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The Combined Effects of Insulin and Cortisol on Surfactant Protein mRNA Levels

STEVEN A. DEKOWSKI AND JEANNE M. SNYDER

Departments of Pediatrics [S.A.D.] and Anatomy [J.M.S.], University of Iowa College of Medicine, Iowa City, Iowa 52242

ABSTRACT

Infants of diabetic mothers are frequently hyperinsulinemic and have an increased incidence of neonatal respiratory distress syndrome, a disease caused by a deficiency in the production of pulmonary surfactant by alveolar type II cells. It has been hypothesized that insulin inhibits fetal lung type II cell differentiation. We have shown previously that insulin inhibits the accumulation of surfactant protein (SP)-A and SP-B mRNA and has no effect on SP-C mRNA levels in human fetal lung tissue maintained *in vitro*. We hypothesized that treatment with glucocorticoids, which are used clinically to accelerate human fetal lung maturation, would overcome the inhibitory effects of insulin on human fetal lung development. In the present study, human fetal lung explants were maintained in the presence or absence of cortisol added alone, or in insulin plus cortisol added together. Cortisol significantly decreased SP-A mRNA levels by approximately 50% at the 100 nM concentration and significantly increased levels by approximately 20% at the 1 nM concentration. Cortisol increased SP-B and SP-C mRNA levels in a dose-dependent fashion (5- and 45-fold at 100 nM cortisol, respectively). The combination of 1 nM cortisol and insulin resulted in inhibition of mRNA levels for SP-A, SP-B, and SP-C at the high insulin concentrations (approximately 50% inhibition for SP-A and SP-B and approximately 25% inhibition of SP-C mRNA levels, in the presence of 40 pmol/L $\times$ 10^{-3} insulin). Surprisingly, 100 nM cortisol plus inhibitory concentrations of insulin increased SP-A mRNA levels (2-fold at 40 pmol/L $\times$ 10^{-3}). SP-B and SP-C mRNA levels in the presence of 100 nM cortisol plus insulin were generally unchanged when compared with levels in explants cultured in cortisol alone. Thus, cortisol modulates the inhibitory effects of insulin on SP mRNA levels in a dose-dependent manner. (***Pediatr Res* 38: 513–521, 1995**)

Abbreviations

SP, surfactant protein
GAPDH, glyceraldehyde phosphate dehydrogenase
PEPCK, phosphoenolpyruvate carboxykinase
ANOVA, analysis of variance

The SP contribute importantly to pulmonary surfactant function and metabolism (1). The SP comprise approximately 5–10% of total surfactant by weight (2). Four SP have been described to date (1). SP-A, the most abundant of the surfactant-associated proteins, mediates the transformation of lamellar bodies into tubular myelin, increases the rate of adsorption of surfactant phospholipids onto an aqueous surface, and regulates surfactant phospholipid synthesis and secretion (3–5). In addition, SP-A may also have immune system related functions (6, 7). SP-B and SP-C are hydrophobic, low molecular weight SP which dramatically increase the rate of spreading of the surfactant phospholipids onto an aqueous surface (8, 9). Furthermore, SP-B, in the presence of SP-A and calcium, contributes to the formation of tubular myelin (10). SP-B may also regulate surfactant metabolism as it has been shown to enhance the uptake of phospholipids by type II cells *in vitro* (11). SP-D, a collagenous protein with structural similarity to SP-A, has recently been identified, and it has been proposed that it also is involved in immune function in the lung (12, 13). Thus, the SP are required for the optimal functioning of surfactant and may perform many other functions in the lung.

SP gene expression has been shown to be regulated by multiple factors both *in vitro* and *in vivo* (1, 14). For example, insulin inhibits SP-A and SP-B gene expression in human fetal lung tissue maintained *in vitro* (15, 16). The levels of SP-A protein in the amniotic fluid of pregnancies complicated by diabetes mellitus are significantly less than in nondiabetic controls (17). Because infants of diabetic mothers have been shown to be hyperinsulinemic, these findings are suggestive that insulin inhibits SP gene expression. In contrast to insulin, glucocorticoids generally stimulate SP gene expression (14, 18). Furthermore, glucocorticoids are used clinically to accelerate fetal lung maturation (19). In the present study, we evaluated the possible opposing effects of these two hormonal regulators on SP gene expression. Our goal was to describe the combined effects of insulin and cortisol on the levels of mRNA for the SP in human fetal lung tissue. We hypothesized that

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Correspondence: Jeanne M. Snyder, M.D., Department of Anatomy, University of Iowa College of Medicine, 1-550 Bowen Science Building, Iowa City, IA 52242.

