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Altered Platelet Alpha$_2$-Adrenoceptors in Patients With Angina Pectoris

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The alpha$_2$-adrenoceptor located on noradrenergic neurons regulates the release of norepinephrine by negative feedback. This receptor is also located on human blood platelets, and the number of these receptors is correlated with the plasma norepinephrine content. The purpose of this study was to determine the status of platelet alpha$_2$-adrenoceptors in patients with symptomatic and asymptomatic coronary artery disease. It was hypothesized that patients with symptomatic coronary disease might have a decrease in alpha$_2$-adrenoceptor number that might be related to increased neuronal norepinephrine release. Specific, high-affinity binding of the alpha$_2$-agonist, ^{3}H-clonidine, and the alpha$_2$-antagonist, ^{3}H-yohimbine, to isolated platelet membranes was used to determine the maximal number of binding sites (in fmol/mg protein ± standard error of the mean) and the dissociation constant (in n*M*) of the alpha$_2$-receptors.

In normal subjects, the number of binding sites for ^{3}H-clonidine was 32 ± 2 and the dissociation constant was 5.5 ± 0.6 (n = 26); the maximal number of binding sites for ^{3}H-yohimbine was 165 ± 12 and the dissociation constant was 4.0 ± 0.5 (n = 16). In patients with symptomatic coronary artery disease, there was a 38% decrease in ^{3}H-clonidine binding (number of binding sites = 20 ± 3; dissociation constant = 6.6 ± 1.2; n = 9; $p < 0.05$) and a 44% decrease in ^{3}H-yohimbine binding (number of binding sites = 93 ± 8; dissociation constant = 4.5 ± 0.3; n = 18; $p < 0.005$). In patients with asymptomatic coronary artery disease, there were no significant changes from the normal population in binding with either ligand (^{3}H-clonidine: number of binding sites = 30 ± 2; dissociation constant = 6.9 ± 1.9; n = 6 and ^{3}H-yohimbine: number of binding sites = 137 ± 11; dissociation constant = 5.1 ± 0.6; n = 10). Five patients were studied during a symptomatic phase of their disease and then restudied during a quiescent period. In this group, the number of alpha$_2$-adrenoceptors increased markedly as shown in ^{3}H-clonidine from 16 ± 3 to 29 ± 3 (44%, $p < 0.005$) and in ^{3}H-yohimbine from 69 ± 22 to 127 ± 17 (46%, $p < 0.05$).

The affinity constants and plasma norepinephrine concentrations did not differ among the three groups. If similar changes in receptor number occur on nerve terminals, this may represent a primary abnormality that permits enhanced release of norepinephrine or a secondary down regulation of receptor number in response to increased synaptic levels of norepinephrine.

Alpha-adrenergic stimulation has been shown to modulate coronary artery tone in conscious dogs (1,2), in canine myocardium (3) and in human subjects (4,5). The importance of the alpha-adrenergic system in this role has been shown after both nerve stimulation (6) and the infusion of norepinephrine (7). Postsynaptic alpha$_2$-adrenoceptors play an important role in coronary vasoconstriction elicited by both sympathetic nerve stimulation and infused norepinephrine (8) and in regulating the tone of both large and small coronary arteries (9). Coronary artery vasoconstriction may be induced by adrenergic stimulation, even in the presence of experimentally produced coronary artery stenosis (10) or adenosine-induced maximal coronary artery dilation (11).

The presynaptic alpha$_2$-adrenoceptor is an autoreceptor that exhibits a negative feedback inhibition on the neuronal release of norepinephrine (12,13). This has been suggested to be important in modulating coronary artery responses to sympathetic nerve stimulation (14). In isolated rat heart, a strong correlation has been found between the sensitivity of alpha$_2$-presynaptic receptor sites and the amount of norepinephrine released at the nerve terminal (15,16). These changes are accompanied by changes in alpha$_2$-adrenoceptor number

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on rat brain neural membranes (17) and on human platelets (18).

It was our hypothesis in the present study that patients with symptomatic coronary artery disease might have fewer of these presynaptic inhibitory receptors. This could lead to increases in norepinephrine release at the nerve terminal and, in turn, to increased vascular tone or spasm. The human blood platelet has been suggested as a model for the adrenergic neuron (19). Its membrane contains the same alpha$_2$-adrenoceptor found on presynaptic nerve terminals (20). This study was designed to characterize the platelet alpha$_2$-adrenoceptor using radioligand binding techniques in patients with both symptomatic and asymptomatic coronary artery disease and to relate any changes in receptor number or affinity to levels of circulating norepinephrine and degree of symptoms.

