Agonists of MAS oncogene and angiotensin II type 2 receptors attenuate cardiopulmonary disease in rats with neonatal hyperoxia-induced lung injury

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Premature infants at risk for neonatal chronic lung disease (CLD) or bronchopulmonary dysplasia (BPD) suffer from lung damage due to mechanical ventilation and/or reactive oxygen species generated by (prolonged) exposure to supplemental oxygen. CLD leads to a permanent simplification of the alveoli, caused by an arrest in alveolar and vascular development, and a subsequent reduction of the alveolar surface and lung function (5, 19). Serious complicating factors in the perinatal period are inflammation and oxidative stress, and at later stages pulmonary arterial hypertension (PAH)-induced right ventricular hypertrophy (RVH) and heart failure (1, 5). PAH is characterized by persistent vasoconstriction and structural remodeling of the pulmonary blood vessels with increased proliferation of vascular smooth muscle cells, leading to a reduction in blood vessel lumen. This process ultimately leads to high mortality due to right heart failure in children and adults (1, 2, 36, 41). Similar to premature infants suffering from CLD, neonatal rats that are exposed to hyperoxia show chronic lung inflammation, persistent alveolar simplification, fibrosis, pulmonary hypertension, and RVH (11, 12).

The renin-angiotensin system plays an important role in the regulation of cardiovascular and renal function and pathogenesis of lung injury (20, 42). The balance between the opposing effector molecules angiotensin II (AngII) and angiotensin-(1-7) [Ang-(1-7)] may play a pivotal role in determining cardiovascular pathogenesis and lung fibrosis (32, 42, 43). After the conversion of angiotensinogen into angiotensin (AngI) by renin, AngII is generated by conversion of AngI by angiotensin converting enzyme (ACE). Ang-(1-7) is generated directly via the conversion of AngII by ACE-2 or indirectly via AngI by ACE-2 and ACE. Binding of AngII to the AngII type 1 receptor (AT1) leads to vasoconstriction, proliferation, and fibrosis in multiple tissues, including the lung, whereas binding of Ang-(1-7) to the MAS oncogene receptor (MAS) counter-balances the detrimental biological actions of AngII, by inducing vasodilation and by inhibiting fibrinogenesis, thrombogenesis, hypertension, cardiac hypertrophy, and lung injury (9, 20, 24, 31, 33, 40, 43). In multiple adult rat models of cardiopulmonary disease Ang-(1-7) treatment protected against monocrotalin-induced PAH and RVH (33), bleomycin-induced pulmonary fibrosis (33), and LPS-induced respiratory distress (49). In addition, protective effects of MAS in cardiac disease are suggested by investigations in MAS-deficient mice that became hypertensive (4). In animal models of bleomycin or cigarette smoke-induced lung injury and neonatal rats with hyperoxia-induced fibrinous lung injury, beneficial effects can be obtained by blocking AT1 or ACE (7, 26, 28, 49) or increasing expression of Ang-(1-7) or ACE-2 (23, 29, 33). Alternatively, the detrimental effects of AngII binding to AT1

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may be counterbalanced by binding to AT2, resulting in reduced proliferation, inflammation, and fibrosis (20, 34, 44). However, knowledge of the role of AT2 signaling in the lung is still incomplete and controversial, because 1) AT2 receptor signaling can be dependent of the presence of AT1 receptors (6 and 2) the switch in receptor function when comparing models with varying hemodynamic conditions or various cell types can be either a physiological antagonism of AT1 receptor or, conversely, a mimic of AT1 signaling (44). A potential beneficial effect of AT2 signaling in pulmonary disease is demonstrated in genetically modified mice, deficient for AT2, in which acute lung and heart injury is aggravated compared with wild-type controls (3, 17), but pharmacological blocking of AT2 attenuates bleomycin-induced fibrosis (47), suggesting an adverse effect of AT2 on pulmonary disease. Additional studies to confirm a beneficial effect of AT2 signaling in cardiopulmonary disease have long been hampered by low expression of the receptor in normal adult tissues and lack of a highly specific agonist for AT2 (34, 35). In this study we have overcome both pitfalls by using a recently developed highly specific agonist that is resistant against degradation in a neonatal rat model for cardiopulmonary disease, in which AT2 expression is expected to be high, because of its high expression in the fetal period (9). This AT2 agonist, termed LP2–3, is fully resistant to ACE. It induces Erk phosphorylation in human bronchial epithelial cells, which is completely inhibited by the AT2 antagonist PD 123319, which indicates high specificity for AT2 (Lanthio Pharma, unpublished data).

