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Original Contribution

Carbonyl modification in rat liver histones: Decrease with age and increase by dietary restriction

Ramesh Sharma [1], Akihiro Nakamura, Ryoya Takahashi, Hideko Nakamoto, Sataro Goto *

Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan

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Abstract

We studied carbonylation, a form of oxidative modification of proteins, of histones in rat livers. Histones H1, H2B/H2A, and H3 were significantly carbonylated but the modification was almost undetectable in H4. Contrary to the generally accepted view of increased protein carbonylation with age, the modification of histones was significantly lower in old (30-month-old) than in young (5-month-old) animals. Dietary restriction of older animals for 2 months resulted in increase in carbonylation comparable to that at the young level. These findings may have physiological implications in chromatin structure/function in aging and beneficial effects of DR by influencing transcription, replication, and/or repair activities.

Keywords: Histones; Carbonylation; Rat liver; Aging; Dietary restriction; Posttranslational modification; Free radicals

Introduction

Histones, a group of highly basic proteins, are essential components of eukaryotic chromatin. The fundamental unit of chromatin, the nucleosome, is formed by wrapping of DNA around the octamer of histone proteins (H2A, H2B, H3, and H4). An equally important histone protein H1 (also called a linker) helps in the high-order compaction of chromatin. In recent years, the influence of histones has been well recognized in a wide range of molecular processes such as replication, gene expression, and DNA repair [1–3]. Their posttranslational modifications (acetylation, phosphorylation, methylation, and ADP-ribosylation) have led to a newer area of histone code/language wherein they recruit many other *trans*-acting factors besides their interaction with DNA, to modulate various molecular processes [4].

Among various posttranslational modifications, only scant information on carbonylation of histones is available [5]. Carbonylation of histones most likely in basic amino acid residues could mask its positive charge and thereby influence the compactness of chromatin and also the recruitment of *trans*-acting factors to modulate gene expression. Carbonylation has been described as a specific marker of oxidative modification of proteins that is reported to increase with advancing age [6–8]. However, carbonylation of histones has not been studied in the aging process so far as we could learn. Having known its richness in basic amino acid residues and also its pivotal role in crucial molecular processes, we attempted to assess the extent of carbonylation in liver histones during aging and also the effects of late-onset dietary restriction (DR) of aged rats [9]. DR is the only nongenetic means known to consistently delay aging and extend the mean and maximum lifespan in various species of animals when initiated early in life [10–12]. While DR initiated later in life has smaller effects of extending the lifespan [13,14], the regimen has been reported to have beneficial effects [9]. Late-onset DR can reduce the age-dependent accumulation of altered proteins [15], shorten half-life, and thereby increase turnover of proteins [16], decrease mitochondrial protein carbonyls [17], upregulate proteasome activity [18], and restore age-related changes of gene expression [19]. Thus, study of the effect of late-onset DR on the carbonylation of histones during the aging process appeared of interest.

Abbreviations: DR, dietary restriction; H1, histone H1 and similarly for H2A, H2B, H3, H4; DNPH, 2,4-dinitrophenylhydrazine; DNP, 2,4-dinitrophenyl; HAP, hydroxylapatite; SDS, sodium dodecyl sulfate.

* Corresponding author. Fax: +81 47 472 1531.
E-mail address: goto@phar.toho-u.ac.jp (S. Goto).
[1] Present address: Department of Biochemistry, North Eastern Hill University, Shillong 793 022, India.

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Materials and methods

Animals, chemicals, and antibodies

Specific pathogen-free (SPF) male rats (F344/DuCrlCrlj) of two different age groups (5 and 30 months) maintained in our University animal facilities were used. They were fed a standard pellet diet (CE-7, Clea Japan, Tokyo) and water ad libitum as per the experimental schedule. A group of old rats was fed on alternate days for 2 months starting at the age of 28 months; however, they had free access to water on all days [17]. Excised livers immediately frozen in liquid nitrogen were stored frozen at −80°C until use. All chemicals used were of analytical grade unless otherwise stated. Rabbit anti-DNPH (2,4-dinitrophenylhydrazine) antibody was obtained by immunization of DNP-hydrazones of oxidatively modified BSA or KLH [20].

