Lung β-Adrenoceptors in Pulmonary Hypertension*

A Study of Biopsy Specimens in Children with Congenital Heart Disease

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Characteristics of β-adrenoceptors were analyzed using radioligand-binding techniques with 3H-dihydroalprenolol in lung specimens from 11 children with pulmonary hypertension (median age, three years) undergoing surgical repair of congenital heart defects and four pediatric control subjects (median age, five years) undergoing thoracotomy for removal of neoplasms or cysts. Scatchard analysis of 3H-DHA binding to lung membranes showed similar values of the dissociation constant in both groups (Kd = 0.72 ± 0.22 nM in patients vs 1.22 ± 0.22 nM in controls; p = NS). The receptor density was significantly increased in patients in comparison with controls, with respective values of 164 ± 10 and 95 ± 13 fmol/mg of protein (p < 0.025), and correlated directly with mean pulmonary arterial pressure (r = 0.82; p < 0.0005). No significant relationship was observed between receptor number and pulmonary arterial medial thickness. Thus, the increase in receptor density in these patients may be related to adaptive changes in cells other than vascular smooth muscle. (Chest 1991; 99:637-41)

\[ 3H-DHA = \text{tritiated dihydroalprenolol}; B_{max} = \text{maximum binding capacity}; K_d = \text{dissociation constant} \]

Beta-adrenoceptors have been demonstrated to modulate many important functions in the lung, including the control of airway and vascular smooth muscle tone, fluid and protein exchange, ion transport, surfactant secretion, release of inflammatory mediators, and microvascular permeability.1-3 Radioligand-binding techniques have made it possible to accurately investigate the receptor characteristics in important pathologic conditions such as chronic obstructive pulmonary disease4,5 and essential hypertension.6,7

As yet, no studies on adrenergic receptors in children with pulmonary hypertension have been performed. We hypothesized that changes in lung β-adrenoceptor characteristics could be associated with the well-known functional and structural abnormalities present in these children. Changes in receptor number or affinity would lead to further vascular tone imbalance and could result in many other functional abnormalities, as stated previously.

Using radioligand-binding techniques with 3H-dihydroalprenolol, we examined the β-adrenoceptor characteristics in lung specimens from children with moderate to severe pulmonary hypertension secondary to congenital heart disease. Results were correlated with hemodynamic data and structural vascular changes observed on histologic examination.

**Materials and Methods**

**Subjects and General Diagnostic Procedures**

Characterization of lung β-adrenoceptors was carried out in 11 children with pulmonary hypertension aged four months to 15 years (median, three years) who were considered candidates for surgical repair of congenital heart defects at the Heart Institute, University of São Paulo. In these children, small lung specimens for receptor characterization were obtained at the time of routine biopsy procedures in order to evaluate the extent of pulmonary vaso-occlusive disorder. At cardiac catheterization the mean pulmonary arterial pressure was above 30 mm Hg in all patients. Measurements were also performed in the presence of vasodilator stimulus. For this purpose, 100 percent oxygen and tolazoline hydrochloride (1 mg/kg) were used. The associated congenital cardiac defects were as follows: ventricular septal defect, isolated (four patients) or associated with patent ductus arteriosus (two patients) or mitral stenosis (one patient); endocardial cushion defect (two patients); transposition of the great arteries (one patient); and isolated patent ductus arteriosus (one patient). The control group included four pediatric subjects aged seven months to eight years (median, five years) without any clinical evidence of pulmonary hypertension. In these subjects a small lung specimen distant from any grossly abnormal area was obtained at thoracic surgery for removal of neoplasms or cysts. Mean pulmonary arterial pressure in the control subjects ranged from 12 to 19 mm Hg. All procedures were carried out after informed consent. The study protocol has been approved by the Scientific Committee on Human Research of the Heart Institute, University of São Paulo.

