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Glucocorticoids both stimulate and inhibit production of pulmonary surfactant protein A in fetal human lung

(dexamethasone/cortisol/cAMP/RNA·cDNA hybridization/explant culture)

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ABSTRACT **Pulmonary surfactant is a mixture of phospholipids and proteins which stabilizes lung alveoli and prevents respiratory failure. The surfactant-associated protein of M_r = 28,000–36,000 (SP-A) influences the structure, function (film formation), and metabolism of surfactant. We have characterized glucocorticoid regulation of SP-A and SP-A mRNA in explants of fetal human lung. The time course of response to dexamethasone was biphasic, with early stimulation and later inhibition of SP-A accumulation. Maximal induction of SP-A occurred with 3–10 nM dexamethasone and ≈300 nM cortisol for 72 hr, and stimulation diminished at higher concentrations. SP-A mRNA accumulation was maximally stimulated at 24–48 hr of exposure to dexamethasone (10 nM) and was generally inhibited by 4–6 days. Stimulation was also observed with cortisone and corticosterone but not with sex steroids, suggesting a receptor-mediated process. When explants were exposed to cortisol for only 24 hr, SP-A content was transiently increased above the level in continuously treated tissue and subsequently was similar to control. The content of SP-A and its mRNA was also increased by dibromo-cAMP, terbutaline, and forskolin, and effects were approximately additive with those of dexamethasone. However, elevated in tracellular cAMP did not alter the biphasic time course or dose–response patterns of dexamethasone. We propose that glucocorticoids have both stimulatory and inhibitory effects on SP-A gene expression. This biphasic regulation is not consistent with generalized toxic effects, product-feedback inhibition, or receptor down-regulation, and it appears to be specific for SP-A among the various surfactant components.**

Pulmonary surfactant, which is composed of both proteins and lipids, lowers surface tension in alveoli and is necessary for normal lung function. The surfactant-associated proteins are thought to have important roles in modifying surfactant phospholipid function and metabolism (1–7). The largest of these proteins, surfactant protein A (SP-A: M_r = 28,000–36,000), accelerates adsorption of phospholipids to the air/aqueous interface *in vitro* (2, 8), regulates the release and recycling of surfactant phospholipids by isolated type II cells (9, 10), and may be critical to the structure of tubular myelin (11). SP-A is developmentally regulated and is not detected in fetal human lung until the third trimester (12, 13).

The development of the surfactant system is of considerable importance in premature infants, who are often born deficient in surfactant and consequently develop respiratory distress syndrome. Glucocorticoids, when administered prenatally, accelerate lung maturation and decrease the incidence of this disease. In studies with fetal animals, these hormones hasten lung morphologic development, increase production of surfactant phospholipids, and improve pulmonary compliance (reviewed in ref. 14).

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The mechanism of glucocorticoid action has been studied in explant cultures of fetal lung, where glucocorticoids accelerate morphologic differentiation of epithelial cells into type II cells, increase the rate of phosphatidylcholine synthesis, and cause accumulation of messenger RNAs for surfactant proteins B (M_r = 7000) and C (M_r = 5000) (15–19). Induction of surfactant phospholipid and protein mRNAs occurs rapidly, is reversible, and appears to be receptor mediated (15, 18, 19).

We have reported that 10 nM dexamethasone also increases levels of SP-A and its mRNA in human fetal lung cultures (13, 20). Phelps and coworkers (21, 22) found that *in vivo* dexamethasone treatment increases SP-A synthesis in lungs of fetal, newborn, and adult rats. However, in other studies with cultured human fetal lung, Whitsett *et al.* (12, 23) have reported that dexamethasone decreased SP-A synthesis and blocked the stimulatory effects of cAMP and epidermal growth factor. This discrepancy with cultured tissue has not been resolved.

In this study, we have further characterized the dexamethasone effect on SP-A production. We used human fetal lung in explant culture to determine the time course, dose response, reversibility, and specificity of the steroid effects on SP-A and its mRNA, and to determine how the glucocorticoid effect is modulated by cAMP. A preliminary report of these studies has appeared (24).

