Desensitization of Postjunctional $\alpha_1$- and $\alpha_2$-Adrenergic Receptor-Mediated Vasopressor Responses in Rat Harboring Pheochromocytoma

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Prolonged stimulation of tissues by adrenergic agonists may lead to diminished responsiveness of the tissues to subsequent activation by catecholamines; this phenomenon has been termed desensitization or tachyphylaxis. We have examined the in vivo consequences of prolonged stimulation of vascular $\alpha$-adrenergic receptors in rats harboring pheochromocytoma, a tumor that secretes catecholamines. In both early (3-4 weeks after implantation) and late (6-7 weeks after implantation) stages of tumor development, New England Deaconess Hospital rats with transplanted pheochromocytomas developed hypertension and tachycardia and had plasma dopamine and norepinephrine concentrations markedly greater than controls. In both these stages of pheochromocytoma, pressor responses to several vasoconstrictors were examined after pithing. Rats with the tumor were found to become progressively less sensitive to $\alpha$-adrenergic agonists. In the early phase of pheochromocytoma, loss of sensitivity was found for both $\alpha_1$- and $\alpha_2$-adrenergic agonists, whereas responsiveness to the nonadrenergic vasoconstrictors Arg-vasopressin and angiotensin-II was intact (homologous desensitization). However, in the later stage of pheochromocytoma, pressor responses to all these vasoconstrictive agents and also to stimulation of the complex sympathetic outflow were found to be subsensitive (heterologous desensitization). In plasma membranes prepared from mesenteric arteries of early stage tumor-bearing rats, [3H]prazosin binding sites were significantly decreased to 150 ± 12 fmol/mg vs. 234 ± 19 fmol/mg in controls. [3H]Yohimbine binding sites were not significantly altered. Our results show that both postjunctional $\alpha_1$- and $\alpha_2$-adrenergic receptor-mediated vasopressor responses can be specifically attenuated in the presence of chronically elevated endogenous catecholamine levels produced by pheochromocytoma and that each $\alpha$-receptor subtype may be differentially regulated in the development of desensitization. (Circulation Research 1987;61:86-98)

The sympathetic nervous system plays an important role in regulating the tone of the peripheral circulation. Catecholamines cause vascular smooth muscle contraction by activating $\alpha$-adrenergic receptors and cause relaxation by interacting with $\beta$-adrenergic receptors. The responsiveness of blood vessels to catecholamines may be modified in a variety of settings, including diseases such as hypertension and altered hormonal states. Additionally, catecholamines may themselves regulate the sensitivity of a vessel to subsequent stimulation by sympathomimetic drugs, these alterations may have important implications for pathophysiology and drug responsiveness.

It has now been clearly demonstrated that both $\alpha_1$- and $\alpha_2$-adrenergic receptors are found postjunctionally in the vasculature of many mammalian species, including humans. Both of these $\alpha$-adrenergic receptor subtypes may mediate vasoconstriction. However, little information is available regarding desensitization of responses mediated by these vascular $\alpha$-receptor subtypes. In particular, the consequences of in vivo chronic exposure to catecholamines are not well characterized. Examination of desensitization in intact animals is of interest because it may not be possible to draw conclusions about the more complex, intact organism from information obtained in isolated cells or vessels. Variables that could modify the desensitization process include metabolism of catecholamines, anatomical localization of receptors relative to sympathetic innervation, effects of altered sympathetic nervous system activity (due to excessive catecholamines), possible counterregulatory mechanisms, and secondary organo-structural alterations.

Pheochromocytoma is a catecholamine-producing tumor that causes a distinctive clinical syndrome usually associated with hypertension. A transplantable pheochromocytoma that secretes predominantly dopamine and norepinephrine and can be carried in the New England Deaconess Hospital (NEDH) strain of rat was developed by Warren and Chute. Rats with transplanted pheochromocytomas provide a valuable model...
sponses. Supported by clinical observations that the vasculature of patients with pheochromocytoma apparently exhibits refractoriness to catecholamines, the present study was designed to characterize the consequences of prolonged elevation of circulating catecholamines on vascular reactivity in pithed rats harboring pheochromocytoma.

Materials and Methods

Animal Model of Pheochromocytoma

NEDH male rats, 8–9 weeks old, were lightly anesthetized with ether and implanted subcutaneously at the base of the neck with several pieces (1 × 1 × 3 mm) of rat pheochromocytoma taken from another NEDH rat harboring the tumor. Several tumor-bearing rats (line P-259 established by Warren and Chute) were generously provided by Dr. William M. Manger, New York University Medical Center. Age-matched male NEDH controls were also lightly anesthetized with ether and received only an intrascapular skin incision. Following the surgery, rats were housed in individual cages with free access to food and water. A palpable tumor mass was generally evident 3–4 weeks after tumor implantation; the tumor was approximately 0.5–0.8 cm in diameter at this time. The animals’ body weights also served as indications of the progression of the tumors. Tumor-bearing animals gain weight at a rate similar to unimplanted age-matched controls for several weeks after tumor implantation. By the time the tumor is palpable, the body weight generally plateaus for 4–7 days and then rapidly decreases. The pheochromocytoma-bearing animals used in this study exhibited body-weight plateaus at 21–28 days after tumor implantation. Animals were studied when their body weight reached a plateau (early stage, 3–4 weeks after tumor implantation) and when a rapid weight loss was apparent (late stage, 6–7 weeks after tumor implantation). Age-matched unimplanted controls were studied at the same time.