**Processing Lung Specimens**

Lung tissue for both histologic analysis and receptor characterization was obtained no later than one week after hemodynamic evaluation. None of the subjects had received β-adrenergic agonists,
antagonists, or corticosteroids within two weeks of obtaining the lung specimens. In patients undergoing surgical repair of congenital heart defects, lung biopsy specimens were obtained at the beginning of the operation, in order to avoid the interference of hypothermia and cardiopulmonary bypass on receptor characteristics. The lung biopsy procedure and tissue preparation for histologic examination followed previously reported techniques. Briefly, inflated specimens of 1 × 2 × 0.5 cm were submerged for 24 hours in formaldehyde solution (10 percent Formalin). After routine paraffin embedding, 4-μm to 6-μm sections were stained with hematoxylin-eosin, Masson trichrome, and elastic Verhoeff stain. Pulmonary vascular lesions were classified according to the criteria of Heath and Edwards. The amount of pulmonary arterial smooth muscle was assessed morphometrically by measuring the arterial wall thickness. Results were expressed as mean percentage wall thickness of terminal bronchiolar, respiratory bronchiolar, and alveolar duct arteries as follows:

\[
\text{% wall thickness} = \frac{2 \times \text{wall thickness}}{\text{external diameter}}
\]

The extent of intimal lesions was expressed as the percentage of preacinar and intra-acinar arteries in each specimen showing intimal proliferation.

**Lung Membrane Preparation**

Peripheral lung tissue (including terminal bronchioli, respiratory bronchioli, alveolar ducts, alveoli, and blood vessels) was homogenized in ten volumes of sucrose solution (0.32 mol/L), in TRIS-HCl buffer (pH 7.5) using a homogenizer (Polytron 10ST). Unhomogenized debris were removed by centrifugation at 500 g for ten minutes (Sorvall RC2-B centrifuge). The supernatant was centrifuged at 50,000 g for 15 minutes (Beckman LS-50 centrifuge), and the resulting pellet was washed in 50 mM TRIS-HCl, 1 mM N-ethylmaleimide, and 1 mM phenylmethyl-sulfonil fluoride (pH 7.5) and centrifuged again. After filtration through a fine nylon mesh, the final pellet was resuspended in the assay buffer at a concentration of approximately 1 mg of protein per milliliter.

**Binding Assay and Specificity Experiments**

Membrane suspensions (100μg) were incubated at 25°C for 15 minutes with 100μl of [3H]-DHA (specific activity, 75 Ci/mmol; from the Radiopharmaceutical Centre, Amersham). Final incubation mixtures contained increased concentrations of [3H]-DHA from 0.5 to 5 nmol/L in the absence and presence of the (±)-propranolol (2μmol/L; Amersham), in order to determine nonspecific binding. Incubation was terminated by vacuum filtration, and membranes were isolated on glass filter filters (Whatman GF/C). Each filter was washed with ice-cold buffer and counted for radioactivity in scintillation fluid (Beckman LS 100C beta counter). Specific binding was determined as the total counts minus nonspecific binding, in the presence of unlabeled propranolol. All binding experiments were carried out in duplicate. The Bmax and the Kd were determined by the analysis of Scatchard plots of the binding curves.

Specificity experiments were performed by the same methods, using 100μl of the membrane suspension and 100μl of [3H]-DHA at the concentration of 5 nmol/L, in the absence and presence of a large excess (500×) of unlabeled competitors: (±)-propranolol (Amersham); (±)-alprenolol; (±)-epinephrine; (±)-norepinephrine (Sigma); atenolol (Wellcome-ICI); and prazosin (Pfizer).

**Statistical Analysis**

The Kd and Bmax values in the different groups were analyzed using the Mann-Whitney rank-sum test. Because of the relatively small size of the pediatric control group, we were not able to assume a normal distribution of the variables under investigation. The correlations between receptor density and age, pulmonary pressures, response to vasodilators, and the amount of vascular smooth muscles were evaluated using regression analysis of data. In all tests a significance level of 0.05 was assumed. Results are reported as the mean ± SE.

**Results**

**Hemodynamic and Histologic Findings**

The mean pulmonary arterial pressure ranged from 33 to 101 mm Hg in children with pulmonary hypertension (Table 1).

In histologic examination of lung biopsy material, increased arterial muscularity was observed in all patients. The medial thickness of terminal bronchiolar, respiratory bronchiolar, and alveolar duct arteries was 21.8 ± 1.8, 22.8 ± 4.3, and 20.1 ± 4.1 percent, respectively.