EXPERIMENTAL PROCEDURES

Explant Culture. Adult lung tissue was obtained at pneumonectomy and fetal tissue from second trimester therapeutic abortuses under protocols approved by the Committee on Human Research at the University of California, San Francisco. Fetal tissue was examined before and after explant culture in serum-free Waymouth's medium and an atmosphere of 95% air/5% CO_2 for up to 8 days (15). Tissue was routinely stored in Waymouth's medium for 18–24 hr at 2°C before culture.

Dexamethasone (10 nM, unless otherwise stated), other hormones, or diluent (control) was added to the medium of treated explants after 24 hr (or at other times where specified). Except when otherwise required by experiment design, explants for measurement of SP-A mRNA were harvested after 72 hr in culture (48-hr exposure to hormone), and explants for SP-A assay were harvested after 96 hr (72-hr exposure to hormone).

Preparation of SP-A and Antibody. SP-A was purified from fluid obtained at lung lavage of a patient with alveolar proteinosis, as described (25). The purified SP-A was used to immunize a rabbit to generate a polyclonal antiserum, from

Abbreviations: SP-A, pulmonary surfactant protein A (M_r = 28,000–36,000); IBMX, 3-isobutyl-1-methylxanthine.

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which an IgG fraction was prepared. The characteristics of this antiserum, which recognizes only SP-A and its multimers on immunoblots, have been described (13, 20).

Enzyme-Linked Immunosorbent Assay (ELISA) for SP-A. The tissue samples were sonicated in distilled water containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride/5 mM *N*-ethylmaleimide/1 mM benzamidine), an aliquot was taken for DNA analysis, and the remainder of the sonicate was diluted in phosphate-buffered saline (0.025 M K_2HPO_4/0.1 M NaCl)/0.5% Tween 20/1% ovalbumin (pH 7.4). The SP-A and SP-A antiserum described above were used for a competitive ELISA, as described (20). This assay detects 0.2 ng or greater amounts of SP-A in 50-μl samples, so that under our usual conditions for preparing sonicates >0.02 μg/mg of DNA can be reliably detected. DNA assays were performed by the fluorometric method of Setaro and Morley (26). Statistical analyses utilized paired *t* tests and analysis of variance (ANOVA).

RNA Assays. RNA was extracted from tissues by the guanidium isothiocyanate technique and specific amounts (generally 0.5–4 μg) were applied to nitrocellulose filters by using a dot-blot apparatus (13). To facilitate comparisons between blots we used adult standards prepared by pooling equal amounts of RNA extracted from four adult lungs.

To determine SP-A mRNA content the blots were probed with the 800-base-pair human cDNA probe pHS10-5 (27), which contains most of the coding sequence for SP-A plus a short region of the 3′ noncoding region; the probe was ^{32}P-labeled by nick translation. Hybridization and washing conditions have been described (13). The blots were subjected to autoradiography and the developed films were densitometrically scanned to yield values for the content of SP-A mRNA in fetal lung samples as percentages of the adult standard.

RESULTS

We have previously reported that the accumulation of SP-A in human fetal lung explants is accelerated in the presence of 10 nM dexamethasone (13, 20). In these and subsequent experiments we consistently observed stimulation of SP-A content by dexamethasone at early times in culture; however, between 4 and 7 days of culture stimulation decreased and was lost in some cases (e.g., Fig. 5).

Dexamethasone also accelerates the accumulation of SP-A mRNA in lung explants, but this effect is not sustained as culture continues. Fig. 1 summarizes results for nine lungs. SP-A levels were increased 0.5- to 10-fold by 1–2 days after addition of 10 nM dexamethasone. However, by 4–6 days, treated levels in 5 of the 9 lungs were below those in their controls. The loss of glucocorticoid stimulation results from decreasing content of SP-A mRNA in treated cultures, whereas message continues to accumulate in control explants in most experiments (13).

To confirm that the observed effects were a function of duration of exposure to dexamethasone and not of time in culture, in three experiments we varied the time of addition of 10 nM dexamethasone (0–76 hr) and kept duration of culture constant at 76 hr (Fig. 2). In these experiments, increases in SP-A mRNA were consistently detected within 10 hr of exposure to dexamethasone. Stimulation was maximal between 30 and 55 hr of exposure and decreased by more than half after 76 hr of exposure.

