Glucocorticoid receptor mRNA and protein in fetal rat lung in vivo: modulation by glucocorticoid and androgen

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Sweezey, Neil B., F. Ghibu, S. Gagnon, E. Schotman, and Q. Hamid. Glucocorticoid receptor mRNA and protein in fetal rat lung in vivo: modulation by glucocorticoid and androgen. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L103–L109, 1998.—Pulmonary glucocorticoid receptor (GR) is essential to timely preparation for the onset of breathing air at birth. We have previously used primary culture of late-gestation fetal rat lung cells to demonstrate differential regulation of GR by glucocorticoid depending on cell type. In this study, we hypothesized that the action of glucocorticoid on GR mRNA expression and protein elaboration in lung cells might be modulated by interactions present in vivo but not in primary culture. Given that male sex hormone (androgen) has an inhibitory effect on antenatal lung development, we also postulated that androgen would decrease antenatal lung GR. We report that antenatal maternal injection of the androgen 5α-dihydrotestosterone (1 mg/kg) enhanced fetal lung cellular levels of GR mRNA and protein and that glucocorticoid increases GR mRNA and protein were enhanced in cells throughout all areas of the lung tissue, suggesting that interactions occurring in intact tissue may override the previously reported direct inhibition by glucocorticoid of GR protein elaboration in isolated fetal rat lung epithelial cells. Furthermore, antenatal administration of the androgen 5α-dihydrotestosterone (0.2 mg/kg) reduced tissue levels of GR mRNA and protein, consistent with androgenic inhibition of antenatal lung development by decreasing GR. We conclude that glucocorticoids and androgens exert opposite effects on fetal lung GR.

dihydrotestosterone; dexamethasone; lung development; in situ hybridization

GLUCOCORTICOID RECEPTOR (GR) mediates glucocorticoid stimulation of maturation of the antenatal lung, which is essential to timely preparation for the onset of breathing air at birth (2). “Knockout” mice congenitally deficient in GR (14) or in corticotropin-releasing hormone (38) and rats treated in utero with glucocorticoid antagonist (20, 21) exhibit lethal lung immaturity at birth. Cellular GR concentration is felt to be a limiting factor in the signal transduction pathway, leading to transcriptional responses to glucocorticoid (41), and cellular sensitivity to glucocorticoid is directly proportional to GR concentration (40). Regulation of cellular GR levels might therefore be expected to be relevant to lung maturation. Indeed, glucocorticoid regulates the expression of numerous genes in the lung (58). Glucocorticoid directly and indirectly regulates production of components of pulmonary surfactant (4, 35, 43), including surfactant proteins (SP-A, SP-B, SP-C, and SP-D; see Refs. 6, 9, 32, 33, and 45). Glucocorticoid regulates the two human SP-A genes (32) in a complex, biphasic dose-dependent manner (33). Glucocorticoid regulation of hydrophobic SP-B and SP-C occurs directly in type II cells, primarily at the level of transcription (5). The recently identified hydrophilic SP-D is also transcriptionally regulated by glucocorticoid in late-gestation fetal rat lung (44). In addition, there is increasing evidence for indirect effects of glucocorticoid on various aspects of lung development. In late gestation, production of surfactant by epithelial cells seems to be augmented by a differentiation factor, fibroblast-pneumocyte factor, elaborated by fetal lung fibroblasts in response to glucocorticoid (42, 47, 48). Glucocorticoid modulates the composition of the extracellular matrix, which in turn regulates SP levels (16). Moreover, glucocorticoid may regulate lung morphological maturation through downregulation of the growth factor transforming growth factor-β (28). Glucocorticoid appears to coordinate the timing of augmented surfactant production through effects on prostaglandin E₂ production and action (56). Glucocorticoid, therefore, appears to modulate all major components of lung maturation. Modulation of GR levels by autologous ligand, termed “autoregulation,” has been described for GR in lung and in other target cells and tissues (7, 15, 22, 29–31, 36). Both up- and downautoregulation have been described (1, 7, 17, 18, 33) via a variety of mechanisms, including transcriptional, posttranscriptional, and posttranslational mechanisms (40, 41). We have demonstrated that glucocorticoid regulates GR mRNA and ligand-binding levels in primary cultures of late-gestation fetal rat lung cells (49). The direction of the effect varies based on cell type, diminishing epithelial cell GR but enhancing fibroblast GR.