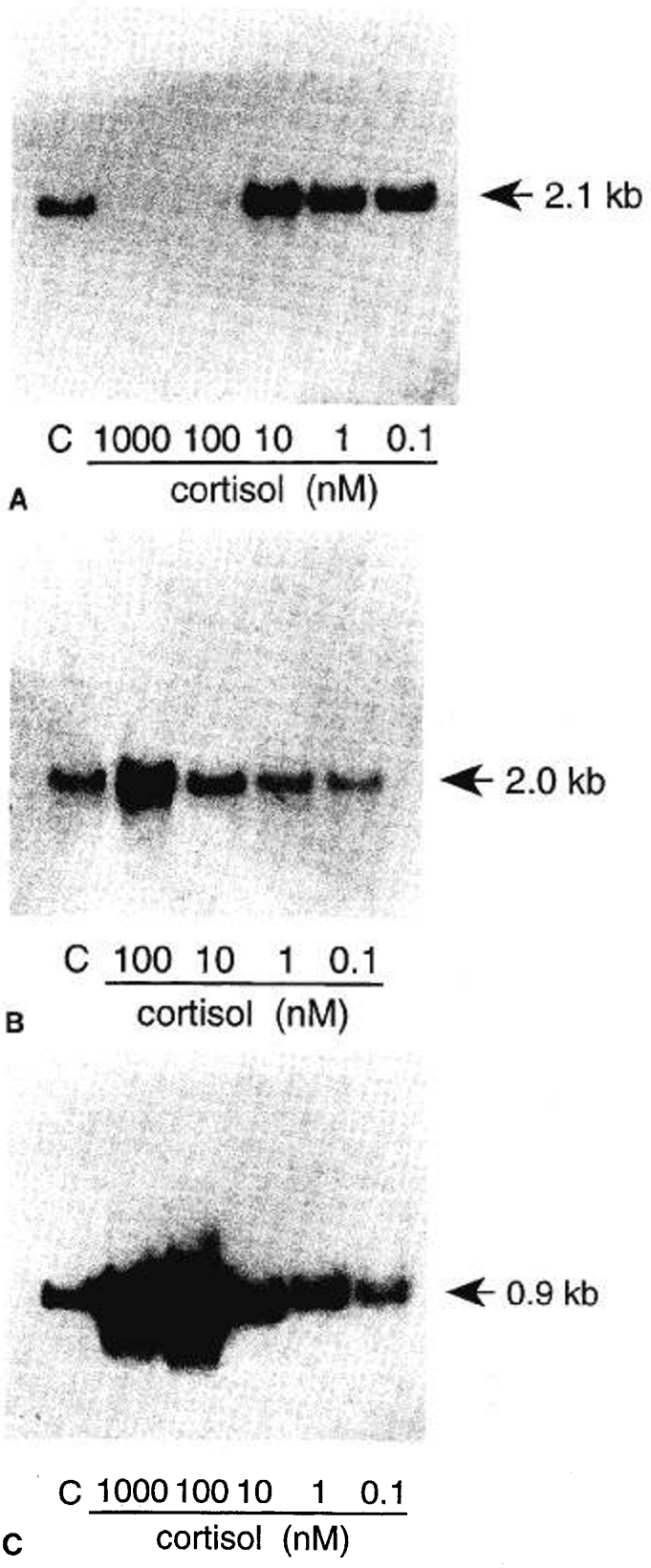

Figure 1. *A*, A representative autoradiogram of a SP-A Northern blot demonstrating the effects of cortisol on the levels of SP-A mRNA in human fetal lung explants. Explants were incubated for 4 d in the absence (*C*) or presence of cortisol at the indicated concentration. *B*, A representative SP-B Northern blot illustrating the effects of cortisol on the levels of SP-B mRNA in human fetal lung explants. Explants were incubated for 4 d in the absence (*C*) or presence of cortisol at the indicated concentration. The 1000 nM cortisol condition is not shown in this Northern blot. *C*, A representative SP-C Northern blot demonstrating the effect of cortisol on SP-C mRNA levels in human fetal lung explants. Explants were incubated for 4 d in the absence (*C*) or presence of cortisol at the indicated concentration.

cortisol would overcome the inhibitory effects of insulin on human fetal lung development.

To study the interactive effects of insulin and glucocorticoids on human fetal lung surfactant-associated protein gene expression, we used an *in vitro* model system (20). Undifferentiated human fetal lung tissue, obtained from midtrimester human abortuses, was minced into explants that were then maintained in culture in serum-free medium. We have shown previously that the epithelial cells of the explants differentiate spontaneously into alveolar type II cells *in vitro* within 4–6 d of culture (20). After a few days of culture, the epithelial cells, which comprise approximately 50% of the cells in the explants, contain abundant lamellar bodies with a phospholipid composition similar to the surfactant produced *in vivo* at approximately 36 wk of gestation (20). We and others have shown that mRNAs for the surfactant-associated proteins increase in the explants with time in culture and that the explants are hormonally responsive (15, 16, 21, 22). In the present study, we characterized the effects of cortisol, added alone, and the effects of insulin plus cortisol on surfactant-associated protein expression in human fetal lung explants maintained *in vitro*.

METHODS

Explant cultures. Approval for the protocols used in this study was obtained from the University of Iowa Human Subjects Review Committee. Lung explants were prepared from tissue obtained from midtrimester human abortuses. The range of gestational ages used in the study was from 17 to 21 wk. Tissues from genetically or morphologically abnormal fetuses were not used for the study. After dissecting away the major airways and blood vessels, the peripheral lung tissue was rinsed several times in serum-free Waymouth's 752/1 medium containing penicillin (100 U/mL), streptomycin (100 mg/mL), and Fungizone (0.25 mg/mL) (GIBCO, Grand Island, NY), minced into 1–2-mm^3 pieces, and placed on pieces of lens paper supported by stainless steel grids in 35-mm culture dishes containing 1 mL of serum-free Waymouth's medium. All of the peripheral lung tissue from one abortus was pooled, then minced. Three different experimental designs were used in the present study: *1*) explants were cultured in the presence of cortisol, added alone, at various concentrations; *2*) explants were cultured in 1 nM cortisol plus or minus insulin at various concentrations; or *3*) explants were cultured in 100 nM cortisol plus or minus insulin at various concentrations. Each of these three experimental designs was repeated four to six times using tissue from one abortus for each experiment. The explants were maintained for 4 d at 37°C in a humidified atmosphere of CO_2 (5%) and air (95%) with media changed daily. Explants were incubated in medium with or without insulin (crystalline porcine, Sigma Chemical Co., St. Louis, MO) at various concentrations (0.04–400 pmol/L $\times$ 10^{-3}) and/or cortisol (0.1–1000 nM). The insulin stock solution (1 mg/mL) was prepared in a dilute solution of HCl (pH 2.75) and stored at −70°C. Like-

wise, cortisol was prepared as a stock solution, 2.0 mg/mL in ethanol, and was stored at −20°C.

RNA isolation. Total RNA was isolated from the cultured lung explant tissue using the single-step method of Chomczynski and Sacchi (23). Approximately 50 mg of lung explant tissue were homogenized in 1.5 mL of RNA extraction mixture. The homogenate was transferred to a microcentrifuge tube containing 150 μL of chloroform, shaken vigorously for approximately 10 s, incubated on ice for 5 min, and then centrifuged at 12 000 × *g* for 15 min at 4°C. The resulting upper aqueous phase was transferred to a microcentrifuge tube containing an equal volume of ice-cold isopropyl alcohol, mixed by inversion, incubated on ice for 30 min, and then centrifuged for 15 min at 12 000 × *g* at 4°C. The precipitated total RNA was washed with 1.5 mL of 75% ethanol, air-dried, resuspended in sterile diethylpropylcarbonate-treated water, and stored at −20°C. RNA was quantitated by determining the absorbance at 260 nm. Ten micrograms of total RNA from each sample were separated by gel electrophoresis on a 1.2% agarose, 5% formaldehyde gel and capillary transferred to a Nytran membrane (Schleider & Schuell, Keene, NH) (24). The RNA was adhered to the membrane by heating at 80°C for 30–60 min.

