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CHANGES IN COLLAGEN CROSS-LINKING AND LYSYL OXIDASE BY ESTROGEN

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Summary

Dermal collagen solubility and lysyl oxidase activity of bones were measured in DDD mice of advancing age. Insoluble fractions of the dermal collagen increased more rapidly in females than in males after 5 weeks of age. Activity of the lysyl oxidase extracted from bones was higher in females than in males after 4 weeks of age. After sexual maturation, such sex differences were always observed in skin as well as in bone tissues. In other experimental animals, dermal collagen solubility was markedly decreased by estrogen treatment and lysyl oxidase was remarkably activated by estrogen in both skin and bone. Thus it is clear that estrogen stimulates the enzyme activity and accelerates the maturation of collagen and elastin in extracellular space.

Introduction

Steroid hormones, in particular estrogen, reportedly have various influence on connective tissue metabolism [1,2]. For example, in the rat uterus, estrogen has been shown to accumulate collagen [3,4] and in the bones of rats to prevent development of immobilization osteoporosis [5]. In preliminary studies, we found that collagen content in mouse bones was increased markedly by giving estrogen, increased slightly by progesterone and not influenced at all by testosterone. Collagen content and diameter of the collagen fibril were significantly decreased in the capsular ligament of rats after estrogen administration, while such were increased after testosterone administration [6].

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Takeda et al. [7] found in histochemical, biochemical and morphological studies that the metabolism of connective tissue in several organs was under hormonal control, with organ specificity. These data suggest that steroid hormones regulate the synthesizing process of fibrous proteins in fibrogenic cells. However, the hormonal regulatory mechanism of extracellular maturation of fibrous proteins has not apparently been documented except for a report on pituitary hormones [8]. The present study was an attempt to clarify the mechanism involved in the hormone regulatory mechanism by determining the influence of sex steroids on collagen solubility and lysyl oxidase activity.

Materials and Methods

DDD mice [9] were fed a commercial diet, and some were gonadectomized under ether anesthesia at 21 days of age. After operation estradiol benzoate (2 μg per mouse), progesterone (1.5 mg per mouse) or testosterone propionate (0.25 mg per mouse) dissolved, respectively, in sesame oil was given subcutaneously twice weekly for 7 weeks. Sham operated animals and some of the gonadectomized animals were injected with sesame oil. The animals were killed by cervical dislocation at 10 weeks of age. For the analysis of the change with advancing age, untreated animals were killed at ages ranging from 10 days to 99 weeks. Abdominal skin and three pairs of bone (ilium, femur and tibia) free of cartilage were employed for biochemical analyses. Collagen solubility was measured by the method of Heikkinen [10] and lysyl oxidase activity was determined by the method reported by Siegel and Martin [11] and Narayanan [12].

Dermal collagen solubility. Abdominal skin (700—1000 mg wet weight) free of subcutaneous fatty tissue was excised and skin collagen was fractionated into the following four parts according to the method described by Heikkinen [10]. NSC, 0.45 M NaCl-soluble fraction. ASC, 0.5 M acetic acid-soluble fraction. ISC_{40}, 0.5 M acetic acid-soluble fraction heated at 40° C. ISC_R, residual insoluble fraction.

Hydroxyproline content in each fraction was determined by Woessner's method [13]. Collagen solubility was expressed as the percentage of each fraction described above.

Preparation of collagen substrate. Thirty pair of calvaria parietal bones from 17-day-old chick embryos were incubated at 37° C in a flask with 6 ml of Eagle's minimal essential medium without lysine, supplemented with 5 mg of ascorbic acid, 2 mg of β-aminopropionitrile fumarate (BAPN) and 200 000 units of penicillin G per 100 ml of medium. To the flask, 400 μCi of DL-[6-^{3}H]-lysine was added. After incubation for 24 h, the calvaria were homogenized in 25 ml of 1 M NaCl/0.05 M Tris · HCl, pH 7.7, in a glass homogenizer and extracted for 1 h at 4° C and then centrifuged at 60 000 × *g* for 25 min. The collagen in the supernatant was precipitated by the addition of NaCl to a 15% concentration. The precipitate was collected by centrifugation, redissolved in 30 ml of 1 M NaCl/0.05 M Tris · HCl, pH 7.7, and then dialysed for 72 h against 0.05 M Tris · HCl/0.16 M NaCl, pH 7.7, at 4° C. The dialysate was used as substrate. The collagen content varied from 150 to 300 μg/ml. The specific activity of the collagen solution was approx. $4.1 \cdot 10^5$—$7.2 \cdot 10^5$ dpm/ml. 0.5 ml of the collagen solution was used per assay tube.

