Characterization of Beta-Adrenoreceptors In Vivo with Iodine-131 Pindolol and Gamma Scintigraphy

Bernadette Hughes*, David R. Marshall, Burton E. Sobel, and Steven R. Bergmann

Cardiovascular Division, Washington University School of Medicine, St. Louis, Missouri

The aim of this study to was to assess the feasibility of using iodopindolol to delineate myocardial beta-adrenoreceptors in vivo. Preliminary biodistribution studies indicated that binding of ^{131}I-d,l-pindolol in the heart was stereospecific, saturable, and displaceable by l-propranolol but not by phenoxybenzamine. However, considerable nonspecific binding was encountered. Subsequently, the stereoisomer, ^{131}I-l-pindolol, was shown to be a high affinity beta-adrenoreceptor antagonist (Kd ~0.37 n*M*) as assessed by Scatchard analysis, and one exhibiting marked specific uptake in lung and heart in rabbits. In contrast, ^{131}I-d-pindolol exhibited no specific binding in rabbit left ventricular membrane preparations nor specific organ uptake. Gamma camera scintigraphy with both isomers demonstrated that the l-isomer accumulated in lung and heart, and that its accumulation was blocked by l-propranolol. In contrast, d-isomer uptake was nonspecific and diffuse. The results indicate that it should be possible to externally visualize receptors by differentiating specific and nonspecific binding components of a ligand in vivo with the use of radiolabeled stereoisomers.

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Alterations of beta-adrenoreceptor number and affinity have been implicated in cardiac disorders associated with ischemia (*1*), reperfusion (*2*), and congestive heart failure (*3*). Analysis of adrenergic density in these studies, however, has required the use of ventricular membrane preparations and procedures that destroy the natural environment of the receptor. Thus, noninvasive assessment of beta-adrenoreceptor density in vivo would be desirable.

Previous studies have demonstrated that it is possible to assess beta-adrenoreceptor occupancy and changes in beta-adrenoreceptor density with the beta-adrenoreceptor ligand iodine-125 (^{125}I) hydroxybenzylpindolol (*4,5*, Hughes et al: unpublished data). Although this ligand is a potent high affinity antagonist of beta-adrenoreceptors, it is highly lipophilic by virtue of its structure and subsequent iodination. Thus, it exhibits considerable nonspecific binding, limiting its use for in vivo noninvasive assessment of beta-adrenoreceptors (Hughes et al: unpublished data).

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For reprints contact: Steven R. Bergmann, MD, PhD, Cardiovascular Division—Box 8086, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110.

* Present address: Pharmacology Department, Wellcome Research Laboratories, Langley Park, Beckenham, Kent, UK.

Recently, iodopindolol ([^{125}I]PIN) has been characterized as a high affinity beta-adrenoreceptor ligand exhibiting minimal nonspecific binding in membrane preparation studies (*6,7*). The purpose of the present study was to determine whether [^{131}I]PIN could be used to noninvasively assess specific beta-adrenoreceptor occupancy in vivo.

MATERIALS AND METHODS

Labeling of Pindolol

Pindolol was iodinated with chloramine T and Na^{131}I* (*7,8*). The reaction mixture was extracted four times with ethyl acetate and the volume reduced under a stream of dry nitrogen. Iodine-131 PIN was purified with the use of high pressure liquid chromatography with a 10-μm PTS-PS-3C18*† reverse phase column developed by isocratic elution with acetonitrile:0.1*M* ammonium bicarbonate (1:1). Elution of [^{131}I]PIN was monitored by radiochromatography and uv spectrophotometry of 254 n*M*. Fractions eluting at ~4–5 min were pooled, extracted into ethyl acetate, dried under nitrogen, redissolved in methanol, and stored at −20°C. The average yield of [^{131}I]PIN was 10–20%. The l and d isomers of pindolol were iodinated similarly.

Receptor Binding Assay

To determine the binding specificity of the d and l isomers of [^{131}I]PIN conventionally, beta-adrenoreceptor binding assays were performed with rabbit left ventricular membrane preparations.

Left ventricles of New Zealand albino rabbits were minced and homogenized with a polytron[‡] in sucrose buffer (pH 7.5). Homogenates were centrifuged at 3,000 *g* for 10 min at 4°, and the supernatant fraction removed, strained through cheesecloth, and centrifuged at 19,000 *g* for 15 minutes at 4°C. The pellets were suspended in Tris buffer (pH 7.5, 50 m*M*) at 4° and centrifuged again at 19,000 *g* at 4°. After final resuspension, the concentration of protein (determined by the method of Lowry) was adjusted to 0.5 mg/ml. Left ventricles from three rabbits were pooled for each assay.

