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Experimental Gerontology 39 (2004) 1061–1067
Experimental Gerontology
www.elsevier.com/locate/expgero

Anti-aging effects of anti-lipolytic drugs

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Received 16 December 2003; received in revised form 3 March 2004; accepted 5 March 2004

Available online 2 April 2004

Abstract

Genetic disruption of insulin and insulin-like signaling pathways may extend lifespan. Hyperinsulinemia and insulin resistance may accelerate aging. The hypothesis was tested that a once-a-week life-long inhibition of insulin secretion by the administration of anti-lipolytic drugs might have anti-aging effects. Groups of 3-month-old male Sprague-Dawley rats were (a) given standard laboratory food ad libitum (AL); (b) fed AL 6 days and fasted 1 day every week (FW); (c) fed AL every other day (EOD), (d) fed like FW and given Acipimox (50 mg/kg b.w.) on the day of fasting (FWA) by the gastric tube. The AL, FW and EOD groups received saline intragastrically. Treatment with ACIPIMOX transiently decreased plasma free fatty acids, glucose and insulin and increased valine plasma levels, and had no long-term effect on food consumption and body weight. By age 6, 12 and 24 months subgroups were taken and the age-related changes in liver dolichol and autophagic proteolysis—which are correlated with life-expectancy—were measured. Liver dolichol levels increased and autophagic proteolysis decreased in mature and older AL rats; EOD and FWA fully counteracted these changes; FW rats had significant but smaller beneficial effects. It is concluded that life-long weekly-repeated transient inhibition of insulin secretion by antilipolytic drugs may have an anti-aging effect, additive to the anti-aging effect of a milder caloric restriction. Speculation is that transiently lower plasma insulin levels might stimulate the anti-aging cell-repair mechanism autophagy, which has longer lasting effects on cell housekeeping.

Keywords: Aging; Antilipolytic drug; Insulin; Autophagy; Dolichol; Rat liver

1. Introduction

A rapidly growing body of evidence shows that the insulin and the insulin-like growth factor-1 (IGF-I) signaling pathways influence longevity in many animal species, from worms to rodents (Bartke, 2001; Kimura et al., 1997; Tatar et al., 2001, 2003). Studies in *C. elegans* demonstrate that disruption of the daf-2 signaling pathway extends longevity (Kenyon et al., 1993; Kenyon, 2001) and similarities with the insulin-like signaling in flies and yeast, and mammalian IGF-I signaling cascade raise the possibility that modifications to the IGF-I signaling could also extend life-span in mammals (Carter et al., 2002). Growth hormone (GH)/IGF-I deficient dwarf mice do live significantly longer than their wild counterparts (Brown Borg et al., 1996). On the other hand, research spanning more than 60 years has shown that diet restriction, which lowers glucose and insulin plasma levels in mammals, consistently extends the median and maximum life-span, and health-span of animals (Masoro, 2003) and perhaps monkeys (Mattison et al., 2003). Evidence was produced that the deleterious effects of insulin on longevity may be mediated by the fat-tissue-insulin-receptor (Bluher et al., 2003).

Antilipolytic drugs like 3,5-dimethylpyrazole or Acipimox are known to decrease insulin secretion (Masiello et al., 2002). In this research, the hypothesis was tested that treatment with antilipolytic drugs might delay the process of aging. For this purpose, we studied the effects of life-long treatment with acipimox (an antilipolytic drug licensed for human use as a hypolipidemic agent) with mild or severe caloric restriction, showing the age-related changes in two parameters (accumulation of the membrane lipid dolichol and the decline in the function of liver autophagy) which are known to correlate with life-expectancy (Cavallini et al., 2001; Dolfi et al., 2003).

