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Lung Angiotensin Receptor Binding Characteristics during the Development of Monocrotaline-Induced Pulmonary Hypertension

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ABSTRACT. Alterations in lung angiotensin converting enzyme (ACE) activity in monocrotaline (MCT)-induced pulmonary hypertension in rats have suggested a pathophysiologic role for angiotensin II (AII) in pulmonary vascular remodeling. ACE inhibitors suppress MCT-induced pulmonary hypertension; however, losartan, an angiotensin type 1 (AT1) receptor antagonist, was without impact. The present study examined AII receptor binding characteristics by radioligand binding during the development of MCT-induced pulmonary hypertension. Saturation binding isotherms for [^{125}I]AII binding to membrane preparations from rat lung were performed at 4, 10, and 21 days following a single injection of MCT (60 mg/kg) or saline vehicle. Right ventricular hypertrophy, an index of pulmonary hypertension, increased at 21 days post-MCT. Saturation binding isotherms revealed a single, high affinity site for [^{125}I]AII binding in lung membranes from MCT-treated and control rats, with no change in receptor affinity or density during the development of pulmonary hypertension. Competition displacement binding demonstrated that the AT1 receptor predominates in lung membranes from control rats, with no alterations in AII receptor subtype distribution following MCT treatment. In summary, these results suggest that the AT1 receptor subtype predominates in rat lung and does not contribute to the development of MCT-induced pulmonary hypertension. BIOCHEM PHARMACOL **54**;1:27–31, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. angiotensin receptor; pulmonary hypertension; monocrotaline; binding

A single subcutaneous injection of MCT§, a pyrrolizidine alkaloid, into rats causes delayed and progressive pulmonary vascular injury, resulting in the development of pulmonary hypertension and right ventricular hypertrophy [1–3]. Although the initial injury is restricted to the vascular endothelium [3, 4], the disease progresses to include medial thickening of muscularized arteries [3–7], extension of smooth muscle into normally nonmuscularized pulmonary arterioles [8], and increased periarterial deposition of matrix tissue proteins [9–11]. Collectively, these changes culminate in a structurally based increase in pulmonary vascular resistance.

Previous studies have implicated AII, a vasoconstrictor peptide, in the pathogenesis of MCT-induced pulmonary hypertension. Research has focused on the initial MCT-induced injury to pulmonary endothelial angiotensin converting enzyme [12, 13]. Previous studies demonstrated that administration of MCT to rats results in a biphasic change in ACE activity, which increases early after MCT [4] but is depressed during the later stages as progressive pulmonary hypertension develops [12]. Moreover, administration of ACE inhibitors suppresses the development of MCT-induced endothelial injury and pulmonary hypertension [14–16]. However, interpretation of the beneficial effect of ACE inhibitors on the development of pulmonary hypertension has been confounded by the inability to differentiate between the antioxidant protective effects of these compounds [16, 17] and the effect of ACE inhibitors on pulmonary and systemic AII production.

Previous studies in our laboratory have demonstrated that chronic daily administration of losartan, a competitive AT1 receptor antagonist, to MCT-treated rats does not prevent the development of pulmonary hypertension and associated right ventricular hypertrophy [18]. These results suggested that the beneficial effects of ACE inhibitors in MCT-induced pulmonary hypertension were unrelated to alterations in lung AII production and function. However, the single daily s.c. dosing regimen with the competitive antagonist, losartan, may not have afforded sufficient antagonism at lung vascular AT1 receptor sites. In the present study, AII receptor binding characteristics were examined

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§ *Abbreviations:* ACE, angiotensin converting enzyme; AII, angiotensin II; and AT1, angiotensin type 1; MCT, monocrotaline.

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at 4, 10, and 21 days following a single s.c. injection of MCT or saline vehicle to determine if alterations in AII receptor density or affinity may contribute to the development of pulmonary hypertension. Competition displacement binding was also performed to determine the angiotensin receptor subtype potentially involved in the development of MCT-induced pulmonary hypertension.

