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Ageing-related changes in the in vivo function of rat liver macroautophagy and proteolysis

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Abstract

Autophagy is a universal, highly regulated mechanism responsible for the degradation of long-lived proteins, cytomembranes and organelles during fasting and may be the cell repair mechanism that mediates the anti-ageing effects of calorie restriction (Bergamini and Gori, 1995). The function of autophagy was studied in vivo on male Sprague Dawley rats fed ad libitum or 40% food restricted. Autophagy was induced in overnight fasted rats by the injection of an anti-lipolytic agent and was investigated by electron microscopy. Changes in regulatory plasma nutrients and hormones were assessed and rate of proteolysis was calculated from the release of $^{14}C_6$-valine from pre-labelled resident proteins. Results in rats fed ad libitum showed that autophagic-proteolytic response to antilypolitic agents was paramount in one month-old rats; was high but delayed in 2 month-old rats, decreased remarkably in 6 month-old rats and almost negligible at older age. Parallel ageing-related changes were observed in the effects of treatment lowering glucose and insulin plasma levels. Calorie restriction prevented all changes. In view of the known suppressive effects of insulin, it may be concluded that the age-changes of autophagy are secondary to the ageing-related alteration in glucose metabolism and hormone levels, whose appearance is delayed by calorie restriction. Data may support the hypothesis that ad libitum feeding accelerates the rate of ageing by raising insulin plasma levels and suppressing autophagy and membrane maintenance, and that calorie restriction may break this vicious circle.

Keywords: Rat liver; Ageing; Autophagy; Proteolysis; Food restriction; Insulin; Antilipolytic agents

1. Introduction

Accumulation of altered DNA (Dizdaroglu, 1999) and protein (Starke-Reed, 1999) and of the structural lipid dolichol (Kalén and Appelkvist, 1989) were reported in different tissues and several animal species. Oxidative stress and the ageing related decline in cell repair mechanisms at the molecular (DNA repair, proteasomal proteolysis etc.), subcellular (autophagy) and cellular level (apoptosis) may cause accumulation and are believed to be major determinants of an organism's life span (Sohal, 2002; Sohal et al., 2002; Stadtman 2002; Honda and Honda 2002; Brunk and Terman 2002).

Autophagy was given very little attention by gerontologists so far. It is a universal, highly conserved and regulated, complex cellular process that involves dynamic membrane rearrangements under a range of physiological conditions leading to cytoplasm sequestration into vesicles and delivery to a degradative organelle, the vacuole in yeast or the lysosome in mammalian cells (Klionsky and Emr, 2000). Autophagy is probably the main mechanism for degradation of long-lived proteins and the only mechanism for the disposal of organelles including mitochondria and peroxisomes (Stromhaug and Klionsky, 2001), and may act as an anti-ageing cell repair mechanism by two different ways: it may cooperate with proteasomes, whose function declines with increasing age (Grune, 2000), in order to

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prevent the accumulation of altered proteins in older cells (Vittorini et al., 1999); it may regulate turnover and prevent alteration of cytomembranes and subcellular organelles like peroxisomes and mitochondria (Locci-Cubeddu et al., 1985). Degradation of altered mitochondria, which are known to accumulate in older cells, occurs in large part by autophagy during normal house-keeping (Tolkovsky et al., 2002). Lipofuscin, the age pigment which accumulates progressively with age in long-lived post-mitotic cells of man and animals forms within secondary lysosomes, due to peroxidative damage of autophagocytosed materials (Brunk et al., 1992; Terman and Brunk, 1998).

Autophagy was originally described as a cellular response to starvation (Stromhaug and Klionsky, 2001). The moment-to-moment function of autophagy is regulated by nutrition (it is inhibited by food intake and it is enhanced by fasting via changes in glucose, amino acid, insulin and glucagon plasma levels (Mortimore et al., 1989). It was suggested that an increase in autophagy caused by the prolongation of fasting periods between meals might account for the anti-ageing effects of food restriction (Bergamini and Gori, 1995; Bergamini et al., 1999).

Autophagy may decline with increasing age. Results of experiments in vitro, with the perfused liver and with isolated liver cells of ad libitum fed (AL) and calorie-restricted (CR) rats (e.g. Ward, 2002; Donati et al., 2001a,b; Cavallini et al., 2001) showed that the function of autophagy and lysosomal degradation peaks by age 6 months (Ward, 1988; Donati et al., 2001a,b), and then decreases because of a deterioration of the control by amino acids (Donati et al., 2001a). CR prevented deterioration (Donati et al., 2001b) and efficacy co-varied with the level and duration very like the effects of CR on longevity (Cavallini et al., 2001). The lipid composition of cell membranes in AL and CR rats exhibited similar changes (Cavallini et al., 2002; Dolfi et al., 2003) with different temporal patterns (surprisingly, changes in lipid preceded the in vitro changes of autophagy).