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doi:10.1016/j.exger.2004.03.025

2. Materials and methods

2.1. Animals

Male 3-month-old rats of the Sprague-Dawley strain were divided in four groups which were maintained (a) on standard laboratory food (Teklad, Harlan Italy, S. Pietro in Natisone, Italy) ad libitum (AL); (b) fed ad libitum and fasted 1 day a week (FW); (c) fed like group (b) and given Acipimox (50 mg/kg b.w. dissolved in saline pH 2.1) on the day of fasting by the gastric tube (FWA); (d) on an every other day (EOD) ad libitum feeding regimen. Groups (a), (b), (d) received saline only. By the ages of 6, 12 and 24 months, subgroups of rats were taken quietly. All rats had free access to water. Food was withdrawn 16 h before experimentation. Rats on the EOD restricted diet were sacrificed on the day of fasting. Rats were anaesthetized by the intraperitoneal injection of pentobarbital (50 mg/kg body weight), the posterior lobule of the right liver lobe was taken for dolichol assay, and liver parenchymal cells were isolated by the collagenase perfusion method of Seglen (1976). The lipid content of the liver was not affected by ageing (6-month-old rats: 65 ± 12.5: 12 month-old-rats: 85 ± 12.3; 24 month-old-rats: 72.1 ± 6.32 mg/g wet tissue). Cell viability was tested by trypan blue exclusion and was always better than 90%.

2.2. Acute effects of acipimox administration

Acipimox (50 mg/kg b.w) or the vehicle only (physiological solution, pH 2.1) was administered to 3-, 6-, 12-, 24-month-old ad libitum fed male Sprague-Dawley rats after 18 h of fasting and blood samples were taken from the tail vein for 6 h after the administration. Free fatty acids (FFA) and glucose in plasma were assayed, respectively, by the *acyl* CoA *synthetase*/oxidase and the glucose oxidase/peroxidase techniques, using commercially available kits (FFA: Free Fatty Acids Half Microtest, *Boehringer*-Manheim KK; glucose: Glucinet, Sclavo ISVT). Plasma insulin was measured by radioimmunoassay according to Herbert et al. (1965) using rat insulin as a standard. Plasma valine was assayed as described below.

2.3. Extraction and HPLC assay of dolichol

Dolichol was extracted and assayed as described (Marino et al., 1998). The sensitivity of the method allowed us to measure at least 1.7 ng total dolichol. A calibration curve of total peak height against quantity (ng) injected, was linear in the 1.7–1000 range. Recovery was determinated by internal standard (dolichol 22) addition to samples (Marino et al., 1990) and was ~95%. Data are given as μg dolichol/g wet tissue.

2.4. Rate of autophagic proteolysis

Hepatocytes were suspended in Krebs–Ringer bicarbonate buffer (3 ml, 1.5×10^6/ml) and incubated in four rows of water-jacketed 10 ml conical flasks enclosed within a Lucite box attached to a Dubnoff apparatus with shaking as described (Venerando et al., 1994). Mixtures of plasma amino acids were added as fraction/multiples of a standard reference mixture of amino acids in rat plasma (Venerando et al., 1994). Cycloheximide (CHX, 10 μM) was added to inhibit reincorporation of amino acids into proteins (Ward and Richardson, 1991) after 30 min as recommended by Venerando et al. (1994). The rate of proteolysis was assessed by the linear release of free valine in the 17 min period following the addition of CHX and was normalised to 10^6 cells/ml. Lysosomal protein degradation was computed by subtracting the valine released in the presence of 5 mM 3-methyladenine (3-MA; Seglen and Gordon, 1982; Blommaart et al., 1997) from total valine release. Previous studies showed that the rate of autophagic sequestration of LDH and the rate of 3-MA-sensitive valine release are correlated positively both with younger and older isolated liver cells (Donati et al., 2001).

2.5. HPLC assay of valine

The acid-soluble supernatants were neutralized with KOH, and then the amino acids were derivatized with dansyl-chloride as described by Taphui et al. (1981). L-Norvaline was added as an internal standard to all samples.

Amino acid separation was carried out on a 4.0×250 mm^2 Bio-Sil ODS-5S column (particle size, 5 μm) in a Beckman (System Gold) HPLC system (Beckman Instruments, Fullerton and San Ramon, CA, respectively). The column was eluted with a linear gradient of eluants A (88: 12 water/acetonitrile, 0.3% glacial acetic acid, 0.035% triethylamine) and B (100% methanol). Valine was determined by measuring the fluorescence of its dansylated derivative with a Jasco spectrofluorometer (340 nm excitation, 525 emission).