Although treatment of lung fibrosis with AT1 blockers attenuates neonatal hyperoxia-induced lung injury, the role of treatment with agonists for the MAS and AT2 receptors is unknown under these conditions. We hypothesize that an active renin-angiotensin system is present and functionally active in the neonatal lung and that stimulation of MAS or AT2 will improve CLD pathology by stimulating alveolar development and reducing lung inflammation, pulmonary hypertension and right ventricular hypertrophy. We tested this hypothesis by treating neonatal rats with experimental CLD after prolonged exposure to hyperoxia with cyclic Ang-(1-7) [cAng-(1-7)] or the AT2 agonist dKcAng-(1-7) and investigating inflammation, alveolarization, and pulmonary hypertension in the lung and right ventricular hypertrophy in the heart as described previously (11).

MATERIALS AND METHODS

Animals

The research protocol was approved by the Institutional Animal Care and Use Committee of the Leiden University Medical Center. Adult Wistar rats (6 mo old; N = 6) were exsanguinated after induction of anesthesia with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (50 mg/kg). Organs were stored at −80°C until isolation of RNA for real-time RT-PCR.

Per experiment newborn rat pups from two to three litters were pooled and distributed over the experimental group. For the MAS agonist experiments neonatal rat pups were distributed over two experimental groups (N = 12): an oxygen and oxygen-agonist group and two room air (RA)-exposed control groups (N = 6) injected with either saline or agonist. For the AT2 agonist experiments neonatal rat pups were distributed over four experimental groups (N = 12), an oxygen, oxygen-agonist, oxygen-Nω-nitro-l-arginine methyl ester (l-NAME), and oxygen-l-NAME-agonist group and four RA-exposed control groups (N = 6) injected either with saline, agonist, and/or l-NAME. Pups were fed by foster dams. Foster dams were rotated daily between the oxygen-exposed pups and two groups of RA-exposed pups to avoid oxygen toxicity: 24 h in 100% oxygen and 48 h in RA. Oxygen concentration, body weight, evidence of disease, and mortality were monitored daily.

Early Concurrent Treatment

Pups were continuously exposed to 100% oxygen for 10 days. From day 2 onward, pups received subcutaneous injections of either 10 µg·kg body wt−1·day−1 of MAS agonist cAng-(1-7) [4,7-lanthionine-stabilized angiotensin-(1-7) (13, 21)] or the AT2 agonist dKcAng-(1-7) [LP2–3: cAng-(1-7) containing a NH2-terminal d-lysine] in 100 µl of 0.9% saline or just 100 µl of 0.9% saline (age-matched control). The linear peptide dKDRvDcIHC, in which dK is a d-lysine and dC is a d-cysteine, was obtained from Pepscan, Lelystad, the Netherlands. dKcAng-(1-7) was obtained from disulfide-bridged dKDRvDcIHC by base-assisted sulfur extrusion.

