Meconium Increases Type 1 Angiotensin II Receptor Expression and Alveolar Cell Death

CHARLES R. ROSENFELD, ALEXANDER M. ZAGARIYA, XIAO-TIE LIU, BRIGHAM C. WILLIS, STEVEN FLUHARTY, AND
DHARMAPURI VIDYASAGAR

*Division of Neonatal-Perinatal Medicine [C.R.R.], Division of Pulmonary and Vascular Biology [B.C.W.], UT Southwestern Medical
Center, Dallas, Texas 75390; Department of Pediatrics [A.M.Z., D.V.], University of Illinois, Chicago, Illinois 60616; and Department of
Animal Biology [S.F.], University of Pennsylvania, Philadelphia, Pennsylvania 19104*

ABSTRACT: The pulmonary renin-angiotensin system (RAS) contributes to inflammation and epithelial apoptosis in meconium aspiration. It is unclear if both angiotensin II receptors (ATR) contribute, where they are expressed and if meconium modifies subtype expression. We examined ATR subtypes in 2 wk rabbit pup lungs before and after meconium exposure and with and without captopril pretreatment or type 1 receptor (AT_1R) inhibition with losartan, determining expression and cellular localization with immunoblots, RT-PCR and immunohistochemistry, respectively. Responses of cultured rat alveolar type II pneumocytes were also examined. Type 2 ATR were undetected in newborn lung before and after meconium instillation. AT_1R were expressed in pulmonary vascular and bronchial smooth muscle and alveolar and bronchial epithelium. Meconium increased total lung AT_1R protein approximately 3-fold ($p = 0.006$), mRNA 29% ($p = 0.006$) and immunostaining in bronchial and alveolar epithelium and smooth muscle, which were unaffected by captopril and losartan. Meconium also increased AT_1R expression >3-fold in cultured type II pneumocytes and caused concentration-dependent cell death inhibited by losartan. Meconium increases AT_1R expression in newborn rabbit lung and cultured type II pneumocytes and induces AT_1R-mediated cell death. The pulmonary RAS contributes to the pathogenesis of meconium aspiration through increased receptor expression. (*Pediatr Res* **63:** 251–256, 2008)

The fetal excretion of meconium into the amniotic fluid occurs in 18–20% of near-term and term pregnancies and is associated with intra-uterine stress (1–5). When episodes are severe, fetal respiratory efforts are altered and meconium contaminated amniotic fluid may be inhaled before, at the time of, or soon after birth in approximately 5% of neonates. These neonates may develop pneumonitis and lung injury secondary to meconium aspiration syndrome (MAS) (2,3,5,6). Thirty percent of neonates with MAS require mechanical ventilation, and many develop respiratory failure requiring inhaled nitric oxide, high-frequency ventilation, or extracorporal membrane oxygenation. It is unclear, however, what contributes to the pathogenesis and severity of MAS.

The clinical manifestations of MAS reflect airway obstruction due to inhaled particulate matter and development of chemical pneumonitis and inflammation, which contribute to the severity of MAS (5). Inflammatory cytokines are elevated in the blood of newborn piglets and the tracheal lavage and lungs of animals after pulmonary instillation of meconium (5,7–10). In most animal studies, homogenized meconium containing particulate matter was instilled into the lungs, making it difficult to separate the obstructive and inflammatory components. We (7) removed the obstructive effects of the particulate matter by using a sterile filtrated supernatant from homogenized term human meconium (MS). In newborn rabbits, MS caused pulmonary inflammation, neutrophil migration, epithelial cell apoptosis, and activation of the pulmonary renin-angiotensin system (RAS) as evidenced by increased angiotensinogen mRNA (7,11). Similar changes occur in models of adult lung disease after exposure to agents such as bleomycin (12); *i.e.*, the lung RAS is up-regulated, as evidenced by increased tissue angiotensinogen and angiotensin II (ANG II), as are various cytokines and cellular apoptosis (12–15). Thus, the pulmonary RAS appears to contribute to the pathogenesis of MAS, and as in the adult, stimulates local inflammatory responses, apoptosis, and lung damage.

