3. P. Wong, J. C. K. Lai, L. Lim and A. N. Davison, *J. inorg. Biochem.* **14**, 253 (1981).
4. J. C. K. Lai, T. K. C. Leung, J. T. Guest, L. Lim and A. N. Davison, *Biochem. Pharmac,* **29**, 2763 (1980).
5. W. J. Nicklas, S. Puszkin and S. Berl, *J. Neurochem.* **20**, 109 (1973).
6. E. K. Silbergeld, *Life Sci.* **20**, 309 (1977).
7. R. W. Colburn and J. W. Maas, *Nature, Lond.* **208**, 37 (1965).
8. J. C. K. Lai, L. Lim and A. N. Davison, *Biochem. Soc. Trans.* **8**, 68 (1980).
9. J. C. K. Lai, L. Lim and A. N. Davison, *Biochem. Soc. Trans.* **8**, 67 (1980).

Biochemical Pharmacology, Vol. 30, No. 22, pp. 3125–3126, 1981.
Printed in Great Britain.

0006-2952/81/223125-02 $02.00/0

Hydralazine as an inhibitor of lysyl oxidase activity

(*Received* 26 *January* 1981; *accepted* 11 *May* 1981)

Many of the unique properties of connective tissue, such as its high tensile strength and the low solubility of its constitutive structural proteins, are due to the oxidative deamination of the ε-amino group of specific lysine and hydroxylysine residues in collagen or elastin by lysyl oxidase to form the aldehyde precursors of the cross-links in these important structural proteins (Ref. 1 and references therein).

Kucharz and Dróżdż [2] found an increase of soluble collagen and a decrease of insoluble collagen in the skins of guinea pigs with a collagen-disease-like syndrome produced by long-term hydralazine administration, and they suggested the possibility of inhibition of lysyl oxidase with hydralazine.

In this paper we demonstrate the *in vitro* inhibition of lysyl oxidase activity by hydralazine.

Materials and methods

Hydralazine hydrochloride was purchased from the Tokyo Kasei Kogyo Co. Ltd., Tokyo. [4,5-^{3}H]Lysine (60–80 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA, and pyridoxal-phosphate from the Sigma Chemical Co., St. Louis, MO. Other chemicals were reagent grade.

Lysyl oxidase was extracted from 16-day-old chick embryo aortas with 4 M urea and 0.16 M NaCl in 0.1 M potassium phosphate buffer, pH 7.4 [3]. After removing the urea by dialysis against 0.05 M potassium phosphate buffer, pH 7.4, overnight at 4°, the enzyme solution was subjected to NaCl fractionation (0–20%) to precipitate and remove the collagen co-extracted with urea into the enzyme solution. The resultant supernatant fraction was dialyzed against the same buffer and used as the enzyme solution for lysyl oxidase assay. Poor linearity of the enzyme dose curve with crude enzyme preparations was greatly improved by removing the collagen (unpublished data). The specific activity of the enzyme preparation was 997 cpm·(mg protein)$^{-1}$·hr^{-1}.

[4,5-^{3}H]Lysine-labeled collagen substrate was prepared from 16-day-old chick embryo calvaria parietal bones by essentially the same method as has been reported [4].

Lysyl oxidase activity was assayed by measuring the tritium released from ^{3}H-labeled collagen substrate following the conversion of specific lysyl residues in collagen into the corresponding δ-semialdehyde, allysine [4]. The incubation mixture contained: 0.3 ml of tritiated collagen substrate (600,000 cpm), 0.4 ml of 1 M NaCl in 0.1 M $KHPO_4$ buffer (pH 7.4), 0.3 ml of enzyme preparation, and water to 2.0 ml. The tritiated substrate was preincubated at 37° for 60 min to reconstitute the fibrils. After a 3-hr incubation at 37° in a shaking water bath, the reaction was terminated by the addition of 0.2 ml of 50% trichloroacetic acid. Tritiated water formed during the incubation was collected by vacuum distillation and 1.5 ml of the distilled water was counted with 10 ml of Bray's solution.

Results and discussion

Figure 1 shows the inhibition of lysyl oxidase activity by hydralazine at different concentrations. Fifty percent inhibition was attained at a concentration of about 30 μM. A similar degree (at about 20 μM) of inhibition was reported on partially purified prolyl hydroxylase from embryonic chick cartilage [5].

To test the reversibility of the inhibition of lysyl oxidase activity, the enzyme was incubated for 2 hr at 0° or 37° in the presence of 5 mM hydralazine and then dialyzed intensively against 0.16 M NaCl in 0.1 M potassium phosphate buffer, pH 7.4. The inhibition of lysyl oxidase activity by hydralazine was, however, essentially irreversible. Furthermore, the labeled collagen substrate was incubated

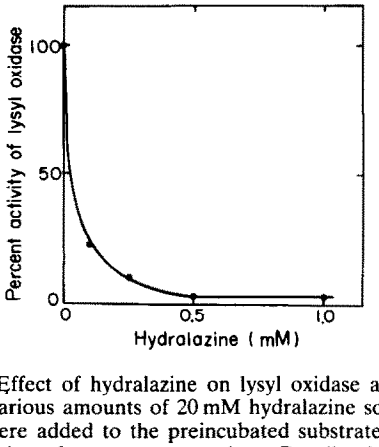

Fig. 1. Effect of hydralazine on lysyl oxidase activity *in vitro*. Various amounts of 20 mM hydralazine solution in water were added to the preincubated substrate prior to the addition of enzyme preparations. Details of the assay procedure are described under Materials and Methods.

with hydralazine and dialyzed against the buffer under the same conditions mentioned above. Only about 8 percent inhibition of lysyl oxidase activity was observed with the hydralazine-treated substrate. These results suggest that irreversible hydralazine action is mainly directed at the enzyme.