Northern blot analysis. The human cDNA probes for SP-A, SP-B, and SP-C were radiolabeled with [^{32}P]cytosine triphosphate (Amersham, Bedford, MA) using a random primer kit (Boehringer Mannheim, Indianapolis, IN). The 0.9-kb SP-A, 1.4-kb SP-B, and 0.8-kb SP-C cDNA probes were obtained from Jeffrey Whitsett, M.D (25–27) (University of Cincinnati, Cincinnati, OH). A human 1.2-kb GAPDH cDNA was obtained from the American Type Culture Collection (Rockville, MD) and was used as an internal control for RNA loading (28). RNA-containing membranes were prehybridized in a sealable plastic bag in 12 mL of hybridization buffer [BSA, (0.2%, wt/vol), polyvinylpyrrolidone (0.2% wt/vol), Ficoll (M_r 500 000, 0.2% wt/vol), Tris-HCl (50 mM, pH 7.4), sodium pyrophosphate (0.1%, wt/vol), SDS (1.0%, wt/vol), dextran sulfate (10%, wt/vol), formamide (50%, wt/vol), sodium chloride (1 M), and heat-denatured herring sperm DNA (0.1 mg/mL)] for 4 h at 42°C. Radiolabeled cDNA probe (1 × 10^6 cpm/mL of hybridization buffer) was injected into the bag containing the prehybridization buffer and membrane. After hybridization for 16 h at 42°C, the membranes were washed two times in 250 mL of 2 × SSC [sodium chloride (0.3 M) and sodium citrate (0.03 M)] at room temperature for 5 min, two times in 250 mL of 2 × SSC and SDS (1%, wt/vol) at 65°C for 30 min, and one time in 250 mL of 0.1 × SSC at room temperature for 15 min. The membranes were wrapped in plastic wrap, exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY) with an intensifier screen (Lighting Plus, Dupont, Wilmington, DE) at −70°C. Reactive bands on the film were quantitated using densitometry. The ^{32}P-labeled cDNA probe was then removed from the membrane by incubating the membrane in 250 mL of Tris-HCl (5 mM, pH 8.0), EDTA (0.2 mM), sodium pyrophosphate (0.5%, wt/vol), polyvinylpyrrolidone (0.002%, wt/vol, M_r 40,000), BSA (0.002%, wt/vol), and Ficoll (0.002%, wt/vol) for 1–2 h at 65°C and then rehybridized to another ^{32}P-labeled SP cDNA probe as de-

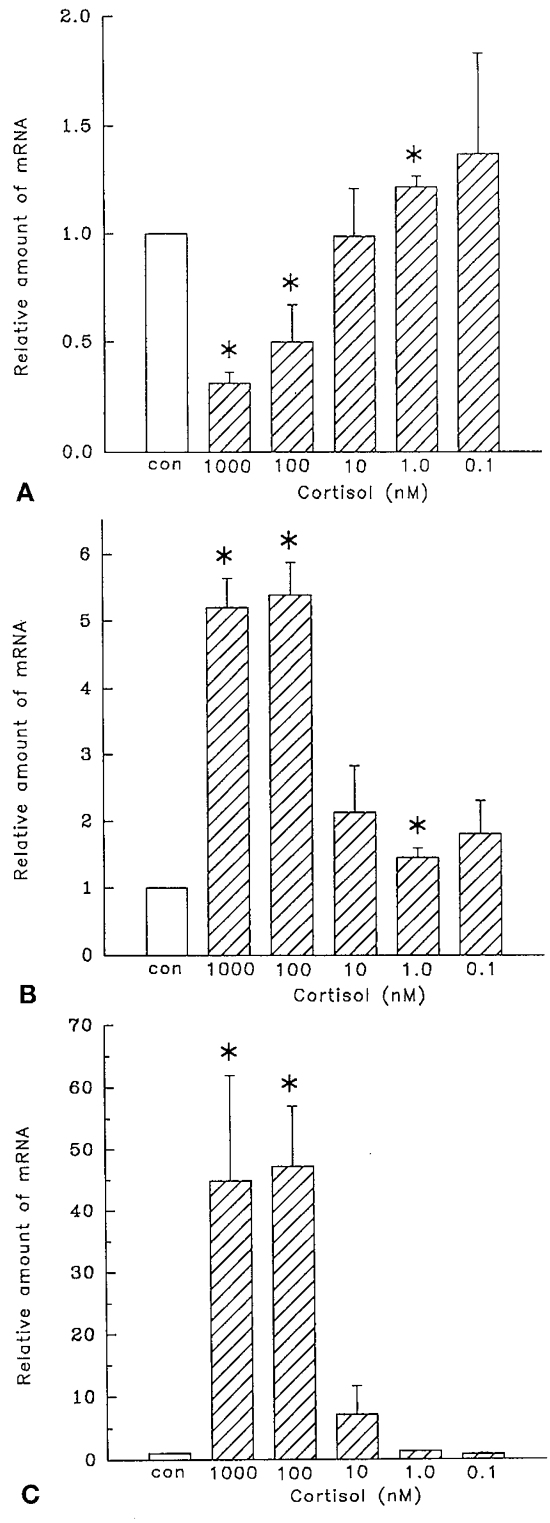

Figure 2. *A*, Densitometric data from four experiments. The data are expressed as the mean ± SEM. Cortisol affected SP-A mRNA levels in a dose-dependent manner ($p < 0.05$, ANOVA). *Asterisks* represent a significant difference from the control (*con*) condition ($p < 0.05$). *B*, Graphic representation of densitometric data from four experiments. The data are expressed as the mean ± SEM. Cortisol increased SP-B mRNA levels in a dose-dependent manner ($p < 0.05$, ANOVA). *Asterisks* represent a significant difference from the control (con) condition ($p < 0.05$). *B*, Densitometric data from four experiments evaluating the effects of cortisol on SP-C mRNA levels. Data are expressed as the mean ± SEM. Cortisol increased SP-C mRNA levels in a dose-dependent manner ($p < 0.05$, ANOVA). *Asterisks* represent a significant difference from the control (*con*) condition ($p < 0.05$).

