

Liver Exposure to Xenobiotics: The Aging Factor and Potentials for Functional Foods

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ABSTRACT

Hepatocytes isolated from 20- and 4-month Wistar rats and cultured with or without α -linolenic acid (LNA) were then added with nutraceutical YHK or silybin before the test with iron or copper. Overall, YHK proved to be more effective than silybin in Fe/Cu-induced peroxidative damage on normal and LNA-loaded hepatocytes ($p < 0.05$). YHK exerted a significant protection against DPPH radical-scavenging activity in the "old" group (p versus silybin) and against lipophilic generators in both age groups ($p < 0.05$ versus silybin). Both compounds were ineffective on age-related increase of surface-charge density. These preliminary data suggest that age *per se* enhances the vulnerability of hepatocytes to xenobiotics, whereas some safe nutraceuticals seem to exert significant protective effects.

INTRODUCTION

IT HAS BEEN SHOWN that metals undergo redox cycling resulting in the production of reactive oxygen species and a number of mechanisms associated with the toxicity of metal ions are very similar to the effects produced by many organic xenobiotics. Indeed, the most important mechanism of oxidative damage to proteins is metal-catalyzed oxidation, which may end up with the loss of enzymatic activity and alteration of protein structure and favor the induction of DNA damage and oncogenesis. On the other hand, with aging process it occurs an increasing exposure to drugs and xenobiotics while facing a decreased detoxifying ability and metabolic impairment,^{1,2} lead-

ing to a higher susceptibility to oxidative damage to DNA, as very recently suggested.³ The present study investigated a novel hepatoprotective compound that recently has been shown to possess significant liver protecting capacity⁴⁻⁶ in *in vitro* testing of hepatocyte oxidative damage by iron and copper while probing the effect of physiologic aging *per se* in this setting.

MATERIALS AND METHODS

Isolation, culture and preparation of hepatocytes

Twenty-month-old and 4-month old male Wistar rats were fed with standard chow and water ad libitum for 2 weeks. Then hepatocytes

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were isolated from "old" (20-month) and "young" (4-month) animals by collagenase perfusion methods. Approximately 1.5×10^6 cells in 3 mL or 5.0×10^6 cells in 10 mL were plated in individual 60- or 100-mm diameter culture dishes and placed in an incubator in an atmosphere of 5% CO₂ 95% air at 37°C. After a 9-hour incubation, the monolayer of hepatocytes was cultured for an additional 12 hours in the medium containing 1.0 mM (-linolenic acid (LNA)-bovine serum albumin (BSA). More than 70% of added LNA was adsorbed by cultured cells after incubation.

Hepatocyte culture test

Hepatocytes were washed and cultured in 60-mm (1.5×10^6 cells/dish) with 200 $\mu\text{g}/\text{mL}$ dilution of YHK sample (panax pseudo-ginseng, Eucommia Ulmoides, polygonati rhizome, glycyrrhiza licorice, panax ginseng, Kyotsu Jigyō, Tokyo, Japan) or sylibin (200 $\mu\text{g}/\text{mL}$) dissolved in dimethyl sulfoxide 10 min before the addition of test salts at a concentration of 100 μM each. Malonildialdehyde (MDA) in the medium was assessed by spectrophotometry.

Preparation of LNA-BSA complex

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LNA was adsorbed to bovine serum albumin and 1 μL of LNA was dissolved in 10 mL of 0.1 N NaOH solution. This solution was serially added to 240 mL of complete Williams' medium E 1 mM BSA, which had a fatty acid/albumin molar ratio of 4. The resulting fatty acid-BSA complex was sterilized by filter-passage through a 0.2- μm Millipore filter.

Lysosome fragility test

Lysosomal enriched fractions were incubated with test compounds and each metal ions and β -galactosidase activity was assessed as described elsewhere,⁷ using 4-methylumbelliferyl- β -galactosidase as a substrate. The results were expressed as percentage of total β -galactosidase released. Lactate dehydrogenase leakage also was measured in the culture medium.⁸

Oxidative damage tests of lysosomes

Assays for the release of acid phosphatase and β -N-acetylglucosaminidase from lysosomes

were performed by incubating lysosomal suspensions with test compounds in the presence of 50mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) or 1mM 2,2'-azobis(2,4-dimethylvaleronitrite) (AMVN). The effect of the test compounds on lysis was calculated as a percentage of control. Quenching activity of YHK and sylibin against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was assessed by spectrophotometry.

Surface charge density of hepatocytes

The electrophoretic mobility of hepatocytes was measured by electrochemical methods. The surface charge density was determined using the equation: $\sigma = \eta u/d$; where u = electrophoretic mobility, η = the viscosity of the solution, d = diffuse layer thickness.⁹ The diffuse layer thickness was determined from the formula: $d = \sqrt{\varepsilon - \varepsilon_0 - R - T/2 - F^2 - I^{10}}$ where R is the gas constant, T is the temperature, F is the Faraday number, I is the ionic strength of 0.9% NaCl, and $\varepsilon\varepsilon_0$ is the permeability of the electric medium.

Statistical analysis

All experiments were performed in triplicate and the significance was determined by employing a Duncan's multiple-range test. Data were expressed in the text as means (SD) and a probability value of <0.05 was set as indicating that a statistically significant difference existed between experimental groups.

