Antenatally administered PPAR-γ agonist rosiglitazone prevents hyperoxia-induced neonatal rat lung injury

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Rehan VK, Sakurai R, Corral J, Krebs M, Ibe B, Ihida-Stansbury K, Torday JS. Antenatally administered PPAR-γ agonist rosiglitazone prevents hyperoxia-induced neonatal rat lung injury. Am J Physiol Lung Cell Mol Physiol 299: L672–L680, 2010. First published August 20, 2010; doi:10.1152/ajplung.00240.2010.—The physiological development and homeostasis of the lung alveolus is determined by the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) by the interstitial lipofibroblast. We have recently shown (Dasgupta C et al., Am J Physiol Lung Cell Mol Physiol 296: L1031–L1041, 2009) that PPAR-γ agonists administered postnatally accelerate lung maturation and prevent hyperoxia-induced lung injury. However, whether the same occurs antenatally is not known. The objective of this study was to test the hypothesis that the potent PPAR-γ agonist rosiglitazone (RGZ), administered antenatally, enhances fetal lung maturation and protects against hyperoxia-induced neonatal lung injury. Sprague-Dawley rat dams were administered either diluent or RGZ (3 mg/kg), at late gestation, to determine its effect on lung maturation and on hyperoxia (95% O2 exposure for 24 h)-induced neonatal lung injury. The lungs were examined for the expression of specific markers of alveolar development (surfactant proteins A and B, cholinephosphate cytidylyltransferase-α, leptin receptor, triglyceride uptake, and [3H]choline incorporation into saturated phosphatidylcholine) and injury/repair, in particular, the markers of transforming growth factor-β signaling (activin receptor-like kinase-5, SMAD3, lymphoid enhancer factor-1, fibroactin, and calponin). Overall, antenatal RGZ accelerated lung maturation and blocked the inhibition of alveolar sacculation and septal wall thinning by hyperoxia. RGZ specifically stimulated the development of the alveolar epithelial type II cell, the lipofibroblast, and the vasculature. The increased expression of the transforming growth factor-β intermediates, such as SMAD3 and lymphoid enhancer factor-1, implicated in hyperoxic lung injury, was also blocked by antenatal RGZ treatment. In conclusion, PPAR-γ agonists can enhance fetal lung maturation and can effectively prevent hyperoxia-induced neonatal lung injury.

METHODS

Animals. Pathogen-free, time-mated Sprague-Dawley rat dams (250–300 g body wt) were obtained from Charles River Laboratories (Hollister, CA). For determining whether antenatal RGZ (Cayman Chemical) administration would enhance fetal lung maturation, pregnant dams were administered either diluent or RGZ (3 mg/kg ip) in 100-μl volumes, at e21 (term day of mating) and e22. The dose of RGZ used is based on the previous work showing

triglyceride diet results in increased triglyceride content in the lungs of their offspring, increased survival, and improved pathological status after prolonged hyperoxic exposure (25). In contrast, newborn offspring of rats fed low-polyunsaturated fatty acid diets are more susceptible to pulmonary oxygen toxicity and early lethality in hyperoxia (24). Furthermore, Kehrer and Autor (14) demonstrated that increasing the saturated fatty acid composition of lung triglycerides in adult rats by dietary manipulation produced increased susceptibility to oxygen toxicity.

Our laboratory has shown that triglycerides from the circulation are stored in lipofibroblasts, and that this biological lipid compartment protects the lung against oxidant injury (26, 34). The trafficking of neutral lipid from the circulation is mediated by the lipofibroblast phenotype (34), which is determined by the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), which plays a critical role in normal lung development and injury/repair (30, 35). Our laboratory has recently shown that administration of PPAR-γ agonist rosiglitazone (RGZ) postnatally enhances lung maturation and can prevent hyperoxia-induced neonatal lung injury, suggesting the potential therapeutic usefulness of PPAR-γ agonists in preventing and/or treating bronchopulmonary dysplasia (4, 20, 36). However, it is not known whether administration of PPAR-γ agonists antenatally could prevent neonatal lung injury. Here, our objective was to determine the effects of antenatal administration of RGZ on fetal lung maturation and on subsequent hyperoxia-induced neonatal lung injury. We hypothesized that antenatal administration of the PPAR-γ agonist RGZ would enhance fetal lung maturation and prevent hyperoxia-induced neonatal lung injury.

