LOCALIZATION OF PROLYL HYDROXYLASE BY THE IMMUNOPEROXIDASE METHOD IN CARDIOVASCULAR TISSUES OF HYPERTENSIVE RATS

AKIRA OOSHIMA, M. D.

An immunohistochemical method and light microscopy were utilized to determine localization of prolyl hydroxylase in the cardiovascular tissues of hypertensive rats. The blood vessels and renal glomerulus from these animals demonstrated an enhanced immunoreaction for prolyl hydroxylase. As the most prominent staining was observed in medial smooth muscle cells of blood vessels, these are probably the major collagen-producing cells in arteriosclerotic lesions induced by hypertension. In the glomerulus, endothelial cells and probably mesangial cells are mainly responsible for the collagen formation. An intense immunoreaction was also found in the fibroblasts which proliferated in the area of myocardial fibrosis in hypertensive rats, while there was no specific staining in myocardial cells in either hypertensive or normotensive rats.

COLLAGEN biosynthesis is markedly increased in the cardiovascular tissues of hypertensive rats and antihypertensive agents prevent and reverse this increased synthesis^{1,2}. In attempts at elucidation, a specific immunoperoxidase method for localization of prolyl hydroxylase (EC 1.14.11.2; proline, 2-oxoglutarate dioxygenase) was developed³ and utilized herein to identify collagen-producing cells in the cardiovascular tissues of hypertensive rats. Prolyl hydroxylase is the enzyme that catalyzes the hydroxylation of certain prolyl residues during the biosynthesis of collagen⁴. The increased activity of this enzyme has been reported in a variety of disease states including hepatic fibrosis⁵, granu-

lation tissues⁶, atherosclerosis⁷ and hypertensive vascular damage^{1,2} which are characterized by an excessive formation of collagen, leading to fibrosis. Thus, prolyl hydroxylase can be used as an excellent biochemical marker of collagen biosynthesis in tissues.

This paper describes that vascular smooth muscle cells are mainly responsible for the increased formation of collagen fibers in the arteriosclerotic blood vessels and that fibroblasts, not myocardial cells, are responsible for the collagen deposition in myocardial fibrosis induced by hypertension.

MATERIALS AND METHODS

Male spontaneously hypertensive rats (SHR)⁸ were sacrificed at 16 and 32 weeks of age. Genetically related Wistar-Kyoto rats served as controls. The other model of hypertensive rat used was the deoxycorticosterone acetate (DOCA)-salt hypertensive animal⁹. Such a state was induced

Key Words:

Prolyl hydroxylase
Hypertensive rats
Immunoperoxidase method
Arteriosclerosis

(Received on April 19, 1978; Accepted on June 12, 1978)

Department of Pathology, Shimane Medical University, Izumo, Shimane, Japan 693

This work was supported in part by Japan Heart Foundation Research Grant for 1977.

in uninephrectomized, 8-week-old, male Wistar rats by twice-weekly subcutaneous injections of DOCA (5 mg per rat) for 5 weeks. These animals were allowed free access to 1% saline as drinking water. Age-matched controls for DOCA-salt hypertensive rats were uninephrectomized and maintained on 1% saline. All animals were provided a regular stock chow diet. The blood pressure was monitored indirectly by the tail cuff method without application of anesthesia.¹⁰ Animals were decapitated and various tissues (aorta, mesenteric artery, heart, kidney, testis and brain) were immediately frozen and cut at 5 μ in a cryostat at $-20 \sim -25^{\circ}\text{C}$. Sections were dried in air for 10 min and fixed in cold acetone for 10 min.

