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AGE-RELATED CHANGES IN THE REDUCIBLE CROSS-LINKS OF CONNECTIN FROM HUMAN SKELETAL MUSCLE

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SUMMARY: After NaB^3H_4-reduction of connectin from human skeletal muscle, the changes in the amounts of the reducible cross-links and specific radioactivity of this elastic protein were followed throughout the whole life-span from embryo to old age. The reducible cross-links, aldimine forms of lysinonorleucine and histidino-hydroxymerodesmosine, and unidentified reducible compounds, which were assumed to be cross-linking amino acids, were found to remarkably decrease with age. A progressive decrease in the incorporation of tritium into the reducible compounds was also observed. We conclude that the conversion of the reducible cross-links derived from lysine and hydroxylysine aldehydes to non-reducible compounds is an essential step in the maturation of connectin fibrils, similar to collagen fibrils.

INTRODUCTION

Connectin, an intracellular elastic protein, has been isolated from myofibrils of rabbit skeletal muscle (1), and its responsibility for passive elasticity and mechanical continuity of myofibrils was demonstrated (1,2). Subsequent electron microscopic studies clearly showed a filamentous network connecting the Z lines of the sarcomere after the removal of other structural proteins, indicating that this filamentous material may be composed of connectin (3,4). We recently reported that connectin from chicken breast muscle contains reducible compounds, aldimine forms of lysinonorleucine and histidino-hydroxymerodesmosine, derived from lysine and hydroxylysine aldehydes (5). These findings provided strong evidence that this elastic protein in myofibrils and the connective tissue proteins, collagen and elastin, share common features of cross-linking.

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In this report we describe the alterations in cross-linking of connectin from human skeletal muscle throughout the whole life-span from embryo to old age.

MATERIALS AND METHODS

Preparation of connectin: Human male gastrocnemius muscles were obtained from ages spanning from a 6 lunar month embryo to a 79-year -old adult. Following several washes with cold distilled water, muscle tissue was homogenized in a Waring blender in 5 volumes (v/w) of 10mM Tris-maleate buffer containing 0.1M KCl, pH 7.2. The homogenate was rapidly stired with a magnetic stirer for 12 h. Fibrous material in the suspension was removed by passing the homogenate through a cheese cloth. The suspension was then centrifuged at 8,000 x g for 30 min. and the residue was extracted with 20 volumes (v/w) of Hasselbach-Schneider solution (6) for 12 h. while stirring. Following filtration through a cheese cloth, insoluble proteins in the filtrate were collected by centrifugation. This extraction was repeated three times followed by extraction with 0.6M KI-0.06M $Na_2S_2O_3$ for 12 h. The latter was repeated twice. The insoluble residue obtained after these extractions was washed with distilled water and then lyophilized. All operations were carried out at 5°C.

Reduction with NaB^3H_4 and cross-link analysis: The dry samples of connectin and human skeletal muscle collagen (8 lunar month embryo, male) were reduced with NaB^3H_4 (7), and lyophilized after dialysis against a large volume of 0.1M acetic acid. The tritiated proteins were hydrolyzed in 3N HCl at 107°C for 48 h, and the hydrolysates were evaporated to dryness. A portion of each hydrolysate was measured for specific radioactivity. The data were expressed in terms of the original weight of labeled sample. Chromatographic fractionation of the radioactive components of each hydrolysate was carried out on a column of Aminex A-4 (8,9,10). Aliquots were counted for radioactivity, and

TABLE I: SPECIFIC RADIOACTIVITY AND AGING

	Lunar month		Years of age					
	6	8	5	16	30	45	58	79
^{3}H cpm x 10^{-2}/mg	223	211	186	131	117	110	88	69
	(100)	(94.6)	(83.4)	(58.7)	(52.5)	(49.3)	(39.5)	(30.9)

Results are expressed as count per minute (cpm) per miligram (dry weight) of $NaB^{3}H_4$-reduced connectin in the hydrolysates. Figures in parentheses represent the per cent of specific radioactivity compared to the 6 lunar month embryo.

values under each radioactive peak were expressed as count per minute (cpm) per miligram of connectin in the hydrolysate loaded on a column (11).

Amino acid analysis: Following 6N HCl hydrolysis at 110°C for 24 h, amino acid composition of connectin was determined by using an amino acid analyzer. Tryptophan content was determined from an alkaline hydrolysate of each sample (12).

