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Effects of Hyperlipoproteinemic Serum and Exogenous Proline Concentration on Collagen Synthesis by Isolated Rabbit Aortas[1] (39036)

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Increased quantities of collagen in the arterial intima is a feature of atherosclerosis in both man (1–3) and experimental animals (4). Recently, we have demonstrated elevated rates of collagen synthesis in the atherosclerotic arteries of dogs fed a cholesterol supplemented, essential fatty acid deficient diet (5), in the atheromatous aortas of rabbits fed a diet containing 2% cholesterol (6), and in the aortic plaques of susceptible pigeons fed a diet containing lard and 0.5% cholesterol (7). In each of these animal models of atherosclerosis, the disease is induced by, and can be related to, high levels of circulating low density or very low density serum lipoproteins. It occurred to us that high concentrations of serum lipoprotein might be directly sclerogenic to arterial tissue, producing fibrosis by direct stimulation of collagen biosynthesis in arterial connective tissue cells.

It is generally agreed that the cells responsible for connective tissue synthesis within the thickened intima are "modified" smooth muscle cells derived from the media (8, 9). The capacity of low density lipoprotein from hyperlipoproteinemic serum to stimulate proliferation in cultured aortic smooth muscle cells has been shown in a number of studies (10, 11). It seemed reasonable to propose that such hyperlipoproteinemic serum might also bring about an augmentation of collagen synthesis by smooth muscle cells from aorta.

The sclerogenic nature of exogenous cholesterol implanted subcutaneously is well recognized. In addition, several other lipids normally present in lipoproteins are known to be sclerogenic. This paper is concerned mainly with the relationship between serum low density lipoprotein concentration and aortic collagen synthesis.

Materials and methods. Aortas for incubation were taken from male New Zealand white rabbits weighing 3 to 4 kg, maintained on Wayne rabbit chow (Allied Mills, Inc., Chicago, Ill.) and allowed food and water *ad libitum*. Rabbits were anesthetized with sodium pentobarbital and killed by air embolism via the marginal ear vein. Aortas were quickly removed, washed in saline and stripped of adventitia. The aorta from each rabbit was divided into four longitudinal strips and each strip was preincubated for 30 min at 37° in screw-capped tubes containing 1.0 ml Earle's balanced salt solution (Grand Island Biochemical, Grand Island, N.Y.) in which was dissolved appropriate concentrations of unlabeled L-proline. In the experiments designed to test the effects of hyperlipoproteinemic serum on collagen synthesis, aortic segments from the same rabbit were paired. One member of each pair was incubated in 1.0 ml Earle's solution to which had been added 1 ml of normolipoproteinemic serum collected by heart puncture from rabbits fed a normal laboratory chow. The other member of each pair was incubated in 1 ml of Earle's solution and 1 ml of hyperlipoproteinemic serum obtained from rabbits fed a diet containing 2% cholesterol. Hyperlipoproteinemic serum always contained cholesterol in concentrations greater than 1500 mg/dl.

After 30 min, 5 μCi[U-^{14}C]L-proline (New England Nuclear, Boston, Mass.) was added to each incubation tube. Following addition of the isotope, the tubes were gassed with 95% O_2:5% CO_2, capped, and incubated with gentle agitation at 37° for 6 hr.

After incubation the segments were re-

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moved and placed in 0.9% NaCl containing 100 μg/ml cycloheximide and 1 m*M* α,α′-dipyridyl for approximately 5 min to inhibit further collagen peptide synthesis and hydroxylation. They were then washed in two changes of iced saline for 30 sec each, blotted dry, and weighed. The segments were then immersed in absolute ethanol: 0.1 *N* HCl (20:1 v/v) and the free proline in the tissue extracted with six changes of this solution for at least 8 hr each at 4° with gentle agitation (12).

The extracted segments were placed in screw-capped tubes and the collagen solubilized by autoclaving in 2.5 ml distilled water at 120° for 2 hr. The extraction was repeated and the pooled gelatin extracts hydrolyzed in 6 *N* HCl at 120° for 18 hr in sealed ampules. The resulting hydrolysates were evaporated and redissolved in 5.0 ml distilled water. Proline and hydroxyproline in the hydrolysates were separated on a 0.9 × 25 cm column of AG 50W-X8 (Bio Rad Laboratories, Richmond, Calif.) as previously described (5). The eluates were evaporated to dryness *in vacuo*, redissolved in 20 ml of a polar liquid scintillation solvent (42 ml Liquifluor, New England Nuclear, Boston, Massachusetts, 25 ml distilled water, and 325 ml absolute ethanol, diluted to 1 liter with toluene) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiencies were determined by automatic external standardization.