In this study, our hypothesis was that glucocorticoid regulation of cellular GR levels might be modulated by interactions present in vivo but not in primary cell cultures highly enriched for a single cell type. Given that male sex hormone (androgen) inhibits maturation of the antenatal lung (18, 51, 52, 54), we also postulated that androgen would decrease antenatal lung GR. We report that exposure to glucocorticoid in vivo enhances GR levels of fetal rat lung cells. GR mRNA (as assessed by in situ hybridization) and GR protein (as assessed by immunocytochemistry) increased throughout all cell types, based on light-microscopic morphology, suggesting that matrix-cell and cell-cell interactions override the direct glucocorticoid inhibition of epithelial cell GR. Androgen exerted the anticipated opposing action, reducing GR mRNA and protein levels.
MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from Pharmacia (Baie D'Urfe, Que.). 5α-Dihydrotestosterone (DHT) was from Steraloids (Wilton, NH). [35S]UTP was from Amersham (Oakville, Ont.), and RNA polymerase was from Promega (Madison, WI). All other chemicals were purchased from Sigma (St. Louis, MO).

Probes

To study GR mRNA using in situ hybridization, we excised a 1.8-kb segment of rat GR cDNA open reading frame between the Xba I and Pst I restriction sites of a construct known as pRD93, a gift of Dr. K. Yamamoto (University of California, San Francisco, CA; see Ref. 37). The cDNA segment was then inserted between the corresponding sites of the plasmid pBluescript II KS. Linearization of this construct with Xba I permitted production of a 1.8-kb sense RNA probe under the direction of the T3 promoter, and linearization by EcoRI allowed production of a 1.1-kb antisense RNA probe using the T7 promoter. A 1.5-kb β-actin cDNA derived from the 2-kb cDNA (A1) of Cleveland et al. (University of California, San Francisco) (13) was used as a control for these studies.

For the study of GR protein using immunohistochemistry, we obtained polyclonal rabbit anti-human GR antibody PA1-511 from Affinity BioReagents (Neshanic Station, NJ). This antibody reacts with rat GR (1), whether bound to ligand or not, and does not interfere with GR binding to DNA. The avidin-biotin complex was from Vector Laboratories (Burlingame, CA).

Animals

Pregnant Wistar rats of known gestational age were obtained from Charles River (St. Constant, Quebec) and maintained in the Laboratory Animal Facilities of the McGill University-Montreal Children’s Hospital Research Institute. The rats were injected daily with 200 μl of the solvent dimethyl sulfoxide, with or without dissolved hormone, from gestational day 15 (day 0 = mating; day 22 = term) until 24 h before they were killed with diethyl ether. For the glucocorticoid group, the final injection included dexamethasone (Dex; 1 mg/kg) as in our previous study (49). The androgen group received 0.2 mg/kg DHT with each injection, a dose known to be effective in increasing fetal lung size in our previous study (26). The androgen group also received a final injection of dexamethasone (1 mg/kg), as in our previous study (49). The control group, the final injection included dexamethasone (Dex; 1 mg/kg) as in our previous study (49). The androgen group also received a final injection of dexamethasone (1 mg/kg), as in our previous study (49). The control group, the final injection included dexamethasone (Dex; 1 mg/kg) as in our previous study (49).

Materials and Methods

In Situ Hybridization

Sections were hybridized as described previously (24–26). Briefly, sections were first treated with 0.1 M glycine-PBS for 5 min and then in Triton X-100 for 10 min. After a brief wash in PBS supplemented with 0.1% diethyl pyrocarbonate-treated water, the slides were incubated in proteinase K (1 mg/ml) in 20 mM Tris-HCl and 1 mM EDTA for 15 min at 37°C. The proteinase K reaction was stopped by 4% paraformaldehyde for 5 min at room temperature. To prevent nonspecific binding of the 35S-labeled probes, the sections were treated with 0.25% acetic anhydride and 0.1% triethanolamine for 10 min followed by 10 mM N-ethylmaleimide with 10 mM DTT at 37°C for 30 min. Prehybridization was performed by 50% formamide in 2× saline-sodium citrate (SSC). For hybridization, sections were incubated with 106 counts/min of radiolabeled riboprobe antisense to GR mRNA diluted in hybridization buffer. Hybridization was performed at 42°C for 12 h. Posthybridization washes were performed with decreasing concentrations of SSC (4× SSC to 0.5× SSC) at 42°C. Next, single-stranded RNA was digested with RNase, and the hybridization signal was developed by standard autoradiography. For negative controls, sections were pretreated with RNase solution or sense riboprobe before hybridization. To ensure that the RNA of each nondegraded sample was intact, sections were hybridized for the housekeeping gene transcript β-actin.