Analysis of Plasma Catecholamine Concentrations

For the analysis of plasma catecholamine concentrations, animals were anesthetized with hexobarbital (150 mg/kg i.p.), and blood (1 ml) from the inferior vena cava below the level of the renal veins was rapidly collected through an abdominal incision and placed in ice-cold tubes containing 0.1% sodium ethylenediaminetetraacetate (EDTA) as an anticoagulant. After centrifugation at 1,500g for 10 minutes, the plasma samples were stored at −80°C until analyzed. Plasma samples were extracted as previously described.

Catecholamine determinations were made by reverse phase high-performance liquid chromatography (HPLC) with electrochemical detection as described previously, using the 88:12 (vol/vol) mixture of 0.1 M potassium phosphate buffer (pH 3.2) and methanol with 2.5 mM octane sulfonate sodium and 10 μM sodium EDTA as the mobile phase. In experiments in which pithed rats were used, plasma catecholamines were not measured so as to avoid any influence of loss of blood volume on the measured hemodynamic parameters.

Hemodynamic Studies

The influence of elevated concentrations of circulating catecholamines produced by pheochromocytoma on blood pressure and heart rate were determined in both intact and pithed rats. Both stages of tumor-harboring rats and age-matched, unimplanted controls were anesthetized with hexobarbital (150 mg/kg i.p.), and the tracheas were cannulated to ensure adequate ventilation. Skin temperature was kept constant (37°C) with a heating pad. Polyethylene catheters (PE-50) were placed in the right common carotid artery and the right femoral vein for direct arterial pressure monitoring and for i.v. administration of drugs, respectively. Systemic arterial blood pressure was measured via a Nihon Kohden TP-200T pressure transducer (Tokyo) and recorded on a Nihon Kohden Model WS-6416 recorder. Heart rate was recorded with a Nihon Kohden AT-600G cardiograph, triggered by the electrocardiograph (ECG). The preparation was allowed to equilibrate for at least 20 minutes before basal values of blood pressure and heart rate were obtained.

After administering atropine (2 mg/kg i.p.), rats were pithed by inserting a stainless-steel rod (1.5 mm diameter) through the right orbit and foramen magnum down through the spinal cord as described by Gillespie and Muir. Immediately after pithing, the tracheal cannula was attached to a Shinano Model SN-480-7 rodent respirator (Japan), and the rat was artificially ventilated with room air at a frequency of 100 cycles/min with a tidal volume of 10 ml/kg body wt. Pithed rats were bilaterally vagotomized in the neck, and d-tubocurarine (3 mg/kg i.v.) and heparin (150 IU/kg i.v.) were injected. The rats were allowed to equilibrate for at least 30 minutes before basal values of blood pressure and heart rate were obtained. In some experiments, the effect of either the α-adrenergic antagonist phentolamine (1 mg/kg i.v.) or the β-adrenergic antagonist (-)-propranolol (1 mg/kg i.v.) on blood pressure and heart rate of the pithed rats was examined.

The effects of various drugs or electrical stimulation of the sympathetic nervous system were determined when the blood pressure had become stable after injections of both d-tubocurarine (3 mg/kg i.v.) and heparin (150 IU/kg i.v.). In the case of experiments involving angiotensin-II (AT-II), an additional pretreatment with indomethacin (15 mg/kg i.v. 30 minutes before administering AT-II) was carried out to prevent possible interference by prostaglandins on the pressor effects of AT-II. Drugs were administered intravenously in increasing doses; complete recovery from the pressor effects of the preceding injection occurred before the next dose was given. Each drug was injected in a volume 1.0 ml/kg. Intravenous injection of this amount of 0.9% NaCl saline generally did not change blood pressure. All the drug solutions were freshly prepared just before the start of the experiment and kept at room temperature. Norepinephrine was
dissolved in 0.9% NaCl solution containing 0.01% l-ascorbic acid. Indomethacin was dissolved in 0.1 M Na2CO3 and diluted with 0.9% saline (final concentration: 15 mg/ml) and the other drugs in 0.9% NaCl saline.

The complete sympathetic outflow was stimulated for 20 seconds by application of rectangular pulses (supramaximal voltage of 80 V, duration 1 msec, frequency 0.1-20 Hz) generated by a Nihon Kohden Model-S-7272A electronic stimulator between the pithing rod and a stainless-steel electrode fixed under the skin of a hind limb. Interestingly, administration of d-tubocurarine, used to eliminate skeletal muscle twitching soon after the pithing, caused a transient increase in blood pressure in the tumor-bearing rats (approximately a 30-40 mm Hg increase, which lasted for 5-10 minutes; data not shown). This response may be due to increased catecholamine release stimulated by d-tubocurarine-induced histamine release. Pretreatment of rats with the histamine antagonist diphenhydramine (1 mg/kg i.v.) or the a-adrenergic antagonist phentolamine (1 mg/kg i.v.) abolished the d-tubocurarine-induced increase in blood pressure seen in rats with pheochromocytoma. Thus, all baseline values shown in Table 2 were obtained after the pressor effect of d-tubocurarine had worn off (at least 25 minutes after the injection). Pressor responses are expressed as increases in diastolic blood pressure in mm Hg.