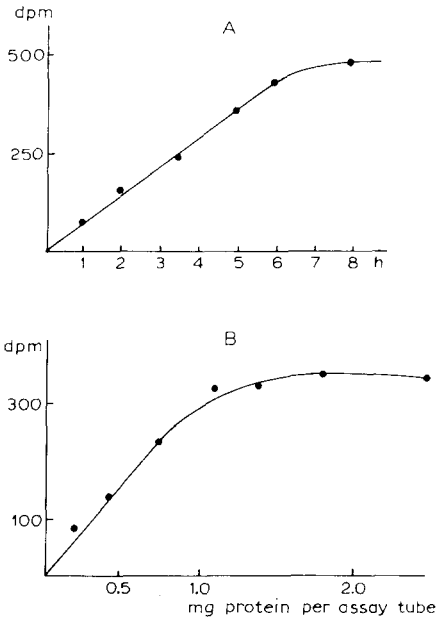

Fig. 1. (A) Dependence of lysyl oxidase from bone homogenate on incubation time. Protein concentration 1.0 mg per assay tube. (B) Relation between lysyl oxidase activity and protein concentration. Incubation time 3.5 h. Incubation conditions were as described in Materials and Methods.

Enzyme sources. Enzyme source of the materials was prepared from skin or bone. Abdominal skin about 500 mg wet weight was homogenized in 8 ml of 0.05 M Tris · HCl/0.16 M NaCl, pH 7.7 (containing 4 M urea) with a Polytron homogenizer and centrifuged at 105 000 × *g* for 60 min. The supernatant was dialyzed against the buffer for 72 h. The dialysate containing 3.0—5.0 mg/ml protein was used as enzyme source. Three pair of ilium, femur and tibia free of cartilage were pooled and homogenized in 4 ml of 0.16 M NaCl/Tris · HCl buffer containing urea. The dialysates contained 1.5—2.0 mg/ml protein as determined by the method of Lowry et al. [14].

Assay of lysyl oxidase. After preincubation of collagen substrate (0.5 ml) at 37° C for 1 h, 1.5 mg protein (skin) or 1.0 mg protein (bone) from each enzyme preparation was added and the total volume of each incubation mixture was adjusted to 1.5 ml with 0.05 M Tris · HCl/0.16 M NaCl, pH 7.7. BAPN (50 μg/ml) was added to some of the tubes. The mixture was then incubated at 37° C for 3.5 h. Substrate blanks were also incubated. After vacuum distillation, 1 ml aliquot of tritium water was analyzed in a Packard liquid scintillation spectrometer. Optimal incubation time and protein concentration were determined according to findings in preliminary experiments (Fig. 1).

Results and Discussion

Soluble fractions (NSC and ASC) of dermal collagen from animals 5 weeks of age were significantly larger than those from 10 days, in both sexes. After 5 weeks of age, insoluble fractions (ISC_{40} and ISC_R) had increased more rapidly

in females than in males to show a distinct sex difference in dermal collagen solubility (Fig. 2).

Lysyl oxidase activity of the bones increased up to 4 weeks and declined gradually thereafter, in both sexes. The enzyme activity was greater in females than in males for the period between 4 and 28 weeks of age (Fig. 3).

There were no significant difference in body weight among the hormone-replaced groups. The proportion of soluble fractions (NSC and ASC) of dermal collagen was greater in ovariectomized mice than in sham operated ones and was remarkably decreased by estrogen administration. On the contrary, progesterone or testosterone administration increased the solubility in ovariectomized mice (Fig. 4). With regard to the solubility of bone collagen, the response to estrogen was similar to that observed in the skin, although the soluble parts occupied only 2% of the total bone collagen in estrogen-treated animals, whereas the rate was 4% in ovariectomized mice.

Lysyl oxidase activity of the skin was markedly elevated by estrogen administration. The specific activity of lysyl oxidase of the bone remarkably increased after estrogen treatment, and the value was as high as that observed in normal 4-week-old mice. Testosterone enhanced the enzyme activity in the skin, but not in the bone. Progesterone administration slightly raised the enzyme activity in the bone. These data suggest that organ specificity is also present in the process of maturation of fibrous proteins.

