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Stimulatory effect of vitamin C on autophagy in glial cells

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Abstract

Intracellular accumulation of damaged or abnormal proteins is a common event associated with numerous neurodegenerative diseases and other age-related pathologies. Increasing the activity of the intracellular proteolytic systems normally responsible for the removal of these abnormal proteins might be beneficial in lessening the severity or development of those pathologies. In this study we have used human astrocyte glial cells to investigate the effect of vitamin C (ascorbate) on the intracellular turnover of proteins. Supplementation of the culture medium with physiological concentrations of vitamin C did not affect protein synthesis, but did increase the rate of protein degradation by lysosomes. Vitamin C accelerated the degradation of intra- and extracellular proteins targeted to the lysosomal lumen by autophagic and heterophagic pathways. At the doses analyzed, vitamin C lowered and stabilized the acidic intralysosomal pH at values that result in maximum activation of the lysosomal hydrolases.

Keywords: ascorbic acid, astrocytes, cathepsins, lysosomes, protein degradation, vitamins.

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Intra- and extracellular deposits of abnormal or modified proteins are a common feature in different neurodegenerative disorders (Benowitz *et al*. 1989; Cataldo *et al*. 1996; Sapp *et al*. 1997). A failure in the mechanisms that normally eliminate the abnormal proteins from the brain has been proposed for many of these diseases (Nixon *et al*. 1994; Cataldo *et al*. 1996; Paresce *et al*. 1996). Similarly, a decrease in the total rates of protein degradation with age seems to contribute to the formation of protein plaques characteristics of the senile brain (Benuck *et al*. 1996; Rattan 1996). Increased protein breakdown may be desirable in all these conditions in which abnormal or damaged proteins accumulate inside cells. Interestingly, many pharmacological compounds have been developed to specifically inhibit different proteolytic systems (Murachi 1984; Seglen *et al*. 1990; Rock *et al*. 1994; Holen *et al*. 1995), but very few interventions have succeeded in improving intracellular protein degradation.

Different proteolytic systems participate in normal protein turnover and in the elimination of damaged proteins from inside cells (Cuervo and Dice 1998). The two main proteolytic systems are the ubiquitin/proteasome (DeMartino and Slaughter 1999), that preferentially degrades proteins with a short half-life, and the lysosomes that, with some exceptions, degrade long half-lived proteins (Seglen *et al*. 1990; Dice 2000). Depending on the cell type and the cellular conditions, lysosomal proteolysis can contribute to as much as 90% of the total intracellular protein breakdown (Seglen *et al*. 1990; Dice 2000).

Studies on different neurodegenerative diseases have focused mainly on the activity of the proteolytic systems inside neurons (Nixon *et al*. 1994; Paresce *et al*. 1996). However, detrimental protein deposits are localized not only inside neurons, but also in the extraneuronal medium. Proteolytic systems in glial cells are normally responsible for eliminating not only their own proteins, but also those secreted by neurons, which otherwise would accumulate in the extraneuronal space. Accordingly, increasing the proteolytic activity of the surrounding glial cells might reduce extraneuronal accumulation of proteins.

In this work, we intended to identify non-pharmacological compounds capable of stimulating the activity of the

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Abbreviations used: FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, human astrocyte; hsc73, heat shock cognate protein of 73 kDa; lamp, lysosome-associated membrane protein; MOPS, 3-(*N*-morpholino) propanesulfonic acid; RNase A, ribonuclease A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; VC, vitamin C.

housekeeping proteolytic systems in glial cells. Poor nutritional status and low levels of antioxidants are common among the elderly population and have been associated with several neurodegenerative diseases (Breitner 2000). New evidence reveals that supplementation with vitamins E and C may protect against dementia and improve cognitive function in late life (Masaki *et al*. 2000). However, the molecular mechanism for these protective effect remains unknown.

The goal of this study was to elucidate whether restoration of physiological levels of vitamin C (VC) inside cells will improve their ability to degraded intra- and extracellular proteins. With that purpose we have evaluated the effect of VC supplementation on the protein turnover in cultured human astrocyte (HA) glial cells. Our results show for the first time that enrichment of HA with physiological concentrations of VC significantly increases their lysosomal proteolytic activity.

Materials and methods

Materials

Sources of chemicals and antibodies were as described previously (Cuervo and Dice 1996; Cuervo *et al*. 1997; Martin and Frei 1997; Martin *et al*. 1999; Cuervo and Dice 2000). The monoclonal antibodies against the human lysosome-associated membrane proteins type 1 and 2 (lamp1 and lamp2) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The polyclonal antibody against cathepsin D was from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The polyclonal antibody against cathepsin L was a generous gift from Dr Gary Sahagian (Department of Physiology, Tufts University, Boston, MA, USA). Ammonium chloride, 3-methyladenine, chloroquine and fluorescein isothiocyanate conjugated to 70 000 mw dextran (FITC-dextran) were from Sigma (St Louis, MO, USA).

Cell culture

HA cells were from Clonetics Laboratories (BioWhittaker, Inc. Walkersville, MD, USA). Normal human astrocytes have been previously shown to be the only cellular component of this preparation. Cells were cultured under humidified 5% CO_2 in neurobasal medium (Gibco Laboratories, Life Technologies, Gaithersburg, MD, USA) supplemented with 8% fetal bovine serum, 20 ng/mL human recombinant growth factor, 6 μg/mL transferrin, 1.4 μg/mL insulin, 50 U/mL penicillin/streptomycin and 0.6 μg/mL anphitericine B (Sigma). Cells were plated in 1% gelatin-coated flasks or six-well plates (Corning Inc., Corning, NY, USA) and the medium was changed every 2 days until they reached confluence. Cells of the fourth to seven passage were used for the experiments. Cell viability was determined by the trypan blue exclusion assay (Freshney 1994). Characterization of the cells as astrocytes was done by glial fibrillary acid protein staining routinely after every two passages. Only cells with preserved staining for the acid protein were used in this study. To deprive cells from serum, plates were extensively washed with Hanks' balanced salts' solution (Life Technologies) and fresh medium without serum was added. We did not detect any changes in cell viability or apoptotic manifestation in HA cells after removal of serum for the periods of time indicated in this study. All experiments were performed in confluent non-dividing HA.