The present study was designed to investigate the effects of ageing on the regulation of rat liver autophagy in vivo. Autophagy was induced in AL and CR rats by intensifying the stimulatory effects of fasting on protein degradation by the injection of an antilipolytic drug (Bergamini and Segal, 1987; Bergamini, 1993). Results showed that older AL rats (but not CR rats) are less sensitive to stimulation of autophagic degradation, that changes occur much earlier than expected from in vitro data, and that the in vivo alteration of autophagy preceeds the accumulation of dolichol in tissues. CR retarded these ageing-related changes.

2. Materials and methods

Animals. Conventionally raised 1-, 2-, 6-, 12-, 18- or 24-month old male Sprague-Dawley rats (Harlan Italy, San Pietro in Natisone) were used as liver donors. Animals were maintained under natural lighting with water continuously available, and were fed a standard laboratory chow ad libitum every day (controls, AL) or given 60% of the amount of the food eaten by ad libitum fed age-matched controls (CR). In order to label long-lived (resident) liver proteins, groups of rats were injected 6 μCi/rat ^{14}C-uniformly labelled valine (288.5 μCi/μmole) intraperitoneally 24 h prior to sacrifice.

Induction of autophagy in vivo. On the morning of the experiment, overnight starved rats were divided into groups at random and were given either the antilipolytic agent 3,5-dimethylpyrazole (DMP) freshly dissolved in saline (12 mg/kg body weight, in 0.2 ml saline) or saline only intraperitoneally. In younger animals, treatment causes a dramatic decrease in the plasma levels of free fatty acids (FFA, in 7.5 min), glucose (in 15 min) and insulin (in 15 min) and a simultaneous increase in glucagon plasma levels, and autophagic vacuoles are visible in liver cells in 30 min in a highly reproducible manner (Bergamini and Segal, 1987).

Liver perfusion. Rats in apparent good health and free of gross pathology were admitted to donation. Histology and electron microscopy did not reveal fatty degeneration and fibrosis. The lipid content of the liver was not affected by ageing (6-month-old rats: 65 ± 12.5; 12 month-old rats: 85.5 ± 12.5; 24 month-old-rats: 72.1 ± 6.32) and was not decreased significantly 60 min after the administration of the antilipolytic drug. Livers were perfused in situ as described by Mortimore and Mondon (1970). Surgery was performed in less than 3 min under anaesthesia (5–10 min after the intraperitoneal injection of 50 mg/kg body weight nembutal, in order to collect the samples of perfusate at the given times: 15, 30, 60 and 150 min after the injection of DMP or saline). Blood samples were taken from the inferior vena cava to assay plasma levels of FFA, glucose, amino acids and hormones, and perfusion was performed in the single-pass mode with a medium free from amino acids. After a 7 min washout, two adjoining 1.5 min fractions of the outflow of the liver (approximately 12 ml each) were taken and centrifuged and the surnatant was used for measurement of $^{14}C_6$ and of amino acid release. At the end of the perfusion, samples of liver tissue were taken to purify proteins and assess specific activity.

Perfusion medium. The perfusion medium was composed of the following: Krebs-Ringer bicarbonate buffer and bovine serum albumin (fraction V, Sigma Chemical, St Louis, MO), 10 mM glucose, 18 μM cycloheximide, and freshly washed no longer usable human erythrocytes (27% vol/vol). Before its addition to the medium, a concentrated aqueous solution of albumin was dialyzed overnight at 5 °C against 4 l of glass-distilled water and was then passed through 3 and 0.2 μm Millipore filters.

Determination of protein degradation and analytic procedure. The plasma-perfusate was deproteinized by