Membrane preparations (600 μl) were incubated in triplicate for 40 min at room temperature with selected concentrations (from 0.05–2.5 n*M*) of ^{131}I-l-PIN or ^{131}I-d-PIN (specific activity 650 μCi/mmol) with and without the displacer l-propranolol (10^{-6} *M*). After incubation, the membranes were filtered under vacuum. Filters were washed with 10 ml of buffer and radioactivity counted in a gamma content[§]. A minimum of six concentrations of ligand were utilized for each Scatchard analysis.

Biodistribution Studies

Rats. To assess the time course of binding, 21 anesthetized male Sprague-Dawley rats (200–300 g) were injected with ~0.1 pmol ^{131}I-d,l-PIN (~100 μCi) i.v. Animals were killed at selected intervals ranging from 3 min to 6 hr after injection of label. Tissues were weighed and radioactivity counted in a gamma well counter.

To assess specificity of binding, additional rats were pretreated with 2 mg/kg (n = 6) or 4 mg/kg (n = 4) of the alpha-adrenoreceptor blocking agent phenoxybenzamine or the beta-adrenoreceptor blocking agents atenolol [0.2 mg/kg (n = 6), 8 mg/kg (n = 4)], d or l propranolol [0.2 mg/kg (n = 6), 8 mg/kg (n = 6)] or ICI 118552 (a selective beta-2 antagonist) [0.2 mg/kg (n = 6), 8 mg/kg (n = 4)], 10 min prior to the injection of ^{131}I-d,l-PIN. All drugs were administered intravenously.

Rabbits. The tissue distribution of ^{131}I-d,l-PIN, ^{131}I-d-PIN, and ^{131}I-l-PIN was determined with and without drug pretreatment in a group of 12 New Zealand White rabbits (2 kg). The radiolabeled ligands were injected through an ear vein, and the rabbits were killed 5–7 min after injection. This time point was selected based on the preliminary studies in rats indicating the peak myocardial accumulation of label at 5–7 min.

Samples of blood, heart, lung, and liver were analyzed. All tissue samples were rinsed thoroughly in saline, blotted dry, and placed in preweighed tubes. Radioactivity in tissue samples was counted in a gamma well counter. Counts/g wet weight of tissue or blood were calculated, and results expressed as tissue:blood ratios.

Gamma Scintigraphy Studies

To determine whether differences in specific and nonspecific binding could be assessed by gamma camera scintigraphy, ten New Zealand white rabbits were anesthetized with thiopental (12.5 mg/kg) and alpha-chloralose (60 mg/kg). A femoral vein was cannulated for injection of labeled pindolol and administration of technetium-99m- (^{99m}Tc) labeled red blood cells (the latter for delineation of the vascular space). To access the specificity of binding, four rabbits injected with ^{131}I-l-PIN, and one injected with ^{131}I-d-PIN were pretreated with 0.2 mg/kg l-propranolol 10 min pror to injection of radiolabeled pindolol.

For scintigraphy, rabbits were placed in the supine position under a gamma camera[¶] with a large field-of-view [^{131}I]collimator. The camera's energy discrimination photopeak was set at 635 keV with a 20% window for ^{131}I and at 140 keV with a 20% window for ^{99m}Tc.

Immediately after i.v. injection of ^{131}I-l-PIN (400–500 μCi, 0.2 ~ 0.4 nmol) or ^{131}I-d-PIN (400–500 μCi, 0.2 ~ 0.4 nmol), 20-sec frames of data were collected for a total of 8–10 min. A total of 400,000–800,000 counts were obtained (50–100,000 counts/min). The camera was interfaced to a digital computer and scintigrams were digitized into a 64 × 64 matrix.

To accurately localize the aorta and heart, red blood cells were labeled with ^{99m}Tc as previously described (*9*). After ^{131}I scintigraphy, 1–2 mCi of ^{99m}Tc-labeled blood cells were injected and four static images of 50,000 counts each collected. Images were processed interactively by outlining regions of interest (ROIs) over the heart and aorta on the [^{99m}Tc]red cells scans and over the right lung, kidney, and liver on the ^{131}I-PIN scans. Counts/pixel/min for each region of interest during the dynamic collection were plotted from the scintigrams.

