C-Receptor Ligand Blocks Pulmonary Clearance of Atrial Natriuretic Peptide in Isolated Rat Lungs (43494A)

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Abstract. **Pulmonary clearance of atrial natriuretic peptide (ANP) was measured by indicator dilution technique in isolated perfused rat lungs with and without ANP clearance receptor (C-receptor) blockade. Approximately 50% of a bolus injection of ^{125}I-ANP was removed during a single pass through the lungs compared with the intravascular marker ^{14}C-dextran. Pulmonary clearance of ^{125}I-ANP was suppressed in a dose-dependent fashion by unlabeled ANP. C-receptor blockade suppressed pulmonary clearance of ^{125}I-ANP to the same degree as unlabeled ANP. High-performance liquid chromatography analysis of the pulmonary venous effluent from lungs treated with C-receptor ligand demonstrated intact ^{125}I-ANP. We conclude that virtually all of the pulmonary vascular uptake of ^{125}I-ANP during a single pass through isolated lungs is secondary to removal by ANP C-receptors.** [P.S.E.B.M. 1992, Vol 201]

Atrial natriuretic peptide (ANP) is a cardiac hormone with potent diuretic, natriuretic, and vasorelaxant properties that helps regulate body fluid homeostasis (1) via interaction with at least three distinct ANP receptors (2, 3). Two of these receptors are thought to mediate the biologic effects of ANP by guanylate-cyclase-linked modulation of intracellular cGMP levels. The third receptor, which is not linked to guanylate cyclase, has recently been proposed to act as a clearance receptor (C-receptor) (4), by removing ANP from the circulation and thereby modulating plasma ANP levels. C-receptor blockade potentiates the biologic activity of endogenous ANP by increasing circulating ANP levels *in vivo* (4).

By virtue of its downstream location from the right atrium and its large pulmonary capillary surface area that receives nearly the entire cardiac output, the lung is ideally situated to influence circulating ANP levels. Recent studies demonstrate that ^{125}I-ANP is readily taken up by the pulmonary vasculature during a single pass in isolated perfused rabbit lungs (5) and that the lung has the highest tissue concentration of specific ANP binding sites in rats (6). Binding studies in rabbit lungs suggest the presence of a single class of high affinity receptors for ANP (7), and purification of ANP receptors in bovine lung reveals a single receptor with a molecular weight similar to that of the ANP C-receptor (8). Based on the above observations, we hypothesized that the majority of ANP receptors in the pulmonary circulation are C-receptors and that C-receptor blockade would reduce pulmonary clearance of ANP. To test this hypothesis, we examined the effect of C-receptor blockade on ANP uptake by isolated perfused rat lungs.

Materials and Methods

Materials. Male Sprague-Dawley rats (300–400 g) were obtained from Charles River Laboratories (Wilmington, MA). ANP_{1-28} was obtained from Peninsula Laboratories, Inc. (Belmont, CA). ^{125}I-ANP_{1-28} (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA) and ^{14}C-dextran (1–5 mCi/g) was obtained from Sigma Chemical Co. (St. Louis, MO). For C-receptor blockade in ANP pulmonary uptake studies,

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we used SC-46452 (des[Phe^{106},Gly^{107},Ala^{115},Gln^{116}]-ANP$_{103-126}$; Searle Laboratories, Inc., St. Louis, MO). This ligand selectively binds to the ANP C-receptor and displaces ^{125}I-ANP$_{103-126}$ from a maximum of 73% of ANP binding sites when incubated with rabbit lung membranes *in vitro* (9). Fifty-percent inhibition of ^{125}I-ANP binding to lung membranes by SC-46542 is achieved at concentrations of 10^{-10} *M*. SC-46542 does not stimulate cGMP production or inhibit cGMP production by ANP in particulate membrane fractions of rabbit lungs *in vitro* (9). Because the availability of SC-46542 was limited, cANP$_{4-23}$ (Peninsula Laboratories) was used for high-performance liquid chromatography studies of the effect of C-receptor blockade on ANP pulmonary uptake.

