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C J Homcy, … , H W Strauss, S Kopiwoda

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Research Article

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Beta Receptor Occupancy

ASSESSMENT IN THE INTACT ANIMAL

CHARLES J. HOMCY, H. WILLIAM STRAUSS, and SUSAN KOPIWODA, *Cardiac Unit, Department of Medicine and the Division of Nuclear Medicine, Department of Radiology, Massachusetts General Hospital, and the Harvard Medical School, Boston, Massachusetts 02114*

ABSTRACT Organ uptake of ^{125}I-hydroxybenzylpindolol, a potent beta adrenergic antagonist, was determined after intravenous administration. Pretreatment with the beta agonist, epinephrine, inhibited an almost identical fraction of ^{125}I-hydroxybenzylpindolol binding as did the antagonist, propranolol. Specific beta receptor binding accounted for 50% of total uptake in the lung and demonstrated the following characteristics. The dose-response curve for propranolol inhibition of ^{125}I-hydroxybenzylpindolol binding duplicated that reported for its physiologic action. Simultaneous serum propranolol levels as determined by a sensitive radioimmunoassay allowed an apparent dissociation rate constant ~7 nM to be obtained that correlated closely with the results reported from membrane binding studies. Alpha blockade had no effect and inhibition of ^{125}I-hydroxybenzylpindolol binding by propranolol demonstrated stereospecificity. After chemical sympathectomy with reserpine or 6-OH dopamine, there was a 100% increase in receptor specific binding. Finally, a scintillation camera was employed to visually and quantitatively detect ^{125}I-hydroxybenzylpindolol displacement from the lung during intravenous propranolol administration in the living animal. Reversal of binding was rapid and an in vivo inhibition curve was generated. Such a method provides the potential for longitudinally assessing beta receptor occupancy and apparent affinity directly in man.

INTRODUCTION

Significant progress has been made in the identification and characterization of cell membrane receptors in the past decade. Compounds have been synthesized that can be labeled to high specific activity and will still bind to receptors with high affinity (1). These agents have been used primarily for the in vitro identification and localization of receptors in specific tissues. In the study of the beta adrenergic receptor, antagonists have been preferentially used in that they may demonstrate less nonspecific membrane binding and do not undergo chemical oxidation in the same manner as catecholamines (2–4). The synthesis, several years ago, of 3-indoloxy-1-(2-*p*-hydroxybenzylpropyl-2-amino) isopropanol, called hydroxybenzylpindolol (5), provided an agent that could readily be labeled with iodine and still maintain its high affinity binding to beta receptors with a reported dissociation rate constant (K_D)[1] of ~0.1 nM (6, 7). In vitro characterization of cell membrane receptors, however, does not provide data concerning receptor occupancy by endogenous agonists in the intact animal and consequently about mechanisms that may be important in the regulation of synaptic activity.

This study was undertaken to define the in vivo distribution of ^{125}I-hydroxybenzylpindolol and to determine whether this could be used to measure beta receptor binding in a particular organ. Specific binding was identified by comparing the distribution data from control animals with that from animals who had previously received the beta blocking agent, propranolol. To further validate that such changes in ^{125}I-hydroxybenzylpindolol distribution are related to beta receptor binding, several lines of evidence were generated. Agonist pretreatment with epinephrine was shown to inhibit ^{125}I-hydroxybenzylpindolol binding in a manner identical to that for the antagonist, propranolol. Propranolol inhibition of ^{125}I-hydroxybenzylpindolol binding followed a dose-response pattern that duplicated its physiological effects. An apparent K_D as determined from simultaneous serum propranolol levels was similar to that obtained from direct membrane binding studies. Alpha blockade had no effect on ^{125}I-hydroxybenzylpindolol binding. Propranolol-

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[1] *Abbreviation used in this paper*: K_D, dissociation rate constant.

inhibitable binding possessed stereospecificity. Furthermore, this approach detected changes in beta receptor occupancy produced by pharmacologic interventions such as chemical sympathectomy. Finally, specific beta receptor binding was externally quantitated in a living animal.