MATERIALS AND METHODS

Administration of Monocrotaline

Male Sprague-Dawley rats (225–250 g; Harlan Laboratories, Indianapolis, IN) were housed in groups of two per cage in a controlled environment under a 12-hr light/dark cycle, and given free access to food and water. Rats were divided into control (equivalent volume of saline vehicle; N = 4) or MCT-treated (N = 4) groups and were examined at 4, 10, and 21 days after injection. MCT-treated rats received a single s.c. injection of MCT (60 mg/kg; Trans World Chemicals, Rockville, MD) on day 1 of the study.

Rat Lung Membrane Preparation

Membranes were prepared from lung taken from individual rats (control, MCT) at each time point. Lungs were dissected and homogenized (Polytron homogenizer, 2 × 30 sec bursts at 4°) in membrane buffer (50 mM Tris, pH 7.4; 250 mM sucrose; 10 mM EDTA). Homogenates (30 mL) were centrifuged at 1100 *g* for 10 min (4°), and the supernatant was transferred to a fresh tube. Supernatants were centrifuged at 48,000 *g* in a Sorvall RC 28S (DuPont, Wilmington, DE) supraspeed centrifuge (rotor F28/36) for 10 min (4°). The supernatant was discarded, and the pellet was resuspended in membrane buffer and processed through centrifugation and resuspension twice more. The final membrane pellet was resuspended (200 mg wet wt/mL; 1 mg protein/mL) in buffer containing 50 mM Tris (pH 7.4), 120 mM NaCl, 1 mM $MgCl_2$.

Radioligand Binding

Initial studies determined optimum membrane protein concentration and time to equilibrium binding in lung tissues. For saturation binding isotherms, membrane protein (50 μg) was incubated with 0.15 nM [^{125}I]AII (2200 Ci/mmol; New England Nuclear, Wilmington, DE) and various concentrations of unlabeled AII (0.02 to 2.5 nM) for 60 min at 26° in buffer (250 μL total volume) containing 120 mM NaCl, 1 mM $MgCl_2$, 0.2% fatty acid free BSA (Sigma), 50 mM Tris, 0.005% (w/v) bacitracin, and 0.24 units/mL aprotonin. For competition studies, membrane protein (50 μg) was incubated with [^{125}I]AII (0.15 nM) in the presence of increasing concentrations (12 concentrations/competitor) of competitors (losartan, PD123319) for 60 min at 26°. Incubations were terminated by rapid filtration (Brandel Cell Harvestor, Gaithersburg, MD) through GF/B glass fiber filters (Brandel) that had been presoaked in 0.2% BSA. Filters were washed three times with ice-cold buffer (50 mM Tris, pH 7.4), and particle-bound radioactivity was assayed in a γ counter. Nonspecific binding was defined as radioactivity bound in the presence of 10 μM AII in the incubation medium and normally was <5% of total binding. All samples were run in duplicate. Protein was determined according to the method of Bradford [19], with BSA as the standard.

Equilibrium binding parameters (*Kd* and B_{max}) and binding isotherms from competition studies were obtained using the LIGAND program. The inhibitory constant (K_i) was calculated from the IC_{50} using the equation of Cheng and Prusoff [20]. Two-tailed Student's *t*-tests were used to determine whether Hill slopes were significantly different from unity (one-sample tests).

TABLE 1. Characteristics of control and MCT-treated rats

Days of treatment	Body weight (g)	Lung protein (mg/g)	Right ventricular hypertrophy (RV/LV + S)
Day 4			
Control	249.3 ± 5.9	3.3 ± 0.4	0.318 ± 0.030
MCT	225.3 ± 4.4*	3.8 ± 0.5	0.386 ± 0.083
Day 10			
Control	288.3 ± 4.9	3.0 ± 0.2	0.296 ± 0.014
MCT	261.3 ± 3.7*	3.1 ± 0.2	0.332 ± 0.010
Day 21			
Control	339.3 ± 1.1	2.7 ± 0.3	0.288 ± 0.016
MCT	301.5 ± 3.1*	3.5 ± 0.7	0.525 ± 0.036*

Values are means ± SEM of N = 4 rats/group/time point.
* $P < 0.05$, significantly different from age-matched control.

RESULTS

Characteristics of Control and MCT-Treated Rats

Body weights were decreased significantly at each time point examined in MCT-treated rats compared with saline controls (Table 1). In contrast, lung membrane protein (mg protein/g wet wt) was not different between MCT-treated and control rats at any time point examined. Right ventricular hypertrophy was elevated significantly at 21 days post-MCT.