Methods

Study patients. Blood was obtained by venipuncture from 26 healthy male and female volunteers (mean age ± standard error of the mean 40 ± 3 years). These control subjects were compared with 28 patients with coronary artery disease as documented by exercise testing and cardiac catheterization. The patients were divided into two groups based on the presence or absence of anginal episodes during the previous 2 weeks. The 18 symptomatic patients in Group I had a mean age of 56 ± 3 years and the 10 asymptomatic patients in Group II had a mean age of 58 ± 4 years. The groups were evenly matched in terms of the anatomic extent of disease, with Group I having 15 patients with multivessel and 3 with single vessel disease and Group II having 8 patients with multivessel and 2 with single vessel involvement. Coronary vessel involvement was defined as diameter narrowing greater than 50%. There was no difference in the two groups between percent luminal stenoses. No patient in either group had a history of congestive heart failure or had a moderately or markedly reduced ejection fraction as determined by cineangiography.

Previous work (20,21) has shown the lack of age or sex dependency of either the total number or the affinity of the platelet alpha$_2$-adrenoceptor. There was no difference in the number of binding sites or the affinity in control subjects or patients over or under the age of 60. For at least 1 month before the study, all patients were free of drugs that might modify the alpha$_2$-adrenoceptor, such as clonidine, alpha methyldopa, tricyclic antidepressants or adrenergic inotropic agents. Two patients in Group I (Cases 38 and 42) and two patients in Group II (Cases 49 and 51) were on stable doses of propranolol. No patient in these groups was being treated with a calcium channel antagonist.

Of the five patients who were studied while symptomatic and then again when asymptomatic, two (Cases 49 and 50) underwent coronary artery bypass grafting before repeat testing. One patient (Case 47) was treated with nifedipine, a calcium channel antagonist, during this period. No patient in this group was receiving beta-adrenergic antagonists.

Informed consent was obtained from all patients. Approval of this study was obtained from the Institutional Review Board of the University of Michigan in May 1980.

Isolation of platelet membranes and radioligand binding assay. Platelet membranes were obtained using the method of Garcia-Sevilla et al. (20). In brief, 50 ml of blood was collected in acid-citrate dextrose (ACD) (8:1, v/v). The blood was centrifuged at 160 × *g* for 10 minutes (25°C), and the platelet-rich plasma was titrated to pH 6.5 with the ACD solution. This was then recentrifuged at 5,100 × *g* for 15 minutes (25°C) to obtain a platelet pellet. The pellet was washed twice with 5 ml of Tyrode's buffer [m*M:* sodium chloride (NaCl), 137; potassium chloride (KCl), 2.7; sodium phosphate, monobasic (NaH_2PO_4), 0.36; magnesium chloride ($MgCl_2$), 0.01; sodium bicarbonate ($NaHCO_3$), 12.0; dextrose, 0.56; pH, 8.0] and recentrifuged for 15 minutes at 5,100 × *g*. The pellet was lysed by homogenization in 2 ml of ice cold hypertonic buffer (Tris-EDTA, 5 m*M*, pH 7.5). The platelet membranes were obtained by centrifugation at 39,000 × *g* for 10 minutes and then resuspended in the Tris incubation buffer (m*M:* Tris-HCl, 50; $MgCl_2$, 10; pH, 7.5) used in the binding assay.

Total radioligand binding, using ^{3}H-clonidine, an alpha$_2$-adrenoceptor agonist, and ^{3}H-yohimbine, an alpha$_2$-adrenoceptor antagonist (New England Nuclear), was measured in 1 ml aliquots of the fresh platelet membranes (0.282 ± 0.22 mg protein), which were incubated in duplicate at 25°C for 20 minutes with the radioligand. Nonspecific binding was determined by adding unlabeled clonidine or yohimbine, 10^{-5} *M,* in addition to the respective tritiated ligand, to a second pair of incubates. Specific binding was defined as the difference between total and nonspecific binding. Incubations were terminated by adding 5 ml of the Tris incubation buffer to the sample. The membrane-bound tritiated ligand was recovered by rapid filtration of the diluted sample under vacuum through Whatman GF/C glass-fiber filters. The filters were washed twice with 10 ml of Tris incubation buffer, air-dried and counted for radioactivity as described by Smith et al. (22). Proteins were determined by the method of Lowry et al. (23).