Nuclear preparation and histone isolation

The nuclei were prepared as described [21,22]. Histones were extracted from the purified nuclei with 0.2 M H_2SO_4 in most of the experiments [23,24]. In some experiments histones were highly purified by hydroxylapatite chromatography essentially as described [25,26]. In brief, crude chromatins were incubated with micrococcal nuclease (Worthington Biochem Co., NJ). The extracts were mixed with hydroxyapatite (HAP, Kishida Chem., Osaka) and the bound proteins were eluted with a buffer containing 3 M NaCl. The eluted proteins were precipitated by ethanol (final concn 90%). The precipitates were dissolved in 6% SDS. The protein concentration was determined by measuring the absorbance at A_{230} of the extracts [27] or with the BCA protein assay kit (Pierce, IL).

DNPH derivatization

The histone preparation was treated with 10 mM DNPH in 2 M HCl or with 2 M HCl alone. The conditions for the derivatization were optimized for the concentration of DNPH, the time of incubation, and temperature to avoid artificial, nonspecific reactions, being slightly modified from the original method [20]. The DNPH-derivatized histones were then precipitated with chilled ethanol but not with acid as usually used because histones are highly basic and therefore cannot be precipitated with acid. The resulting precipitates were dissolved in 8 M urea for Western blotting. In some experiments (Fig. 3) the histones were subjected to protein carbonyl assay according to Levine's protocol [28].

SDS-PAGE and Western blot analysis

Carbonylation of histones was studied by Western blot using anti-DNP antibody [20]. Five micrograms of proteins was loaded on each lane of 15% SDS-PAGE. After the electrophoresis histones were transferred to nitrocellulose membrane using a Bio-Rad semidry transfer apparatus. PVDF membrane was not used because basic proteins were not efficiently trapped on the membrane when transferred. Blocking of the membrane was performed in skimmed milk and then incubated with anti-DNP-hydrazone antibody. The blots were incubated with anti-rabbit IgG-HRP in the buffer, washed, and then developed with the ECL-Plus immunoblotting system (Amersham Biosciences, UK). Immunological signals were quantified using ImageQuant with the Storm 860 image analyzer (Molecular Dynamics).

Oxidative modification in vitro

The isolated histones or purified nuclei were incubated with oxidizing medium (50 mM sodium phosphate buffer, pH 7.5/1 mM $FeSO_4$/1 mM EDTA/ 0.15% H_2O_2/25 mM ascorbic acid) for 1 h at 37°C as described [29] with slight modifications. The reaction was terminated by the addition of chilled ethanol for isolated histones or by centrifugation for nuclei.

Sodium borohydride reduction of histones

Isolated histones were incubated in reducing medium (86 mM Tris-HCl buffer, pH 8.5/40 mM sodium borohydride/0.86 mM EDTA) for 1 h at 37°C essentially as described [20,30]. The reaction was stopped by the addition of chilled ethanol and the precipitated histones were processed for DNPH derivatization and Western blotting.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Statistical significance between means was determined using analysis of variance (ANOVA), followed by Fisher's least-significant difference test. The significance level was set at $P < 0.05$.

Results and discussion

Protein carbonylation has been studied extensively as a cause of possible detrimental consequences in aging and age-related diseases, but carbonyl modification of histones has not been reported except one study [5] as far as we know. We report here carbonylation with age of rat liver histones under in vivo conditions and also under in vitro oxidative and reductive conditions. Our observations indicate that all the histone types except H4 are carbonylated in vivo in rat liver (Fig. 1, lane 1), although the extent of this modification of each histone subtype was variable; i.e., H1 and H2B/H2A appeared more carbonylated than H3. The possible reason for H4 being not carbonylated in vivo could be because of its location in the higher order chromatin and/or its varied amino acid composition. H4 is known to have a lower percentage of basic amino acid residues than other histones [1,4]. It could also be possible that the in vivo extent of H4 carbonylation is so meager that it is beyond the detection limit. The inner core of the nucleosome (having H3 and H4) was relatively more resistant to the oxidation. Other histone types, such as H2B/H2A and H1, are rapidly and/or slowly exchanged depending on the transcrip-

