Increased turnover of arterial collagen in hypertensive rats

(hydroxyproline)

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ABSTRACT   The turnover of total collagen in several tissues of 12-week-old normotensive and hypertensive rats was estimated by using tritium-labeled proline as a precursor. The effect of reutilization of the label was minimized by treatment with large doses of unlabeled proline subsequent to administering the radioactive imino acid. The collagen from skin, tail tendon, aorta, and mesenteric artery in normotensive animals had a half-life of about 60–70 days. In hypertensive animals the half-lives of skin and tail tendon collagen were unchanged but the half-lives of collagen in the aorta and mesenteric artery were reduced to 17 days.

In previous studies from this laboratory we reported that hypertension in rats leads to an increased synthesis and deposition of collagen in arteries (1) and microvessels (2). In one set of experiments, treatment of hypertensive rats with β-aminopropionitrile, an inhibitor of collagen crosslinking, resulted in a lowering of the blood pressure within 3 weeks (3). If collagen deposition itself leads to a maintenance of the elevated pressure as we have proposed (3), then this reversal in such a short time may indicate that in hypertension collagen breakdown as well as synthesis may be accelerated. To investigate this possibility, we set about to measure the decay in specific activity of protein-bound hydroxyproline (collagen) after rats were labeled with L-[2,3-^{3}H]proline. Many other investigators have attempted to measure collagen turnover using radioactive amino acids (4–9); however, no correction was made for reutilization of the labeled amino acid that was used. In this report we have utilized an *in vivo* pulse–chase experiment to minimize the problem of reutilization and have been able to estimate the turnover of collagen in several tissues of normal and hypertensive rats.

MATERIALS AND METHODS

Desoxycorticosterone acetate (DOCA) was purchased from ICN Pharmaceuticals, Plainview, NY; L-proline from Sigma Chemical Co., St. Louis, MO; and L-[2,3-^{3}H]proline from New England Nuclear, Boston, MA. Male Wistar rats were obtained from Marland Breeding Farms, Hewitt, NJ, and fed Purina rat chow, Ralston Purina Co., St. Louis, MO.

Normotensive rats (12-weeks-old) with an average body weight of 373 g were kept in disposable cages in a hood. DOCA/salt hypertension was produced in uninephrectomized 6-week-ole male Wistar rats by twice weekly subcutaneous injection of DOCA (5 mg per rat) (10, 11). Rats were maintained on the standard diet and allowed free access to drinking water containing 1% NaCl. After 6 weeks of treatment their average blood pressure was 200 mm Hg (1 mm Hg = 133 Pa) and their average body weight was 416 g. During the experiments, the normotensive animals gained 120 g in 53 days and

250 g in 111 days, while the hypertensives gained 90 g in 53 days. Of the twenty rats started on the DOCA/salt regimen, five died from respiratory infections and three others could not be used because they were also infected. For this reason only three time points could be measured for the hypertensive group. Blood pressure was monitored weekly by the tail cuff microphone method with an instrument made by Hoffmann-La Roche & Co., Basel, Switzerland.

Blood was obtained by either cardiac puncture or tail vein bleeding. Serum proteins were precipitated by the addition of 3 ml of 10% trichloroacetic acid per ml of serum and removed by centrifugation at 13,000 × g for 20 min. Proline was measured (10) in the supernatant solution after neutralization with 10 M KOH. Urine samples were treated in an identical manner.

Each rat, whether normotensive or hypertensive, was injected intraperitoneally with 1 mCi of L-[2,3-^{3}H]proline (20–40 Ci/mmol). Starting 24 hr after the injection of label, 1.5-g injections of L-proline in 10 ml of H_2O were administered twice daily and continued for 12 days. At the times indicated, the animals were killed with ether and the tail tendon, skin from the abdominal area, the aorta, and the mesenteric artery were removed. The vascular tissues were prepared as previously described (1). Hair and subcutaneous fat were removed from the skin with a scalpel and the tendon was carefully separated from surrounding connective tissue. All tissues were kept at −20° until assay.

One-hundred-milligram samples of skin, tendon, and aorta and 25-mg samples of the mesenteric artery were homogenized at 0° in 4 ml of 10% trichloroacetic acid in a glass-to-glass Duall 23 tissue grinder (five 30-sec pulses). The precipitated proteins were then separated from the supernatant solution by centrifugation at 13,000 × g for 20 min. The supernatant solution was neutralized with 10 M KOH and set aside for assay of the specific activity of free proline. The precipitates were hydrolyzed in 6 M HCl at 120° for 18 hr and neutralized with 10 M KOH prior to assay of the specific activity of hydroxyproline. Proline was assayed colorimetrically by the method of Troll and Lindsley (12) and hydroxyproline by the method of Kivirikko *et al.* (13). Proline and hydroxyproline were isolated for measurement of radioactivity by a modification of the method of Peterkofsky and Prockop (14). Potassium borate buffer with KCl was used in place of the sodium pyrophosphate buffer with NaCl, and alanine was added to increase the sensitivity (13). Extractions were performed with 11 ml of toluene, and 10 ml of the toluene extract was added to a vial containing 10 ml of ACS (aqueous counting scintillant, Amersham-Searle), and the radioactivity was measured in a Packard 3380 scintillation counter. The counting efficiency was 30% and there was no quenching. All determinations were performed in duplicate