Ascorbate quantification

Intracellular levels of ascorbate were measured by paired-ion reversed-phase HPCL coupled with electrochemical detection as described before (Martin *et al*. 1999). In brief, cells scraped from culture dishes were washed twice with phosphate buffer saline, to remove any residual gelatin from the plate coat, and then homogenized in a Dounce homogenizer with a tightly fitting pestle. Proteins in the cellular homogenate were precipitated with 5% (w/v) metaphosphoric acid, 1 mM diethylenetriaminepentaacetic acid and supernatants were subjected to chromatography on a LC8 column (Supelco, Bellefonte, PA, USA) using 1% methanol, 40 mM sodium acetate, 1.5 mM dodecyltriethylammonium phosphate (Q12 ion pair cocktail; Regis, Morton Grove, IL, USA) as the mobile phase. Samples were injected with an autosampler, 1100 series (Hewlett Packard, Palo Alto, CA, USA). Ascorbate was detected at an applied potential of +0.6 V by an LC 4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN, USA). Ascorbate was eluted as a single peak with a retention time of 5.5 min Peaks were integrated with a ChemStation (Hewlett Packard). Ascorbate concentration was calculated based on a calibration curve, and was expressed in nmol/mg protein.

Measurement of protein synthesis

Rates of protein synthesis were measured in confluent cells grown in six-well plates as the incorporation of [^{3}H]leucine into acid-insoluble material in the presence of an excess (2.8 mM) unlabeled leucine in the medium. Under those conditions incorporation of radioactivity into protein accurately reflects rates of proteins synthesis (Gulve and Dice 1989) and minimizes differences due to alteration of amino acid transport and/or intracellular amino acid pool sizes (Schneible *et al*. 1981). At different times after incubation with 10 μCi/mL [^{3}H]leucine cells were extensively washed, collected and precipitated with 10% trichloroacetic acid. The amount of acid-precipitable radioactivity in each sample 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. To study the electrophoretic pattern of the newly synthesized proteins cells were incubated as above but with 0.2 mCi/mL of [^{35}S]methionine/cysteine mixture (Easy Tag-Express; NENLife Science Products, Boston, MA, USA) and then extensively washed and lysed in lysis buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)]. Lysated were cleared by centrifugation, and supernatants were processed for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and fluorography. Proteins were visualized after exposure to a PhosphorImager screen in a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA, USA). Where indicated VC was added to the cells 7 h before labeling and the same concentration was maintained during the labeling.

Measurement of protein degradation

Protein degradation was measured as described previously (Cuervo and Dice 1996). Briefly, confluent cells labeled with 2.5 μCi/mL [^{3}H]leucine for 48 h were then extensively washed and maintained in medium containing an excess of leucine and supplemented or not

with 10% fetal calf serum. Aliquots of the medium were taken at the indicated times, and acid-soluble (amino acids) and acid-precipitable (proteins and peptides) radioactivity was measured as described above. Degradation rates of short-half life proteins were determined by the same procedure but after a labeling period of 1 h at 37°C. Where indicated cells were supplemented with VC for the last 7 h of the labeling time, as well as throughout the chase period.

Isolation of lysosomes

Lysosomes from cultured cells were isolated in percoll/metrizamide discontinuous gradients as previously described (Storrie and Madden 1990). β-Hexosaminidase (EC 3.2.1.52) latency was measured as an index for the integrity of the lysosomal membrane after isolation (Storrie and Madden 1990). Preparations with more than 10% broken lysosomes were systematically discarded. Lysosomal matrices and membranes were isolated after hypotonic shock of the lysosomal fraction followed by centrifugation as previously described (Ohsumi *et al*. 1983).

Degradation of proteins by isolated lysosomes

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.13) was radiolabeled with [^{14}C]formaldehyde by reductive methylation (Jentoft and Dearborn 1983) to a specific activity of 1.2×10^6 dpm/nmol, and ribonuclease A (RNase A; EC 3.1.27.5) with [NaB_3H_4] to 1.1×10^7 dpm/nmol. The pool of cytosolic proteins was obtained by metabolic labeling of NIH3T3 cells with [^{3}H]leucine as above (5000 dpm/μg protein). Degradation of radiolabeled proteins by isolated intact lysosomes or lysosomal matrices was measured as described before (Cuervo and Dice 1996; Cuervo *et al*. 1997). Briefly, lysosomes were incubated in MOPS buffer [10 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS) pH 7.3, 0.3M sucrose, 1 mM dithiothreitol, 5.4 μM cysteine] with 260 nM radiolabeled protein for 30 min at 37°C. Reactions were stopped with 10% trichloroacetic acid and filtered through a Millipore Multiscreen Assay System (Millipore, Bedford, MA, USA) using a 0.45-μm pore filter. Radioactivity in the flow through and in the filter was measured as described above. Proteolysis was expressed as the percentage of the initial acid-precipitable radioactivity transformed to acid-soluble radioactivity at the end of the incubation. Degradation of proteins by isolated lysosomal matrices was measured as above, but incubations were performed for 15 min and in 1 mM dithiothreitol supplemented water to maintain the final pH close to the one in the lysosomal lumen.

Endocytosis of proteins by cultured cells

[^{3}H]RNase A was added into the medium of confluent HA cells and aliquots of the medium were taken at different times. After precipitation with trichloroacetic acid, the amount of acid-precipitable radioactivity remaining in the medium for each time was measured. To measure the degradation rates of endocyted proteins once in the lysosomal compartment, cells were allowed to endocyte [^{3}H]RNase A for 12 h and then, after extensive washing, fresh medium was added, and aliquots of the medium were removed at different times. The amount of acid-soluble radioactivity in each sample was determined after precipitation with trichloroacetic acid as above.

Measurement of intralysosomal pH

The intralysosomal pH was monitored as described before (Ohkuma *et al*. 1982) by measuring changes in the fluorescence of endocyted FITC-dextran. FITC-dextran (1 mg/mL) was added to the culture media of confluent HA cells for 20 h and then lysosomes were isolated as above. The FITC-fluorescence in the lysosomal fraction was measured at 495 nm (pH-sensitive fluorescence) and 450 nm (pH insensitive fluorescence) excitation and 550 nm emission wavelengths. Double standard curves were prepared by measuring the ratio of fluorescence intensities at 495 nm/450 nm for 2 mg of FITC-dextran at different pH and in different buffers, and by comparing the fluorescence intensities at 495 nm excitation, in intact lysosomes and after disruption with 0.1% Triton X-100. Where indicated, VC was administered 7 h before adding the fluorescent probe, and maintained until the lysosomal isolation. As described in this work, VC supplementation does not modify rates of fluid-phase endocytosis, and consequently the amount of probe internalized was comparable in treated and control cells. In some experiments, after FITC-endocytosis cells were fixed and processed for confocal microscopy as described before (Cuervo and Dice 1996). Images were acquired as single scans on a Bio-Rad MRC-1024 confocal microscope (Bio-Rad Laboratories, Hercules, CA, USA) equipped with an argon laser. All digital microscopic images were prepared using Adobe PHOTOSHOP 5.0 software (Adobe Systems Inc., Mountain View, CA, USA).

