

## Differences in Adrenergic Nerve and Receptor Function in Dog Internal Thoracic, Coronary and Mesenteric Arteries

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**ABSTRACT**—Isolated dog internal thoracic arteries (ITA) responded to norepinephrine and phenylephrine with concentration-related contractions, which were suppressed by prazosin, but not by yohimbine. Clonidine did not contract ITA. In coronary arterial strips, norepinephrine produced a relaxation. Isoproterenol relaxed coronary arterial strips contracted with serotonin but did not alter the tone of ITA. Forskolin and beraprost, an analog of prostaglandin I<sub>2</sub>, relaxed coronary and ITA strips to a similar extent. The  $\beta$ -adrenoceptor density, assayed by [<sup>3</sup>H]dihydroalprenolol binding, was markedly less in ITA than in coronary arteries. Nicotine and transmural electrical stimulation did not alter the tension of ITA. Immunohistochemical study indicated that nerve fibers containing tyrosine hydroxylase immunoreactivity were markedly less in ITA than in coronary and mesenteric arteries. These results indicate that  $\beta$ -adrenoceptor function and adrenergic innervation are considerably reduced in dog ITA. Norepinephrine-induced vasoconstriction appears to be mediated by  $\alpha_1$ -adrenoceptors in the arteries.

**Keywords:** Internal thoracic artery, Adrenergic function, Immunohistochemistry,  $\beta$ -Adrenoceptor binding

The internal thoracic artery (ITA) is at present the vessel of choice for most surgical revascularization procedures of the heart. ITA grafts display better long term patency than saphenous vein grafts (1–3). However, perioperative infarction due to a restriction of blood flow caused by vasospasm of the ITA has been reported (4–6). The incidence of cardiac death is significantly greater in patients with ITA grafts than in those with saphenous vein grafts. Knowledge about receptor function in these blood vessels may provide a clue for analyzing the mechanism of vasospasm and a rationale for the prophylaxis and reversal of post operative vasospasm.

Catecholamines play an important role in the control of vascular tone. The vasorelaxant response to epinephrine was found to be mediated by  $\beta_2$ -adrenoceptors in human ITA (7). Weinstein et al. (8) and He et al. (9) suggested from functional studies on human ITA strips that adrenergic vasoconstriction is primarily mediated through  $\alpha_1$ -adrenoceptors, and Bevilacqua et al. (10) reported by radioligand assays that ITA possesses  $\alpha_1$ -adrenoceptors that are involved in the vasoconstrictor response.

The present study was performed to characterize adrenergic functions in dog ITA by comparing them with

those in coronary or mesenteric arteries by measurement of the mechanical arterial response, radioligand binding assay of adrenoceptors and immunohistochemical demonstration of adrenergic innervation.

### MATERIALS AND METHODS

The studies review board at our university approved the use of dog arteries for this study. Mongrel dogs of either sex weighing 6 to 15 kg were anesthetized with intravenous injections of sodium pentobarbital (30 mg/kg) and sacrificed by bleeding from the common carotid arteries. The sternum, mesentery and heart were rapidly removed, and then internal thoracic (ITA: 1.5–2.5 mm outside diameter (OD)), mesenteric (0.5–1.5 mm OD) and coronary arteries (1.0–2.5 mm OD) were carefully isolated within 30 min after death. The arteries were immediately used for tension experiments, stored in the prefixed solution for the immunohistochemical study and the remainder was kept frozen at  $-80^{\circ}\text{C}$  for the binding assay.

#### Tension recording

The arteries were cut helically into strips approximately 20 mm long. The endothelium of the strips was not intentionally removed. The specimens were fixed vertically be-

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tween hooks in a muscle bath containing the modified Ringer-Locke solution kept at  $37 \pm 0.3^\circ\text{C}$  and aerated with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The hook anchoring the upper end of the strip was connected to the lever of a force-displacement transducer (Nihon Kohden Kogyo Co., Tokyo). The resting tension was adjusted to 1.5 g, which is optimal in inducing the maximum contraction. Constituents of the solution were as follows: 120 mM NaCl, 5.4 mM KCl, 2.2 mM  $\text{CaCl}_2$ , 1.0 mM  $\text{MgCl}_2$ , 25.0 mM  $\text{NaHCO}_3$  and 5.6 mM dextrose. The pH of the solution was 7.35 to 7.42. Before starting the experiments, all of the preparations were allowed to equilibrate for 60 to 90 min in the bathing media, during which time the solutions were replaced every 10 to 15 min.

