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Differential hepatic protein tyrosine nitration of mouse due to aging – Effect on mitochondrial energy metabolism, quality control machinery of the endoplasmic reticulum and metabolism of drugs

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ABSTRACT

Aging is the inevitable fate of life which leads to the gradual loss of functions of different organs and organelles of all living organisms. The liver is no exception. Oxidative damage to proteins and other macromolecules is widely believed to be the primary cause of aging. One form of oxidative damage is tyrosine nitration of proteins, resulting in the potential loss of their functions. In this study, the effect of age on the nitration of tyrosine in mouse liver proteins was examined. Liver proteins from young (19–22 weeks) and old (24 months) C57/BL6 male mice were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotted onto nitrocellulose membranes. Proteins undergoing tyrosine nitration were identified using anti-nitrotyrosine antibody. Three different protein bands were found to contain significantly increased levels of nitrotyrosine in old mice (Wilconxon rank-sum test, $p < 0.05$). Electrospray ionization liquid chromatography tandem mass spectrometry (ESI-LC–MS/MS) was used to identify the proteins in these bands, which included aldehyde dehydrogenase 2, Aldehyde dehydrogenase family 1, subfamily A1, ATP synthase, H^+ transporting, mitochondrial F1 complex, β subunit, selenium-binding protein 2, and protein disulfide-isomerase precursor. The possible impairment of their functions can lead to altered hepatic activity and have been discussed.

1. Introduction

Aging is a universal phenomenon observed in all living organisms but the detailed physiological mechanisms implicated with aging is still not clear. Evidently, aging leads to progressive loss of functions of various organs accompanied by increasing mortality and disability. The effect of age observed in the structure and function of liver include increase in serum and biliary cholesterol, decrease in the rate of liver regeneration, and decreased ability to metabolize drugs. This leads to many health problems including coronary disease, gallstones and inability to tolerate pharmaceuticals. Unfortunately, high prevalence of different diseases such as arthritis, hypertension, cancer, diabetes and stroke due to aging leads to high use of medications in older people. Although there are distinct benefits of appropriate medications, the harmful effects of such medications in older people are well documented. In fact, the occurrence of adverse drug reactions correlates with age (for references see [1]).

Recent research has attempted to determine the physiological mechanism underlying the development of the aging process. One well-established theory of aging is the free radical theory originally proposed by Harman [2]. According to this theory, reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radical interfere with normal physiological functions by oxidizing proteins, lipids and other biomolecules. ROS are generated endogenously, as by product of metabolic processes, and exogenously, from pollutants, radiation, and other sources (for references see [3]).

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In addition to ROS, reactive nitrogen species (RNS) also oxidatively modify macromolecules and have been implicated in aging and age-associated diseases [4]. Like ROS, RNS are generated both endogenously and exogenously, and they include nitric oxide and peroxynitrite [4]. Nitric oxide is a naturally occurring messenger molecule that plays a key role in blood flow, inflammation, and neurotransmission, but is cytotoxic in excess [5]. It may be absorbed from exogenous sources such as cigarette smoke, or synthesized endogenously by neutrophils and macrophages [5]. In addition to auto oxidation, a reaction between nitric oxide and superoxide anion synthesizes peroxynitrite [4]. Although it has been proposed [6] that peroxynitrite leads to the formation of 3-nitrotyrosine, it must be mentioned that the importance of peroxynitrite in nitrotyrosine formation is somewhat contested [7,8].

In fact, Ischiropoulus [9] has suggested that tyrosine nitration is due to a variety of mechanism, rather than one simple pathway. Regardless of the mechanism, nitration of tyrosine is a key modification, observed in normal aging process and diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and heart failure [4,10,11]. Unfortunately, although protein tyrosine nitration can be involved in a number of pathological changes and associated with various diseases, very little is known about the specific protein targets so far.

The present study aims to find out the effect of RNS on the liver due to aging. The goal is to identify the proteins which are modified by RNS and the possible effect(s) of such modification on the function of liver.

2. Materials and methods

2.1. Young and old mouse liver sample collection

Young (19–22 weeks) and old (24 months) C57BL//6 male mice were obtained from Harlam Sprague Dawley, Indianapolis, IN, maintained as a part of the National Institute of Aging Rodent Colonies. The mice were rested for one to two weeks at the animal facility of the Joint Sciences Department of Claremont Universities Consortium in Claremont, CA. The animals were euthanized by cervical dislocation. Livers were collected, frozen immediately, and stored in liquid nitrogen until further use. Five old and five young livers were used.

