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Mineralocorticoid receptor antagonists attenuate pulmonary inflammation and bleomycin-evoked fibrosis in rodent models

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ABSTRACT

Accumulating evidence indicates protective actions of mineralocorticoid antagonists (MR antagonists) on cardiovascular pathology, which includes blunting vascular inflammation and myocardial fibrosis. We examined the anti-inflammatory and anti-fibrotic potential of MR antagonists in rodent respiratory models. In an ovalbumin allergic and challenged Brown Norway rat model, the total cell count in nasal lavage was 29,348 ± 5451, which was blocked by spironolactone (0.3–60 mg/kg, p.o.) and eplerenone (0.3–30 mg/kg, p.o.). We also found that MR antagonists attenuated pulmonary inflammation in the Brown Norway rat. A series of experiments were conducted to determine the actions of MR blockade in acute/chronic lung injury models. (1) *Ex vivo* lung slice rat experiments found that eplerenone (0.01 and 10 μM) and spironolactone (10 μM) diminished lung hydroxyproline concentrations by 55 ± 5, 122 ± 9, and 83 ± 8%. (2) In in vivo studies, MR antagonists attenuated the increases in bronchioalveolar lavage (BAL) neutrophils and macrophages caused by lung bleomycin exposure. In separate studies, bleomycin (4.0 U/kg, i.t.) increased lung levels of hydroxyproline by approximately 155%, which was blocked by spironolactone (10–60 mg/kg, p.o.). In a rat Lipopolysaccharide (LPS) model, spironolactone inhibited acute increases in BAL cytokines with moderate effects on neutrophils. Finally, we found that chronic LPS exposure significantly increased end expiratory lung and decreased lung elastance in the mouse. These functional effects of chronic LPS were improved by MR antagonists. Our results demonstrate that MR antagonists have significant pharmacological actions in the respiratory system.

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1. Introduction

The renin-angiotensin-aldosterone system (RAAS) is a fundamental constituent in the governance of blood pressure homeostasis, as well as fluid and salt balance (Werning and Siegenthaler, 1969; Jones, 1970; Rudnicki and Mayer, 2009; Hsueh and Wyne, 2011). However, inappropriate activation of RAAS adversely influences several cardiovascular diseases (Covic and Gusbeth-Tatomir, 2009). Consequently, drugs that attenuate RAAS such as, angiotensin converting enzyme (ACE-I) inhibitors which block the conversion of the inactive angiotensin I to the active vasoconstrictor angiotensin II, angiotensin receptors blockers which block angiotensin II type I receptors and mineralocorticoid receptor antagonists that antagonize the actions of the mineralocorticoid aldosterone, have found significant utility for conditions such as hypertension, left ventricular dysfunction, acute myocardial infarction, diabetic nephropathy and atherosclerosis (Pitt et al., 1999, 2003 ; Rajagopalan et al., 2002; Jacoby and Rader, 2003; Burchill et al., 2012; Lund et al., 2012; Mentz et al., 2012). Interestingly, scientific evidence is amassing indicating that increased RAAS activity may also be an important contributing factor to inflammation and remodeling pathology associated with chronic cardiovascular and perhaps other diseases (Pitt, 1995; Pitt et al., 2003; Stegbauer et al., 2009). With this being said, in contrast to the well established association between RAAS and cardiovascular pathology, the link between increased RAAS activation and respiratory pathology/disease remains less understood. In animals studies, angiotensin II, a key peptide in RAAS, has been shown to elicit LTD4 mediated bronchoconstriction in guinea pigs, and promotes antigen-induced airway hyperresponsiveness, eosinophilic inflammation and airway remodeling in ovalbumin sensitized guinea pigs and rats (Myou et al., 2000; Wang et al., 2008). In human asthmatics

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angiotensin II produces weak bronchoconstriction (Millar et al., 1994). Moreover, the use of ACE inhibitors has been demonstrated to decrease mortality of patients hospitalized with acute COPD exacerbations by unclear mechanisms (Mortensen et al., 2009).

Like angiotensin II, aldosterone is a principal component of RAAS. In recent years there has been a shift in the dogma surrounding aldosterone from a steroid mainly responsible for salt and water homeostasis to a more ubiquitous effector hormone with extended biology contributing to pro-inflammatory and pro-fibrotic outcomes in multiple target organ systems (Hu et al., 2005; Pearce et al., 2003). For example, several reports have demonstrated that MR activation in animal models elicits perivascular and interstitial fibrosis in the aorta, heart and kidneys (Brilla and Weber, 1992; Lacolley et al., 2002; Marney and Brown, 2007). The aforementioned pathologies are likely mediated by direct binding of aldosterone to MR and the transcription of pro-inflammatory genes such as MCP-1, IL-6, IL, IL-1β, NADPH oxidase, hemooxygenase-1 subunits, cyclooxygenase-2, osteopontin, TGF-β, orosomucoid, tenascin-X, type I and Type III collagens (Marney and Brown, 2007). There may also be important transcription-independent pathways that contribute to deleterious biology engendered by excessive aldosterone exposure (Sønder et al., 2006; Marney and Brown, 2007). Nonetheless, MR antagonism with drugs like spironolactone and eplerenone effectively attenuate inflammation and fibrosis produced by aldosterone in in vitro biological assays and on in vivo animal models.