The arterial strips were placed between a pair of stimulating electrodes made of platinum plate, approximately 2 mm apart from each other. The gaps between the electrodes and strip were wide enough to allow undisturbed vascular contractions or relaxations and yet sufficiently narrow to permit effective stimulation of intramural nerve terminals. The preparations were stimulated transmurally by a train of 0.2-msec square pulses of supramaximum intensity applied at frequencies of 5 and 20 Hz for periods of 40 and 10 sec, respectively. Electrical pulses were delivered from an electronic stimulator (Nihon Kohden Kogyo Co.).

Isometric contractions and relaxations were displayed on an ink-writing oscillograph. The contractile response to 30 mM  $\text{K}^+$  was first obtained, and the preparations were washed 3 times with control media and equilibrated for 40–50 min. Responses to agonist were obtained under resting conditions or in arterial strips partially precontracted with prostaglandin (PG)  $\text{F}_{2\alpha}$  ( $[4-9] \times 10^{-7}$  M) or serotonin (5-HT,  $[1-6] \times 10^{-7}$  M), the contraction being in a range between 20% and 30% of the contraction induced by 30 mM  $\text{K}^+$ . Contractions induced by agonists were presented as relative values to those induced by 30 mM  $\text{K}^+$ . Relaxations were presented as relative values to those induced by  $10^{-4}$  M papaverine. Concentration-response curves for isoproterenol, forskolin, beraprost, norepinephrine, phenylephrine and clonidine were obtained by adding the drug directly to the bathing media in cumulative concentrations. In assessing the effects of blocking agents, the responses to the agonist were examined repeatedly until stabilization, and then the artery strips were treated with the blocking agents for approximately 30 min before the response to the agonist was obtained. The dissociation constant ( $K_B$ ) of prazosin was calculated from the equation:  $K_B = [\text{B}]/(\text{dose ratio} - 1)$ , where  $[\text{B}]$  is the concentration of the antagonist. The dose ratio is the ratio of the median effective concentrations of norepinephrine or phenylephrine in the presence and absence of the antagonist.

### Receptor binding assay

On thawing of the ITA, mesenteric and coronary arteries which were stored at  $-80^\circ\text{C}$ , the vessels (approximately 12 vessels for one assay) were placed in an ice-cold buffer (50 mM Tris-HCl, pH 7.4), carefully trimmed of fatty adventitial tissues, coarsely minced with scissors, and then homogenized 5 times using a Brinkmann Polytron tissue disruptor (Westburg, NY, USA) for 20 sec at setting 7. The resulting tissue homogenate was filtered through two layers of gauze. The filtrate was then centrifuged at  $800 \times g$  for 10 min at  $4^\circ\text{C}$ . The supernatant was recentrifuged at  $12,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The supernatant was centrifuged again at  $100,000 \times g$  for 60 min at  $4^\circ\text{C}$ . The supernatant was discarded, and the remaining membrane pellet was resuspended in Tris-HCl buffer and used for the  $\beta$ -adrenoceptor binding assay. Protein concentration was determined by the method of Bradford with human serum albumin as the standard.

Binding assays were generally performed in duplicate by incubating 100  $\mu\text{l}$  (approximately 100  $\mu\text{g}$  protein) of membrane suspension with 100  $\mu\text{l}$  of [ $^3\text{H}$ ]dihydroalprenolol (DHA, specific radioactivity 3.85 TBq/mmol; NEN Research Products, Boston, MA, USA) and 100  $\mu\text{l}$  of various drugs in a final volume of 300  $\mu\text{l}$  in polypropylene test tubes. Assays were initiated with the addition of membrane suspension and were carried out in the buffer containing 50 mM Tris-HCl (pH 7.4). Membranes were incubated for 30 min at  $37^\circ\text{C}$  with different concentrations of [ $^3\text{H}$ ]DHA in a shaking water bath (120 cycles/min). The bound and free radioligands were then separated by rapid (less than 5 sec) filtration over GF/B glass fiber filters (Whitman, Clifton, NJ, USA) on a filtration manifold (Millipore, Bedford, MA, USA). The filters were washed with 15 ml of 50 mM Tris-HCl at room temperature. The radioactivity retained on the filters was counted in a gamma counter at 70% efficiency. Nonspecific binding was defined as the amount of [ $^3\text{H}$ ]DHA binding measured in the presence of  $10^{-5}$  M ( $\pm$ )propranolol. This concentration was chosen because it occupied essentially all of the binding sites for [ $^3\text{H}$ ]DHA under these conditions but was low enough not to cause more general membrane perturbations. Subtraction of the nonspecific binding from the total binding yielded the specific binding. Specific binding of the radioligand was linear with the amount of protein applied.