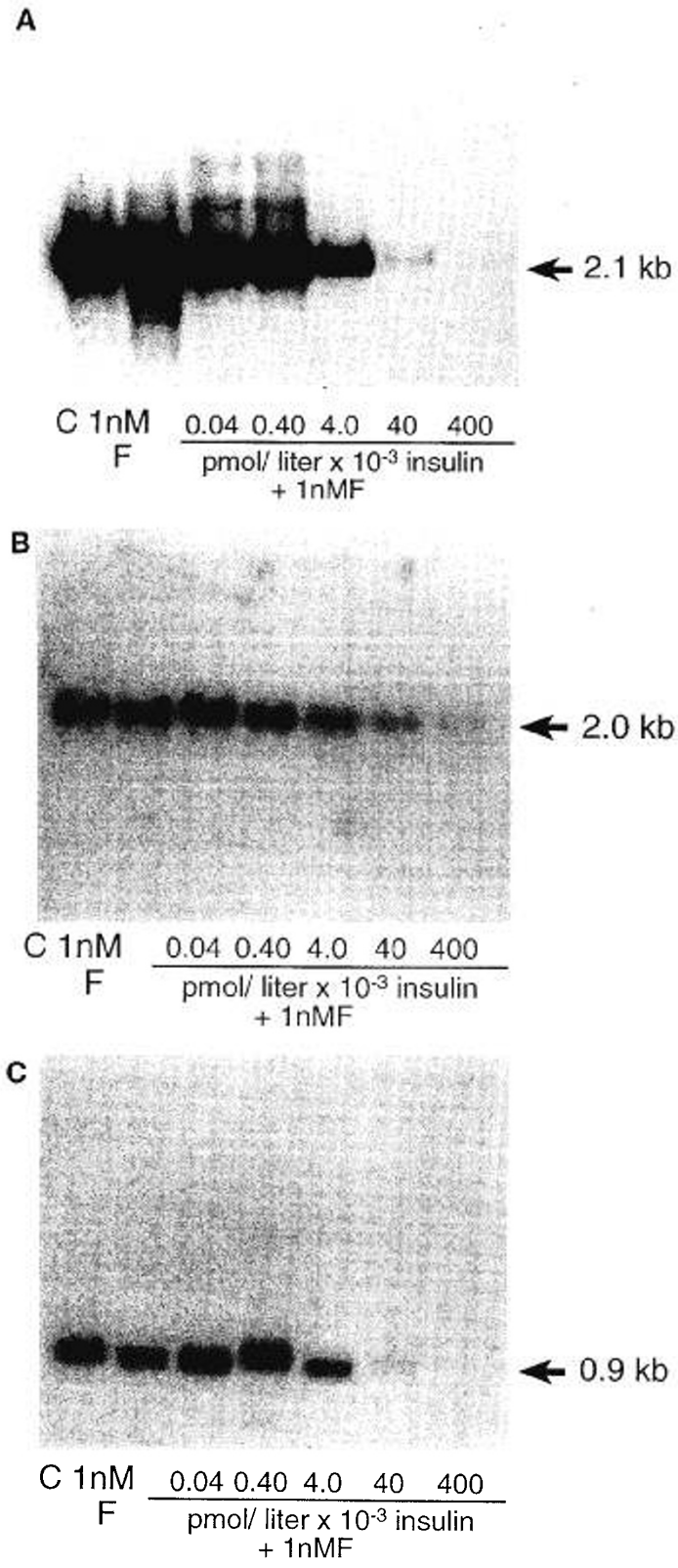

Figure 3. *A*, The effect of 1 nM cortisol (*F*) plus insulin on SP-A mRNA levels in human fetal lung explants. Explants were maintained in serum-free medium that contained either no additions (*C*), 1 nM cortisol (*1 nM F*), or 1 nM cortisol plus insulin at the indicated concentrations. A representative SP-A Northern blot is shown. *B*, The effect of 1 nM cortisol (*F*) and insulin on SP-B mRNA levels in human fetal lung explants. Explants were maintained for 4 d in the absence (*C*) or presence of 1 nM cortisol (*1 nM F*) or 1 nM cortisol plus insulin at the indicated concentration. A representative SP-B Northern blot is shown. *C*, The effect of 1 nM cortisol (*F*) and insulin on SP-C mRNA levels in human fetal lung explants. Explants were maintained for four days in the absence (*C*) or presence of 1 nM cortisol (*1 nM F*) or in 1 nM cortisol plus insulin at the indicated concentration. A representative SP-C Northern blot is shown.

scribed above. Membranes were stripped and reprobed up to three times without appreciable loss of RNA from the membrane. In a previous study, we have shown that these methods result in linear relationship between the amount of SP mRNA and the intensity of the reactive band (29).

Data analysis. The mRNA densitometric data are expressed as the ratio of the absorbance of the individual surfactant-associated protein mRNA reactive bands to the absorbance of the GAPDH mRNA reactive bands on the same membrane. In a previous study, we determined that insulin had no effect on GAPDH mRNA levels in the human fetal lung explants (16). In the present study we found that, likewise, cortisol had no effect on GAPDH mRNA levels (data not shown). Data for each Northern blot were normalized such that the control value was equal to one. Experiments were repeated three to five times. Each experiment was performed using tissue from one abortus. Data are expressed as the mean ± SEM. Statistical comparisons were made using one-way ANOVA and a two-tailed Dunnett's test (30). Because the variance of the untreated control condition was always zero, the ANOVA did not include the control condition. A two-tailed Dunnett's test was used to compare the untreated control condition and, in the combination experiments, the cortisol alone condition, to the other experimental conditions.

RESULTS

We initially evaluated the effects of cortisol, at concentrations ranging from 0.1 to 1000 nM, on the mRNA levels for SP-A, SP-B, and SP-C in the human fetal lung explants (Figs. 1 and 2). Northern blot analysis detected bands of 2.1 kb for SP-A, 2.0 kb for SP-B, and 0.9 kb for SP-C, consistent with the previously reported sizes of these mRNAs in human lung (Fig. 1) (25–27). Treatment of the explants with cortisol altered SP-A mRNA levels in a dose-dependent manner (Fig. 1*A*, $p < 0.05$, ANOVA). Cortisol at the 1000 and 100 nM concentrations significantly decreased SP-A mRNA levels by approximately 70 and 50% relative to controls, respectively (Fig. 2*A*). SP-A mRNA levels were significantly increased by approximately 20% at the 1 nM cortisol concentration when compared with untreated controls.

Cortisol increased SP-B mRNA levels in the human fetal lung explants in a dose-dependent fashion (Fig. 1*B*, $p < 0.05$, ANOVA). SP-B mRNA levels were significantly increased approximately 5-fold at cortisol concentrations of 100-1000 nM when compared with controls (Fig. 2*B*). At the remaining cortisol concentrations of 0.1, 1, and 10 nM, SP-B mRNA levels were increased by approximately 50–100%, with statistical significance reached only at the 1 nM concentration.

An even greater, dose-dependent, stimulatory effect of cortisol was observed on SP-C mRNA levels (Fig. 1*C*, $p < 0.05$, ANOVA). SP-C mRNA levels were significantly increased approximately 45-fold at the 1000 and 100 nM cortisol con-