RESULTS AND DISCUSSION

In the present study we have attempted to prepare connectin without using the urea-SDS method (1), since some of the reducible cross-links of collagen are known to be labile to dilute acid and alkali, heat and protein denaturants (13,14). The amino acid composition of connectin prepared in this study was found to be very similar to preparation made by the urea-SDS method (5) except for hydroxyproline content; hydroxyproline was entirely absent in all samples, which indicates that the removal of collagen fibrils was effectively achieved. Perhaps the collagen fibrils were easily entangled during rapid stirring and then removed by the filtration procedures.

Age-related changes in specific radioactivity of $NaB^{3}H_4$-reduced connectin are shown in Table I. The results showed a significant decrease in specific radioactivity with advancing age; the sample

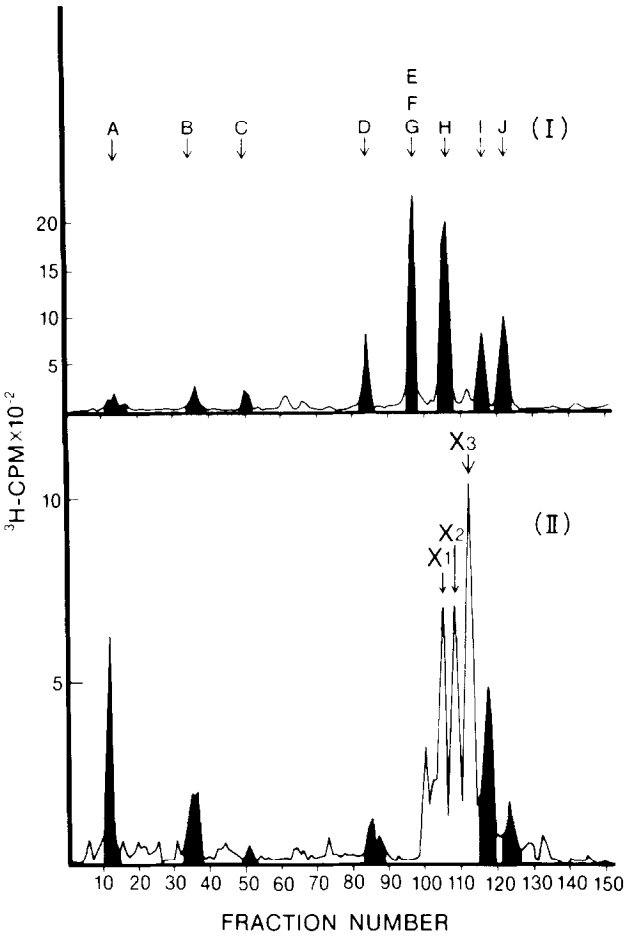

Fig. 1. Chromatographic patterns of the radioactive components in acid hydrolysates of NaB^{3}H$_4$-reduced human skeletal muscle collagen (I) and connectin (II) from 8 lunar month embryo. Fractionation was carried out on a column of Aminex A-4 (0.9 x 58 cm). The peaks are: A, unknown (fall through); B, dihydroxynorleucine; C, hydroxynorleucine; D, N$^{\epsilon}$-hexosyl-hydroxylysine; E, N$^{\epsilon}$-hexosyllysine; F, aldol histidine; G, dihydroxylysinonorleucine; H, hydroxylysinonorleucine; I, lysinonorleucine; J, histidino-hydroxymerodesmosine; X1,X2,X3, unknown.

from 79 years of age incorporated only 30.9% of the tritium taken up by the sample from 6 lunar month embryo.

The chromatographic patterns of the radioactive components in acid hydrolysates of NaB^{3}H$_4$-reduced connectin and collagen are shown in Figure 1. Although connectin was found to contain dihydroxy-norleucine, hydroxynorleucine, N$^{\epsilon}$-hexosylhydroxylysine, lysino-