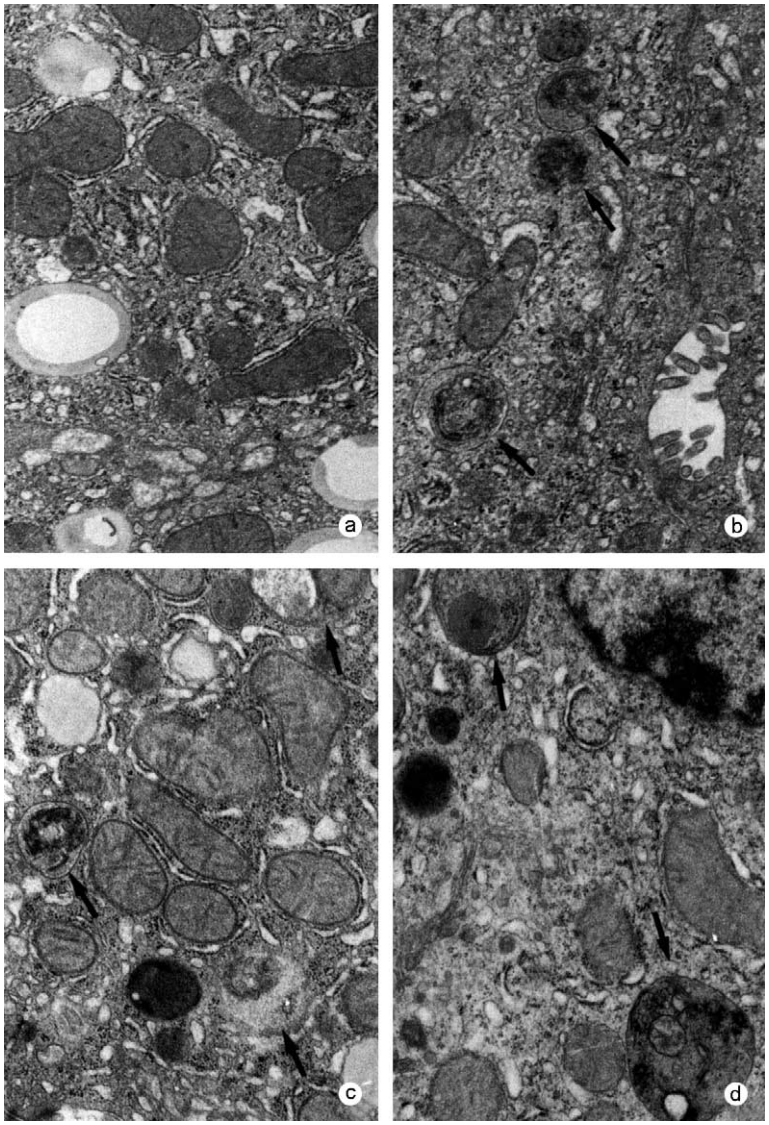

Fig. 1. Ultrastructure of the liver cell (peribiliar area) of 2-month-old ad libitum fed rats fasted overnight (× 18,400). (a) Control; (b) Treated with 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w.) intraperitoneally 30 min before sacrifice. A few autophagic vacuoles (arrows) are seen in the peribiliar area; (c) Treated with DMP (12 mg/kg b.w.) intraperitoneally 60 min before sacrifice. Many autophagic vacuoles are seen (arrows) containing organelles and partially degraded material; (d) Treated with DMP (12 mg/kg b.w.) intraperitoneally 150 min before sacrifice. Large autophagic vacuoles (arrows) contain unidentifiable material.

the addition of 0.25 vol ice-cold 25% TCA, and radioactivity in the surnatant was counted in a Beckman LS TD 500 liquid scintillation counter to an error less than 3%. Approximately 0.5 g liver tissue was homogenized in ice-cold 5% TCA and proteins were centrifuged, washed three times with ice-cold 5% TCA, the nucleic acids were removed by hydrolysis in 5% TCA at 90 °C for 15 min, and lipid were extracted three times with ethanol/diethylether (2:1, v/v) at 65 °C for 5 min. Diethylether-washed proteins were dried at 60° for one hour, weighed, dissolved in 1N KOH at 100 °C for 30 min and neutralized by the addition of a stechiometric amount of 2N $HClO_4$, centrifuged and counted as described to an error less than 1%. Total values of liver protein degradation were computed from the released acid-soluble radioactivity in the perfusate divided by the specific activity of liver proteins.

Electron microscopy observations. Very small fragments of liver tissue were taken approximately 5 min after the beginning of perfusion and were fixed in glutaraldehyde in 0.1 M phosphate buffer for a few hours, freed from gross connective tissue, reduced to smaller pieces, postfixed in OsO_4 in 0.1 M phosphate buffer, dehydrated, and embedded with Polybed 812 by standard procedures. Thin sections were stained with uranyl acetate and lead citrate and were observed with a Siemens Elmiskop 1 A or with a Zeiss EM 203 electron microscope. Quantitative analysis of