**Radioligand Binding Studies**

**Preparation of Plasma Membranes from Rat Mesenteric Arteries**

Plasma membrane vesicles were prepared from rat mesenteric arteries using modifications of the method described by Kwan et al. and Agrawal and Daniel. Rats were anesthetized with hexobarbital (150 mg/kg i.p.) and killed by cervical dislocation. The small intestine was doubly ligated at the proximal end of the intestine and was perfused retrogradely with buffer A, which contained 3.0 ml each of 46.5 and 38.5% sucrose and 2.0 ml each of 32.5 and 26.5% sucrose solutions and then centrifuged at 113,000g for 100 minutes. Five distinct bands of proteins were obtained after centrifugation. The top-most thick band (F1), which has been found to contain highly enriched plasma membrane vesicles, was removed with a Pasteur pipette, diluted with buffer A, and recentrifuged at 105,000g for 30 minutes. The sedimented plasma membrane vesicles were suspended in buffer A and mixed with a hand-driven Teflon-pestle homogenizer with 7 strokes up and down. The purity of the plasma membrane fraction was checked routinely by measuring the 5'-nucleotidase enzyme activity. The 5'-nucleotidase enzyme activity in the F1 plasma membrane fraction of the rat mesenteric arteries was in the range of 40-60 μmol/mg protein/hr. All membrane preparations used in the present study contained the 5'-nucleotidase enzyme activity in this range. Protein content of the membranes was determined by the method of Lowry et al.

**Receptor Binding Assays**

Radioligand receptor binding assays were done using plasma membrane fractions (F1) obtained from mesenteric arteries of controls and rats bearing pheochromocytoma. Preliminary studies of the radioligand receptor binding were done also in the various fractions obtained from the density gradient. Also, we found that membranes stored frozen (in buffer A, pH 7.4, at -70°C) up to 1 month gave results comparable to those obtained with freshly prepared membranes.

In the radioligand binding assays, buffer B (50 mM Tris-HCl, 10 mM MgCl2, and 1 mM EDTA, pH 7.5) was used in the incubation media. [3H]Prazosin (α1-receptors) and [3H]Yohimbine (α2-receptors) were used as the radioligands and were freshly diluted from stock solution with cold buffer B. The incubation media contained 100 μl of buffer B with or without unlabeled drug and 50 μl of diluted radioligand. The reaction was started by adding 100 μl of membrane suspension to make a final volume of 250 μl. Incubations were performed in a gyroratory shaker water bath at 25°C for 25 minutes, at which time equilibrium was reached for all the concentrations of each radioligand used in this study. The reaction was terminated by the addition of 5 ml of the incubation buffer (4°C). The bound and free radioligands were separated by rapid filtration over glass fiber filters (Whatman GF/C, Maidstone, England). The filters were washed with an additional 15 ml of incubation buffer at 4°C. The radioactivity retained on the filters was counted in a Packard Model TRI-CARB 300 liquid scintillation counter at 50% efficiency (Packard Instrument Co., Inc., Downers Grove, Ill.). Nonspecific binding was defined as the amount of [3H]Prazosin or [3H]Yohimbine binding measured in the presence of 10 μM phentolamine. The specific binding of each radioligand generally represented 80-95% of total counts bound at concentrations near their Kd. Data is expressed as...
amount of specific binding/mg protein. Experiments were done in triplicate.

Data Analysis

The pressor responses for each drug were measured, and the resulting dose-pressor response curves were analyzed simultaneously using the four parameter logistic equation on an APPLE II computer. These data were fit based on the law of mass action using a general program for the analysis of data in terms of models. The Kₘ,ₜ and maximal responses (Emax) were analyzed for significant differences using the ALLFIT program. (The ALLFIT program is a modification of the DeLean et al program written by Martin H. Teicher and was obtained from the Biomedical Computing Technology Information Center, Nashville, Tenn.)

The saturation curves in the radioiodinated binding studies were analyzed using nonlinear regression on an NEC9801F computer. These data were fit based on the four parameter logistic equation to the data as described above.

The experimental data given in the text and figures are the mean ± SEM of number of experiments as indicated. Overall changes between groups were analyzed statistically by two-way analysis of variance (factor 1 = stage of tumor, factor 2 = the presence of pheochromocytoma). Student’s t test for unpaired observations was also used to determine the significance of difference between values of controls and those of tumor-bearing animals within the same age group. Critical value of statistical significance was a p value of less than 0.05. In the several figures (Figures 2–4 and 6–8) where no significant difference was obtained between the younger and older age-matched control NEDH rats, these 2 groups of control data were pooled; however, the statistical comparison of pressor responses obtained from either the early or late stage of pheochromocytoma rats was always made with the corresponding age-matched controls.

Materials

[¹H]Prazosin (specific activity, 19.8 Ci/mmol) and [¹H]Yohimbine (specific activity, 80.5 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Phenolamine (Regitine mesylate; Ciba-Geigy Corp., Summit, N.J.), UK-14,304 (Pfizer, Sandwich, U.K.), clonidine HCl (C.H. Boehringer Ingelheim Ltd., Elmsford, N.Y.), and methoxamine (Burroughs Wellcome, London) were generously supplied by each company. Angiotensin-II (AT-II), indomethacin, (-)norepinephrine bitartrate, (+)propranolol HCl, (-)phenylephrine HCl, and Arg-vasopressin (ADH) were purchased from Sigma Chemical Co., St. Louis, Mo. d-Tubocurarine chloride and atropine sulfate were obtained from Yoshitomi Pharmaceutical, Osaka, Japan, and Fusco Pharmaceutical, Osaka, respectively. All other chemicals and reagents used were from standard commercial sources.