Prolyl hydroxylase activity was restored maximally (about 80 percent) by the addition of Fe^{2+} to the incubation mixtures containing hydralazine because of its chelating reaction with Fe^{2+} [5]. The hydralazine-induced inhibition of dopamine β-hydroxylase activity was almost reversed by the addition of Cu^{2+} which is essential for the inactivation of the endogenous inhibitors present in the enzyme preparations [6]. The inhibitory effect of hydralazine on the collagen secretion was also reversed by Fe^{2+} alone or Fe^{2+} together with Mn^{2+} [7].

However, hydralazine-induced inhibition of lysyl oxidase activity could not be reversed even by the intensive dialysis of inhibited enzyme preparation against 0.05 M potassium phosphate buffer containing 0.1 mM $CuCl_2$.

It is now almost established that a pyridoxine derivative [8, 9], probably pyridoxal-phosphate, is another essential cofactor of lysyl oxidase, in addition to copper ion [10–12]. Since lysyl oxidase is inhibited by some carbonyl reagents [11], hydralazine might react with pyridoxal cofactor of lysyl oxidase. We failed, however, to restore the activity of inhibited enzyme by dialyzing intensively against the phosphate buffer containing 0.05 mM pyridoxal-phosphate.

Since hydralazine combined with proteins as hydralazones [13] and with sulfhydryl compound [14], hydralazine might combine directly with the protein moiety of lysyl oxidase. As for the sulfhydryl group, it has been demonstrated that chick cartilage lysyl oxidase contains a sufficient number (30/1000 residues) of half cysteines which could contribute to the stability of the enzyme molecules [15].

Further investigations on the mechanism of hydralazine inhibition are under way along these lines in our laboratory.

In summary, lysyl oxidase activity was inhibited by hydralazine *in vitro* (50 percent inhibition at about 30 μM). Hydralazine seems to bind to the enzyme fairly tightly, because the inhibition was not reversed by intensive dialysis against phosphate buffer. The activity could not be restored even by the dialysis of inhibited enzyme against copper or pyridoxal-phosphate solution.

Department of Biochemistry
School of Dentistry
Aichi-Gakuin University
Nagoya 464, Japan

YUKIKO NUMATA
TATSUO TAKEI
TARO HAYAKAWA*

REFERENCES

1. M. L. Tanzer, in *Biochemistry of Collagen* (Eds. G. N. Ramachandran and A. H. Reddi), p. 137. Plenum Press, NewYork (1976).
2. E. Kucharz and M. Dróżdż, *Explle. Path.* **15**, 63 (1978).
3. T. Hayakawa, M. Hino, H. Fuyamada, T. Nagatsu, H. Aoyama and Y. Izawa, *Clinica chim. Acta* **71**, 245 (1976).
4. R. C. Siegel, *Proc. natn. Acad. Sci. U.S.A.* **71**, 4826 (1974).
5. R. S. Bhatnagar, S. S. R. Rapaka, T. Z. Liu and S. M. Wolfe, *Biochim. biophys. Acta* **271**, 125 (1972).
6. T. Z. Liu, J-T. Shen and H. F. Loken, *Proc. Soc. exp. Biol. Med.* **145**, 294 (1974).
7. R. S. Rapaka, R. W. Parr, T. Z. Liu and R. S. Bhatnagar, *Teratology* **15**, 185 (1977).
8. J. C. Murray and C. I. Levene, *Biochem. J.* **167**, 463 (1977).
9. J. C. Murray, D. R. Fraser and C. I. Levene, *Expl. molec. Path.* **28**, 301 (1978).
10. R. C. Siegel, S. R. Pinnell and G. R. Martin, *Biochemistry* **9**, 4486 (1970).
11. E. D. Harris, W. A. Gonnerman, J. E. Savage and B. L. O'Dell, *Biochim. biophys. Acta* **341**, 332 (1974).
12. J. K. Rayton and E. D. Harris, *J. biol. Chem.* **254**, 621 (1979).
13. M. A. Paz and S. Seifter, *Am. J. med. Sci.* **263**, 281 (1972).
14. H. M. Perry, Jr., H. A. Schroeder, G. S. Goldstein and E. M. Menhard, *Am. J. med. Sci.* **228**, 396 (1954).
15. F. L. H. Stassen, *Biochim. biophys. Acta* **438**, 49 (1976).

* Address all correspondence to: Dr. Taro Hayakawa, Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, 2-11 Suemori-dori, Chikusa-ku, Nagoya 464, Japan.

Biochemical Pharmacology, Vol. 30, No. 22, pp. 3126–3128, 1981.
Printed in Great Britain.

0006-2952/81/223126-03 $02.00/0

Cyclic AMP response to norepinephrine in the limbic forebrain of male and female rats: effect of desipramine

(*Received* 26 *January* 1981; *accepted* 18 *May* 1981)

Cytoplasmic receptors exist in certain brain areas for steroid hormones [1] which could act directly or indirectly as endocrine regulators of neuronal activity. Through the combined use of autoradiography and fluorescence histochemistry, Heritage *et al.* [2] have identified estradiol target sites in nuclei of the major norepinephrine (NE) containing cell bodies in the brain stem, thus providing a neuroanatomical basis for physiological interactions between sex steroid hormones and NE in brain. Moreover, chronic exposure of ovariectomized rats to 17α-ethynylestradiol has been reported to decrease beta adrenergic cyclic AMP responses in the cortex linked to a reduction in the density of beta adrenergic membrane receptors [3]. Since beta adrenergic receptors in brain represent a subpopulation of noradrenergic receptors coupled to adenylate cyclase [4, 5] and since most if not all antidepressant treatments down-regulate the NE receptor coupled adenylate cyclase system in brain [6], it was of interest to determine whether