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that, when administered in a dose of 3 mg/kg, it accelerates lung maturation and blocks hyperoxia-induced lung injury in neonatal rats (4, 18, 36). After spontaneous delivery, on postnatal day 1, the pups were placed in either 21 or 95% O2 for 24 h, following which their lungs were collected to examine specific markers of lung injury and repair. All animal procedures were performed following National Institutes of Health guidelines for the care and use of laboratory animals, and approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee.

Lung fibroblast culture. To elaborate on the mechanism of the effects of hyperoxia on lung injury and how it is mediated by RGZ, e20 fetal rat lung fibroblasts were cultured according to previously described methods (20). At 80% confluence, cells were pretreated for 1 h with either transforming growth factor (TGF)-β1, -β2, or -β3 antibody (10 μM, R&D System, Minneapolis, MN) or RGZ (10 μM, St. Louis, MO). The cells were then exposed to hyperoxia (95% O2 + 5% CO2) for 30 min, 6 h, or 24 h at 37°C. Control cells were kept in 21% O2 + 5% CO2. At the specified time points, Western analysis and immunocytochemistry was performed for markers of TGF-β activation [phosphor- and total SMAD3, lymphoid enhancer factor-1 (LEF-1), α-smooth muscle actin (α-SMA)] using previously described methods (4, 20, 21, 36). For labeling SMAD3, rabbit monoclonal antibody Alexa Fluor 488 green and, for labeling α-SMA monoclonal antibody, Alexa Fluor 568 red were used.

Western analysis for relevant proteins. Protein extraction and Western blot analysis for surfactant protein (SP)-A, SP-C, cholinephosphate cytidylyltransferase-α (CCT-α), leptin receptor (LR), parathyroid hormone-related protein (PTHrP) receptor, PPAR-γ, vascular endothelial growth factor (VEGF) and its receptor FLK-1, SMAD3, LEF-1, and GAPDH were performed using methods described previously (4, 20, 21, 36). Primary antibodies used included SP-A (1:200), SP-C (1:500), VEGF (1:350), FLK-1 (1:350), CCT-α (1:200), LR (1:200), LEF-1 (1:400; all from Santa Cruz, San Diego, CA); PTHrP receptor (1:100; Upstate, Temecula, CA); PPAR-γ (1:2,000; Alexis Biochemicals, San Diego, CA); SMAD3 (1:200; Cell Signaling, MA); and GAPDH (1:10,000; Chemicon, Temecula, CA). The density of the protein bands was quantified using a scanning densitometer (Eagle Eye, Stratagene) and expressed normalized to GAPDH levels.

Measurement of rate of surfactant phospholipid synthesis. To determine de novo surfactant phospholipid synthesis, [3H]choline incorporation into saturated phosphatidylcholine was performed as described previously (21).

Triglyceride uptake assay. The rate of [3H]triolein uptake was assayed as a marker for triglyceride uptake, as described previously (13).

Transmission electron microscopy. For ultrastructural examination, rat lungs were removed from the chest cavity, fixed in 2% paraformaldehyde containing 0.2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After a brief wash in 0.1 M cacodylate buffer, lungs were postfixed with 1% osmium tetroxide (EMS) for 1 h. Specimens were then dehydrated in a series of graded ethanol and were embedded in Epon812 (EMS). Initially, 1-μm semithin sections were stained with toluidine blue and examined under light microscopy to determine the orientation of the lungs for ultrathin sectioning. Ultrathin sections were stained with uranyl acetate and lead citrate to determine the orientation of the lungs for ultrathin sectioning. Yeast ultrathin sections were stained with uranyl acetate and lead citrate before being observed under a Jeol 1010 electron microscope. Epithelial cell height, lamellar body diameter, and basement membrane thickness were measured using the transmission electron microscopy (TEM) images using AMTV 542 software (Advanced Microscopy Technologies, Danvers, MA). For random sampling, two individual

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**Fig. 1. Antenatal rosiglitazone (RGZ, 3 mg/kg) administration once at embryonic day (e) 18, and 24 h later once more at e19, to pregnant dams increased markers of fetal lung alveolar epithelial, mesenchymal, and vascular differentiation, when examined at e20. A: increase in the expression of epithelial markers, surfactant proteins A (SP-A) and C (SP-C), cholinephosphate cytidylyltransferase-α (CCT-α), and leptin receptor (LR). B: increase in the expression of mesenchymal markers, parathyroid hormone-related protein receptor (PTHrPR) and peroxisome proliferator-activated receptor-γ (PPAR-γ). C: increase in the expression of vascular markers, VEGF and FLK-1. +, With RGZ; -, without RGZ. Values are means ± SE; n = 4. *P < 0.05 vs. control.**
samples per experimental condition were examined from comparable areas of lungs embedded in Epon blocks for ultrathin sectioning. For cell height measurement, TEM images were made at a 2,500-fold magnification (40 μm × 50 μm/image), with each image containing an average of 10 cells, and 10 different images per block were examined for this measurement. For lamellar body diameter measurement, TEM images were made at a 12,000-fold magnification (8 μm × 10 μm/image), with each image containing one entire cell, and 30 different images per block were examined for this measurement. For basement membrane measurement, TEM images were made at a magnification of 25,000-fold magnification (4 μm × 5 μm), and five different areas of the basement membrane per field were chosen to examine the thickness.

