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Carbonylation of mitochondrial proteins in *Drosophila melanogaster* during aging

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Abstract

Age-related changes in carbonylation of mitochondrial proteins were determined in mitochondria from the flight muscles of *Drosophila melanogaster*. Reactivity with antibodies against (i) adducts of dinitrophenyl hydrazine (DNP), commonly assumed to react broadly with derivatized carbonyl groups, (ii) malondialdehyde (MDA) or (iii) hydroxynonenal (HNE), was compared at five different ages of flies. MDA and HNE are carbonyl-containing products of lipid peroxidation, which can form covalent adducts with proteins. Specific objectives were to address the following inter-related issues: (1) what are the sources of adducts involved in protein carbonylation in mitochondria during aging; (2) is carbonylation by different adducts detectable solely by the DNP antibodies, as assumed widely; (3) can the adducts formed by lipid peroxidation products *in vivo*, be used as markers for monitoring age-associated changes in oxidative damage to proteins. The total amounts of immunoreactive proteins, detected by all three antibodies, were found to increase with age; however, the immunodensity of individual reactive bands and the magnitude of the increases were variable, and unrelated to the relative abundance of a protein. While some protein bands were strongly immunopositive for all three antibodies, others were quite selective. The amounts of high molecular weight cross-linked proteins (>200 kDa) increased with age. In general, the anti-HNE antibody reacted with more protein bands compared to the anti-MDA or -DNP antibody. The results suggest that sources of the carbonyl-containing protein adducts vary and no single antibody reacts with all of them. Overall, the results indicate that HNE shows robust age-associated increases in adduction with mitochondrial proteins, and is a good marker for monitoring protein oxidative damage during aging.

Keywords

Carbonylation; hydroxynonenal; malondialdehyde; oxidative stress; aging; *Drosophila melanogaster*; antibodies

INTRODUCTION

A primary manifestation of the aging process is the progressive loss in the functional competence of various physiological systems, which diminishes the ability to maintain homeostasis, with a consequent exponential rise in the rate of mortality [1,2]. Although a cause-

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and-effect relationship has yet to be firmly established, a pro-oxidizing shift in the cellular redox state and progressive accumulation of macromolecular oxidative damage, are often postulated to underlie the age-associated attrition in the maximal capacity of physiological systems (reviewed in [3–6]). Mitochondria are often implicated in the causation of oxidative stress and senescent changes because: (i) their inner membrane is the primary site of generation of superoxide anion radical, a progenitor of an array of downstream reactive oxygen species (ROS) [7,8]; (ii) mitochondrial respiratory activity, particularly the ADP-stimulated state 3 rate of oxygen consumption, declines in the aged organisms [9,10]; and (iii) mitochondria themselves undergo an age-associated increase in macromolecular oxidative damage, which has been experimentally demonstrated to lead to an increase in rates of mitochondrial ROS generation [11] and a decline in respiratory activity [12].

Although virtually all types of macromolecules undergo oxidative damage *in vivo*, protein damage is thought to have direct physiological consequences due to their role as bio-catalysts. Attacks by ROS on proteins can cause a variety of structural modifications including, formation of disulfide cross-links, methionine sulfoxide, dityrosine cross-links, nitrotyrosine, and carbonyls [3,13]. The addition of carbonyl groups is a ubiquitous and well-characterized post-translational modification. Protein carbonylation can occur by at least three different mechanisms (reviewed in [3,14,15]): (i) metal-catalyzed oxidation (MCO), which introduces carbonyls in to the side-chains of certain amino acids; (ii) reactions with lipid peroxidation products containing aldehyde functional groups, such as HNE and MDA, and (iii) interaction between glycine residues and sugars (glycation) or their oxidation products (glycoxidation). Membrane proteins are thought to be particularly susceptible to modification by the products of lipid peroxidation, because of the presence of the polyunsaturated fatty acids in the phospholipid bilayer, whereas glycation tends to be largely restricted to the extracellular proteins [16].