RESULTS

Metal-induced lipid peroxidation

MDA generation in the medium exhibited a direct time-course increase peaking at 5 hour after the addition of metal catalysts and this effect was more pronounced in the "old" group (0.01 versus "young"). The amount of MDA concentration for Fe and Cu ions was 2.9 and nmol/mg protein/5 hour in normal hepatocytes and 8.4 and 9.5 nmol/mg protein/5 hour in loaded hepatocytes, respectively. Both sylibin and YHK significantly decreased at the same MDA generation in the medium in the

“young” group ($p < 0.05$). However, YHK showed significant protective effect against metal ions in “old” group, as expressed by the half inh concentration IC_{50}) of lipid peroxidation on either normal hepatocytes ($FeSO_4$ 14.2 ± 2.4 18.9 ± 3.2 ; $CuSO_4$ 9.5 ± 0.7 versus 11.3 ± 0.3) and in LNA-loaded cell ($FeSO_4$ 71.2 ± 8.3 78.8 ± 5.1 ; $CuSO_4$ 19.5 ± 1.2 versus 21.3 ± 1.5 , $p < 0.01$ versus sylibin).

When challenged with metal ions, lysosomal fractions expressed a significant increase of LDH leakage and β -galactosidase release (between 22% and 31% from untreated group, $p < 0.01$) at the same extent in both age groups. Both compounds significantly reduced the LDH concentration recovered in the medium of lysosomal fractions (36% to 42% decrease from untreated group, $p < 0.05$).

Tests of lysosomal oxidative stress

As compared to untreated lysosomal fractions challenged with the two peroxide radicals generators, either YHK and sylibin exerted a significant protection in “young” group ($p < 0.01$). In particular, such protection was comparably effective between hydrophilic and lipophilic generated free radicals. However, YHK showed a significantly more protective effect than sylibin against lipophilic generators and higher DPPH radical-scavenging activity in “old” group (Fig. 1) ($p < 0.05$).

Surface charge density

The surface charge density was $12\% \pm 3\%$ higher in the “old” group ($p < 0.05$ versus

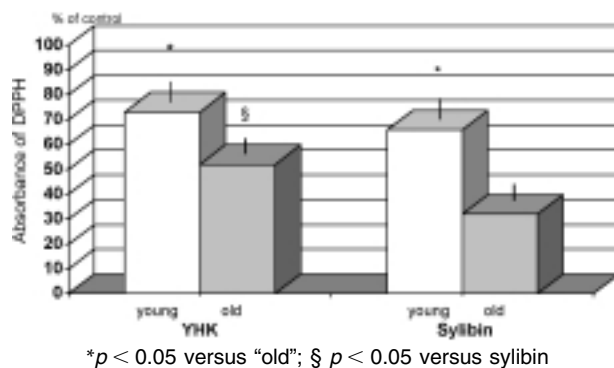


FIG. 1. DPPH radicals-scavenging activity of YHK and sylibin in lysosomal fractions (young versus old) (mean \pm standard deviation).

“young”) and this parameter was unaffected by both test compounds, irrespective of age group.

DISCUSSION

The most relevant mechanism of oxidative damage to proteins is metal-catalyzed oxidation,¹¹ which causes the loss of enzymatic activity and alteration of protein structure.¹² This process involves the binding of such metals to specific sites with the reduction of Fe or Cu by a suitable electron donor like NADH, NADPH, ascorbate and, through the generation of H_2O_2 , a further cascade burst of highly reactive oxygen species perpetuating tissue damage. In particular, the accumulation of divalent metal ions has been proposed to contribute to aging, neurodegenerative disorders, and enhanced cancer risk.^{13,14} Among biologic systems, iron preferably accumulates within the liver with derangement of hepatic microsomal enzyme activity, electron transport, respiration and lysosomal function.¹⁵ Indeed, the oxidant stress damage is preferentially targeted to the lysosomal compartment, which is rich in low molecular weight redox-active iron and the rupture of lysosomes, followed by relocation of labile iron to the nucleus, could be an important intermediary step in the generation of oxidative DNA damage, as has been recently demonstrated.¹⁶ In this study it was interesting to note that hepatocytes of “old” mice were most susceptible to transitional metals–oxidative stress with altered electrokinetic properties as compared to “young” control, thus confirming the concept of the “aging liver.”¹¹ This suggests that the oxidative damage observed in older animals probably results from the accumulation of endogenous damage, which worsens the injury caused by exposure to xenobiotics. Although known hepatoprotective agents such as sylibin confirmed its antioxidant efficacy in “young” hepatocytes, it appeared that only YHK was able to maintain such properties also in “old” cells. It is conceivable that some YHK components endowed by potent antioxidant property^{17–20} might have further contributed to such effect. Such preliminary data suggest the potential of some functional foods to beneficially affect the homeostasis of

free radicals balance also in senescent organisms. Further studies are in progress to analyze the genomic expression of YHK in view of modifying its hydrophobic/hydrophilic characteristics, given its lack of effect on the electrochemical membrane properties, which are likely to rely also on nonoxidative mechanisms.

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