Both agonists were prepared by Lanthio Pharma, Groningen, The Netherlands. Linear Ang-(1-7) interacts with MAS, but at high concentrations it may also interact with AT1, which exerts opposite effects. Tyr4 is essential for interaction of AngII with AT1 (25). This amino acid is absent in cAng-(1-7) and dKcAng-(1-7) and thereby precludes their agonistic interaction with AT1. As a result they will be more specific for MAS and AT2, respectively. Lung and heart tissue was collected on day 10. To find the optimal dosing of both agonists we performed a pilot experiment in which hyperoxia-exposed rat pups were treated with 10–30 µg·kg−1·day−1 of cAng-(1-7) (MAS agonist), or 5–20 µg·kg−1·day−1 of dKcAng-(1-7) (AT2 agonist) or saline (N = 6). Both agonists were studied in separate experiments. We used the ratio of right to left free ventricular wall thickness (RV/LV) in histological sections of the heart as a readout. This parameter was selected for two reasons: 1) hyperoxia-induced neonatal CLD results in severe experimental CLD with persistent PAH-induced RVH (11, 12), and 2) stimulation of MAS or AT2 is expected to be a potent treatment option for PAH and cardiac hypertrophy in adults (33, 34). Pups exposed to hyperoxia developed RVH, which could be completely prevented by administration of MAS agonist or AT2 agonist (5 µg·kg−1·day−1; Fig. 1). Separate experiments were performed for 1) histology (N = 8), 2) lung tissue homogenates (N = 10), and 3) collection of bronchoalveolar lavage fluid (N = 12). To quantify the degree of RVH, hearts were harvested, followed by removal of the atria. Next, the right ventricular (RV) wall free was dissected, weighed separately from the interventricular septum (IVS) and left ventricle (LV), frozen immediately in liquid nitrogen, and stored at −80°C for RNA isolation. As an indicator of RVH the weight ratio RV/LV + IVS was calculated (N = 8). In an additional experiment, the effect of specific nitric oxide synthase inhibition with 25 mg·kg−1·day−1 of l-NAME (Sigma, St. Louis, MO) in 0.9% saline on AT2 stimulation was investigated (N = 8) in RA- and oxygen-exposed pups, injected daily with saline, LP2–3, l-NAME, or LP2–3 and l-NAME. A concentration of 25 mg·kg−1·day−1 of l-NAME in 0.9% saline completely abolishes the beneficial effects of apelin, which are dependent on eNOS activation, in experimental BPD (11). In a separate experiment lung tissue was collected from neonates on days 1, 3, 6, and 10 and from adult rats (6 mo) for RT-PCR (N = 8).

Experiments with agonists for AT2 and MAS and the effect of l-NAME on AT2 stimulation were performed in different nonoverlapping time frames. This may contribute to the biological variation that was observed in some of the parameters studied in the response to hyperoxia with the agonists for AT2 and MAS. We included the own control groups for both interventions in RA- and oxygen-exposed pups to correct for potential differences in the biological response.
MAS AND AT2 STIMULATION IN HYPEROXIC LUNG INJURY

Table 1. Sequences of oligonucleotides for forward and reverse primers for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE1</td>
<td>5'-CCGCCACCTCCTCGGTACA-3'</td>
<td>5'-TGGGCAAGATCCCCTGATACT-3'</td>
</tr>
<tr>
<td>ACE2</td>
<td>5'-ACCAGAAAATGTGTCTGACATCAT-3'</td>
<td>5'-GATAGCCTCCCAGAGACATC-3'</td>
</tr>
<tr>
<td>AGT</td>
<td>5'-CTGGGCAAGATGCGTACA-3'</td>
<td>5'-GGAGTTCAAGGAGGATGCTGTT-3'</td>
</tr>
<tr>
<td>AT1a</td>
<td>5'-AGGAAGCTGTCAGAAATGCTGCTG-3'</td>
<td>5'-TGTTTTTCTGTTGGTTGAAGTGTT-3'</td>
</tr>
<tr>
<td>AT2</td>
<td>5'-CTGGGCAAGATGCGTACA-3'</td>
<td>5'-AGGGAAAAGCCAGAAATGCTGTT-3'</td>
</tr>
<tr>
<td>CINC1</td>
<td>5'-AGAAGCTGTCAGAAATGCTGCTG-3'</td>
<td>5'-GGAGTTCAAGGAGGATGCTGTT-3'</td>
</tr>
<tr>
<td>MAS</td>
<td>5'-CTGGGCAAGATGCGTACA-3'</td>
<td>5'-AAAAGTTGGGCGGCTGCTA-3'</td>
</tr>
<tr>
<td>MCP1</td>
<td>5'-ATGGCAGAATGCCCAGAGACATC-3'</td>
<td>5'-TTTCTCCAGGCAACTGATTG-3'</td>
</tr>
<tr>
<td>TF</td>
<td>5'-CCGCCACCTCCTCGGTACA-3'</td>
<td>5'-GGAGTTCAAGGAGGATGCTGTT-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-TGGGCAAGATCCCCTGATACT-3'</td>
<td>5'-GATACGCCGCGGAGACATC-3'</td>
</tr>
</tbody>
</table>

between all intervention experiments (MAS, AT2 agonist, and AT2 agonist and L-NAME).