Isolated Perfused Rat Lungs. Rats were anesthetized with pentobarbital (50 mg/kg, ip). The chest was opened by medium sternotomy and cannulae were placed in the pulmonary artery, trachea, and left atrium. The heart and lungs were removed en bloc and suspended in a perfusion apparatus. Lungs were ventilated at 64 breaths per minute with 5% CO_2 in air and perfused at 10 ml/min with Earle's balanced salt solution (Sigma), equilibrated with 95% O_2 and 5% CO_2, and warmed to 39°C. Pulmonary artery pressure was monitored continuously.

Pulmonary ANP Clearance. Single pass pulmonary clearance of ^{125}I-ANP in isolated lungs was determined using an indicator dilution technique described previously (5). ^{125}I-ANP (0.06 μCi) and ^{14}C-dextran (0.15 μCi) were co-injected into the pulmonary artery. Simultaneously, pulmonary venous effluent was directed into tubes in a fraction collector advancing at 0.02 min/tube (1.2 sec). Radioactivity in each tube was determined by standard scintillation spectrometry and corrected for counting efficiency, energy channel spillover, and dilution to yield dpm/ml of effluent. In order to compare the radioactivity of ^{125}I to ^{14}C, the activity in each sample was converted to fractional concentration (FC), defined as:

$$FC\times = (\text{activity of } X \text{ in sample})/(\text{total activity injected}) \quad [1]$$

Fractional concentration from each sample was plotted versus the appropriate time (or fraction) to generate effluent FC versus time curves. Percentage of instantaneous ^{125}I-ANP uptake was determined for each outflow sample as:

$$\%\ \text{Uptake} = (1-(FC[^{125}\text{I}]/FC[^{14}\text{C}])) \times 100 \quad [2]$$

ANP uptake changes were assessed by comparing the percentage of integral uptake at the end of FC versus time curves (approximately 18 sec) generated under different experimental conditions. Percentage of integral uptake was calculated as shown above using the sum of FC measured for ^{125}I and ^{14}C. Nonspecific binding by the perfusion apparatus was assessed by perfusing the apparatus without a lung and repeating the single-pass uptake determination as described above. Slow release of ^{125}I material from the lung was assessed by collecting effluent samples every 30 sec for approximately 15 min after the bolus injection of ^{125}I-ANP and ^{14}C-dextran.

Isolated Vessels. Following anesthesia, as described above, the heart and lungs were removed en bloc. Extrapulmonary and proximal intrapulmonary segments of the left pulmonary artery were isolated and cut into rings 2–3 mm in length. Each ring was attached to a myograph (Grass FT03) by two parallel wires (32 μm in diameter), passed through the lumen, and suspended in a vessel bath filled with Earle's balanced salt solution and sodium bicarbonate at 37°C and bubbled with 5% CO_2 and 95% O_2. Vascular ring segments were allowed to equilibrate for 45 min and then were given a resting tension of 500 mg. Contractility was tested by a dose-response curve using 10^{-8} *M* to 10^{-5} *M* phenylephrine. Constricted vessels were relaxed with 10^{-5} *M* acetylcholine to confirm the presence of a functional endothelium. Vessel baths were then washed with fresh Earle's balanced salt solution and the vascular rings were constricted with 10^{-6} *M* phenylephrine. A dose-response curve was then performed with 10^{-9}-10^{-6} *M* ANP, SC-46542, or equivalent volumes of saline in time controls. The ability of SC-46542 to inhibit the vasorelaxant effect of ANP was tested by repeating dose-response curves to ANP after SC-46542 (10^{-6} *M*) was added to the vessel bath. Vasorelaxation was expressed as percentage of peak tension measured immediately before the first dose of the agent tested.