2.6. Statistical analysis

The analysis of variance (ANOVA) test was used to evaluate differences among multiple conditions. If positive, the Tukey test was employed to test for statistical significance. Student's-*t* test was used for two means comparisons. Values of $P > 0.05$ were considered not to be significant.

2.7. Materials

Dansyl chloride was obtained from Pierce (Pierce Europe, Beijerland, Netherlands). Amino acids, collagenase (type IV) and cycloheximide were obtained from Sigma

(SIGMA-Aldrich, Milan). All other reagents were of the highest quality commercially obtainable.

3. Results

The intragastric administration of the antilypolitic drug Acipimox (50 mg/kg b.w.) to overnight fasted 3-month-old rats caused a dramatic decrease in the concentration of plasma FFA (Fig. 1A: lower FFA levels were found for at least 4 h after the administration of the drug), glucose (Fig. 1B, for at least 3 h) and insulin (Fig. 1C, for 4 h). An increase in the concentration of valine was also observed and lasted for at least 6 h (Fig. 1D). At the later times, when FFA and glucose levels were back to basal values and valine was still higher, insulin levels rebounded. The effects of Acipimox were dose-dependent, and were significant at a dosage as low as 1 mg/kg. The effects of Acipimox were similar in adult, mature and older rats.

A life-long weekly administration of Acipimox had no significant effect on body weight and food intake (Table 1). Body weight was maximum in AL rats; the FW diet had a barely significant effect on body weight and food consumption. EOD reduced body weight (−25%) and food consumption (−35%) significantly.