2.2. SDS–PAGE and western blot analysis

The protein samples for livers of young and old mice were prepared as described earlier for kidney and heart [12,13]. Separation of the proteins was carried out by SDS–PAGE using linear gradient polyacrylamide gels (7.5–12.5% w/v, acrylamide, Bio-Rad Laboratories) and the proteins were transferred electrophoretically onto a nitrocellulose membrane. Following blocking and washing of nitrocellulose membranes as described earlier [14], the nitrocellulose membranes were treated with (i) anti-nitrotyrosine antibody (Millipore), (ii) HRP conjugated rabbit anti-mouse antibody and (ii) Chemiluminescence detection kit (Visualizer Western Blot Detection Kit). Digital images were captured and analyzed using a CCD-based AutoChemi Bioimaging system and VisionWorksLS software (UVP, LLC, Upland, CA). Statistical significance ($p \leqslant 0.05$) for differential expression between young and old samples was carried out using the Wilcoxon Rank Sum Test.

2.3. Staining of nitrocellulose membrane, band excision and trypsin digestion

The proteins were separated by SDS–PAGE, electroblotted onto nitrocellulose membranes and stained with Memcode protein stain (Bio-Rad Laboratories). Typically, for band excision and trypsin digestion, the nitrocellulose membrane to which proteins have been transferred electrophoretically, contained one lane of young liver sample, one lane of old liver sample, one lane of prestained molecular weight marker and nine lanes of pooled liver samples. The membrane was cut through the middle of the lane containing prestained molecular weight marker and the section containing two lanes of young and old samples was developed for detection of nitrotyrosine containing proteins for western blot analysis as described above. The remaining nine lanes were developed with Memcode protein stain reagent, a total protein stain (Bio-Rad Laboratories) according to manufacturer's instruction. The protein bands corresponding to the bands showing in upregulation in protein tyrosine nitration were excised, washed, cut into small pieces and digested with trypsin (Promega, 20 μg/mL in 50 mM NH_4HCO_3, pH 8.0). The membrane was incubated overnight at 37 °C. The samples were vacuum dried, 1 mL acetone was added and the supernatant was collected. The membrane was further washed two times with 2% acetonitrile with 0.1% formic acid, vortexed and the supernatant was collected. The above supernatants containing tryptic peptides were combined, dried and stored at −20 °C.

2.4. Tandem mass spectrometry of tryptic peptides, database searching and data processing

The tryptic digested proteins were analyzed by nano-ESI-LC–MS/MS using LCQ Deca XP Proteome X System (Thermo Electron Corporation, San Jose, CA). The conditions used were described in detail [13]. The mass spectrometer was set to acquire a full MS scan between 450 and 1800 *m/z* followed by a full MS/MS scan. All MS/MS spectra were searched with SEQUEST algorithm based Bioworks 3.3 (Thermo-Fisher) against a database created by extracting mouse entries from NCBI ftp site. Proteins with four or more spectra were accepted as positive identification.

3. Results

Western blot analysis identified three bands containing nitrotyrosine containing proteins (lanes 1–10, cf. Fig. 1). Total protein present in the same extract used for Western blot analysis was detected using Memcode protein stain (cf. Fig. 2). The top two protein bands formed a doublet while the bottom band was identified as a single band. They were labeled as TT (top–top), TB (top–bottom) and B (bottom) bands. As evident from Table 1, area density analysis and a Wilcoxon rank-sum showed a significantly higher level

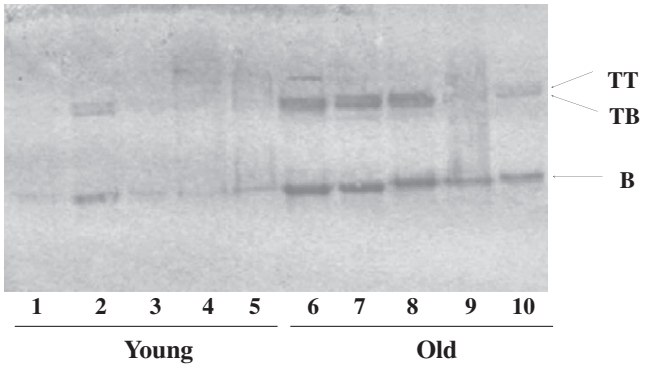

Fig. 1. Effect of aging on tyrosine nitration of proteins isolated from mouse liver. Lanes 1–5 are liver samples from five different young mice and lanes 6–10 are liver samples from five different old mice. Approx. 50 μg of protein was loaded onto each lane. Anti-nitrotyrosine antibody was used for detection of nitrotyrosine containing proteins. TT denotes top–top band, TB denotes top–bottom band and B denotes bottom band containing nitrotyrosine proteins.