It is now well established that the amount of carbonylated protein in tissue homogenates of different species increases during aging [15,17]. In initial studies, where amounts of protein carbonyls, derivatized with DNPH, were measured spectrophotometrically, it was estimated that >10% of the protein in tissue homogenates of aged organisms was carbonylated [3]. It was also inferred from such data that carbonylation was a ubiquitous and random process. However, later studies, initially conducted in this laboratory, involving immunoblotting with anti-DNP antibodies, indicated that protein carbonylation during aging largely targets specific proteins and is not a random occurrence [18,19]. Since carbonylation can potentially affect protein catalytic activity, it is of interest to understand the mechanisms of carbonylation, particularly the intracellular sources of the carbonyl adducts, e.g. whether they are formed by MCO or are products of lipid peroxidation, such as MDA and HNE. This issue is also of practical importance as it is presently unclear whether anti-DNP antibody, which is most commonly used for the localization of carbonylated proteins is indeed versatile and specific enough to react with different carbonyl-containing epitopes, including MDA and HNE.

Another important issue concerning anti-DNP antibodies is that their use for quantification of carbonylated proteins has been widely reported to yield erratic results. For instance, while some studies have found aging-related increases in protein carbonyl content [4,11,17], others reported the absence of such increases (e.g. see [20] and references therein). Some of the reported limitations of this technique include artifactual formation of carbonyls during tissue handling, batch-to-batch variation in specificity of commercially available antibodies and alterations in the electrophoretic and electrofocusing mobility of DNPH-derivatized proteins compared to underivatized samples. Such variations can complicate quantitative comparisons between immunoblots. The reliability of the DNP-based carbonyl assays has been repeatedly questioned and caution advised in the interpretation of data [21]. Accordingly, in this study the patterns of protein carbonylation were investigated in mitochondria from the flight muscles of

Drosophila melanogaster at different ages, using antibodies against DNP, MDA or HNE. The main aim was to compare the different immunoprobe to localize carbonylated proteins in mitochondria during aging.

MATERIALS and METHODS

Chemicals

Sources of the various chemicals were: 2,4-Dinitrophenylhydrazine (DNPH), Sigma Chemical Co. (St Louis, MO); anti-HNE antibodies, Alpha Diagnostics (San Antonio, TX); anti-MDA antibodies, Academy Bio-Medical Company Inc., (Houston, TX); anti-porin antibodies, Mitosciences (Eugene, OR); polyclonal rabbit anti-dinitrophenyl (DNP) antibodies, Zymed (San Francisco, CA); ECL plus Western blotting detection system, Amersham Biosciences, (Arlington Heights, IL); horse radish peroxidase-labeled goat anti-rabbit IgG secondary antibodies, Pierce (Rockford, IL). All other chemicals used were of the highest purity available commercially.

Animals

Larvae of *y w Drosophila melanogaster* were raised on a standard mixture of agar, cornmeal, and yeast as described previously [22]. After eclosion, flies were anaesthetized with a gentle stream of CO₂, and grouped in batches of twenty-five. They were subsequently maintained at 25°C under constant light. Studies were conducted on male flies.

Isolation of mitochondria and detection of DPN, HNE and MDA modified proteins

Mitochondria were isolated from thoracic flight muscles, as described previously [10,22]. Briefly, thoraces from 200 flies were excised using a razor blade and gently pounded in a chilled mortar, containing 300–400 µl of ice-cold isolation buffer (0.32M sucrose, 10 mM EDTA and 10 mM Tris/HCl, pH 7.3), to which 2% (w/v) BSA (fatty acid content 0.003%) had been added. The brei was filtered through Spectra/Mesh[®] nylon (pore size=10µm). After centrifugation for 10 min at 2200 g, the pellet was rinsed briefly in BSA-free isolation buffer, resuspended in 500 µl of the BSA-free buffer and stored at –80°C until used.

To detect protein carbonyls (C=O) immunochemically with anti-DNP antibodies, mitochondrial samples were treated with dinitrophenyl hydrazine (DNPH) according to the methods of Levine [23] and resolved by SDS-PAGE (10% v/v), as described by Laemmli [24], and transferred onto PVDF membrane at 4°C overnight. The blots were then incubated overnight at 4°C with anti-DNP antibodies, diluted at 1:5,000 in TBS-T containing 5% fat-free milk. The membranes were subsequently treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies at 1:10,000 dilution in TBS-T overnight at 4°C. Immunostained proteins were visualized using ECL-Plus Western blotting detection reagent. HNE and MDA blots were processed identically but without the DNPH derivatization. Following SDS-PAGE separation, gels were stained with Coomassie to visualize total protein loading onto the gels. The amount of protein in a specific band was quantified densitometrically on dried SDS-PAGE gels. Total protein amounts were determined prior to gel loading using the sodium BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL), according to the manufacturers instructions. Band intensity data were statistically analyzed by pair-wise comparisons to the youngest samples by Student's t-tests on Microsoft Excel 2002 software. P values of <0.05 were considered statistically significant.