METHODS

Hydroxybenzylpindolol was supplied as a reagent grade chemical from Sandoz Ltd. (Basel, Switzerland). The compound was iodinated using a modification of the chloramine-T method (6). The labeling efficiency averaged 40%. The iodinated product was purified to a theoretical specific activity of ~2,000 Ci/mmol using a paper chromatographic technique previously described (6). The retardation factor (R_f) (0.50) of the iodinated product by thin-layer chromatography on silica gel plates in diethylamine:toluene (5:6) was similar to the starting material.

Biodistribution studies were performed in type CD-1 mice weighing 30–40 g, obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass. In all studies, the amount of ^{125}I-hydroxybenzylpindolol injected was ~1 pmol (activity of 1–2 μCi). The propranolol-treated animals received the indicated dose of propranolol intravenously either 10 or 15 min before the administration of ^{125}I-hydroxybenzylpindolol.

An initial study was performed in control and propranolol-pretreated animals to define the distribution of ^{125}I-hydroxybenzylpindolol over time. Tracer distribution was determined following sacrifice of the animals with an overdose of ether; samples of blood, kidney, heart, liver, spleen, stomach, small bowel, thyroid, pancreas, bone, fat, skeletal muscle, and brain were obtained, weighed, and counted in a gamma counter with a window of 20–60 keV. In addition to the tissue samples, an aliquot of injected dose was counted at the same time and results expressed as percent injected dose per organ. Data is expressed as mean±its standard duration. Comparison of groups was made using the Student t test.

In other experiments, agonist-inhibitable binding was determined following pretreatment with epinephrine. 50-μg doses of epinephrine were used because higher doses resulted in a significant mortality rate among the treated animals. To insure that the serum level of the drug was maximal, experiments were performed in which the epinephrine was administered simultaneously with the labeled hydroxybenzylpindolol. The same degree of inhibition (50%) of hydroxybenzylpindolol binding in the lung occurred as when the epinephrine was administered 15 min before hydroxybenzylpindolol injection. Reserpine-pretreated animals received three 15-μg doses intraperitoneally at 48, 36, and 24 h before the study; 6-OH dopamine was administered intraperitoneally in two doses of 5 mg per animal at 36 and 24 h before the study (8–10).

External counting. Imaging and external measurement of ^{125}I-hydroxybenzylpindolol in an anesthetized rabbit was accomplished with an Ohio-Nuclear 420/550 gamma camera (Ohio-Nuclear, Inc., Subsid. Technicare Corp., Solon, Ohio) using a low-energy parallel hole collimator. High voltage was maximized and a 50% window was used that included the gamma and x-ray emission of ^{125}I. Data was analyzed on a nuclear medicine data system VP450, Ohio-Nuclear, Inc. Regions of interest in the lungs were outlined with a light pen program. Curves representing pulmonary ^{125}I-hydroxybenzylpindolol activity vs. time after injection were then generated. Nonspecific binding was determined by measuring lung accumulation of ^{125}I-hydroxybenzylpindolol in control rabbits of identical weight that were pretreated with intravenous propranolol (1.5 mg/kg) 15 min before the injection of the label. The curves in Fig. 6 were computed after subtracting this value for each time point.

Radioimmunoassay. Antibodies that recognize propranolol with high affinity were raised in the following manner. The bromohydrin of alprenolol was synthesized according to the method of Hoebeke et al. (11). This was confirmed by nuclear magnetic resonance which showed loss of the olefin proton signal and by elemental analysis of the product (Galbraith Laboratories, Knoxville, Tenn.). The bromohydrin was then reacted with bovine serum albumin that had previously been modified by reaction with *N*-acetylhomocysteinethiolactone. This resulted in a product with 2 mol of alprenolol bound per mol of albumin (as later determined by radioimmunoassay of the hydrolyzed product). New Zealand White rabbits were immunized with 0.5 mg of this immunogen every 3 wk. Approximately 3–4 mo later, antiserum was harvested that bound both [^{3}H]alprenolol and [^{3}H]propranolol with an apparent K_D of 5 nM similar to that previously reported (11, 12). The final assay employed 100 μl sera, 50 μl 4 nM [^{3}H]propranolol (final concentration, 1 nM) and 50 μl of a 1:10 or 1:50 dilution of antiserum.[2] Separation of bound from free ligand was performed by a double antibody precipitation technique. In the following manner, it was also shown that these antibodies primarily recognized the propranolamine side chain. Thus 4-OH propranolol (an effective beta blocker), alprenolol, and propranolol were recognized with similar affinities despite their different ring structures (apparent K_D ~ 5 nM) whereas naphthoxyacetic acid, which possesses an identical ring structure to propranolol, but lacks the requisite side chain, was not recognized by this antibody class. Catecholamines were recognized as a result of the similarity of their ethanolamine side chain but with a 500-fold lower affinity. A variety of other drugs (aldomet, digoxin, and tricyclic antidepressants) did not react at concentrations at least 10,000-fold greater or not at all.