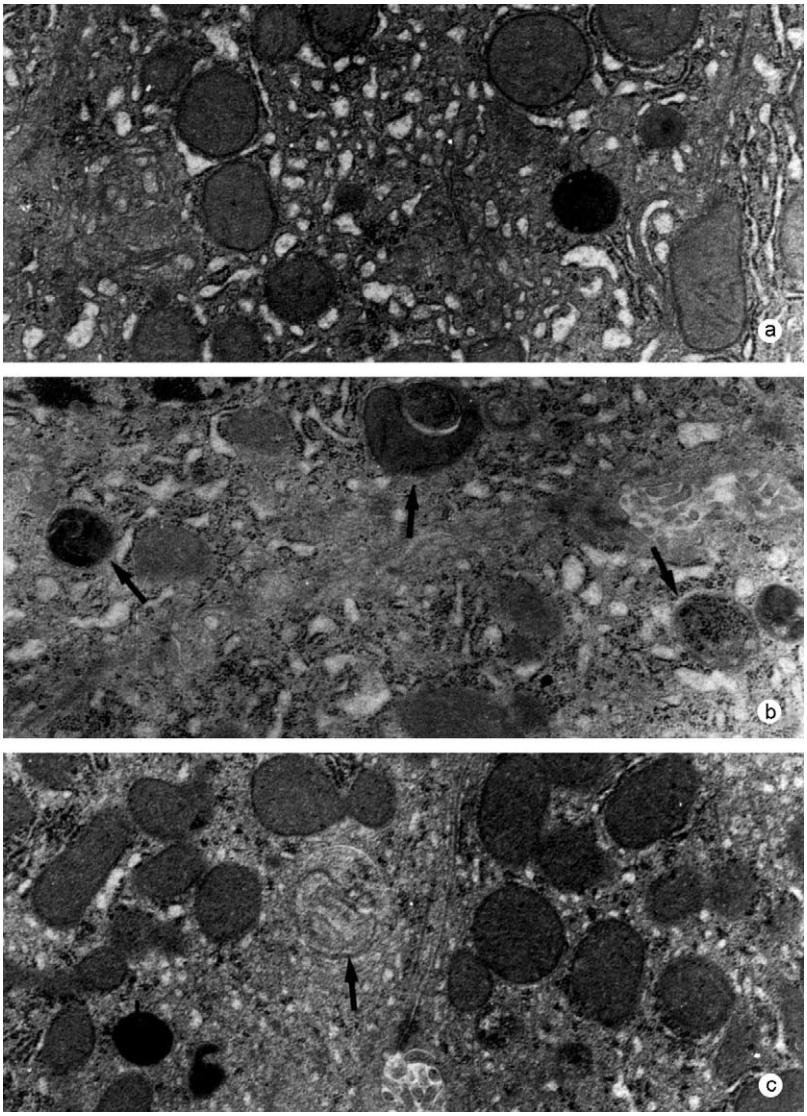

Fig. 2. Ultrastructure of the liver cell (peribiliar area) of 6-month-old ad libitum fed rats fasted overnight (× 18,400). (a) Control. A lysosome (L) is visible. (b) Treated with 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w.) intraperitoneally 60 min before sacrifice. Many autophagic vacuoles are seen (arrows) containing identifiable material (mitochondria and rough endoplasmic reticulum). (c) Treated with DMP (12 mg/kg b.w.) intraperitoneally 150 min before sacrifice. Large autophagic vacuoles (arrows) contain degraded material.

ultracellular components (the volume density of lysosomes, autophagic vacuoles and dense bodies) was performed by morphometric techniques (Weibel, 1979).

Materials. The following materials were purchased from the following companies: 3,5-dimethyl pyrazole, from Fluka AG; amino acid standards, glutamine, cycloheximide, bovine serum albumin and methionine sulphonate from Sigma Italy, Milano; glucagon from Novo industry (Bagsvaerd, Denmark). $^{14}C_6$-valine was purchased from Amersham International, Amersham, UK. ^{125}I-insulin was a generous gift of Prof. E. Navalesi.

Statistical analysis. One- or two-way analysis of variance (ANOVA) test were used as appropriate to evaluate differences among multiple conditions. If positive, the Tukey test was used to test for their statistical significance. Values of $P > .05$ were considered not to be significant.

3. Results

3.1. Age-changes of autophagy and proteolytic function in AL rats

Effects of the administration of DMP on the lysosomal vacuolar system of liver cells. Figs. 1–3 depict the changes in the vacuolar system of liver cells from 2-month, 6-month, 12-month old AL rats at different times after the intraperitoneal injection of the antilipolytic agent. In the controls (injected with saline) fasted rats of any age, lysosomal structures of varying appearances were encountered frequently and increased in number with increasing age (A). The effect of the administration of DMP is smaller the older the age: in 2-month old rats, a number of autophagic vacuoles was seen 30 min after

