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EFFECT OF PROPRANOLOL ON PROLYL HYDROXYLASE ACTIVITY IN BLOOD VESSELS OF RATS

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Abstract—The effect of propranolol on prolyl hydroxylase activity in blood vessels of rats was investigated *in vitro* and *in vivo*. Propranolol at a concentration of 6.7×10^{-6} M inhibited 50 per cent of the activity of prolyl hydroxylase in the aorta *in vitro*. The inhibition was recovered when excessive amounts of ferrous iron and ascorbate were added to the reaction mixture. Propranolol showed a competitive type of inhibition with respect to varying concentrations of substrate. Hypertensive rats were made by treatment with desoxycorticosterone acetate and 1% sodium chloride (DOCA–salt). When propranolol was given daily to hypertensive rats for 4 weeks simultaneously with DOCA–salt, no effect on blood pressure was detected, but the activity of prolyl hydroxylase in the aorta and mesenteric artery was significantly inhibited. Prolyl hydroxylase activity in the blood vessels in normotensive rats treated with propranolol was also inhibited. These results suggest that propranolol inhibits prolyl hydroxylase activity *in vitro* and *in vivo* at the site of action where an oxygen intermediate is formed from the interaction of ferrous iron and ascorbate.

Prolyl hydroxylase (EC 1.14.11.2; proline, 2-oxoglutarate dioxygenase) catalyses the synthesis of hydroxyproline in collagen by the hydroxylation of certain prolyl residues in peptide linkages [1]. The enzyme requires 2-oxoglutarate and molecular oxygen as cosubstrates, ferrous iron as a cofactor and ascorbate as a reducing agent [1, 2]. In previous reports it has been shown that hypertension accompanied an increase in prolyl hydroxylase activity in blood vessels [3, 4] and the increased activity can be prevented by antihypertensive drugs, reserpine and guanethidine [5, 6]. The inhibition of prolyl hydroxylase activity leads to the synthesis of an unhydroxylased form of collagen which is more susceptible to tissue protease degradation [7]. Therefore, drugs which inhibit prolyl hydroxylase might have a clinical application in the treatment of fibrotic disease processes or hypertension. Recently, it has been reported that epinephrine [8] and catechin [9] inhibited prolyl hydroxylase activity. In this study, we have found that propranolol, which is used in the treatment of hypertension in man [10, 11], inhibits prolyl hydroxylase activity using the enzyme of the homogenate of blood vessels of rats *in vitro* and *in vivo*.

MATERIALS AND METHODS

In vitro *experiments*. Male Wistar rats (seven weeks old) were decapitated. The aorta was excised and homogenized in 30 vol. 0.25 M sucrose containing 10 mM Tris–HCl buffer (pH 7.4), 100 mM dithiothreitol and 10 μM EDTA. The homogenate was centrifuged at 15,000 *g* for 30 min. Prolyl hydroxylase activity was measured in a supernatant by the tritium release assay of Hutton *et al.* [2]. The control assay mixture consisted of 0.1 ml of supernatant enzyme (0.1 mg protein), 5×10^{-2} M Tris–HCl buffer (pH 7.4), 10^{-4} M ferrous ammonium sulfate, 10^{-3} M sodium ascorbate, 10^{-4} M 2-oxoglutarate, 2 mg bovine serum albumin, 0.4 mg catalase and 10^6 dpm of [4-^{3}H]proline-labeled collagen substrate in a final volume of 1 ml. The assay mixture was incubated for 30 min at 30°. The reaction was stopped by addition of trichloroacetic acid. The tritiated water of the reaction system was separated by vacuum distillation of the reaction mixture. The radioactivity was measured by a Packard 3330 scintillation counter. A minimum volume for the prolyl hydroxylase assay was 5 μg of enzyme protein. All determinations were done in triplicate. Protein concentration was determined by the method of Lowry *et al.* [12], with bovine serum albumin as the standard. Propranolol hydrochloride was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

In vivo *experiments*. Male Wistar rats (seven weeks old) were subjected to uninephrectomy under anesthesia. One week later, these rats were made hypertensive by s.c. twice-weekly injection of 5 mg per rat of desoxycorticosterone acetate (DOCA, Tokyo Kasei Kogyo Co., Tokyo, Japan) suspended in corn oil [13]. The sham-operated rats were used as controls. Rats were maintained on a standard diet and allowed unlimited drinking water containing 1% sodium chloride. Propranolol (50 mg/kg) was administered i.p. once daily to some of the rats for 4 weeks simultaneously with or without DOCA–salt. The systolic blood pressure was measured by a tail-cuff method without anesthesia once a week (Narco Biosystem Inc.). The body weight of rats was measured at the same time of the determination of the blood pressure. Rats were decapitated 4 weeks after the treatment. The aorta and mesenteric arteries were excised and prolyl hydroxylase activity was measured by the same method as *in vitro* experiments.