AII Receptor Binding Characteristics in Lungs from Control and MCT-Treated Rats

Binding of [^{125}I]AII to lung membranes was saturable and of high affinity. Initial experiments determined the time to equilibrium (60 min) and the optimum membrane protein (50 μg from the linear portion of the membrane protein curve) for saturation binding isotherms. Nonspecific binding of [^{125}I]AII to lung membranes was routinely 5% of specific binding.

In lung membranes from both control and MCT-treated rats, [^{125}I]AII interacted with a single population of high

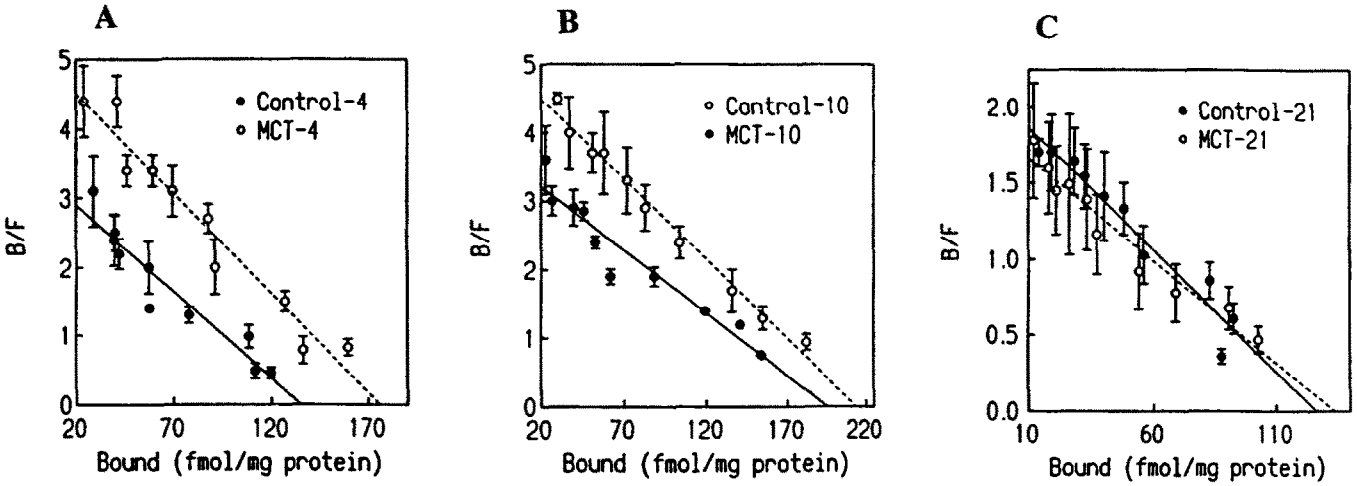

FIG. 1. Scatchard analysis of [^{125}I]AII binding to lung membranes from day 4 (A), day 10 (B), and day 21 (C) MCT-treated and control rats. Lung membranes were prepared after rats were treated with either MCT or saline vehicle for each time point. Membranes were incubated with [^{125}I]AII for 60 min at 26°. Scatchard analysis revealed a single, high affinity AII receptor binding site in lung membranes from control and MCT-treated rats. There was no significant difference in the affinity or density of AII receptor binding in lung membranes from control and MCT-treated rats at any time point examined. Values are means ± SEM for N = 4 rats/group at each time point.

affinity binding sites at 4, 10, and 21 days post-treatment (Fig. 1, A–C, respectively). Hill coefficients for lung binding were not different from unity, indicating a single class of AII binding sites and the absence of positive or negative cooperativity.