**Table 1—Clinical, Hemodynamic, and Biochemical Data from Children with and without Pulmonary Hypertension**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Defect</th>
<th>Cardiac PAP</th>
<th>PAP-PAW</th>
<th>β-Adrenoceptor Density, fmol/mg of protein</th>
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<td></td>
<td></td>
<td>mm Hg</td>
<td>mm Hg</td>
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<tr>
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<td>PDA</td>
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<td>29</td>
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<tr>
<td>2</td>
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<td>30</td>
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<tr>
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<td>11mo</td>
<td>VSD</td>
<td>35</td>
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<tr>
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<td>33</td>
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<tr>
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<td>96</td>
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<tr>
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<td>TGA</td>
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<td>26</td>
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<tr>
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<td>19mo</td>
<td>VSD/MS</td>
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**Controls**

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*PAP and PAP-PAW, Mean pulmonary arterial pressure and transpulmonary pressure gradient, respectively; ECD, endocardial cushion defect; PDA, patent ductus arteriosus; TGA, transposition of great arteries; VSD, ventricular septal defect; MS, mitral stenosis.

Figure 1. Lung biopsy section from 15-year-old patient with severe pulmonary hypertension, showing artery with plexiform lesion at entrance of pulmonary aicuni (arrow). Parent vessel shows important intimal hyperplasia. This patient had mean pulmonary arterial pressure of 101 mm Hg and β-adrenoceptor density of 300 fmol/mg of protein (Verhoeff elastic stain, original magnification × 200).

Beta-adrenoceptors in Pulmonary Hypertension (Lopes et al)
tively. Intimal lesions compatible with Heath-Edwards grade 2, 3, or 4 were observed in seven patients. The percentage of terminal bronchiolar and respiratory bronchiolar arteries showing intimal proliferation ranged from 15 to 39 percent. Plexiform lesions were observed in two patients (Fig 1). In all biopsy specimens, no significant microscopic parenchymatous abnormalities were observed regarding alveolar septa and bronchial structures.

\[3H-DHA\] Binding to Lung Tissue

Binding of \(^{3}H\)-DHA to lung membranes was saturable, reaching a plateau at an average ligand concentration of 5 nM, with specific binding representing 60 percent of total counts (Fig 2). In all patients, as well as in control subjects, Scatchard analysis of data showed a single class of high-affinity binding sites. The order of displacement of \(^{3}H\)-DHA binding by unlabeled adrenergic competitors (agonists and antagonists), which is represented in Figure 3, indicated the predominance of \(\beta_2\)-adrenergic receptors.

Scatchard analysis of \(^{3}H\)-DHA binding to lung tissue showed similar Kd values in both patients and controls (Kd = 0.72 ± 0.22 and 1.22 ± 0.22 nM, respectively; p = NS); however, the Bmax was significantly increased in children with pulmonary hypertension (Table 1), in comparison with values from those with normal pulmonary pressure (Bmax = 164 ± 19 vs 95 ± 13 fmol/mg of protein; p < 0.025). The relationships between Bmax and hemodynamic variables are represented in Figure 4. The Bmax was significantly influenced by the mean pulmonary arterial pressure, both in the whole group of 15 pediatric subjects (r = 0.82; p < 0.0005) and within the group of 11 patients (r = 0.80; p < 0.005). The receptor number also correlated with the transpulmonary pressure gradient (r = 0.88; p < 0.0005). A negative correlation was observed between Bmax and the magnitude of pulmonary vascular response to vasodilators (r = -0.73; p < 0.01). No significant decrease (>10 percent of baseline value) in systemic arterial pressure was observed during tolazoline administration. In children with pulmonary hypertension, Bmax was also influenced by age (r = 0.76; p < 0.005). No significant correlation was observed between Bmax and arterial medial thickness. Interestingly, the highest Bmax value was observed in a 15-year-old patient with mean pulmonary arterial pressure of 101 mm Hg and severe plexiform lesions in

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**Figure 2.** Graphic representation of \(^{3}H\)-DHA binding to lung membranes. **A (left).** Binding of \(^{3}H\)-DHA as function of ligand concentration. TB, NB, and SB represent total, nonspecific, and specific binding, respectively; NB was obtained in presence of 2μM (±)-propranolol; SB was determined as difference between TB and NB. **B (right).** Scatchard analysis of data corresponding to specific \(^{3}H\)-DHA binding; B/F is ratio of bound to free, represented as function of bound \(^{3}H\)-DHA. Slope of regression line and x-axis intercept correspond, respectively, to -1/Kd and Bmax. In this case, Kd = 0.29 nM, and Bmax = 93 fmol/mg of protein.

