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Stimulation of Macroautophagy Can Rescue Older Cells from 8-OHdG mtDNA Accumulation: A Safe and Easy Way to Meet Goals in the SENS Agenda

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ABSTRACT

Reduction of oxidative stress within mitochondria is a major focus and important part in the SENS agenda. The age-related accumulation of mitochondria rich in oxidatively altered DNA may be a biomarker of malfunctioning and increased oxidative stress. Macroautophagy is the cell repair mechanism responsible for the disposal of excess or altered mitochondria under the inhibitory control of nutrition and insulin, and may mediate the antiaging effects of caloric restriction. The authors investigated the effects of stimulation of macroautophagy by the injection of an antilipolytic agent on the age-related accumulation of oxidatively altered mitochondrial DNA (mtDNA) in rat liver cells. Results showed that treatment rescued older cells from the accumulation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the mtDNA in less than 6 hours. It is concluded that the age-related changes in mtDNA and function are likely to be the consequence of a failure of macroautophagy in the recognition and disposal of a small number of severely injured mitochondria, and that easy and safe ways are available to counteract this change.

INTRODUCTION

AN AGENDA WAS PROPOSED quite recently (the SENS agenda) to combat the aging process[1,2] based on repairing the molecular and cellular damage that accumulates throughout life, including rescue of mtDNA mutations, so as to prevent age-related illness and frailty. However, severe criticism was raised that the proposed remedies fall in the realm of dreams[3] at the present time. Therefore, it might be appropriate to report here very recent results showing that accumulation with increasing age of mtDNA mutations in liver cells can be rescued in less than 6 hours by the stimulation of macroautophagy.

Macroautophagy is an important degradation/recycling system ubiquitous in eukaryotic cells that involves the rearrangement of subcellular membranes to sequester cytoplasm and organelles for delivery to the lysosome, where the sequestered cargo is degraded and recycled to generate nutrients during fasting under the control of amino acids and hormones.[4] It is well known that function of macroautophagy is essential for the turnover and rejuvenation of cellular components (including mitochondria) and declines with increasing age.[5–7]

Macroautophagy can be induced in liver cells by the injection of an antilipolytic agent to overnight fasted rats.[8] Treatment causes a highly reproducible and timeable sequence of events:

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plasma-free fatty acids, glucose, and insulin levels decrease in less than 10, 15, and 20 minutes, respectively.[9] Glucagon levels increase by 20 minutes. Vacuolation of liver cell lysosomes and activation of macroautophagy can be detected with an electron microscope as early as 30 minutes after treatment.[10] It was shown that the process can be monitored easily by measuring the changes in valine plasma levels.[11]

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were maintained on a standard rodent chow (Harlan TEKLAD, San Pietro al Natisone, Italy) and given drinking water ad libitum. Groups of six rats were sacrificed by the age of 3 and 16 months after 24 hours of fasting.

Acute effects of 3,5-dimethylpyrazole (DMP) administration

The antilipolytic agent DMP (12 mg/Kg bw) or vehicle only (physiologic solution) was injected intraperitoneally to older rats 6 and 3 hours before their sacrifice. The effects of treatment were assessed by assaying free fatty acids and glucose in plasma (by the acyl CoA synthetase/oxidase and the glucose oxidase/peroxidase techniques, respectively, using commercially available kits: FFA: Free Fatty Acids Half Microtest, Boehringer-Manheim KK; glucose: Glucinet, Sclavo ISVT, Siena, Italy).[9] Effects on plasma insulin were measured by radioimmunoassay using rat insulin as a standard.[12] The stimulatory effect of the antilipolytic drugs on the rate of liver autophagic proteolysis was monitored by assaying the levels of plasma valine by HPLC.[13] Norvaline was added as an internal standard to all samples.

Assay of oxidative mtDNA damage

The animals were anesthetized and livers were removed and homogenized in ice-cold 0.25 M sucrose solution supplemented with 10 mM EDTA (pH 7.2 to 7.4; 1 g wet tissue per 10-mL solution) by using a Potter-Elvehjem apparatus (Thomas Scientific, Philadelphia, PA). Crude mitochondrial preparations were obtained from liver homogenates by differential centrifugation. mtDNA was isolated according to the selective alkaline denaturation[14] modified procedure,[15] and mtDNAs (15 to 25 μg) were digested to the deoxynucleoside level by incubation at 37°C with 5 U of nuclease P1 (in 20 μL of 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) for 30 minutes and 1 U of alkaline phos-