Lung morphometry. For morphometry, the lungs were inflation fixed in situ with 4% paraformaldehyde in phosphate buffer at 5-cm inflation pressure. The trachea was ligated, and the lungs removed and placed in 4% paraformaldehyde for 4 h at 4°C. The lung was subsequently transferred to phosphate-buffered saline containing 30% sucrose (weight/volume) until equilibrated in the cold (4°C). The lung tissue was subsequently examined morphometrically. An investigator unaware of the treatment groups performed lung morphometry following the method described previously (4, 20, 21, 36).

Measurement of RGZ in plasma. RGZ was measured by a high-performance liquid chromatography (HPLC) gradient program of a 50:50 mixture of acetoniitride and methanol (solvent A), and 50 mM ammonium acetate buffer, pH 8.0 (solvent B). The HPLC column was a Whatman μBondapak C18 analytic column (3.9 × 150 mm). Elution of RGZ was accomplished with a timed program beginning with 100% solvent B, 5 min; then changed to 50% solvent B for 20 min and held for 20 min; followed by 95% solvent B for 5 min and held for 10 min. RGZ had a retention time of 29 ± 0.5 min in this system, and the amount of RGZ was calculated from a standard curve of authentic RGZ run on each day of the assay.

Statistics. The experimental data were analyzed using either ANOVA with Newman Keuls post hoc analysis or an unpaired t-test, as appropriate. The results are expressed as means ± SE. P < 0.05 was considered indicative of statistically significant differences between the experimental groups.

RESULTS

We initially evaluated the effects of antenatal RGZ administration on alveolar epithelial, lipofibroblast, and endothelial differentiation. Western blot analysis on protein extracts from whole lungs demonstrated that RGZ administration significantly increased SP-A, SP-C, CCT-α, and LR levels (Fig. 1A), which are key functional markers of alveolar epithelial type II cell differentiation. Similarly, antenatal RGZ administration increased both PTHrP receptor and PPAR-γ levels significantly (Fig. 1B), which are key lipofibroblast differentiation markers. Additionally, RGZ also increased the expression of VEGF and FLK-1 (Fig. 1C), key functional markers of endothelial differentiation. Triolein uptake and choline incorporation into disaturated phosphatidylcholine by lung explants ex vivo, two important functional markers for alveolar lipofibroblast and epithelial type II cell maturation, respectively, also increased significantly compared with the diluent-treated controls (Fig. 2). Enhanced lung maturation by antenatal administered RGZ was also confirmed electron microscopically (Fig. 3). As expected, and consistent with increased alveolar maturation, epithelial cell height decreased (7.13 ± 0.24 vs. 5.12 ± 0.29 μm, control vs. RGZ; P < 0.0001), and the number of lamellar bodies/cell increased (0.75 ± 0.25 vs. 1.92 ± 0.38, control vs. RGZ; P < 0.05) in RGZ-treated animals compared with the diluent-treated controls, even though the lamellar body size was not different between the control and RGZ-treated groups (659 ± 55 vs. 730 ± 62, control vs. RGZ; P = 0.0535) (Fig. 3A). Furthermore, though not statistically significant, with RGZ treatment, alveolar basement membrane thickness showed a trend toward thinness (60.22 ± 2.0 vs. 52.09 ± 3.2 nm, P = 0.0535) (Fig. 3B).

Having demonstrated that antenatal administration of RGZ enhanced fetal rat lung maturation, we next examined whether it provided any protection against hyperoxia-induced neonatal lung injury. For this, we administered either RGZ or diluent to pregnant dams on e19, i.e., 24 h before the expected delivery at term. Following delivery, 24-h exposure to hyperoxia from postnatal day 0 (day of delivery) to postnatal day 1 had a significant inhibitory effect on the whole lung PTHrP receptor and PPAR-γ protein levels (Fig. 4). However, in the hyperoxia exposure + antenatal RGZ administration group, both of these decreases were attenuated. In fact, in the RGZ-treated group, PPAR-γ levels were significantly increased compared with both the 21 and 95% O2-exposed groups. Similarly, SP-C and CCT-α levels in the 95% O2-exposed group were significantly decreased (Fig. 4), whereas antenatal RGZ treatment not only blocked the decrease in SP-C levels, but actually significantly increased SP-C levels compared with both the 21% and the 95% O2-exposed groups.