**Figure 3.** Representative competition of unlabeled adrenergic agonists and antagonists for \(^{3}H\)-DHA binding sites on lung membranes. Determinations were carried out using \(^{3}H\)-DHA at concentration of 5 nM in presence of large excess (500 x) of unlabeled competitors. Absence of significant \(^{3}H\)-DHA displacement by atenolol (\(\beta_1\)-adrenergic antagonist) and prazosin (\(\alpha_1\)-adrenergic antagonist) indicates predominance of \(\beta_2\)-adrenoceptors.
pulmonary vessels (Fig 1).

**DISCUSSION**

A major limitation of the present study was the relative difficulty in obtaining lung specimens for receptor characterization from children with normal pulmonary pressures. For ethical reasons, we were only able to include in the study subjects from whom lung specimens could be obtained during routine diagnostic or therapeutic procedures.

Information concerning adrenoceptor behavior in pulmonary vaso-occlusive disorders remains limited. Most reports focusing on β-adrenoceptor alterations in lung disease include subjects with chronic airways obstruction. Although some degree of heightened pulmonary arterial pressure might certainly have occurred in many subjects in these studies, results cannot be easily compared with ours because of remarkable differences in the mechanisms of the disorders under investigation.

The influence of age on lung β-adrenoceptor density in our patients is in agreement with previous observations in humans, as well as in other mammals.

Our results clearly suggest that lung β-adrenoceptor concentration correlates directly with the severity of pulmonary hypertension in children with congenital heart disease. We were not able to examine the relationship between receptor density and pulmonary vascular resistance, because this variable could not be obtained in all patients; however, β-adrenoceptor number significantly correlated with transpulmonary pressure gradient, which indirectly reflects peripheral resistance. In one patient with severe mitral stenosis, pulmonary arterial hypertension was essentially the consequence of a marked increase in mean left atrial and pulmonary wedge pressure of 30 mm Hg. In this patient the maximum 3H-DHA binding capacity of 100 fmol/mg of protein was not different from that observed in normal controls.

A further evidence that receptor concentration correlates with the severity of the disorder is the inverse relationship between Bmax and the magnitude of pulmonary vascular response to vasodilators, which represents the reversible component of pulmonary hypertension. The highest Bmax value (ie, 300 fmol/mg of protein) was observed in a patient with only an 8 percent fall in pulmonary arterial pressure after vasodilators. This patient had advanced pulmonary vascular damage and was considered unsuitable for cardiac surgery on the basis of Heath-Edwards grade 4 lesions observed on histologic examination.

Although it seems attractive to correlate the increase in receptor number with medial hypertrophy of pulmonary arteries, we did not observe such a relationship in our patients. Previous autoradiographic studies on β-adrenoceptor distribution in normal lung tissue have shown a much lower receptor concentration in the smooth muscle of airways and vessels than in other structures such as the epithelium of large and small airways, alveolar walls, submucosal glands, and vascular endothelium. It is reasonable therefore to suppose that receptor changes may be related to
cellular elements other than vascular smooth muscle.

Because no significant correlation was observed between receptor number and arterial medial thickness and because no important microscopic abnormalities were detected regarding alveolar septa and bronchial structures, we speculate that vascular intimal proliferation may have accounted at least in part for the increased receptor density. Autoradiographic localization of β-adrenoceptors in human lung has demonstrated that the receptor density over the vascular endothelium is high, with grain density ranging from 20 to 75 percent that of alveoli. It has also been reported that children with pulmonary hypertension have changes in surface characteristics and intracytoplasmic components of endothelial cells, which indicate enhanced metabolism in response to hemodynamic abnormalities. Furthermore, we observed intimal lesions in 15 to 39 percent of all terminal bronchiolar and respiratory bronchiolar arteries in seven patients.

Because lung β-adrenoceptors modulate many metabolic functions in the lung, the increase in receptor concentration may represent one of the several adaptive changes that occur in these patients. The present study is a preliminary attempt at investigating adrenoceptor behavior in children with pulmonary hypertension, and further studies will be necessary to determine the exact localization and biologic significance of receptor changes.

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