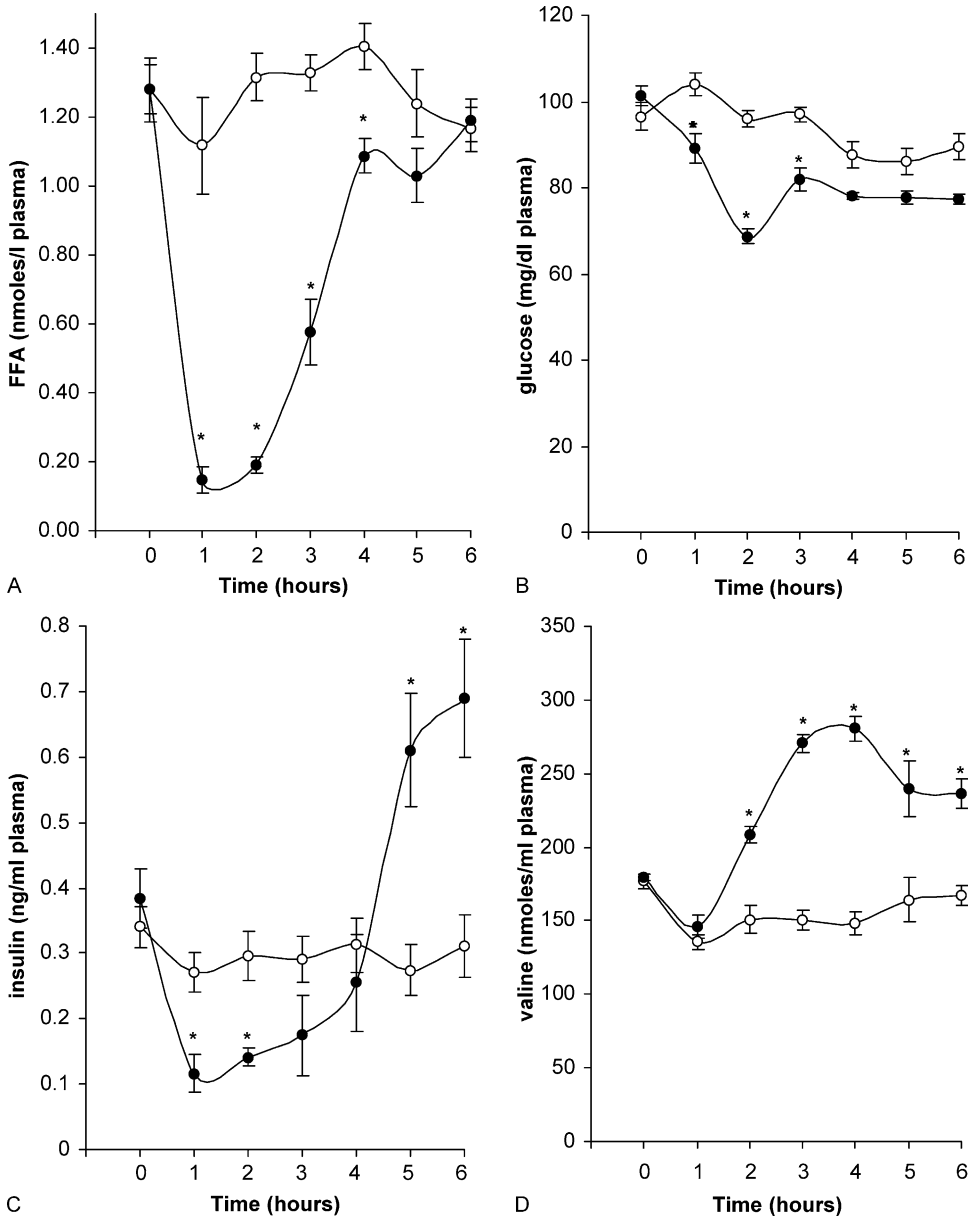

Fig. 1. Effects of the intragastric administration of Acipimox (50 mg/kg body weight) on the plasma levels of free fatty acids (FFA, (a)), glucose (b), insulin (c) and valine (d) in young male Sprague Dawley rats (3 months) submitted to a 18 h fasting period. Legend: open circle, controls, administration of physiologic solution, pH 2.1; closed circle, administration of Acipimox. Results are given as mean ± SE of at least four cases. *significatively different from control at the same time (t test, $P < 0.05$).

Table 1
Effects of increasing age and different treatments on the body weights (a) and on the daily caloric intakes (b) of male Sprague Dawley rats

	Six months	Twelve months	Twenty four months
(a) Body weight (g)			
AL	501 ± 8.5	538 ± 3.6	580 ± 10.8
FW	464 ± 9.2	544 ± 16.4	552 ± 19.4
FWA	452 ± 10.1	501 ± 22.7	544 ± 22.8
EOD	380 ± 12.9	445 ± 17.6	456 ± 29.5
(b) Caloric intake (kcal) per day			
AL	78.4 ± 2.61	77.6 ± 1.48	79.2 ± 1.87
FW	69.8 ± 3.39	70.6 ± 1.29	71.4 ± 3.28
FWA	69.8 ± 2.34	65.6 ± 1.25	71.0 ± 1.68
EOD	53.4 ± 1.01	52.6 ± 2.89	56.6 ± 0.86

Results are given as mean ± SE of at least four cases. (a) Statistical analysis (two way ANOVA). Age main effect: $P < 0.0001$. (Tukey test—6 vs. 12 and 24-month: $P < 0.05$ all). Treatments main effect: $P < 0.0001$ (Tukey test—AL vs. EOD and FWA; FW and FWA vs. EOD: $P < 0.05$ all). Age by treatments interaction: NS. (b) Statistical analysis (two way ANOVA). Age main effect: NS. Treatments main effect: $P < 0.0001$ (Tukey test—AL vs. FW, EOD and FWA; FW and FWA vs. EOD: $P < 0.05$ all). Age by treatments interaction: NS.

Fig. 2 shows that the regulation of liver autophagic proteolysis by extracellular amino acid faded gradually with increasing age down to very low levels in older AL rats; EOD and FWA counteracted age-changes almost fully; FW counteracted only in part the age-dependent loss of the amino acid control of autophagic proteolysis. Quite interesting, EOD started by age 3 months caused at first a transient increase in maximum autophagic proteolysis (as measured in the absence of amino acid): maximum levels were reached by age 6 months, whereas FW and FWA did not.