Catecholamine determination. Serum epinephrine and norepinephrine were measured by the radioisotopic enzymatic method of DaPrada and Zurcher (13), using an ethanolamine-chloroform-methanol solvent system. In this system, both compounds are O[^{3}H]methylated and then separated by thin-layer chromatography providing resolution from each other and from circulating precursors. Some hemolysis of samples occurred because of the inability to obtain adequate volumes from the same mice used in the above protocols. However, these measurements were aimed at simply showing a major reduction in catecholamine concentrations following these protocols. Norepinephrine levels in peripheral blood were in the range of 10 ng/ml with epinephrine concentrations averaging 1–3 ng/ml which, to some extent, represents the stress response that these animals undergo (14).

RESULTS

The blood disappearance of ^{125}I-hydroxybenzylpindolol was rapid, and at 5 min after administration in the control animals, <6% of the injected dose was present in the entire blood volume of the mouse. Data on the distribution of ^{125}I-hydroxybenzylpindolol are summarized in Fig. 1. The liver was the organ with the

[2] Homcy, C., S. Rockson, R. Cody, and E. Haber. 1980. A sensitive radioimmunoassay potentially applicable to all β-blocking drugs. American Heart Association Meeting. Accepted for presentation.

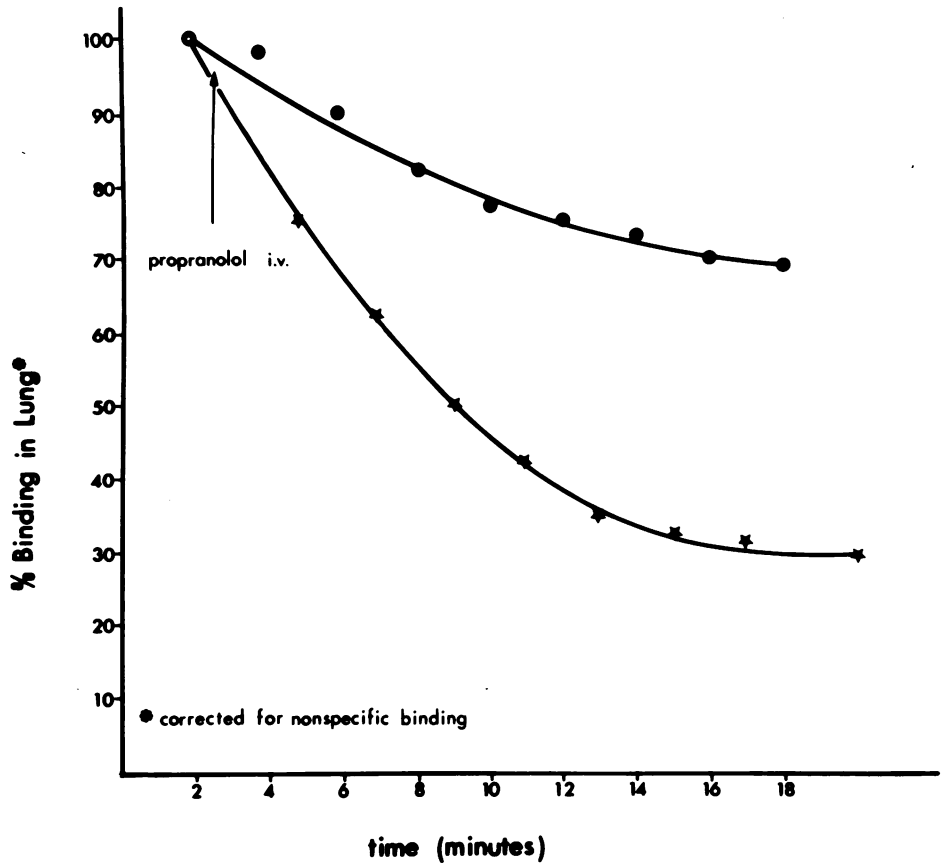

FIGURE 1 Mice were pretreated either with normal saline, D,L-propranolol (15 mg/kg), or epinephrine (1.5 mg/kg) in an identical volume (0.1 ml); 15 min later, ^{125}I-hydroxybenzylpindolol (1 μCi) was injected intravenously and 15 min thereafter, the animal was killed. The organs were removed and their activity determined in a Packard gamma counter (Packard Instrument Co., Inc.). At least three experiments, each with six mice in the control and six in the propranolol group, were performed. Activity (percent dose per organ) has been normalized with the control being 100%. Standard deviation bars are shown. Each bar had included within it the percent dose per organ under control conditions (lung, $P < 0.001$; spleen, $P < 0.05$; pancreas, $P < 0.01$; no significant changes were detected in other organs shown; $n = 18$). □, total; ▨, propranolol; ■, epinephrine.