Estradiol benzoate added to the assay tube in a concentration of 10^{-2} or

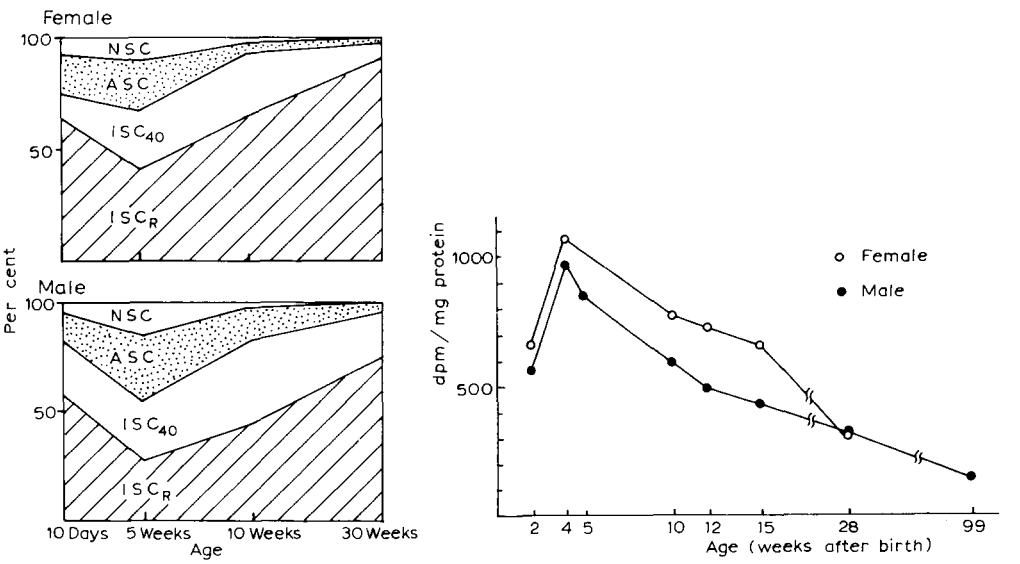

Fig. 2. Dermal collagen solubility with advancing age. Three animals in each experimental group were subjected to analysis at 5, 10 and 30 weeks of age. In 10-day-old mice, the pooled samples of whole skin from three animals were examined and the results were averaged.

Fig. 3. Lysyl oxidase activity of bone with advancing age. The number of male animals at 2, 4, 5, 10, 12, 15, 28 and 99 weeks of age included 6, 5, 2, 3, 3, 3, 3 and 2, respectively. The number of female animals at 2, 4, 10, 12, 15 and 28 weeks of age included 6, 5, 6, 3, 3 and 2, respectively. Values were corrected for substrate blanks.

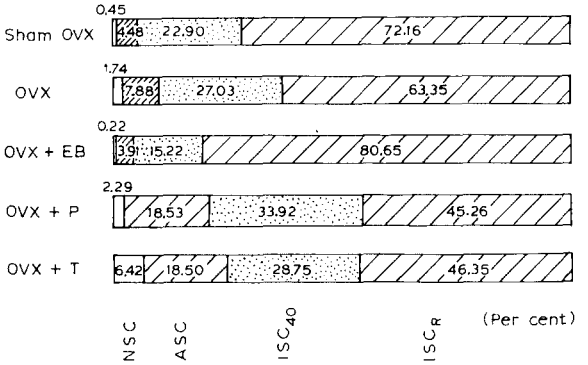

Fig. 4. Dermal collagen solubility in treated mice. In each experimental group, three mice were used to determine the solubility of skin collagen. The results of three experiments were averaged. Sham OVX, sham ovariectomy; OVX, overiectomy; EB, estradiol benzoate; P, progesterone; T, testosterone propionate.

10^{-3} μg/ml did not elevate the enzyme activity (Table I).

Fibrous proteins synthesized in and secreted from fibrogenic cells are considered to have a distinct characteristic i.e. formation of intra- and inter-molecular cross-links. Through such chemical modification, collagen and elastin become more stable. Lysyl oxidase catalyses the formation of lysine-derived aldehyde, allysine, this being the first step in the cross-linking process of both collagen and elastin. After this enzymic step, cross-links are formed by non-

TABLE I

LYSYL OXIDASE ACTIVITY IN ORCHIECTOMIZED SEX STEROID REPLACED MICE

TX, orchiectomy; T, testosterone propionate; EB, estradiol benzoate; P, progesterone. Values are presented as means ± S.E. Numbers in parentheses indicate number of animals used.

Experimental group		Lysyl oxidase activity (dpm/mg protein)	
		Bone	Skin
Substrate Blank		195 ± 5 (4)	496 (1)
Sham TX		424 ± 36 (5)	1232 ± 64 (3)
	Enzyme + BAPN		515
TX		445 ± 35 (6)	789 ± 22 (4)
	Enzyme + Buffer	433 *	
	Enzyme + EB 10^{-2} μg/ml	437	
	Enzyme + EB 10^{-3} μg/ml	377	
	Enzyme + prop. glycol	383	
TX + T		448 ± 34 (3)	1209 (1)
TX + EB		1109 ± 175 (4)	1858 ± 325 (3)
	Enzyme + BAPN	339	
TX + P		607 ± 65 (2)	

P values: $P < 0.01$ = TX + EB vs. Sham TX (bone), TX + EB vs. TX (bone), TX + EB vs. TX + T (bone), TX vs. Sham TX (skin),; $P < 0.05$ = TX + EB vs. TX (skin).

* Enzyme was preincubated for 1 h with estradiol benzoate prior to combining with the substrate. Estradiol benzoate dissolved in propylene glycol (100 μg/ml) was diluted with the buffer.

enzymic reactions and collagen gradually becomes insoluble or elastin aggregates. Morphometric studies using the electron microscope revealed that elastin fibrils aggregated to form thick elastic fibers, with advancing age, in the hip joint capsule of rats. The proportion of these matured elastic fibers increased after estrogen administration (data not included). Our findings in the skin and bone, where most of whole body collagen is located indicate that estrogen physiologically enhances lysyl oxidase activity and accelerates the cross-linkage of fibrous proteins promoting their maturation. As estradiol benzoate did not directly activate the enzyme in the assay tube, it is assumed that estrogen stimulates the production of lysyl oxidase in fibrogenic cells. The precise mechanism of this action of estrogen is now being investigated.

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