Table 1.   Specific activity of [³H]proline in tissues of rats with
and without a chase with unlabeled proline

Tissue	Specific activity of [³H]proline, cpm/µmol	
	Untreated	Treated
Skin	14,200 ± 1,600	12 ± 14
Tendon	14,300 ± 1,100	5 ± 9
Aorta	17,800 ± 1,400	2 ± 1
Mesenteric artery	17,700 ± 2,000	1 ± 1
Serum	12,700 ± 2,400	5 ± 3

Five animals in each group were labeled by intraperitoneal injection
with 1 mCi of L-[2,3-³H]proline. Starting 24 hr after the injection of
the label, the treated group received 3.0 g of unlabeled L-proline daily
for 11 days. On the 12th day both groups were killed and the specific
activity of the free proline in the tissues was measured.

and specific activities are reported as cpm/µmol of imino acid.
The specific activity of hydroxyproline in the turnover studies
was corrected for dilution due to growth of the animals as
suggested by Neuberger *et al.* (4, 5).

RESULTS

Radioactive L-proline was used as the source of label because
on incorporation into collagen it appears as labeled hydroxy-
proline. Because the latter is present essentially only in collagen,
the hydroxyproline can be isolated from total tissue protein to
yield data on the specific turnover of collagen. In initial studies,
when L-[2,3-³H]proline was administered, the specific activity
of free proline in the blood and tissues were found to remain
relatively constant for several weeks at a level 30-80 times that
of the specific activity of the peptidyl hydroxyproline in tissues
(data not shown). Under such circumstances, relabeling of
hydroxyproline would result in unreliable turnover values for
collagen. To overcome this problem, 3 g of unlabeled L-proline
was administered per day over a 12-day period after injection
of the label to lower the specific activity of the free proline
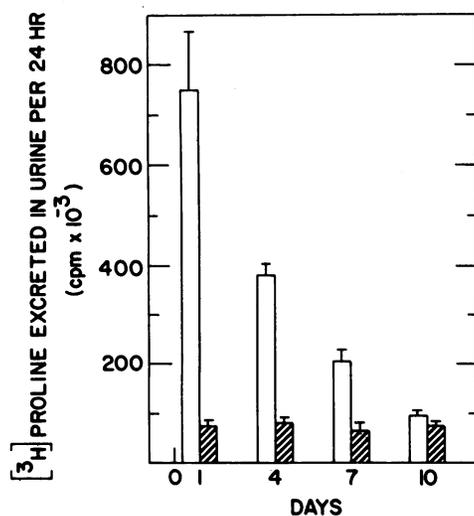pools. Because the turnover of collagen was known to be longer

FIG. 1.   Urinary excretion of [³H]proline in rats given unlabeled
proline and in untreated controls. Twelve-week-old Wistar rats were
injected with 1 mCi of L-[2,3-³H]proline. After 24 hr, three of the rats
were injected daily with 3 g of unlabeled L-proline. Twenty-four-hour
urine samples were collected and radioactive proline was measured.
Values are mean ± SD for three rats. Hatched bars, untreated; open
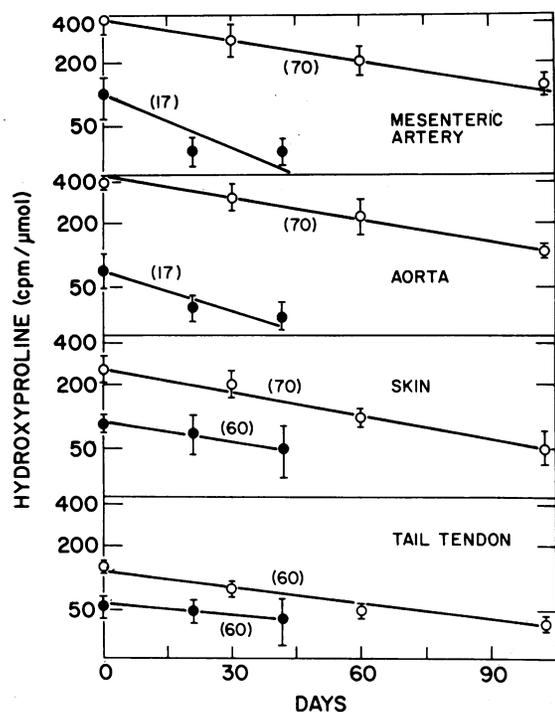bars, treated.

