay buffer (see legend to Fig. 1). Diluted samples (50 µl; 1/125) were preincubated for 30 min at 20°C with various concentrations of inhibitor (50 µl; 0.0, 0.3, 1.6, 8, 40, 200, 1000, and 5000 n*M*). After addition of 100 µl of 200 µ*M* Z-Arg-Arg-NHMec, the initial velocity of product formation at 37°C in the presence of inhibitor (v_i), was recorded. Results were expressed as residual enzyme activity ($v_i/v_o \times 100\%$) after normalization for the initial velocity of product formation in the absence of inhibitor (v_o)

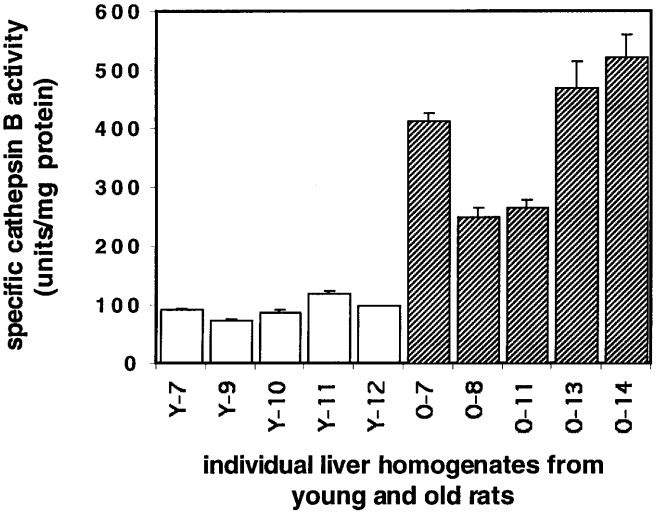

Fig. 3 Levels of active cathepsin B in liver homogenates of young and old animals. Each of five individual liver homogenates from young and old rats was analyzed in triplicate for cathepsin B activity. Liver homogenates (2 µl) were preincubated for 30 min at 20°C in 498 µl assay buffer (see legend to Fig. 1). After this preincubation period, 500 µl of fresh assay buffer containing 200 µ*M* Z-Arg-Arg-NHMec was added and incubation continued for 20 min at 37°C. Controls containing 5 µ*M* (final concentration) CA-074 were prepared for each sample. After subtraction of control values, results were expressed as specific enzyme activities in units/mg protein (means ± SEM; *n*=3). *Clear bars*: young animals (Y-7 to Y-12); *hashed bars*: old animals (0-7 to 0-14)

related cathepsins such as cathepsins F, K, L2, and S vary between 80 and 5000 (Mason et al. 1985; Assfalg-Machleidt et al. 1990; Wang et al. 1998). Cathepsin L, for example, because of the tightness of its interaction with endogenous inhibitors or cystatins (for a review, see Abrahamson 1994), requires a tissue autolysis step at pH 4.2 in order to be released from enzyme-inhibitor complexes (Mason et al. 1985).

When 250-fold diluted pools of rat liver homogenates were preincubated with either E-64 or CA-074 and then assayed with Z-Arg-Arg-NHMec, a dose-dependent inhibition of enzyme activity was observed (Fig. 2). At a 0.5 µ*M* concentration, E-64 completely blocked enzyme activity in samples from both young and old animals (Fig. 2). This result shows that under our assay conditions, only papain-like cysteine peptidase activities are measured. CA-074, the selective and irreversible inhibitor of cathepsin B (Murata et al. 1991), was more efficient than E-64 at low inhibitor concentrations. However, at a 2.5 µ*M* concentration, there still remained 5.7% and 3.7% residual enzyme activity in liver homogenates of young and old rats, respectively (Fig. 2). This residual enzyme activity could be due to the presence of small amounts of some other cysteine peptidase capable of cleaving Z-Arg-Arg-NHMec. Alternatively, it could be due to the slow binding kinetics of inhibitor at low enzyme concentrations (<1 n*M*), as we have observed with purified cathepsin B (Linebaugh et al. 1999).

Five individual rat liver homogenates from each age category were assayed for cathepsin B activity at the optimal dilution of 1:500, along with parallel controls containing 5 µ*M* CA-074. All five samples from old animals were found to have two- to sevenfold higher activity than the five samples from young animals (Fig. 3).