Glucocorticoid Dose Response. Dose–response studies were performed with both dexamethasone and cortisol. Maximal induction of SP-A on day 4 of culture occurred at 3–10 nM dexamethasone and there was no stimulation at 100 nM; half-maximal response was observed at 1–2 nM (Fig. 3*a*). The response curve for SP-A mRNA (day 3 of culture) was shifted slightly to the left with maximal response (5-fold) at 3 nM dexamethasone and apparent half-maximal effect at <1 nM (Fig. 3*b*). Induction of SP-A mRNA by cortisol was maximal (5-fold) at 300 nM and half-maximal at about 200 nM; stimulation was lost at 10 μM (Fig. 3*c*). Data for SP-A content were similar (not shown).

Effect of Other Steroids. We exposed explants of three lungs to either no hormones (control) or to various other steroids at 1 μM. Stimulation of SP-A mRNA by cortisol, cortisone, and corticosterone was similar to that by dexamethasone (10 nM), but levels were not different from controls in explants exposed to estradiol, dihydrotestosterone, and 17-hydroxyprogesterone (Fig. 4). Similar results were observed for content for SP-A (data not shown).

Effect of Removing Glucocorticoid. Most receptor-mediated effects of glucocorticoids, including stimulation of various surfactant components (ref. 15; H.G.L. and P.L.B., unpublished data), are reversible upon removal of hormone. To examine reversibility of SP-A stimulation, we exposed explants to cortisol for the second 24 hr in culture and then removed the hormone by replacing the medium with hormone-free medium three times at 2-hr intervals. Cortisol was used in these experiments because it dissociates faster from glucocorticoid receptors than does dexamethasone. As shown in Fig. 5, removal of cortisol on day 2 of culture produced a transient increase in SP-A content (day 3) compared with cultures continuously treated with hormone. On subsequent days, SP-A levels were similar in explants from which cortisol had been removed and in control cultures, whereas the rate of accumulation decreased continuously in cortisol-treated explants. The pattern for SP-A

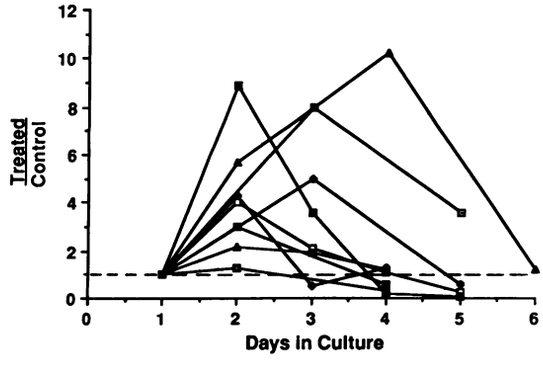

FIG. 1. Time course of SP-A mRNA in dexamethasone-treated vs. control cultures. Data from nine separate experiments with 10 nM dexamethasone are shown as the treated-to-control ratio. The broken line indicates a ratio of 1 (no hormone effect).

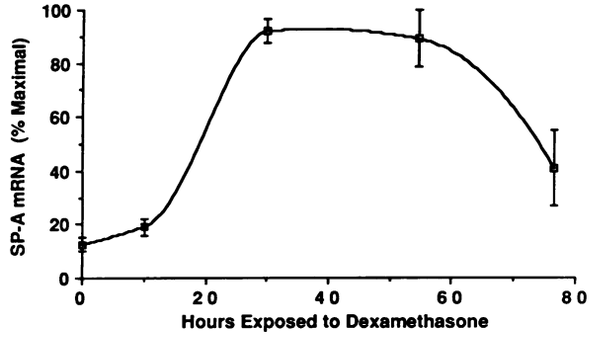

FIG. 2. Time course of SP-A mRNA after exposure to 10 nM dexamethasone. Explants were cultured for 76 hr with addition of hormone at initiation of the cultures (76-hr exposure) or after 22, 46, and 66 hr of culture (54-, 30-, and 10-hr exposure, respectively). Data are mean ± SEM for three experiments and are expressed as the percent of the maximal value in each experiment.