### Immunohistochemistry of tyrosine hydroxylase

The internal thoracic, coronary and mesenteric arteries were rapidly removed and fixed in ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.4) containing 0.3% glutaraldehyde and 4% paraformaldehyde for 10 min; Then they were postfixed overnight in 0.1 M PBS with 4% paraformaldehyde followed by cryoprotection in 15%

sucrose. Free-floating thick sections were then cut by a blade. These sections (20- $\mu$ m-thick) were cut on a cryostat ( $-18^{\circ}\text{C}$ ) (Cryotom, Nakagawa Seisakusyo Co., Tokyo) and kept with 0.1 M PBS, containing 0.3% Triton X-100 at  $4^{\circ}\text{C}$  for 4 days. The specimens were exposed to purified rabbit antiserum against bovine adrenal tyrosine hydroxylase (1 : 6000; Eugene Tech. Int. Inc., Ramsey, NJ, USA) in PBS with 0.3% Triton X-100 for 4 days at  $4^{\circ}\text{C}$ . Subsequently, biotinylated goat antirabbit immunoglobulin G antibody and avidin-biotinylated peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA) were conjugated to the primary antibody at room temperature for 1 hr each. Immunolabeled peroxidase was visualized by incubation at room temperature for 3 to 5 min with 0.56 mM 3,3-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto), 1.3  $\mu$ M hydrogen peroxide and 10 mM nickel ammonium sulfate. The specimens were mounted onto gelatin/chrome-alum-coated glass slides. After several washes with distilled water, the sections were air-dried and cover-slipped with Entellan (Merck, Darmstadt, Germany). An immunohistochemical control experiment, in which the antiserum against tyrosine hydroxylase was excluded from the reaction mixture, gave no positive staining.

#### Statistics and drugs used

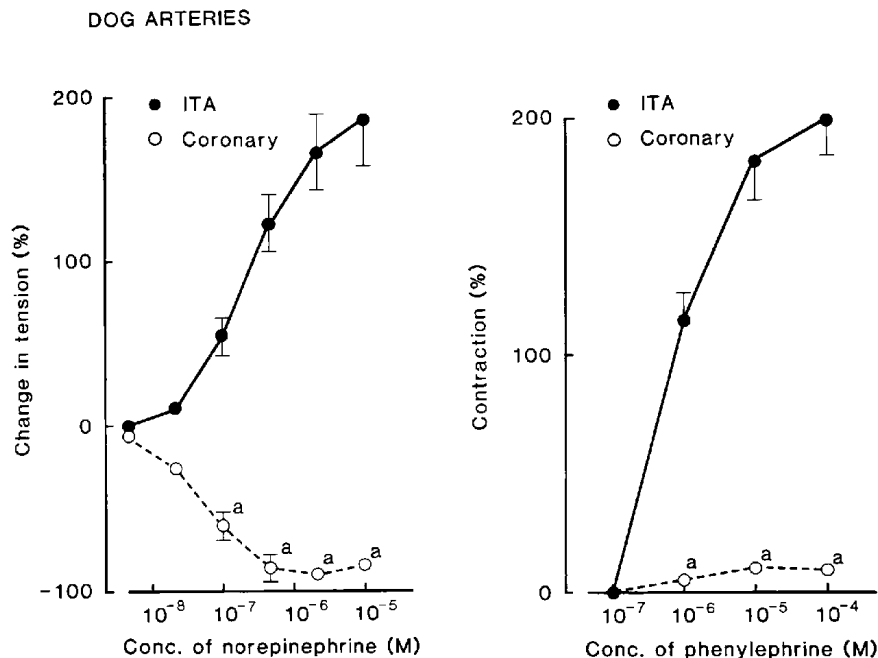
The results shown in the text and figures are expressed

as mean values  $\pm$  S.E.M. Statistical analyses were made by Student's *t*-test. Drugs used were *dl*-norepinephrine hydrochloride, cocaine hydrochloride (Sankyo Co., Tokyo); phenylephrine hydrochloride, *dl*-isoproterenol hydrochloride, forskolin (Sigma Chemical Co., St. Louis, MO, USA); clonidine hydrochloride (C.H. Boehringer Ingelheim Ltd., Elmsford, NY, USA); prazosin hydrochloride (Pfizer-Taito Co., Tokyo); yohimbine hydrochloride, nicotine (Nacalai Tesque, Kyoto); phen-tolamine mesylate, *dl*-propranolol hydrochloride (Sumitomo Pharmaceutical Co., Osaka); beraprost sodium (Kaken, Tokyo); serotonin creatinine sulfate (5-HT), hexamethonium bromide (Merck); prostaglandin  $\text{F}_{2\alpha}$  (Ono Pharmaceutical, Osaka); and papaverine hydrochloride (Dainippon Pharmaceutical Co., Osaka).