Two angiotensin II (ANG II) receptor (ATR) subtypes mediate ANG II signaling (16). They are derived from separate genes yet share 40% homology (17). The type 1 ANG II receptor (AT_1R) predominates in adults and mediates most biologic responses to ANG II (16). The type 2 receptor (AT_2R) is primarily expressed in fetal tissues, and other than the female reproductive tissues (18) it is down-regulated soon after birth (19). Its role in ANG II-mediated events remains unclear, but may include attenuation of AT_1R-mediated effects (16,20,21). The expression, localization, and regulation of ATR subtypes in the developing lung are unknown. The AT_1R mediates pulmonary epithelial apoptosis in adult lung and contributes to pulmonary responses to MAS (12,13); the role of the AT_2R is unknown. Furthermore, it is unclear if meconium alters subtype expression. Therefore, we (1) characterized ATR subtype distribution in newborn rabbit lung, (2) determined if meconium exposure modifies pulmonary sub-

Abbreviations: ACE, Angiotensin converting enzyme; **ANG II,** Angiotensin II; **ATR,** Angiotensin II receptors; **AT_1R,** Angiotensin II type 1 receptor; **AT_2R,** Angiotensin II type 2 receptor; **MAS,** Meconium Aspiration Syndrome; **MS,** Meconium supernatant; **RAS,** Renin-Angiotensin System

type expression and in which cells, (3) examined the role of pulmonary ANG II in ATR subtype expression (22), and finally, (4) examined the effects of meconium on ATR expression in cultured alveolar type II pneumocytes and cell viability. We hypothesized that meconium would up-regulate the lung AT_1R, which would be involved with cellular apoptosis.

METHODS

In vivo *studies*. Studies were performed in 2 wk postnatal New Zealand White rabbit pups (Kuiper Rabbit Ranch, Gary, IN) to characterize baseline pulmonary ATR subtype expression and regulation after MS exposure. Six groups of newborns ($n = 4$) were studied: Group 1 had pulmonary instillation with saline and served as controls; Group 2 underwent MS instillation alone; Group 3 received the angiotensin converting enzyme (ACE) inhibitor captopril in the drinking water for 48 h (500 mg/L; Sigma Chemical Co., St. Louis, MO) followed by saline instillation to determine whether inhibition of tissue ANG II synthesis modified basal ATR expression since changes in ANG II modify ATR expression (22); Group 4 received captopril for 48 h + MS instillation to determine whether MS-induced changes in ATR expression were due to local ANG II synthesis; Group 5 received saline instillation with the AT_1R specific antagonist losartan to determine whether basal ATR expression is altered by AT_1R activation (22); and Group 6 received MS + losartan instillation to determine whether MS-induced changes in ATR occur through AT_1R activation. The experimental protocol was approved by the Animal Care and Use Committee of Michael Reese Hospital, Chicago.

The MS was prepared as reported (7). First pass human meconium was obtained from term, healthy neonates, homogenized on ice [1 g in 9 mL of 0.9% NaCl to a 10% (wt/vol) final concentration], centrifuged at 5000 RPM for 20 min at 4°C, and the supernatant filtered using a glass filter and sterilized with a 0.2 μm filter (Millipore Co., Bedford, MA), which removes all bacteria. This debris-free supernatant allowed us to determine whether soluble factors, *e.g.*, bile salts and proteases, in MS contribute to ATR expression in newborn rabbit lung (7–9,11). Rabbits were anesthetized with intraperitoneal ketamine hydrochloride (10 mg/kg) and xylazine (1 mg/kg), the trachea exposed *via* a midline incision, and 1.2 mL/kg of 0.9% NaCl, 10% sterile MS, 0.9% NaCl plus 50 mg losartan or MS plus 50 mg losartan was instilled into the lungs followed by 5 mL air to disperse the solutions throughout the lungs. The incision was closed with 4–0 suture, and pups spontaneously breathed room air. They were euthanized with intraperitoneal nembutal (100 mg/kg) 8 h after lung instillation of each solution, the chest opened, and lungs removed and processed as described below (7–9,11). Captopril was added to the drinking water for 48 h after which either saline ($n = 4$) or MS ($n = 4$) was instilled.

Western immunoblot analysis. At the time of tissue collection, pieces of left lung were frozen on dry ice and stored at −20°C. At the time of assay, sodium dodecyl sulfate (SDS) homogenates were prepared from 15–20 mg samples (23). The homogenate was centrifuged at $10,000 \times g$ for 2 min, and the supernatant containing soluble protein was used to determine protein contents (BCA reagent, Pierce, Rockford, IL); 30 μg were subjected to electrophoresis in 7.5% polyacrylamide gels and transferred to nitrocellulose paper (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked in buffer containing powdered milk (5% wt/vol) and incubated overnight at 4°C with antiserum against AT_1R (1:1500; N-10, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or AT_2R (1:3000). Both have been validated (23–25). The nitrocellulose paper was incubated with donkey anti-rabbit IgG conjugated with affinity purified horseradish peroxidase diluted at 1:5000 with TTBS. Regions containing receptor proteins were visualized by enhanced chemiluminescence. Densitometry was performed, and values were expressed as arbitrary units. Ovine umbilical artery smooth muscle at 145 and 116 d of ovine gestation served as positive controls for AT_1R and AT_2R, respectively (23).