A significant accumulation of the membrane-lipid dolichol was seen in the liver tissue; EOD and FWA counteracted these age-changes almost fully; the milder food restriction FW counteracted accumulation only in part (Fig. 3).

4. Discussion

These results show that the administration of an antilipolytic drug to fasted rats can counteract age-related changes in membrane lipids and lysosomal proteolytic function which are known to be correlated with life-expectancy. In the present experiment, rats were given Acipimox intragastrically once a week, on the day of fasting starting by age 3 months and subgroups were sacrificed by age 6-, 12- and 24-months. Groups of age-matched ad libitum fed and food restricted rats were used as controls. Age-related changes in autophagic proteolysis and dolichol content of the liver tissue in ad libitum fed and food restricted rats are in very good agreement with the previously reported data (Donati et al., 2001; Marino et al., 1998). Age-changes in lysosomal autophagic degradation and in the lipid composition of liver cells were shown to co-vary with life-expectancy and were proposed to be good biomarkers of aging and longevity (Cavallini et al., 2001;

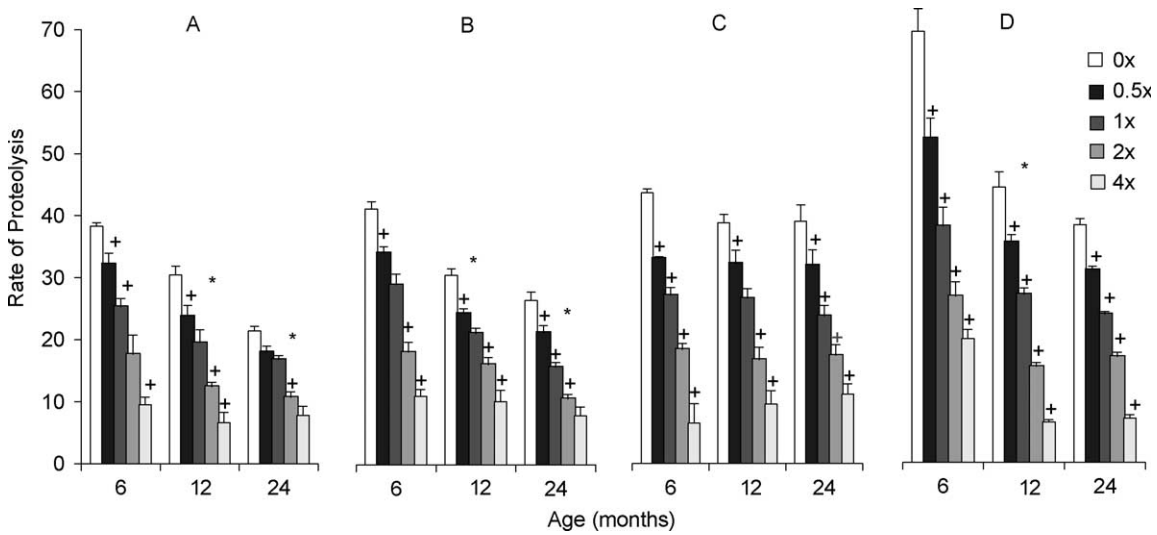

Fig. 2. Effect of increasing age on the amino acid regulation of autophagic proteolysis in liver cells isolated from rats submitted to different treatments: (A) AL, rats fed ad libitum; (B) FW rats fasted 1 day a week; (C) FWA rats fasted 1 day a week and given Acipimox on the day of fasting; (D) EOD, rats fed every-other-day ad libitum. Results are given as nmoles valine/min per gram of wet tissue, mean ± SE. + Significative different from previous (lower) amino acid concentration (Tukey test within each age group, $P < 0.05$); *significative different from previous age (Tukey test within each treatment group, $P < 0.05$). Statistical analysis (three way ANOVA): Age main effect: $P < 0.0001$ (Tukey test—6 vs. 12 and 24-month; 12 vs. 24-month: $P < 0.05$ all). Treatment main effect: $P < 0.0001$ (Tukey test—AL vs. FW, FWA and EOD; FW vs. FWA and EOD; FWA vs. EOD: $P < 0.05$ all). Amino acid concentration main effect: $P < 0.0001$. (Tukey test—0x vs. 0.5x, 1x, 2x and 4x; 0.5x vs. 1x, 2x and 4x; 1x vs. 2x and 4x; 2x vs. 4x: $P < 0.05$ all). Age by treatment interaction: $P < 0.0001$. Age by amino acid concentration interaction: $P < 0.0001$. Treatment by amino acid concentration interaction: $P < 0.0001$. Age by treatment by amino acid concentration interaction: NS.