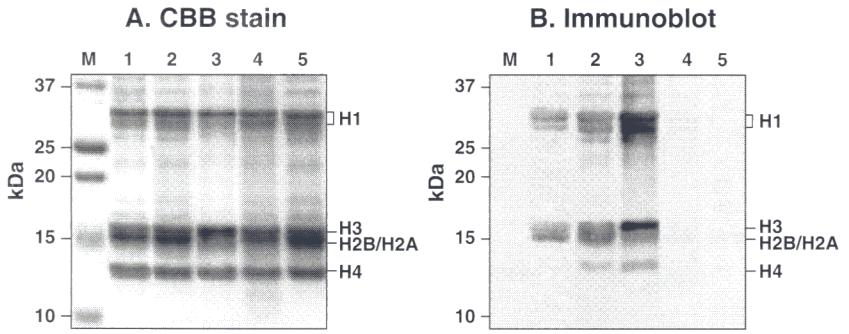

Fig. 1. Carbonylation of rat liver histones isolated by acid extraction. (A) Coomassie brilliant blue (CBB)-stained gels. (B) Western blot for carbonyls after DNPH derivatization. Lane M, molecular weight markers; lanes 1–5, immunoblot for protein DNP-hydrazones; lane 1, histones isolated from the liver; lane 2, histones isolated after in vitro oxidation of nuclei for 1 h; lane 3, isolated histones oxidized in vitro for 1 h; lane 4, sodium borohydride-treated histones isolated from the liver; and lane 5, histones isolated from the liver and treated with HCl alone without DNPH. Rats used in these experiments were 5 months old.

tional state of the chromatin [3,4]. However, in vitro metal-catalyzed oxidation of histones leads to a much higher degree of carbonylation including H4 (both in isolated histones or histones in the nuclei) (Fig. 1, lanes 2 and 3). This suggests that all types of histones undergo oxidative modifications, albeit to varying degrees in the subtypes. Histone preparations when treated with reducing agent gave very poor carbonyl signals, indicating that our assay system is highly specific to carbonyls because reducing the carbonyls to alcohols should abolish the reactivity with DNPH and therefore its specific antibody recognition (Fig. 1, lane 4; Fig. 2D; see also [20]). There were no carbonyl signals observed when the histones were treated with HCl alone, indicating that the antibody specifically recognizes DNP-hydrazones of the proteins (Fig. 1, lane 5). Our findings are in contrast with an earlier study that used histones from bovine tissues in which H1 was preferentially carbonylated in vivo [5]. The reason for this discrepancy may be due to the difference of animals used: Wondrak et al. [5] studied bovine tissues (thymus, liver, and spleen) while we examined rat livers.

In the experiments described above histones were extracted with H_2SO_4 by virtue of their highly basic nature. We confirmed the age-related change of the relative extent of carbonylation of histones isolated by HAP chromatography (Fig. 3). The patterns of histone carbonylation were essentially identical between results obtained by the two methods (see lanes Y and O in Figs. 2A and 3). It should be noted that we used two independent methods of histone extraction, obtaining virtually identical results of the carbonylation pattern. The former method allows us to isolate histones quickly, avoiding the possibility of degradation in the presence of the acid [23] in view of oxidatively modified proteins being generally highly susceptible to protease digestion. In the latter method, however, chromatins were first bound to HAP, being dependent on DNA