#### RESULTS

##### *Mechanical responses to norepinephrine, phenylephrine and clonidine*

The addition of norepinephrine in concentrations ranging from  $5 \times 10^{-9}$  to  $10^{-5}$  M produced a concentration-related contraction of ITA strips (Fig. 1, left). Further increasing the concentration to  $5 \times 10^{-5}$  M produced very few or no additional contractions. The median effective concentration ( $\text{EC}_{50}$ ) was  $(4.98 \pm 1.02) \times 10^{-7}$  M ( $n=6$ ). The contractions were significantly suppressed by treat-



**Fig. 1.** Dose-response curves for norepinephrine (left panel) and phenylephrine (right) in canine internal thoracic (ITA) and coronary arteries ( $n=6$ ). Responses to norepinephrine in coronary arteries were obtained in the strips precontracted with 5-HT. Contractions and relaxations are presented as relative values to those induced by 30 mM  $\text{K}^+$  and  $10^{-4}$  M papaverine, respectively. Significantly different from the values obtained from ITA: <sup>a</sup>,  $P < 0.01$ . Vertical bars represent the S.E.M.

ment with prazosin in a dose-dependent manner (Fig. 2 left). However, yohimbine in concentrations up to  $10^{-7}$  M did not significantly inhibit the norepinephrine-induced contractions (Fig. 2 right). The amine-induced relaxations were attenuated by treatment with  $10^{-6}$  M yohimbine. The  $K_B$  values at  $10^{-8}$  M and  $10^{-7}$  M prazosin were  $(1.97 \pm 0.27) \times 10^{-9}$  M ( $n=8$ ) and  $(1.29 \pm 0.12) \times 10^{-9}$  M ( $n=8$ ), respectively. In mesenteric arterial strips, norepinephrine produced contraction. The maximal contraction and  $EC_{50}$  were  $201.3 \pm 24.1\%$  and  $(1.83 \pm 0.83) \times 10^{-6}$  M ( $n=6$ ), respectively. On the other hand, norepinephrine did not produce contractions in coronary arterial strips under resting conditions. When the strips were partially contracted with  $PGF_{2\alpha}$ , norepinephrine produced relaxations in a concentration-related manner (Fig. 1, left). The norepinephrine-induced relaxations were abolished or reversed to a contraction by  $10^{-6}$  M propranolol.

The addition of phenylephrine in concentrations ranging from  $10^{-7}$  to  $10^{-4}$  M contracted the ITA strips (Fig. 1, right). The magnitude of the maximal contraction induced by  $10^{-4}$  M phenylephrine was almost the same as that induced by  $10^{-4}$  M norepinephrine ( $208 \pm 25\%$  vs  $211 \pm 19\%$ ;  $n=6$ ). The contractile response was significantly inhibited by prazosin ( $10^{-8}$  and  $10^{-7}$  M), but it was not influenced by yohimbine ( $10^{-8}$  and  $10^{-7}$  M). The  $K_B$  values of  $10^{-8}$  M and  $10^{-7}$  M prazosin were  $(7.40 \pm 3.12) \times 10^{-10}$  M and  $(8.11 \pm 2.69) \times 10^{-10}$  M ( $n=6$ ), respectively. In coronary arteries, phenylephrine pro-

duced only slight contractions (Fig. 1, right); the magnitude was markedly less than that of ITA ( $6.5 \pm 1.5\%$  at  $10^{-5}$  M vs  $211.0 \pm 18.8\%$  at  $10^{-4}$  M,  $n=6$ ).

Clonidine in concentrations ranging from  $10^{-7}$  M to  $10^{-4}$  M produced no contraction in ITA strips.

#### Responses to isoproterenol, forskolin and beraprost

The addition of isoproterenol in concentrations ranging from  $10^{-9}$  to  $10^{-5}$  M produced a concentration-related relaxation in coronary arterial strips partially contracted with 5-HT (Fig. 3). The apparent  $EC_{50}$  values of isoproterenol averaged  $(3.2 \pm 0.5) \times 10^{-8}$  M ( $n=7$ ). The isoproterenol-induced relaxations were not influenced by treatment with  $10^{-5}$  M phentolamine (Fig. 3). On the other hand, isoproterenol produced no relaxations in ITA strips partially contracted with 5-HT. Slight contractions were elicited at  $10^{-6}$  M or higher, which were abolished by treatment with  $10^{-6}$  M phentolamine (Fig. 3).

The addition of forskolin ( $10^{-9}$  to  $10^{-5}$  M) produced concentration-related relaxations in ITA and coronary arterial strips (Fig. 4, left); the magnitude of relaxations was significantly greater in coronary arteries than in ITA ( $P < 0.01$ ). The apparent  $EC_{50}$  values of forskolin in the ITA and coronary arteries were  $(1.93 \pm 0.47) \times 10^{-6}$  M ( $n=9$ ) and  $(6.45 \pm 0.98) \times 10^{-7}$  M ( $n=9$ ), respectively ( $P < 0.01$ ).