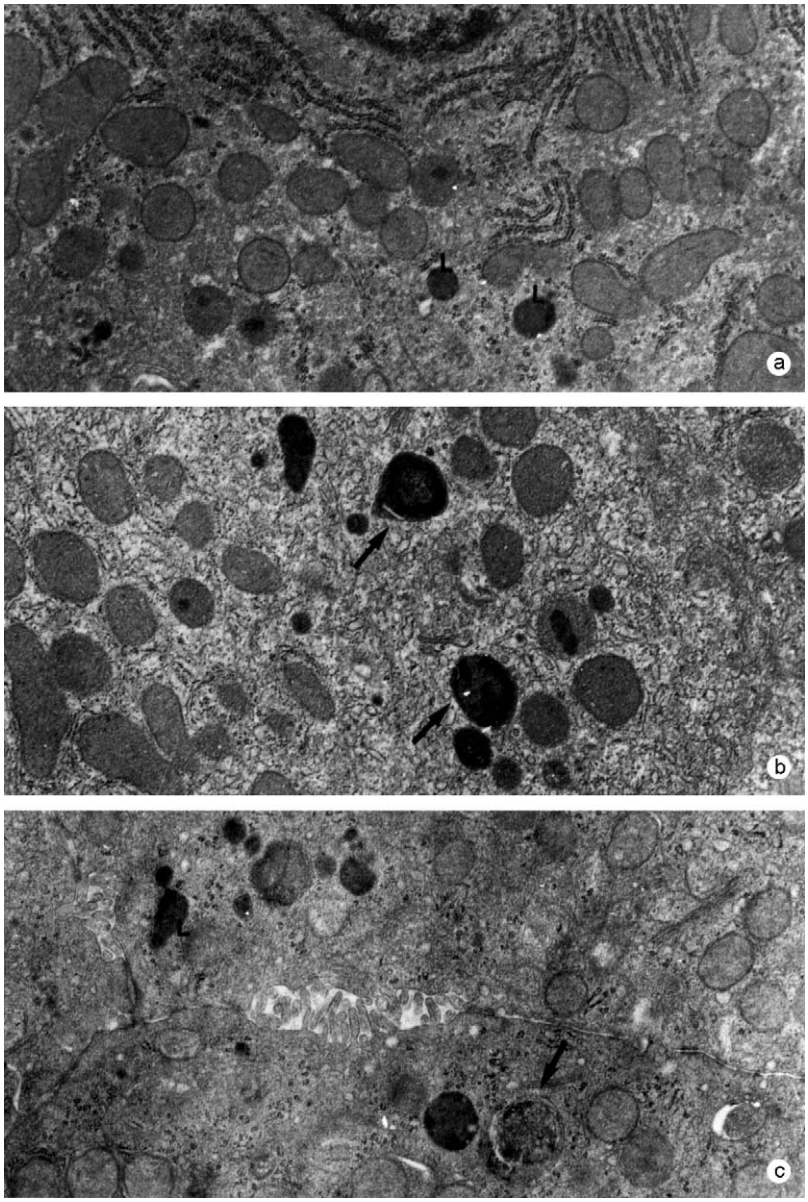

Fig. 3. Ultrastructure of the liver cell (peribiliar area) of 12-month-old ad libitum fed rats fasted overnight (× 18,400). (a) Control. Lysosomes (L) are visible. (b) Treated with 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w.) intraperitoneally 60 min before sacrifice. Many lysosomes are seen, differing in shape. (c) Treated with DMP (12 mg/kg b.w.) intraperitoneally 150 min before sacrifice. Many activated lysosomes (L) and an autophagic vacuole containing rough endoplasmic reticulum are visible.

DMP administration; after 60 min vacuoles were often seen to contain partially digested material; after 150 min, digestion was far more advanced and the large autophagic vacuoles often contained unidentifiable material (Fig. 1); in 6-month-old rats, the autophagic response to DMP was blunted but still clearly visible (Fig. 2): by 60 min after the injection, autophagic vacuoles were increased in number and contained identifiable material; by 150 min content was almost completely digested. In 12-month-old rats no autophagic vacuoles could be seen by 60 min, and only a few autophagic vacuoles containing identifiable material were seen by 150 min (Fig. 3). A similar response to DMP was seen in 24-month-old rats. Morphometric analysis showed that the volume density of autophagic vacuoles by 150 min after the DMP injection declined from 0.80/100 ml in 2 month-old rats down to one half and to 1/8 that value in 6-month and 24-month-old rats, respectively.

Effects of the administration of DMP on the autophagic-lysosomal proteolysis of liver cells. These effects were explored by measuring the rate of degradation of long-lived (resident) proteins as the release of ^{14}C-valine from $^{14}C_6$-valine-labelled liver proteins into the liver perfusate (Fig. 4). The valine release was measurable in control fasted rats and values per unit of

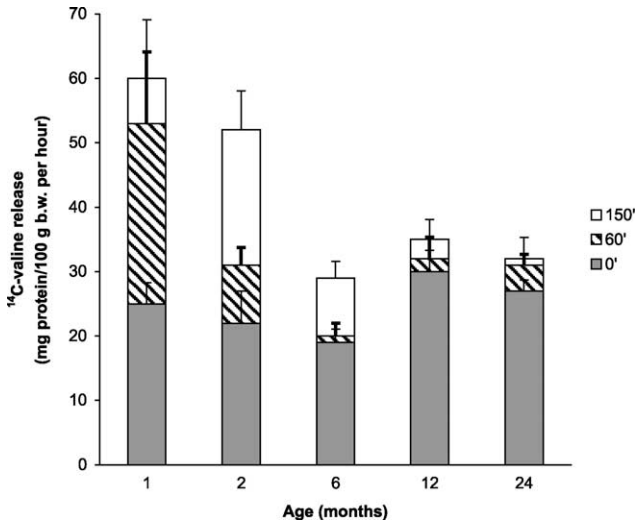

Fig. 4. Effects of increasing age on the liver proteolysis of fasted rats 0, 60 and 150 min after the administration of 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w. intraperitoneally). Liver protein had been pre-labelled by a single injection of ^{14}C-uniformly labelled valine (6 μCi/rat). Means of 7 (1,2,6,12 month-old rats) or 5 cases (24 month-old rats) ± S.E.M. are given. On the ordinate: release of ^{14}C labelled valine is given as mg of labelled protein degraded/100 g body weight per hour. On the abscissa: age (months). Statistical analysis (ANOVA) showed that the basal release of valine was not affected by age, and that the effect of DMP was highly significant ($P < 0.01$) and changed significantly with increasing age (DMP × age interaction, $P < 0.01$).