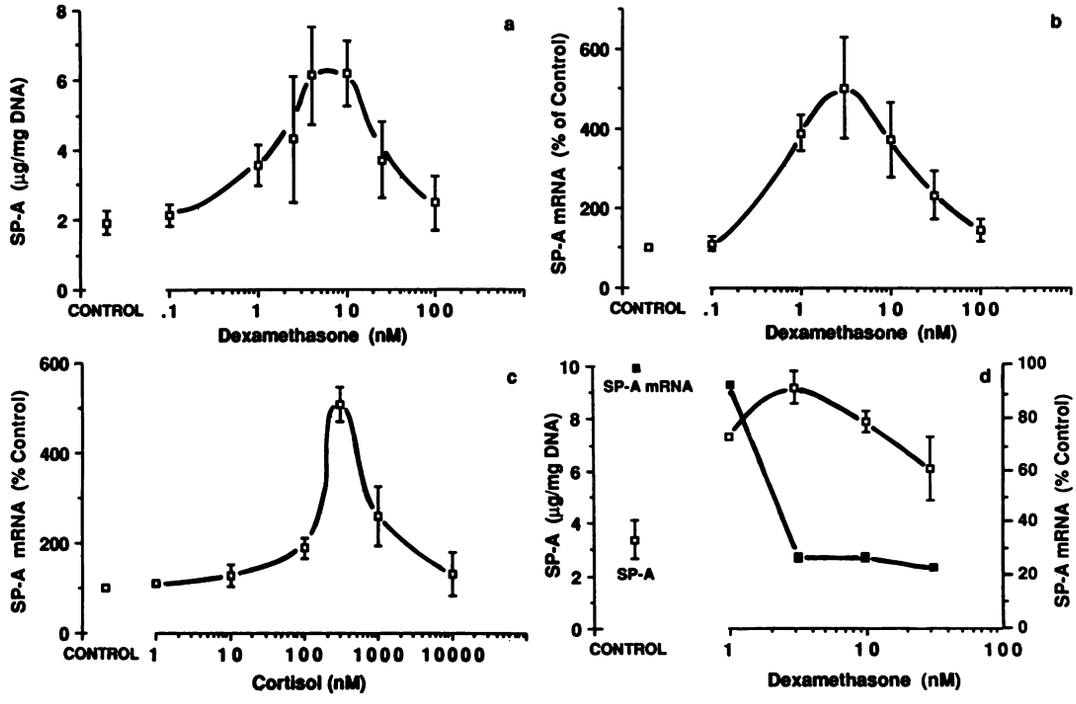

FIG. 3. Dose–response studies with dexamethasone and cortisol. (*a*) Dexamethasone and SP-A content on day 4; (*b*) dexamethasone and SP-A mRNA content on day 3; (*c*) cortisol and SP-A mRNA on day 3; (*d*) effect of dexamethasone in presence of forskolin (10 μM) and 3-isobutyl-1-methylxanthine (IBMX; 0.1 mM) on day 4. Data are mean ± SEM for three to six experiments in *a–c* and mean ± SEM of four replicates (SP-A) or single values (mRNA) in *d*.

mRNA in these experiments was similar except that content of message in continuously treated explants declined on days 4 and 5 (not shown).

Effect of cAMP. It has been reported that cAMP increases synthesis of SP-A (12, 23, 28) and that this effect is inhibited by dexamethasone (12). Because we found a stimulatory component of glucocorticoid treatment, we examined the effects of cAMP and interaction with dexamethasone. Under conditions of hormone exposure that were optimal for dexamethasone stimulation, we found similar stimulation of SP-A content by dibromo-cAMP and by terbutaline and forskolin, agents which activate intracellular adenylate cyclase (Table 1). All three substances also increased SP-A mRNA but less so than dexamethasone. The response to forskolin plus dexamethasone was approximately additive for both SP-A and its mRNA.

Fig. 6 shows the time course for SP-A accumulation in explants treated with forskolin plus IBMX (an inhibitor of cAMP phosphodiesterase). Compared with control, forskolin treatment was stimulatory (2- to 6-fold) at all time points examined. These findings indicate that the response to cAMP (forskolin + IBMX) does not have an inhibitory component.