HPLC. Fractions analyzed for ANP content by HPLC were obtained as described above in separate experiments. Lungs were injected with 0.2 μCi of ^{125}I-ANP alone or with 10^{-10} moles of cANP$_{4-23}$. Five fractions immediately after the peak FC of ^{125}I were pooled to obtain sufficient radioactive counts and extracted on C_{18} columns (Fisher Scientific, Pittsburgh, PA). Pooled fractions were eluted with 96% ethanol and 4% acetic acid, and evaporated to dryness by vacuum centrifugation. They were reconstituted in 100 μl of 0.1% trifluoroacetic acid, passed through a 2-μm filter, and loaded onto a Nucleosil C_{18} column (5 μ, 250 × 5 mm; Alltec Assoc. Inc., Deerfield, IL) attached to an Isco HPLC. ANP was eluted using an acetonitrile gradient as described previously (10). Eluted fractions were collected every 60 sec and counted on a gamma counter. Using this method, intact ^{125}I-ANP$_{(99-126)}$ can be distinguished from 3-iodotyrosine and the major metabolite ^{125}I-ANP$_{(104-126)}$ formed by enzymatic cleavage of the ^{103}Ser–^{104}Ser bond (10). Further metabolism of ^{125}I-ANP$_{(99-126)}$ and ^{125}I-ANP$_{(104-126)}$ results in diminution of their respective radioactive peaks while the 3-iodotyrosine peak remains unchanged (10).

Statistics. Analysis of variance was used to determine the statistical significance of differences between mean values of the percentage of inhibition of uptake. Post hoc analysis was performed using the Student-Newman-Keuls test. Data are shown as mean ± SE. Differences were considered significant at $P < 0.05$.

Results

FC ^{125}I curves from a representative experiment (Fig. 1) demonstrate that approximately 50% of a bolus injection of ^{125}I-ANP is taken up by the perfused lung relative to the intravascular marker ^{14}C-dextran. Co-injection of increasing amounts of unlabeled ANP inhibits the removal of ^{125}I-ANP in a dose-dependent manner, indicating displacement of ^{125}I-ANP from specific removal sites. In five experiments, the highest dose of unlabeled ANP used (10 nmol) reduced pulmonary clearance of ^{125}I-ANP to 7.3 ± 2.0% (Fig. 2). Approximately 3% of injected ^{125}I-ANP was removed in a single pass through the tubing and cannulae of the perfusion apparatus, in the absence of an isolated lung.

FC ^{125}I versus time curves from a representative experiment in which ^{125}I-ANP was co-injected with the C-receptor ligand SC-46542 indicate that C-receptor blockade also inhibited ^{125}I-ANP removal in a dose-dependent fashion (Fig. 3). In five experiments, the highest dose of SC-46542 (100 nmol) reduced pulmonary uptake of ^{125}I-ANP to 10.2 ± 1.9%, similar to the highest dose of unlabeled ANP (10 nmol) (Fig. 2).

Slow release of ^{125}I from the perfused lungs persisted for at least 15 min (Fig. 4). Fractional concentration of both ^{125}I and ^{14}C decreased to approximately 1% of the peak value within 1 min following injection. ^{14}C activity decreased to zero within 2 min, but ^{125}I activity remained slightly elevated for the duration of the experimental period (approximately 15 min), suggesting a prolonged, slow release of ^{125}I-ANP or radiolabeled fragments from the lung.

Pulmonary venous effluents were analyzed for the

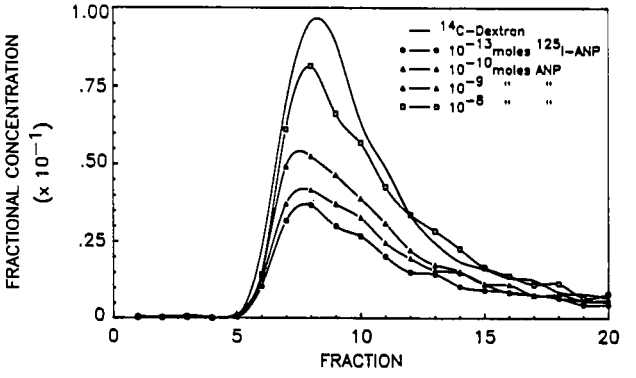

Figure 1. Fractional concentration versus fraction (or time) curves from a representative experiment with unlabeled ANP. FC of ^{125}I in the effluent from isolated perfused rat lungs injected with 10^{-13} moles of ^{125}I-ANP is increased by co-injection with progressively higher doses of unlabeled ANP.