After first pass mixing, ^{131}I-PIN counts/pixel in the aortic ROI did not vary over the imaging interval (see Results) and were significantly lower than the ^{131}I counts in lung and heart. Thus, blood-pool activity was not subtracted. Results were expressed as tissue/blood ratios to correct for the amount of label injected.

Statistic Analysis

The Mann-Whitney U test for nonparametric data was used to test for significant difference between groups.

RESULTS

The specific activity of the ^{131}I-d,l-PIN, ^{131}I-d-PIN and ^{131}I-l-PIN varied between 600–1,100 μCi/nmol depending on the specific activity of Na^{131}I used. Pico-

nanomolar concentrations were administered for imaging and biodistribution studies. For the receptor-binding studies, appropriate dilutions were made such that an accurate determination of the affinity constant of the ligand for the receptor could be obtained.

Receptor Binding Assays

The specific binding of ^{131}I-l-PIN in the rabbit ventricle represents ~40–50% of total binding. Specific binding of ^{131}I-l-PIN was saturable (Fig. 1A), reversible, and stereospecific with a high affinity for the beta-adrenoreceptor (Kd = 0.37 ± 0.18 (s.d.) n*M*, n = 3 complete analyses, Fig. 1B). There was no cooperative binding.

Iodine-131-d-PIN demonstrated no affinity for the beta-adrenoreceptor in the concentrations studied (ranging from 0.05–10 n*M*, Fig. 1C). Thus, it labeled nonspecific binding sites.

Biodistribution Studies

Rats. Preliminary biodistribution studies were conducted with the labeled d,l-isomer. Maximum binding of ^{131}I-d,l-PIN in rats occurred in the lung, with significantly less binding in the liver and heart. Binding decreased markedly over the first hour in both lung and heart (data not shown), and negligible concentrations of the tracer were present in these organs at 3 hr. Liver uptake decreased at 6 hr presumably reflecting increased concentrations of metabolites of iodopindolol. Blood activity decreased sharply after the first 2 hr but then remained relatively constant. Since maximal binding occurred at 5 min, this interval was selected for the other biodistribution studies.

To assess the specificity of the d,l isomer for the beta-receptor, distribution studies were conducted after specific blockade. Pretreatment with d,l-propranolol or ICI 118552, a selective beta-antagonist, significantly reduced binding in the lung in comparison with blockade achieved with either d-propranolol, phenoxybenzamine, or the cardioselective beta-antagonist, atenolol (Fig. 2).

The affinity of ^{131}I-d,l-PIN for cardiac tissue was ~ sixfold lower than that for the lung. Although atenolol and propranolol reduced cardiac uptake of label, the reduction was not statistically significant (Fig. 2). The alpha-antagonist, phenoxybenzamine, and to some extent, d-propranolol, increase cardiac uptake of ^{131}I-d,l-PIN. This enhanced binding was significantly reduced by atenolol and by l-propranolol but not by ICI 118552.

Increased doses (8 mg/kg) of propranolol or ICI 118552 did not decrease lung binding further (data not shown) indicating that specific binding was saturable. Uptake of ^{131}I-d,l-PIN in the heart, however, was increased in the presence of 8 mg/kg propranolol.

Liver uptake of ^{131}I-d,l-PIN was increased by all the beta-adrenoreceptor antagonists evaluated and most significantly by l-propranolol. In contrast, phenoxybenzamine did not significantly affect liver uptake of ^{131}I-d,l-PIN.

Rabbits. Since it was apparent that the d,l-isomer exhibited significant nonspecific binding, we thus evaluated labeled stereoisomers in rabbits. Rabbits were selected as the smallest animal practical for gamma imaging studies. Results of rabbit biodistribution studies with the isomers ^{131}I-l-PIN, ^{131}I-d-PIN, and ^{131}I-d,l-PIN are presented in Table 1. All rabbits were injected with ~50 pmol. Pretreatment with l-propranolol (0.2 mg/kg) significantly reduced binding of the l-isomer, but did not affect the distribution of ^{131}I-d-PIN.

Scintigraphy Studies

To assess whether cardiac beta-adrenoreceptors could be assessed noninvasively, we evaluated rabbits after administration of labeled stereoisomers of pindolol. Specificity was evaluated in some animals by administering specific antagonists prior to label administration. Scintigrams from representative rabbits injected with ~400–500 μCi ^{131}I-l-PIN (0.2–0.4 nmol) and ^{131}I-d-PIN (~450 μCi 0.2–0.4 nmol) are shown in Fig. 3. Iodine-131-l-PIN accumulated in the lung and heart within 4 min (Fig. 4A). This accumulation was blocked by 0.2 mg/kg l-propranolol (Fig. 3). The distribution of ^{131}I-d-PIN was more diffuse and was not affected by pretreatment with propranolol. Both isomers appeared to be cleared rapidly by the kidney (Figs. 3 and 4B) rather than by the liver (as in the rat). The blood levels of both isomers, assessed from counts/pixel in the aortic ROIs, were not significantly different and did not vary markedly over the first 8 min of scanning (Fig. 4B).