At an optimal concentration of 0.1 mM, IBMX consistently increased the content of SP-A in explants treated for 72 hr with dexamethasone (2.4-fold with range 1.3–3.5, $n = 8$) or terbutaline/forskolin (SP-A, 2.9-fold; SP-A mRNA, 1.9–fold, $n = 3$). Treatment with dexamethasone + IBMX for longer times was inhibitory as seen with dexamethasone alone (data not shown). When IBMX was added to control explants, SP-A content increased in four experiments (2.3- to 5.2-fold) but not in five other experiments; this variability was not related to tissue age or responsiveness to other hormones and remains unexplained.

To further examine the relationship between glucocorticoid and cAMP, we performed dexamethasone dose–re-

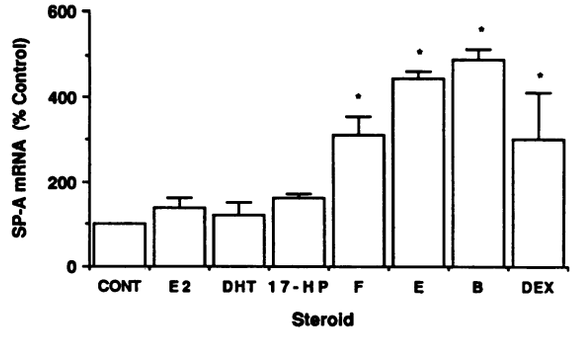

FIG. 4. Effect of various steroids on content of SP-A mRNA. Explants were exposed to dexamethasone at 10 nM or other steroids at 1 μM for 48 hr. Data are mean ± SEM for three experiments. CONT, control; E2, 17β-estradiol; DHT, dihydrotestosterone; 17-HP, 17α-hydroxyprogesterone; F, cortisol; E, cortisone; B, corticosterone; and DEX, dexamethasone. *, $P < 0.05$ vs. control.

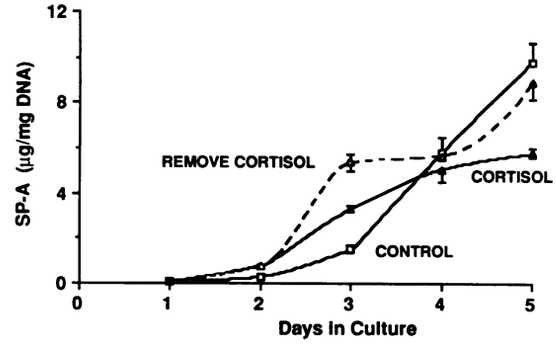

FIG. 5. Effect of removing cortisol on SP-A content. Explants of a 19-week-gestation lung were cultured without hormone (control), or with 1 μM cortisol added on day 1. Cortisol was removed from some culture dishes on day 2 by repeated medium changes (broken line). Data are mean ± SEM for four replicates.

Table 1. Effect of cAMP on SP-A and SP-A mRNA

Hormones added	SP-A, μg/mg of DNA	SP-A mRNA, % control
None (control)	1.85 ± 0.25	100
Dexamethasone (10 nM)	3.74 ± 0.5	371 ± 57
Dibromo-cAMP (0.1 mM)	4.20 ± 0.85	204 ± 17
Terbutaline (10 μM)	3.54 ± 0.44	131 ± 9
Forskolin (10 μM)	5.02 ± 0.93	254 ± 95
Forskolin + dexamethasone	7.98 ± 1.5	665 ± 44

Levels of SP-A mRNA and SP-A were determined after 3 and 4 days of culture, respectively, with addition of hormones on day 1. Data are mean ± SEM for five lungs (four for dibromo-cAMP) for SP-A and three lungs for SP-A mRNA. All treated values are greater than control ($P < 0.05$) except for forskolin and SP-A mRNA ($P = 0.15$); forskolin + dexamethasone vs. forskolin: $P < 0.05$ for both SP-A and mRNA.

sponse experiments in the presence of forskolin and IBMX (Fig. 3*d*). The response curve for SP-A was biphasic and similar to results with dexamethasone alone (Fig. 3*a*). Levels of SP-A mRNA, determined after 72 hr of exposure (rather than 48 hr as in most other experiments), were decreased at all concentrations of dexamethasone.