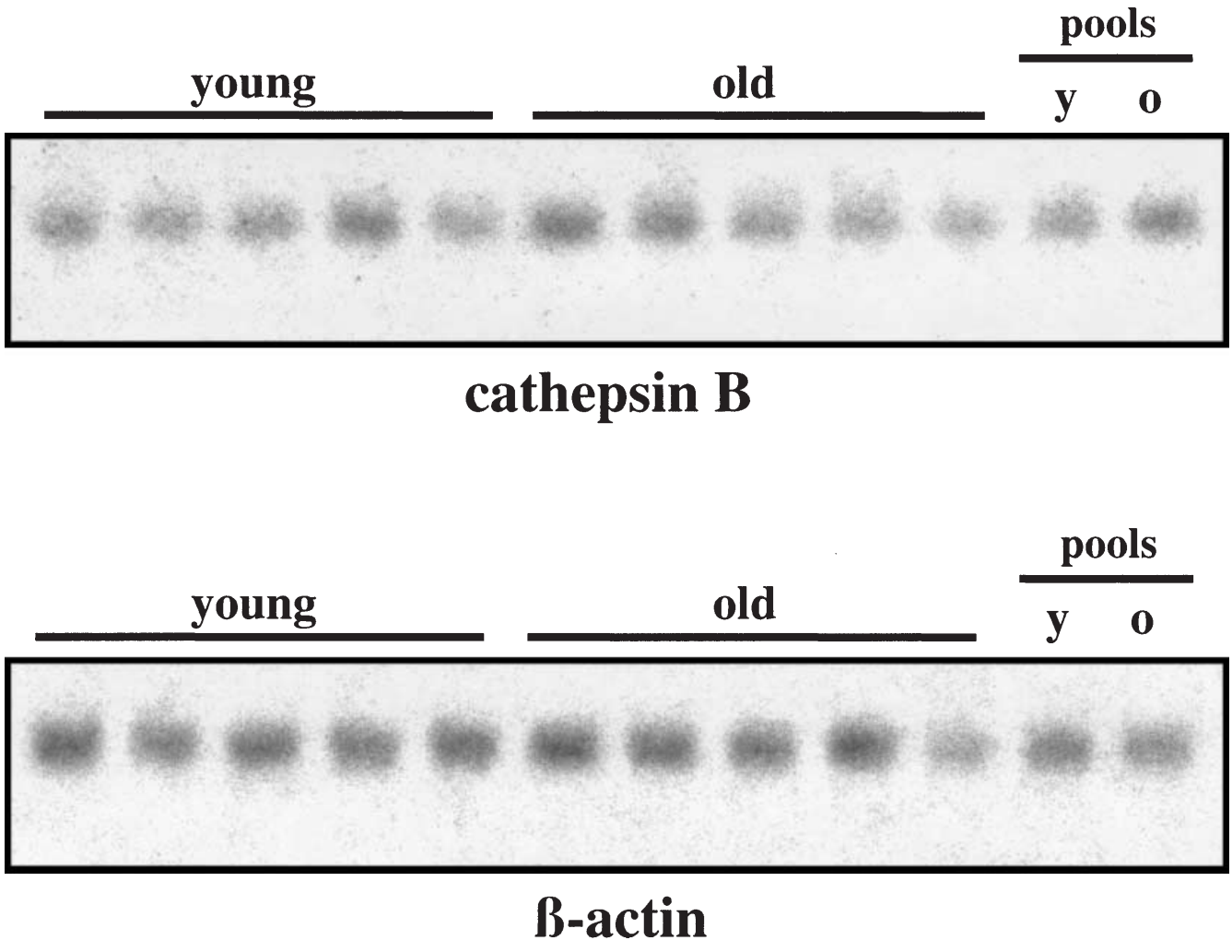

Fig. 4 Northern blot analysis of cathepsin B mRNA in the liver of young and old animals. Total liver RNA (25 µg) was fractionated on 1.5% agarose/glyoxal gels, transferred to GeneScreen, and hybridized to random prime-labeled human cathepsin B cDNA (*top*), and, after probe removal, to rat β-actin cDNA (*bottom*) as described in Materials and methods. We analyzed five individual animals of each age, as well as pools obtained from these same animals

The mean (± SEM) specific activity of cathepsin B for both age classes was 385±122 (range 250–522) and 96±16 (range 75–120) U/mg protein, respectively. This fourfold increase in the specific activity of cathepsin B in liver homogenates from old animals was found to be highly significant ($P \leq 0.001$) using Student's *t* test. There are several mechanisms that can account for increased cathepsin B activity in tissue homogenates from old rats. These include: (a) an increased synthesis, processing, and/or maturation of the proenzyme; (b) a delayed or reduced turnover of active enzyme; (c) a decreased expression of endogenous inhibitors; and (d) infiltration of the tissue by inflammatory cells expressing high levels of cathepsin B (Graf and Strauli 1983; Keppler et al. 1988). The last-mentioned possibility could be ruled out as we verified an absence of expression of α_1-acid glycoprotein, an early marker of inflammation (Sierra et al. 1989), in each rat. Mechanism (c) was also ruled out since we chose a preincubation time and dilution of tissue homogenates in which there was complete dissociation of enzyme-inhibitor complexes. Thus, potential differences in the levels of endogenous inhibitors, as we have previously shown to exist between liver homogenates of young and old rats (Keppler et al. 1997), did not interfere here with the determination of total cathepsin B activity.

