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Protein oxidation seems to be linked to constitutive autophagy: A sex study

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ABSTRACT

Aim: Although constitutive autophagy is linked to redox state and participates in cell homeostasis, it is scarcely known if redox state, autophagy, and lysosomal function depend on sex, a factor that largely influences health and diseases. Therefore, we evaluated the existence of sex differences in redox state and constitutive autophagy in rat tissues.
Main methods: 7 week old Sprague–Dawley rats were used to obtain organs. Malondialdehyde (MDA), and carbonylated proteins were measured by spectrophotometric methods for redox state assessment. The autophagy biomarkers Beclin-1, and microtubule-associated protein 1 light chain 3 (LC3), the mammalian target of rapamycin (mTOR; checkpoint in autophagic process), and the lysosomal associated membrane protein (LAMP-1; biomarker of lysosomes) were evaluated by Western blotting. Immunofluorescence analysis was also performed for LC3 and LAMP-1 colocalization.
Key findings: In the heart, Beclin-1, and LC3-II/LC3-I were higher in males than in females suggesting that the male heart has a major constitutive autophagy and this was linked with higher levels of carbonyl groups, indicating that protein oxidation could play a role. In the liver, it was found that LAMP-1 was higher in males and greatly colocalized with LC3 indicating a larger number of autophagolysosomes. None of the above parameters was significantly different in the kidneys of both sexes with the exception of MDA, which was significantly higher in females.
Significance: The above results suggest that sex differences exist in redox state and autophagy and they occur in an organ-specific way. Importantly, it seems that the protein oxidation is more linked with constitutive autophagy, at least in cardiac ventricles, in comparison with lipid peroxidation.

Introduction

Living cells produce reactive oxygen species (ROS) that can act either as signaling molecules or as destructive molecules resulting in autophagy and cell death (Kurz et al., 2007). Under normal conditions, there is a balance between formation and neutralization of ROS and, consequently, a minimal modification of biomolecules (Laher, 2013). Proteins and lipids are among molecules that undergo oxidative stress being the carbonylation one of the most important oxidative modification of proteins (Dalle-Donne et al., 2006), while lipid peroxidation leads to the generation of unsaturated lipid aldehydes such as malondialdehyde (MDA) when ROS attack polyunsaturated fatty acids (Mylonas and Kouretas, 1999). Some authors consider carbonyl groups of proteins and unsaturated lipid aldehydes as the best markers of oxidative stress (Dalle-Donne et al., 2006; Valle et al., 2007). They are linked with numerous diseases and conditions such as cardiovascular diseases, diabetes mellitus and its complications, liver diseases, and aging (Laher, 2013) which are characterized by numerous sex differences (Legato, 2009).

Evidence seems to suggest that redox state might be different in males and females (Malorni et al., 2007). However, the sex influences on carbonyl groups and MDA content are scarcely known and controversial, at least in healthy animals, probably due to the different methods used, and the different ages and species of animals. In particular, in aged male rats (24 months old), cardiac protein oxidation, measured as protein carbonyl, is higher than in females whereas the cardiac lipid peroxidation, analyzed with the N-methyl-2-phenylindole reaction, is not different between sexes (Kayali et al., 2007). In aged C57BL6 mice (10 months of age) and in rats (24 months or 3 months old Wistar rats), brain oxidative stress is bigger in females than in males (Ali et al., 2006; Guevara et al., 2009; Katalinic et al., 2005). Indeed, in male and female livers obtained from 3 months

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aged rats, the levels of carbonyl groups and lipid peroxidation detected with our own methods are not different (Valle et al., 2007).

Autophagy is crucial for maintaining cellular homeostasis process whereby cytoplasmic components are delivered to lysosomes for degradation (Rabinowitz and White, 2010). Alteration in constitutive autophagy is implicated in many pathological conditions, including heart diseases (Wang et al., 2012), diabetes mellitus and its complications, and liver diseases (Murrow and Debnath, 2012).

A cross-talk between ROS and autophagy has been described (Lee et al., 2012; Scherz-Shouval et al., 2007): ROS can induce directly autophagy affecting LC3 lipidation and autophagosome formation, and indirectly modulating mammalian target of rapamycin (mTOR), the master autophagic regulator (Jung et al., 2010). Specific roles of different ROS-derived molecules are complex and sometimes controversial: carbonylated proteins can enhance autophagy to ensure they are removed (Essick and Sam, 2010), while lipid peroxidation products are able to inhibit autophagy contributing to cell dysfunction (Krohne et al., 2010).

However, it is still unclear whether the autophagic process is influenced by sex. In particular, in isolated rat vascular smooth muscle cells, in starved neurons, in starved rat embryonic fibroblasts, and in the atrophic hearts of tumor-bearing mice, constitutive or induced autophagy appears to be sex-dependent (Cosper and Leinwand, 2011; Du et al., 2009; Straface et al., 2009).