FIG. 2.   Decay of tritiated hydroxyproline in several tissues of
normotensive and hypertensive rats. Twelve-week-old normotensive
and DOCA/salt hypertensive rats were injected with 1 mCi of L-
[2,3-³H]proline. During the next 12 days all animals received 3 g of
unlabeled L-proline per day by subcutaneous injection. The rats were
killed at the times indicated and the specific activity of bound hy-
droxyproline was measured. Each point is the mean ± SD determined
from three to five rats. The numbers in parentheses are the calculated
half-lives in days for the various tissues. O, Normotensive; ●, hy-
pertensive.

than 12 days, such a procedure was considered acceptable.
Table 1 compares the specific activities of free proline in several
tissues with and without the chase regimen. Fig. 1 shows the
increased excretion of tritiated proline in the urine of the ani-
mals given the nonradioactive proline compared to untreated
controls. It is apparent that the injections with unlabeled L-
proline effectively removed the labeled imino acid from the
tissues.

To measure collagen turnover and to determine the effects
of hypertension, 20 Wistar controls and 20 DOCA/salt hy-
pertensive rats were given L-[2,3-³H]proline by intraperitoneal
injection. Twenty-four hours after the administration of the
label, each rat was injected with 3 g of unlabeled L-proline per
day for 12 days. The first groups of five normal and five hy-
pertensive rats were killed on the 12th day and the remainder
at the times indicated in Fig. 2. As shown, the half-life of col-
lagen hydroxyproline in all the tissues of the normotensive
animals ranged from 60 to 70 days. Hypertension had no effect
on collagen turnover in skin and tendon. However, the half-life
was decreased to 17 days in the aorta and mesenteric artery of
the hypertensive animals. During the course of these experi-
ments, the specific activity of the free proline in tissues never
exceeded the low values of the treated group shown in Table
1. It should be noted that the incorporation of label in the hy-
pertensive animals was ⅓ that in the normotensive controls.
This was due to the increased urinary excretion of proline in
the DOCA/salt rats, 14.3 µmol/100 g body weight per 24 hr,
compared to a value of 4.1 in the normotensive animals. Nev-
ertheless, skin and tail tendon yielded the same half-life values
in both instances.

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DISCUSSION

The half-life of a labeled precursor influences the calculation of the turnover of metabolic products (15–17). When the specific activity of the precursor pool is high compared to the specific activity of the product, errors due to reutilization must be considered. This is especially true in the measurement of proteins with long half-lives, where the label used is an amino acid with a slow turnover in the free pools, as with proline, leucine (18), and tyrosine (19). Unless relabeling from a highly radioactive pool is taken into account, values for protein turnover reflect the turnover of the amino acid, which can be a very slow process and, as shown above, hardly measureable over a study of several weeks. The extremely long half-lives reported for collagen by Neuberger et al. (4, 5), Kao et al. (6, 7), Gerber et al. (8), and Popenoe and Van Slyke (9) were apparently determined without taking relabeling into account. The data of Jackson and Heininger (20), who utilized $^{18}O_2$ to label the hydroxyproline, are comparable to those reported here. Because the free oxygen pool turns over within minutes, there is no possibility of relabeling with the $^{18}O_2$. Although Jackson and Heininger labeled weanling rats, it is possible to examine their data and calculate a turnover number for insoluble skin collagen in 4-week-old animals. The turnover time we have calculated from their data is 30 days. By contrast, with rats of the same age, Gerber et al. (8) reported a turnover time for insoluble skin collagen of 150 days. The discrepancy is most likely explained by the fact that Gerber et al. (8) did not make the necessary corrections for reutilization of the [^{14}C]proline label. Our values of 60–70 days for the turnover of skin and tail tendon collagen in 12-week-old rats are reasonable compared to those calculated from the data of Jackson and Heininger because collagen synthesis is more rapid in young, rapidly growing animals (21).

A most important finding is that in normotensive rats collagen turnover in the aorta and mesenteric artery is the same as in skin and tail tendon. The significance of this may be related to the need for maintaining normal body proportions and suggests some centralized regulatory mechanism, perhaps mediated by growth hormone (22). However, the specific increase in turnover of arterial collagen (about 4-fold) as a result of hypertension indicates that whatever central regulation there may be can be overridden by a local need. This is well known from studies of skin wounds (23), liver fibrosis (24), and lung fibrosis (25). In all the above cases a local insult or injury to the tissue specifically turns on collagen synthesis in that tissue. In the case of the skin, the initiation of collagen synthesis by an incision was actually localized to within a few millimeters of the wound (23). Hypertension may be considered another type of insult or injury, analogous to the above examples, which impinges on the entire vascular bed exposed to the increased pressure (2). In support of this localization of response, we have recently found that veins of hypertensive animals, which are not exposed to increased pressure, do not show increased collagen synthesis (26).

Measurement of arterial collagen turnover will be most useful in studies on other models of hypertension and on the effects of antihypertensive drugs. Because hypertension specifically affects arterial collagen, skin and tail tendon may serve as initial controls when turnover studies are carried out on vascular collagen in hypertensive animals.

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