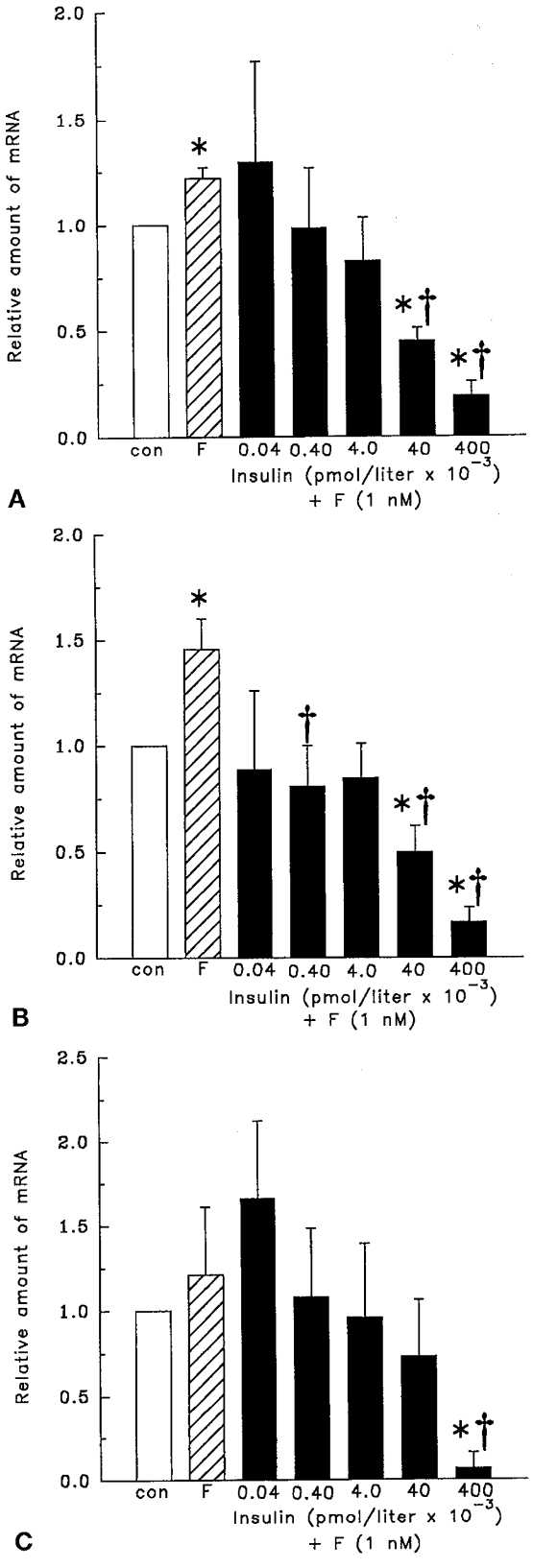

Figure 4. *A*, Densitometric data from five experiments demonstrating the effects of insulin and 1 nM cortisol (*F*) on SP-A mRNA levels. The data are expressed as the mean ± SEM. Insulin, in the presence of 1 nM F, decreased SP-A mRNA in a dose-dependent manner ($p < 0.05$, ANOVA). *Asterisks* represent a significant difference from control explants (*con*) ($p < 0.05$). *Daggers* represent a significant difference from the 1 nM cortisol alone condition (F, $p < 0.05$). *B*, Densitometric data from four experiments demonstrating the effects of insulin and 1 nM cortisol on SP-B mRNA levels. Data are expressed as mean ± SEM. Insulin, in the presence of 1 nM cortisol, decreased SP-B mRNA in a dose-dependent manner. *Asterisks* represent significant difference from controls (*con*) ($p < 0.05$). *Daggers* represent a significant difference from the 1 nM cortisol alone condition (*F*, $p < 0.05$). *C*, Densitometric data from 3 experiments demonstrating the effects of insulin and 1 nM cortisol on SP-C mRNA levels. Data represent mean ± SEM. The *asterisk* represents a significant difference from the serum-free control condition (*con*) ($p < 0.05$). The *dagger* represents a significant difference from the 1 nM cortisol alone condition (F, $p < 0.05$).

centrations (Fig. 2*C*). At the 10 nM cortisol concentration, SP-C mRNA levels were increased approximately 7-fold relative to controls, although this difference was not statistically significant. No significant effects of cortisol on SP-C mRNA levels were observed at the remaining concentrations.

We then evaluated the effects of insulin (0.04–400 pmol/L × 10^{-3}), in combination with cortisol (1 or 100 nM), on mRNA levels for SP-A, SP-B, and SP-C in the human fetal lung explants (Figs. 3–7). We chose the 100 nM cortisol concentration because it produced prominent inhibitory (SP-A) or stimulatory (SP-B and SP-C) effects on the levels of mRNA for the SP when added alone (Figs. 1 and 2), and it is the same order of magnitude as serum cortisol concentrations reported in premature human newborns (approximately 200 nM) (31). The concentration of cortisol equivalents in steroid-treated newborns has been estimated as approximately 500 nM (31). The 1 nM cortisol concentration was selected to provide contrast to the 100 nM concentration because it produced opposite or less dramatic effects on SP mRNA levels when added alone, *i.e.* slightly stimulatory effects on SP-A, SP-B, and SP-C mRNA levels (Figs. 1 and 2).

Insulin, in the presence of 1 nM cortisol, decreased SP-A mRNA levels in a dose-dependent manner (Fig. 3*A*, $p < 0.05$, ANOVA). In the presence of 1 nM cortisol, insulin at concentrations of 400 and 40 pmol/L × 10^{-3} ng/mL significantly decreased SP-A mRNA levels when compared with levels in control and in 1 nM cortisol treated explants (Fig. 4*A*). No significant effect of 1 nM cortisol on SP-A mRNA levels was observed at the remaining insulin concentrations (4.0–0.04 pmol/L × 10^{-3}).

SP-B mRNA levels were also decreased in a dose-dependent fashion in the presence of insulin and 1 nM cortisol (Fig. 3*B*, $p < 0.05$, ANOVA). At insulin concentrations of 400 and 40 pmol/L × 10^{-3}, SP-B mRNA levels were decreased by approximately 85 and 50%, respectively, relative to controls (Fig. 4*B*). When compared with the 1 nM cortisol alone condition, insulin significantly decreased SP-B mRNA levels at the 0.4, 40, and 400 pmol/L × 10^{-3} concentrations (Fig. 4*B*).

In combination with 1 nM cortisol, insulin decreased SP-C mRNA levels in a dose-dependent manner, although the effect was not statistically significant (Figs. 3*C* and 4*C*, $p < 0.11$, ANOVA). SP-C mRNA levels were decreased by 95% at the 400 pmol/L × 10^{-3} insulin concentration when compared with the untreated controls and to the 1 nM cortisol-treated condition (Fig. 4*C*). The effects at the remaining insulin concentrations were not statistically significant.