The significance of the difference between values for the control and treated groups was determined with Student's *t*-test.

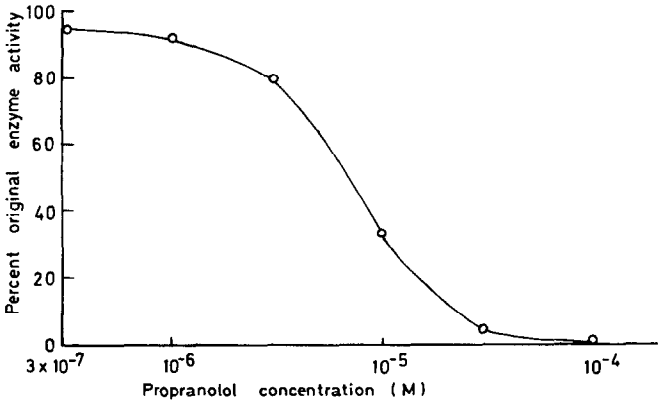

Fig. 1. Inhibition of prolyl hydroxylase activity by varying concentrations of propranolol.

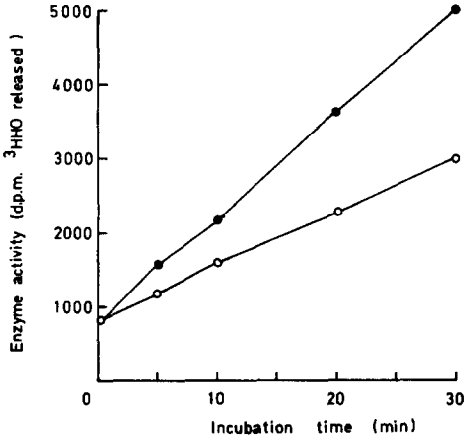

Fig. 2. Time-course of inhibition of prolyl hydroxylase by propranolol. The enzyme was incubated in the presence (○) or in the absence (●) of 6×10^{-6} M propranolol.

RESULTS

In vitro *experiments*. As shown in Figure 1, the activity of prolyl hydroxylase was reduced in the presence of propranolol in the concentration range of 3×10^{-6} to 10^{-4} M. The enzyme activity was inhibited 50 per cent at a concentration of about 6.7×10^{-6} M of propranolol. Maximum inhibition of the enzyme activity was obtained at a propranolol concentration of 10^{-4} M. The time-course of inhibition of the prolyl hydroxylase activity by propranolol showed that the inhibition occurred immediately after the start of the incubation, and approx. 40 per cent inhibition of the enzyme activity was consistently observed throughout the incubation period (Fig. 2). In order to investigate the mechanism of the inhibitory action of propranolol, experiments were performed in which excessive ferrous iron and ascorbate were added to the reaction mixture prior to the addition of propranolol (Fig. 3). The addition of varying concentrations of ferrous iron recovered partially the inhibition of prolyl hydroxylase by propranolol. A maximum of 76 per cent of the original activity was restored with 6×10^{-4} M ferrous iron. Ascorbate at the concentrations of 10-fold excess of control assay mixture was unable to restore the original activity of the enzyme. However, excessive amounts of both ferrous iron and ascorbate resulted in the recovery of 88 per cent of the original activity as shown in Fig. 4. Double-reciprocal plots of the inhibition of prolyl hydroxylase are shown in Fig. 5.