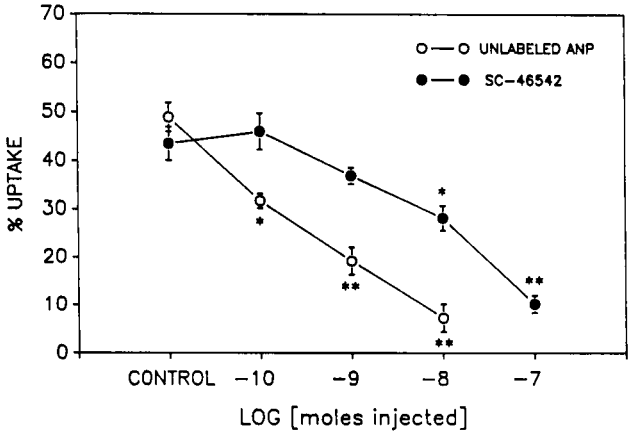

Figure 2. Dose-response curves comparing the effect of unlabeled ANP and the ANP C-receptor ligand SC-46542 on percentage of uptake of ^{125}I-ANP in isolated perfused rat lungs. Control represents 10^{-13} moles of ^{125}I-ANP injected in the absence of unlabeled ANP or SC-46542. (Mean ± SE, n = 3–5, $^{*}P < 0.05$ vs control; $^{**}P < 0.05$ vs proceeding point.)

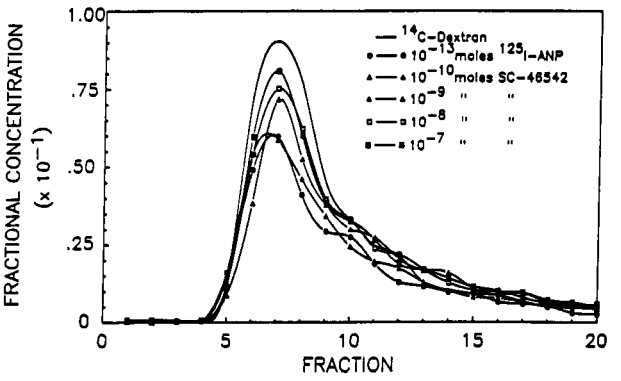

Figure 3. Fractional concentration versus fraction (or time) curves from a representative experiment with the ANP C-receptor ligand SC-46542. FC of ^{125}I in the effluent from isolated perfused rat lungs injected with 10^{-13} moles of ^{125}I-ANP is increased by co-injection with progressively higher doses of C-receptor ligand.

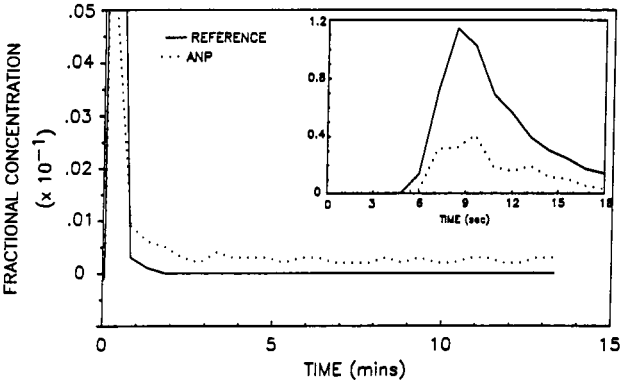

Figure 4. Slow release of ^{125}I-ANP from isolated perfused rat lungs. Initially, ^{125}I-ANP is removed by the pulmonary vasculature and FC of ^{125}I is less than that of the intravascular marker ^{14}C-dextran (inset). After 1 min, FC of ^{125}I exceeds FC of ^{14}C, demonstrating release of ^{125}I-labeled material into the effluent.

presence of ANP by HPLC to determine whether radioactive counts recovered represented intact ^{125}I-ANP or radiolabeled breakdown products. Effluents from four lungs injected with ^{125}I-ANP alone and two lungs co-injected with ^{125}I-ANP and the C-receptor ligand $cANP_{4-23}$ were analyzed. Total radioactivity in the late fractions (after the first 20 sec) was too low for detection by HPLC analysis. HPLC elution profiles from representative experiments with and without C-receptor blockade demonstrate a single predominant peak that is identical to that of the parent ^{125}I-ANP compound, indicating minimal metabolism during a single pass (Fig. 5). As anticipated, radioactivity in the pooled fractions is increased during co-injection with $cANP_{4-23}$, demonstrating that C-receptor blockade prevents removal of intact ANP.