Increased cathepsin B protein, but not mRNA, in aged rat liver

To discriminate between mechanisms (a) and (b) above, we compared the steady-state levels of hepatic cathepsin B mRNA and protein in young versus old animals. Northern blot analysis of total RNA extracted from individual rat livers indicated that the average level of expression of the 2.3-kb cathepsin B transcript did not change as a function of age (Fig. 4). The β-actin transcript used for normalization similarly did not change with age (Fig. 4). Extensive analysis of more than a hundred individual animals has shown that actin mRNA lev-

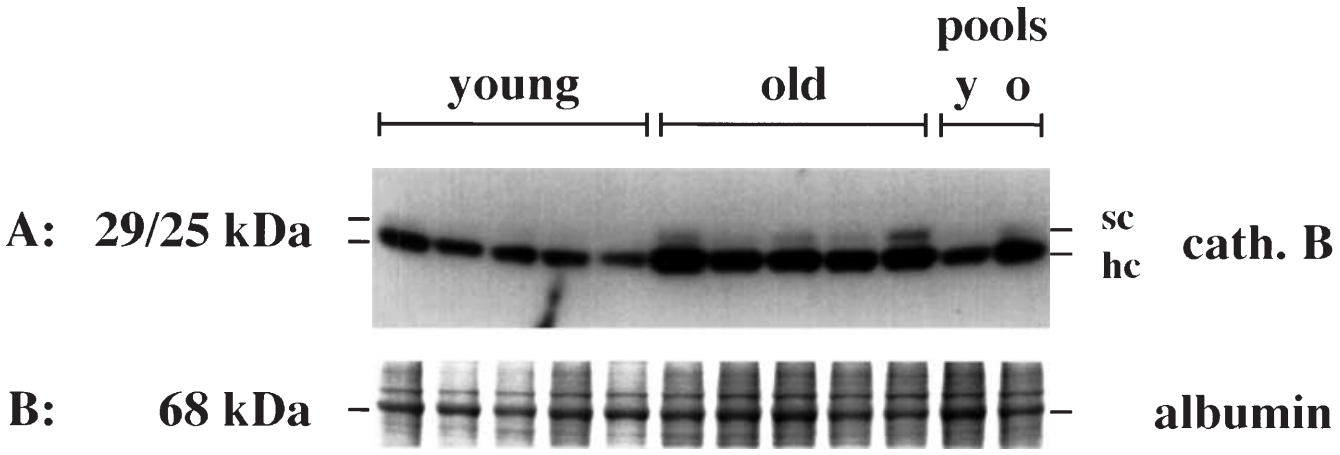

Fig. 5A, B Immunoblot analysis of cathepsin B forms in the liver of young and old animals. Fifty micrograms of proteins from whole liver homogenates was reduced and denatured before electrophoresis on 16% SDS-polyacrylamide gels. The resolved proteins in gel **A** were transferred to nitrocellulose membranes, and cathepsin B detected by immunoblotting with rabbit polyclonal antibodies and visualized by ECL. The abbreviations *sc* and *hc* stand for single-chain and heavy chain of mature cathepsin B forms (*cath. B*). In gel **B**, the separated proteins were stained with Coomassie Brilliant Blue G-250 to evaluate equal loading of hepatic proteins. Electrophoretic mobility was calibrated with prestained proteins of known molecular mass (rainbow markers)

els do not change as a function of age in rat liver (data not shown). Likewise, pools of total RNA did not show any changes in expression of the two transcripts upon aging of animals (Fig. 4). This suggests that there was no increase in hepatic expression of the cathepsin B gene or post-transcriptional stabilization of its mRNA upon aging of rats. In contrast to the Northern blots, immunoblotting for cathepsin B showed that the steady-state levels of the mature protein increased in old animals as compared to younger ones (Fig. 5A). Both the 29-kDa single chain (sc) and 25-kDa heavy chain (hc) of double-chain cathepsin B (Takio et al. 1983) were detected with the rabbit polyclonal antibody (Fig. 5A). Semiquantitative image analysis of protein bands indicated that both mature cathepsin B forms increased at least twofold when normalized against levels of albumin (the prominent Coomassie-stained 68-kDa band on Fig. 5B). Altogether, our results establish that the stability and/or turnover [point (b), above] of mature liver cathepsin B was most likely altered in aging rats when compared with young animals.