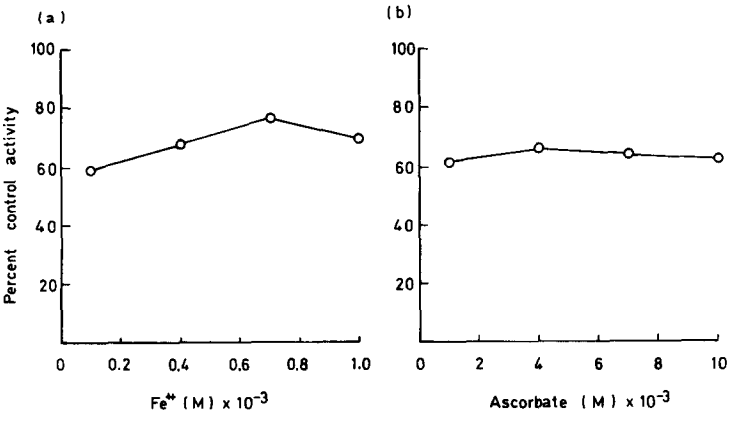

Fig. 3. Effects of excessive concentrations of Fe^{2+} (a) or ascorbate (b) on the inhibition of prolyl hydroxylase by 6×10^{-6} M propranolol. The percentage of the control activity was calculated with respect to the control assay mixtures, containing the same amount of cofactor and no inhibitor.

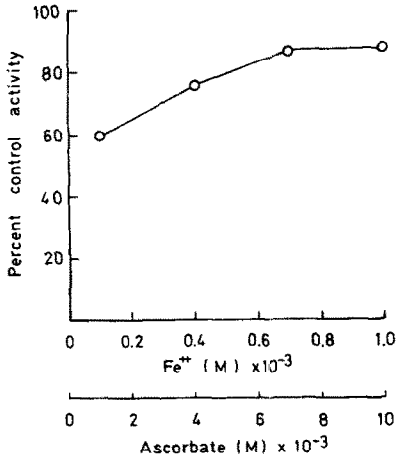

Fig. 4. Effects of both excessive concentrations of Fe^{2+} and ascorbate on the inhibition of prolyl hydroxylase by 6×10^{-6} M propranolol. The percentage of the control activity was calculated with respect to the control assay mixtures, containing the same amounts of both cofactors and no inhibitor.

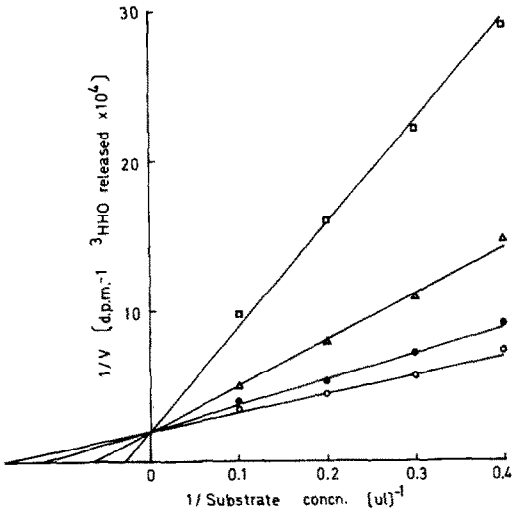

Fig. 5. Competitive inhibition of propyl hydroxylase by propranolol. The prolyl hydroxylase activity was determined under the control assay conditions in the presence of 3×10^{-6} M (●), 6×10^{-6} M (△), 10^{-5} M (□) propranolol and in its absence (○).

Table 1. Final body weight and blood pressure*

Group	Body weight (g)	Blood pressure (mg Hg)
Control	228 ± 8	119 ± 7
Control + propranolol	225 ± 6	115 ± 8
DOCA–salt	210 ± 7	196 ± 5†
DOCA–salt + propranolol	208 ± 6	190 ± 7†

* Propranolol (50 mg/kg) was administered i.p. once daily for 4 weeks. Each value is the mean ± S.E. of 5 rats per group.

† Statistically different from control, $P < 0.01$ (Student's *t*-test).

Propranolol showed a competitive type of inhibition with respect to varying concentrations of substrate.

In vivo *experiments*. The blood pressure of the rats treated with DOCA–salt for 4 weeks was increased much more than the control. Propranolol lowered the blood pressure of the hypertensive or normotensive rats but the results were not statistically significant. No significant difference in body weight was seen among 4 groups (Table 1). As shown in Table 2, prolyl hydroxylase activity in the aorta and mesenteric artery was decreased by the treatment with propranolol in normotensive and hypertensive rats. The inhibition of prolyl hydroxylase activity by propranolol in the aorta in DOCA–salt hypertensive rats was observed more significantly than that in normotensives, being 74 per cent activity

Table 2. Effect of propranolol on prolyl hydroxylase activity in the aorta and mesenteric artery*

Group	Prolyl hydroxylase activity (dpm/mg of protein)	
	Aorta	Mesenteric artery
Control	13,290 ± 922	18,165 ± 1311
Control + propranolol	10,554 ± 730	14,060 ± 1025†
DOCA–salt	22,613 ± 1422‡	29,572 ± 1651‡
DOCA–salt + propranolol	16,772 ± 1211†§	20,902 ± 1018‖

* Propranolol (50 mg/kg) was administered i.p. once daily for 4 weeks. Each value is the mean ± S.E. of 5 rats per group.