highest concentration of radiopharmaceutical, followed by skeletal muscle, kidney, bone, and bowel. Over the course of time, the concentration in most organs except thyroid, fatty tissue, and small bowel decreased. In general, maximal uptake in all organs occurred within 5 min and gradually decreased over the next 25 min. The effects of propranolol were greatest in the lung, spleen, pancreas, thyroid, and blood. Approximately 50% of ^{125}I-hydroxybenzylpindolol binding in the lung was consistently blocked by propranolol whereas only 10–20% was consistently inhibited in these other organs. Therefore, further studies detailed below concentrated on ^{125}I-hydroxybenzylpindolol binding in the lung only.

It was expected that maximal agonist (epinephrine) inhibition of ^{125}I-hydroxybenzylpindolol binding would duplicate the effects of the antagonist, propranolol. Therefore, the same protocol was employed with the exception that the beta agonist, epinephrine, was used to define specific binding. As can be seen in Fig. 1, the same fraction and distribution of receptor specific binding resulted as had been obtained utilizing propranolol.

Inhibition of ^{125}I-hydroxybenzylpindolol binding by propranolol pretreatment was a saturable process and occurred at doses of propranolol at which the physiological effects of the drug are observed (15). A competitive inhibition curve relating serum propranolol concentrations to hydroxybenzylpindolol binding is shown in Fig. 2. It can be seen that 50% inhibition of hydroxybenzylpindolol binding occurs at a D,L-propranolol concentration of 7 nM. It should be noted

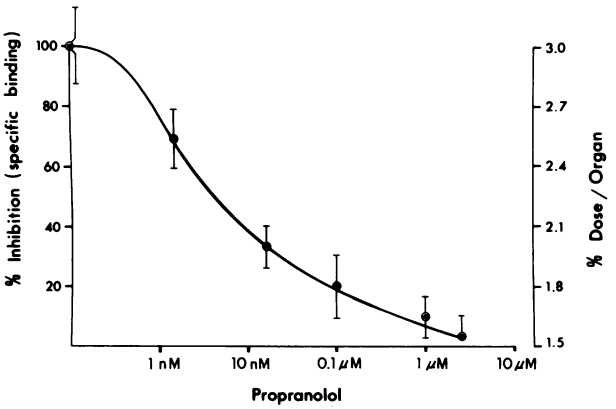

FIGURE 2 Doses of 0.001 mg/kg, 0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, and 1 mg/kg D,L-propranolol were used. The mean propranolol concentration from the three experiments was used for each dosage. The standard error at 0.001 mg/kg, 0.01 mg/kg, 0.05 mg/kg, and 0.1 mg/kg was <10% and at 1.0 mg/kg was <15%. Blood samples were collected before sacrifice and serum propranolol levels were determined by radioimmunoassay. Standard deviation bars for ^{125}I-hydroxybenzylpindolol binding are shown. Five animals were used at each dose in every experiment. The results from three individual experiments are included.