General methods

Protein concentration was determined by the Lowry method (Lowry *et al*. 1951) using bovine serum albumin as a standard. Lysosomal enzymatic activities (β-hesosaminidase, cathepsin L (EC 3.4.22.15) and cathepsin B (EC 3.4.22.1) were measured as reported previously (Storrie and Madden 1990; Cuervo *et al*. 1997). After SDS–PAGE (Laemmli 1970) gels were subjected to silver staining (Wray *et al*. 1981) or immunotransferred to nitrocellulose membranes using a Min-TransBlot SD wet transfer cell (Bio-Rad, Richmond, VA, USA). Immunoblotting of the nitrocellulose membranes was performed following standard procedures (Towbin *et al*. 1979). The proteins recognized by the specific antibodies were visualized by chemiluminescence methods (RenaissanceR; NEN-Life Science Products, Boston, MA, USA). Densitometric quantification of the immunoblotted membranes was performed with an Image Analyzer System (Inotech S-100; Inotech, Sunnyvale, CA, USA). The Student's *t*-test was used for statistical analyses.

Results

Incorporation of VC into HA cells in culture

We first measured the uptake of VC by HA cells normally maintained in low VC (1–5 μM) culture medium. As shown in Fig. 1, these cells contained very low endogenous levels of VC (0.5 ± 0.2 nmol/mg protein, less than 0.1 mM), compared with the 1–3 mM normally present in astrocytes in different brain regions (Oke and Adams 1987; Rice and Russo-Menna 1998). Supplementation with 100 μM VC led to a time-dependent intracellular accumulation of the vitamin (Fig. 1a). Intracellular levels of VC reached a maximum of 5.2 ± 0.65 nmol/mg protein (1.3 mM) after 7 h of incubation. Using this incubation time we found that the intracellular levels of VC increase linearly to the increase of VC

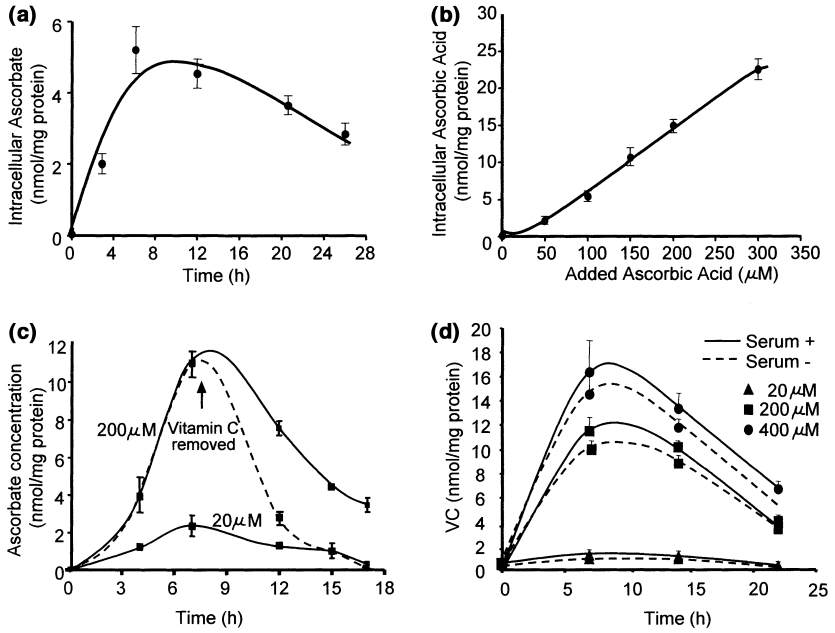

Fig. 1 Time- and dose-dependent uptake of VC by HA in culture. (a) Confluent human astrocyte (HA) were supplemented with 100 μM vitamin C (VC) in the medium for up to 24 h. At indicated times levels of intracellular VC were determined as described in Material and methods. (b) HA cells were incubated with increasing concentrations of VC (as indicated), and after 7 h the concentration of intracellular VC was determined as above. (c) HA cells were incubated with the indicated concentrations of VC. After 7 h the medium of part of the cells supplemented with 200 μM VC was removed and replaced by fresh medium without VC (dashed line). VC content was measured at the indicated times as above. (d) HA cells were incubated with the indicated concentrations of VC in medium supplemented (serum +, solid line) or not (serum –, dashed line) with 8% fetal bovine serum. Intracellular content of VC was measured at the indicated times as above. Results shown en each panel are mean ± SE of three different experiments. VC levels are expressed as nmol/mg protein.

supplemented in the extracellular medium. Under basal conditions, the concentration of VC in the extracellular brain fluid is approximately 200–400 μM (Svensson *et al*. 1998). We found intracellular concentrations of VC similar to physiological levels (12.6 ± 0.9 nmol/mg protein; 3.1 mM) after supplementation of the medium with 200 μM VC for 7 h (Fig. 1b).

Interestingly, intracellular levels of VC rapidly decreased when the vitamin was removed from the culture medium. We found a 76% decrease in the intracellular levels of VC 5 h after its removal from the culture medium (Fig. 1c). Therefore, to analyze the effect of VC on protein turnover we supplemented the cells with the desired concentration of VC in the medium for 7 h, and maintained those concentrations for the duration of the experiment.

VC is normally sensitive to rapid oxidation into dehydroascorbic acid, however, we could detect 69.2 ± 2.5% and 84.9 ± 1.5% of the VC initially added in the extracellular medium after 7 h of incubation with 200 or 400 μM VC, respectively. Components of the fetal bovine serum added to the medium seem to have a potent stabilizing effect on the extracellular VC, because the half-life of VC was significantly shorter in serum-free medium (VC levels decreased to 12.1 ± 0.4% and 21.0 ± 2.8% of its initial values after 7 h incubation in cells supplemented with 200 or 400 μM VC, respectively). Interestingly, as shown in Fig. 1(d), for each dose of VC analyzed, intracellular levels of VC were comparable in cells maintained in the presence and in the absence of serum during the whole incubation period.

Effect of VC on intracellular protein turnover

When we measured protein synthesis in HA cells in culture we found that the total amount of a radiolabeled amino acid mixture ([^{35}S]methionine and cystein) incorporated into proteins at different times was similar in cells maintained in the presence or absence of VC (Fig. 2a). In addition, we detected similar electrophoretic patterns for the radiolabeled proteins in both groups of cells (Fig. 2b).