Histology

Formalin-fixed, paraffin-embedded, 4-μm-thick heart and lung sections were stained with hematoxylin and eosin (HE). Lungs were immunostained additionally with anti-ED1 (monocytes and macrophages; 1:5), anti-myeloperoxidase (MPO, RB-373-A1, Thermo Fisher Scientific, Fremont, CA; diluted 1:1,500), anti-α smooth muscle actin (ASMA, A2547, Sigma-Aldrich, St. Louis, MO; diluted 1:10,000) or anti-von Willebrand factor (vWF, A0082, Dako Cyto-mation, Glostrup, Denmark; diluted 1:4,000), stained with EnVision-HRP (Dako, Glostrup, Denmark), by using the chromogenic substrate NovaRed as recommended by the manufacturer (Vector, Burlingame, CA), and counterstained briefly with hematoxylin by standard methods (10, 45). For morphometry of the lung, an eyepiece reticle with a coherent system of 21 lines and 42 points (Weibel type II ocular micrometer; Olympus, Zoeterwoude, The Netherlands) was used (45). We used different (immuno)histochemically stained lung sections for each quantification, except for alveolar crest and pulmonary arteriolar wall thickness, which were determined on the same ASMA-stained section. To investigate alveolar enlargement in experimental BPD we studied the number of alveolar crests to exclude potential effects of heterogeneous alveolar development. The number of alveolar crests (50), determined on lung sections stained immunohistochemically for ASMA, were assessed in 10 nonoverlapping fields at a ×400 magnification for each animal and were normalized to field. The density of ED1-positive monocytes and macrophages or MPO-positive neutrophilic granulocytes was determined in the alveolar compartment by counting the number of cells per field. Results were expressed as cells per millimeter squared. Per experimental animal 20 fields in one experiment were studied at ×400 magnification. Pulmonary alveolar septum thickness was assessed in HE-stained lung sections at a ×400 magnification by averaging 100 measurements per 10 representative fields. Capillary density was assessed in lung sections stained for vWF at a ×200 magnification by counting the number of vessels per field. At least 10 representative fields per experimental animal were investigated. Results were expressed as relative number of vessels per millimeter squared. Pulmonary arteriolar wall thickness was assessed in lung sections stained for ASMA at a ×1,000 magnification by averaging at least 10 vessels with a diameter of less than 30 μm per animal. Medial wall thickness was calculated from the formula “percent wall thickness = 2 × [wall thickness – 100”]. Fields containing external diameter wall thickness were excluded from the analysis. Thickness of the right and left ventricular free walls was assessed in a transversal HE-stained section taken halfway the long axis at a ×40 magnification by averaging six measurements per structure. RVH was calculated for each heart by dividing average RV free wall thickness and average LV free wall thickness. For morphometric studies in lung and heart eight rat pups per experimental group were studied. Quantitative morphometry was performed by two independent researchers blinded to the treatment strategy using the NIH Image J program (10–12, 50).

Fibrin Detection Assay

Quantitative fibrin deposition was determined in lung tissue homogenates by Western blotting (11, 45; N = 10). Lung tissue homogenates for quantitative fibrin deposition by Western blotting were pretreated as described previously (45). Tissue samples, dis-
glycerol, 5% β-mercaptoethanol, and 0.4 mg/ml of bromophenol blue) were subjected to SDS-PAGE (7.5% gel; 5% stacking gel) and blotted onto PVDF membrane (Immobilon-FL, Millipore, Bedford, MA). The 56-kDa fibrin β-chains were detected with monoclonal 59D8 (Oklahoma Medical Research Foundation, Oklahoma City, OK; diluted 1:1,000), infrared labeled goat-anti-mouse secondary antibody (IRDye 800CW; Licor Biosciences, Lincoln, NE, diluted 1:5,000), and quantified by use of an infrared detection system (Odyssey infrared imaging system, Fig. 2. Growth (A) and survival (B) on day 10 (N = 12) in RA- and age-matched O2-exposed pups injected daily with saline or agonist for MAS [cAng-(1-7), 5 μg/kg twice a day] or AT2 [dKcAng-(1-7), 5 μg/kg twice a day]. Data are expressed as means ± SE. Open bar, RA-NaCl; hatched bar, RA-agonist; solid bar, O2-NaCl; shaded bar, O2-agonist. Data are expressed as means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. age-matched O2-exposed controls. ΔΔΔP < 0.001 vs. own RA-exposed controls.