Results

Body Weight, Tumor Weight, and Plasma Catecholamines

Table 1 compares the body weight, tumor weight, and concentration of plasma catecholamines in the pheochromocytoma-bearing (both early and late stages) rats with age-matched, unimplanted NEDH controls. The body weights of tumor-harboring (PHEO) and control rats were similar at about 4 weeks after tumor implantation (early stage), while a marked loss of body weight was noted in PHEO rats at 7–8 weeks after tumor transplant (late stage). There was a marked increase in the plasma levels of dopamine and norepinephrine in rats with the tumor, irrespective of their stages, compared with controls, but plasma epinephrine levels were not significantly altered (Table 1). The high concentrations of plasma catecholamine were not correlated with disease stage or tumor size. However, the tumors weighed significantly more in

Table 1. Comparison of Control and Pheochromocytoma (PHEO)-Implanted Rats

<table>
<thead>
<tr>
<th></th>
<th>Control NEDH rats (11–12 weeks old)</th>
<th>PHEO-bearing rats (early stage)</th>
<th>Control NEDH rats (14–15 weeks old)</th>
<th>PHEO-bearing rats (late stage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>294 ± 3 (n = 36)</td>
<td>286 ± 4 (n = 33)</td>
<td>348 ± 3 (n = 37)</td>
<td>224 ± 3* (n = 39)</td>
</tr>
<tr>
<td>Tumor weight (g)</td>
<td>...</td>
<td>1.3 ± 0.1 (n = 9)</td>
<td>...</td>
<td>4.5 ± 0.3* (n = 8)</td>
</tr>
<tr>
<td>Plasma dopamine (pg/ml)</td>
<td>149 ± 20 (n = 8)</td>
<td>55,800 ± 4,670* (n = 7)</td>
<td>188 ± 32 (n = 8)</td>
<td>60,800 ± 5,420* (n = 8)</td>
</tr>
<tr>
<td>Plasma norepinephrine (pg/ml)</td>
<td>163 ± 27 (n = 8)</td>
<td>33,600 ± 4,120* (n = 7)</td>
<td>201 ± 58 (n = 8)</td>
<td>28,500 ± 2,680* (n = 8)</td>
</tr>
<tr>
<td>Plasma epinephrine (pg/ml)</td>
<td>98 ± 26 (n = 8)</td>
<td>128 ± 32 (n = 7)</td>
<td>108 ± 29 (n = 8)</td>
<td>146 ± 46 (n = 8)</td>
</tr>
</tbody>
</table>

*p < 0.01 vs age-matched control NEDH rats, †p < 0.01 vs early stage PHEO-bearing rats, no symbol indicates no significant difference (p > 0.05)

Plasma catecholamine measurements were done as described in "Materials and Methods." Values are mean ± SEM, n refers to the number of rats.
the late stage than in the early stage. The tumors were usually solitary, highly vascular, and well encapsulated. In larger tumors (weighing more than 2 g), areas of necrosis and fibrosis were generally observed. No metastases were visually evident.

Baseline Arterial Blood Pressure and Heart Rate in Intact and Pithed Rats

Baseline values of arterial blood pressure and heart rate for the various groups of intact, anesthetized rats are shown in Table 2A. Both stages of PHEO rats showed significantly elevated blood pressure and heart rate compared with age-matched controls; these values were more elevated in the late-stage PHEO rats. The consequences of the high concentrations of circulating catecholamines on cardiovascular responses were further examined after these rats had been pithed. Baseline values of the blood pressure and heart rate for each group of rats 30 minutes after pithing are summarized in Table 2B. Pithing caused a drop of blood pressure in each of the groups. The decrease in blood pressure was more significant in PHEO rats compared with the age-matched, unimplanted rats; decrease in DBP in early stage, 51 vs. 26 mm Hg, p < 0.01; decrease in DBP in late stage, 77 vs. 27 mm Hg, p < 0.01; decrease in SBP in early stage, 61 vs. 38 mm Hg, p < 0.01. decrease in SBP in late stage. 87 vs. 43 mm Hg, p < 0.01. In pithed rats bearing pheochromocytoma, basal values of SBP (in both stages) and DBP (only in late stage) were significantly elevated compared with age-matched, pithed controls. However, pithing tended to equalize the blood pressures of the rats harboring early and late stages of pheochromocytoma. Baseline heart rate was unaffected by pithing; consequently, heart rates remained significantly higher in both groups of pithed rats harboring pheochromocytoma.

To assess the contribution of α- and β-adrenergic receptor-mediated responses in maintaining the hypertension and tachycardia seen in PHEO rats, we next examined the effect of the β-receptor antagonist propranolol (1 mg/kg i.v.) and the α-receptor antagonist phentolamine (1 mg/kg i.v.) on the blood pressure and heart rate (Tables 2C and 2D). The administration of (±) propranolol did not significantly change blood pressure or heart rate in pithed control rats; however, in PHEO rats it evoked a rapid persistent drop in heart rate and a transient pressor response (generally 40–80 mm Hg maximum elevation in DBP) that lasted 8–12 minutes (Figure 1). Values for blood pressure and heart rate shown in Table 2 were obtained at least 30 minutes after propranolol administration, during which time the pressor response to (±) propranolol had worn off. As summarized in Table 2C, propranolol caused a marked decrease of heart rate in both PHEO groups. Indeed, after treatment with propranolol, heart rates of pithed PHEO rats were not significantly different from those of the age-matched controls treated with propranolol. However, propranolol only partially decreased the elevated SBP in the pheochromocytoma rats. In contrast, phentolamine caused a rapid and marked drop in blood pressure of pithed PHEO rats without any significant influence on heart rate. Phen-

Table 2. Arterial Blood Pressure and Heart Rate in Control NEDH and Pheochromocytoma(PHEO)-Implanted Rats: Baseline Values in Intact, Anesthetized and Pithed States Before and After Administration of Propranolol or Phentolamine

<table>
<thead>
<tr>
<th>Control</th>
<th>PHEO- bearing NEDH rats</th>
<th>Control</th>
<th>PHEO- bearing rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>106 ± 5</td>
<td>175 ± 10†</td>
<td>116 ± 4</td>
</tr>
<tr>
<td>DBP</td>
<td>67 ± 4</td>
<td>93 ± 9*</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>HR</td>
<td>337 ± 7</td>
<td>470 ± 12†</td>
<td>345 ± 5</td>
</tr>
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</table>

(A) Intact, anesthetized rats

(B) Pithed rats without any pretreatment of antagonists

(C) Pithed rats (after propranolol 1 mg/kg i.v administration)

(D) Pithed rats (after phentolamine 1 mg/kg i.v administration)

* p < 0.05 vs. age-matched control NEDH rats; † p < 0.01 vs age-matched control NEDH rats; ‡ p < 0.05 vs. early stage PHEO-bearing rats; ‡‡ p < 0.05 vs. early stage of NEDH rats, no symbol indicates no significant difference (p > 0.05).

