Effect of Methylprednisolone in Epinephrine–Thyroxine Induced Arteriosclerosis (36919)

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Collagen is abundant in both medial and intimal regions of blood vessels and is recognized as a dominant component of human arteriosclerosis. Recent studies (1) of rabbits with epinephrine–thyroxine induced arteriosclerosis have demonstrated that the enzyme responsible for hydroxylation of proline in collagen, proline hydroxylase, is significantly elevated in diseased aortas before visual evidence of extensive aortic disease was observed. Since proline hydroxylase may be involved in the control of collagen synthesis (2, 3) these data suggest that collagen biosynthesis has an active role in the formation of arteriosclerotic lesions.

Glucocorticoids have been shown to cause a decrease in collagen content in both normal and inflamed connective tissues (4, 5). The exact biochemical mechanism responsible for the reduction in tissue collagen content is unknown. Houck, Patel and Gladner (4) observed a decrease in tissue collagen content and correlated this with an increase in free collagenolytic activity. They hypothesized that the loss of tissue collagen in response to glucocorticoid treatment was the result of increased collagen catabolism through the induction of collagenase activity in skin fibroblasts.

Nakagawa, Fukuhara and Tsurufuji (5) reported that a single injection of betamethasone decreased the rate of ^{3}H-proline incorporation in rat carrageenin granuloma but did not alter proline hydroxylase activity. These authors proposed that the steroid either inhibited the formation of protocollagen indirectly or inhibited the transport of proline across cell membranes or both, but did not affect the conversion of proline to hydroxyproline. However, Cutroneo, Costello and Fuller (6) found that administration of glucocorticoids for 4 days caused a significant reduction in enzymatic protocollagen proline hydroxylase activity in rat liver and granuloma tissue grown on sponge implants, suggesting that glucocorticoids decreased tissue collagen content through a direct effect on the rate of collagen synthesis.

The literature cited above indicates that glucocorticords decrease tissue collagen content but the biochemical mechanism remains in doubt. This study was conducted to determine whether methylprednisolone treatment will inhibit protocollagen proline hydroxylase activity in normal and in epinephrine–thyroxine treated rabbit aortic tissue and to determine whether inhibition of collagen synthesis would prevent the development of arteriosclerotic lesions in the epinephrine–thyroxine treated rabbits.

Materials and Methods. Male New Zealand rabbits weighing approximately 3 kg were used throughout the study. The rabbits were divided into the following four groups: A. controls; B. epinephrine–thyroxine treated (epinephrine was injected iv with an infusion pump: 0.025 mg/kg for the first 5 days, 0.050 mg/kg for the remaining 4 days; thyroxine was injected ip 0.050 mg/kg); C. methylprednisolone treated (Medrol, Upjohn Co., Kalamazoo, MI, injected im 1.0 mg/kg as a suspension in 1.5% methyl cellulose) and D. epinephrine–thyroxine–methylprednisolone treated (drugs were administered as described in B and C above). Rabbits were treated for either 4 or 9 days and were sacrificed by cervical dislocation 24 hr after the last injection. The aortas were quickly removed and chilled, and the degree of aortic disease was estimated using the 0 to 4 grading system described by Lorenzen (7). Aortas were sectioned into thoracic and abdomi-

TABLE I. Aortic Proline Hydroxylase Activity of Rabbits Treated with Epinephrine–Thyroxine and Methylprednisolone for 9 Days.

Treatment	Proline hydroxylase,[b] mean ± SE (*N*)		No. of rabbits with lesions graded
	Thoracic aorta	Abdominal aorta	0-1-2-3-4
Control	8385 ± 831 (6)	4704 ± 787 (6)	4-2-0-0-0
Epi-Thy[a]	19,960 ± 2381 (8)[f]	14,130 ± 1844 (8)[f]	5-0-2-0-1
Methyl[a]	6398 ± 1522 (8)[c]	2165 ± 435 (8)[e]	6-2-0-0-0
Epi-Thy-Methyl[a]	13,733 ± 2519 (9)[d]	5971 ± 1716 (9)[c]	2-3-2-1-1

[a] Rabbits were treated with epinephrine–thyroxine (Epi-Thy), methylprednisolone (Methyl) or epinephrine–thyroxine and methylprednisolone (Epi-Thy-Methyl) as described in the Methods section.

[b] Estimated by the formation of ^{3}HHO from incubation at 30° of 3,4-^{3}H-proline rich substrate with 15,000*g* enzyme preparation and is reported as dpm/mg protein of enzyme preparation/30 min.

[c] NS, $p > .05$.

[d] $p < .05$.

[e] $p < .01$.