Prolyl hydroxylase was purified from newborn rat skins according to the method of Rhoads and Udenfriend.¹¹ The highly purified enzyme was subjected to 7% polyacrylamide gel electrophoresis.¹¹ The enzyme band was visualized with 1-anilino-8-naphthalene sulfonate¹³ and cut from the gels. Slices of gels containing electrophoretically homogenous enzyme were homogenized in 0.5M cacodylate buffer, pH 7.0, with 10^{-5}M EDTA and 10^{-4}M dithiothreitol. The homogenate was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into the shaved back of a rabbit. The injection was repeated 3 times at 3 week intervals. The rabbit received approximately 1.2 mg of the enzyme and was bled 2 weeks after the last injection. The monospecificity of antibody obtained with this procedure was confirmed by double immunodiffusion and immuno-

electrophoresis.³

Immunoperoxidase staining included the use of monospecific rabbit antiserum to prolyl hydroxylase and goat anti-rabbit IgG (IgG fraction) conjugated with horseradish peroxidase (Miles Lab). Fixed sections were briefly immersed in phosphate-buffered saline (0.02M sodium phosphate, pH 7.5, containing 0.16M NaCl) and incubated for 30 min at 37°C in a constant humidity chamber with rabbit antiserum diluted to 1/20 with the phosphate-buffered saline. Sections were then washed by immersion and agitation in 3 changes of the phosphate-buffered saline. The washed sections were incubated again for 30 min at 37°C with horseradish peroxidase-labeled goat anti-rabbit IgG diluted to 1/50. The sections were washed as before and peroxidase was localized by the use of 3-, 3'-diaminobenzidine as substrate.¹⁴ Control sections consisted of adjacent sections as above, except for the substitution of control rabbit serum obtained before the immunization or antiserum absorbed with purified prolyl hydroxylase. Some of the frozen sections were fixed in 10% formalin solution and stained with hematoxylin and eosin for histological examinations. Student's t-test for group comparison was used for statistical analysis of the data.

RESULTS

As shown in Table I, the blood pressure of spontaneously hypertensive rats (SHR) was elevated over 200 mmHg either at the age of 16 or 32 weeks. Severe hypertension was induced within 5 weeks by treatment with deoxycorti-

TABLE I BLOOD PRESSURE (mm Hg) OF SPONTANEOUSLY HYPERTENSIVE RATS (SHR), DEOXYCORTICOSTERONE ACETATE (DOCA)-SALT HYPERTENSIVE RATS AND NORMOTENSIVE CONTROLS

<i>Weeks of Age</i>	16	32
SHR	208 ± 10 (10)*	214 ± 12 (9)*
Control	117 ± 4 (4)	122 ± 5 (7)
<i>Weeks of Treatment</i>	5	
DOCA-salt	227 ± 13 (6)*	
Control	123 ± 5 (5)	

Each value represents the mean $\pm$ S.D.

(): Number of rats examined.

* : Statistically significant compared to control, $p < 0.01$

Fig.1. Localization of prolyl hydroxylase in aortas of 16-week-old spontaneously hypertensive rats (SHR) (a) and normotensive rats (b). c, control section from a hypertensive rat reacted with preimmune serum. Note the more intense immunostaining in the SHR (a) as compared to normotensive rat (b). No specific staining is seen in control sections (c). Horizontal bar=20 μ

Fig.2. Localization of prolyl hydroxylase in mesenteric arteries of 32-week-old SHR (a) and normotensive rats (b). c, control section from a normotensive rat reacted with preimmune serum. Note the marked immunostaining is seen in medial smooth muscle cells of the SHR (a). No specific staining is detected in the control section (c). Horizontal bar = 20 μ

Fig.3. Localization of prolyl hydroxylase in brain blood vessels (circle of Willis; inset, small artery and arteriole) of 16-week-old SHR (a) and normotensive rats (b). A more intense immunoreaction is observed in the SHR (a) compared to the normotensive rat (b). c, control section for SHR; specific immunostaining is abolished when antiserum absorbed with purified prolyl hydroxylase is used. Horizontal bar = 20μ

Fig.4. Localization of prolyl hydroxylase in coronary arteries of 16-week-old SHR (a) and normotensive rats (b). c, control section from a hypertensive rat reacted with preimmune serum. Note the intense immunostaining in the SHR (a) as compared to the normotensive rat (b). No specific staining is seen in the control section (c). Horizontal bar = 20μ