Immunohistochemistry. To determine the sites of ATR subtype expression, we performed immunohistochemistry. At tissue collection, lungs were removed, ligated at the hilus, and the right bronchus cannulated and instilled with 4% formaldehyde in PBS at 20 cm H_2O. Samples were embedded in paraffin and 0.6 μm sections mounted on glass slides (8). Hematoxylin-eosin staining was performed and sections analyzed under 50 and 100 μm/cm magnification by light microscopy.

Additional lung segments were fixed in 4% paraformaldehyde and embedded in paraffin (23). Sections were mounted on positive slides, deparaffinized, hydrated, incubated with avidin-biotin blocking agent, and incubated overnight with 1:200 AT_1R antibody, 1:3000 AT_2R antibody or diluent. After endogenous peroxidases were quenched with 3% H_2O_2 in H_2O, immunostain-

ing was detected with strepavidin-biotin-horseradish peroxidase and hematoxylin counter staining. Controls were done using nonimmune rabbit sera.

Reverse transcription-polymerase chain reaction. We performed semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays to examine subtype mRNA in lung tissue. Samples stored at −80°C had total RNA extracted and concentration and purity measured as described (23). RT was performed with 2 μg total RNA in 50 μL of reaction solution (23). The reaction was incubated at room temperature for 10 min, 37°C for 1 h, and terminated at 95°C for 5 min. The RNA loaded, 2 μg, was on the linear portion of a loading curve for each species, which extended between 0 and 4 μg (data not shown).

PCR was performed on 1.0 μL of RT product with primers designed from nucleotide sequences for AT_1R and AT_2R in sheep (Life Technologies, Inc., Gaithersburg, MD). Malate dehydrogenase (MDH) was the reference gene and was unaffected by the treatments studied. The PCR primers were: AT_1R, 5′-CTTTGTGGTGGGGCTATTTGG-3′ (forward) and 5′-AAAAGT-GAATATCTGGTGGGGA-3′ (reverse), 671 bp; AT_2R, 5′-CCTGTTCTCTAT-TACATTAT-3′ (forward) and 5′-GCTATAACTTCACAGCTATTA-3′ (reverse), 741 bp; MDH, 5′-CAAGAAGCATGGC-GTATACAACCC-3′ (forward) and 5′-TTTCAGCTCAGGGAGGCC-TC-3′ (reverse), 369 bp. The methods are described (23).

PCR products were size fractionated with 10 μL of the PCR reaction mixture on 1.5% agarose gels containing 25 μg/μL ethidium bromide and visualized under UV light. Optical densities of DNA bands were scanned and quantified using Scion Image software (Scion Corp., Frederick, MD). The accuracy of amplified sequences was verified by purifying the PCR products from agarose gels and sequencing them (UT Southwestern Medical Center DNA Sequencing Facility Core). To compare values across experiments, targeted PCR products were run on the same gel.

Alveolar epithelial cells. AT_1R were detected in rabbit alveolar epithelium by immunohistochemistry (see Results); thus, we examined the effects of MS on AT_1R expression and cell viability in cultured type II alveolar pneumocytes. Alveolar type II cells were isolated from adult male Sprague-Dawley rats, followed by differential adherence on IgG-coated bacteriological plates (26). Protocols were approved by the UT Southwestern Medical Center, Institutional Animal Care and Use Committee. Enriched type II cells were resuspended in minimal defined serum-free medium and seeded on tissue culture-treated polycarbonate (Nucleopore) filter cups (Transwell; Corning Costar, Cambridge, MA) at 1.0×10^6 cells/cm^2 (27). Cell were maintained in a humidified 5% CO_2 incubator at 37°C for 24 h before treatment and were >85% pure by lamellar body immunofluorescent staining; viability of >95% was assured with trypan blue dye exclusion.