DISCUSSION

We have shown that glucocorticoids both stimulate and inhibit accumulation of SP-A and its mRNA in cultured human fetal lung. Stimulation by dexamethasone is maximal at low doses (<10 nM) and after short exposures (24–72 hr) and diminishes as dosage or duration of exposure increases. Inhibition of SP-A mRNA accumulation is often observed after 4 days of exposure to 10 nM dexamethasone and occurs more rapidly with higher concentrations of glucocorticoid. These findings would appear to explain the conflicting reports regarding glucocorticoid effects on SP-A in human fetal lung. Whitsett *et al.* (12) reported only inhibition of SP-A synthesis, but most of their studies used 10 μM dexamethasone and culture for 4–5 days. Our previous studies describing stimulation of SP-A were performed with 10 nM dexamethasone (13, 20). A similar biphasic response to dexamethasone has also been reported in an abstract by Mendelson *et al.* (29).

Several properties of the stimulatory effect support a receptor-mediated mechanism of glucocorticoid action. We found that both dexamethasone and natural glucocorticoids (cortisol, cortisone, and corticosterone) increased SP-A and its mRNA, but representative sex steroids had no effect. Although cortisone itself lacks appreciable glucocorticoid activity, in fetal lung it is reversibly metabolized to cortisol, which is biologically active (30). This interconversion may account for the relatively steep slope of the cortisol dose–response curve: until enzyme saturation is reached most

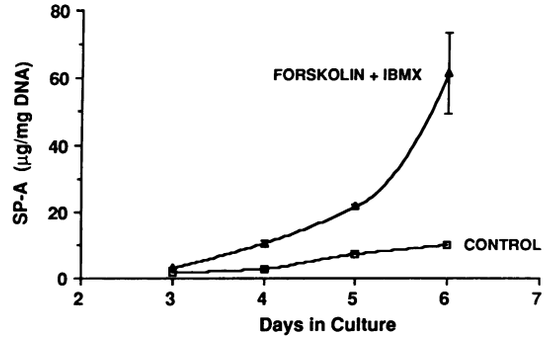

FIG. 6. Time course of SP-A accumulation in the presence of forskolin (10 μM) plus IBMX (0.1 mM). Hormone was added after 1 day of culture. Data are mean ± SEM for four replicates.

cortisol that is added may be metabolized to cortisone. Removal of cortisol from cultures eventually reversed the stimulation, as would be predicted by dissociation of receptor–steroid complex.

Stimulation of SP-A and its mRNA accumulation occurred at a dexamethasone concentration of ≤1 nM and was maximal at ≈5 nM. The concentration for apparent half-maximal effect (1–2 nM) is somewhat less than the K_d for dexamethasone–receptor binding at 37°C in intact lung cells and other human tissues (5–16 nM) (14), raising the possibility that the glucocorticoid effect is indirect. Alternatively, the half-maximal increase may actually occur at a higher concentration than indicated by the data of Fig. 3 *a* and *b* due to a superimposed inhibitory response curve.

Although the properties of the inhibitory effect of glucocorticoid are not yet fully defined, some features of the process are evident from our study and a previous report. Inhibition of SP-A and SP-A mRNA accumulation is similar, indicating that the effect is pretranslational. At a dexamethasone concentration of 10 nM, onset of the inhibitory process is considerably slower than for the stimulatory effect. In studies with 10 μM dexamethasone, Whitsett *et al.* (12) noted inhibition of SP-A accumulation by 24–48 hr and no apparent stimulatory phase. When we removed cortisol from explants, levels of SP-A and its mRNA increased above values for continuously treated cultures. By contrast, removal of cortisol decreased mRNA for both surfactant protein B and surfactant protein C in the same experiments (H.G.L. and P.L.B., unpublished data). The transient increase in SP-A after removal of cortisol suggests that the inhibitory effect of glucocorticoids is more rapidly reversed than the stimulatory action. Inhibition of SP-A mRNA was observed with nanomolar dexamethasone (with longer times of culture) and with cortisol, suggesting that inhibition, like stimulation, is receptor mediated. As also observed by Whitsett *et al.* (12), inhibition of SP-A accumulation by dexamethasone can be seen in the presence of increased cAMP. Finally, inhibition may be species specific; glucocorticoid treatment of lung explants from rabbit (31) and 18-day rat (I. Gross and J. Floros, personal communication) produced only stimulation of SP-A and its mRNA at all doses tested.