Scatchard analysis of the saturation isotherms was used to determine the maximal number of binding sites (x-intercept of a plot of specifically bound ligand versus bound/free ligand) and the dissociation constant (negative reciprocal of the slope of the regression line) (24).

Catecholamine determinations. Catecholamine determinations were performed using the radioenzymatic assay of Passon and Peuler (25). The rat liver catechol-O-methyltransferase was isolated according to the method of Axelrod and Tomchik (26). All samples were obtained by

relatively atraumatic venipuncture with the patient recumbent for at least 30 minutes, a method that does not alter the catecholamine concentration (27). The blood was collected in ACD, and the serum was immediately frozen at −70°C.

Statistical analysis. Statistical analysis was performed using Student's *t* test. Correlation coefficients (r) were obtained by linear regression analysis using the method of least squares. Significance was defined as a probability (p) value less than 0.05.

Results

Binding data in normal control subjects. The specific binding of both ^{3}H-clonidine and ^{3}H-yohimbine to platelet membranes from both normal subjects and patients with coronary artery disease was both saturable and of high affinity (Fig. 1 and 2). The lack of correlation between age and sex and the number of binding sites or the affinity of the radioligand for the receptor site was confirmed as seen in previous studies (20,21). The individual data for the control population are shown in Table 1A for ^{3}H-clonidine (n = 26; maximal number of binding sites = 32 ± 2; dissociation constant = 5.5 ± 0.6) and in Table 1B for ^{3}H-yohimbine (n = 16; number of binding sites = 165 ± 12; dissociation constant = 4.0 ± 0.5).

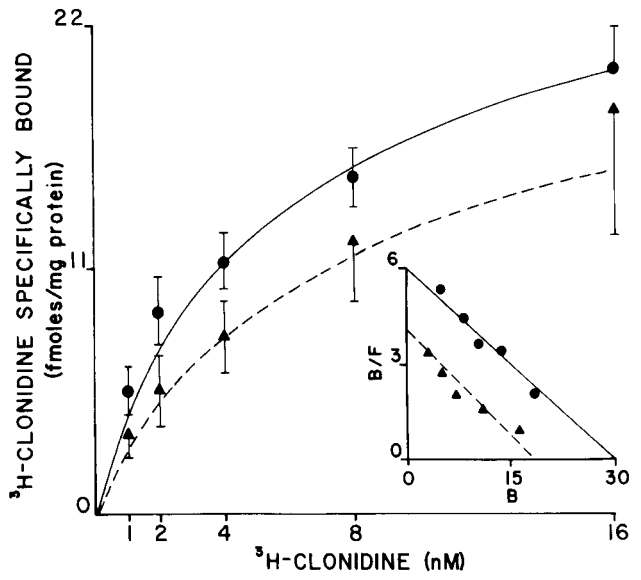

Figure 1. Specific binding of ^{3}H-clonidine to platelet membranes from normal subjects (**closed circles**) and from patients with symptomatic coronary artery disease (**closed triangles**) as a function of increasing concentrations (1 to 64 n*M*) of the ligand. **Ordinate:** ^{3}H-clonidine specifically bound (fmol/mg protein). **Abscissa:** concentration of ^{3}H-clonidine (n*M*). **Inset:** Scatchard plot showing the difference in the number of high-affinity binding sites (normal subjects: maximal number of binding sites = 32 ± 2; dissociation constant = 5.5 ± 0.6 versus patients with symptomatic disease: maximal number of binding sites = 20 ± 3.3; dissociation constant = 3.3; $p < 0.05$). Each point represents the mean ± standard error of the mean. B = specifically bound ligand; B/F = bound/free ligand.