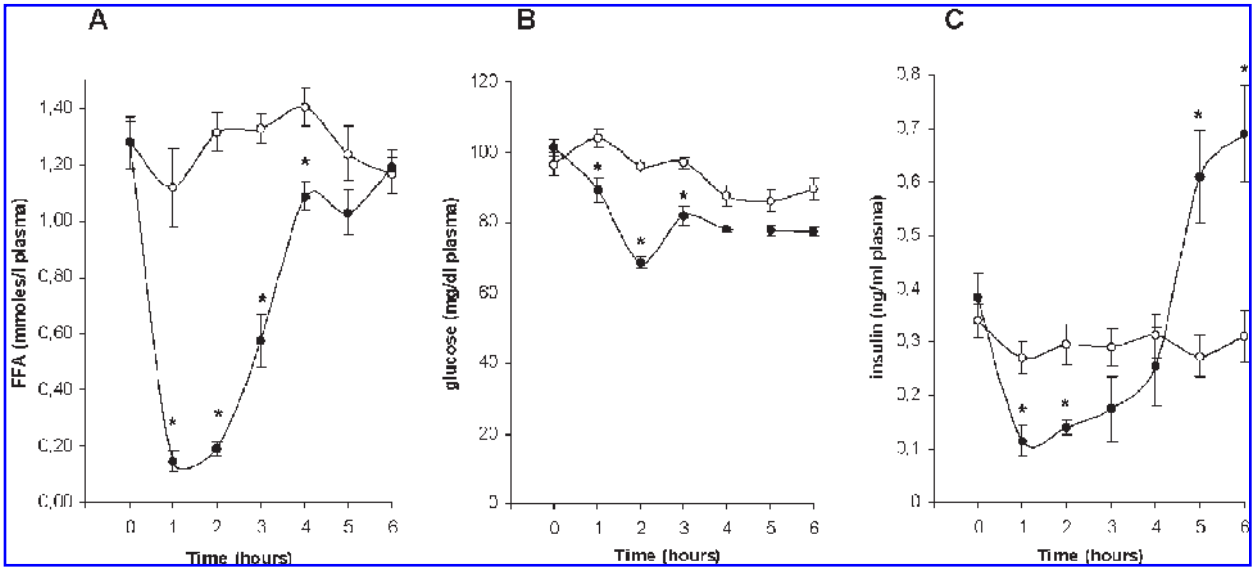

FIG. 1. Effect of the administration of an antilipolytic drug to fasted young rats on the plasma levels of FFA **(A),** glucose **(B),** and insulin **(C)**.[9] Means ± SEM are given for six cases. *Open circles:* control rats; *closed circles:* rats treated with the antilipolytic drug. * Denotes a statistically significant different with respect to control (*t*-test, $p < 0.05$).

Table 1. The Effects of the Administration of an Antilipolytic Drug on Valine Plasma Levels

	Time after the first injection (hours)					
	0 h		*3 h*		*6 h*	
Age	*CTRL*	*DMP*	*CTRL*	*DMP*	*CTRL*	*DMP*
3 months	177 ± 4.9	180 ± 2.7	151 ± 6.6	271 ± 6.3	167 ± 6.3	281 ± 8.5
16 months	216 ± 7.63	220 ± 4.5	189 ± 5.4	308 ± 10.8	217 ± 9.7	373 ± 9.8

Results are expressed as nmol/mL plasma. Means ± SEM of at least six cases are given. Statistical analysis (ANOVA): The effect of age, drug, and time were highly significant ($p < 0.0001$ all). Interactions were not significant.

phatase (in 20 μL of 1M Tris-HCl, pH 8.0) for 1 hour.[16] 8-OHdG and deoxyguanosine (dG) were measured by HPLC with online electrochemical and ultraviolet detection, respectively. For analysis, the nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μm, 4.6 mm × 25 cm) (Beckman-Coulter, Milano, Italy), and was eluted with a mobile phase containing 2.5% acetonitrile and 50 mM phosphate buffer pH 5.0.[17] A Beckman 126 pump at 1 mL/minute was used. 8-OHdG was detected with an ESA Coulochem III electrochemical coulometric detector (ESA, Inc., Bedford, MA) with a 5011 analytical cell run in the oxidative mode (E1 = 0 mV, E2 = 200 mV), and dG was detected with a Beckman UV detector model 166 at 254 nm. For quantification peak areas of dG standards and three-level calibration pure 8-OHdG standards (Sigma-Aldrich, Schnelldorf, Germany) were analyzed during each HPLC run.