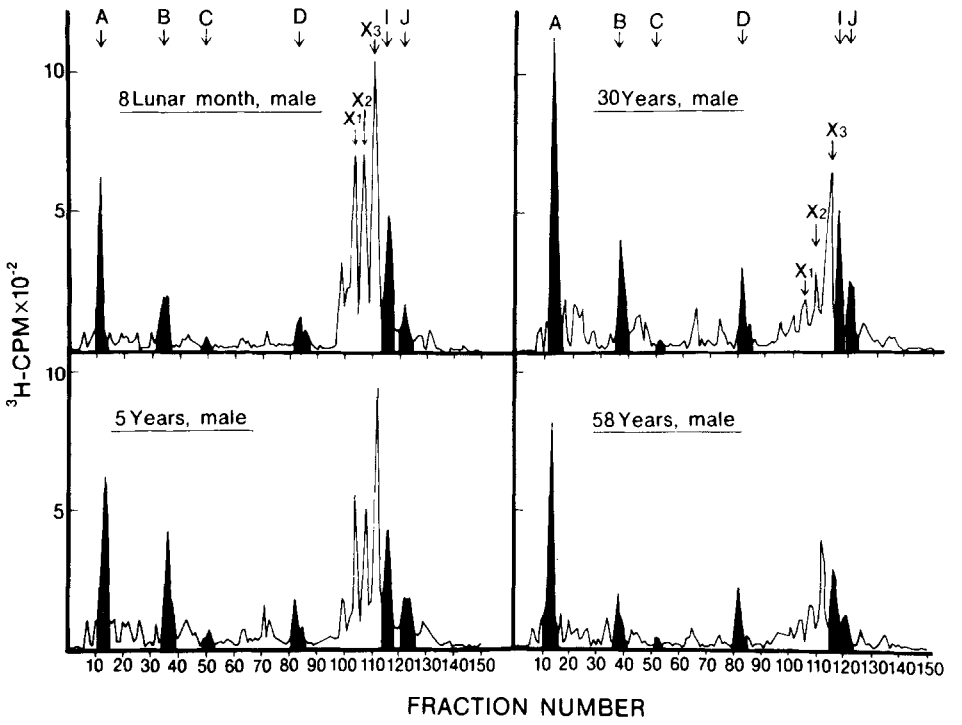

Fig. 2. Chromatographic patterns of the radioactive components in acid hydrolysates of NaB^{3}H$_4$-reduced connectin from human skeletal muscle of various ages. The peaks are the same in Fig. 1.

norleucine and histidino-hydroxymerodesmosine, another three radioactive peaks, which appeared just prior to the elution of lysinonorleucine, did not seem to occur in the radioactive profile of reduced collagen. These unknown components, which we designate as X1, X2 and X3, were found to significantly diminish after treatment with either 8M urea, 1% SDS and dilute alkali (chromatograms are not shown). These results may account for these components being less prominent in earlier preparations made by the urea-SDS method (5) and suggest that X1, X2 and X3 are labile reducible cross-links derived from lysine and hydroxylysine aldehydes.

Figure 2 shows that there are marked differences in the elution profiles of four representative samples. In all of the profiles the

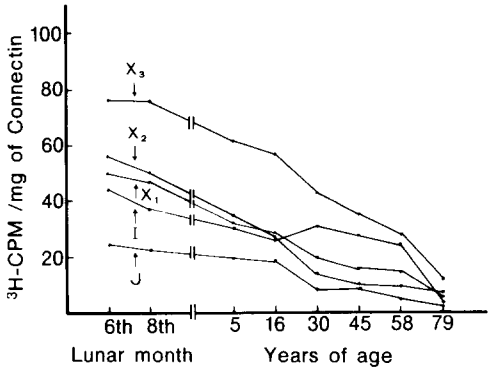

Fig. 3. Age-dependent changes in the most prominent reducible components eluted by ion-exchange chromatography (Fig. 2). Results are expressed as count per minute (cpm) under each radioactive peak per miligram (dry weight) of NaB^3H_4-reduced connectin loaded on the column. Abbreviations used are those in Fig. 1.

peak of X3 was found to be the most prominent one and to decrease significantly with age (Figure 3). In similar fashion, relative abundance of the major components, which appeared in the region of the reducible cross-links of collagen (fractions 95-125), was determined and shown in Figure 3. The results showed that all of the identified cross-links and the unknown components, Xl, X2 and X3, progressively decrease with age.

Previous studies on aging of collagen showed that the amounts of NaB^3H_4-reducible cross-links decrease with increasing age of experimental animals (15) and human (16,17). These facts implied that the reducible cross-links act only as intermediates and are converted to non-reducible and more stable compounds during the maturation process of collagen. This implication was strongly supported by our previous findings that with increasing age there is a progressive decrease in the incorporation of tritium into the reducible compounds of human tendon collagen (16).

The present study demonstrates significant decrease in both the amounts of the reducible cross-links and the specific radioactivity

of connectin with age. Thus, it seems that the maturation of connectin fibrils may well follow the same stabilization mechanisms as the reducible cross-links of collagen.

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