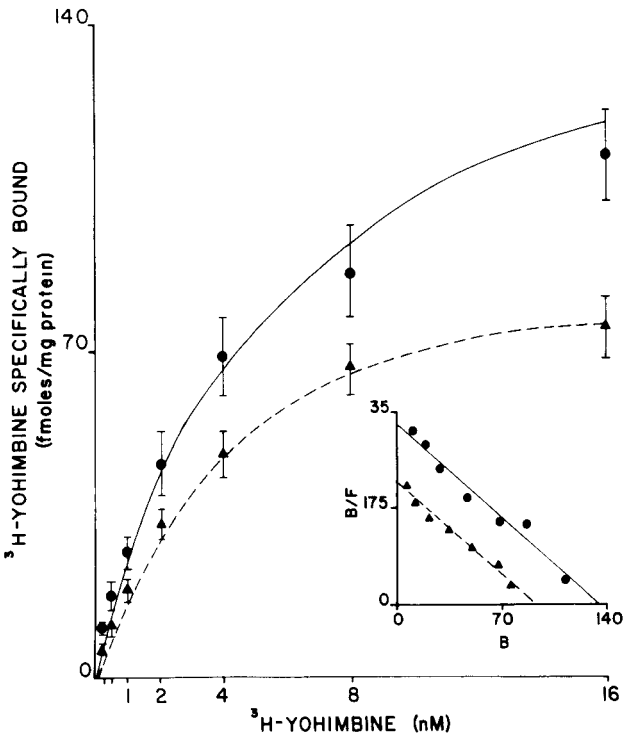

Figure 2. Specific binding of ^{3}H-yohimbine to platelet membranes from normal subjects (**closed circles**) and from patients with symptomatic coronary artery disease (**closed triangles**) as a function of increasing concentrations (0.25 to 16 n*M*) of the ligand. **Ordinate:** ^{3}H-yohimbine specifically bound (fmol/mg protein). **Abscissa:** concentration of ^{3}H-yohimbine (n*M*). **Inset:** Scatchard plot showing the difference in the number of high affinity binding sites (normal subjects: maximal number of binding sites = 165 ± 12; dissociation constant = 4.0 ± 0.5 versus patients with symptomatic disease: maximal number of binding sites = 93 ± 8; dissociation constant = 4.5 ± 0.3; $p < 0.005$). Each point represents the mean ± standard error of the mean. B = specifically bound ligand; B/F = bound/free ligand.

Binding data in patients with symptomatic and asymptomatic coronary disease. Patients with symptomatic coronary artery disease (Group I, n = 18, Table 2A) had binding data that showed highly significant decreases in maximal number of binding sites, as defined by both ^{3}H-clonidine binding (20 ± 6.6, 38%; $p < 0.05$; Fig. 1) and by ^{3}H-yohimbine binding (93 ± 8, 44%; $p < 0.005$; Fig. 2). The number of binding sites in patients with asymptomatic coronary artery disease (Group II, Table 2B) did not differ significantly from that in the control subjects (^{3}H-clonidine binding: 30 ± 2, n = 6 and ^{3}H-yohimbine binding: 137 ± 11, n = 10).

The dissociation constant of the ligands for the binding sites did not differ significantly between the control group and Groups I and II. For ^{3}H-clonidine, the dissociation constant was 6.6 ± 1.2 in Group I and 6.9 ± 1.9 in Group

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II. For ^{3}H-yohimbine, this constant was 4.5 ± 0.3 in Group I and 5.1 ± 0.6 in Group II.

Table 1. ^{3}H-Clonidine and ^{3}H-Yohimbine Binding to Platelet Membranes of Normal Subjects

Case	Age (yr) & Sex	High Affinity Binding: B_{max} (fmol/mg protein)	High Affinity Binding: K_D (n*M*)
A. ^{3}H-Clonidine Binding			
1	34M	33	4 8
2	19M	29	4.8
3	23M	32	6.9
4	43M	28	8.7
5	27M	40	5.2
6	44F	51	4.8
7	36F	36	4 1
8	23M	48	5 7
9	26F	29	5.1
10	22M	20	6.9
11	28M	48	5.8
12	31M	52	5.8
13	20F	39	4.7
14	27F	29	4.6
15	30F	18	1 7
16	31M	21	0 3
17	57M	22	1.4
18	67M	32	4.5
19	55F	44	9.9
20	69F	44	8.0
21	70F	32	2.8
22	48M	28	13 2
23	56M	35	12.9
24	39F	23	5 4
25	45M	19	1 8
26	62M	31	6 8
Mean	40	33	5.5
± SEM	3	2	0.6
B. ^{3}H-Yohimbine Binding			
1	34M	245	3.1
6	44F	214	3.6
11	28M	166	2.5
12	31M	160	2.5
13	20F	186	3 0
14	27F	133	3 2
22	48M	57	2.9
23	56M	174	5.0
24	39F	124	7.8
26	62M	138	4 3
27	49M	123	4.3
28	60F	146	9 2
29	20M	168	2.5
30	28M	166	3.1
31	40F	208	3.4
32	24F	238	2.9
Mean	37	165	4.0
± SEM	4	12	0 5

B_{max} = maximal number of binding sites; K_D = dissociation constant; SEM = standard error of the mean.