The combination of insulin and 100 nM cortisol produced a very different pattern of regulation of the SP mRNAs (Figs. 5 and 6). In general, SP-A mRNA levels were increased by

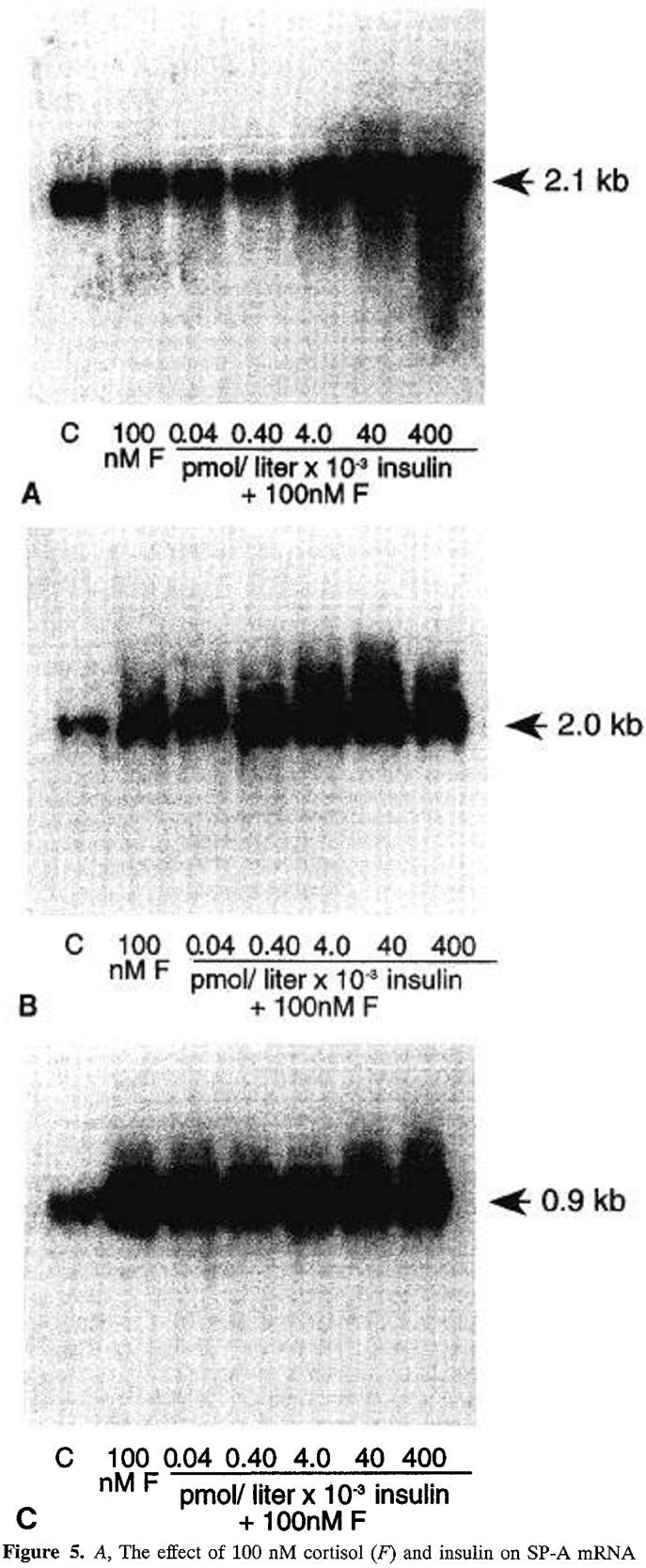

Figure 5. *A*, The effect of 100 nM cortisol (*F*) and insulin on SP-A mRNA levels in human fetal lung explants. Explants were maintained in serum-free medium that contained no additions (*C*), 100 nM cortisol (*100 nM F*), or 100 nM cortisol plus insulin at the indicated concentrations. A representative SP-A Northern blot is shown. *B*, The effect of 100 nM cortisol and insulin on SP-B mRNA levels in human fetal lung explants. Explants were maintained for four days in the absence (*C*) or presence of 100 nM cortisol (*100 nM F*) or in 100 nM cortisol plus insulin at the indicated concentration. A representative SP-B Northern blot is shown. *C*, The effect of 100 nM cortisol and insulin on SP-C mRNA levels in human fetal lung explants. Explants were maintained for four days in the absence (*C*) or presence of 100 nM cortisol (*100 nM F*) or in 100 nM cortisol plus insulin at the indicated concentration. A representative SP-C Northern blot is shown.

insulin in the presence of 100 nM cortisol in a dose-dependent manner, although the effect was not statistically significant (Figs. 5*A* and 6*A*, $p < 0.06$, ANOVA). SP-A mRNA levels were increased by approximately 150% at the 400 pmol/L $\times$ 10^{-3} insulin concentration in the presence of 100 nM cortisol when compared with the 100 nM cortisol alone condition (Fig. 6*A*). The increases at other insulin concentrations were not statistically significant when compared with levels in the 100 nM cortisol condition or when compared with levels in the untreated controls (Fig. 6*A*).

The dose-dependent effect of insulin, in the presence of 100 nM cortisol, on SP-B mRNA levels was not significant (Figs. 5*B* and 6*B*, $p < 0.17$, ANOVA). In the presence of insulin and 100 nM cortisol, SP-B mRNA levels were increased by a minimum of 100% (2500 ng/mL) to a maximum of 380% (0.4 pmol/L $\times$ 10^{-3}) relative to untreated controls with a statistically significant stimulation observed at every insulin concentration except 4 pmol/L $\times$ 10^{-3} (Fig. 6*B*). Comparing the insulin plus cortisol treatment to the 100 nM cortisol alone condition revealed a statistically significant decrease at the 400 pmol/L $\times$ 10^{-3} insulin concentration (Fig. 6*B*).

We also evaluated the effects of 100 nM cortisol and insulin on SP-C mRNA levels (Fig. 5*C*). After 4 d in culture in the presence of 100 nM cortisol, SP-C mRNA levels were significantly increased 40–50-fold at each insulin concentration (0.04–400 pmol/L $\times$ 10^{-3}) tested when compared with levels in control explants. However, this increase was not different from that seen with 100 nM cortisol added alone (Fig. 6*C*). Thus, insulin, in the presence of 100 nM cortisol, had no significant, dose-dependent effects on SP-C mRNA levels ($p < 0.99$, ANOVA).