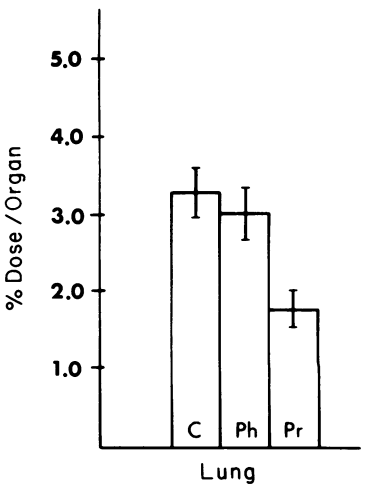

FIGURE 3 A similar protocol was also employed in these experiments with a phentolamine dose of 3.0 mg/kg and a propranolol dose of 1.5 mg/kg. The mean and its standard deviation is shown ($P < 0.001$ for propranolol-treated groups (Pr) vs. control groups (C); $P < 0.1$ for phentolamine-treated groups (Ph) vs. control groups; $n = 10$).

that the concentration of the active isomer is in the range of 3 to 4 nmol. These serum levels were achieved by increasing the dose from 0.001 to 0.1 mg/kg. Thereafter, a 10-fold increase in the dose to 1.0 mg/kg resulted in a serum level of 5 μM with no further inhibition of binding. Residual binding at this serum propranolol concentration was considered to represent nonspecific binding and was subtracted from total binding at each point to determine specifically bound counts.

Alpha blockade did not affect ^{125}I-hydroxybenzylpindolol binding. Fig. 3 demonstrates that high dose pretreatment with an alpha blocker, phentolamine (3 mg/kg), using the same protocol as that for propranolol, did not significantly alter lung ^{125}I-hydroxybenzylpindolol binding. A simultaneous experiment involving propranolol pretreatment is shown for comparison.

Specific ^{125}I-hydroxybenzylpindolol binding demonstrates stereospecificity as seen in Fig. 4. Doses of 0.1 mg/kg of L-propranolol inhibit ~45–50% of ^{125}I-hydroxybenzylpendolol binding, whereas the D-isomer blocks only 10% of the binding at a dose fivefold greater.

Additional studies were performed in chemically sympathectomized animals to determine whether changes in ^{125}I-hydroxybenzylpindolol binding in vivo might reflect alterations in beta receptor occupancy or number. Fig. 5 depicts the result obtained following such a chemical sympathectomy. In these experiments, groups of mice were treated either with reserpine or 6-OH dopamine. Then the typical protocol outlined previously was followed whereby ^{125}I-hydroxybenzylpindolol binding in saline-treated controls was compared with that in propranolol-treated animals. After the reserpine treatment, the concentrations of ^{125}I-hydroxybenzylpindolol in the lungs rose markedly as shown in Fig. 5. Furthermore, propranolol-inhibitable binding compromised all of this increase, consistent with the proposal that beta receptor binding was being delineated. Additional confirmation of this finding is that identical results were obtained after 6-OH

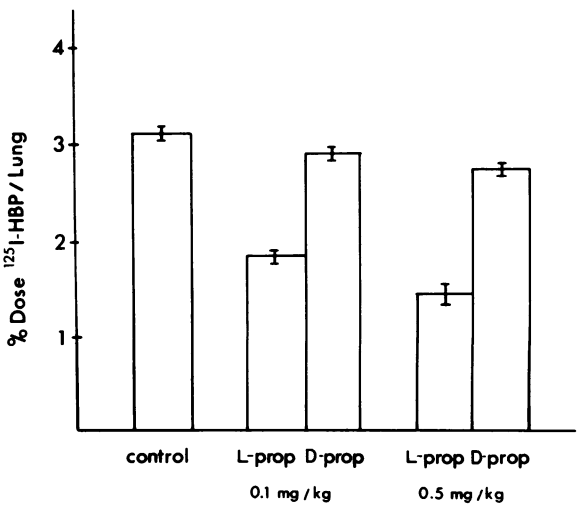

FIGURE 4 The protocol described previously was used with the doses of the individual isomers shown. The mean and its standard error is shown. Differences between L-propranolol-treated and control groups were significant at the $P < 0.001$ level ($n = 10$). HBP, hydroxybenzylpindolol.