Binding data in patients during and after a period of symptomatic disease. In the five patients studied both during and after a period of sympotomatic disease, the number of binding sites changed significantly in the same manner as in the patient group comparisons (Fig. 3 and 4). When the patients were symptomatic, the maximal number of binding sites for specific binding of ^{3}H-clonidine was 16 ± 3; whereas it increased to 29 ± 3 on follow-up testing ($p < 0.005$). For specific binding of ^{3}H-yohimbine, the initial number of binding sites was 93 ± 8 and was 127 ± 17 ($p < 0.05$) on repeat testing. Again, the affinity constants were unchanged (^{3}H-clonidine: 7.3 ± 2.1 to 5.9 ± 2.0 and ^{3}H-yohimbine: 4.4 ± 0.6 to 5.9 ± 0.9).

Catecholamine values. Plasma norepinephrine, epinephrine and dopamine were measured in all control subjects and patients. In normal subjects, the norepinephrine concentration was 385.1 ± 63.9 pg/ml (range 54 to 908). There was no significant difference from control values in norepinephrine content in Group I (symptomatic) patients (483.5 ± 82.1 pg/ml; range 199 to 1,495) or in Group II (asymptomatic) patients (379.8 ± 85.6 pg/ml; range 58 to 897). Although there was a trend toward an increase in epinephrine concentration in patients in Group I (104.0 ± 22.5 pg/ml; range 0 to 274) and Group II (93.6 ± 16.8 pg/ml; range 33 to 189), these values did not differ significantly from those of the normal group (53.6 ± 12.7 pg/ml; range 8 to 163). There was no significant difference in dopamine content among the three groups (normal: 13.9 ± 7.8 pg/ml, range 0 to 100; Group I: 34.8 ± 10.3 pg/ml, range 0 to 143; Group II: 25.8 ± 7.8 pg/ml, range 0 to 81).

Discussion

Alpha-adrenergic activity in coronary arteries. The importance of the alpha-adrenergic system in determining coronary vascular tone has been suggested by several investigators (1–11). Even in the presence of local metabolic vasodilation, sympathetic vasoconstriction was capable of limiting oxygen delivery to the myocardium (10,11). The increase in coronary vascular resistance after cold pressor testing has been shown to be inhibited by alpha-adrenergic blockade with phentolamine (5). The increase in resistance was greater in patients with coronary artery disease than in patients with normal coronary arteries. This suggested that changes in the alpha-adrenergic system's response to stimuli may be involved in determining vascular tone, particularly in atherosclerotic vessels.

Bassenge et al. (8) examined the presence and role of vascular alpha$_2$-adrenoceptors in the coronary bed of dogs. Coronary constriction was elicited by both infused norepinephrine and sympathetic nerve stimulation that was inhibited by alpha$_2$-blockade with rauwolscine. The increased release of norepinephrine during nerve stimulation after alpha$_2$-

Table 2. ^{3}H-Yohimbine and ^{3}H-Clonidine Binding to Platelet Membranes of Patients With Symptomatic and Asymptomatic Coronary Artery Disease

Case	Age (yr) & Sex	^{3}H-Yohimbine Binding		^{3}H-Clonidine Binding	
		B_{max} (fmol/mg protein)	K_D (n*M*)	B_{max} (fmol/mg protein)	K_D (n*M*)
A. Patients With Symptomatic Coronary Artery Disease					
33	57M	100 4	4 5		
34	36M	138 4	4 9		
35	57M	104 6	2.6		
36	62M	92 3	2.4		
37	55M	99 7	5.1		
38	60M	36 8	3.8	42 2	3 5
39	47M	116 0	4.8		
40	60M	125 0	3.8		
41	54M	117 8	3.0	18 8	5 4
42	72F	85 0	5.3	12 7	7 6
43	56M	110 1	4 1	26 5	6 5
44	47M	114 2	7.3		
45	62F	96 7	3 3		
46	60F	120.7	4 3	17.1	3.0
47	40M	11 0	2.6	10.0	2 0
48	42M	31 0	5.0	10.9	8 8
49	65F	113 4	4 1	22.7	13.4
50	68F	66 7	6.0	20.9	9.4
Mean	56	93 3	4 5	20 2	6.6
± SEM	3	8 3	0 3	3.3	1.2
B. Patients With Asymptomatic Coronary Artery Disease					
46	60F	185.4	4 4	25 8	2.9
47*	40M	92 1	5.3	22.5	1.6
48	42M	91 3	3.9	37 0	7 4
49	65F	137 2	9.0	29 5	4 5
50	68F	129 4	6 6	32 1	13.1
51	49M	151 9	4 3		
52	77M	192 6	2 5		
53	59M	150 0	3 7		
54	59M	100 4	4 0		
55	57M	139 6	7.6		
Mean	58	137 0	5 1	29.6	6.9
± SEM	4	11 2	0 6	2.1	1.9