We then compared the total rates of protein degradation in HA cells treated or not with VC. Supplementation of cells with 200 μM VC significantly increased the degradation rate of proteins with long half-life (20% increase in percentage protein degraded per hour; Fig. 3a), but did not change the degradation rate of short half-life proteins (Fig. 3b). When the vitamin was removed from the cultured medium, protein degradation rates returned to values close to non-treated cells (Fig. 3a). The dose-dependent effect of VC on the degradation of individual proteins identified after electrophoretic

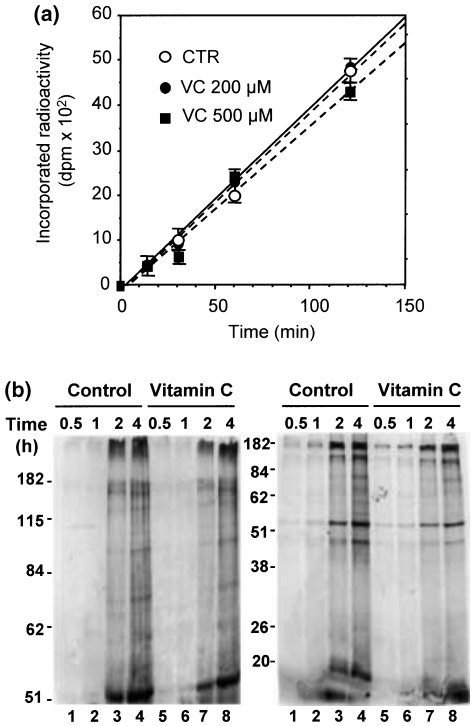

Fig. 2 Effect of vitamin C (VC) on protein synthesis in human astrocyte (HA). Confluent HA cells were maintained without additions (CTR) or supplemented with the indicated concentrations of VC. After 7 h at 37°C radiolabeled amino acids [[^{3}H]leucine (a) or a mixture of [^{35}S]cysteine/methionine (b)] were added to the medium, and at the indicated times cells were collected and precipitated with trichloroacetic acid (a) or processed for SDS–PAGE and fluorography (b). Values in (a) indicate the radioactivity incorporated in the acid-precipitable fraction and are mean ± SE of three different experiments.

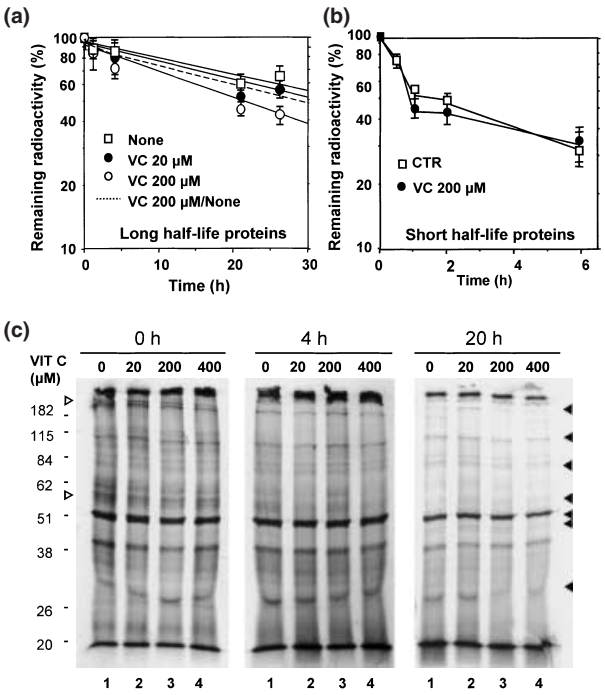

Fig. 3 Effect of vitamin C (VC) on total intracellular protein degradation. (a and b) Confluent HA were labeled with [^{3}H]leucine for two days (a) or 1 h (b) as described under Material and methods. During the last 7 h of the labeling period (a) or 7 h before the labeling (b) part of the cells were supplemented with the indicated concentrations of VC. After extensive washing, fresh medium with or without (CTR) VC was added, and protein degradation was measured as described in Material and methods. Values are mean ± SE of three different experiments. (c). Confluent HA cells were labeled with a mixture of [^{35}S]cysteine/methionine for 2 days. Samples were collected as in (a) and processed for SDS–PAGE and fluorography. Arrowheads indicate proteins with accelerated degradation in the VC group.

separation is shown in Fig. 3(c). VC supplementation did not modify the degradation of proteins with a half-life shorter than 4 h, but accelerated, in a dose-dependent manner, the degradation of proteins still present inside the cell 20 h after synthesis (long half-life proteins).

Proteolytic systems activated by VC

With some exceptions, proteins with a long half-life are predominantly degraded in lysosomes. We found that in the presence of ammonium chloride, a classical lysosomal inhibitor, the stimulatory effect of VC on the degradation of proteins in HA cells was almost completely abolished (Fig. 4a).

As described for many other cell types (Seglen *et al.* 1990; Dice 2000), we also found in confluent HA cells that rates of protein degradation increased during serum deprivation (Fig. 4b). We found that concentrations of VC as low as 20 µM, that did not change degradation rates in serum-supplemented cells (Fig. 3a), significantly increased protein breakdown in HA cells maintained in the absence of serum (Fig. 4b). The predominant effect of VC on the degradation of proteins with a long half-life, the blockage of that effect by ammonium chloride, along with the higher stimulatory effect of VC on cells deprived of serum, strongly suggest that VC preferentially stimulates protein degradation in lysosomes.

The VC-mediated increase in lysosomal protein degradation in HA results from the simultaneous activation of several lysosomal pathways. In serum-deprived HA treated with 3-methyladenine, a specific inhibitor of macroautophagy (Seglen *et al.* 1990), we only observed a partial decrease in the stimulatory effect of VC on protein degradation (Fig. 5a). In contrast, when we blocked the degradation of proteins in the lysosomal lumen with ammonium chloride, the stimulatory effect of VC was completely abolished (Fig. 5a). These results suggest that VC stimulates macroautophagy but also other lysosomal pathways active under these conditions, such as chaperone-mediated autophagy.