SBP, systolic blood pressure (mm Hg); DBP, diastolic blood pressure (mm Hg); HR, heart rate (beats/min)

Values are mean ± SEM, n refers to the number of rats.
tolamine did not significantly affect the blood pressure or heart rate of control pithed rats. After the injection of phentolamine in both groups of PHEO rats, the drop in blood pressure was so marked that DBP values in these rats were significantly lower than those in age-matched controls injected with the drug.

Effect of Pheochromocytoma on the Pressor Response of Pithed Rats to α-Adrenergic Agonists

Dose-response curves for the pressor effects of injected (-)-norepinephrine are presented in Figure 2. In the early-stage PHEO rats, the curves were displaced in a rightward direction; the ED50s were increased ninefold for methoxamine and fourfold for phenylephrine without significant changes in the maximum pressor response (p > 0.05) compared to controls. However, in the late-stage PHEO rats, not only were there more pronounced rightward displacements of the dose-response curves, but also there were significant (p < 0.05) reductions of Emax of 47 ± 6 mm Hg for methoxamine and 35 ± 5 mm Hg for phenylephrine, respectively. Dose-response curves for α2-selective adrenergic receptor agonists clonidine and UK-14,304 in pithed early and late PHEO rats were both characterized by marked reductions in Emax as shown in Figure 3. Emax values in control rats were 92 ± 5 mm Hg for clonidine and 81 ± 4 mm Hg for UK-14,304, and those for clonidine and UK-14,304 in early stage of PHEO rats were 25 ± 5 mm Hg and 23 ± 3 mm Hg, respectively. In the late stage of pheochromocytoma, there was no pressor response to either agonist at any dose examined; rather, a fall of blood pressure was observed at the highest doses, particularly for clonidine. The fall in blood pressure with the partial agonist clonidine could relate to its antagonizing the effects of the endogenous, full agonist, norepinephrine.

Specificity of the Desensitization in the Pressor Responses of the Pithed PHEO Rats

Further experiments were performed to determine if the desensitization in responsiveness to α-adrenergic agonists was homologous or heterologous. In this case, homologous desensitization would involve loss of responsiveness only to catecholamines, whereas responsiveness to drugs or hormones causing their action through other receptors would not be impaired. Conversely, heterologous desensitization involves loss in responsiveness to a multiplicity of agonists acting on different receptors. To address this issue, we have examined the pressor responses to nonadrenergic vasopressors, AT-1 and Arg-vasopressin (ADH) (Sigma Chemical Co.).

Administrations of ADH produced dose-dependent pressor responses in both pithed control and PHEO rats. In both stages of pithed PHEO rats, doses of ADH between 10 and 100 mIU/kg frequently (over 90% of rats examined) evoked a biphasic pressor response as shown in Figure 5; an initial pressor response to ADH...
injection was followed by a relatively long-lasting, delayed, and further rise in pressure. Both components of the pressor response in PHEO rats to ADH were found to be modified by pretreatment with the $\alpha$-adrenergic antagonist phentolamine (1 mg/kg i.v.); namely, the initial pressor response was decreased and later secondary response was abolished in the presence of phentolamine. This result suggests that the early pressor response to ADH administration was partially due to catecholamines while the secondary response was entirely mediated by $\alpha$-adrenergic stimulation, possibly due to ADH-stimulated release of catecholamines from the tumor.

To minimize the potentially confounding role of catecholamines in the response to ADH, we constructed dose-response curves to ADH in the presence and absence of phentolamine. In Figures 6A and 6B, the initial pressor responses to ADH are illustrated. In the absence of $\alpha$-blockade, the pressor response to ADH was enhanced in the early PHEO rats and suppressed in the late PHEO rats compared with controls. Pretreatment with phentolamine eliminated the enhanced pressor response in the early stage PHEO rats, but the pressor responses for the late stage PHEO rats remained markedly suppressed. The response to ADH in the controls was largely unaffected by phentolamine.

Pressor responses to another nonadrenergic vasoconstrictor, AT-II, are shown in Figure 7. As in the case of ADH, the dose-response curves were constructed in both the presence and absence of phentolamine (1 mg/kg i.v.). The dose-response curve for AT-II in the early-stage PHEO rats was found to be similar to controls, whereas in the late-stage PHEO rats, pressor responses to AT-II were significantly ($p<0.05$) decreased at all doses examined, either in the presence or absence of phentolamine. The results are similar to those seen with ADH.