Immunoblotting controls

Anti-porin monoclonal antibodies (Mitosciences, Eugene, OR), at a concentration of 1:10000, were always used to check protein loading and blotting. Primary and secondary antibodies

were tested for specificity with immuno-blots performed in the presence or absence of each antibody.

RESULTS

Life spans of the *y w* flies under conditions similar to those in this study have been reported previously (e.g. see [25], amongst many others). Typically, the average life span is about 70 days and the maximum around 100 days. Samples were taken from flies ranging from 15 to 60 days of age.

Comparison of protein amounts at different ages

Initially, aliquots of flight muscle mitochondrial proteins from 15-, 22-, 30-, 45- and 60-day-old-flies were separated by SDS-PAGE and stained with Coomassie in order to ascertain whether the levels of proteins, indicated by densities of the various bands, were altered during aging (Fig. 1). Although the separation by SDS-PAGE cannot adequately resolve the low-abundance proteins, information about the potential age-related changes in the densities of the readily visible bands is deemed essential for the comparisons of western blots at different ages. Nonetheless, no notable age-related differences in the densities of protein bands in Coomassie stained gels were apparent (Fig. 1A).

Epitope specificity of the antibodies

An important criterion used for comparisons between the three antibodies was the Signal-to-Noise ratio (SNR). As shown in Fig. 1A-D, the SNR was relatively high, which (i) permitted the identification of distinct immunopositive bands with all of the three antibodies, and (ii) established the age-related variations in the immunodensities of different protein bands. However, it needs to be pointed out that immunoblot analysis is essentially a semi-quantitative analytical technique, which is potentially vulnerable to several complicating factors, including the batch-to-batch variation in titer and epitope specificity. In particular, the anti-DNP antibodies seem to be quite problematic for quantitative comparisons, and also to be less specific compared to the antibodies against HNE and MDA adducts. The blots shown here are from experiments where the conditions were rigorously controlled. The differences in immunoreactivity of protein bands with anti-DNP antibodies, between successive ages, were found to be relatively subtle; however, distinct differences were discernable between the 15- and 60-day-old flies.

Age-related changes in levels of proteins with MDA-adducts

Mitochondrial proteins from flight muscles of 15-, 22-, 30-, 45- and 60-day-old flies were resolved by SDS-PAGE and processed for western analysis using anti-MDA antibodies (Figs. 1 and 2). Comparison of the combined density of the immunopositive proteins indicated that there was a progressive age-associated increase of ~110% in the total amount immunoreactivity between 15- and 60- days of age. Four distinct protein bands, labeled A–D (Fig. 2), were found to exhibit positive immunostaining at all of the five ages, but only two, A (~60 kDa) and B (~200 kDa), showed an age-related increase in immunodensity. The other two, C (~100 kDa) and D (~37 kDa), did not. The age-associated increase in the density of bands A and B was nearly linear, but different in magnitude, being ~85% in A and ~150% in B.

Age-related changes in amounts of proteins with HNE-adducts

Comparison of western blots of the mitochondrial proteins, using anti-HNE antibodies, was made between 15-, 22-, 33-, 47- and 60-day-old flies. During 15 and 60 days of age, the combined densities of the various immunoreactive proteins showed a progressive increase of ~80%. Seven distinct immunoreactive bands, ranging from 7 to 100 kDa in MW, were

consistently observed at all the five ages. Two bands, J (~7 kDa) and K (~19 kDa), were designated as “minor”, because compared to the others they had relatively low levels of protein (Fig. 1A), however, the largest increase, per unit of protein in the band, occurred in the ~19 kDa “minor” band, J (Fig. 2C). All of the protein bands that were reactive with HNE-antibody, except one (K), showed an age-related increase in immunodensity, ranging from 65% to 200%.