Fig. 3. Representative lung sections stained for von Willebrand factor (vWF; A–D), α-smooth muscle actin (ASMA; E–H), the monocyte and macrophage marker ED1 (I–L), or myeloperoxidase (MPO) as a marker for neutrophilic granulocytes (M–P) of RA (A, E, I, and M) and O2-exposed pups (O2; B–D, F–H, J–L, and N–P) injected daily with saline (A, B, E, F, I, J, M, and N), MAS agonist [cAng-(1-7), 5 μg/kg twice a day; C, G, K, and O] agonist [dKcAng-(1-7), 5 μg/kg twice a day; D, H, L, and P] until 10 days of age. Values are expressed as means ± SE (N = 8). a = alveolus. Arrows in A–D indicate vWF-positive blood vessels.
Administration of both agonists for 10 days had no adverse effects on mean body weight in RA controls and oxygen-exposed pups. Exposure to hyperoxia resulted in a 50% survival on day 10 and was not affected by administration of agonist (Fig. 2B). RA-exposed pups showed no morbidity or mortality during the experimental period of 10 days.

**Effects of MAS and AT2 Agonists on Lung Airway Development and Inflammation**

In rat lungs development proceeds from the saccular stage at birth toward the alveolar stage in 10 days (Fig. 3A). Administration of both agonists did not have adverse effects on the number of alveolar crests (Fig. 4A), pulmonary vessel density (Fig. 4B), alveolar septum thickness (Fig. 4C), arterial medial wall thickness (Fig. 4D), and influx of macrophages (Fig. 4E) and neutrophilic granulocytes (Fig. 4F). Oxygen exposure for 10 days resulted in edema, a heterogeneous distribution of enlarged air spaces with a decreased number of alveolar crests (2-fold, \( P < 0.001 \); Fig. 4A), surrounded by septa with increased thickness (2-fold, \( P < 0.001 \); Fig. 4B and 4C), reduced pulmonary vessel density (2- to 3-fold, \( P < 0.001 \); Fig. 4B and 4D), and increased pulmonary arterial medial wall thickness (2.6-fold, \( P < 0.001 \); Fig. 3F and 4D). Hyperoxia led to a massive inflammatory reaction, characterized by an overwhelming influx of inflammatory cells, including macrophages (4-fold, \( P < 0.001 \); Fig. 3F and 4E) and neutrophils (3-fold, \( P < 0.001 \); Fig. 3N and 4F), compared with RA-exposed controls. Administration of the MAS agonist reduced alveolar septal thickness (1.4-fold, \( P < 0.001 \); Fig. 4C), arterial medial wall thickness (1.4-fold, \( P < 0.001 \); Fig. 4D), and the influx of neutrophils (2.1-fold, \( P < 0.005 \); Fig. 3O and 4F) compared with oxygen-exposed controls but had no beneficial effects on hyperoxia-induced inhibition of alveolarization and

**Statistical Analysis**

Values are expressed as means ± SE. Differences between groups were analyzed by one-way ANOVA for independent samples, followed by Tukey’s multiple comparison test, by use of the GraphPad Prism, version 5, software package (San Diego, CA). Differences at \( P \) values < 0.05 were considered statistically significant.

**RESULTS**

**Effects of MAS and AT2 Agonists on Growth and Survival**

On day 10, mean body weight of pups was comparable in all RA groups (18 g; Fig. 2A) and all oxygen groups (13–14 g). Administration of both agonists for 10 days had no adverse effects on mean body weight in RA controls and oxygen-exposed pups. Exposure to hyperoxia resulted in a 50% survival on day 10 and was not affected by administration of agonist (Fig. 2B). RA-exposed pups showed no morbidity or mortality during the experimental period of 10 days.

**Fig. 4. Lung morphometry, including the quantifications of alveolar crests (A), number of pulmonary vessels (B), septal thickness (C), arterial medial wall thickness (D), and influx of macrophages (E) and neutrophilic granulocytes (F), was determined on paraffin sections in RA pups injected daily with saline (open bar) or agonist (hatched bar) and \( O_2 \) pups injected daily with saline (solid bar) or agonist (shaded bar): 5 \( \mu g \) kg twice a day of MAS agonist [cAng-(1-7)] or AT2 agonist [dKcAng-(1-7)] until 10 days of age. Values are expressed as means ± SE (N = 8). *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \) vs. age-matched \( O_2 \)-exposed controls. \( \Delta P < 0.05 \), \( \Delta \Delta P < 0.01 \), and \( \Delta \Delta \Delta P < 0.001 \) vs. own RA controls.
angiogenesis and influx of macrophages. Administration of the AT2 agonist reduced alveolar septal thickness (1.5-fold, \( P < 0.001 \); Fig. 4C), arterial medial wall thickness (1.4-fold, \( P < 0.01 \); Fig. 3H and 4D), and the influx of macrophages (1.5-fold, \( P < 0.05 \); Fig. 3L and 4E) compared with oxygen exposed controls but had no beneficial effects on hyperoxia-induced inhibition of alveolarization and angiogenesis and the influx of neutrophils.