**Effect of Pheochromocytoma on the Pressor Responses to Stimulation of the Sympathetic Nervous System**

Electrical stimulation of the complete sympathetic outflow induced a sharp rise in blood pressure in a frequency-dependent manner in both control and PHEO rats. As shown in Figure 8, pressor responses of early stage PHEO rats were significantly smaller than those of controls at the relatively lower frequencies tested (0.5–2.0 Hz), but for higher frequencies tested, the pressor responses were similar. In the late stage PHEO rats, the pressor responses were significantly smaller compared with those of controls at all the frequencies examined. To obtain the same rise in arterial pressure, pithed PHEO rats required approximately 5 times higher frequencies of electrical stimulation compared with control rats.
Characterization of \[^3H\]Prazosin and \[^3H\]Yohimbine Binding in Rat Mesenteric Artery

Specific binding for both \[^3H\]prazosin and \[^3H\]yohimbine to the Fl fraction of mesenteric arteries was saturable (Figure 9). Computer analysis of the saturation curves indicated that \[^3H\]prazosin was binding to a single class of sites with a dissociation constant of 0.60 ± 0.1 nM and a calculated total binding capacity of 226 ± 17 fmol/mg protein (n = 3). The specific binding of \[^3H\]prazosin reached equilibrium in 20 minutes and remained stable for at least 20 more minutes (data not shown). \[^3H\]Yohimbine was also found to be binding to a single class of sites with a dissociation constant of 29.8 ± 4.8 nM and a calculated total binding capacity of 490 ± 36 fmol/mg protein (n = 3). The specific binding of \[^3H\]yohimbine reached equilibrium in 15 minutes and remained stable for at least 20 more minutes (data not shown).

Catecholamines displaced both \[^3H\]prazosin and \[^3H\]yohimbine with the expected \(\alpha\)-adrenergic potency order (Table 3). Also, unlabelled prazosin was almost 2,400 times more potent in competing at the \[^3H\]prazosin binding sites than at the \[^3H\]yohimbine sites, whereas yohimbine was about 120 times more potent at the \[^3H\]yohimbine binding sites than at the \[^3H\]prazosin sites (Table 3), suggesting that \[^3H\]prazosin and \[^3H\]yohimbine were labelling \(\alpha_1\)- and \(\alpha_2\)-receptors, respectively. Also, the slopes of these antagonist-competition curves were not different from unity, confirming binding to a homogeneous class of sites for each radioligand.

Influence of Pheochromocytoma on \[^3H\]Prazosin and \[^3H\]Yohimbine Binding

We examined the possibility that an alteration in \(\alpha_1\)- or \(\alpha_2\)-receptor number or affinity might be associated with the desensitization of vascular contraction induced by pheochromocytoma. Saturation binding experiments performed with mesenteric artery membrane preparations from rats with pheochromocytoma (early stage) showed a 36% decrease in total \[^3H\]prazosin binding (control = 234 ± 19 fmol/mg protein, PHEO rats = 150 ± 12 fmol/mg protein; p < 0.01, n = 4), but the affinity of \[^3H\]prazosin binding was not altered by the presence of the tumor (control, \(K_D = 0.65 ± 0.1\) nM; PHEO rats, \(K_D = 0.56 ± 0.1\) nM, n = 4). The total number of \[^3H\]yohimbine binding sites, on the other hand, was not significantly altered by the presence of pheochromocytoma. The number of \[^3H\]yohimbine binding in controls and in PHEO rats were 453 ± 34 fmol/mg and 486 ± 47 fmol/mg protein (p > 0.05), respectively. The \(K_D\) of the \(\alpha_2\)-receptors for \[^3H\]yohimbine was, likewise, not altered in PHEO rats (28.3 ± 4.2 nM) compared with that of the controls (32.6 ± 6.5 nM; n = 4 each).

**Discussion**

Utilizing pithed preparations of rats harboring pheochromocytoma, we have examined the effects of chronic in vivo exposure to increased concentrations of catecholamines on vascular responsiveness. In both early (3–4 weeks after implantation) and late (6–7 weeks after implantation) stages of tumor development, New England Deaconess Hospital rats with transplanted pheochromocytomas developed hypertension and tachycardia and had dramatically increased concentrations of plasma dopamine and nor-epinephrine. Pithing caused a more marked drop of blood pressure in the hypertensive tumor-bearing rats than in age-matched, unimplanted rats. The pressor response to \(\alpha\)-adrenergic agonists was found to be progressively diminished with time in the pithed tumor-harboring rats. Also, the desensitization in the vascular system of the rats with pheochromocytoma was ho-
FIGURE 8  Pressor response to stimulation of complete sympathetic outflow in pithed rats. Three groups of animals were studied: age-matched controls (O), early stage (△), or late stage (△) pheochromocytoma-bearing rats. Ordinate: increase in diastolic blood pressure (DBP). Abscissa: frequency of electrical stimulation Each point with a vertical bar represents mean ± SEM of 5-6 experiments. Asterisks: significant difference (p < 0.05) from corresponding values obtained from age-matched NEDH rats.

mological for α-adrenergic receptor-mediated contraction in the early stage of the disease, while it became heterologous in the late stage. Radioisotope binding studies showed that the α-adrenergic receptor number decreased 36% in mesenteric artery plasma membranes from rats with pheochromocytoma, while α-receptor number was unaltered.

The results of hemodynamic studies before and after pithing suggest the possibility that the sympathetic nervous system (SNS) is actively involved in the maintenance of hypertension in the NEDH rat model of pheochromocytoma. In several other experimental models of secondary hypertension, such as deoxycorticosterone-salt (DOCA-salt) and renal hypertension,28 an increase in sympathetic nerve activity has been related to the maintenance of hypertension. On the other hand, in pheochromocytoma, one might expect that sympathetic activity would be depressed reflexly due to the high circulating catecholamines. However, it has been suggested that the SNS may play a role in the maintenance of hypertension in patients with pheochromocytoma since clonidine, an antihypertensive agent that acts by decreasing SNS activity, was effective in lowering blood pressure.9,10 Also, Johnson et al12 have reported that sympathetic nerve impulse activity was elevated paradoxically during chronic catecholamine infusion. Additionally, we recently observed that both clonidine and chlorisondamine, a ganglion blocker, markedly decreased blood pressure in intact rats with pheochromocytoma (P. Prokoheimer, M. Maze, and B.B. Hoffman, unpublished observations). In the present study, eliminating SNS activity by pithing caused a markedly greater reduction of blood pressure in pheochromocytoma-bearing rats, and the blood pressure in pithed pheochromocytoma rats was found to be further suppressed by an α-adrenergic antagonist phentolamine. These observations suggest that not only high concentrations of circulating catecholamines but also intact sympathetic nerve function are involved in the maintenance of the elevated blood pressure. Consequently, hypertension in pheochromocytoma is a complex process influenced by both the sympathetic nervous system and circulating catecholamines, as well as alterations in cardiovascular responses to catecholamines.