Finally, when we compared the rates of fluid-phase endocytosis in control and VC supplemented cells (Fig. 5b,

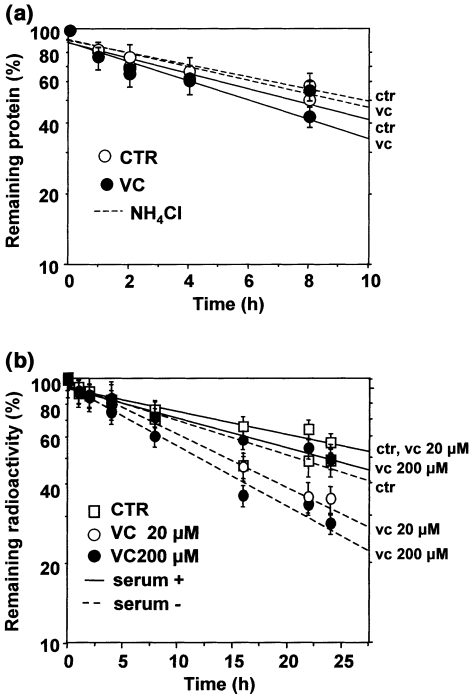

Fig. 4 Proteolytic system(s) involved in the stimulatory effect of vitamin C (VC) on intracellular protein degradation.(a) Total rates of protein degradation were measured in confluent control (CTR) and VC 200 µM supplemented human astrocyte (HA) as described in Fig. 3(a). Where indicated ammonium chloride (10 mM) was added during the chase period. Values are mean ± SE of four different experiments. (b) Total rates of protein degradation were measured as in Fig. 3(a) in control (CTR) and 200 µM VC-treated cells maintained in medium supplemented (serum +) or not (serum –) with 10% fetal calf serum, as indicated. Values are mean ± SE of three different experiments.

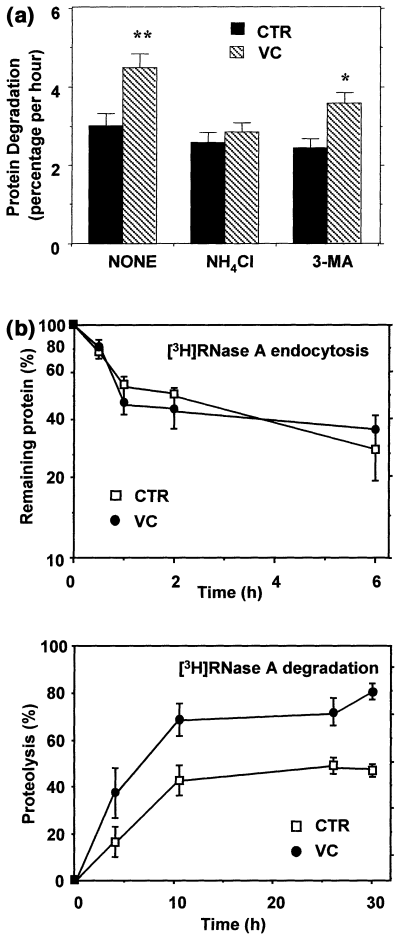

Fig. 5 Effect of vitamin C (VC) on different lysosomal pathways. (a) Control human astrocyte (HA) (CTR) and HA supplemented with 200 µM VC were labeled with [^{3}H]leucine for two days and then maintained in serum-free medium without additions (NONE) or with 10 mM ammonium chloride (NH_4Cl) or 10 mM 3-methyl adenine (3-MA), as indicated. The rate of protein degradation in each group was measured as described in Material and methods. Values are mean + SE of triplicate samples in two different experiments. Differences from control values are significant with $^*p < 0.05$ and $^{**}p < 0.005$. (b) Upper panel: [^{3}H]RNase A was added into the incubation medium of confluent control (CTR) and 200 µM VC-treated HA. At the indicated times the percentage of protein internalized by the cells was measured as described in Material and methods. The percentage of initial protein (acid-precipitable radioactivity) remaining in the medium at each time is shown. Values are the mean ± SE of triplicate samples from two different experiments. Lower panel: The degradation of [^{3}H]RNase A endocyted during 10 h by control (CTR) and 200 µM VC supplemented HA was measured as described in Material and methods. Values are the mean ± SE of triplicate samples from two different experiments and are expressed as percentage of initial acid-precipitable radioactivity transformed in acid-soluble radioactivity for each time.

upper), we did not find any significant effect of VC on the internalization of radiolabeled RNase A, even at the highest concentrations of VC tested. However, once in the lysosomal compartment, RNase A was degraded faster in VC-supplemented cells than in control cells (Fig. 5b, lower). It is unlikely that VC directly modifies the sulfhydryl groups of RNase A, making it more susceptible to degradation by lysosomal proteases, since we obtained similar results using a synthetic peptide lacking any of those groups (data not shown). Thus, the effect of VC seems to be mostly targeted to the lysosomal activity.

Direct effect of VC on the lysosomal compartment

We did not find significant differences in the purity of the lysosomal fractions isolated from control and VC supplemented HA cells (data not shown). The total protein content in lysosomes isolated from cells supplemented with VC was lower than in control cells (Table 1). Interestingly, the total activity of the two lysosomal proteases analyzed (cathepsin B and cathepsin L) did not change after treatment with VC, though their specific activities, as expected from their differences in protein content, were higher in the lysosomes from VC-treated HA (Table 1). For unknown reasons, but in agreement with previous studies (Hoehn and Kanfer 1980;

		β-HEX		CATH B		CATH L	
	Protein (μg)	Units	U/mg	Units	U/mg	Units	U/mg
Control	523 ± 2	390 ± 1	745 ± 3	385 ± 2	736 ± 4	268 ± 1	512 ± 3
VC (200 μM)	335 ± 3	490 ± 3	1462 ± 6	376 ± 3	1122 ± 7	300 ± 2	895 ± 5

Table 1 Enzymatic characterization of lysosomes from control and vitamin C (VC) supplemented human astrocyte (HA)

Lysosomes were isolated from non-supplemented (control) and 200 μM VC supplemented HA as described in Material and methods. Total protein content, and the activities of β-hexosaminidase (β-HEX) and cathepsins B and L (CATH B, CATH L) were measured by standard procedures (Lowry *et al.* 1951; Storrie and Madden 1990). Enzymatic activities are expressed as total units (total activity) and units per mg of protein (specific activity).

Rathi *et al*. 1984), the total activity of hexosaminidase was significantly higher in cells supplemented with VC.

We found similar electrophoretic patterns for the lysosomal proteins in both groups of cells, but differences in the content of specific proteins (Fig. 6a, left, indicated by arrowheads). Using specific antibodies we detected a higher content on both, membrane (lamp2a and lamp1) and matrix proteins (cathepsin L and D) in lysosomes isolated from HA supplemented with VC (Fig. 6a, right, lanes 3–6). These differences were not evident when we compared the total levels of the same proteins in homogenates (Fig. 6a, right, lanes 1, 2), suggesting that the content of exogenous non-lysosomal proteins is lower in lysosomes from VC treated cells, probably due to their more rapid degradation inside lysosomes from treated cells.