After 24 h, cells were exposed to carrier solution or MS (pH 7.0) diluted 1:10 for 20 h. Monolayers were lysed in 2% SDS sample buffer and protein concentrations determined (Bio-Rad, Hercules, CA). Cellular protein from cells with and without MS exposure was resolved by SDS-polyacrylamide gel electrophoresis and transferred to membranes (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBST) at pH 7.5 and incubated for 2 h with AT_1R polyclonal rabbit antibody (see above) in TBST. Immunoblots were incubated and antibody complexes visualized as described above. Blots were imaged using a computerized imaging station (UVP, Inc., Upland, CA), and processed using Photoshop 8.0 (Adobe Systems, San Jose, CA). Intensities were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

To determine whether MS affected type II cell viability, MS (0, 1:3, 1:10, and 1:100) in serum-free media was added to cultured cells for 20 h, at which time we counted the adherent cells in monolayers maintained in media with or without MS. Nonadherent cells are considered to have died (27). Filters were washed with cold PBS, cells lysed, and nuclei stained and counted using a hemocytometer. We examined the role of AT_1R in cell death by exposing cultured cells to MS (1:10), MS + losartan (10^{-5} M), MS + the AT_2R-specific antagonist PD-123319 (10^{-5} M), or either receptor antagonist alone, performing studies in quadruplicate.

Statistical analysis. Data were analyzed using analysis of variance for multiple groups (ANOVA) and nonpaired *t* test where indicated. Data are presented as means ± SEM.

RESULTS

ATR subtypes. We initially examined AT_1R and AT_2R expression in lung samples from saline- and MS-instilled newborn rabbits and animals receiving captopril without and with MS. Notably, AT_2R protein was undetected in any group of animals, but was present in the control umbilical artery

A Western Analysis
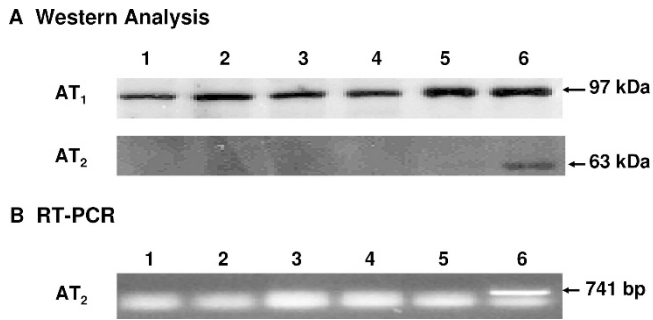
Figure 1. AT$_1$R and AT$_2$R expression in (A) 2 wk rabbit lung after instillation with saline (lane 1), MS (lane 2), saline after captopril pretreatment (lane 3) and MS after captopril (lane 4). Positive controls for AT$_1$R (lane 5) and AT$_2$R (lane 6). RT-PCR (B) for AT$_2$R mRNA for each sample are in the lower half of the figure.
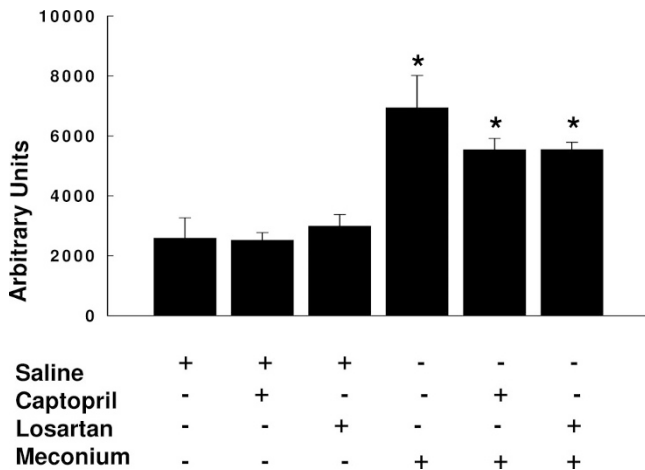

Figure 2. Summary of immunoblot analyses for AT$_1$R in 2 wk rabbit lung after saline or meconium instillation in the absence and presence of captopril pretreatment or AT$_1$R blockade. Values are densitometry in arbitrary units (see Methods). Data are means $\pm$ SEM, $n = 4$ for each group. *$p < 0.001$ versus saline.

(Fig. 1A). Since AT$_2$R protein was not detected, we performed RT-PCR to further probe for AT$_2$R expression. AT$_2$R mRNA also was undetectable in any sample except the control and was unaffected by meconium (Fig. 1B). In contrast, AT$_1$R protein was detected in all treatment groups (Fig. 1A), and meconium increased expression 2.7-fold (Fig. 2; $p = 0.006$). Neither ACE inhibition nor AT$_1$R blockade with losartan affected basal or MS-stimulated increases in AT$_1$R protein ($p > 0.1$, ANOVA), values increasing 2.6-fold after meconium exposure ($p < 0.001$, ANOVA).