The specific activity of free proline in each incubation medium was determined at the end of the 6 hr incubation period. Following removal of the aortic segments, 2.0 ml of 10% trichloroacetic acid (TCA) was added to each tube containing 2 ml of incubation media at 4°. The total volume was brought to 5.0 ml with 5% TCA and the samples spun at 1000*g* for 10 min at 4° to remove the precipitated protein. After the removal of interfering substances with Permutit (Fisher Scientific Co., Pittsburgh, Pa.), proline was determined in an aliquot of the supernatant by the method of Troll and Lindsley (13). An equivalent aliquot was taken for the determination of proline radioactivity. In a similar fashion, the specific activity of tissue free proline was determined by measuring proline concentration and non-protein radioactivity in the acidic ethanol tissue extracts which had been pooled, evaporated to dryness, and redissolved in 5.0 ml distilled water.

We assumed that the specific activity of the tissue free proline provides a better approximation of the specific activity of the intracellular pool from which peptidyl proline is derived than does the specific activity of the medium free proline. Therefore, aortic collagen synthesis was calculated by dividing the radioactivity of aortic peptidyl hydroxyproline by the specific activity of free proline in the tissue, yielding the amount of proline hydroxylated. Since hydroxyproline accounts for about 14% of the weight of collagen, multiplication of this value by 6.94 (14) gives the weight of collagen synthesized per aortic segment during the incubation period.

Results and discussion. As we have observed in previous *in vitro* experiments with rabbit arterial segments removed from the animal immediately after death (6), collagen synthesis was initially rather slow but increased progressively throughout incubation. Fig. 1 is a plot of the amounts of collagen synthesized by rabbit aortic segments incubated in buffer containing normal rabbit serum for various time periods. The low rates of synthesis at early time intervals gives

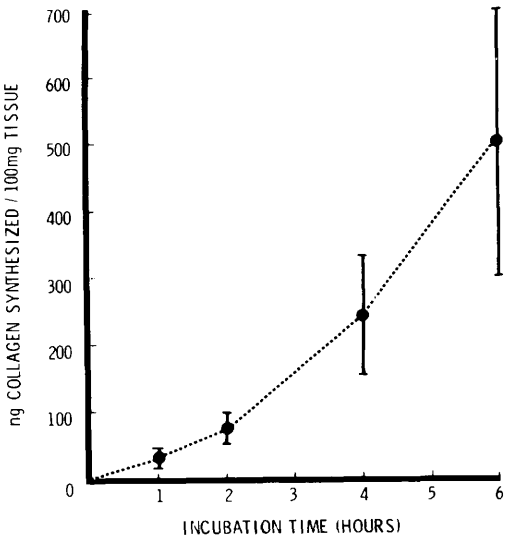

FIG. 1. Time course of collagen synthesis *in vitro* by normal rabbit aortic segments incubated in Earle's solution and normocholesterolemic rabbit serum. Mean ± SD of four segments at each time interval.

TABLE 1. COLLAGEN SYNTHESIS IN AORTAS INCUBATED IN NORMOLIPOPROTEINEMIC AND HYPERLIPOPROTEINEMIC SERA.

Pair number	Normolipoproteinemic serum[a] (ng collagen synthesized/100 mg tissue/6 hr)	Hyperlipoproteinemic serum[b] (ng collagen synthesized/100 mg tissue/6 hr)
1	125.68	39.34
2	44.67	58.68
3	35.67	48.56
4	24.71	37.16
5	40.19	37.96
6	63.66	47.75
7	58.10	22.98
8	39.17	43.67
9	117.46	136.65
10	118.60	91.18
11	92.01	140.09
12	88.40	87.51
13	884.53	920.01
14	1636.53	1599.94
15	655.89	662.03
16	872.62	837.76
17	607.04	699.45
18	626.36	562.72
19	314.25	270.75
20	678.18	457.78

Paired t test: $\bar{D} = 16.06$, $S_D2 = 3931$; $S_{\bar{D}}2 = \sqrt{S_D2/n} = 14.01$; $t = \bar{D}/S_{\bar{D}} = 1.15$, df = 19; $P > 0.3$.