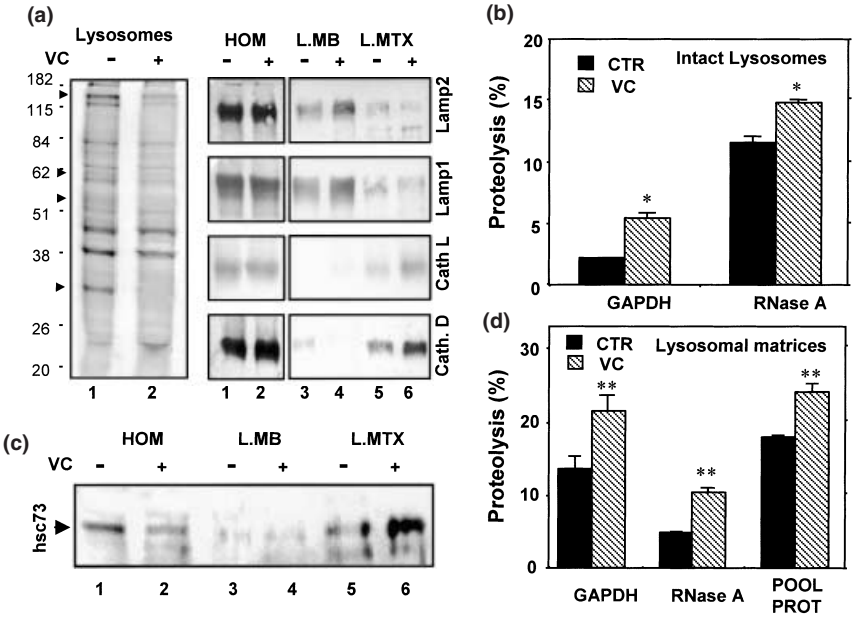

Fig. 6 Effect of vitamin C (VC) on the uptake and degradation of proteins by isolated lysosomes. (a) Homogenates (HOM; 100 μg protein), and the membranes (L.MB) and matrices (L.Mtx) from lysosomes (25 μg protein) isolated from HA cells supplemented (+) or not (–) with 200 μM VC, were subjected to SDS–PAGE and immunoblot with specific antibodies for the lysosome associated membrane proteins 1 and 2 (lamp1 and 2) and for cathepsin D and L (Cath D and L), as labeled. Left panel shows the electrophoretic pattern of lysosomes (10 μg protein) visualized after silver staining of the gel. Arrowheads indicate bands with different intensity in both groups. (b) Lysosomes (50 μg protein) isolated from control (CTR) and 200 μM VC-supplemented human astrocyte (HA), were incubated with the indicated radiolabeled substrates [glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribonuclease A (RNase A)] in MOPS buffer. Proteolysis was measured as described in Material and methods. Values are mean + SE of three different experiments. Differences from control are significant for $^{*}p < 0.05$. (c) Immunoblot for hsc73 of homogenates (100 μg protein) and lysosomal membranes and matrices (15 μg protein) isolated from cells supplemented (+) or not (–) with 200 μM VC. (d) Lysosomal matrices (25 μg) isolated from lysosomes of control (CTR) and 200 μM VC-supplemented HA were incubated with the indicated substrate proteins in water. Protein degradation was calculated as in (b). Values are mean + SE of three different experiments and differences from control are significant for $^{**}p < 0.005$.

Previous studies reported a dose-dependent destabilizing effect of VC on the lysosomal membrane (Hoehn and Kanfer 1980; Rathi *et al*. 1984). However, we found that the concentrations of VC that stimulated protein degradation in HA did not affect the stability of the lysosomal membrane, measured as β-hexosaminidase latency (data not shown).

We then used an *in vitro* system, previously developed in our laboratory, to analyze the effect of VC on the ability of isolated intact lysosomes to take up and degrade different substrate proteins (Cuervo and Dice 1996; Cuervo *et al*. 1997). We found that the degradation rates of both, GAPDH and RNase A, previously identified as substrate for chaperone-mediated autophagy, were higher in lysosomes isolated from VC supplemented cells (Fig. 6b). We have shown previously that the limiting step in this process is the uptake of substrate proteins through the lysosomal membrane, rather than their degradation once in the lysosomal lumen (Cuervo and Dice 2000). Consequently, VC has a moderate stimulatory effect on the chaperone-mediated transport of proteins into lysosomes. Uptake of substrate proteins by this pathway depends on the lysosomal levels of lamp2a, the receptor at the lysosomal membrane, and of the heat shock cognate protein of 73 kDa (hsc73), an intralysosomal chaperone (Cuervo *et al*. 1995; Cuervo *et al*. 1997; Cuervo and Dice 2000). We found that lysosomes from VC supplemented cells contained higher levels of hsc73 in their lumen (Fig. 6c).

Interestingly, when we eliminated the transport step by incubating the same substrates with broken, instead of intact lysosomes, the differences between the control and the VC-supplemented group were even higher (Fig. 6d). These results suggest that though VC moderately increases the uptake of cytosolic proteins into lysosomes, its most striking effect is to accelerate their degradation once in the lysosomal lumen.

VC-mediated changes in the intralysosomal pH

After subcellular fractionation we found that a portion (0.5%) of the total intracellular VC could be detected inside the lysosomal compartment. Supplementation of the culture medium with VC significantly increased intralysosomal levels of VC in a dose-dependent manner. We found a four-fold increase in the levels of VC in lysosomes from cells supplemented with 200 μM VC compared with cells supplemented with 20 μM VC.

VC might thus increase the lysosomal proteolytic activity by directly activating the lysosomal proteases or by inducing changes in the intralysosomal environment that facilitate protein degradation. As we have described before, we did not find a significant increase in the enzymatic activity of cathepsin B or cathepsin L in VC treated cells (Table 1).

We next compared the intralysosomal pH in lysosomes from control and VC supplemented cells. As shown in Fig. 7(a), the morphology and intracellular distribution of the acidic vesicular compartment, labeled by endocytosis of FITC-dextran, was similar in control and VC-supplemented cells. However, the fluorescence signal was brighter in the group of cells supplemented with VC (note the different gain settings in both pictures), suggesting higher acidification. We did not find changes on the fluorescence signal when FITC-dextran was incubated in a pH-neutralized solution of VC, discarding a possible direct effect of VC on FITC fluorescence. To quantify the differences in the intralysosomal pH we isolated the FITC-loaded lysosomes from both groups of cells. The basal pH values of the lysosomes from VC treated cells were slightly lower than from control cells (pH of 5.5 and 5.3 in control and VC, respectively). Interestingly, the lysosomal pH of VC-supplemented cells remained acidic even in the presence of agents such as NH_4Cl or cloroquine, that raised the pH toward almost neutral values in control cells (Fig. 7b). This stabilizing effect of VC on the pH of isolated lysosomes was also evident in the cultured cells. Thus, the inhibitory effect on protein degradation observed during treatment with ammonium chloride in both, control and VC supplemented HA, was gradually lost after 10 h in the group receiving VC but not in the control group (Fig. 7c).