The specificity of the C-receptor ligand SC-46542 for C-receptors, as opposed to guanylate cyclase-linked A- or B-receptors, was tested on isolated pulmonary artery segments. In comparison to saline, SC-46542 had no direct vasorelaxant effect, nor did it alter the dose-dependent vasorelaxant activity of ANP (Fig. 6).

Discussion

Our study confirms previous findings that ANP is taken up by the pulmonary circulation during a single pass through isolated, perfused lungs (5). In addition, we have shown that pulmonary clearance of radiolabeled ANP is almost completely suppressible by co-injection with unlabeled ANP, which demonstrates that ANP is cleared from the pulmonary circulation by binding to specific ANP receptors. Furthermore, virtually all pulmonary clearance of radiolabeled ANP was blocked with the C-receptor ligand SC-46542, which suggests that specific uptake of circulating ANP by the lung occurs almost entirely as the result of binding to ANP C-receptors.

These observations support the concept that the lung plays an important role in clearing ANP from the circulation. This hypothesis was first proposed by Turrin and Gillis (5), who found that 67% of a bolus injection of ANP was taken up by isolated rabbit lungs during a first pass. More recent studies (6) have demonstrated that the lung has a higher tissue concentration of specific binding sites for ANP than the kidney or other vascular tissues in the rat. Considering the large pulmonary capillary surface that is exposed to almost the entire cardiac output and recent evidence that ANP C-receptors are involved in the regulation of circulating ANP levels (4), our observations suggest that the lung may play a major role in modulating plasma ANP levels.

Our inference that C-receptors constitute the vast majority of specific ANP receptors in the pulmonary circulation assumes that the C-receptor ligands employed in this study are highly specific and do not cross-react with guanylate-cyclase-linked ANP receptors. This assumption is supported by previous studies (9) that have shown that SC-46542 does not stimulate particulate guanylate cyclase activity *in vitro* or interfere with ANP-induced cGMP production. In addition, the present study demonstrates that SC-46542 has no intrinsic vasodilatory effect on preconstricted pulmonary artery segments and does not block the vasorelaxant properties of ANP (Fig. 6).

Additional supportive evidence for C-receptors constituting the major portion of ANP receptors in the lung derives from studies demonstrating the isolation of a single class of ANP receptor from bovine lung with a molecular weight similar to that of the C-receptor (8). Although ANP A- and B-receptors have not yet been isolated from lung tissue, guanylate-cyclase-linked receptors are undoubtedly present in the lung because ANP stimulates guanylate cyclase activity in particulate membrane fraction of rabbit lung (9) and relaxes preconstricted pulmonary vessels (11).