AT$_1$R mRNA. To examine mechanisms regulating lung AT$_1$R expression, we performed RT-PCR on samples from saline- and MS-instilled newborn rabbits and those pretreated with captopril. As with the immunoblots, AT$_1$R mRNA was detected in the lungs of all animals (Fig. 3), values increasing 29% ($p = 0.006$, ANOVA) after MS exposure. The increase in mRNA was unaffected by ACE inhibition or losartan (data not shown).

Immunohistochemistry. We next examined the cellular distribution of ATR subtypes in newborn lung. AT$_2$R immunostaining was absent throughout the newborn lung before and after MS instillation (not shown), confirming its absence.
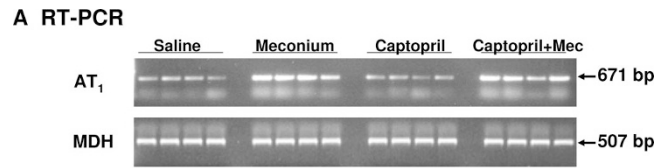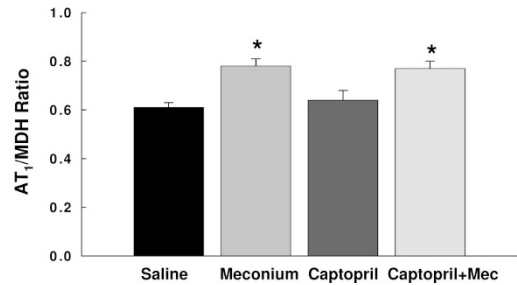
A RT-PCR

Figure 3. RT-PCR (A) for AT$_1$R and MDH mRNA in newborn lungs instilled with saline or MS with and without captopril pretreatment. Summary data (B) are presented as AT$_1$R:MDH ratio. Data are means $\pm$ SEM, $n = 4$ for each group. *$p = 0.006$ versus saline.
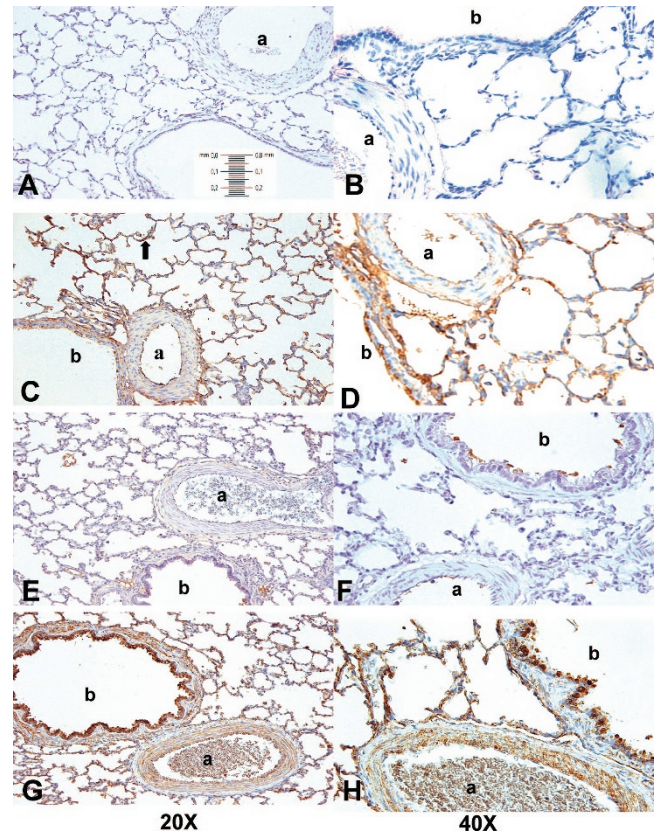

Figure 4. AT$_1$R immunohistochemistry in 2 wk rabbit lungs after instillation of saline or MS. A and B represent saline controls with nonimmune rabbit serum; C and D are saline controls incubated with anti-AT$_1$R antiserum. E and F are meconium exposure incubated with NIRS; G and H are incubated with anti-AT$_1$R antiserum. Arterial lumen is "**a**" and bronchial lumen "**b**". Arrows designates alveolar septum. A, C, E, and G are 20×; remainder are 40× magnification.