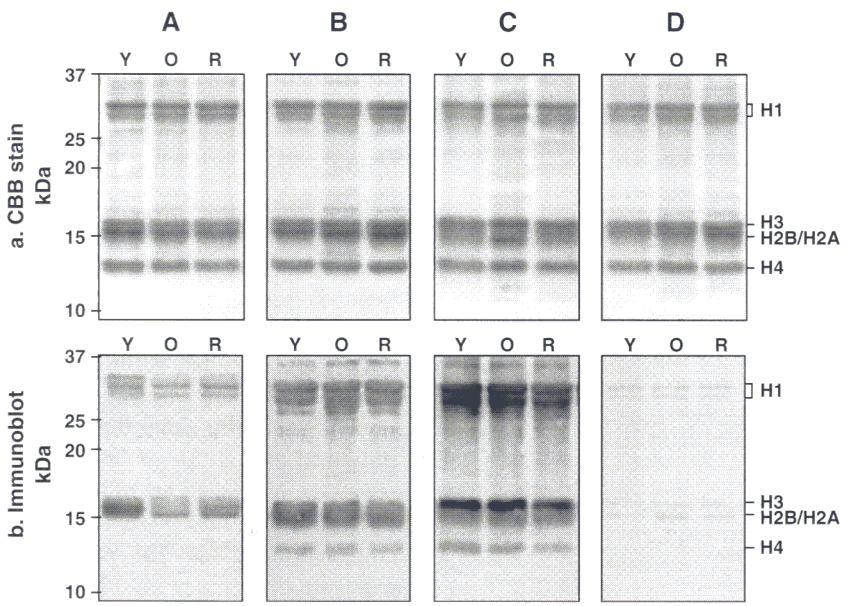

Fig. 2. Representative patterns of carbonylation of histones from the liver of young (Y), old (O), and old dietary restricted (R) rats. (a) Coomassie brilliant blue (CBB) stained gel; (b) Immunoblot for carbonyls. (A) Histones isolated from the liver of young (Y, 5-month-old), old (O, 30-month-old), and old (30-month-old) dietary restricted (R) rats for 2 months and subjected to Western blotting for carbonyls. (B) Histones isolated from the liver of same age group rats after in vitro oxidation in purified nuclei. (C) Oxidation of isolated histones from the same age group rats. (D) Sodium borohydride reduction of isolated histones from the same age group of rats. Representatives of 5 animals in each group are shown here.

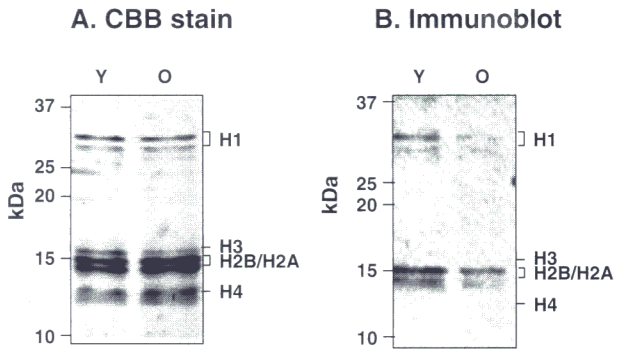

Fig. 3. Confirmation of carbonylation of histones isolated by hydroxylapatite chromatography. Histones purified from livers of young (Y) and old (O) rats by hydroxyapatite chromatography (see Materials and methods for details) were derivatized with DNPH and then subjected to Western blot with anti-DNP antibody. (A) CBB-stained gel. (B) Western blot for carbonyls.

fragments on the nucleosomes from which histones were selectively eluted with salt. We have verified the validity of the method in the isolation of histones using specific antibodies against histones in Western blots (Biogenesis, UK; data not shown). Thus, carbonylation of histones and its age-related changes were confirmed in two independent methods of extraction. It should also be noted that because of the highly basic nature of histones the conventional method of carbonyl measurement that involved precipitation with TCA could not be used and we therefore used ethanol for the precipitation.

Most interestingly, we observed a significantly higher carbonylation of histone proteins in vivo from the liver of younger animals compared to older ones (Fig. 4, Table 1). The lower degree of histone carbonylation in the liver of older animals could be because of the more compact chromatin configuration [31–33], masking the availability of histones for such modification. Conversely, more highly carbonylated histones in younger animals may allow less histone interaction with DNA and maintain chromatin in a more relaxed state. At this stage, it is not clear whether this is a cause or an effect of

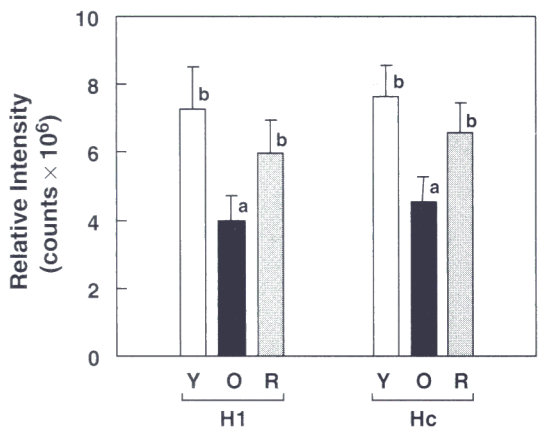

Fig. 4. Summary of carbonylation of histones from young (Y), old (O), and old dietary restricted (R) rats. The ECL-generated signals (Fig. 2A-b) for 5 animals in each group were quantified using the ImageQuant program with Strom 860. Histone H1 and core histones (H3/H2B/H2A) are expressed as H1 and Hc, respectively. Bars represent mean ± standard deviation. [a] Statistically significant ($P < 0.05$) from young rats; [b] statistically significant ($P < 0.05$) from old rats.