The desensitization of vascular responsiveness in rats with pheochromocytoma was found to increase with time. In the early stage of tumor development, the desensitization is characterized by a specific loss of α-adrenergic receptor-mediated vasopressor response ("homologous desensitization"), but in the later stage, the desensitization becomes more general ("heterologous desensitization"). The possibility that early changes in the sensitivity of vessels specific to α-adrenergic agonists are related to the hypertension associated with pheochromocytoma rather than catecholamine production by the tumor is unlikely since the alterations in the α-adrenergic agonist/smooth muscle interaction observed in PHEO rats is different from that observed in several other animal models of hypertension.28 On the other hand, the nonspecific alteration of the vascular responsiveness observed in the late

FIGURE 9  Saturation of [3H]prazosin (top panel) and [3H]yohimbine (bottom panel) binding sites from the plasma membrane of rat mesenteric artery. The membrane preparations for experiments and assays are described in "Materials and Methods." Specific binding (●) is the difference between total binding (○) and nonspecific binding (△) determined in the presence of 10 μM phentolamine. Number of α-receptors labelled by [3H]prazosin was 236 fmol/mg protein and by [3H]yohimbine was 518 fmol/mg protein, respectively. The experiments shown are representative of 3 such experiments.
Stage may be partially associated with adaptive vascular structural changes that result from chronic hypertension. There is experimental evidence suggesting that chronically elevated vascular tension results in striking desensitization in vitro. Also, we have found that isolated aortic ring segments from rats with pheochromocytoma exhibited heterologous desensitization in vitro. Desensitization was found in the vascular system of rats with pheochromocytoma for both α₁- and α₂-receptor-mediated contraction. However, the pattern of desensitization was different for both receptors. The desensitization of the α₁-adrenergic receptor-mediated pressor response in the early stage PHEO rats is characterized by a rightward shift in agonist dose-response curves with an intact maximal response. On the other hand, desensitization of the α₂-adrenergic receptor-mediated pressor effect exhibits a blunted maximal response. One possible explanation for this different pattern of desensitization is that there is greater receptor reserve for α₁-receptor-mediated contraction. Of particular interest is the recent finding of the difference in receptor reserve for the postjunctional vascular α₁- and α₂-adrenergic receptors, which is modulated by presynaptic neural pressor responses in the pithed rat. Examining the occupancy-response relation by the phenoxylbenzamine inactivation method, Ruffolo and Yaden have recently demonstrated that a large receptor reserve exists for postjunctional vascular α₁-adrenergic receptors but not for postjunctional vascular α₂-adrenergic receptors in pithed rats. These workers observed that a maximum pressor response induced by α₁-agonists occurred when only a fraction of the receptors were occupied. However, for α₂-receptors, there was a linear relation between receptor occupancy by agonists and response, indicating a lack of receptor reserve. In view of these results, receptor or postreceptor alterations that may occur after prolonged exposure to catecholamines released by pheochromocytoma might be predicted to have a greater effect in maximal responses to α₂-agonists rather than α₁-agonists. Consequently, it is possible that these results can be explained by differences in receptor reserve although other factors may also be involved.

Our radioligand studies have quantitated the number of α₁- and α₂-receptors in rat mesenteric artery plasma membranes, using [3H]prazosin and [3H]yohimbine, respectively, and are in agreement with previous results in this tissue. Also, there is agreement between our receptor-binding studies and measurement of pA₂ values for prazosin (pA₂, 9.7–9.9) and yohimbine (pA₂, 6.8–7.3) at α₁- and α₂-receptors in experiments measuring contraction of rat mesenteric artery, suggesting these binding sites are of physiologic significance. Another important consideration with respect to these binding sites is the possibility of contamination of the plasma membrane fraction by presynaptic neural elements since mesenteric artery is a densely innervated tissue. Agrawal and Daniel observed that in 6-hydroxydopamine-denervated rats, the Bmax and the Kᵦ of the [3H]prazosin binding was unaffected, but the number of [3H]yohimbine binding sites was reduced by approximately half without any change in the affinity, suggesting that sympathectomy lowers the number of yohimbine binding sites either by removing contaminating neural membranes or by modulating the number of postjunctional sites. Thus, possible contamination of the plasma membrane fraction by presynaptic neural elements in the [3H]yohimbine binding sites cannot be neglected, and this must be kept in mind when evaluating the results involving receptor measurements in vascular smooth muscle.