Administration of \( \text{l-NAME} \) did not have adverse effects on the number of alveolar crests (Fig. 5A), pulmonary vessel density (Fig. 5B), alveolar septum thickness (Fig. 5C), arterial medial wall thickness (Fig. 5D), and influx of macrophages (Fig. 5E) and neutrophilic granulocytes (Fig. 5F). The beneficial effects of LP2–3 treatment of hyperoxia-induced lung injury on septal thickness (Fig. 5C), medial wall thickness (Fig. 5D), and influx of macrophages (Fig. 5E) and neutrophils (Fig. 5F) persisted in the presence of \( \text{l-NAME} \).

**Effects of MAS and AT2 Agonists on Lung Coagulation and Vascular Leakage**

Pulmonary fibrin deposition is a sensitive marker for tissue damage in hyperoxia-induced neonatal lung disease (45) and was studied in homogenates as a readout for lung damage (Fig. 6A). The protein concentration on postnatal day 10 increased 15-fold after hyperoxia (cAngI–7 experiment, \( P < 0.001 \)) and was not affected by agonist administration under normoxia or hyperoxia.

**mRNA Expression in Lung Tissue**

Renin-angiotensin system during neonatal lung development and experimental BPD. Directly after birth on day 1 the presence of a functional renin-angiotensin system was demonstrated by mRNA expression of the receptors AT1, AT2, and MAS (Fig. 7, A–C), angiotensinogen (Fig. 7D), and the converting enzymes ACE-1 and ACE-2 (Fig. 7, E and F). During normal lung development mRNA expression of AT1, AT2, and ACE-2 decreased gradually, whereas expression of angiotensinogen and ACE-1 increased gradually. This resulted in a 1.8-fold (\( P < 0.001 \)), 9.3-fold (\( P < 0.001 \)), and 2.7-fold (\( P < 0.001 \)) decrease in mRNA expression for AT1, AT2, and ACE-2, and an 1.6-fold (\( P < 0.05 \)) and a 2.7-fold (\( P < 0.001 \)) increase in mRNA expression for angiotensinogen and ACE-1, respectively, on day 10 compared with day 1. During normal lung development MAS mRNA expression was decreased on day 6 (2.5-fold; \( P < 0.001 \)) compared with day 1. In adult lung,
expression of all three receptors was decreased compared with day 10: 1.3-fold ($P < 0.05$) for AT1, 10.2-fold ($P < 0.001$) for AT2 and 2.2-fold ($P < 0.01$) for MAS, and expression was increased compared with day 10 for angiotensinogen (2.2-fold, $P < 0.001$), ACE-1 (2.5-fold, $P < 0.001$), and ACE-2 (1.9-fold, $P < 0.001$). Exposure to 100% oxygen for 10 days resulted in an increase in expression of AT2 (3.2-fold, $P < 0.001$) and a decrease in expression of AT1 (1.5-fold, $P < 0.001$), angiotensinogen (2.2-fold, $P < 0.001$), and ACE-1 (4.6-fold, $P < 0.001$) compared with RA controls on day 10. MAS and ACE-2 did not show differential mRNA expression during early neonatal lung development compared with oxygen-exposed rat pups.