*Patient treated with nifedipine. Abbreviations as in Table 1.

adrenoceptor blockade suggested a physiologically significant role for the presynaptic alpha$_2$ site in the whole animal. Johannsen et al. (14) have shown that the infusion of clonidine, an alpha$_2$-agonist, into dogs inhibited coronary vasoconstriction due to sympathetic nerve stimulation but not to infused norepinephrine. This response to clonidine presumably resulted from the stimulation of the presynaptic alpha$_2$-adrenoceptor and inhibition of norepinephrine release. These observations are consistent with the importance of alpha-adrenergic control of coronary vascular tone and with the ability of the presynaptic alpha$_2$ site to modulate the release of norepinephrine from the sympathetic nervous system.

Platelet alpha$_2$-adrenoreceptors. The alpha-adrenoceptor on the human platelet has been well characterized as being of the alpha$_2$ subtype (20). Stimulation of presynaptic alpha$_2$-receptors inhibits the neuronal release of norepinephrine (12,13). Thus, a decrease in number of this inhibitory site would be expected to permit increased norepinephrine release with nerve stimulation. Changes in the number of platelet alpha$_2$-receptors have been shown to correlate with changes in similar neuronal receptors in the rat brain after various therapies such as antidepressant drug treatment (17,18,20) and electroconvulsive shock therapy (28). Similar interventions caused decreases in receptor sensitivity, which resulted in increased norepinephrine release after nerve stimulation as measured by the tension developed by rat atrial strips during field stimulation (15,16). There is

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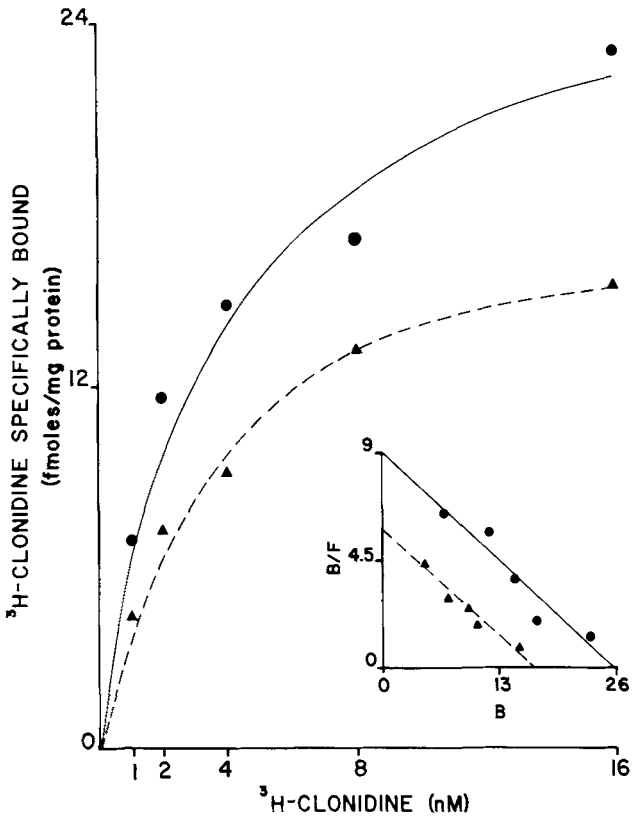

Figure 3. Comparison of specific binding of ^{3}H-clonidine to platelet membranes as determined by Scatchard analysis for a representative patient (Case 46) when symptomatic (**closed triangles**) and again when asymptomatic (**closed circles**). **Abscissa:** maximal number of binding sites in fmol/mg protein. B = specifically bound ligand; B/F = bound/free ligand.