Our results suggest that supplementation of HA with VC stimulates intralysosomal protein degradation, probably by lowering and stabilizing the intralysosomal pH, at values at which the lysosomal proteases reach their maximal proteolytic activity.

Discussion

Cells in culture, including HA, can readily be enriched in VC using physiological concentrations of the vitamin in the culture medium. Supplementation of HA cells with those concentrations of VC increases the intracellular rates of protein degradation. Lysosomes are the main target for the VC effect since: (i) VC supplementation preferentially stimulates degradation of long half-life proteins, typical substrates for lysosomal degradation (Fig. 3); (ii) the stimulatory effect of VC can be inhibited by blocking lysosomal degradation with ammonium chloride (Fig. 4a); (iii) the effect of VC on protein degradation is even more marked under conditions of nutritional deprivation, when lysosomes are maximally activated (Fig. 4b); and (iv) lysosomes isolated from VC supplemented cells show lower content of non-lysosomal proteins (Fig. 6a and Table 1) and higher proteolytic ability (Figs 6b and d). VC seems to stabilize the intralysosomal pH at acidic values optimal for the lysosomal hydrolases (Fig. 7).

A reduction of the levels of VC in serum has been described in patients with dementia and in different neurodegenerative diseases (Sinclair *et al*. 1998; Foy *et al*. 1999). In addition, VC has been shown to have cerebroprotective effects against toxic-mediated and ischemic damage (Plotnikov *et al*. 2000; Polidor *et al*. 2001). Some of the mechanisms for this protective effect of VC in the brain start now to be elucidated (i.e. its role as neuromodulator of

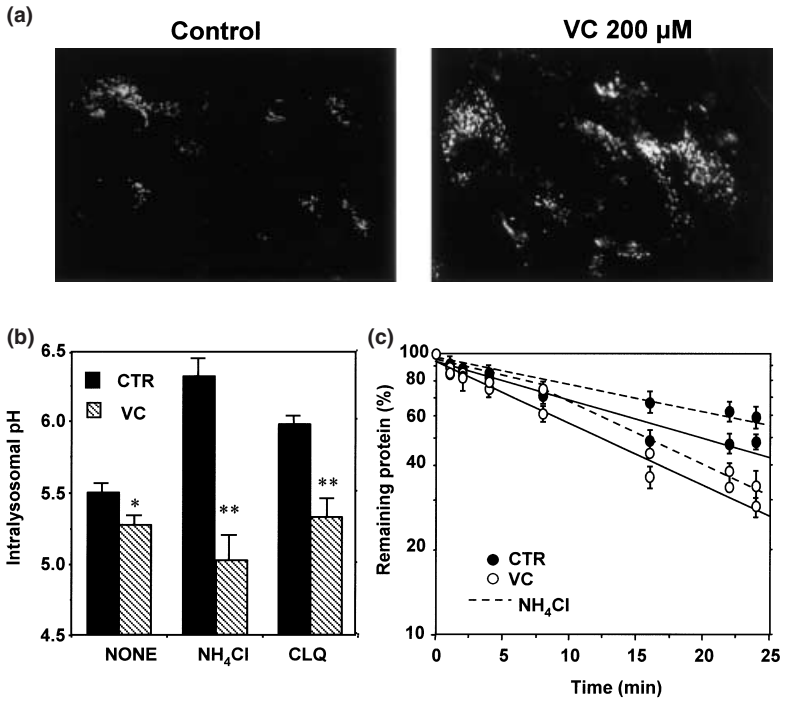

Fig. 7 Effect of vitamin C (VC) on intralysosomal pH. (a) The acidic vesicular compartment of control (left) and human astrocyte (HA) cells supplemented with 200 µM VC (right) was visualized by confocal microscopy after FITC-dextran internalization. Gain in right was set at 1/50 of gain in left. Bar = 1 µm (b) FITC-dextran loaded lysosomes from control (CTR) or 200 µM VC treated HA were incubated in an isotonic medium without additions (None) or in the presence of 10 mM ammonium chloride (NH_4Cl) or cloroquine (CLQ) for 20 min at 25°C. At the end of the incubation intralysosomal pH was calculated from the changes in FITC-dextran fluorescence as described in Material and methods. Values are mean + SE of triplicate samples from two different experiments. Differences from control are significant for $^*p < 0.05$ and $^{**}p < 0.005$. (c) Total rates of protein degradation were measured as in Fig. 3 (a) in control (CTR) and 200 µM VC-treated cells maintained in serum-free medium. Where indicated, 10 mM NH_4Cl was added during the chase. Values are mean ± SE of three different experiments.

glutamate-mediated neurotransmission, protecting against glutamate toxicity, or its unique antioxidant effect in the brain extracellular microenvironment (Grunewald 1993; Kiyatkin and Rebec 1998; Rice 2000). However, other physiologic roles for VC, including its possible modulatory effect on the function of different intracellular organelles, remain unknown.

At the concentrations of VC used in this work we did not observe any VC-mediated toxic effects related to oxidative stress, as determined by cell viability and morphological characteristics of the cells (data not shown). Both a stabilizing (Chari *et al*. 1983; Grabarczyk *et al*. 1983) and a destabilizing (Imai *et al*. 1995) effect of VC on the lysosomal membrane have been reported. We did not find significant changes in the stability of the lysosomal membrane in HA supplemented with 20–200 µM VC. Furthermore, parallel studies in our laboratory have shown that HA supplemented with 200 µM VC are significantly better protected than non-supplemented control cells against pro-inflammatory cytokines, ethanol or oxidized lipids, such as oxidized LDL (Martin and Frei 1997; A. Martin and N. Nguyen, unpublished observations).

Similar to what has been described in plasma (Dhariwal *et al*. 1991) VC is mostly present in its reduced form in the medium of cells supplemented with serum. Surprisingly, in the absence of serum, despite the decreased half-life of VC in the culture medium, the intracellular levels of VC are similar to the ones reached in serum-supplemented cells (Fig. 1d). It is possible that under those conditions part of the oxidized VC in the medium is transported into the cells in the form of dehydroascorbic acid, and then converted again to ascorbic acid through glutathione and NAD(P)H-dependent process (Siushansian *et al*. 1997). However, this does not seem to be the main mechanism by which intracellular VC levels are maintained in serum deprived cells, because we did not detected any signal of cellular stress resulting from abnormal consumption of glutathione or NAD(P)H in those cells. It is thus more likely that the replenishment of the intracellular storage of VC might be achieved during the first hours of VC supplementation, and that the small amount of non-oxidized

VC present in the medium after that might be enough to maintain the intracellular pool.