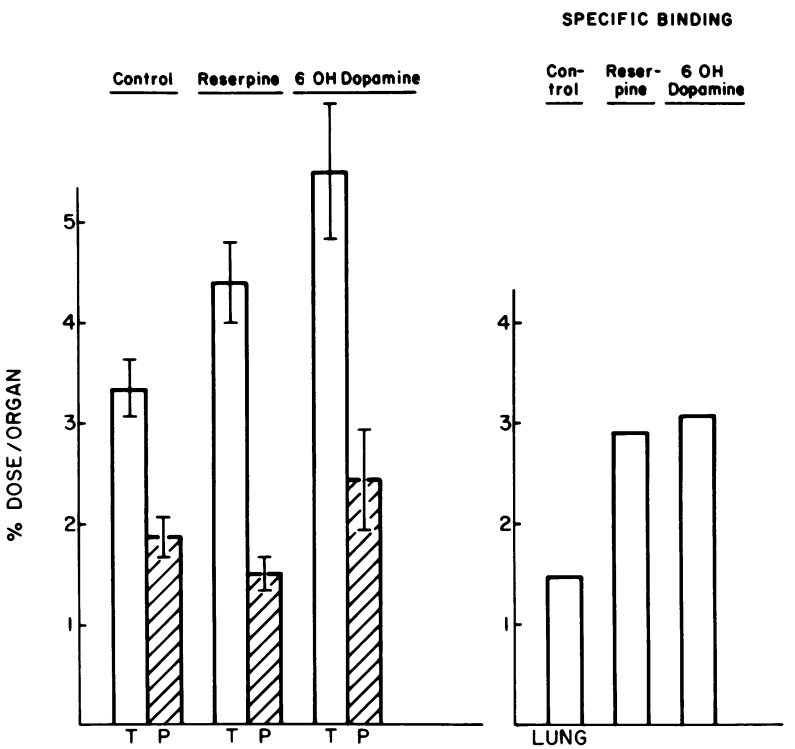

FIGURE 5 Mice were pretreated with either reserpine or 6-OH dopamine as described in Methods. The same protocol as described previously was then used. The first graph depicts data from control, reserpine, and 6-OH dopamine-treated animals with and without propranolol pretreatment (1.5 mg/kg). The second graph shows specific binding only for each of these groups. Differences between the propranolol-treated and control animals in each group were significant at the $P < 0.001$ level ($n = 10$).

dopamine infusion which produced a chemical sympathectomy via a different mechanism. Both drugs resulted in a significant depletion of norepinephrine levels measured from peripheral blood samples (reserpine 17% of control and 6-OH dopamine 19% of control). Reserpine also decreased serum epinephrine levels to 24% of control, whereas 6-OH dopamine actually resulted in an increase of 50%. However, epinephrine only accounted for 10–20% of total serum catecholamines.

Detection of beta receptor binding was also attempted in a living animal and an in vivo competitive inhibition curve was generated. A 3-kg rabbit was anesthetized and injected with 150 μCi of ^{125}I-hydroxybenzylpindolol via an ear vein. At 2 min after injection, the lungs were identified from the whole body image utilizing a scintillation camera and total counts were collected for 1 min from a defined area of interest within the lung fields. 2 min after the injection, a 200-μg bolus of propranolol was injected and again counts were collected for 1 min from the previously defined area of interest. This procedure was repeated every 2 min over a 20-min period. The counts remaining in the defined area of interest at each time point were first corrected for nonspecific binding as indicated in Methods and then expressed as a percentage of the initial activity recorded at 2 min after ^{125}I-hydroxybenzylpindolol injection and plotted vs. time. The count collection recorded from the initial 2-min image was consistently in the range of 2,500–3,000 cpm. A control curve from an experiment in which no propranolol was injected is shown for comparison. The greatest effect was seen after the first dose as might be predicted in that a relatively high serum propranolol concentration resulted.

DISCUSSION

The distribution of the beta antagonist ^{125}I-hydroxybenzylpindolol in the mouse in these experiments is significantly different from that of the ^{11}C-labeled catecholamine, norepinephrine, as studied by Ansari et al. (16). Ansari found the highest concentration of [^{11}C]norepinephrine in the heart, followed by lung, liver, and kidney. Because the binding of such agents could be based on either specific binding to the beta receptor or nonspecific binding to other sites on the cell membrane or within the cell, receptor binding was defined in our studies by comparing control binding

with that following pretreatment with the beta receptor antagonist propranolol. The most significant changes in the distribution of ^{125}I-hydroxybenzylpindolol following such pretreatment occurred in the lung with lesser changes in thyroid, spleen, and blood. These data indicate that the total organ distribution reflects primarily nonspecific binding.