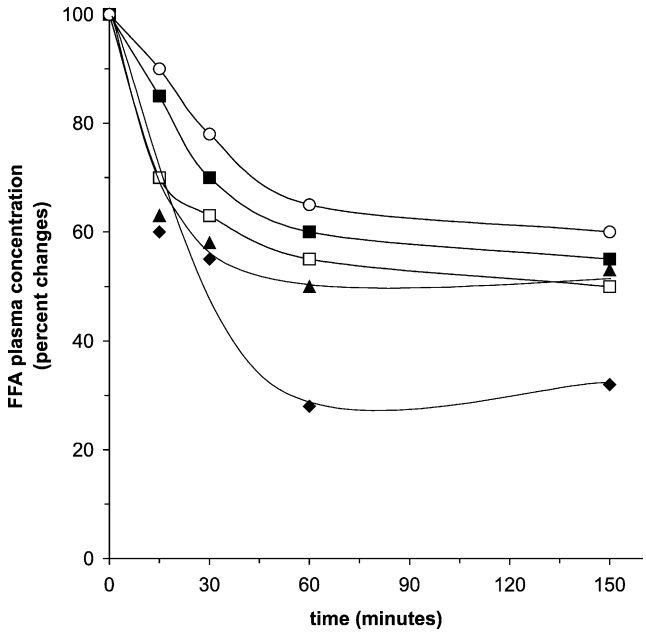

Fig. 5. Effects of the intraperitoneal administration of 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w.) on the FFA plasma concentrations of rats aged 1 (◆), 2 (▲), 6 (□), 12 (■) and 24 months (○). On the ordinate: plasma FFA concentration, per cent changes. On the abscissa: time (min) after the DMP injection. Means of 7 (1,2,6,12 month-old rats) or 5 cases (24 month-old rats) ± S.E.M. are given. Statistical analysis (ANOVA) on the original data showed that in 1-, 2- and 6-month-old rats the decreasing effect of DMP on plasma FFA was highly significant within 15 min. In 12 and 24 month-old rats the effect was highly significant in 30 min.

body weight were similar at any tested age. The administration of DMP increased valine release after a very short time-lag (30 min) in the youngest (one-month-old) age-group (the highest values of proteolysis were attained in 60 min). A slower but equally high stimulation of autophagic proteolysis was seen in 2-months old rats. The effect of DMP administration on ^{14}C-valine release was much smaller by age 6 months and was almost negligible at older ages.

Metabolic and endocrine effects of the administration of DMP. The ageing-related decline in the rate of autophagic proteolysis in 6 months old rats was not expected (maximum rate of rat liver proteolysis in vitro was known to peak by this age). Therefore, we decided to investigate the effects of ageing on the metabolic and endocrine effects of DMP, which mediate the stimulation of rat liver autophagy. DMP was almost equally effective on FFA plasma levels in all age-groups of rats, but the effects on insulin levels depended on age (Figs 5 and 6): in general, the older the animal's age the smoother and smaller were the effects on the hormone.

3.2. Age-changes of autophagic-proteolytic function in CR rats

The effects of the administration of DMP on proteolysis of liver cells of 12-month-old rats fed a 40% food-restricted diet are depicted in Fig. 7: no significant effect of the drug on the release of ^{14}C-valine from pre-labelled resident protein was detected in AL animals and a significant stimulation was seen in CR rats both 60 and 150 min after the injection. In CR rats, treatment with DMP decreased plasma glucose and insulin levels significantly (Fig. 8).

4. Discussion

Regulation of liver macroautophagy and protein degradation in vivo was studied on young AL male Sprague-Dawley rats (200 g b.w.; approximate age: 7 weeks) by electron microscopy and short-term single-pass liver perfusion and valine assay in the perfusate (Bergamini and Kovacs, 1990; Bergamini et al., 1993, 1994). By this age, the injection of an antilipolytic agent (DMP) at a very high dosage (12 mg/kg b.w.) to rats fasted overnight enlarged the lysosomal-autophagic compartment in the pericanalicular area of liver cells in 30 min. The stimulatory effects of DMP on autophagic proteolysis were shown to be secondary to metabolic and endocrine effects of the drug, which lowered FFA plasma levels in less than 7.5 min and glucose and insulin in 15 min, and increased glucagon and corticosterone plasma levels in 15 min (Locci-Cubeddu et al., 1985; Bergamini et al., 1993, 1994). The present data on younger rats are in good agreement with the previous results. In addition, they show that in AL rats the size and timing of the autophagic-proteolytic response to