DISCUSSION

The effects of cortisol, added alone, on SP mRNA levels in cultured human fetal lung tissue observed in the present study are similar to previously reported data. Liley *et al.* (32) examined the effects of cortisol on SP-A mRNA levels and observed dose- and time-dependent effects with maximal stimulation observed at the 300 nM cortisol concentration after 3 d in culture. This observation differs somewhat from our findings, although the differences may be explained by different culture techniques, time in culture, and length of exposure to cortisol. In another study using dexamethasone, a synthetic glucocorticoid, a dose-dependent, biphasic pattern of SP-A mRNA regulation was described, with stimulation of SP-A mRNA levels observed at concentrations less than 10^{-9} M and inhibition observed at concentrations greater than 10^{-8} M (21). Allowing for differences in potency between dexamethasone and cortisol, this pattern of regulation is consistent with our observations. Both cortisol and dexamethasone have been shown to increase SP-B and SP-C mRNA levels in human fetal lung explants in

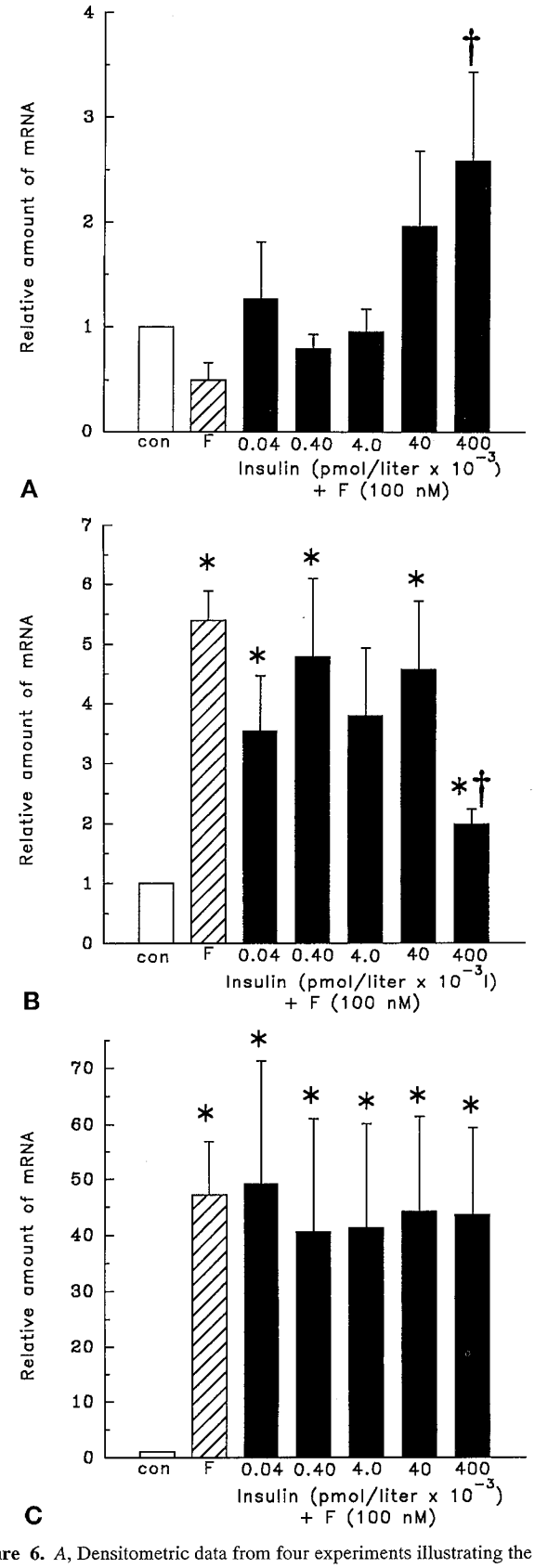

Figure 6. *A*, Densitometric data from four experiments illustrating the effects of insulin and 100 nM cortisol (*F*) on SP-A mRNA levels. Data are expressed as the mean ± SEM. The *dagger* represents a significant difference from cortisol alone condition (*F*) ($p < 0.05$). *B*, Graphic representation of densitometric data from four experiments demonstrating the effects of insulin and 100 nM cortisol (*F*) on SP-B mRNA levels. Data are expressed as mean ± SEM. Asterisks and daggers represent significant difference from the control (*con*) and cortisol alone condition (*F*), respectively ($p < 0.05$). *C*, Densitometric data from four experiments showing the effect of 100 nM cortisol (*F*) and insulin on SP-C mRNA levels. Data represent mean ± SEM. *Asterisks* represent a significant difference from the control condition (*con*) ($p < 0.05$).

a dose-dependent fashion with maximal levels of induction of 3–4-fold and 20–30-fold, respectively (22). This pattern of regulation is similar to our observations.

The effects of insulin and cortisol, each added alone, on SP mRNA levels in human fetal lung explants are dose-dependent and quite different (16) (Figs. 1 and 2). However, in combination, the effects of the hormones were opposite to what might have been anticipated based on the effects of either hormone studied individually. The most striking example of this unexpected response was the effect of 100 nM cortisol in combination with insulin on SP-A mRNA levels (Figs. 5*A* and 6*A*). Cortisol at 100 nM, added alone, decreased SP-A mRNA levels by greater than 50%. Insulin (400 and 40 pmol/L × 10^{-3}) has previously been shown to also decrease SP-A mRNA levels (by approximately 73 and 62%, respectively) (16). Therefore, the observation that the combination of these two hormones, at inhibitory concentrations, resulted in an increase of SP-A mRNA levels was surprising. To understand the potential mechanisms by which this interactive effect is achieved, studies concerning the molecular mechanism involved, *i.e.* an assessment of the effects of the hormone combinations on SP gene transcription and mRNA stability, will be required. Also unexpected was the observation that 1 nM cortisol, which when added alone had a slightly stimulatory effect on SP mRNA levels, appeared to alter the effects of insulin on SP-B and SP-C mRNA levels. In previous studies we have shown that insulin, added alone, inhibits SP-A mRNA levels in a dose dependent manner (16). The addition of 1 nM cortisol had no effect on the insulin-mediated inhibition of SP-A mRNA. In contrast, whereas insulin added alone inhibited SP-B mRNA levels by approximately 25% at the highest concentration tested (400 pmol/L × 10^{-3}), it had no effect on SP-C mRNA levels. In the presence of 1 nM cortisol, however, SP-B mRNA levels were decreased by 50 and 75% at the 40 and 400 pmol/L × 10^{-3} insulin concentration. Likewise, whereas insulin had no effect on SP-C mRNA levels when added alone, in the presence of 1 nM cortisol, levels of SP-C mRNA were depressed 90% when explants were exposed to 400 pmol/L × 10^{-3} insulin.

Another experimental model in which the effects of insulin on gene expression have been studied extensively is the expression of mRNA for PEPCK in rat liver epithelial cells (33, 34). Insulin decreases PEPCK mRNA levels, whereas glucocorticoids increase PEPCK mRNA levels in rat liver. In contrast to the interactive effects of insulin and glucocorticoids on SP mRNA levels, it has been shown that the inhibitory effects of insulin predominate over the stimulatory effects of glucocorticoids on PEPCK mRNA levels in rat liver epithelial cells (35). The combined effect of the hormones is mediated primarily at the level of PEPCK gene transcription (34).