We have not yet determined whether specific binding in the lung is primarily in vascular endothelium or parenchymal epithelium. That it does not represent binding to blood elements is based on the simple observation that a much greater degree of specific binding was present in the lung than either in blood or in a densely vascular organ such as spleen. In addition, Bylund et al. (17) have demonstrated in a perfused mouse lung model that the great majority of specifically bound ^{125}I-hydroxybenzylpindolol is associated with tissue elements other than blood. In contrast, the relatively low uptake in the heart and other organs as well as the lack of effect of propranolol pretreatment may be the result of a variety of factors. To some extent this probably reflects the relatively small fraction of the total cardiac output that these organs receive as compared with the lung. The actual dose of ^{125}I-hydroxybenzylpindolol administered is extremely small (~1 pmol) and thus the relatively high uptake in the lung as compared with the heart and other organs may reflect a bolus effect after intravenous administration. Furthermore, Bylund et al. (17) reported that nonspecific binding in heart tissue was significantly higher (approximately fivefold) than in lung, even when determined during in vitro binding experiments using crude membrane preparations. In particular, the high nonspecific uptake of ^{125}I-hydroxybenzylpindolol in the liver almost certainly reflects the role of this organ in metabolizing drugs of this nature. High first-pass extraction and metabolism of propranolol and other beta blockers by the liver is a well-known phenomenon.

Specific and nonspecific binding was also evaluated by Bylund et al. (17) in mice by comparing the tissue concentration of ^{125}I-hydroxybenzylpindolol 5 min after intravenous injection in control mice with that in mice treated with 1 mg/kg of propranolol. In those studies, specific binding was assessed by removing the tissues and performing in vitro analyses of ^{125}I-hydroxybenzylpindolol displacement by propranolol on crude membrane preparations. Using this approach, specific binding in the brain averaged 66%, whereas in the heart this was 58% and in the lungs 92%. Their results also indicate that as the amount of ^{125}I-hydroxybenzylpindolol injected was increased, specific binding decreased. Furthermore, such a method suffers in that some loss of receptor-bound label will occur as the equilibrium is disturbed during the minutes required for the preparation of even a crude membrane fraction. In an attempt to keep nonspecific binding to a minimum, the amount of ^{125}I-hydroxybenzylpindolol administered to each animal in our studies was held constant at ~1 pmol (1–2 μCi). We have also observed that by determining specific binding at earlier time points (5–10 min) after injection, one can consistently inhibit 50% of total binding with propranolol pretreatment. Our initial experiments, as demonstrated in Fig. 1, consistently indicated that 40–50% of total binding was specific when determined at 15 min after ^{125}I-hydroxybenzylpindolol administration. Later experiments using an earlier time point demonstrated a higher degree of specific binding in the 50% range (Figs. 2 and 4). The basis for this finding becomes more obvious after examination of the in vivo binding curves in Fig. 6. Most of the decrease in total binding over the first 10–20 min appears to represent depletion of the receptor-bound ligand pool.

The marked increase in specific binding in the lung after chemical sympathectomy could have been the result of an actual increase in the number of receptors although physiologic data suggest that this may occur over a longer time period (10). In vitro studies have documented this phenomenon of upregulation following either chemical sympathectomy with 6-OH dopamine which produced a decrease in tissue norepinephrine concentrations similar to ours (18), or after chronic receptor blockade (19). Alternatively, in simply depleting endogenous agonists, a greater fraction of the receptor population may have been available for ^{125}I-hydroxybenzylpindolol binding. Recently, a functional parallel for these observations in the lung was reported by Jengo et al. (20) who showed that a greater degree of supersensitivity to exogenous catecholamines occurred in the pulmonary vascular bed than in the systemic bed after chemical sympathectomy. This led them to postulate that the lung has a greater concentration of sympathetic nervous system innervation or of catecholamine receptors than does the systemic vasculature.