DISCUSSION

The aim of this study was to evaluate whether specific beta-adrenoreceptor occupancy could be determined noninvasively. In previous studies, biodistribution of radiolabeled adrenoreceptor ligands has been expressed as the total radioactivity in a particular organ (*4,10*), or radioactivity in each organ expressed as percentage of injected dose (*5*). However, preliminary studies with ^{131}I-PIN demonstrated that the total radioactivity in each organ varies with injected dose (data not shown). Absolute quantification of the injected dose is difficult to achieve. In contrast, tissue:blood ratios remain quite constant over a 200-fold range of injected dose (data not shown).

Results of our biodistribution studies in rats indicated that in vivo, ^{131}I-d,l-PIN exhibits characteristics typical of a beta-adrenoreceptor ligand. Binding was reduced in the lung and to a lesser extent in the heart in a dose-related manner by beta-adrenoreceptor blockade with l-propranolol, ICI 118552, and atenolol but not by d-propranolol or the alpha-adrenoreceptor antagonist, phenoxybenzamine. Maximal specific binding of ^{131}I-

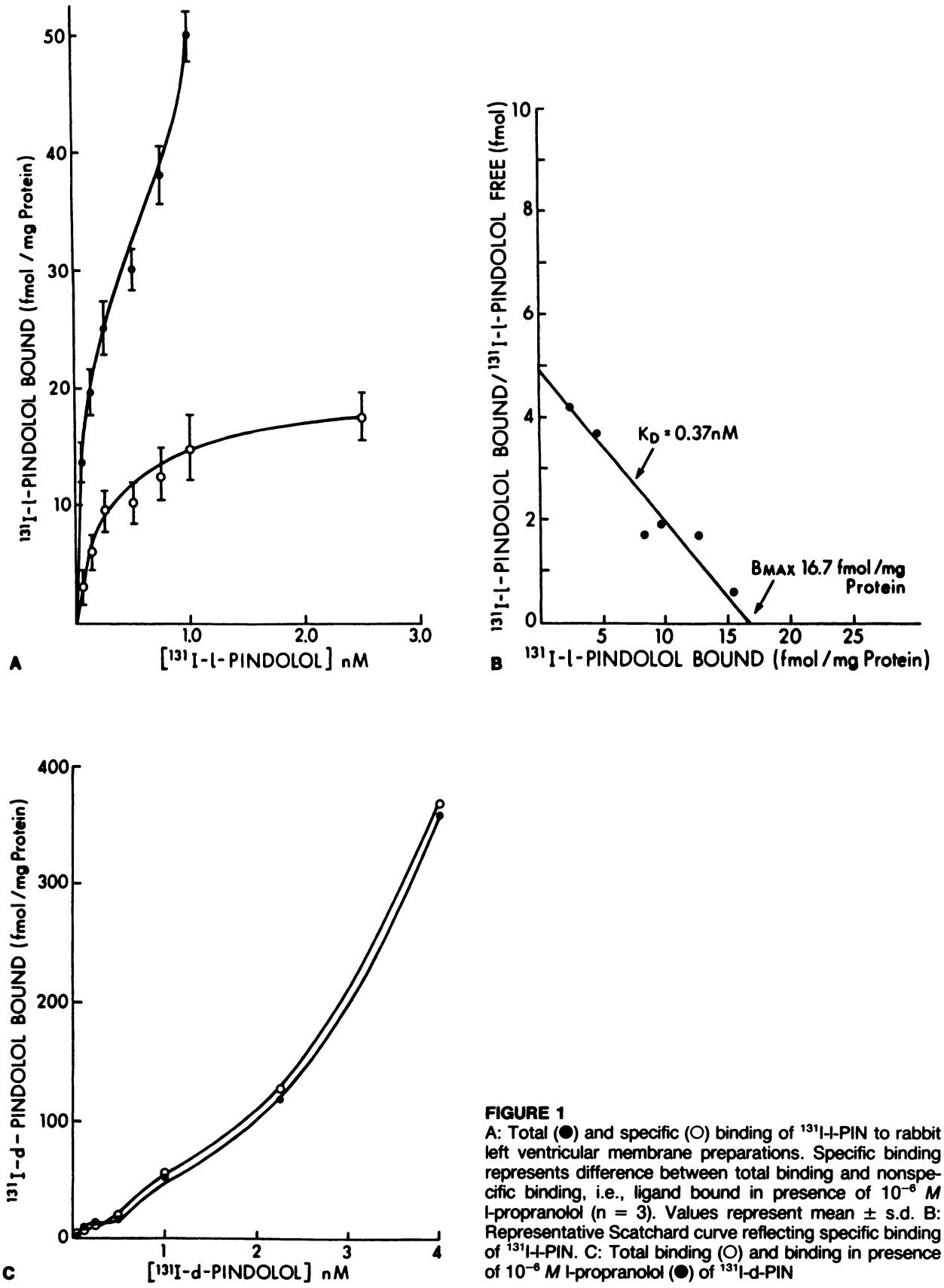

FIGURE 1
A: Total (●) and specific (○) binding of ^{131}I-l-PIN to rabbit left ventricular membrane preparations. Specific binding represents difference between total binding and nonspecific binding, i.e., ligand bound in presence of 10^{-6} *M* l-propranolol (n = 3). Values represent mean ± s.d. B: Representative Scatchard curve reflecting specific binding of ^{131}I-l-PIN. C: Total binding (○) and binding in presence of 10^{-6} *M* l-propranolol (●) of ^{131}I-d-PIN

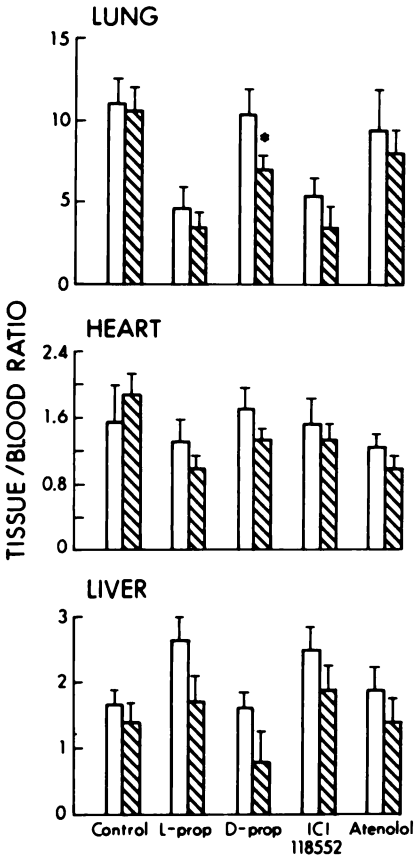

FIGURE 2