Numerous health problems are influenced by sex (Legato, 2009) and many of them concern the heart, the liver and the kidney (Legato, 2009) thus, the current study has been designed to establish whether in rat hearts, livers and kidneys sex affects: a) the formation of protein carbonyl groups and MDA, and b) autophagic response through the measure of Beclin-1, required for the initiation of the autophagosome (Rabinowitz and White, 2010), the soluble form of LC3 (LC3-I) and its converted form (LC3-II) localized in autophagosomal membranes (Rabinowitz and White, 2010), the autophagic hub mTOR (Jung et al., 2010) and the lysosomal associated membrane protein (LAMP-1), which mediates the fusion of mature autophagosomes with cytosolic lysosomes leading to the autophagolysosome (Eskelinen, 2006).

Materials and methods

Animals

Male and female Sprague–Dawley rats (7 weeks old) were purchased from Harlan, Italy. Rats (2–3 per cage) were maintained on a 12 h light/dark cycle and allowed food and water ad libitum till the sacrifice. The experimental protocols were carried out in accordance with the Italian law (DL 116, 1992) and the National Institute of Health principles of laboratory animal care (NIH 80-33, revised 1996).

Animals were decapitated, and thoracic and abdominal cavities were opened to expose the hearts, livers and kidneys. Organs were rapidly removed and washed with ice-cold PBS. Parts of each tissue (ventricles for the hearts, all lobes for the livers, one kidney) were weighed and homogenized in ice-cold PBS, stored at −80 °C and used within 1 month, while the other parts from the same animals were used for immunofluorescence analyses, after formalin fixation and paraffin embedding.

Protein detection

Total tissue protein content was measured by the Lowry method (Lowry et al., 1951).

MDA detection

MDA levels were measured according to Esterbauer and Cheeseman (1990) with slight modifications as previously described (Campesi et al., 2012).

Carbonyl assay

Carbonylated proteins were quantified as described by Fagan et al. (1999) with minor modifications using 2, 4-dinitrophenylhydrazine (DNPH) (10 mM in HCl 2 N). The absorbance was recorded at 370 nm and the carbonyl concentration was calculated using the extinction molar coefficient of DNPH ($\varepsilon = 22\,000$). Carbonyl concentration was normalized using the blank protein concentration measured at 280 nm.

Selection of the reference protein for normalization of western blot data

Previously, in rat liver, it was shown that the reference proteins most frequently used to normalize Western Blot data were sexually divergent (Campesi et al., 2012; Verma and Shapiro, 2006). Therefore, we evaluated α-actin, β-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the hearts, livers and kidneys to select the suitable protein for normalization. The procedure of protein determination was described below.

Western blot analysis

80 μg of proteins was resolved by SDS-polyacrylamide gel electrophoresis, transferred to PVDF and membranes blocked with 5% skim milk in TBST (150 mM NaCl, 20 mM Tris–HCl, pH 7.2) for 2 h. The membranes were, then probed overnight at 4 °C with specific polyclonal antibodies, (α-actin (1:1000), β-tubulin (1:1000), GAPDH) (1:1000), mTOR (1:1000), Beclin-1 (1:1000), LAMP-1 (1:1000) and LC3 which reacts with both LC3-I and LC3-II (1:500). Antibodies were obtained from Cell Signaling Technology (DBA Italia, Italy) with the exception of an anti-LC3 antibody that was purchased from Eppendorf Italia (Italy).

After washing in TBST, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1 h, RT, 1:2000; Cell Signaling Technology, DBA Italia, Italy). Specific proteins were detected by the chemiluminescence reaction (Luminata TM Classic Western Blot HRP substrate, Millipore, Italy), followed by densitometric analysis by dedicated software (Labworks, Canada).

Immunofluorescence microscopy

For immunofluorescence microscopy analyses in the hearts, livers and kidneys a double labeling of LAMP-1 and LC3 was performed as LC3-II and LAMP-1 co-localization indicates autophagolysosome formation (Wu et al., 2006). 5 μm thick sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. After distilled water washings, sections were incubated with polyclonal anti-LC3 (Eppendorf Italia, Italy), which reacts with both LC3-I and LC3-II, and monoclonal anti-LAMP-1 (DBA Italia, Italy) for 30 min at 37 °C. After extensive washing, sections were exposed to the appropriate TRITC- and FITC-conjugated secondary antibody (Sigma-Aldrich, Italy) for 30 min at 37 °C. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich, Italy). Each sample was tested in triplicate and at least 3 different animals for each sex were analyzed and examined with an Olympus BX51 microscope and immunofluorescence analyses were carried out by intensified video microscopy (IVM) by a Charge-coupled device camera (Carl Zeiss, Germany) at 100× magnification.

Statistical analysis

For statistical analysis of data, male and female were compared. Continuous parametric variables were analyzed using the Student *t*-test. Non-parametric variables were compared with the Mann–Whitney rank test. For all tests, a P-value ≤ 0.05 was considered as statistically significant.