Beraprost, a stable analog of  $PGI_2$ , relaxed both arteries (Fig. 4, right). The relaxations in coronary arteries were significantly greater than those in ITA ( $P < 0.01$ ).

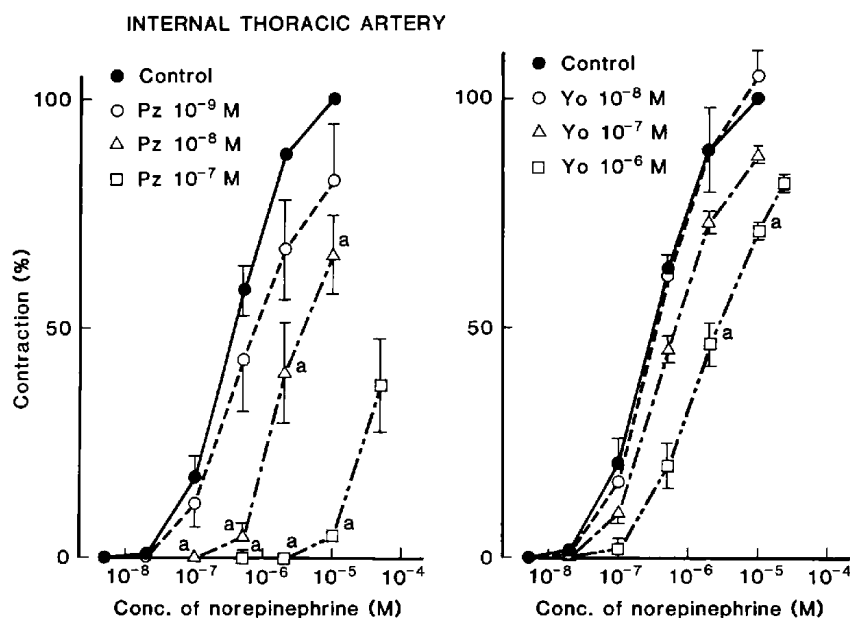


Fig. 2. Modification by prazosin (Pz,  $10^{-9}$ – $10^{-7}$  M) (left panel) and yohimbine (Yo,  $10^{-8}$ – $10^{-6}$  M) (right) of the response to norepinephrine of canine internal thoracic arteries ( $n=8$ ). Contractions induced by  $10^{-5}$  M norepinephrine in the control strips were taken as 100%. Significantly different from the control: <sup>a</sup>,  $P < 0.01$ . Vertical bars represent the S.E.M.

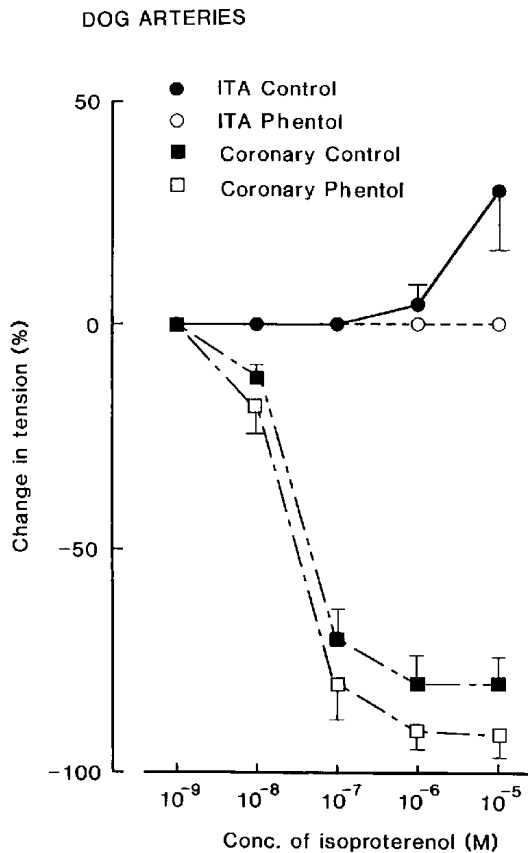


Fig. 3. Modification by phentolamine (Phentol  $10^{-6}$  M) of the response to isoproterenol of canine internal thoracic (ITA) and coronary arteries contracted with 5-HT ( $n=8$ ). Relaxations induced by  $10^{-4}$  M papaverine were taken as 100%. Vertical bars represent the S.E.M.