Tissue/blood ratios of ^{131}I-dl-PIN in control and drug treated, male Sprague-Dawley rats, in absence (□) and presence (▨) of 2 mg/kg phenoxybenzamine to reduce the nonspecific binding of ^{131}I-d,l-PIN (n = 6). Values represent mean ± s.d.

d,l-PIN occurred in the lung, similar to the findings of Homcy et al. (*5*) and Bylund et al. (*4*). Relative to lung, this ligand showed lower affinity for cardiac beta-adrenoreceptors, perhaps because of its lipophilicity. In contrast, many cardioselective $beta_1$-antagonists such as atenolol, practolol, and sotalol are hydrophilic (*11*). Cardiac binding of pindolol was increased in the presence of the alpha-adrenoreceptor antagonist phenoxybenzamine. This enhanced binding was significantly reduced by the beta-blocker, l-propranolol; the cardioselective antagonist, atenolol; and to a lesser extent, by the beta-antagonist, ICI 118552. Enhanced cardiac binding of ^{131}I-PIN in the presence of phenoxybenzamine is likely to be specific since phenoxybenzamine should saturate nonspecific binding sites. Displacement with agonists was not attempted since these agents (epinephrine, norepinephrine, and isoproterenol) are rapidly removed from the circulation. To achieve high effective concentrations at the receptor site, potentially lethal concentrations would have to be injected and such concentrations would also have affected the basal circulatory state.

TABLE 1
Biodistribution of ^{131}I-dl-PIN, ^{131}I-d-PIN, and ^{131}I-l-PIN in New Zealand White Rabbits*

Organ	^{131}I-d-PIN (n = 4)	^{131}I-l-PIN (n = 2)	^{131}I-l-PIN and PRO† (n=2)
Lung	3.26 ± 0.6	11.3 ± 1.8	5.1 ± 1.2
Liver	0.45 ± 0.05	0.6 ± 0.1	0.52 ± 0.07
Heart	0.99 ± 0.36	2.2 ± 0.2	1.08 ± 0.1

*Results are expressed tissue/blood ratios. Values represent the mean ± s.d.