Cells with specific deposit of silver grains were considered to be positive for mRNA. GR hybridization signal was assessed semiquantitatively on a scale of 0 to 3 as previously described (19, 26), where 0 represents no hybridization signal, 0.5 very weak, 1 weak, 1.5 weak to moderate, 2 moderate, 2.5 moderate to strong, and 3 strong.

Immunocytochemistry

The avidin-biotin complex technique was used, as previously described (19, 26). Cryostat sections of unfixed tissue were fixed in acetone-methanol for 10 min, dried, and then treated with 3% hydrogen peroxide in PBS to eliminate endogenous peroxidase activity. Sections were washed in PBS three times for 3 min each time and then incubated in 10% normal goat serum for 30 min. The sections were incubated with the GR antibody overnight at 4°C. After three washes of 5 min each in PBS, sections were incubated with biotinylated goat anti-rabbit IgG antiserum for 45 min, washed in PBS, and then incubated with avidin-biotin peroxidase complex for 45 min. Immunoreactivity was visualized by diaminobenzidine, and sections were counterstained with hematoxylin. Sections serving as negative controls were incubated with normal goat serum instead of the primary antibody. Immunostaining was graded as previously described (19, 26) on a semiquantitative scale of 0 to 3 analogous to the grading scale for in situ hybridization.

Statistical Analyses

All sections were graded on two separate occasions by a single observer (Hamid) blinded to the gestational age and treatment group of the tissue sections. All data are reported as means ± SE of at least five independent experiments. Each evaluation of GR mRNA or protein staining was performed using independent blocks of lung tissue from unrelated fetuses. Data were tested for normality of distribution using the Kolmogorov-Smirnov test before differences in means were assessed using ANOVA, followed by comparisons between subgroups by the Student-Newman-Keuls test, as appropriate, using SigmaStat for Windows, version 2, statistical software.

RESULTS

In Situ Hybridization

Effect of glucocorticoid on GR mRNA levels. We determined the effect of Dex on GR mRNA levels in fetal rat lung tissue. On each of gestational days 18–21, there was a diffuse increase in the GR mRNA hybridization signal obtained from tissue sections of fetuses whose mothers had been injected with Dex 24 h before being killed. Representative photomicrographs of in situ hybridization of sections from gestational day 20 are illustrated in Fig. 1. Mean scores ± SE are as fol-
Effect of androgen on GR mRNA levels. We determined the effect of DHT on GR mRNA levels in fetal rat lung tissue. On gestational days 20 and 21, there was a decrease in GR hybridization signal obtained from tissue sections of fetuses whose mothers had been injected with DHT before death. Mean scores ± SE were as follows: day 18 DHT (0.6 ± 0.3) vs. control (0.7 ± 0.3), P < 0.01; day 19 DHT (0.4 ± 0.2) vs. control (0.8 ± 0.1), P < 0.01; day 20 DHT (0.1 ± 0.1) vs. control (1.1 ± 0.3), P < 0.01; day 21 DHT (0.3 ± 0.2) vs. control (1.4 ± 0.1), P < 0.01 (Fig. 2).

Effect of development on GR mRNA levels. Within the control group, GR mRNA levels progressively increased between days 19 and 21 of gestation (P < 0.05, day 21 vs. 20 and day 20 vs. 19; Fig. 2).