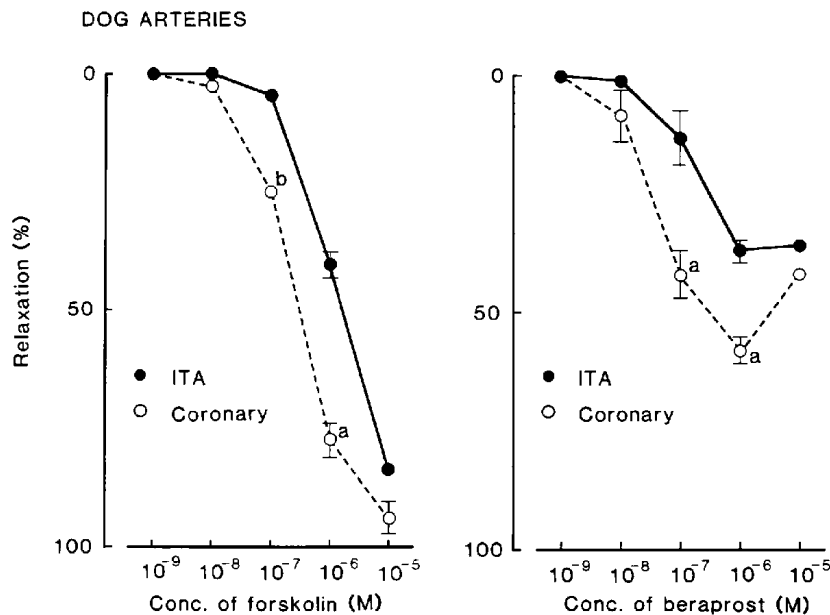


Fig. 4. Dose-response curves for forskolin (left panel,  $n=7$ ) and beraprost (right,  $n=8$ ) in canine internal thoracic (ITA) and coronary arteries contracted with 5-HT. Relaxations induced by  $10^{-4}$  M papaverine were taken as 100%. Significantly different from the values obtained from ITA: a,  $P<0.01$ ; b,  $P<0.05$ . Vertical bars represent the S.E.M.

The apparent  $EC_{50}$  values of beraprost in ITA and coronary arteries were  $(3.39 \pm 0.65) \times 10^{-7}$  M ( $n=6$ ) and  $(1.04 \pm 0.45) \times 10^{-7}$  M ( $n=6$ ), respectively ( $P<0.01$ ).

#### Receptor binding assay

The specific binding of [ $^3$ H]DHA was saturable with ligand concentration in ITA and coronary arterial strips. A typical saturation curve and Scatchard plot for ITA are illustrated in Fig. 5. In four experiments, the mean  $K_D$  values of ITA and coronary arteries were  $1.66 \pm 0.27$  and  $0.75 \pm 0.19$  nM, respectively. The maximal number of binding sites,  $B_{max}$ , of ITA and coronary arteries were  $7.1 \pm 1.8$  and  $137.9 \pm 18.2$  fmol/mg protein, respectively; the difference was statistically significant ( $P<0.01$ ).

#### Responses to nicotine and transmural electrical stimulation

Nicotine ( $10^{-4}$  M) elicited a transient relaxation in coronary arteries partially contracted with 5-HT ( $48.4 \pm 5.2\%$ ,  $n=5$ ) and a transient contraction in mesenteric arteries ( $67.2 \pm 10.8\%$ ,  $n=5$ ). The nicotine-induced relaxation and contraction were abolished by  $10^{-5}$  M hexamethonium. However, neither contractions nor relaxations were induced by nicotine in ITA contracted with 5-HT ( $n=8$ ).

The application of transmural stimulation at a frequency of 20 Hz elicited a transient relaxation in coronary arterial strips partially contracted with 5-HT ( $22.4 \pm 3.5\%$ ,  $n=4$ ), which was abolished or reversed to a contraction by treatment with  $10^{-6}$  M propranolol. In mesenteric ar-