Zeleznik and Roth (21) have recently detected in vivo binding of ^{125}I-insulin to a high affinity receptor in rabbits. In results analogous to ours, administration of unlabeled insulin before injection of tracer decreased the distribution space of the labeled insulin, consistent with inhibition of binding resulting from prior saturation of available receptors with the unlabeled hormone. This process was dose dependent and saturable paralleling our observations with propranolol-mediated inhibition of ^{125}I-hydroxybenzylpindolol binding. Furthermore, by monitoring serum tracer levels they were able to detect apparent reversal of ^{125}I-insulin binding to its receptor during infusion of unlabeled insulin. Our findings utilizing external imaging techniques to quantitate ^{125}I-hydroxybenzylpindolol binding to pulmonary beta receptors are in line with their observations. The decrease in hydroxy-

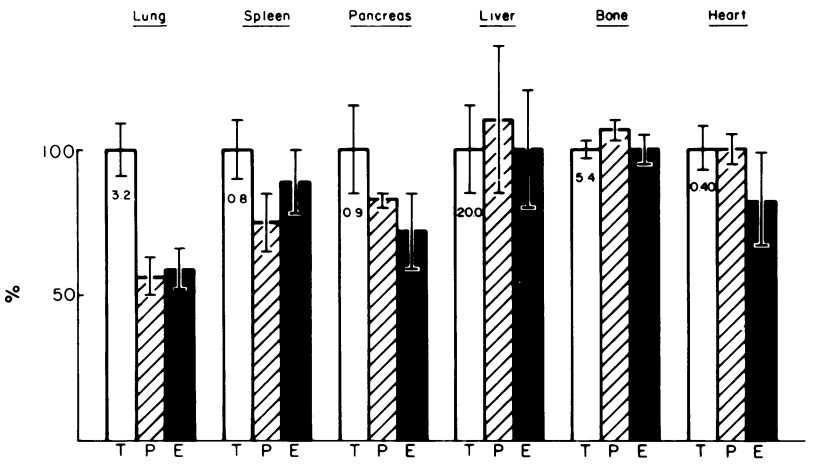

FIGURE 6 A 3-kg pentobarbital-anesthetized rabbit was injected with 150 μCi of ^{125}I-hydroxybenzylpindolol having previously been positioned under a parallel hole collimator. Counts were collected for 1 min from a defined area of interest within the lung using a light pen program. This procedure was repeated every 2 min in the control animal or after a 200-μg bolus of propranolol which was injected every 2 min between repeat images. The counts collected at each time point were first collected for nonspecific binding as indicated in Methods and then expressed as the percentage of activity recorded at the initial image 2 min after ^{125}I-hydroxybenzylpindolol injection and plotted against time. Nonspecific binding accounted for ~50% of total binding at the 2-min time point and remained relatively stable over the next 18 min. At the 20-min time point, it consistently amounted to 65–70% of total binding. Similar results were obtained in three separate experiments, (n = 3 for each group, control and treated). Plasma concentrations of hydroxybenzylpindolol were estimated to be in the range of 10 to 100 pmol during the first 10–15-min post intravenous administration as determined by sampling of blood aliquots for radioactivity. ●, control; ⋆, propranolol.

benzylpindolol binding following propranolol administration is probably best explained as reversal of ^{125}I-hydroxybenzylpindolol binding to lung beta receptors resulting from competition with the large excess of unlabeled propranolol.

Potential applications for this technique include the quantitation of beta receptor blockade and the longitudinal profiling of changes in receptor occupancy and apparent affinity of the intact animal. Our preliminary attempt at generating an in vivo competitive inhibition curve (Figs. 2 and 6) suggests that these are reasonable goals when coupled with simultaneous measurement of serum propranolol concentrations. The competitive inhibition curve obtained in mice indicates an apparent K_D for propranolol in the nanomolar range that is quite consistent with results obtained using purified membrane preparations (22). The inhibition curves obtained with external scanning techniques were aimed at showing that external quantitation of the displacement of receptor-bound ^{125}I-hydroxybenzylpindolol was feasible in a living animal and that it duplicated the results obtained in the propranolol-pretreated animals (Fig. 2). This technique can only complement in vitro binding studies that are necessary to directly measure receptor concentration and affinity. This method, however, adds a different dimension in that it may be a more direct measure of postsynaptic receptor activity than any presently available. Traditional approaches that have included measurements of plasma catecholamines, catecholamine turnover, and determination of degradation products (23, 24) suffer from the fact that they are quantitating events distant from the synapse or are a reflection of only a minor portion of synaptic activity (25).

ACKNOWLEDGMENTS

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