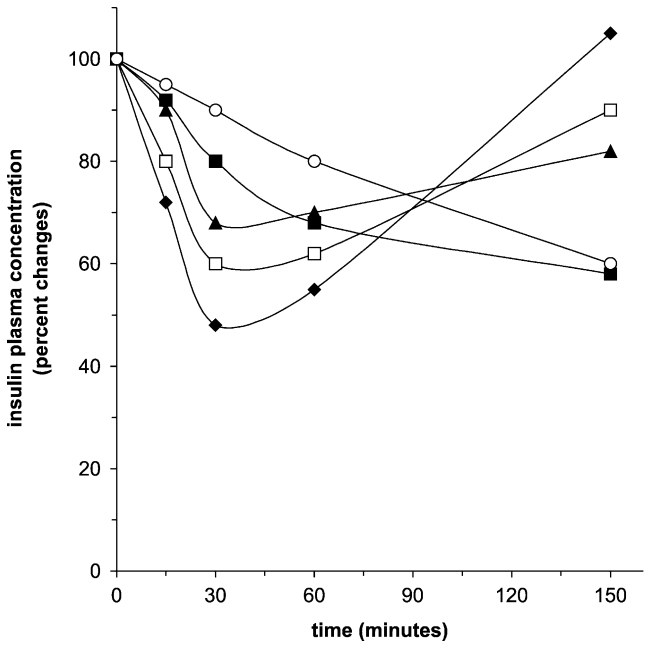

Fig. 6. Effects of the intraperitoneal administration of 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w.) on the insulin plasma concentrations of rats aged 1 (◆), 2 (▲), 6 (□), 12 (■) and 24 months (○). On the ordinate: per cent changes in the insulin plasma concentration. On the abscissa: time (min) after the DMP injection. Means of 7 (1,2,6,12 month-old rats) or 5 cases (24 month-old rats) ± S.E.M. are given. Statistical analysis (ANOVA) on the original data showed that the main effects of DMP, age and time and interactions age × DMP, time × DMP and age × time × DMP were highly significant all. By 15 min after DMP administration, changes in plasma insulin levels were highly significant in 1-month-old rats; significant in 2-month-old rats; not significant in 6-month-old rats.

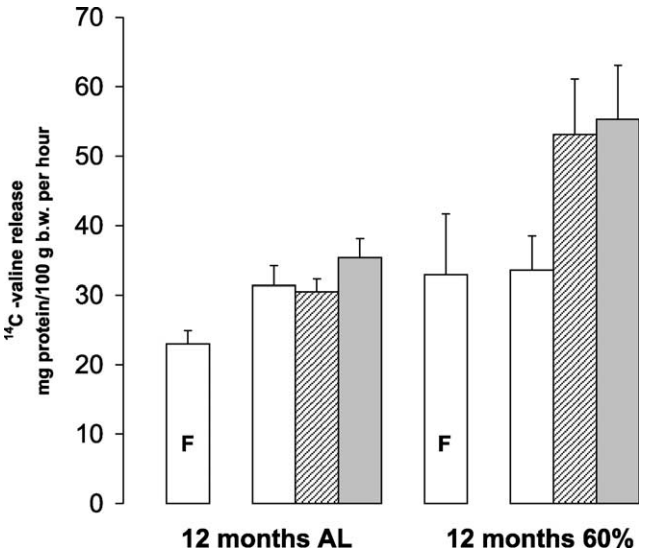

Fig. 7. Effects of the administration of 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w. intraperitoneally) on the liver proteolysis of 18 hours-fasted rats rose on ad libitum feeding (AL) or on an anti-aging calorie-restricted regimen (namely 40% calorie-restriction, 60%). Data obtained with ad libitum fed non-fasted rats are given as an additional control (F). Liver long-lived protein had been pre-labelled by a single injection of ^{14}C-uniformly-labelled valine (6 μCi/rat) 24 h before sacrifice. On the ordinate: release of ^{14}C labelled valine is given as mg of labelled protein degraded/100 g body weight per hour. On the abscissa: feeding regimen. Means of 7 (AL) or 5 (60%) rats ± S.E.M. are given. White columns: DMP 0 min; shadowed column: DMP 60 min; grey columns: DMP 150 min. Statistical analysis showed that DMP had no effect on AL rats, and significantly increased protein degradation in calorie restricted rats ($P < 0.01$).

the administration of DMP change remarkably with increasing age: in 1-month-old rats the autophagic response is very prompt (a maximal increase of proteolysis is attained in 30 min); in 6-month-old rats response is delayed and smaller; in older rats, it is negligible. In essence, the older the age the fewer and smaller are the autophagic vacuoles formed in response to the injection of a maximally active dose of DMP, and the smaller the volume of cytoplasm and its constituents that are sequestered and digested by liver cell lysosomes to release amino acids. These changes were prevented by CR, and a juvenile proteolytic response was seen in 12-month-old rats on a 40% food-restriction.

In most tissues of the ageing organisms and in most ageing model systems overall proteolysis declines with age (Ward and Shibatani, 1994; Cuervo and Dice, 2000) most likely as a consequence of a reduction of macroautophagic and chaperone mediated lysosomal proteolysis (Cuervo and Dice, 2000). Changes in lysosomes of senescent tissues and organisms are common and have been used as biomarkers of ageing. A variety of age-associated changes in hydrolase activities were described but none was shown to cause abnormal lysosome function; rather, delivery of substrates to the lysosomal matrix may limit protein degradation rates in older cells (Cuervo and Dice, 2000). Formation of autophagic vacuoles decreases in older age (Bergamini and