Notably, AT$_1$R immunostaining was diffuse and evident in bronchial and vascular smooth muscle and epithelial cells in the bronchial mucosa and alveolar septa of saline-treated animals (Fig. 4C, D) and markedly increased in all cell types
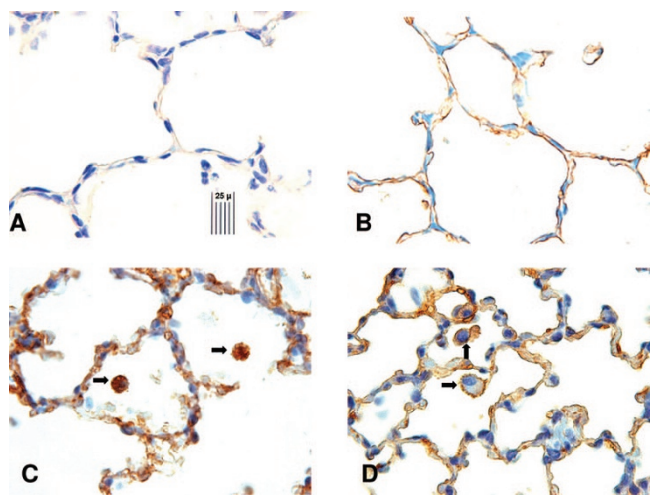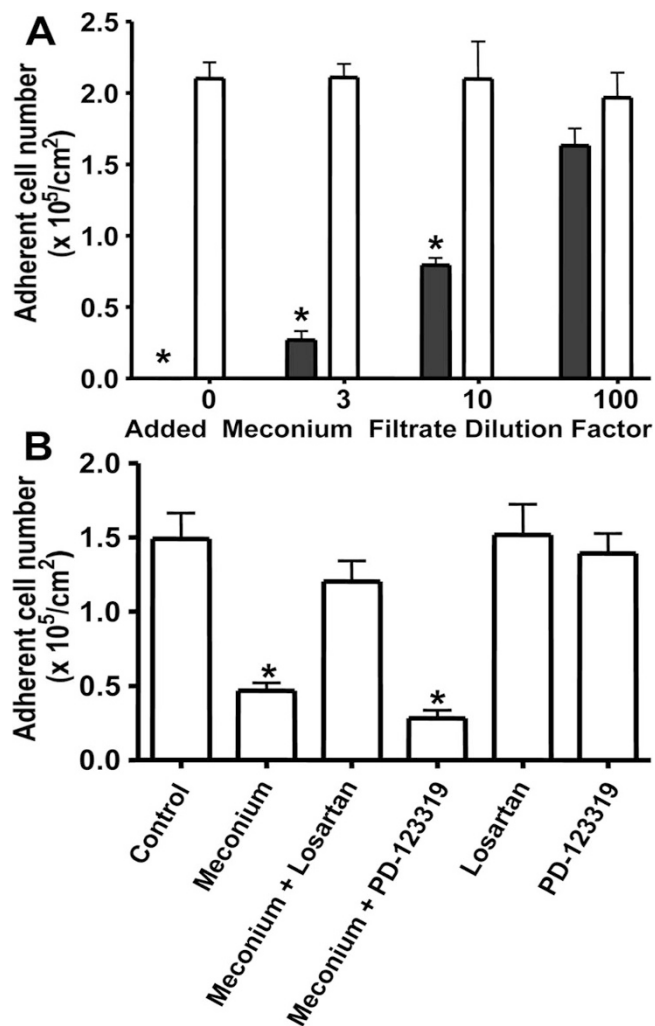
Figure 5. AT$_1$R immunohistochemistry for alveolar septae and epithelial cells in 2 wk rabbit lungs after instillation of saline (*B*), MS (*C*) and MS after captopril pretreatment (*D*). (*A*) Incubated with NIRS after saline. Arrows designate nonalveolar cells within air spaces. Magnification is 100×.
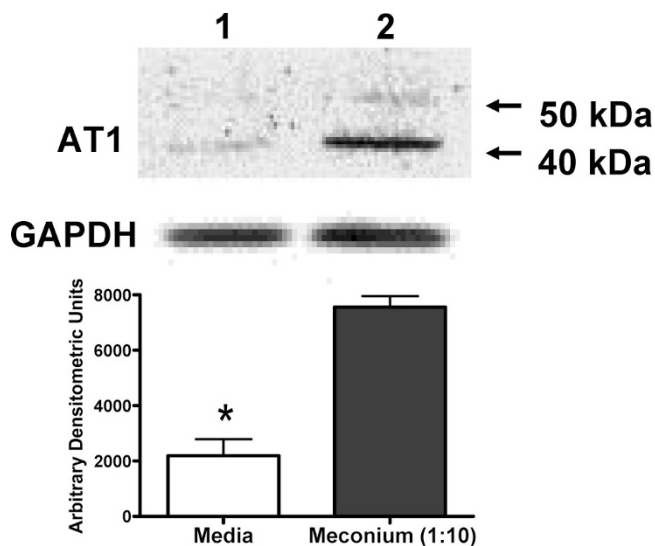
Figure 6. AT$_1$R expression in primary cultured rat type II pneumocytes without and with meconium. The upper portion is representative immunoblot for AT$_1$R and GAPDH after 20 h exposure to carrier solution + media (lane 1) and meconium at 1:10 dilution (lane 2). The lower portion is results of densitometry, $n = 3$. *$p < 0.01$.