The inhibition of SP-A accumulation caused by dexamethasone could have several explanations. First, under these conditions glucocorticoids might cause a generalized inhibition of protein synthesis. However, dexamethasone treatment does not affect total RNA and protein recovered from explants, rate of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable proteins, or content of actin mRNA (12, 13, 23, 32). Second, the epithelial cells might have lost most of their differentiated type II cell characteristics with continued culture. However, electron micrographs of day 6 explants demonstrate mature-appearing type II cells containing large lamellar bodies (15, 16). Furthermore, the glucocorticoid-induced accumulation of mRNAs for SP-B and SP-C is sustained for at least 7 days (18, 19), as is choline incorporation into phosphatidylcholine (15) and activity of fatty acid synthetase (P.L.B. and L. G. Gonzales, unpublished data). A third possibility is that glucocorticoid receptors in the explants are down-regulated at higher doses or longer durations of exposure to dexamethasone. However, the sustained increase in mRNAs for SP-B and SP-C and in the rate of choline incorporation, all of which are reversible events, argue against this hypothesis. Finally, increasing levels of SP-A could inhibit further accumulation of SP-A mRNA through a feedback mechanism. However, induction of SP-A by forskolin, interferon γ (33), and epidermal growth factor (23) is sustained for at least 4 days after hormone addition.

Since none of the above explanations seems likely, we therefore suggest that glucocorticoids have direct stimulatory

and inhibitory effects on SP-A gene expression and that glucocorticoid inhibition is specific for SP-A among surfactant components. Like various other glucocorticoid-regulated genes (34), the human SP-A gene described by White *et al.* (27) contains a consensus sequence for glucocorticoid receptor binding. In view of our results, this regulatory element could exert either positive or negative transcriptional control on the gene. In addition, SP-A gene expression may be influenced indirectly by glucocorticoid-induced factor(s) in either type II cells or other cell types (e.g., fibroblasts).

The inhibitory action of glucocorticoids may result from increased degradation of SP-A mRNA. Lee *et al.* (35) have reported that glucocorticoids decrease the stability of interleukin 1β mRNA. A conserved sequence (AUUUA) is found in the 3′ untranslated region of this and other mRNAs which have short half-lives (36) and is also present in SP-A mRNA (27). It seems unlikely that glucocorticoids are primarily acting at the level of translation or protein processing, since both the positive and negative responses were similar for SP-A and its mRNA.

Dexamethasone might stimulate SP-A production by increasing cAMP. However, three observations are not consistent with this possibility. Accumulation of SP-A and SP-A mRNA is approximately additive in the presence of dexamethasone plus forskolin; if dexamethasone acted via cAMP, an additive response would be unlikely, since forskolin alone is a potent activator of adenylate cyclase (37). Second, addition of forskolin and IBMX to increase cAMP did not shift the dexamethasone dose response. Third, the ratio of SP-A to SP-A mRNA stimulation for dibromo-cAMP, terbutaline, and forskolin is considerably higher than for dexamethasone, suggesting differences in the action of cAMP and glucocorticoid on expression of the SP-A gene. However, these considerations do not rule out the possibility of indirect effects of glucocorticoids on the response to cAMP.

Although glucocorticoids are known to exert both positive and negative gene regulation, there is a paucity of examples of both stimulatory and inhibitory effects on synthesis of the same protein. Ono and Oka (38) have demonstrated a biphasic cortisol dose–response curve for α-lactalbumin synthesis in mouse mammary explants in culture, which is quite different from that for casein synthesis in the same tissue. Thus, glucocorticoid effects on SP-A may involve a unique form of regulation. The functional significance of our findings awaits *in vivo* correlation, but the data suggest that levels of SP-A could be low in infants treated with prolonged courses of high dose steroids.

In summary, we have presented evidence that glucocorticoids have both early stimulatory and late inhibitory effects on production of SP-A. Stimulation is receptor mediated, both effects occur at a pretranslational level and are independent of cAMP, and the inhibitory action appears to be specific for SP-A among surfactant components.

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