[f] $p < .001$.

nal segments at the celiac artery and homogenized in nine volumes of 0.25 *M* sucrose using a glass coaxial homogenizer. Noninjected controls were used throughout the study since previous investigations had shown that ip and iv saline injection for 14 days had no effect on the parameters investigated in this study.

The rabbits in Tables I and II were assayed for proline hydroxylase activity using different batches of labeled substrate. The chick embryos used in preparing the substrate will unavoidably incorporate varying amounts of labeled proline. This variation in substrate specific activity makes it necessary to assay control and experimental animals with the same batch of substrate and prevents a comparison of total enzyme activity between groups assayed with a different batch of substrate.

Proline hydroxylase activity was measured in the 15,000*g* supernatant of aortic homogenates, using the method described by Hutton, Tappel and Udenfriend (8). This assay

TABLE II. Aortic Proline Hydroxylase Activity of Rabbits Treated with Epinephrine–Thyroxine and Methylprednisolone for 4 Days.

Treatment	Proline hydroxylase,[b] mean ± SE (*N*)		No. of rabbits with lesions graded
	Thoracic aorta	Abdominal aorta	0-1-2-3-4
Control	10,072 ± 962 (8)	7718 ± 711 (8)	4-4-0-0-0
Epi-Thy[a]	13,594 ± 2748 (7)[c]	8785 ± 794 (8)[c]	3-4-1-0-0
Methyl[a]	3482 ± 393 (8)[e]	2093 ± 208 (8)[e]	5-3-0-0-0
Epi-Thy-Methyl[a]	9818 ± 3471 (9)[c]	3908 ± 480 (8)[d]	7-2-0-0-0

[a] Rabbits were treated with epinephrine–thyroxine (Epi-Thy), methylprednisolone (Methyl) or epinephrine–thyroxine and methylprednisolone (Epi-Thy-Methyl) as described in the Methods section.

[b] Estimated by the formation of ^{3}HHO from incubation at 30° of 3,4-^{3}H-proline rich substrate with 15,000*g* enzyme preparation and is reported as dpm/mg protein of enzyme preparation/30 min.

[c] NS, $p > .05$.

[d] $p < .01$.

[e] $p < .001$.

system is based upon the stoichiometric formation of ^{3}HHO and ^{3}H-hydroxyproline when a substrate consisting of a polypeptide rich in 3,4-^{3}H-proline is incubated with aortic enzyme and cofactors (9). An aliquot of the 15,000g supernatant of aortic homogenates was incubated under air in the presence of 3,4-^{3}H-proline-rich chick embryo substrate (approximately 350,000 dpm), 7.5 μmole of ascorbic acid, 0.9 μmole of α-ketoglutarate, 0.45 μmole of ferrous ammonium sulfate and 0.5 *M* Tris–HCl buffer (pH 7.5) to a final volume of 3 ml. The reaction was terminated after 30 min by the addition of 0.3 ml of 50% trichloroacetic acid and the resulting tritiated water was collectected by vacuum distillation, and counted in a liquid scintillation spectrometer. All samples were corrected for counting efficiency by use of an automatic external standard.

Hydroxyproline content of aortic homogenates was estimated by the method of Kivirikko, Laitinen and Prockop (10). Protein concentration in aortic homogenates and the 15,000*g* supernatant were determined by the method of Lowry *et al.* (11) using bovine serum albumin as a standard.

Results. Rabbits injected with epinephrine–thyroxine for 9 days (Table I) demonstrated a significant increase in collagen synthetic rate and an increase in the number of arteriosclerotic lesions. A similar increase was noted in animals treated with epinephrine–thyroxine for 4 days (Table II), however, at this time the changes were not significant. Rabbits injected with methylprednisolone for 4 days (Table II) showed a significant decrease in proline hydroxylase activity in both the thoracic and the abdominal aortas compared to control. Rabbits treated for 9 days with methylprednisolone (Table I) did not show a significant decrease in proline hydroxylase activity in the thoracic aorta, but still exhibited a significant decrease in the abdominal aorta compared to control values.

When the rabbits were given epinephrine–thyroxine and methylprednisolone for 4 days (Table II) proline hydroxylase activity of the thoracic aorta was the same as the controls but significantly higher than the methylprednisolone treated rabbits. The abdominal aorta, however, was significantly lower than the controls but had the same activity as rabbits which received just methylprednisolone. When the epinephrine–thyroxine and methylprednisolone treatment was continued for 9 days (Table I), the thoracic aorta proline hydroxylase activity was significantly greater than the controls and the methylprednisolone treated rabbits. In the abdominal aorta, however, enzyme activity was the same as the controls but was significantly elevated when compared to the abdominal aortas of the methylprednisolone treated animals. The rabbits which received epinephrine–thyroxine and methylprednisolone for 9 days had a similar degree of aortic disease using the visual grading system. Histologically, the changes observed in both the epinephrine–thyroxine and the epinephrine–thyroxine–methylprednisolone treated animals were medial lesions similar to those previously reported (7). Both tissues demonstrated a localized injury characterized by spreading and fragmentation of the elastic fibers.