Figure 7. Effects of MS on cell adherence in cultured type II pneumocytes. The upper portion (*A*) illustrates concentration-dependent decreases (*$p < 0.001$, ANOVA) in adherent cells with MS. Each dilution is the mean ± SEM for 9–12 monolayers from three preparations. The lower portion (*B*) shows the effects of AT$_1$R inhibition with losartan (10^{-5} M) and AT$_2$R inhibition with PD123319 (10^{-5} M) on meconium-induced decreases in cell adherence ($n = 4$). *$p < 0.01$ *versus* control.

after MS instillation (Fig. 4*G, H*), including cells within the alveolar septa (Fig. 4*H*). At 100× magnification (Fig. 5*A*), control lungs demonstrated thin alveolar septae, well defined alveolar epithelial cells, modest AT$_1$R immunostaining and few cells within air spaces (Fig. 5*A, B*). After MS instillation, alveolar septae were thickened, cellularity increased and AT$_1$R immunostaining of alveolar epithelium more intense (Fig. 5*C*). Cells with membrane immunostaining were now evident within the air spaces (Fig. 5*C, D*). AT$_1$R immunostaining was unaffected by captopril pretreatment (Fig. 5*D*), providing additional evidence that local ANG II synthesis does not contribute to AT$_1$R regulation.

Cultured type II pneumocytes. Having identified AT$_1$R in cells within the alveolar septae of newborn lungs and increased AT$_1$R immunostaining after MS, we sought to deter-

mine whether this involved type II pneumocytes. Exposure of cultured type II pneumocytes to MS increased AT$_1$R >3-fold ($p < 0.01$; Fig. 6). Since MS is associated with increased apoptosis in bronchial lavage of newborn rabbits (7,11), we examined the effects of MS on type II pneumocyte viability by measuring cellular adherence. MS exposure dose-dependently decreased adherence, *i.e.*, increased cell death (Fig. 7*A*; $p < 0.01$, ANOVA). Furthermore, MS (1:10 dilution) decreased cell adherence 67% (Fig. 7*B*; $p < 0.01$, ANOVA, $n = 4$), and this was inhibited by 10^{-5} M losartan, but unaffected by PD123319, an AT$_2$R antagonist. Neither antagonist affected cell viability.

DISCUSSION

MAS continues to be associated with substantial morbidity in near-term and term neonates (4,5). Understanding its pathogenesis could result in new strategies to decrease this morbidity. We (7–9,11) previously instilled particulate-free MS in

lungs of newborn rabbits to focus on inflammatory and cellular responses to the soluble constituents in meconium. The tissue RAS was observed to contribute to the cellular responses to meconium, including ATR-mediated epithelial apoptosis that resembled adult models of lung damage (7,9,11,12). Although the AT$_1$R appeared to be involved, it was unclear if expression was altered and if AT$_2$R also contributed. In the present study, AT$_2$R were not detected in newborn lung and expression was unaffected by meconium exposure. AT$_1$R, however, were expressed in several cell types throughout the newborn lung, including epithelium and smooth muscle, and increased after meconium exposure. Importantly, cultured alveolar type II pneumocytes also demonstrated basal AT$_1$R expression and MS-induced increases that were associated with decreased cell viability blocked by AT$_1$R inhibition. Thus, we provide new evidence of involvement of the pulmonary RAS in the cellular responses to MAS through increased AT$_1$R expression and enhanced ANG II-mediated cell death.