[a] Normolipoproteinemic serum cholesterol concentration was 39 mg/dl for incubations involving the first eight pairs of aortic strips and 31 mg/dl for the remaining 12 pairs.

[b] Corresponding hyperlipoproteinemic serum cholesterol concentrations were 2325 and 1874 mg/dl.

the curve a nonlinear appearance. From other experiments we know that if the aortas were preincubated for 120 min in unlabeled medium before the addition of ^{14}C-proline, this early inhibition is not seen. We assume that the inhibition is due to temporary impairment of metabolic processes during the interval between removal from the animal and incubation. However, continuation of the incubation for much longer than 6 hr also leads to tissue damage and for this reason we did not extend the preincubation stage for longer than 30 min.

It will also be seen from Fig. 1 that there was an inherently large variation in the amount of collagen synthesized at 6 hr by apparently similar normal aortic segments. To remove the effects of this variation and allow us to detect the possible effects of changes in the serum supplement we adopted a paired experimental approach.

TABLE II. COLLAGEN SYNTHESIS BASED ON TISSUE FREE PROLINE SPECIFIC ACTIVITY[a].

Medium proline concentration	Net flux medium to tissue (μg pro/g wet wt)	Rate of collagen synthesis (ng/100 mg tissue/6 hr ± SE)
0.02 m*M*	−27.5	471 ± 125
0.04 m*M*	−18.0	558 ± 153
0.08 m*M*	12.8	489 ± 92
0.16 m*M*	54.6	456 ± 137

[a] The specific activity of free proline in the arterial segments at the end of the 6 hr incubation period was used to calculate rates of collagen synthesis in various concentrations of medium free proline. Four aortic segments were incubated at each concentration.

Effect of hyperlipoproteinemic serum. Twenty pairs of aortic segments were used to investigate whether medium supplemented with serum from hyperlipoproteinemic rabbits could stimulate aortic collagen synthesis under these conditions. There is a considerable variation between pairs but very little between individual members of each pair (Table I). The sensitive paired *t* test (15) detected no significant difference in collagen synthesis rates between the members of each pair. Hyperlipoproteinemic serum caused no detectable increase in aortic collagen synthesis when compared with normal serum, despite a 60-fold difference in the concentration of cholesterol in these two sera.

Effects of variations in medium proline concentration. In the foregoing experiments medium proline concentration was adjusted to 0.2 m*M* in each incubation. At this concentration we found there was relatively rapid uptake of proline by the tissue. In preliminary investigations we noted that at proline concentrations below 0.08 m*M* there was measurable net efflux of proline from the tissue segment during the incubation (Table II).

It is reasonable to suppose that proline flux between medium and tissue is partly concentration dependent, and it has been

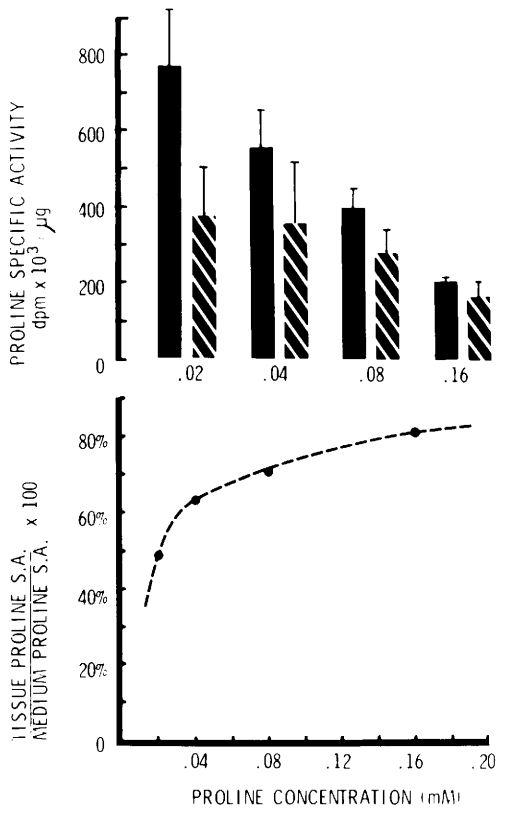

FIG. 2. Effects of medium proline concentration on medium tissue proline equilibration. *Top diagram*: Mean proline specific activity in medium (solid bars) and tissue (cross hatched bars) after 6 hr incubation in media containing varying concentrations of free proline. Vertical lines indicate standard deviation of four incubations. *Bottom diagram*: Change in tissue proline specific activity expressed as a percentage of medium proline specific activity (same data as top).