Fig.5. Localization of prolyl hydroxylase in renal glomeruli of 32-week-old SHR (a) and normotensive rats (b). c, control section from a hypertensive rat reacted with preimmune serum. Sclerotic glomerulus of SHR (a) shows increased immunostaining compared to that of the normotensive rat (b). No specific reaction is seen in control section (c). Horizontal bar = 20μ

Fig.6. Localization of prolyl hydroxylase in the mesenteric artery with periarteritis nodosa in a deoxycorticosterone acetate (DOCA)-salt hypertensive rat. Proliferating smooth muscle cells show the marked immunostaining of prolyl hydroxylase. Horizontal bar = 20μ

Fig.7. Localization of prolyl hydroxylase in the testicular artery of a 32-week-old SHR. Medial smooth muscle cells show enhanced immunostaining. Horizontal bar = 20μ

corticosterone acetate (DOCA)-salt. Blood pressure in control animals for both models of hypertensive rats remained at normal levels.

Cardiac hypertrophy and increased thickness of the vascular wall (aorta and mesenteric artery) were observed macroscopically in all hypertensive animals. Microscopically, myocardial fibrosis and severe arteriosclerotic changes with occasional fibrinoid necrosis were noted in 32-week-old SHR, however, such changes were more frequent in DOCA-salt hypertensive rats. On the other hand, in the 12-week-old SHR, a mild arteriosclerosis, representing the early stages of vascular damages induced by hypertension, was

evident. Histological findings in SHR were the same as reported previously.¹⁶

Immunohistochemical findings: Non-specific staining was not detected throughout this experiment in control sections reacted with either pre-immune rabbit serum or antiserum absorbed with purified prolyl hydroxylase. As shown in Figs. 1–4, increased immunostaining of prolyl hydroxylase was evident in blood vessels of hypertensive rats in which caliber sizes varied

Fig. 8. Localization of prolyl hydroxylase in the renal artery of a 32-week-old SHR. The vascular lumen is completely occluded and proliferating adventitial fibroblasts show positive staining. Horizontal bar = 20 μ

(aorta, mesenteric artery, circle of Willis and coronary artery). It is of great interest that this increase was also detected in the small artery and arterioles of brain, heart, kidney and testis. The immunostaining in the small artery and arterioles of the brain is demonstrated in Fig. 3. In normotensive rats, the staining was less intense. In addition to these blood vessels, glomerular capillaries of hypertensive rats showed a more intense staining than those of normotensive rats (Fig. 5). The elevated immunoreaction for vascular prolyl hydroxylase was observed in 73% (8/11) of 16-week-old SHR, 89% (8/9) of 32-week-old SHR and 100% (6/6) of DOCA-salt hypertensive rats. Intensity of the immunostaining was much greater in DOCA-salt hypertensive rats and 32-week-old SHR than in 16-week-old SHR.

Although endothelial cells and adventitial fibroblasts yielded positive staining, medial smooth muscle cells showed the most prominent immunoreaction in the blood vessels of hypertensive rats. Blood vessels with severe arteriosclerosis found in some of 32-week-old SHR and DOCA-salt hypertensive rats demonstrated marked immunostaining (Figs. 6–9). Smooth muscle cells showed the most prominent immunoreaction in the vascular wall. Proliferating fibroblasts at the site of myocardial fibrosis

Fig. 9. Localization of prolyl hydroxylase in the heart with coronary arteriosclerosis and myocardial fibrosis in a DOCA-salt hypertensive rat. Note the intense immunostaining in the sclerotic coronary artery. Fibroblasts proliferating in the fibrotic area show positive staining, while there was no reaction in myocardial cells. Horizontal bar = 20 μ

yielded marked immunostaining, while no specific staining was noted in myocardial cells (Fig. 9). In addition, adventitial fibroblasts which proliferated around sclerotic blood vessels showed the positive staining (Fig. 9).