Increasing evidence suggests that the mechanism of glucocorticoid regulation of the SP genes is different for each of the SP. The biphasic effects of glucocorticoids on SP-A mRNA

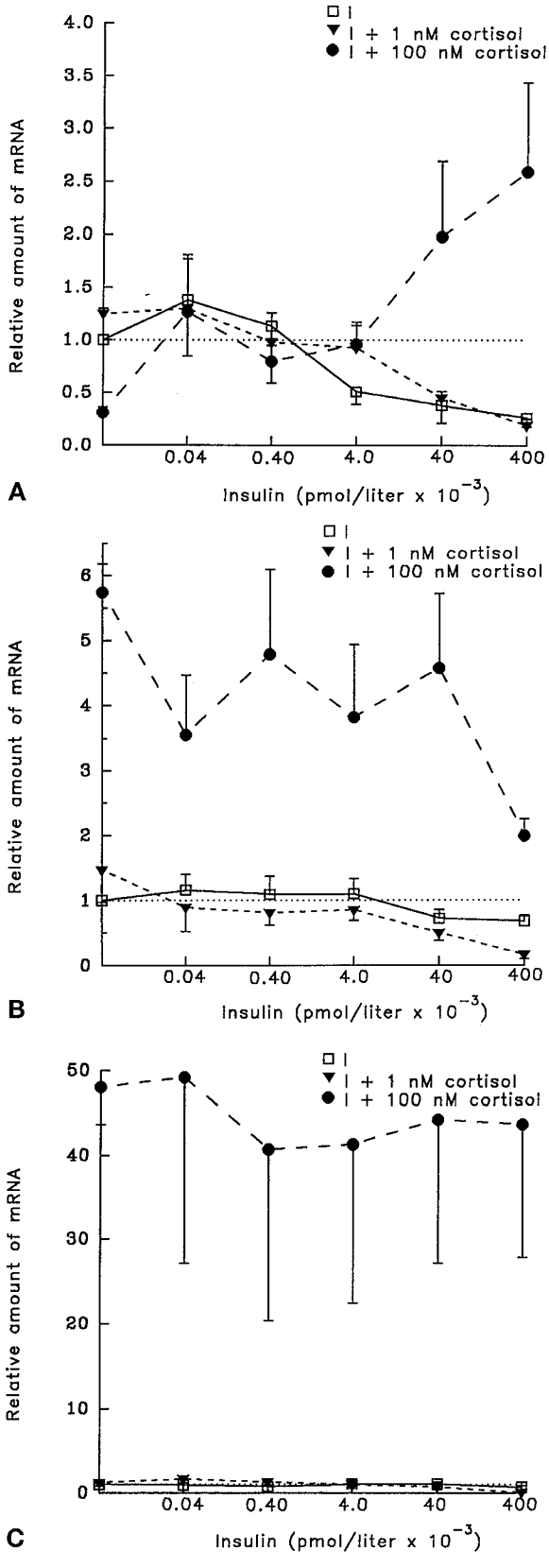

Figure 7. *A*, Graphic representation illustrating the effects of insulin with or without added cortisol (1 and 100 nM) on SP-A mRNA levels. (Insulin alone data for *A–C* are adapted from Ref. 16.) *B*, The effect of insulin with or without added cortisol (1 and 100 nM) on SP-B mRNA levels. *C*, The effect of insulin with or without added cortisol (1 and 100 nM) on SP-C mRNA levels.

levels are probably the result of differential actions of glucocorticoids on SP-A mRNA transcription and stability (36). Glucocorticoid induction of SP-B in human fetal lung has been shown to be the result of an increase in both transcription rate and mRNA stability as recently reported by Venkatesh *et al.* (37). In contrast, the induction of SP-C mRNA by glucocorticoids is the result of an increased transcription rate without a change in mRNA stability, and, unlike the glucocorticoid effects on SP-A and SP-B, this increase in the rate of transcription is dependent upon ongoing protein synthesis (37).

The effects of insulin on mRNA levels for the SP in human fetal lung explants have previously been described (16). However, the molecular mechanisms involved in insulin regulation of the SP genes are not yet understood. From investigations in other systems, regulation of gene expression by insulin may occur at the level of transcription, posttranscription, or translation (38). The two genes that encode human SP-A are approximately 5 kb in length and are comprised of seven exons and six introns (39, 40). We did not assess the differential effects of insulin on the two human SP-A genes in the present study. A consensus sequence for glucocorticoid receptor binding has been identified in the 5′-flanking region of both human SP-A genes (25, 41). Review of SP-A gene structure also reveals a sequence with high homology to insulin regulatory elements that have been described in other insulin responsive genes (34). Both the glucocorticoid and the proposed insulin regulatory elements are in close proximity to each other and within 300 bp of the site of transcription initiation. Their adjacent location suggests the possibility of interaction of the binding of respective regulatory proteins. The 5′-flanking region of the SP-B gene also includes several potential GREs as well as a sequence with similarity to the proposed insulin regulatory element (42). Review of the SP-C genes, however, does not reveal any potential glucocorticoid or insulin regulatory sequences (43).

Insulin and glucocorticoid actions are initiated after binding to specific receptors, and the degree of response to either hormone is influenced by the concentration and affinity of these receptors. The insulin receptor appears early in fetal life, and although the specific pattern varies depending upon the tissue, the concentration of insulin receptors generally increases with gestation (44). Insulin receptors have been described in fetal rat and rabbit lung tissue and in isolated fetal rabbit type II cells (45–47). Glucocorticoids have been observed to modulate the normal development of the fetal lung insulin receptor (48). In fetal rabbit lung, betamethasone increased insulin receptor numbers by 250% without altering their affinity (48).

Human fetal lung explants have been used by several investigators to study various aspects of lung development. Because the accelerated differentiation of the human fetal lung tissue that has been observed in culture occurs in the absence of serum, the effect of individual hormones and regulatory factors on fetal lung development and alveolar type II cell differentiation can be studied *in vitro*. The concentrations of cortisol added to the cultures represent physiologic levels in the sense that our added concentrations encompass ranges that have been described for normal newborn infants and infants whose mothers received antenatal steroids (31, 49–51). In this system, the

addition of cortisol modulated the effect of insulin on SP mRNA levels in a dose-dependent and dramatic fashion (Fig. 7). However, we do not know if our culture conditions correspond to the environment present in the developing fetus which is further complicated by the presence of numerous additional regulatory factors.

Lung development is a product of multifactorial regulation. In our experiments we have examined SP gene expression in an *in vitro* model. The combination of insulin and cortisol had dose-dependent and, at times, unpredictable effects on mRNA levels for all of the SP. Unresolved questions include whether these changes in mRNA levels are reflected in changes in SP content, the mechanisms by which insulin, and insulin in combination with glucocorticoids, regulate SP gene expression, and if these hormonal interactions are similar to those that occur *in vivo*. The unpredictability of the responses we observed in this study reminds us of the complexity of the developing fetal lung and may help us understand the success or failure of our attempts to influence lung maturation.

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