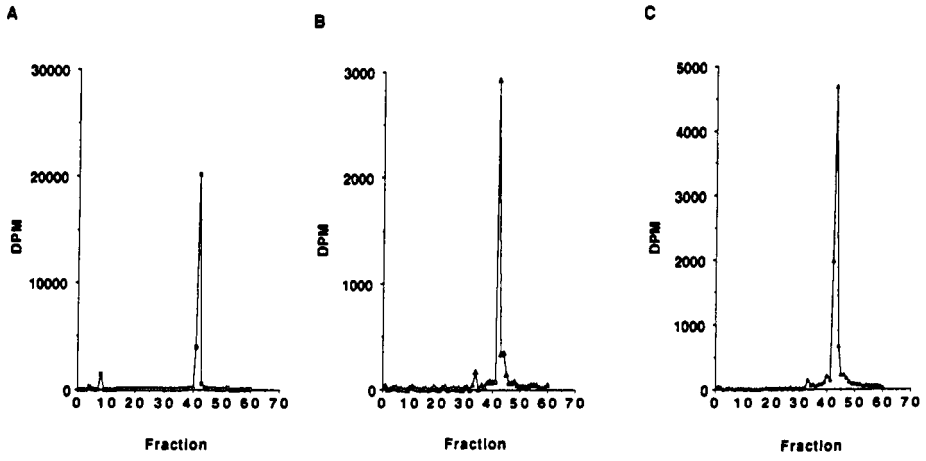

Figure 5. HPLC profiles of (A) ^{125}I-ANP, (B) effluent from isolated perfused rat lungs injected with 4×10^5 dpm of ^{125}I-ANP, and (C) effluent from lungs co-injected with 4×10^5 dpm of ^{125}I-ANP and 10^{-8} moles of $cANP_{4-23}$.

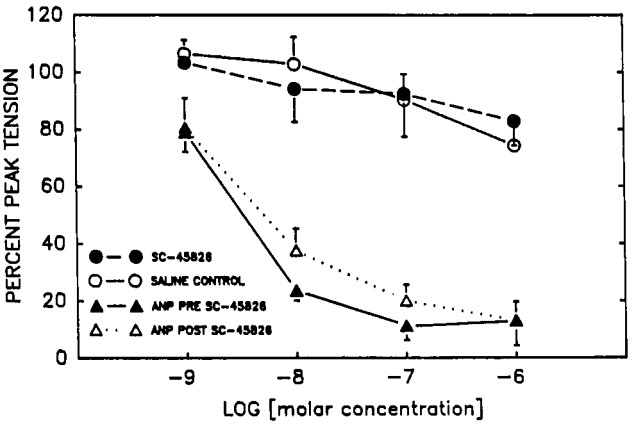

Figure 6. Dose-response curves of SC-46542 compared with ANP and saline in isolated pulmonary artery segments preconstricted with 10^{-6} *M* phenylephrine. ANP dose-response curves were done before and after additions of SC-46542 (10^{-6} *M*) to vessel baths. Values shown are mean ± SE (n = 4–6).

ANP metabolism occurs by a variety of peptidases, including the metalloendopeptidase 24.11, aminopeptidases, and carboxypeptidases (10). Our HPLC analysis of lung effluent following injection of ^{125}I-ANP into the pulmonary artery demonstrates no detectable metabolism during a first pass. Furthermore, HPLC analysis of lung effluent during C-receptor blockade shows that the increased radioactivity is due to intact ^{125}I-ANP. These findings suggest that binding to the C-receptor is more important than metabolism in first pass removal of ANP by lungs.

Whether or not pulmonary clearance of circulating ANP plays a role in ANP metabolism is unknown. In the present study, we used a serum-free preparation to avoid the possibility of ANP degradation by serum peptidases. Although serum peptidases are not known to readily metabolize ANP *in vitro* (12), it is possible that they could have a greater effect on ANP bound to C-receptors in the pulmonary vasculature. Also, because we were unable to determine the identity of the radiolabeled material that was released late, it remains possible that ANP bound to the C-receptor, or other nonspecific binding sites, undergoes metabolism by membrane-bound peptidases and subsequent release into the circulation, as proposed by others (13).

The effect of C-receptor blockade on circulating ANP levels has only recently been described (4). Recent experiments show that by increasing plasma ANP levels, specific C-receptor ligands have the same pulmonary hemodynamic effects as chronic ANP infusion in intact animals (14). Thus, modulation of C-receptor abundance in different pathophysiologic states could be an important means of regulating the bioactivity of ANP. In this study, we demonstrate that specific ANP binding during a single pass through the pulmonary circulation can be blocked with virtual completeness by C-receptor blockade, which suggests that the majority of pulmonary vascular ANP receptors are C-receptors. These findings lend support to our hypothesis that pulmonary C-receptors modulate circulating levels of ANP and suggest that through this mechanism, the lung may play an important role in mediating the biologic effects of ANP.

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