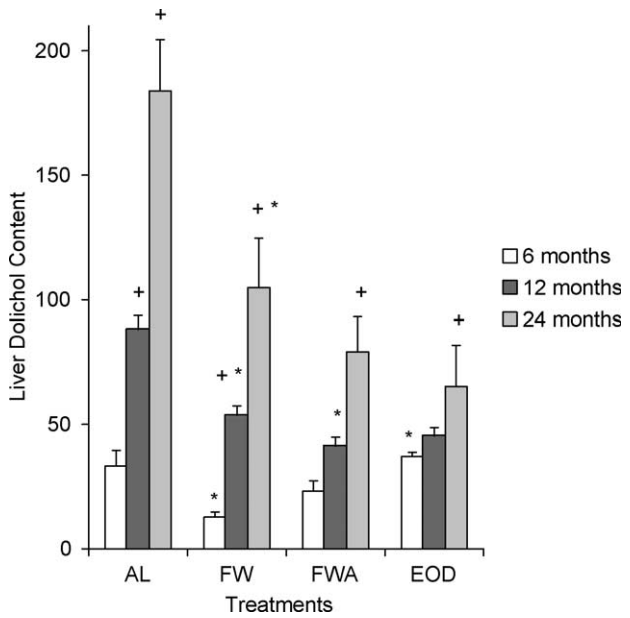

Fig. 3. Age-dependent changes in the levels of dolichol in the liver of rats submitted to different treatments: (A) AL, rats fed ad libitum; (B) FW rats fasted 1 day a week; (C) FWA rats fasted 1 day a week and given Acipimox on the day of fasting; (D) EOD, rats fed every-other-day ad libitum. Results are given μg dolichol/g wet tissue; mean ± SE of at least four cases are given. ± Significative different from previous age (Tukey test within each treatment group, $P < 0.05$); *significative different from previous treatment at the same age (*t*-test, $P < 0.05$). Statistical analysis (two way ANOVA): age main effect: $P < 0.0001$ (Tukey test—6 vs. 12 and 24-month; 12 vs. 24-month: $P < 0.05$ all). Treatment main effect: $P < 0.0001$ (Tukey test—AL vs. FW, FWA and EOD: $P < 0.05$ all). Age by treatment interaction: $P < 0.0005$.

Dolfi et al., 2003). Furthermore, present data show that a milder (10%) regimen of food restriction, which is known to have a significant, even though submaximal, beneficial effect on animal's longevity (Duffy et al., 2001), has submaximal beneficial antiaging effects both on age-changes in autophagic proteolysis and on dolichol accumulation (Cavallini et al., 2002). Therefore, a very interesting finding is that life-long once-a-week administration of the antilipolytic drug Acipimox had significant antiaging effects similar to those of calorie restriction, without any adverse effect on food consumption and animal's body weight. To our knowledge, no drugs had been reported so far to beneficially influence biomarkers of the aging, which are bound to change with life-expectancy.