Although an understanding of MR mediated pulmonary related pathology is not fully realized there is evidence that the lung, and in particular the epithelium, is a target for aldosterone and MR activation (Fischer and Clauss, 1990; Illek et al., 1990; Agostoni et al., 2005). Moreover, Zhao et al., (1998) have provided results indicating that MR blockage with spironolactone is protective in a rat pulmonary fibrosis model. The aim of the present study was to further explore the pharmacological profile of MR antagonists in animal models of pulmonary inflammation and fibrosis, using well characterized aldosterone antagonists, spironolactone and eplerenone (Sutanto and de Kloet, 1991; Takeda, 2004). We also studied the effects of spironolactone on respiratory inflammation in an acute lipopolysaccharides (LPS) rat model and pulmonary function in a chronic mouse LPS model.

2. Materials and methods

2.1. Animal care and use

These studies were performed in accordance with the guidelines of the Institute for Laboratory Animal Research (ILAR). All studies were part of an institutional animal care and use committee (IACUC)-approved protocol and animals were housed in an AAALAC International accredited research facility. Since all experimental key readouts were terminal, all study animal groups were used once only and were euthanized at the end of each study.

2.2. Effect of MR blockade on allergic-mediated nasal inflammation

Male Brown Norway rats (200–250 g) were purchased from Charles River (Wilmington, MA, USA) and housed conventionally in an animal room with temperature of 21 °C and a daily light-dark cycle (0700 to 1900 light). Chow and water were supplied ad libitum. The animals were studied between 80 and 85 days of age. To examine allergic-mediated nasal inflammatory responses, rats were actively sensitized to ovalbumin over a 14 day regimen. On day 1, animals were administered 20 μg of ovalbumin (i.p.) and 8 mg/kg of aluminum hydroxide (i.p.) in 1 ml of physiological saline. Seven days later, an i.p. booster containing ovalbumin (20 μg) and aluminum hydroxide (8 mg/kg) was administered. Non-sensitized rats received two doses of aluminum hydroxide (8 mg/kg) administered on days one and eight. Spironolactone (0.3–60 mg/kg, p.o.) and eplerenone (0.3–30 mg/kg) were given orally in 0.4% methylcellulose 1 h before antigen provocation. The doses of spironolactone and eplerenone used in this study and subsequent experiments were selected based on historical and literature dose levels of these drugs typically employed in in vivo rodent models. In a second set of experiments, we sought to rule out the possibility that anti-inflammatory effects of eplerenone were mediated by glucocorticoid receptor (GR) activation. Thus, the activity of eplerenone on total nasal lavage fluid cell counts was determined in the absence and presence of the GR antagonist RU486. Specifically, eplerenone (10 mg/kg, p.o.) was given 1 h before antigen challenge. RU486 (15 mg/kg, i.p.) or vehicle was administered 30 min before eplerenone. As a comparator for eplerenone we also ran the GR agonist betamethasone (3 mg/kg, p.o.) alone and with RU486.

Antigen provocation consisted of applying 10 μl of 1% ovalbumin solution in physiological saline directly to each naris. Non-sensitized animals received an equal volume of saline. Twenty-four hours after nasal challenge, rats were surgically prepared for nasal lavage. Following anesthesia with ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.) the animals were placed in the supine position on a heating pad (37 °C). Trachea was isolated and then cannulated with polyethylene tubing (PE-240), which was inserted retrograde from the trachea into the nasopharynx. A 1.5-mm length of PE-240 tubing was also introduced into the opposite open end of the trachea, which allowed spontaneous breathing throughout the experiment. The esophagus was ligated with 3-0 suture silk and the oral cavity was sealed with 0.5–1.0 ml of reprosil (DENTSPLY International, Milford, DE) to prevent leakage. The nasal cavity was perfused with saline (0.9% NaCl) using a perfusion pump (150 μl/min for 20 min). The perfusion fluid was collected through the nose and total inflammatory cells were manually counted using methods previously described (Celly et al., 2006).

2.3. Effect of MR blockade on allergic-mediated pulmonary inflammation

Male Brown Norway rats (200–250 g) were sensitized to ovalbumin as described above. On day 14 of the sensitization protocol, rats were lightly anesthetized with 3% isoflurane (supplemented with 100% oxygen) and 3 mg of the vehicle or drug, micronized spironolactone (0.001–3.0 mg/kg), eplerenone (0.3–3.0 mg/kg) or hydrocortisone (GR agonist; 0.3–10 mg/kg) were admixed with micronized powdered lactose vehicle. Drugs were injected directly into the trachea using a hand-operated DP-4 insufflator delivery device (PennCentury, Philadelphia, PA). Animals were kept on a heating pad until they recovered from anesthesia, then returned to their cages and allowed food and water ad libitum. All animals survived these manipulations and no additional interventions were required to ensure their survival. Five hours after i.t. dosing, animals were placed in a plexiglas chamber (21 l) and exposed to aerosolized ovalbumin (1%) for 30 min. Non-sensitized rats were exposed to isotonic saline for 30 min. Using an ultrasonic nebulizer (Model Ultra-Neb 99; DeVilbiss, Somerset, PA, USA), ovalbumin aerosol was generated and circulated through the chamber at a flow rate of approximately 8 l/min. Total bronchioalveolar lavage (BAL) inflammatory cell counts were measured 24 h after the antigen challenge (Celly et al., 2006). The activity of spironolactone and eplerenone on pulmonary cell influx also included an assessment on BAL eosinophil and neutrophil cell populations (i.e. differential cell counts).

2.4. Effect of MR antagonist on collagen formation in an ex vivo lung fibrosis model

The effects of eplerenone (0.01 and 10 μM), spironolactone (10 μM), pirfenidone (10 μM) and captopril (10 μM) on collagen formation in an *ex vivo* lung slice fibrosis model which was