Immunocytochemistry

Effect of glucocorticoid on GR protein levels. We determined the effect of Dex on GR protein levels. On gestational day 20, there was a diffuse increase in GR staining of tissue sections of Dex-exposed fetuses but not on days 18, 19, or 21. Representative photomicrographs of immunocytochemistry of sections from gestational day 20 are illustrated in Fig. 3. Mean scores ± SE were as follows: day 18 Dex (1.0 ± 0.2) vs. control (1.2 ± 0.3), P > 0.05; day 19 Dex (1.2 ± 0.2) vs. control (0.9 ± 0.1), P < 0.05; day 20 Dex (1.8 ± 0.2) vs. control (1.3 ± 0.3), P < 0.05; day 21 Dex (2.1 ± 0.3) vs. control (2.0 ± 0.2), P > 0.05 (Fig. 4).

Effect of androgen on GR protein levels. We determined the effect of DHT on GR protein levels in fetal rat lung tissue. On gestational days 20 and 21, there was a decrease in staining in the DHT-exposed group. Mean scores ± SE were as follows: day 18 DHT (0.8 ± 0.1) vs. control (1.2 ± 0.3), P > 0.05; day 19 DHT (0.8 ± 0.3) vs. control (0.9 ± 0.1), P > 0.05; day 20 DHT (0.5 ± 0.2) vs.
Effect of development on GR protein levels. Within the control group, GR protein levels increased between days 20 and 21 of gestation (P < 0.05; see Fig. 4).

DISCUSSION

We report that glucocorticoid and androgen have opposing effects on GR levels in late-gestation fetal rat lung cells in vivo. GR mRNA and protein, as assessed by in situ hybridization and immunocytochemistry, respectively, were enhanced by glucocorticoid and diminished by androgen. This report is in contrast to our previous findings that glucocorticoid decreases GR ligand binding in primary lung cell cultures highly enriched for epithelial cells (49), where the influences of cell-cell interactions are substantially reduced. Based on light-microscopic morphology and localization, maximal levels of GR mRNA and protein occurred in epithelial and mesenchymal cells on the same gestational day.
in further contrast to our previous in vitro findings (49). In addition, glucocorticoid increased GR mRNA and protein levels in epithelial and nonepithelial cells alike. This contrasts with the observation by Torday (50) of fibroblast-specific GR regulation in fetal rat lung cells isolated ex vivo. Moreover, the glucocorticoid-induced increase of cellular GR mRNA levels in vivo is also in contrast to our previous report of GR mRNA stability after incubation of primary cell cultures in the presence of glucocorticoid (49). These differences suggest that interactions between epithelium and nonepithelial components modulate GR levels in fetal lung.

Our present findings are also in contrast to those of Brönnegård and Okret (12). They observed a 35–50% decrease in fetal day 16–21 rat whole lung GR mRNA levels (as assessed by solution hybridization) after maternal administration of much lower doses (0.2 mg/kg) of a less potent glucocorticoid, betamethasone. This may suggest that a high level of glucocorticoid stimulation is required to induce increased GR levels. The doses of Dex (and of DHT) employed in the present study were the same as those used in our previous work (49) to permit comparisons of the effects on cellular GR depending on the cellular environment: in vivo or in vitro (highly enriched for a single cell type). Other lower doses were not used in this work.

Local bioavailability of both glucocorticoids and androgens is subject to sensitive regulation by enzymatic interconversion between active and inactive forms. Androgens are reversibly interconverted by sulfotransferases and sulfatases (27). Hydroxysteroid sulfotransferase is an androgen-inactivating enzyme thought to modulate the role of androgens in retarding the maturation of fetal human lung. It is expressed in respiratory epithelium in rapidly increasing amounts as gestation progresses (27), providing a potential mechanism by which to control the timing of lung maturation in late gestation. Furthermore, the metabolism of steroid hormones by isolated lung epithelial cells differs from that in the whole animal or in tissue slices in important ways. Human lung tissue converts (active) hydrocortisone to (inactive) cortisol by the action of 11-hydroxysteroid dehydrogenase (46). In fetal rat lung, inactivation predominates in the epithelial cell, whereas activation predominates in the fibroblast (55). Therefore, low intracellular levels of active glucocorticoid would be expected in primary cultures highly enriched for epithelial cells compared with epithelial cells exposed to elevated local levels of active glucocorticoid due to surrounding fibroblast activation. Such differences might account for the observed differential effect of exogenous glucocorticoid on GR levels of epithelial cells in relative isolation vs. in intact lung.