Curiously, the anti-aging effects of antilipolytic agents can be obtained by giving the drug once a week. Present and previous experiments show that a single administration of a potent antilipolytic drug to fasted rats has very intense though transient metabolic and endocrine effects. These effects include a decrease in circulating FFA, which may begin in a few minutes (Gerritsen and Dulin, 1965; Locci-Cubeddu and Bergamini, 1983; Schein et al., 1971) and last for hours (Locci-Cubeddu et al., 1985), and a rapid decrease in plasma glucose levels (Gerritsen and Dulin, 1965; Locci-Cubeddu and Bergamini, 1983; Stein et al., 1996). Basal insulinemia is also rapidly and transiently decreased upon 3,5-dimethylpyrazole or Acipimox treatment, concomitantly with the reduction of plasma FFA and glucose (Locci-Cubeddu et al., 1985). The transient reduction of plasma insulin levels after the administration of antilipolytic drugs may be secondary to hypoglycaemia and is compatible with the concept that glucose-stimulated insulin secretion relies upon circulating FFA in the fasting state (Stein et al., 1996; Dobbins et al., 1998). Alternatively and not exclusively, the inhibitory effect of antilipolytic agents on insulin secretion may be secondary to a decrease in endogenous beta-cell lipolysis and to alteration of lipid signaling (Masiello et al., 2002). In conclusion, in view of the transiency of changes of plasma insulin, it is unlikely that the anti-aging effects of antilipolytic agents could be attributed to hypoinsulinemia by itself.

Besides the effects on insulin secretion, treatment with antilipolytic drugs may influence secretion of other hormones, including glucagon, glucocorticoid (Bergamini et al., 1993) and growth hormone (Peino et al., 1996). As an important consequence, the sudden, dramatic increase in the plasma glucagon/insulin ratio may stimulate autophagy and lysosomal proteolysis (Blommaart et al., 1997; Parrilla et al., 1974). It has been reported that antilipolysis during fasting may increase protein degradation (Norrelund et al., 2003). Higher autophagic proteolysis in liver cells stimulates the release of long branched chain amino acids from liver cells (Donati et al., 2001; Mortimore et al., 1996), and may account for the increase in the concentration of plasma valine, which is seen in 1–2 h and may last for several more hours after Acipimox administration. Autophagy is the universal and highly regulated mechanism responsible for the degradation of long-lived proteins, cytomembranes and organelles during fasting (e.g. Klionsky and Emr, 2000). Autophagy is involved in cell re-modeling and is the only mechanism for the degradation of altered membranes and cell organelles which can help cells to get rid of altered membranes and organelles. The function of autophagy declines in older animals fed ad libitum (Donati et al., 2001; Ward, 2002; Cuervo, 2003) and the declination is prevented by caloric restriction (Cavallini et al., 2001; Donati et al., 2001). In vivo, the action of autophagy becomes weaker and weaker in older ad libitum fed animals, and retains strength in food restricted animals (Del Roso et al., 2003). A recent paper provides evidence that autophagy is an essential downstream pathway for one of the mutations known to extend life-span in *C. elegans* (Melendez et al., 2003). It was proposed that suppression of autophagy in the ad libitum fed animal or human, may interfere with the maintenance of cell membranes and cause a progressive alteration of free-radical metabolism and accumulation of dolichol (Bergamini et al., 2003). Rats on a caloric restricted regimen spend most of the day in a state of fasting and higher autophagic proteolysis. The administration of antilipolytic drugs suppresses the release of FFA by the adipose tissue and stimulates autophagy. We have observed that Acipimox

does not affect autophagy and biomarkers of aging if given to rats fed ad libitum (unpublished). Present observation shows that treatment with the antilipolytic drug intensifies the anti-aging effects of submaximal calorie restrictions and makes them maximal.

It has already been shown that lower insulin and IGF-1 levels are associated with a longer life-expectancy (Tatar et al., 2001). Genetic interventions, which tune down insulin plasma levels (or hormone functioning in peripheral tissues) for life, extend lifespan by a 50% (Bluher et al., 2003). Life-long restriction of food intake (e.g. every-other-day ad libitum feeding) may decrease insulin plasma levels during the time of fasting only (e.g. Reaven et al., 1983), and still extends longevity by a 50% (Yu et al., 1985). This report may indicate that both frequency and level of hypoinsulinemia may affect the rate of aging: the anti-aging effects of caloric restriction decreased with the decrease in the frequency of fasting, but were restored if level of lower-frequency hypoinsulinemia were further decreased by the administration of an antilipolytic drug.

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