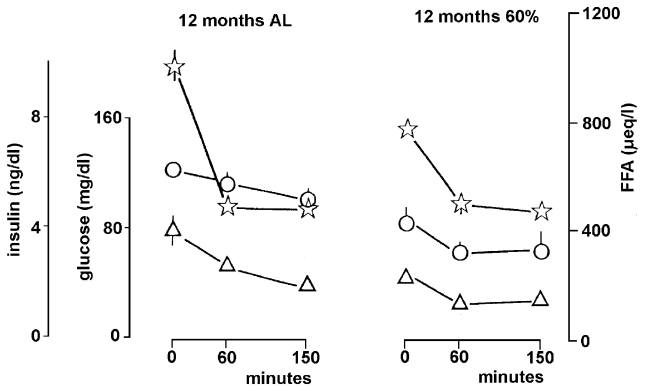

Fig. 8. Effects of the intraperitoneal injection of 3,5-dimethylpyrazole (DMP, 12 mg/kg b.w.) on the FFA (☆), glucose (○) and insulin (△) plasma concentrations. Rats: see Fig. 7. On the ordinate: plasma glucose and insulin (left) and plasma FFA (right). On the abscissa: time from DMP administration (min). Means of 7 (AL) or 5 (60%) rats ± S.E.M. are given. DMP caused a highly significant decrease in FFA plasma levels in all diet groups, and caused small, slow changes in plasma glucose and insulin in AL rats. On the other hand, DMP caused a highly significant decrease in plasma glucose and insulin in 60% fed rats in 60 min. Plasma concentrations in ad libitum fed non fasted rats were: 12 months AL (6 cases). FFA: 133 ± 13.2; Glucose: 144 ± 4.8; Insulin: 8.14 ± 0.56. 12 months 60% (5 cases): FFA: 98 ± 12.2; Glucose: 126 ± 8.6; Insulin: 4.14 ± 0.82.

Kovacs, 1990). Decreased formation of autophagic vacuoles and an even more striking delay of fusion of autophagic vacuoles with lysosomes were reported by Terman (1995). In the perfused liver, the maximum rate of autophagic proteolysis peaked by age 6-months both in AL and in CR rats and declined thereafter (Ward, 1988). Hepatocytes isolated from AL male Sprague-Dawley rats aged 12, 18 and 24 months (Donati et al., 2001a) showed a loss of the aminoacidic control of autophagic vacuoles formation which was prevented by calorie restriction (Donati et al., 2001b), efficacy depending on grade (unpublished) and duration (Cavallini et al., 2001). Evidence in this article shows that stimulation in vivo of liver autophagy becomes smaller and time-lag longer by adult age; besides that, difference in the temporal pattern indicates that in vivo and in vitro age-dependent changes should have different causes, and gives further support to the statement that one must be cautious when extrapolating to liver in vivo, observations obtained with isolated liver cells or primary culture of hepatocytes (Thirion and Wattiaux, 1991).

In the in vitro experiments, liver autophagy was induced by lack of amino acids. The in vivo induction of liver macroautophagy and protein degradation by the physiologic mechanism involves more subtle and complex (unbalanced and asynchronous) changes in the levels of selected nutrients, and in modulation by hormones (Bergamini et al., 1994). In other words, the slow and smooth metabolic and endocrine changes normally occurring during transition from the fed to the fasted state are intensified by the sudden lack of plasma FFA secondary to the administration of the antilipolytic agent. New evidence in this article shows that the size of the DMP effect decreases and time lag increases with increasing age. Several factors may contribute in making adult and older AL (but not CR) rats more tolerant to a shortage of plasma FFA during fasting: for example, in AL (but not in CR rats) triglycerides accumulate in muscles by adult age (Gupta et al., 2000) and might supply locally for the missing plasma fatty acid. The turnover rate of plasma FFA is slower in older rats (Del Roso, 1991). It appears that ad libitum feeding suppresses autophagy and prevents autophagy from being activated by fasting (at adult age); alteration of membrane(s) composition and function (by mature and older age) may be a secondary effect. Anti-ageing food restriction keeps animals on fasting for a great part of the day (as the average, 16 h a day in case of 40% CR; by definition 24 h every two days in the case of every-other-day ad libitum feeding, unpublished) and makes autophagy work for many hours every day and may improve membrane maintenance (Bergamini et al., 1999). It has been shown recently that weekly life-long pharmacological stimulation of autophagy intensifies the sub-maximal anti-ageing effects of a 10% calorie restriction to make them maximal (i.e. similar to those of every-other-day ad libitum feeding (Bergamini et al., 2002a,b). Current evidence indicates that adult and older ad libitum fed rats may not make good use of the cell repair mechanism autophagy, and that food restriction may break the vicious circle.

The respective roles of protein and membrane degradation in the anti-ageing action of autophagy may deserve a final comment. The dramatic decline in liver autophagy in very old age may contribute to accumulation of altered proteins in the liver of AL rats in the last 10% part of life (Vittorini et al., 1999). Present evidence shows that decreased autophagy may cause the alteration of the lipid composition of membranes and accumulation of dolichol in the liver tissue by adult and older age. Anti-ageing dietary restrictions prevent both of these changes effectively (Vittorini et al., 1999; Marino et al., 1998; Dini et al., 2001; Dolfi et al., 2003).

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