Since we have previously observed that TGF-β activation contributes significantly to hyperoxia-induced neonatal lung injury, we next examined the levels of key intermediates in the TGF-β signaling pathways (Fig. 5). 95% O2 exposure caused significant increases in the levels of p-SMAD3, activin receptor-like kinase-5 (ALK-5), LEF-1, fibronectin, and calponin,
Fig. 3. A: antenatal RGZ (3 mg/kg) administration once at e18, and 24 h later once more at e19, to pregnant dams increased lung maturation, as evidenced by the increased number of lamellar bodies/epithelial cell (0.75 ± 0.25 vs. 1.92 ± 0.38, control vs. RGZ; \( *P < 0.017 \); arrows point to lamellar bodies; bar = 2 μm), even though the lamellar body size was not different between the control and RGZ-treated groups (659 ± 55 vs. 730 ± 62, control vs. RGZ; \( P > 0.05 \)).

B: with RGZ treatment, there was trend toward decrease in alveolar basement membrane thickness (60.22 ± 2.0 vs. 52.09 ± 3.2 nm, control vs. RGZ; \( P = 0.0535 \)). Values are means ± SE.
all of which, except ALK-5, were significantly blocked by antenatal RGZ administration. ALK-5 levels were not different between the hyperoxia alone and hyperoxia + RGZ-treated groups. In addition, and even more importantly, antenatal RGZ administration blocked the 24-h hyperoxia exposure-induced decrease in alveolar sacculations (185 ± 10 vs. 195 ± 15 sacculles/mm²; 21% O₂ vs. 95% O₂ vs. 95% O₂ + RGZ; P < 0.05; Fig. 6). The hyperoxia-induced decrease in alveolar septal wall thickness (4.9 ± 0.2 vs. 4.2 ± 0.4 vs. 5.5 ± 0.5 μm; 21% O₂ vs. 95% O₂ vs. 95% O₂ + RGZ, P < 0.05) was also blocked by antenatal RGZ administration (Fig. 6).

Since our previous work has demonstrated a key role of alveolar interstitial fibroblasts in mediating hyperoxia-induced lung injury and how it can be blocked by modulating PPAR-γ expression by fibroblasts, we next determined if hyperoxia-induced activation of TGF-β signaling was blocked by pretreatment of cultured e20 alveolar interstitial fibroblasts with either TGF-β neutralizing antibody or RGZ (Fig. 7). As expected, the hyperoxia-induced increase in p-SMAD3 and LEF-1 levels was blocked by pretreatment with either TGF-β neutralizing antibody or RGZ, and this blockage seems to be more robust with RGZ. Blockage of hyperoxia-induced TGF-β activation by RGZ was also corroborated by immunocytochemistry. Figure 8 shows that 24-h exposure to hyperoxia resulted in markedly increased expression of SMAD3 (nuclear) and α-SMA by alveolar interstitial fibroblasts, both of which were blocked by pretreatment with RGZ.
Finally, to demonstrate that antenatally administered RGZ to pregnant dams crosses the placenta and is detectable in the neonatal pup blood, we measured RGZ levels in the plasma of the blood collected from neonatal rat pups from RGZ-treated rat dams at the time of death. Figure 9 shows the level of RGZ measured from the plasma of pups following 24-h exposure to hyperoxia, which was 0.35 ± 0.10 μg/ml (mean ± SE).