**Effects of agonist administration on mRNA expression in lung tissue.** Administration of MAS agonist, but not AT2 agonist, for 10 days during normal neonatal development in RA resulted in an increase in mRNA expression (Fig. 8) of AT1a (Fig. 8D; 2.1-fold, $P < 0.001$) and ACE-1 (Fig. 8H; 1.8-fold, $P < 0.001$). Expression of the proinflammatory factors monocyte chemoattractant protein (MCP)-1 (Fig. 8A) and the chemokine-induced neutrophilic chemoattractant-1 (CINC1; 8B), the procoagulant protein tissue factor (TF; 8C), AT2 (Fig. 8E), MAS (Fig. 8F), and angiotensinogen (Fig. 8G), and ACE-2 (Fig. 8I) did not change during normoxia. Ten days of oxygen exposure resulted in an increase in mRNA expression of MCP-1 (5.0-fold; MAS experiment, $P < 0.001$ and 10.6-fold;
AT2 experiment, \( P < 0.001 \), and 16.1-fold; AT2 experiment, \( P < 0.001 \), TF (2.8-fold; MAS experiment, \( P < 0.001 \) and 5.1-fold; AT2 experiment, \( P < 0.001 \)), and AT2 (2.9-fold; MAS experiment, \( P < 0.001 \) and 3.2-fold; AT2 experiment, \( P < 0.001 \)), whereas mRNA expression was decreased in lungs of oxygen-exposed pups for AT1a (1.9-fold; AT2 experiment, \( P < 0.001 \)), angiotensinogen (1.7-fold; MAS experiment, \( P < 0.01 \) and 1.8-fold; AT2 experiment, \( P < 0.001 \)), and ACE-1 (2.9-fold; MAS and AT2 experiment, \( P < 0.001 \)) compared with RA controls.

Treatment of oxygen-exposed pups with MAS or AT2 agonist for 10 days did not result in changes in mRNA expression of MAS (Fig. 8F) and ACE-2 (Fig. 8I) compared with oxygen-exposed pups.

**Right Ventricular Hypertrophy**

Administration of MAS or AT2 agonists for 10 days during normal neonatal development had no adverse effect on the heart (Fig. 9). Exposure to hyperoxia for 10 days resulted in RVH, affected by an 1.3-fold increase in the ratio RV/LV free wall thickness (Fig. 9, A and B) and an 1.5-fold increase in weight ratio RV/(LV + IVS; Fig. 9C) compared with RA controls (\( P < 0.001 \)). Administration of agonists prevented RVH, demonstrated by a normalization in relative RV/LV free wall thickness (\( P < 0.001 \)) and a decrease in RV/(LV + IVS) weight ratio after AT2 agonist treatment (\( P < 0.05 \)) compared with oxygen-exposed pups.

Administration of L-NAME did not have adverse effects on cardiac development and did not prevent the beneficial effects of LP2–3 treatment on hyperoxia-induced RVH (Fig. 9D).

**DISCUSSION**

Administration of agonists for MAS or AT2 to neonatal rat pups exposed to prolonged hyperoxia, an in vivo model for experimental CLD (45), reduced cardiopulmonary injury by attenuating arterial medial wall thickness (PAH), alveolar septal thickness and inflammation in the lung, and RVH. Both agonists had no beneficial effects on lung alveolarization and vascularization and capillary alveolar leakage and no adverse effects on normal lung and heart development. These findings...
demonstrate that agonists for MAS [cAng-(1-7)] and AT2 [dKcAng-(1-7)] may be suitable candidates to prevent PAH-induced RVH in preterm infants with severe CLD.

The receptors AT1, AT2, and MAS, the precursor of their ligands angiotensinogen, and the converting enzymes ACE-1 and ACE-2 are differentially expressed during lung development and/or in hyperoxia-induced lung injury, suggesting a role for angiotensin-angiotensin receptor signaling in the pathophysiology of severe experimental CLD, in which arrested alveolarization and pulmonary hypertension play a pivotal role. However, the role of the renin-angiotensin system in experimental CLD is still unclear. The relatively high expression of AT2 in the neonatal lung and the adaptive response in mRNA expression in the neonatal lung toward hyperoxia, resulting in a relative increase in AT2 expression, is in favor of increased AT2 signaling and may contribute to the beneficial cardiopulmonary effects that we observed after AT2 agonist treatment of rat pups with experimental CLD in this study.