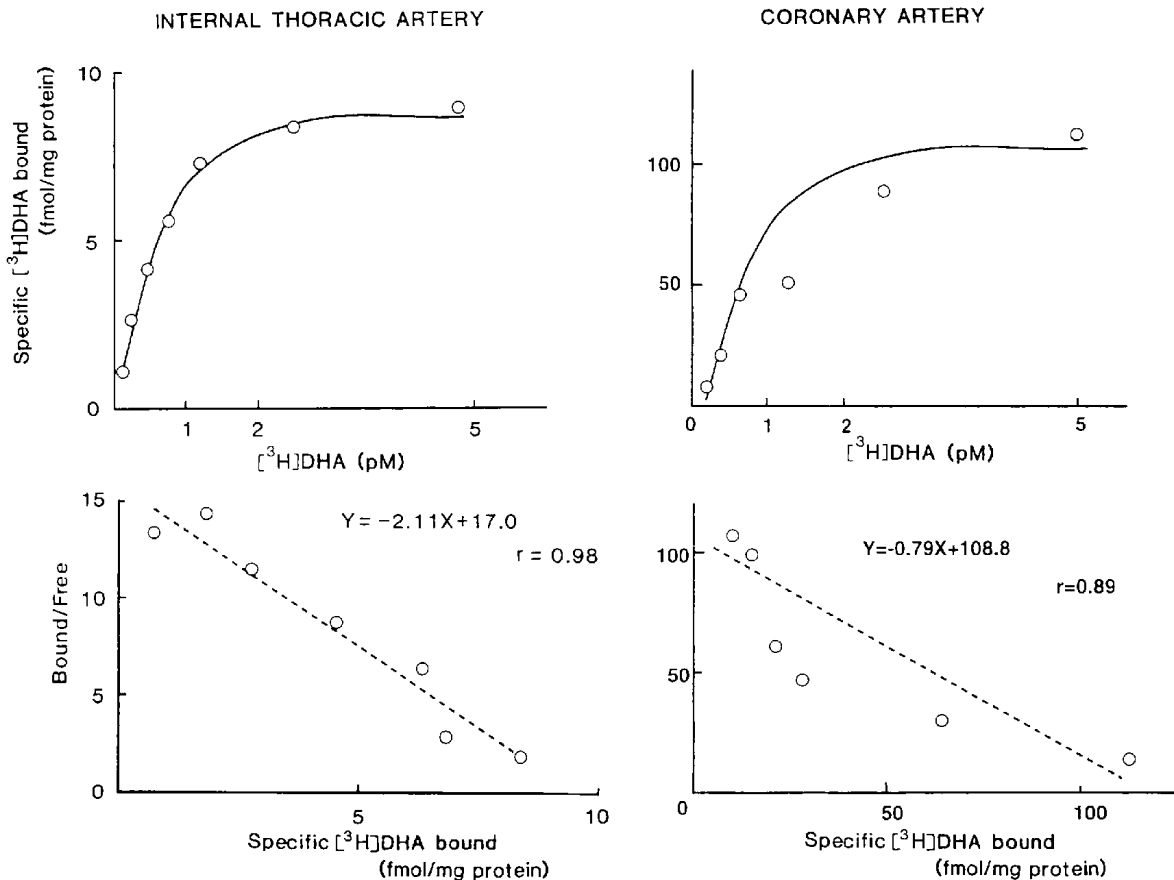


Fig. 5. Saturation curve (upper panel) and Scatchard plot (lower) for specific binding of [ $^3\text{H}$ ]dihydroalprenolol (DHA) to plasma membranes isolated from canine internal thoracic arteries (left) and coronary arteries (right).

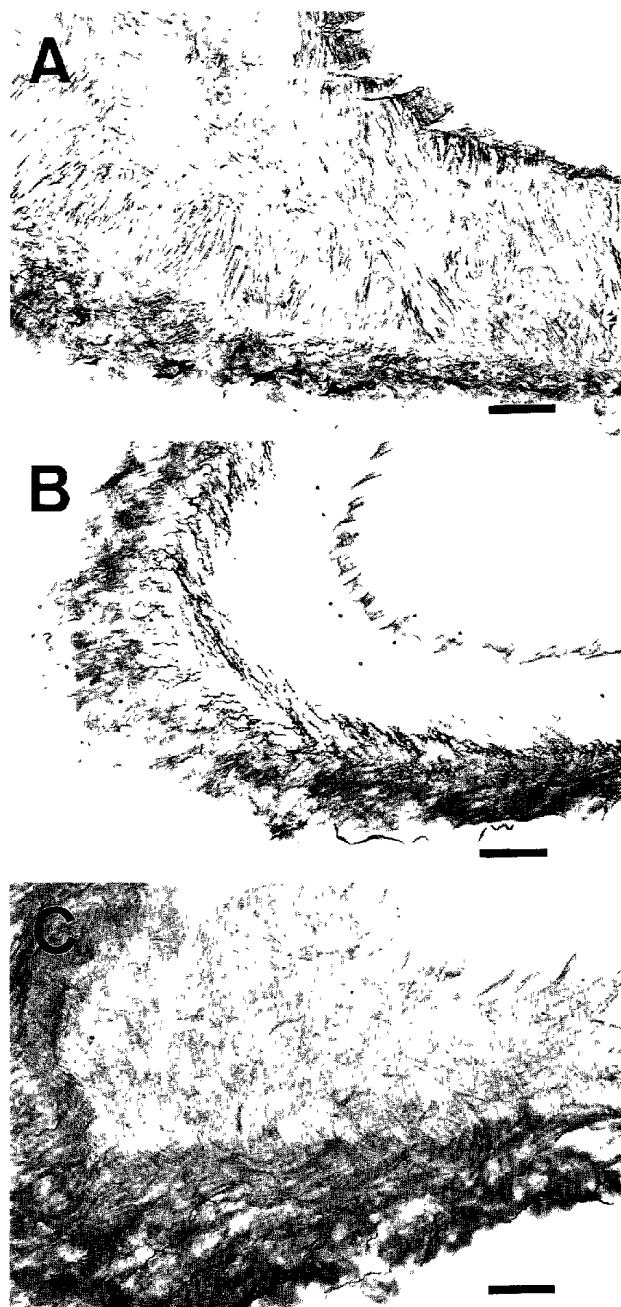
terial strips, the stimulation elicited a transient contraction ( $48.5 \pm 5.8\%$ ,  $n = 4$ ), which was markedly suppressed or abolished by  $10^{-6}$  M phentolamine. The application of transmural stimulation at frequencies of 5 and 20 Hz elicited neither relaxations nor contractions in all of seven ITA strips partially contracted with 5-HT.