The affinity of [^{125}I]AII binding was not altered in lung membranes from MCT-treated rats compared with controls at any time point examined (Table 2). Additionally, there was no significant difference in AII receptor density between MCT-treated and control lungs at any time point examined (Fig. 1, A–C; Table 2). To determine if the AII receptor subtype was altered during the development of MCT-induced pulmonary hypertension, competition displacement binding was performed in lung membranes from day 10 and day 21 MCT-treated and control rats using the AT1 selective antagonist losartan and the AT2 ligand PD123319 (Fig. 2). Losartan displaced [^{125}I]AII binding to lung membranes from both MCT-treated and control rats by greater than 90% over two orders of magnitude of losartan concentration, indicative of a single class of AT1 receptors in lung. The potency (K_i) of losartan displacement was not significantly different between control and MCT-treated rats at either time point examined (control, day 10 and day 21, respectively: 8.3 ± 3.3, 4.5 ± 1.5 nM; MCT, day 10 and day 21, respectively: 7.8 ± 2.2, 3.9 ± 0.7 nM). PD123319, the AT2 ligand, did not displace [^{125}I]AII binding from lung membranes of control or MCT-treated rats (Fig. 2).

TABLE 2. Characteristics of AII receptor binding in lungs from control and MCT-treated rats

Days of treatment	K_d (nM)	B_{max} (fmol/mg protein)	Hill coefficient
Day 4			
Control	0.37 ± 0.08 (3)	147.5 ± 31.7 (3)	0.99 ± 0.01 (3)
MCT	0.43 ± 0.12 (4)	180.7 ± 37.2 (4)	1.03 ± 0.04 (4)
Day 10			
Control	0.49 ± 0.14 (4)	175.7 ± 23.5 (3)	0.90 ± 0.03 (3)
MCT	0.47 ± 0.09 (4)	179.0 ± 28.7 (4)	0.93 ± 0.01 (4)
Day 21			
Control	0.63 ± 0.05 (4)	128.4 ± 15.7 (4)	0.93 ± 0.02 (4)
MCT	0.75 ± 0.16 (5)	124.9 ± 35.6 (5)	0.96 ± 0.03 (5)

Values are means ± SEM; the number of determinations is given in parentheses.

DISCUSSION

Results of the present study indicated that alterations in lung AII receptor binding density or affinity do not contribute to the development or maintenance of MCT-induced pulmonary hypertension. Competition displacement binding studies demonstrated that the AT1 receptor predominates in control and MCT-treated rat lungs, with no alterations in angiotensin receptor subtype expression during the development of MCT-induced pulmonary hypertension.

The present study revealed a high affinity, moderate capacity AII receptor binding site in lung membranes prepared from control and MCT-treated rats. The binding was rapid, saturable, and reversible. The dissociation constant determined in saturation experiments was consistent with previously reported data obtained in several different organs of the rat, as well as in rat lung [21]. The K_i for competition displacement with losartan in lung membranes from control and MCT-treated rats was in agreement with previously reported competition studies using this antagonist [22]. Additionally, PD123319, the selective AT2 receptor ligand, did not displace specific [^{125}I]AII binding in rat lung membranes. These results are in agreement with previous studies demonstrating the AT1 receptor as the