DISCUSSION

The monospecific antibody to prolyl hydroxylase used in this experiment cross-reacts with both active and inactive forms of the enzyme.^{16,17} The inactive form may be a precursor of the active enzyme.¹⁸ Using experimental atherosclerotic rabbits and hypertensive rats, it was demonstrated that an increase in vascular prolyl hydroxylase activity paralleled a net increase in prolyl hydroxylase related antigen.^{1,19} Therefore, the immunohistochemical localization of prolyl hydroxylase was assumed to be a sensitive indicator for cellular fibrogenetic activity in cardiovascular tissues. Endothelial cells, medial smooth cells and adventitial fibroblasts were suggested to play a role in vascular collagen formation.³ Since the medial smooth muscle cells showed the most prominent immunostaining in the vascular wall of hypertensive rats, they may be the major collagen-producing cells responsible for the increased deposition of vascular collagen. This result supports previous findings by other workers that medial smooth muscle cells in culture actually produce connective tissue components including collagen^{20,21} and may play a principal role in the initiation and progression of vascular diseases, particularly atherosclerosis.²² In blood vessels with severe arteriosclerotic changes such as periarteritis nodosa of the mesenteric artery, the proliferating adventitial fibroblasts also showed an intense immunoreaction, suggesting they are actively synthesizing vascular collagen. Glomerular capillaries of hypertensive rats yielded an enhanced immunoreaction for prolyl hydroxylase. This suggests that endothelial cells and probably mesangial cells of the glomerulus may produce collagen and may induce sclerotic changes in the glomerulus of hypertensive rats.¹⁵ It is of great interest that small arteries and arterioles showed an elevated immunostaining in various tissues of hypertensive rats. The increased collagen deposition in small arteries and arterioles may, to some extent, account for the structural hypertrophy of the resistance vessels in hypertension.²³ The immunohistochemical findings in small blood vessels of the brain of hypertensive rats correlate well with the biochemical data published pre-

viously.² In the fibrotic area of the heart probably induced by local ischemia with coronary arteriosclerosis, it was noted that locally proliferating fibroblasts were responsible for collagen deposition, while myocardial cells have no fibrogenetic activity. Since the formation of collagen is an important step in cardiovascular pathology, the immunohistochemistry developed here may provide a greater insight into the pathogenesis of arteriosclerosis and myocardial fibrosis, and can be utilized in early detection of cardiovascular lesions.

Acknowledgement

The author wishes to express his gratitude to Drs. Sidney Udenfriend and George Cardinale (Roche Institute of Molecular Biology, Nutley, N.J. 07110, U.S.A.) for helpful advice and encouragement. Thanks are due to M. Ohara for assistance with the manuscript.

REFERENCES

1. OOSHIMA, A., FULLER, G. C., CARDINALE, G. J., SPECTOR, S., & UDENFRIEND, S.: Increased collagen synthesis in blood vessels of hypertensive rats and its reversal by antihypertensive agents. *Proc. Natl. Acad. Sci. U.S.A.* 71: 3019-3023, 1974.
2. OOSHIMA, A., FULLER, G. C., CARDINALE, G. J., SPECTOR, S., UDENFRIEND, S.: Collagen biosynthesis in blood vessels of brain and other tissues of the hypertensive rat. *Science* 190: 898-900, 1975.
3. OOSHIMA, A.: Immunohistochemical localization of prolyl hydroxylase in rat tissues. *J. Histochem. Cytochem.* 25: 1297-1302, 1977.
4. CARDINALE, G. J., & UDENFRIEND, S.: Prolyl hydroxylase. *Adv. Enzymol.* 41: 245-299, 1974.
5. TAKEUCHI, T., KIVIRIKO, K. I., & PROCKOP, D. J.: Increased procollagen proline hydroxylase activity in the liver of rats with hepatic fibrosis. *Biochem. Biophys. Res. Commun.* 28: 940-944, 1967.
6. MUSSINI, E., HUTTON, J. J. Jr., & UDENFRIEND, S.: Collagen proline hydroxylase in wound healing, granuloma formation, scurvy, and growth. *Science* 157: 927-929, 1967.
7. FULLER, G. C., & LANGNER, R. O.: Elevation of aortic proline hydroxylase: a biochemical defect in experimental arteriosclerosis. *Science* 168: 987-988, 1970.
8. OKAMOTO, K., & AOKI, K.: Development of a strain of spontaneously hypertensive rats. *Jap. Circ. J.* 27: 282-293, 1963.
9. DE CHAMPLAIN, J., KRAKOFF, L. R., & AXELROD, J.: Relation between sodium intake and norepinephrine storage during the development of experimental hypertension. *Circ. Res.* 23: 497-491, 1968.
10. WILLIAMS, J. R., HARRISON, T. R., & GROLLMAN, A.: A simple method for determining the systolic pressure of the unanesthetized rat. *J. Clin. Invest.* 18: 373-376, 1939.