Comparison of mitochondrial protein carbonyls between 15- and 60-day-old flies using anti-DNP antibodies

Anti-DNP antibodies are widely assumed to react with protein carbonyls originating from various sources, including MCO, HNE or MDA. To determine whether the protein bands showing positive immunoreactivity with anti-MDA and anti-HNE antibodies also react with anti-DNP antibodies, a comparison was made between 15 and 60 day-old-flies. All of the five protein bands, detected by anti-DNP antibodies, labeled L, M, N, O, and P, showed age-related increases in immunodensity, ranging from ~41% in band L (~200 kDa), ~71% in band M (~90 kDa), ~77% in band N (~50 kDa), ~107% in band O (~40 kDa) and ~83% in band P (~37 kDa). Comparatively, more protein bands reacted with HNE-antibodies and also showed an age-related increase in immunodensity than with anti-DNP or anti-MDA antibodies. The rank order of the percent increase in total immunoreactivity was anti-MDA > anti-HNE > anti-DNP.

Comparison of levels of high molecular weight proteins with DNP HNE, and MDA adducts

One consequence of ROS attacks on proteins is inter-molecular cross-linking. As shown in Fig. 3B, the ratios of high-molecular-weight (HMW) to total immunoreactivity of the various proteins increased with age, with the rank order anti-DNP > anti-MDA > anti-HNE antibody.

Densities of individual immunopositive bands are not dependent on their protein levels

An important issue in the localization of proteins that are targets of oxidative modifications is whether the intensity of the immunostaining is dependent upon the relative abundance of the specific proteins or on the frequency of the adducts. The former will favor the concept of random damage, while the latter would imply selectivity of the damage. A comparison of staining intensities of various bands, obtained by Coomassie blue staining, with those of western blots indicated that there was no obvious correspondence between them (compare the 116 kD band in Figs. 1 and 3). This is consistent with the concept that protein oxidative damage during aging is not dependent upon the relative amount of a specific protein.

DISCUSSION

A novel feature of this study is that it compared the age-related pattern of carbonylation of mitochondrial proteins using three different antibodies, namely anti-DNP, anti-MDA and anti-HNE, which either localize epitopes solely comprised of carbonyl groups on the side chains of particular amino acid residues (anti-DNP), or recognize a larger epitope that contains carbonyl groups (anti-MDA and anti-HNE). Based on the historical view gained from the literature, our initial expectation was that the anti-DNP antibody would broadly react with the various carbonyl-containing protein bands. The results indicated that while the total amount of protein, reacting positively with of the three antibodies increased with age, the specific protein bands were not identical. A relatively greater variety of mitochondrial proteins showed reactivity with HNE, than with anti-DNP antibody. The patterns of HNE- and MDA-positive bands were also different from each other, suggesting that such adducts are formed with specific proteins. Thus on the basis of this study it may be suggested that multiple immunoprobes should be used for the localization of carbonylated proteins.

Carbonylation of proteins has been shown to arise by at least three different mechanisms: (i) by metal-catalyzed oxidation (MCO), (ii) by reaction with lipid peroxidation end-products

containing aldehyde functional groups, such as HNE and MDA, and (iii) by interaction between glycine residues and sugars (glycation) or their oxidation products (glycoxidation). It has been suggested that MCO may be the dominant mechanism for the carbonylation of soluble proteins *in vivo* [3,15]. Early studies on the quantification of carbonylated proteins were based on derivatization of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to hydrazone, followed by spectrophotometric detection of the resultant DNP-protein [26]. It was estimated that up to 30% of the protein from cells of old organisms versus 10% in the young may be carbonylated. Another concept also prevalent in the early literature was that oxidative damage to macromolecules, including proteins, was ubiquitous and random, as the reactions with free radicals are usually non-enzymatic in nature. However, later studies indicated that spectrophotometric estimates of the relative abundance of carbonylated proteins may be an overestimate, resulting from the presence of unreacted DNPH and nonprotein carbonyls [17–19]. More relevantly, studies involving immunological detection of DNP-proteins, where the proteins in the homogenate are electrophoretically separated, indicated that carbonylation was a selective phenomenon, whereby relatively few proteins showed positive immunoreaction with DNP-antibodies. Mitochondrial aconitase and adenine nucleotide translocase in the housefly were among the first proteins identified to exhibit an age-associated increase in carbonylation during aging [18,19]. Later studies have considerably augmented this list, e.g. see [27]. Results of this study accord with the concept that protein carbonylation during aging is a selective rather than a ubiquitous or random phenomenon. For instance, a comparison of the Coomassie blue stained gels (Fig. 1A) with those stained immunologically (Figs. 1B–D) demonstrates that only some of the bands observed in the former exhibit immunoreactivity in the latter.