DISCUSSION

Recognizing that there are intrinsic developmental mechanisms that protect the lung against oxidant injury (6, 8), we have tested the hypothesis that, by targeting the maturation of the lipofibroblast, we could protect the lung against oxidant injury. To that end, we observed that antenatal administration of RGZ-enhanced fetal lung maturation and virtually blocked 24-h hyperoxia-induced lung molecular and morphometric changes postnatally, suggesting a novel antenatal intervention to protect against hyperoxia-induced neonatal lung injury. Although there was some heterogeneity in the cytoprotective effects of antenatal RGZ administration on different alveolar cell-type markers affected by hyperoxia, alveolar epithelial type II cell (SP-A, SP-C, CCT-α, LR), fibroblast (PTHrP receptor and PPAR-γ), vascular (VEGF and FLK-1), and functional differentiation (triolein uptake and [3H]choline incorporation into saturated phosphatidylycholine) markers, which have previously been shown to promote lung alveolar development and homeostasis (31, 32) on the one hand, and prevent oxidant injury (3, 4, 20, 28, 34) on the other, clearly increased following antenatal RGZ administration. Although the rates of uptake of triolein and incorporation of choline into saturated phosphatidylycholine are potentially susceptible to the effects of endogenous substrates, the...
increased rate of triglyceride uptake is consistent with the effects of RGZ on protection of the lung against oxidant injury (34) and on increased surfactant synthesis (26); the increased rate of choline incorporation into saturated phosphatidylcholine is consistent with the increased CCT-α expression. These data provide an alternative approach to the standard contemporary antenatal steroid administration for enhancing fetal lung maturation.

It should not be surprising that RGZ stimulation of lipofibroblast differentiation would be cytoprotective against oxidant injury, since lipofibroblasts have intrinsic antioxidant properties, and they produce specific growth and differentiation factors that have both direct and indirect effects on alveolar differentiation and homeostasis (27, 29, 35). The uptake of neutral lipids by lipofibroblasts, which protects them against oxidant injury, is specifically dependent on PPAR-γ induction of adipocyte differentiation-related protein, which determines the uptake and storage of neutral lipid (9, 22). Lipofibroblasts produce specific growth factors that mediate the epithelial-mesenchymal interactions that promote alveolar epithelial growth and differentiation (27, 33, 35). Alveolar epithelial cells, in turn, express growth factors, such as VEGF, which are necessary for alveolar capillary growth and differentiation. Therefore, the lipofibroblast is a complete source of growth factors for alveolar growth and differentiation (27, 35).

Although we believe that RGZ-mediated attenuation of the hyperoxia-induced neonatal lung injury is mainly mediated by its effect on lipofibroblasts, it is also possible that, at least to some extent, this effect might be mediated by its effects on other PPAR-γ-expressing cell types, such as macrophages and alveolar epithelial cells. Furthermore, although the signaling pathways involved were not studied in great detail, we do document that postnatal hyperoxia-induced activation of TGF-β/Wnt signaling was blocked by antenatal RGZ administration. Since TGF-β, Wnt, and PPAR-γ signaling pathways are normally active in the developing lung, it is not surprising that we found the expressions for the key markers of these pathways, i.e., p-SMAD3, LEF-1, and PPAR-γ, respectively, under nor-
moxic conditions. However, on exposure to hyperoxia, the downregulation of PPAR-γ signaling and the upregulation of TGF-β/Wnt signaling alters the balance in these critical pathways in favor of TGF-β/Wnt dominance.

Antenatal glucocorticoids have been the “gold standard” for antenatal acceleration of lung development for more than 3 decades, yet we know that it is not the ideal agent for this purpose (1, 15, 17). The most salient features of the Collaborative Trial of Antenatal Steroids for Acceleration of Lung Maturation (2) were that, although the treatment was effective, it only significantly affected females and promoted maternal infection. In the absence of any other such agent, antenatal steroids have remained the drug of choice for the prevention of Respiratory Distress Syndrome until now. Studies in our laboratory have shown the efficacy of PPAR-γ agonists in preventing the pulmonary complications of preterm birth due to lung immaturity and/or hyperoxic exposure (3, 4, 20, 28), fetal smoke exposure (15a, 18, 19), and infection (16). The current study is proof of principle that antenatally administered PPAR-γ agonist RGZ is effective in accelerating normal lung development and the consequences of postnatal oxygen exposure. Obviously, head-to-head studies comparing the effects of antenatal steroids and PPAR-γ agonists, along with extensive safety, pharmacokinetic, and pharmacodynamic studies of PPAR-γ agonists, need to be performed before this class of drugs can be considered for human trials. However, animal studies (7) and limited human literature documenting that fetal exposure to RGZ during the first (10) and second (12) trimesters provide no evidence to suggest that RGZ is teratogenic. In line with the data presented here, transplacental transfer of RGZ has previously also been documented in an ex vivo human placental perfusion model (11).

In summary, antenatal RGZ administration effectively blocked the molecular and morphological changes following 24-h postnatal exposure to hyperoxia. Antenatal administration of PPAR-γ agonists is a novel strategy to enhance fetal lung maturation and effectively prevent neonatal lung injury.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the author(s).

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