The thoracic aortas from rabbits in all experimental groups treated for either 4 or 9 days (Table III) did not show a significant difference in hydroxyproline of hydrolysates of whole aorta homogenates.

TABLE III. Effect of Epinephrine–Thyroxine and Methylprednisolone on Aortic Hydroxyproline.

	Thoracic aorta (μg hydroxyproline[a]/mg protein)	
Treatment	4 Day	9 Day
Control[b]	42.15 ± 2.20 (8)	31.93 ± 3.15 (5)
Methyl	43.54 ± 0.91 (8)[c]	38.70 ± 1.57 (8)[c]
Epi-Thy	43.16 ± 1.60 (8)[c]	37.75 ± 1.12 (8)[c]
Epi-Thy-Methyl	44.51 ± 0.99 (9)[c]	37.56 ± 1.18 (9)[c]

[a] Hydroxyproline (μg/mg of total protein) in the whole homogenate of a 9.1 sucrose homogenate of the thoracic aorta. Reported as mean ± SE (*N*).

[b] Rabbits were treated with epinephrine–thyroxine (Epi-Thy), methylprednisolone (Methyl) or epinephrine–thyroxine and methylprednisolone (Epi-Thy-Methyl) as described in the Methods section.

[c] NS, $p > .05$.

Discussion. The data in Table I and the microscopic observations clearly indicate that methylprednisolone will not protect against the formation of epinephrine–thyroxine induced arteriosclerotic lesions. Alper *et al.* (*12*) reported that methylprednisolone protected their rabbits from cholesterol induced atherosclerosis. Our data may differ from that of Alper *et al.* because of the use of epinephrine–thyroxine which induces medial fibrous plaques. Lorenzen (13) and others have shown that thyroxine potentiates the ability of epinephrine to cause aortic disease, presumably by potentiating the effects of epinephrine on aortic tissue. Kalsner (14) has shown that glucocorticoids will also potentiate the response of rabbit aorta to epinephrine. This potentiation of epinephrine's ability to constrict rabbit aorta may explain why we observed no protection from the methylprednisolone treatment.

Glucocorticoids will cause a decrease in collagen content in various tissues. The biochemical mechanism is unknown, but could be related to a decreased rate of synthesis or an increased rate of degradation. The data presented in this study demonstrate that methylprednisolone causes a significant decrease in aortic proline hydroxylase activity in rabbits receiving only glucocorticoid. The mechanism whereby methylprednisolone causes a decrease in enzyme activity is not known. Rabbits which received epinephrine–thyroxine and methylprednisolone (Tables I and II) had thoracic aorta proline hydroxylase values which were significantly higher than rabbits treated with methylprednisolone but were not statistically different from rabbits treated with epinephrine–thyroxine. These data indicate that the inhibition of thoracic aorta proline hydroxylase by methylprednisolone is reversed by epinephrine–thyroxine treatment. In the abdominal aorta the inhibiting effects of methylprednisolone were only partially reversed by 9 days (Table I). The difference between the response of the thoracic and the abdominal aorta to epinephrine–thyroxine treatment in the presence of methylprednisolone can be explained by the observation that epinephrine–thyroxine treatment has its primary site of action in the thoracic aorta (1, 13).

The collagen content of the thoracic aortas as estimated by hydroxyproline content was unchanged in all experimental groups of rabbits (Table III) even though the tissues had an increased rate of proline hydroxylase activity. Takeuchi, Kivirikko and Prockop (15) in a study of hepatic fibrosis in rats also observed an increase in proline hydroxylase activity before observing any changes in liver hydroxyproline content. Our data supports the suggestion of Takeuchi, Kivirikko and Prockop that under conditions of altered collagen synthesis changes in proline hydroxylase activity occur before changes in tissue hydroxyproline content, and may serve as an early indicator of increased collagen synthesis.

Summary. Rabbits treated with methylprednisolone had decreased levels of aortic proline hydroxylase activity, indicating that glucocorticoids decrease collagen content by affecting the rate of synthesis. The inhibitory effects of methylprednisolone on enzyme activity were reversed by treatment of rabbits with epinephrine–thyroxine. Methylprednisolone which is reported to inhibit aortic sclerosis in cholesterol fed rabbits also did not protect epinephrine–thyroxine treated rabbits from aortic sclerosis.

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