11. RHOADS, R. E., & UDENFRIEND, S.: Purification and properties of collagen proline hydroxylase from newborn rat skin. *Arch. Biochem. Biophys.* 139: 329–339, 1970.
12. DAVIS, B. J.: Disc electrophoresis – II, Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121: 404–427, 1964.
13. HARTMAN, B. K., & UDENFRIEND, S.: A method for immediate visualization of proteins in acrylamide gels and its use for preparation of antibodies to enzymes. *Anal. Biochem.* 30: 391–394, 1969.
14. NAKANE, P. K. & PIERCE, G. B. Jr.: Enzyme-labeled antibodies: Preparation and application for the localization of antigens. *J. Histochem. Cytochem.* 14: 929–931, 1967.
15. OOSHIMA, A., YAMORI, Y., & OKAMOTO, K.: Cardiovascular lesions in the selectively bred group of spontaneously hypertensive rats with severe hypertension. *Jap. Circ. J.* 36: 797–812, 1972.
16. McGEE, J. O'D., LANGNESS, U., & UDENFRIEND, S.: Immunological evidence for an inactive precursor of collagen proline hydroxylase in cultured fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* 68: 1585–1589, 1971.
17. STASSEN, F. L. H., CARDINALE, G. J. McGEE, J. O'D., & UDENFRIEND, S.: Prolyl hydroxylase and an immunologically related protein in mammalian tissues. *Arch. Biochem. Biophys.* 160: 340–345, 1974.
18. CHICHESTER, C. O. III, Fuller, G. C., & CARDINALE, G. J.: In vitro labeling and turnover of prolyl hydroxylase and a related immunoreactive protein. *Biochem. Biophys. Res. Commun.* 73: 1056–1062, 1976.
19. FULLER, G. C., MATONEY, A. L., FISHER, D. O., FAUSTO, N., & CARDINALE, G. J.: Increased collagen synthesis and the kinetic characteristics of prolyl hydroxylase in tissues of rabbits with experimental atherosclerosis. *Atherosclerosis.* 24: 483–490, 1976.
20. ROSS, R., & KLEBANOFF, S. J.: The smooth muscle cell I. In vitro synthesis of connective tissue proteins. *J. Cell Biol.* 50: 159–171, 1971.
21. ROSS, R.: The smooth muscle cell II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell Biol.* 50: 172–186, 1971.
22. ROSS, R., & GLOMSET, J. A.: The pathogenesis of arteriosclerosis. *New Engl. J. Med.* 295: 369–377 & 420–425, 1976.
23. FOLKOW, B., HALLBÄCK, M., LUNDGREN, Y., SIVERTSSON, R., & WEISS, L.: Importance of adaptive changes in vascular design for establishment of primary hypertension, studied in man and in spontaneously hypertensive rats. *Circ. Res. Suppl. 1* 32, 33: 2–16, 1973.