#### *Immunohistochemistry of tyrosine hydroxylase in ITA, mesenteric and coronary arteries*

In the coronary arterial section (Fig. 6A), there were abundant, fine nerve fiber networks of adrenergic nerves and thick spindle fiber bundles. In the mesenteric arterial section (Fig. 6B), fine nerve fiber networks were evident.

dose-dependently by prazosin, an  $\alpha_1$ -adrenoceptor antagonist (11), but not by the  $\alpha_2$ -adrenoceptor antagonist yohimbine in concentrations upto  $10^{-7}$  M (12). Phenylephrine contracted ITA, whereas clonidine did not alter the arterial tone. On the other hand, coronary arterial strips responded to norepinephrine only with a relaxation, which was depressed by  $\beta_1$ -adrenoceptor antagonists (13). These findings suggest that norepinephrine contracts ITA by acting on  $\alpha_1$ -, but not  $\alpha_2$ -, adrenoceptors but relaxes coronary arteries due to predominant activation of  $\beta_1$ -adrenoceptors.

Isoproterenol relaxed coronary arterial strips contracted with 5-HT but did not alter the tone of ITA strips.



**Fig. 6.** Immunohistochemical demonstration of tyrosine hydroxylase in canine arteries. The adrenergic nerve fibers were stained evidently in canine coronary (A) and mesenteric (B) arteries, whereas they were sparsely stained in the internal thoracic (C) artery. Bars = 50  $\mu$ m.

magnitudes of relaxation in ITA and coronary arteries. Therefore, it appears that the population of  $\beta$ -adrenoceptors is markedly less in dog ITA than in coronary arteries; however, metabolic processes to generate cyclic AMP in smooth muscle cells similarly function in both arteries. The radioligand binding assay with [ $^3$ H]DHA supports the paucity of  $\beta$ -adrenoceptors in ITA. Relative un-

responsiveness to isoproterenol has been demonstrated in human (7, 19) and monkey ITA (unpublished data, S. Shiraishi et al.).

Nicotine and transmural electrical stimulation did not alter the tension of canine ITA but produced a relaxation in coronary arteries and a contraction in mesenteric arteries. Norepinephrine released from stimulated adrenergic nerves is involved in the relaxation and contraction, since the responses are depressed by  $\beta$ - and  $\alpha$ -adrenoceptor antagonists, respectively, and abolished by tetrodotoxin (for electrical stimulation) and hexamethonium (for nicotine) (20–22). Release of [ $^3$ H]norepinephrine during electrical stimulation of perivascular nerves was also demonstrated (23). Therefore, adrenergic nerves do not seem to function in dog ITA strips under the experimental conditions used, while the nerve function is well retained in coronary and mesenteric arteries. Immunohistochemical studies indicated that nerve fibers containing tyrosine hydroxylase were markedly less in the ITA wall than in the coronary and mesenteric arterial wall. Similar results were obtained by the use of anti-dopamine  $\beta$ -hydroxylase antibody in these arteries (unpublished data, S. Shiraishi et al.). Therefore, the relative unresponsiveness of ITA to adrenergic nerve stimulation appears to be due mainly to a paucity of adrenergic innervation.

In dog ITA, the  $\alpha_1$ -adrenoceptor subtype is involved in contractions caused by norepinephrine, and there are only few  $\beta$ -adrenoceptors responsible for relaxation. Despite the presence of postsynaptic  $\alpha_1$ -adrenoceptors, adrenergic neurogenic vasoconstriction is if any slight in dog ITA. If this is the case in humans, the vasospasm in ITA grafts for coronary artery bypass grafting when evoked by increased sympathetic efferent discharge would be caused by catecholamines in the plasma that are liberated from the adrenals but not by norepinephrine from the adrenergic nerves innervating the ITA. Furthermore, special care has to be taken for the infusion of norepinephrine to patients with an ITA graft during the perioperative period.

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