Previous studies have reported VC-dependent changes in lysosomal morphology, and in the activity of several lysosomal enzymes (Leveille and Schwartz 1982; Grigor'ev 1988). High concentrations of ascorbic acid in plasma increase the number of lysosomes and the formation of vesicular structures in sites where neurons are adjacent to astrocytes (Grigor'ev 1988). However, we did not observe changes in the number or morphology of the components of the lysosomal system in HA cells after VC supplementation (Fig. 7a). Whether the lack of effect is related to the concentrations of VC reached in glial cells compared to neurons, or to peculiarities of the lysosomal system in these cells requires further investigation.

An inverse relationship between tissue concentrations of VC and the activity of several lysosomal enzymes such as hexosaminidase, arylsulfatase A and B and acid phosphatase, has been described (Leveille and Schwartz 1982). Interestingly, similar to our findings in HA, the total activity of most cathepsins remains unchanged after VC supplementation (Hoehn and Kanfer 1980). Our results suggest that VC, rather than directly activating the lysosomal proteases, might instead contribute to creating an intralysosomal environment ideal for the activity of these enzymes.

To the best of our knowledge the effect of VC on protein degradation has been analyzed only for specific proteins involved in the intracellular metabolism of iron. VC supplementation retarded the autophagic uptake of ferritin, decreasing intracellular levels of free iron (Bridges 1987; Hoffman *et al*. 1991). However, this seemed to be a specific effect for ferritin, rather than a generalized effect on autophagy rates, because degradation of other proteins, such as hemoglobin, was not affected (Bridges 1987).

VC enhances the degradation in isolated lysosomes of purified proteins (i.e. GAPDH or RNase A) that have not been previously in contact with the vitamin. Furthermore, in cultured HA, VC increases the degradation after endocytosis, not only of full size proteins, but also of short peptides that lack sulfhydryl groups and are thus susceptible to modification by VC. Thus, though we can not discard that VC might modify the proteolytic susceptibility of other proteins inside the cells, it is clear that the higher degradation rates of the substrates used in this work resulted directly from VC-mediated changes in the lysosomal compartment.

According to our results, VC might have a buffering effect stabilizing the lysosomal pH at acidic values (Fig. 7). The underlying mechanism for this effect remains unknown. A percentage of intracellular VC can be detected in lysosomes after VC supplementation. This selective accumulation of ascorbic acid in lysosomes might directly decrease the intralysosomal pH by the same principle that the selective accumulation of amines, such as ammonium chloride, raises it (Fig. 7b). Lipid peroxidation of the lysosomal membrane by hydroxyl radicals, formed via Fenton reactions in the lysosomal lumen, results in dissipation of the proton-gradient (Zdolsek and Svensson 1993). The antioxidant properties of VC (Hoffman *et al*. 1991; Imai *et al*. 1995) may prevent lipid peroxidation from occurring at the lysosomal membrane, and in this way contribute to the stabilization of the acidic lysosomal pH. Whether or not VC decreases the intralysosomal pH directly by stimulating the activity of the vacuolar ATPase requires further investigation.

The stimulatory effect of VC on protein degradation observed in intact cells can be reproduced in isolated lysosomes (Fig. 6). Consequently, part of the stimulatory effect might result from the direct action of the intracellular pool of VC on these organelles, possibly through the described pH changes. However, we cannot discard the possibility of an additional effect of VC at the extracellular level. In that case, based on the effect observed in serum-deprived cells despite of the shorter VC half-life in the medium, the oxidized form rather than the reduced VC could be the active agent.

The acidification of the lysosomal lumen increases their proteolytic activity, but it might also indirectly augment the transport of some cytosolic proteins into lysosomes (Fig. 6b). We have previously demonstrated that lysosomes with a more acidic pH show higher efficiencies in the uptake of proteins by chaperone-mediated autophagy (Cuervo *et al*. 1997). A decrease in the intralysosomal pH from 5.6 to 5.3 is enough to stabilize the hsc73 located in the lysosomal lumen by making it resistant to degradation. Higher levels of hsc73 inside lysosomes increase their ability for protein uptake (Cuervo *et al*. 1997). In agreement with those previous studies, we have shown here that the lysosomes from VC-supplemented cells also contain higher levels of hsc73 in their lumen (Fig. 6c). Only specific cytosolic proteins containing a targeting motif (KFERQ-like) are degraded in lysosomes by chaperone-mediated autophagy (Cuervo and Dice 1998). The activation of this pathway by VC might be of special value in diseases, such as Alzheimer's or Huntington's disease, in which the accumulated protein contains a KFERQ-like targeting motif (A.M. Cuervo and J.F. Dice, unpublished results).

Though many intracellular proteins are normally degraded by proteolytic systems other than lysosomes (i.e. proteasome or calpains), there is increasing evidence that, once proteins aggregate, autophagy is probably the alternative mechanism used by the cells for their elimination (Kopito 2000). Recently, organized formations of aggregated proteins known as aggresomes have been shown to be localized to specific cytosolic regions where a very active autophagic process takes place (Waelter *et al*. 2001). Aggregates might thus only accumulate when their production rate overpass the capabilities of the autophagic system. In this context, stimulating the proteolytic ability of lysosomes would presumably facilitate the elimination of the aggregates.

One of the main advantages of the effect of VC is that, it mostly increases the degradation of proteins that have been already transported into lysosomes, rather than stimulating a non-selective delivery of proteins to lysosomes by autophagy or endocytosis. Maintained stimulation of autophagocytosis might result in undesired degradation of cellular components essential for cell survival. In addition, the endocytic uptake of small aggregates of fibrillar amiloid protein by microglia cells seems preserved in Azheimer's disease (Chung *et al*. 1999). Instead, the problem arises from its poor degradation inside lysosomes after internalization, leading to their release from the microglial cells back into the extraneuronal medium (Chung *et al*. 1999). Increasing the degradation of the internalized protein in lysosomes by VC supplementation would prevent, or at least significantly reduce, this release of undigested proteins from glial cells.

Together our data show that VC supplementation, a simple nutritional intervention, improves the ability of lysosomes to degrade proteins. The effect of VC on protein degradation is not limited to HA. We have observed increased rates of intracellular protein degradation after VC supplementation in other cell types such as human and mouse fibroblasts, and human and hamster epithelial cells (data not shown). Interestingly, the stimulatory effect of VC was significantly higher in HA than in any other cellular type analyzed. The cell type-dependence of the VC effect will require further investigation.

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