Although we previously reported (49) that Dex treatment increases fetal rat whole lung GR binding levels, we had failed to demonstrate any change in GR mRNA levels by Northern blot analysis, in apparent disagreement with the present results by in situ hybridization. This may have been due to a type II statistical error, reflecting insufficient power in the previous study. Alternatively, although the enhanced GR mRNA levels in situ are present in some cells of all morphological types, the levels may not be elevated in each individual cell, such that studies of whole lung homogenate could miss an enhancement that was in fact present.

Cell-cell and matrix-cell interactions have previously been implicated in the modulation of steroid hormone effects in the lung and other organs. Dunsmore et al. (16) have reported evidence that adrenal steroid hormones modulate extracellular matrix metabolism by rat lung epithelial type II cells. They suggest that consequent effects on extracellular matrix biological activity may modulate epithelial cell differentiation or growth, which is under steroid hormone control. Recent evidence from studies of fetal rat lung suggests that the combined effects of glucocorticoid and alveolar fluid distension may coordinate the timely transfer of triglycerides from fibroblasts to type II cells for augmented surfactant production through their effects on the production and action of prostaglandin E2 as term approaches (56). Fetal rat lung extracellular matrix contains immunoreactive steroid hormone-binding globulin, which has been suggested to modulate the actions of androgen in embryonic stroma, thereby regulating the development of the epithelium in hormone-modulated tissues (8), such as the lung. Agonist-free transformation of GR in human B lymphoma cells suggests that cell-cell interactions are capable of transforming GR in the absence of ligand (57).
these changes occur in the context of increasing circulating levels of glucocorticoid hormones: glucocorticoid inhibits GR in most published reports (29, 30).

Androgen reduced GR mRNA and protein levels, in keeping with the hypothesis that androgenic inhibition of antenatal lung maturation may in part be mediated by reduction of GR activity. We report effects of DHT on GR mRNA on days 20 and 21 and on GR protein on day 20 during the saccular stage of lung development. At 20 days, fetal rat lung expresses androgen receptor (10), which is required for transduction of androgen action in a fashion strictly analogous to the role of GR in glucocorticoid action. Human fetal lungs express both isoforms of androgen receptor at levels much higher than adult human lungs (59). In the period from days 17 to 22, male (but not female) fetal rats experience a surge in circulating testosterone levels (23), most pronounced on days 18–19 (34). Moreover, it is around this saccular stage of lung development that prematurely born human boys tend to lag behind girls in lung development (53), making it attractive to speculate that this lag may be due in part to the fetal surge in androgen levels. We did not detect any DHT-mediated the hormonal regulation of GR, suggesting that this may be due in part to the fetal surge in plasma androgen levels. We did not detect any DHT-induced reduction in lung GR at earlier gestational ages (days 18 or 19), possibly due to an insensitivity of the measurement at low levels. Alternatively, at these stages, lung GR may be still expressed at an irreducibly low baseline level. However, the effect of DHT on GR might also reflect a cumulative dose effect rather than a differential response to DHT based on later gestational age. The rats were injected with DHT daily from day 15 until death, so that rats killed on day 18 had been injected on days 15–17 (3 days), whereas those killed on day 21 had been injected on days 15–20 (6 days) for two times the total dose.

Androgen also inhibits lung maturation by other mechanisms apart from diminution of lung cell GR. Androgen effects on lung maturation are in part medi-ated by the actions of transforming growth factor-β (53). Moreover, androgen interferes with lung maturation by inhibition of fibroblast-pneumocyte factor at a pretranslational level (18). Diminution of lung cell GR, like inhibition of fibroblast-pneumocyte factor, may contribute to the relative insensitivity of male fetuses to stimulation of lung maturation by antenatal glucocorticoid (3). We did not attempt to block the effects of androgen on GR using the nonsteroidal androgen antagonist flutamide because, in preliminary studies of whole lung GR, flutamide acted as a partial androgen agonist.

In summary, we report that glucocorticoid enhances, but androgen diminishes, GR mRNA and protein levels in late-gestation fetal rat lung cells in vivo. Matrix-cell or cell-cell interactions would appear to be important in mediating the hormonal regulation of GR, suggesting that further study of such interactions may be relevant to our understanding of lung development.

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REFERENCES


GLUCOCORTICOID RECEPTOR IN FETAL RAT LUNG