suggested that the rate of proline transport and diffusion into the cell influences the rate of collagen synthesis (12). We considered it worthwhile to investigate this phenomena in more detail and to determine whether changes in medium proline concentration had any effect on *in vitro* aortic collagen synthesis under the conditions described.

Table II contains the results of incubations carried out at four different medium proline concentrations. At low proline concentrations there was substantial loss of proline from the tissue into the incubation medium. In spite of this the rate of collagen synthesis appeared to be stable and not readily influenced by proline concentration. As before, collagen synthesis rates were calculated, from the amount of tissue peptide-bound hydroxyproline radioactivity and the specific activity of *tissue* free proline.

Figure 2 shows that at low medium proline concentrations there is a failure to obtain equilibrium between tissue and medium free proline, and the isotopic tracer is still present predominantly in the medium. The failure of equilibration of medium and tissue free proline pools at low levels of exogenous proline is important for it indicates the difficulties of determining rates of protein synthesis by isotopic methods. If the tissue proline pool is ignored and rates of collagen synthesis in these experiments calculated on the basis of medium proline specific radioactivity, an artifactual relationship between medium proline concentration and collagen synthesis is created.

Finerman *et al.* (12) reported the occurrence of such a relationship between proline concentration and collagen synthesis in fetal rat calvaria, which was found to disappear at concentrations of proline above 0.15 m*M*. If fetal rat calvaria behave similarly to rabbit aortic segments *in vitro*, the relationship is probably not real, being an artifact of expressing the results on the basis of medium proline radioactivity. Certainly in rabbit aortas, we could find no evidence that collagen synthesis was influenced by proline concentrations or proline flux. However, we recognize that our analysis is also incomplete in that it does not take into account the possible compartmentalization of the tissue proline pool.

It is well established that patients with certain forms of hyperlipoproteinemia are more prone to severe atherosclerosis than those with normal serum lipoprotein levels. The experiments we have described provide no evidence for the suggestion that part of that relationship is mediated by a direct sclerogenic effect of hyperlipoproteinemic serum. Aortic collagen synthesis was not affected by the type or quantity of exogenous lipoprotein. This does not imply that lipoprotein entrapped *within* the arterial wall, or lipid split from the apoprotein carrier, may not be directly sclerogenic in arteries. We have previously commented on the increased density of collagen staining material around

deposited cholesterol crystals in experimental atherosclerotic plaques (5). Such sequestered lipid may well be a potent stimulant to fibrosis in this situation.

Experimental rabbit atherosclerotic lesions commence with intimal cell hyperplasia. Moreover, a proliferative response of arterial smooth muscle cells to exposure to hyperlipoproteinemic serum can be demonstrated in culture (10, 11). We have not investigated whether a similar proliferative response can be detected in short term incubation experiments with intact aortic tissue, similar to those described here, but we feel it would be worthwhile to do so. Similarly, although we have failed to demonstrate any enhancement of collagen synthesis by serum lipoprotein in these experiments it cannot be inferred that similar experiments with cultured aortic smooth muscle cells would also be negative. We believe that such experiments are necessary before it can be said with confidence that serum lipoproteins have no direct effect on collagen synthesis.

Summary. Collagen synthesis was measured in segments of normal rabbit aorta, incubated *in vitro*, by monitoring the formation of peptidyl-^{14}C-hydroxyproline from [U-^{14}C]-L-proline added to the incubation medium. The effect of hyperlipoproteinemic rabbit serum on the rate of collagen synthesis was compared with the effect of normal rabbit serum. No differences in the rates of synthesis were detected between the two batches of serum, despite a 60-fold difference in serum cholesterol concentration.

Increases in free proline concentration in the incubation medium resulted in changes in proline flux between medium and tissue pools of free proline, but medium proline concentration had no effect on the rate of collagen synthesis.

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