Table 1
Relative amount of carbonylated histones from young, old, and old dietary restricted rats

	Histone H1	Core histones (H3/H2B/H2A)
Young	100 ± 17.2	100 ± 11.8
Old	55.0 ± 10.0[a]	59.3 ± 9.5[a]
Old dietary restricted	82.0 ± 13.9[b]	86.0 ± 11.8[b]

Relative amount of protein carbonyl was determined by Western blot. The values are expressed relative to those in the young animals for histone H1 and core histones (H2A/H2B/H3). Mean ± standard deviation, $n = 5$.
[a] Statistically significant ($P < 0.05$) from young rats.
[b] Statistically significant ($P < 0.05$) from old rats.

greater compactness of chromatin during aging. Two months of DR in older animals led to an increase in carbonylation, the level of which is similar to that of younger animals (Fig. 2A, Fig. 4, Table 1). DR may thus cause relaxation of chromatin and hence give rise to an increase in carbonylation of histones or vice versa. Such an age-dependent difference of histone carbonylation was largely abolished in histones oxidized in purified nuclei (Fig. 2B) or in isolated histones oxidized in vitro (Fig. 2C). Under in vitro oxidation, H1, a more exposed linker protein in the nucleosomal chromatin organization, is likely more susceptible to such modification. It is also possible that H1 is highly Lys rich and, therefore, could have more sensitive sites for carbonyl modification. Remarkably, histone H3 was highly carbonylated in histones oxidized in vitro and in those from isolated nuclei oxidized in vitro. The observation that H2A and H2B dimers are more exposed than the H3 and H4 tetramers in the core nucleosome [1,3] may explain why the former appear to be more carbonylated than the latter. The protein that is most likely to be histone H3 appeared relatively more highly carbonylated than other core histones in in vitro oxidation of isolated histones (Fig. 2C) compared to histones isolated directly from the liver (Fig. 2A) or from oxidatively treated nuclei (Fig. 2B). This is probably because of the isolated histones having a more open structure that makes H3 more susceptible to the oxidation.

Regarding possible implications of the carbonylation of histones, we suggest that it may have a role in modulating the chromatin structure and function such as gene expression and repair in aging. It is now well appreciated that histone acetylation/deacetylation plays a crucial role in modulating gene expression by masking and demasking positive charges of Lys residues that influence its interaction with DNA [34]. Analogous to this modification, which is enzymatic and reversible, carbonylation occurs mostly in Lys and Arg residues nonenzymatically either directly or by reaction with aldehydes produced as a result of lipid peroxidation or sugar oxidation [6,8,35]. Since carbonylation may also mask positive charges of Lys and Arg residues and contribute to changes in chromatin compaction, it may be plausible that there could be less exposure of histones in older chromatin compared to younger ones wherein chromatin is known to be more relaxed, as evident from greater nuclease digestion [22,30–33]. Alternatively, higher carbonylation of histones at a younger age may act as a sink and scavenge reactive oxygen and aldehyde species to serve as a nucleosomal defense that might help DNA

protection [36]. It was earlier reported that nucleosomal histones might protect DNA from iron-mediated damage [37]. Higher carbonylation of histones in the liver of younger animals may be an indication of higher protection of the chemical integrity of DNA.

Lifelong DR can extend the mean and maximum lifespan in many animal species [38]. The regimen has a variety of beneficial effects such as retardation of onset of age-related diseases and decline of physiological functions. DR initiated late in life is less well studied but has also been shown to "rejuvenate" cellular parameters [9,39]. In the present study, we examined the effect of short-term DR in old rats on histone carbonylation that decreased with age. The carbonylation of histones was increased significantly by the DR regimen. This finding cannot be explained from the reported mechanism of the effects of DR that suggests reduction of oxidative stress [10].

Our paradoxical finding of reduced carbonylation of histones in the liver of older animals diversifies a common view that protein carbonylation increases with aging as a result of increased oxidative stress possibly causing a decline of physiological functions [6–8]. To our knowledge, this is the first report to show that there is a reduced carbonylation of specific proteins during aging and increase by late-onset DR. These observations may point to a new physiological role of protein carbonylation. Further characterization of histone carbonylation and study of the possible role of carbonylated histones are required to specify their exact role in chromatin structure and function.

Finally, although we have not studied the chemical nature of the histone carbonylation yet, a brief further discussion may be valuable on this issue. In addition to the possibility of the proteins being oxidized to generate carbonyl moieties directly or indirectly as discussed so far, it is possible that poly-ADP-ribosylation known to occur on histones may be responsible for the carbonylation as discussed by Wondrak et al. [5]. In this regard it is interesting to note that Quesada et al. reported that ADP-ribosylation of histones H1 and H2B is inhibited during aging in rat ventral prostate, discussing the relation with age-related failure of DNA repair in senescent animals [40].

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