Discussion

The liver is one of the rat tissues showing the highest expression of lysosomal cathepsin B. This is true at the mRNA (San Segundo et al. 1986), protein (Kominami et al. 1985), and activity levels (Higashi et al. 1986). Based on the differential expression of cathepsin B transcripts in various rat tissues, San Segundo et al. (1986) proposed that the high expression in organs such as the kidney, the liver, and the brain could reflect a specialized function of cathepsin B in these organs. This is in agreement with early immunohistochemical studies showing that the great majority of hepatic cathepsin B is not present in hepatocytes but in the cells lining the sinusoidal walls (Graf and Strauli 1983; Ii et al. 1985). Sinusoidal cells, consisting of Kupffer cells (liver macrophages) and endothelial cells, are known to be important in the clearance of abnormal, foreign substances or glycoproteins from the circulation (Hubbard et al. 1979).

In the present study, we compared the steady-state levels of enzyme activity, protein, and mRNA of cathepsin B in the livers of young (15 months) and old (25 months) rats. Measurement of enzyme activity showed that the specific activity of cathepsin B was increased fourfold in liver homogenates of old animals when compared with young animals. This increase was paralleled by at least twofold changes in cathepsin B protein. In contrast, no significant changes were observed in the steady-state levels of cathepsin B transcripts. At this point, we cannot exclude the possibility that an increased translation rate of cathepsin B transcripts and/or processing/maturation rate of the proenzyme might be responsible for the observed increases in mature protein and enzyme activity in the liver of old rats. There are several concurrent observations which suggest that the increased cathepsin B levels in aged rat liver tissue might be the result of increased stability and/or impaired turnover of the mature protein. According to our present understanding of aging, there is a general decline in the rate of intracellular protein degradation (Reznick et al. 1981; Lavie et al. 1982; Ward 1988). There have not been many studies addressing the mechanism of catabolism of lysosomal enzymes to date. Given the high concentrations of peptidases in lysosomes, an intralysosomal pathway of (auto)digestion has been proposed (Tsujinaka et al. 1995). In the periportal reticuloendothelial system, the basal turnover rate of cathepsin B could be regulated by the amount of phagocytosed and/or endocytosed material.

We have previously identified and characterized a gene product, T-kininogen, whose expression is increased nine to tenfold during aging in the rat liver

(Sierra et al. 1989). T-kininogen is a plasma glycoprotein and its expression is strongly induced during inflammation (Chao et al. 1988). We have shown, however, that the increase in liver mRNA levels during aging of rats is not linked to inflammation since expression of other markers of the acute phase response are unchanged (Sierra et al. 1989). Increases in liver T-kininogen mRNA lead to a concomitant, albeit quantitatively less significant, increase in serum T-kininogen levels (Sierra et al. 1992). Rat T-kininogen accumulates at its site of synthesis, which is primarily the liver (Sierra et al. 1992), and also at many distant sites. Thus, kininogen has been detected by immunoenzymatic techniques in the perfused rat brain, heart, and kidney (Chao et al. 1988), organs which do not express detectable mRNA (Sierra et al. 1989). This suggests that rat T-kininogen, like other vasoactive kinin precursors (Muller-Esterl et al. 1986; Herwald et al. 1995), may be able to bind to specific cell surface receptors and undergo endocytosis. Since the best-characterized function of T-kininogen is that of an inhibitor of papain-like cysteine peptidases (Ohkubo et al. 1984), it is tempting to speculate that endocytosed T-kininogen could drastically impair the lysosomal protein degradation machinery. Indeed, inhibition of lysosomal cysteine peptidases, through injection of either leupeptin or E-64 into the brain or other organs of young mice, leads to ultrastructural changes that closely resemble the typical morphology of similar tissues derived from older animals (Ivy et al. 1991). These in vivo observations provide support for the "protease inhibitor model of lipofuscin formation" in aging processes. Since the liver is the primary site of synthesis of T-kininogen, it does not lend itself well for studies aimed at identifying the subcellular site of accumulation of the protein in aging rats. However, we have initiated studies on the effect(s) of high circulating T-kininogen in aging animals on nonproducing tissues such as the spleen. The immunofluorescent localization of T-kininogen and cathepsin B and the direct measurement of the proteolytic capacity of cells that have been grown in the presence of high concentrations of the inhibitor are also in progress in our laboratories.

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