MATERIALS

All chemicals used were of analytic grade.

RESULTS

The injected antilipolytic agent had a full effect, rapidly lowering free fatty acids (FFA), glucose, and insulin plasma levels for almost 3 hours (Fig. 1). Therefore, drug was reinjected after 3 hours in the following experiment.

Table 1 shows that treatment increased the plasma levels of valine through experimentation both in younger and 16-month-old rats to a similar extent.

Figure 2 shows the effects of older age and of the antilipolytic drug on 8-OHdG level in mtDNA. The stimulation of autophagy rescued liver cells from the age-associated accumulation of altered mtDNA and tuned levels down to the juvenile value in less than 6 hours.

DISCUSSION

Macroautophagy is the universal mechanism responsible for maintaining and cleaning cells,[5] which helps to get rid of altered or excess organelles and membranes (including mitochondria and peroxisomes).[6,18,19] The in vivo function of macroautophagy is regulated by nutrition. (Degradation was shown to occur

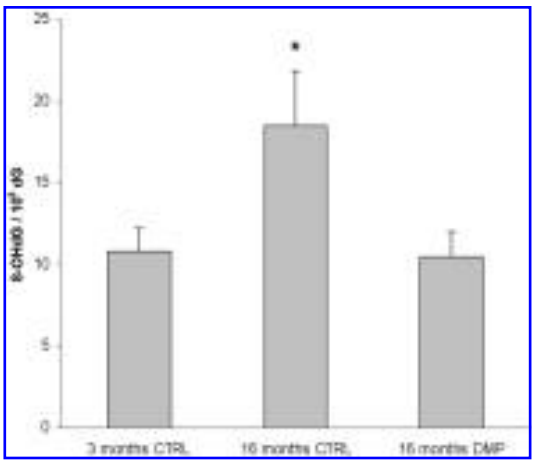

FIG. 2. Effect of age and of the pharmacologic stimulation of macroautophagy on the abundance of 8-OHdG in mtDNA (on the ordinate) in the liver of male Sprague-Dawley rats. Means of eight cases are given. Vertical bars = SEM. The restorative effect of the pharmacologic treatment was highly significant. (* Denotes a statistically significant different with respect to 3-month-old controls; *t*-test, $p < 0.05$).

with lack of exogenous nutrients in the intervals between meals.)[20] It was suggested that macroautophagy might be involved in aging and the antiaging mechanism of caloric restriction.[21] Several pieces of evidence may support the hypothesis: Increasing deterioration in functioning of macroautophagy is known to occur with increasing age starting before age 12 months.[7,22] The age-related decline in macroautophagy is counteracted by caloric restriction;[23] both extension of lifespan and protection of rat liver autophagic proteolysis from the age-related decline covary with the duration of antiaging food restriction in a similar way;[24] lifelong intermittent stimulation of autophagic proteolysis by antilipolytic agents leads to the pharmacologic intensification of suppression of aging (PISA) by caloric restriction.[9,25]

It is well known that altered mitochondria progressively accumulate with increasing age,[26,27] and accumulation may be part of a vicious circle leading to an increased rate of reactive oxygen species production and aging.[25,28] Caloric restriction can prevent the accumulation at least in part.[29,30] It was proposed that the beneficial effects of caloric restriction decreasing mitochondrial electron flow and proton leaks to attenuate damage caused by reactive oxygen species could depend on an induction of mitochondrial biogenesis.[31,32]

In view of the role of macroautophagy in the action mechanism of caloric restriction, these data prompted the authors to explore the effects of macroautophagy stimulation in altered mtDNA removal. The obtained data, showing that two timed injections of an antilipolytic drug stimulate autophagy and fully suppress the accumulation of oxidatively damaged mtDNA in the aging liver in an astonishingly short time, indicate that the age-related decline of macroautophagic degradation may be behind the age-related accumulation in mammalian cells of mitochondria bearing high levels of oxidatively damaged mtDNA.

The mitochondrial turnover rate in rat liver cells is rather slow.[33] It is conceivable that the age-related accumulation of damaged mtDNA might be a consequence of failure of macroautophagy in the selective disposal of a small number of severely injured mitochondria.[5] It has been shown that incubation of primary cells and cell lines in the absence of serum promotes autophagy of mitochondria with deleterious mtDNA mutations but spares their normal counterpart.[34]

In conclusion, easy and safe ways to meet the goals of the SENS agenda are already available. The SENS agenda also may be a useful guideline to focus antiaging biology on the real targets.

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