The RAS mediates its effects through AT$_1$R and/or AT$_2$R subtypes. There are no reports of ATR distribution or regulation in newborn lung. We now report that AT$_2$R are not expressed in 2 wk lung before or after MS exposure and thus, do not contribute to MAS. AT$_1$R, however, are present in bronchial and alveolar epithelium and bronchial and vascular smooth muscle. This is consistent with undetectable AT$_2$R mRNA, but substantial AT$_1$R mRNA throughout the lung of developing rats (28). The broad distribution of AT$_1$R suggests that ANG II could contribute to the regulation of bronchial and vascular smooth muscle tone in newborn lung as in lungs of adult animals (29). Although speculative, increases in bronchial and vascular smooth muscle AT$_1$R and tissue angiotensinogen, resulting in increased ANG II after meconium exposure, could enhance local RAS activity and contribute to the increases in airway and/or vascular resistance that complicate MAS (5). Further, the increases in circulating ANG II after birth (30,31) could facilitate pulmonary hypertension in the presence of increased pulmonary smooth muscle AT$_1$R in MAS. This has not been examined and warrants study.

Meconium increases tissue angiotensinogen mRNA (11), suggesting that increases in pulmonary ANG II synthesis might contribute to the pathophysiology of MAS. This occurs in adult lung and cultured alveolar epithelium after exposure to toxic or inflammatory agents, suggesting a common pathway resulting in lung damage (12,32). For example, bleomycin-induced lung injury increases pulmonary ANG II synthesis and epithelial apoptosis in adult lung, which is blocked by ACE inhibition or AT$_1$R blockade (12,32). However, localization and regulation of lung AT$_1$R/AT$_2$R expression were not examined. We observed AT$_1$R in several cell types throughout the newborn lung and diffuse up-regulation by MS. Thus, meconium-induced increases in tissue angiotensinogen could increase lung ANG II synthesis and AT$_1$R expression (11,22,33); however, neither ACE inhibition nor AT$_1$R blockade altered basal or MS-stimulated AT$_1$R expression. Thus, meconium-induced increases in lung AT$_1$R expression are not due to increased tissue ANG II synthesis or AT$_1$R activation,

but rather a soluble constituent, *e.g.*, bile salts or proteases, in meconium.

AT$_1$R expression was observed in alveolar epithelial cells in intact newborn rabbits along with meconium-induced increases by immunohistochemistry; but the specific cell type was unclear. Therefore, we examined AT$_1$R expression in cultured type II pneumocytes. As in intact newborn animals, basal receptor expression was seen and values increased >3-fold after MS exposure, confirming that a soluble constituent in meconium activates the lung RAS and increases AT$_1$R protein *via* transcriptional mechanisms unrelated to local ANG II synthesis (22). It is unclear if similar mechanisms account for enhanced expression in other lung cells.

Increased caspase 3 and evidence of apoptosis are reported in cells within the bronchial lavage of MS exposed newborn rabbits (7) and both decrease after ACE inhibition or nonspecific ATR inhibition with saralasin, suggesting ATR activation contributes to apoptosis through local autocrine or paracrine mechanisms (9,34). AT$_1$R were detected in several cell types within the newborn lung and cultured type II pneumocytes. In the latter, increases in AT$_1$R expression were associated with dose-related increases in cell death inhibited by losartan, confirming prior studies. It is tempting to speculate that other cells in the newborn lung that express the AT$_1$R are similarly affected. If so, receptor blockade or even ACE inhibition may represent new strategies for preventing the cellular responses to MAS.

The RAS elicits inflammatory responses in several tissues (12,35,36). As previously reported (7,11,34), there was cellular infiltration into the air spaces associated with meconium-induced increases in AT$_1$R and activation of the tissue RAS. Interestingly, cells in the alveolar spaces also exhibited AT$_1$R immunostaining after MS. It is unclear if they were attracted to the air spaces by activation of membrane AT$_1$R and if they contribute to inflammatory responses mediated by local ANG II synthesis. It is notable that ACE inhibition is associated with decreased pulmonary cytokines after in meconium exposed newborn rabbits (9). Further studies are needed to address these findings.

In the current study, we have extended investigations of the pulmonary responses to MAS. We (7,9,11) previously reported that meconium activated the pulmonary RAS, evidenced by increases in angiotensinogen mRNA associated with apoptosis and cytokine release. We now report that AT$_2$R are not involved, and AT$_1$R are not only expressed in several cell types in newborn lung, including bronchial and alveolar epithelium and bronchial and vascular smooth muscle, but are also up-regulated after meconium exposure. We confirmed this in cultured alveolar type II pneumocytes and demonstrated AT$_1$R-mediated apoptosis. These observations support the conclusion that the tissue RAS contributes to the pathophysiology of MAS and suggests that inhibitors of the RAS may be useful in the treatment of MAS.

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