† Statistically different from control, $P < 0.05$ and ‡$P < 0.01$.

§ Statistically different from DOCA–salt hypertension, $P < 0.05$ and ‖$P < 0.01$ (Student's *t*-test).

of DOCA–salt hypertension. Almost the same results were also obtained from the mesenteric artery.

DISCUSSION

The present experiments demonstrated that propranolol inhibited the activity of prolyl hydroxylase in the aorta of the rat *in vitro*. The inhibition was observed immediately after the addition of propranolol. Futhermore, preincubation of enzyme and propranolol prior to the addition of substrate did not increase the inhibition, which suggests that propranolol is not a tight binding inhibitor of prolyl hydroxylase. Increases in ferrous iron partially reversed the inhibition of prolyl hydroxylase activity by propranolol. On the other hand, the effect of epinephrine which has been reported to inhibit prolyl hydroxylase by an iron chelating mechanism was inverted completely when a ferrous concentration was increased [8]. Ascorbate did not modify the decreasing activity of the enzyme. This finding indicates that propranolol does not inhibit prolyl hydroxylase by preventing the oxidation of ascorbate. Bhatnagar and Liu [14] suggested that ferrous iron, ascorbate and oxygen interact at a reducing site in the enzyme. If an oxygen intermediate is involved in the hydroxylation process, propranolol might inhibit the reaction by removing it. Addition of excessive amounts of both ferrous iron and ascorbate caused the recovery of 88 per cent of the original activity of prolyl hydroxylase. Furthermore, propranolol showed a competitive type of inhibition with respect to substrate. Therefore, it seems likely that propranolol exerts its action at the site where an oxygen intermediate is formed from the interaction of ferrous iron and ascorbate, as described by Bhatnagar and Liu [14]. Similar results have been reported that nitro-blue tetrazolium [8, 15] and catechin [9] caused a competitive type of inhibition of prolyl hydroxylase activity by the interaction of ferrous iron, ascorbate and molecular oxygen.

The antihypertensive effect of propranolol on hypertension in man has been well documented [10, 11]. In experimental hypertension in rats, however, the effects of propranolol on the blood pressure have shown conflicting results. There are reports of either a reduction [16, 17] or no effect [18, 19] on the blood pressure of DOCA–salt hypertensive rats. In this study, I used 5 rats in a group according to previous reports which described the effect of drugs on the blood pressure [4–6], although it seems that the number of observations was small. Thus, it was observed that high doses of propranolol did not significantly lower the blood pressure. Similar results were obtained by Dusting and Rand [16], who reported that low doses of propranolol decreased the blood pressure but high doses of propranolol failed to lower the blood pressure. Without affecting the blood pressure, propranolol decreased prolyl hydroxylase activity in blood vessels of rats *in vivo*. Increased prolyl hydroxylase activity in hypertensive rats was more susceptible to propranolol than that in normotensives. Moreover, the inhibition which was observed to be more potent in mesenteric artery than in aorta may be due to the route of the administration of propranolol.

We have previously reported that the vascular prolyl hydroxylase activity is increased in hypertensive rats [3, 4]. Increased activity is decreased by reserpine or guanethidine, concomitant with the decrease in the blood pressure [5, 6]. The effect of propranolol on prolyl hydroxylase activity, however, did not associate with the change in the blood pressure. Mylecharane and Raper [20] showed that propranolol had a typical but weak guanethidine-like effect in the isolated tissue. But reserpine or guanethidine did not inhibit prolyl hydroxylase activity *in vitro* [5, 6]. From those, it is concluded that the mode of action of propranolol inhibiting prolyl hydroxylase activity is a different mechanism to that of reserpine or guanethidine which is related to the lowering of blood pressure but acts at the site of action where oxygen interacts with cofactors, as shown by *in vitro* experiments.

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