†PRO = propranolol (0.2 mg/kg injected i.v. 10 min prior to injection of radiolabeled ligand). Ether anesthesia was used in these studies.

The enhanced hepatic uptake of ^{131}I-PIN in the presence of beta-adrenoreceptor antagonists is probably due to increased free circulating ^{131}I-PIN resulting in displacement of ligand from plasma protein binding sites and from specific beta-adrenoreceptor sites. This interpretation is supported by the results obtained with propranolol. Propranolol, which binds avidly to plasma proteins, resulted in the greatest enhancement of liver uptake (Fig. 2). The increased concentration of free ^{131}I-PIN may be responsible for the paradoxical increase of ^{131}I-PIN in the heart after beta-adrenoreceptor blockade since an increased concentration of free ^{131}I-PIN will result in maximal nonspecific binding. Increased uptake in the heart in the presence of propranolol has been observed previously (*4,12*). In disease states, increased or decreased uptake in an organ may not merely reflect increased or decreased receptor binding, but may be influenced markedly by changes in free drug availability and metabolism in the liver. These considerations may not influence binding assays in vitro but must be taken into account if receptors are to be quantified in vivo.

Although the initial studies with ^{131}I-d,l-PIN were encouraging, considerable nonspecific binding was evident. Thus, we attempted to resolve specific and nonspecific binding components. Since the physical as well as the pharmacologic properties of a compound play important roles in its biodistribution, the radiolabeled d-isomer of the receptor ligand served to represent a useful moiety for studying nonspecific binding characteristics. Isomers possess identical physical and chemical properties, and thus the distribution should be identical except that the l-isomer will also bind to the stereospecific receptor sites.

To ascertain pharmacologic purity of the isomers, receptor-binding assays were performed with both ligands in rabbit left ventricular membrane preparations. The specific binding component of ^{131}I-l-PIN was reversible, reached saturation at concentration 1.2–2.5 n*M*, and had a Kd of 0.37 ± 0.18 n*M* similar to values reported for the ligand ^{125}I-l-PIN (*6,7*). Iodine-131-d-PIN did not exhibit any specific binding in concen-