In the present study, chronic in vivo exposure to high circulating concentrations of catecholamines caused a reduction in the number of α₁-adrenergic receptors. Colucci et al previously reported that epinephrine injection of rats for 48 hours resulted in a 45% decrease in mesenteric artery α₁-adrenergic receptors. Similarly, Wikberg et al and Colucci and Alexander have observed that in vitro chronic exposure to norepinephrine of cultured rabbit aorta smooth muscle cells results in an up to 80% decrease in α₁-adrenergic receptor number without a change in norepinephrine binding affinity. Thus, changes in vascular α₁-adrenergic receptor number have been observed in response to increases in agonist concentrations and could partly account for the regulation of α₁-adrenergic responsiveness. However, it is likely that other types

<table>
<thead>
<tr>
<th>Competing drug</th>
<th>[3H]Prazosin binding sites</th>
<th>[3H]Yohimbine binding sites</th>
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<tbody>
<tr>
<td></td>
<td>Kᵦ (nM)</td>
<td>Hill slopes</td>
</tr>
<tr>
<td>(-)Epinephrine</td>
<td>1,600</td>
<td>0.61</td>
</tr>
<tr>
<td>(+)Norepinephrine</td>
<td>4,200</td>
<td>0.69</td>
</tr>
<tr>
<td>(+)Epinephrine</td>
<td>33,000</td>
<td>0.49</td>
</tr>
<tr>
<td>(-)Isoproterenol</td>
<td>&gt;100,000</td>
<td>0.63</td>
</tr>
<tr>
<td>Prazosin</td>
<td>25</td>
<td>0.98</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>28</td>
<td>0.96</td>
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</tbody>
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Membranes were incubated with either [3H]prazosin or [3H]yohimbine and varying concentrations of adrenergic agonists and antagonists. Competition curves were analyzed by computer as described in "Materials and Methods." Data shown are the mean values obtained in two or three experiments.
of regulation occur at one or more postreceptor steps that could lead to an alteration in the occupancy-response coupling relation. We have previously demonstrated that exposure of intact rabbit aorta to epinephrine for 7 hours results in a marked decrease in the sensitivity to norepinephrine-induced contraction without a decrease in the maximal contractile response. This desensitization of contraction was not due to a change in α, -adrenergic receptor number or affinity for agonists, but there was a blunting of norepinephrine-stimulated phosphatidylinositol turnover. Furthermore, it was recently observed that the coupling of α, -adrenergic receptor occupancy to calcium efflux in rabbit aortic smooth muscle cells can be altered by in vitro exposure to norepinephrine in the absence of a change in α, -adrenergic receptor number or affinity. This result raises the possibility that an alteration in occupancy-response coupling at a step proximal to Ca++ mobilization, rather than a reduction in receptor number, may be of primary importance in the process of agonist-induced α, -adrenergic receptor desensitization of vascular smooth muscle cells. Therefore, not only is the down-regulation of receptors but also some other agonist-induced alteration in occupancy-response coupling may contribute to the desensitization of α, -adrenergic receptor-mediated vasoconstrictor responses demonstrated in the vascular system of pheochromocytoma rats. Such postreceptor alterations may be of primary importance for desensitization of the α,-adrenergic receptor-mediated vasoconstrictor response since we did not detect any change in the number of these receptor sites.

In contrast to α, -adrenergic receptors, very little information is available regarding the mechanism by which postjunctional vascular α, -adrenergic responsiveness is regulated. Development of selective tolerance to α, -adrenergic agonists has been demonstrated in the vascular system of the rat after chronic treatment with clonidine. Our present results show that in vivo desensitization of the postjunctional vascular α, -adrenergic receptor-mediated vasoconstrictor response can be caused not only by high concentrations of synthetic agonists but also by endogenous catecholamines. We found that desensitization develops without any change in the number of α, -receptors. The finding that agonist-induced selective desensitization of α, -adrenergic receptor-mediated physiologic responses can occur without any detectable alteration in the number of α, -adrenergic receptors, as measured in radioligand binding experiments, has been reported for α, -receptors in human platelets.

It is notable that pressor responses to exogenously administered norepinephrine and to the higher frequencies of electrical stimulation of the complete sympathetic outflow are relatively intact in the early stage of pheochromocytoma rats, while in the same animals, pressor responses to separately administered specific α, - and α, -adrenergic agonists were markedly reduced. Since β-adrenergic and muscarinic-cholinergic receptors were blocked by (±)propranolol and atropine, respectively, the pressor responses induced by these sympathomimetic drugs are due to stimulation of α, -adrenergic receptors on vascular smooth muscle. Also, it has been demonstrated that vascular responses to endogenous and exogenous norepinephrine stimulate both α, - and α, -adrenergic receptors, although the relative contribution of each subtype may be different. Our present results, therefore, imply that neuronally released or exogenously administered higher concentrations of norepinephrine can produce pressor response to the same extent as controls even when there are reduced individual activities of α, - and α, -adrenergic receptors. Alabaster and Davey observed that a combination of selective α, - and α, -antagonists caused a greater shift of the norepinephrine vasoconstrictor dose-response curve to the right than occurred with either antagonist alone. This result suggests that norepinephrine can interact with α, - or α, -adrenergic receptors and still elicit a certain vasoactive effect even after selective blockade of the alternate subtype. This potentially additive effect of norepinephrine might explain our observation of the relatively intact nerve-induced or exogenously applied norepinephrine-induced pressor response in pheochromocytoma rats.

This study demonstrates that the α, -adrenergic receptor-mediated vasoconstrictor responses can be modulated in response to prolonged stimulation by high levels of circulating catecholamines in pheochromocytoma. These alterations might be relevant to problems relating to vascular instability in pheochromocytoma patients. In this respect, the rat with pheochromocytoma that secretes large amounts of norepinephrine is not only a good experimental model of the human disease but also provides a valuable model for studying the cardiovascular consequences of in vivo chronic exposure to catecholamines. Animals with transplant ed pheochromocytoma should offer a useful model to explore the pathogenesis for these less well understood in vivo events as well as give an ideal opportunity to study the potential reversibility or prevention of these changes.

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