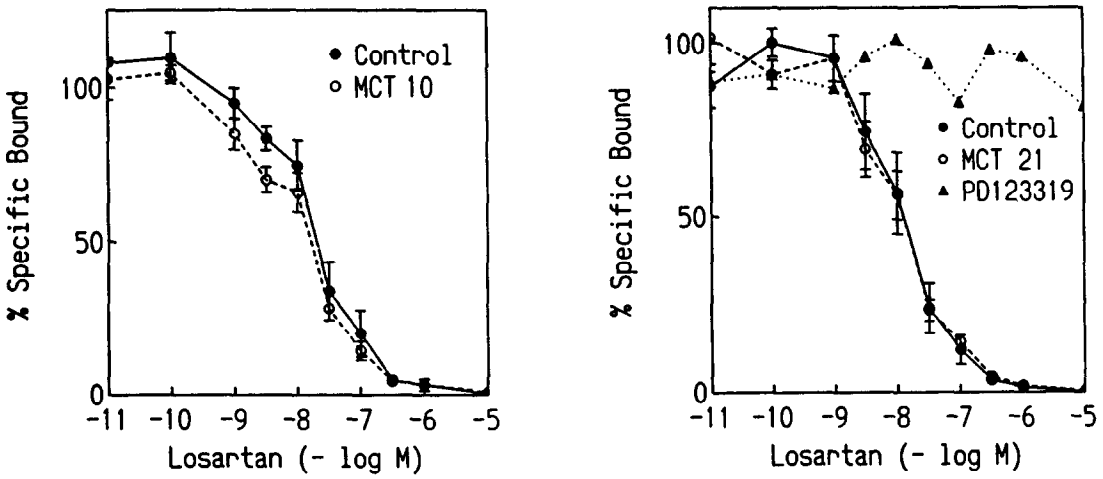

FIG. 2. Competition displacement of [^{125}I]AII binding in lung membranes from day 10 (left) and day 21 (right) MCT-treated and control rats. Lung membranes were incubated with [^{125}I]AII in the presence of increasing concentrations of losartan (AT1 antagonist; day 10 and 21) or PD123319 (AT2 antagonist; day 21 only). Data is expressed as percent specific bound (100% specific binding: control, 102.8; MCT-treated, 110.4 dpm × 10^3/mg protein). Values are means ± SEM for N = 4 rats/group.

only AII receptor subtype in rat lung [21]. Moreover, these results demonstrated that the AII receptor subtype population is not altered during the development of MCT-induced pulmonary hypertension.

Previous studies have demonstrated that ACE inhibitors can partially prevent the development of MCT-induced pulmonary hypertension [14, 15, 17]. The thiol-containing ACE inhibitor, captopril, ameliorates MCT-induced pulmonary hypertension to a greater extent than non-thiol ACE inhibitors [17], suggesting a nonspecific interaction of the thiol-containing compounds in the prevention of pulmonary hypertension. However, additional studies have demonstrated that several non-thiol-containing ACE inhibitors are capable of preventing MCT-induced pulmonary hypertension [16], ruling out nonspecific thiol effects as major therapeutic factors. Recent studies have demonstrated that high concentrations of MCT decrease ACE activity in cultured endothelial cells [23]. However, MCT-induced decreases in ACE activity were thought to be an indirect effect of MCT, related to delayed endothelial cell injury. Moreover, previous studies in our laboratory demonstrated that losartan, a selective, competitive AT1 receptor antagonist, does not prevent increases in pulmonary artery pressure or the development of right ventricular hypertrophy in MCT-treated rats [18]. One major difference between AT1 antagonists and ACE inhibitors is the bradykinin-potentiating ability of ACE inhibitors. Thus, the previously demonstrated ability of ACE inhibitors to prevent or alleviate MCT-induced pulmonary hypertension may result from their ability to increase lung bradykinin levels, rather than decrease local AII production. Collectively, these results suggest that while alterations in endothelial ACE activity occur after MCT treatment, associated alterations in lung AII concentrations may be unrelated to the development of pulmonary hypertension.

One of the potential mechanisms whereby AII could contribute to the pathogenesis of MCT-induced pulmonary hypertension is via its ability to promote vascular smooth muscle hypertrophy [24] or hyperplasia [25], including neointima formation at the site of vascular injury [26]. Previous studies have demonstrated that the AT1 receptor antagonist losartan completely prevents AII-induced increases in smooth muscle cell protein synthesis [27] and DNA synthesis [25]. In vascular injury models, both AT1 (losartan, TCV-116 [28, 29]) and AT2 (CGP42112A [28]) antagonists have proven beneficial in preventing neointima formation after vascular injury, suggesting a potential role for both AII receptor subtypes in smooth muscle migration and proliferation. In the present study, in rat whole lung membrane preparations from control and MCT-treated rats, an AT2 receptor antagonist did not displace [^{125}I]AII binding, demonstrating that MCT-induced pulmonary hypertension is not associated with alterations in AII receptor subtype distribution.

Among many changes accompanying vascular remodeling in pulmonary hypertension, alterations in vascular reactivity to AII have been reported to be associated with the development of MCT-induced pulmonary hypertension [30–34]. Depending on the preparation used and the time after MCT treatment, vascular reactivity to AII has been reported either to increase initially followed by a gradual decline [30], or to be decreased consistently throughout the development of pulmonary hypertension [34]. Results from the present study would suggest that alterations in vascular reactivity to AII may be the result of structural alterations, rather than changes in AII receptor number or affinity.

In summary, the present study demonstrated that alterations in AII receptor density or affinity of binding do not contribute to the development of MCT-induced pulmonary hypertension. However, the role of AII in hypoxia-induced pulmonary vascular remodeling and hypertension remains to be elucidated.

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