The beneficial effects of MAS and AT2 agonist administration to rat pups with experimental CLD are mainly mediated via prevention of PAH-induced RVH and are in agreement with observations in multiple adult rodent models, including decreased pulmonary hypertension and lung fibrosis (33) and acute LPS-induced lung injury in ventilated rats after Ang-(1-7) treatment (48), and cardiac hypertrophy in Ang-(1-7) overexpressing rats (4). Analogous to findings in apoE-deficient mice, in which diet-induced atherosclerosis could be attenuated by Ang-(1-7)-induced improved endothelial function via NOS-dependent vasorelaxation (30, 38), reduced pulmonary hypertension in experimental CLD may be explained by a vasoprotective effect of Ang-(1-7). In addition, the protective effects of Ang-(1-7) on the vasculature may involve stimulation of NO and bradykinin (16, 46). Controversy exists about the role of NO and how various NO synthases, which are expressed in multiple cell types, including inflammatory and vascular cells, should be studied in lung models (27). Conflicting data may arise from stimulation of NOS in different cell types of the diseased lung. Furthermore, NO may lead to inflammation (18) and reduced vascular remodeling. Inhaled NO has been used as an experimental therapy to improve vascular and alveolar structure in various models of lung injury, including bleomycin and hyperoxia-induced neonatal lung injury in rats (39, 37), and is supported by our previous data on the beneficial effects of eNOS-NO-cGMP signaling in this animal model for experimental CLD (10, 11, 37). Together these data demonstrate the therapeutic potential of Ang-(1-7) in cardiopulmonary disease in adults and neonates.

Inflammation and coagulation play an important role in experimental CLD as demonstrated by the massive influx of macrophages and neutrophils to the lung and the upregulation of genes that are involved in the activation and migration of leukocytes in vivo, including uPA/uPAR and chemokines (45) and the beneficial effects of reduced neutrophil influx after anti-chemokine treatment (50) and of PDE-4 inhibitors, which...
are potent anti-inflammatory agents (12). The beneficial effects of treatment with both agonists on hyperoxia-induced neonatal lung injury may, at least in part, be explained by reduced inflammation, demonstrated by a reduced influx of inflammatory cells (neutrophilic granulocytes for MAS agonist, and neutrophils and macrophages for AT2 agonist) and reduced fibrin deposition (AT2 agonist) in the lung. The differing effects of each agonist on inflammation clearly suggest a different mechanism than AT1 antagonism alone. Indeed cAng-(1-7) and dKcAng-(1-7) function fundamentally differently. cAng-(1-7) stimulates MAS, whereas dKcAng-(1-7) acts via AT2, which receptor seems to heterodimerize with AT1, thereby inhibiting AT1 receptor signaling (14, 44). Apparently these functional differences may cause the differential effects on inflammation. However, this was not confirmed by mRNA expression of proinflammatory cytokines (MCP1 and CINC1) and the pulmonary presence of macrophages. Conflicting data on the role of MAS and Ang-(1-7) are reported on inflammation in multiple tissues. In mice with renal ischemia-reperfusion injury Ang-(1-7) infusion aggravated the inflammatory response, whereas kidney injury in MAS-deficient mice was less severe (15). In contrast, an anti-inflammatory effect of Ang-(1-7) was reported in lungs of mice with experimental asthma by ovalbumin-induced allergic lung inflammation and antigen-induced arthritis (8, 14). In both animal models MAS activation attenuated the inflammatory response by decreasing neutrophil accumulation, whereas in mice deficient for MAS arthritis was aggravated because of an increased influx of neutrophils and proinflammatory cytokines. This discrepancy in the role of MAS in inflammation may be due to the complex pathogenesis of multiple models of tissue injury in different organs and the activation of multiple signal transduction pathways, including NO-dependent signaling as discussed above.

Biological variation was observed in some of the parameters studied in the response to hyperoxia, with the highest variation in fibrin deposition and vascular leakage, which may be explained by the fact that interventions with the agonists for AT2 and MAS were performed in different experiments in nonoverlapping time frames.

Our findings in AT2 agonist-treated neonatal rats with hyperoxia-induced lung injury are in line with experiments in AT2-deficient mice, in which acute lung disease is aggravated (17), but in sharp contrast with the beneficial effects of pharmacological blocking of AT2 on bleomycin-induced fibrosis (47). This discrepancy may be due to differences in maturation (neonatal vs. adult), with relatively high AT2 levels of expression in the neonatal period and different triggers for injury.

When the absence of adverse effects of treatment with both agonists in rats is confirmed in patients, extrapolation of the beneficial effects of AT2 and MAS agonist treatment in rat pups with experimental CLD to preterm infants with respiratory failure may result in a beneficial effect of both agonists on PAH and RVH, which are the major reasons for mortality or severe morbidity in preterm infants with severe BPD.

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