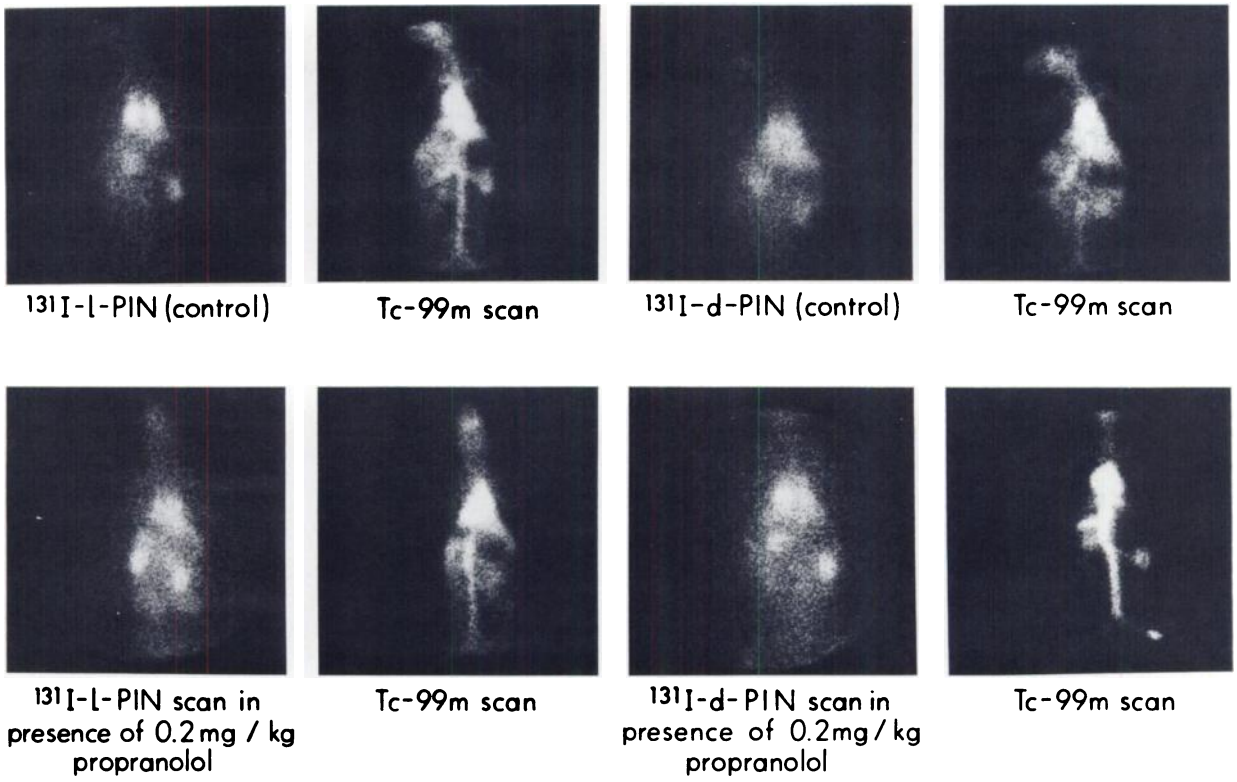

FIGURE 3
Representative scintigrams obtained from rabbits 2 min after injection with 400–500 μCi of ^{131}I-l-PIN or ^{131}I-d-PIN in absence or presence of 0.2 mg/kg l-propranolol. Technetium-99m-labeled red cells were used to localize heart and aorta

trations ranging from 0.05–10.0 n*M*. Total and nonspecific binding did not appear to be saturable.

Biodistribution studies in rabbits (Table 1) confirmed that uptake of the ^{131}I-l-PIN in comparison to the ^{131}I-d-PIN was greater in the lung and heart and selectively displaced by propranolol. Species differences in uptake of this ligand were observed between rabbits and rats. The species differences may result from different metabolic pathways, with clearance predominantly hepatic in the rat, and renal in the rabbit (*13*).

It is not possible to compare directly the results of the biodistribution studies with those obtained by analysis of ROIs in scintigrams since the actual volume of tissue in each ROI interrogated with planar imaging is not known. However, conclusions from scintigraphic studies are similar to those from biodistribution studies. In rabbits injected with ^{131}I-l-PIN, marked uptake was seen in lung and heart (Figs. 3 and 4) which was blocked by pretreatment with propranolol. These findings are in contrast to those in animals injected with a similar amount of ^{131}I-d-PIN in which binding was more diffuse and the distribution was not affected by propranolol pretreatment. Blood levels of both isomers were not significantly different judging from counts/pixel in aortic ROIs and did not vary markedly over the 8-min scanning period (Fig. 4). Results from biodistribution studies showed little accumulation in the aortic walls. Both isomers rapidly accumulated in kidney, reflecting the predominantly renal clearance of the compound in the rabbit (Figs. 3 and 4). In the presence of 0.2 mg/kg l-propranolol, ^{131}I-l-PIN was concentrated in the kidney and the spleen (Figs. 3 and 4), probably reflecting an increase in "free" ^{131}I-l-PIN.

The major difficulty encountered in analysis of planar scintigrams was distinguishing lung from heart (Fig. 3). With the aid of ^{99m}Tc-labeled red cells, it was possible to label the blood pool. The [^{99m}Tc]RBC scans were used to define the aorta and the cardiac blood pool. However, overlap between lung and cardiac areas is inevitable especially in small animals. Overlap was reduced by selecting the right lung field for the lung ROI. It can be reduced further by using large experimental animals, but studies with such animals would require large and expensive quantities of ^{131}I.

Our results suggest that it should be possible to delineate noninvasively with gamma scintigraphy nonspecific binding and define specific receptor binding by subtraction from total binding using a subtraction approach developed previously for assessment of platelet aggregation (*9*) and tumor localization (*14*). For conventional gamma scintigraphy, ^{123}I-d-PIN and ^{131}I-l-PIN could be used.