A pertinent issue is: what is the physiological significance of protein carbonylation? Carbonyl groups are chemically reactive and act thermodynamically and kinetically as targets for nucleophilic attacks and the formation of Schiff bases with amines, which leads to protein cross-linking [28]. It is apparent from Fig. 3 that cross-linking of proteins indeed occurs in mitochondria during aging. MDA seems to be involved in the formation of a higher proportion of high molecular weight (>200 kDa) proteinaceous material than HNE, suggesting that the dialdehydic nature of MDA may be relatively more efficient in forming protein cross-links. On the other hand, anti-DNP immunoreactivity was also apparent in proteins with molecular weights of >200 kDa (Fig. 3). Cross-linking can block access to protease cleavage sites, thereby decreasing the rate of turnover and leading eventually to protein insolubility [29]. Another factor that may also contribute to the accumulation of cross-linked proteins is that activity of multicatalytic proteases tends to decline during aging [30]. Whatever the underlying factors may be, accumulation of cross-linked proteins has been widely hypothesized to be potentially cytotoxic.

Although it was initially widely held that carbonylation leads to a loss in protein catalytic activity, our recent studies on mitochondria from mouse heart and skeletal muscles have indicated that the presence of an oxidative modification in a protein is not an *a priori* evidence of loss of protein function. Similarly the absence of an age-associated increase in immunoreactivity does not suggest that there may be no further loss of protein function [31]. For instance, in mouse heart and kidney mitochondria, aconitase, very long chain acyl coenzyme A dehydrogenase (VLCAD), the β -polypeptide of the mitochondrial F_1 complex of ATP synthase, and the E2 component of α -ketoglutarate dehydrogenase complex were identified to exhibit MDA modification. Although there was no age-related increase in the amount of the MDA-modified proteins, aconitase and ATP synthase showed an age-related decrease in activity, while VLCAD and α -ketoglutarate dehydrogenase did not. Such data suggest that no assumptions about the loss of function of an oxidatively modified protein can be made solely based on the demonstration of carbonylation, without actual measurement of the specific activity. Data presented in this study form our initial characterization of targeted

proteins for oxidative damage in *Drosophila* mitochondria, with subsequent aims being protein identification and determination of possible functional alterations.

In conclusion, results of this study indicate that mitochondrial proteins in *Drosophila* undergo a notable degree of carbonylation, particularly by forming adducts with lipid peroxidation products, 4HNE and MDA. These changes were selective and of sufficient magnitude to be potential indicators of oxidative stress.

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Figure 1. Immunological determination of MDA-, HNE- and DNP-modified mitochondrial proteins from the flight muscles *Drosophila melanogaster* (15-, 22-, 30-, 45- and 60-day-old-flies)

DNP treated total mitochondrial proteins were resolved by SDS-PAGE and blotted onto PVDF membrane. HNE- and MDA-modified proteins were detected immunochemically, as described in Materials and Methods. Panel A shows Coomassie stained proteins; Panel B, immunolabelling with anti-MDA proteins; Panel C, immunolabelling with anti-HNE proteins; Panel D, immunochemical detection of DNP derivatized proteins; Panels E, F and G show MDA, HNE and DNP total reactive quantities respectively. Panel G shows means of data obtained from duplicate western analyses.

Figure 2. Immunochemical quantitation of individual HNE, MDA and DNP-protein bands from mitochondria from 15-, 22-, 30-, 45- and 60-day-old flies

Panel C is based on duplicate western blots; D is from a typical immunoblot, where $n=5$; other data are average of triplicate western analyses.

Figure 3. Typical profile plots of immunoreactivity from Western analyses of MDA, HNE and anti-DNP post-translational modifications in *Drosophila melanogaster* mitochondrial proteins (A).

Comparison of the ratio of high molecular weight (HMW) to Total immunostaining for HNE, MDA and anti-DNP protein modifications in *Drosophila* mitochondria (B)

Proteins were resolved and analyzed as described in "Materials and Methods". ImageJ was used to determine the coordinates for individual profiles. Data are from typical samples of 33 day old *D. melanogaster* mitochondria, with the exception of the C=O bar in panel B, which compares 15 and 60 day-old-flies. Vertical bars represent standard error of the mean.