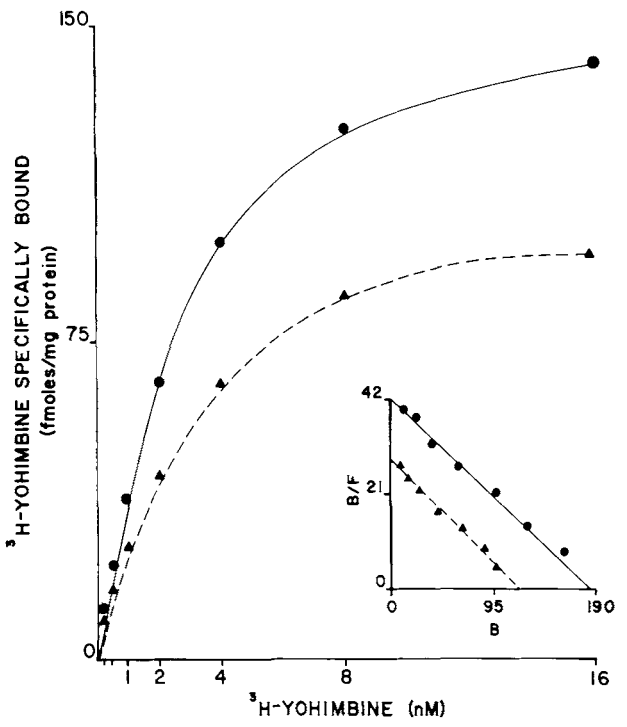

Figure 4. Comparison of specific binding of ^{3}H-yohimbine to platelet membranes as determined by Scatchard analysis for a representative patient (Case 46) when symptomatic (**closed triangles**) and again when asymptomatic (**closed circles**). **Abscissa:** maximal number of binding sites in fmol/mg protein. B = specifically bound ligand; B/F = bound/free ligand.

an inverse relation between the number of alpha$_2$-adrenoceptors on platelets and circulating norepinephrine levels in patients with chronic congestive heart failure (21).

It is not absolutely certain from the present study whether the decrease in receptor number is a primary change related to the underlying disease or a secondary change related to transient changes in the concentrations of circulating catecholamines. Increased plasma concentrations of circulating norepinephrine, epinephrine or dopamine were not found in either of our patient groups with angina. This is consistent with work done by Schwartz et al. (29), who found no change in the peripheral catecholamine concentration of patients after pacing to angina. Robertson et al. (30) also found no abnormalities in plasma catecholamines or their urinary metabolites in patients with variant angina. Any increase in plasma norepinephrine may be very transient. The half-time of ^{3}H-norepinephrine has been shown to be 5 minutes in mice and 2 minutes in cats (31,32). It is possible that changes in platelet alpha$_2$-adrenoceptor number reflect transient changes in the norepinephrine concentration in a manner similar to that in which glycosalated hemoglobin reflects transient changes in glucose concentration (33). Small changes in the amount of norepinephrine released, perhaps even within the normal range, may be important in affecting vascular tone if atherosclerotic vessels are supersensitive to catecholamines, as has been suggested (34).

Clinical implications. Abnormalities of the adrenergic nervous system have been found in the variant angina syndrome (35–37). It has been suggested that vasospasm may underlie much of myocardial ischemia (38). Several studies (38) have shown no increase in myocardial oxygen demand before an episode at rest or, in some cases, during exercise-induced angina. Circumstantial evidence for the importance of spasm in patients with unstable angina is found in propranolol's reported lack of efficacy in decreasing the number of ischemic episodes at rest (39) or in actually causing an increase in the duration of such episodes (40).

Changes in vasomotor tone may be important in causing stable angina. In this study, the change in the number of platelet alpha$_2$-adrenoceptors reflected changes in the symptomatic activity of the coronary artery disease, with a decrease in receptor number correlating with increased symptoms. If the decrease in alpha$_2$-adrenoceptor number found during periods of symptomatic coronary artery disease is primary, this could permit an increased release of neuronal norepinephrine during nerve stimulation. This increase could cause coronary artery vasoconstriction, even at times of

maximal vasodilation due to local metabolic factors. If the decrease is secondary to increased concentrations of norepinephrine, then it might be an important marker of increased adrenergic activity that may be related to episodes of angina.

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