Detection with positron emission tomography would eliminate the problems of differentiation between heart

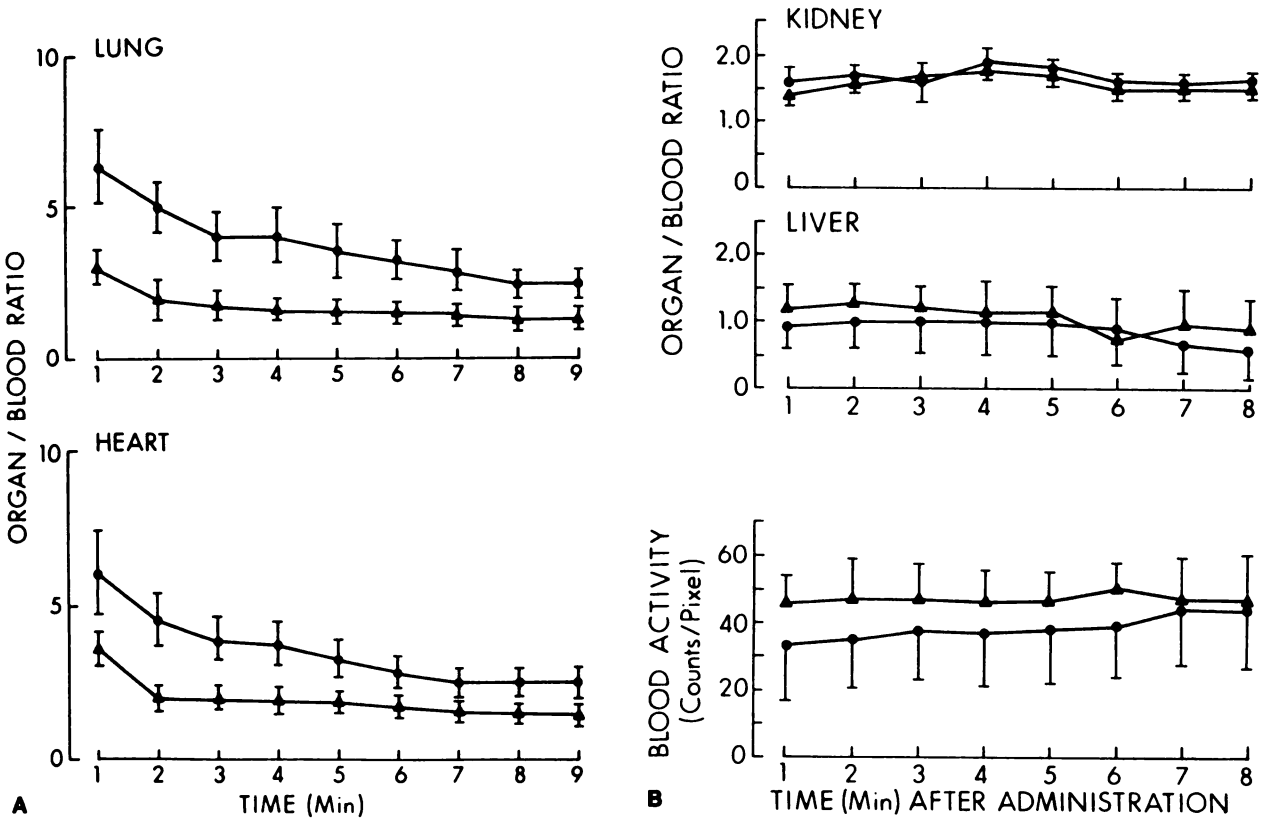

FIGURE 4

A: Average time-activity curves of ^{131}I-l-PIN and ^{131}I-d-PIN in lung and heart and (B) in kidney, liver, and blood obtained from digitized scintigrams. Values are expressed as mean ± s.d. of tissue/blood ratios for data from organ data, and as counts/pixel from aortic blood pool. (●) = ^{131}I-l-PIN; (▲) = ^{131}I-d-PIN

and lung. Dynamic acquisition of tomographic data would additionally facilitate detection of differences in the kinetics of specific and nonspecific binding. Carbon-11-labeled (^{11}C) beta-adrenoreceptor antagonists have been produced already (*15*) and ^{11}C can be incorporated into many ligands. However, to date, the problem of nonspecific binding has hampered external detection of cardiac receptor occupancy (*5,13*, Hughes et al: unpublished data). The results of the present study demonstrate the feasibility of delineating binding of biologically active l-isomer by assessing nonspecific binding with d-isomer and subtraction, thus permitting quantitative detection of specific receptor binding. The applications of this approach are not necessarily limited to receptors but may extend to external detection of any compound that exhibits stereospecificity as a prerequisite for biologic activity.

FOOTNOTES

* Du Pont Diagnostic Imaging Div., No. Billerica, MA. (600–12,000 Ci/mmol.)

† Chromanetics Corp., Gaithersburg, MD.

‡ Polytron Bunkmann Instrument.

§ Micromedics Inc., Horsham, PA.

¶ Siemens Medical Systems, Iselin, NJ.

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