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Modeling Glucocorticoid Mediated Fetal Lung Maturation: II. Temporal Patterns of Gene Expression in Fetal Rat Lung

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ABBREVIATIONS**:** DEX, dexamethasone; PK/PD, pharmacokinetic/pharmacodynamic; GR, glucocorticoid receptor; RT-PCR, reverse transcription polymerase chain reaction; SR, steroid receptor complex; CRH, corticotrophin releasing hormone

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ABSTRACT

Our previous report describes the temporal steroid patterns during pharmacokinetic (PK) studies with dexamethasone (DEX) where doses of six 1 umol/kg injections were given during gestational ages 18-20 days in rats. DEX PK was used in conjunction with the endogenous corticosterone profile to understand the regulation of fetal lung pharmacodynamics (PD). Expression of the glucocorticoid receptor (GR) and surfactant proteins A and B mRNA were chosen as lung maturational markers. GR appeared to be insensitive to the circulating glucocorticoids indicating that unlike the adult situation, GR was not under negative feedback control of its ligand. Surfactant protein B exhibited ~ 400-fold induction in control fetal lung during the last days of gestation and the inductive effect was even greater in the treatment group. Surfactant protein A displayed ~ 100-fold induction in control fetal lung during late gestation. However, the treatment group exhibited biphasic stimulatory and inhibitory effects for surfactant protein A. The inhibitory effect indicated that the chosen dosing scheme for DEX was not an optimal regimen. These data were used to determine by simulation the DEX regimen that would reproduce the temporal pattern of lung maturation observed in control animals. PK/PD modeling indicated that maintaining steroid exposure at approximately twice the equilibrium dissociation constant for the steroid/receptor interaction should produce optimal stimulation of both surfactant proteins. The simulations illustrate that administering smaller quantities of steroids over extended periods of time that produce sustained steroid exposure might be the optimal approach for designing dose-sparing antenatal corticosteroid therapy.

INTRODUCTION

Glucocorticoids accelerate fetal lung maturation in almost every animals species studied (Ballard, 1986). Glucocorticoids are clearly involved in normal fetal lung maturation because knockout animals for either GR or the glucocorticoid precursor corticotrophin releasing hormone (CRH) die at birth due to lack of lung development (Muglia et al., 1995; Cole et al., 1995). The importance of the steroid/receptor interaction is exemplified by the fact that the CRH knockouts are rescued by glucocorticoid treatment, while the GR knockouts are not.

Glucocorticoids induce lung maturation by stimulating the production of pulmonary surfactant (Kotas and Avery, 1971). Lung surfactant, composed of glycerophospholipids and proteins, helps to reduce the surface tension at the alveolar air-liquid interface. Enzymes involved in glycerophospholipid synthesis as well as surfactant proteins are under the transcriptional control of glucocorticoid-bound receptor molecules. Evidence supporting GR-gene mediated process regulating fetal lung maturation has been reviewed by Ballard (1995).

In spite of the rich literature concerning glucocorticoid induced fetal lung maturation, most studies have been performed using isolated cells and explant cultures of fetal lung. Only sparse information is available on glucocorticoid induced surfactant production after in vivo treatment. The few studies (Phelps and Floros, 1991; Schellhase and Shannon, 1991) that have examined in vivo effects involve administration of one or multiple DEX doses and a single time point analysis at 24 hr after the last DEX dose. Such a study design has somewhat limited value given the fact that corticosteroids produce markedly diverse multi-phasic temporal patterns of gene expression (Jin et al.,

2003). The objective of this work was to develop models that quantitatively describe the in vivo mechanism behind glucocorticoid mediated fetal lung maturation. Understanding the system in a quantitative manner provides the unique benefit of using mathematical modeling to design optimal dosing regimens for antenatal corticosteroids. These models describe fetal lung maturation being initiated by the free plasma concentration of exogenous and endogenous steroids in fetal plasma (for detailed plasma glucocorticoid temporal patterns, see Samtani et al., 2005, companion paper).

The markers selected for fetal lung maturation and the rationale for their choice are as follows: a) Appreciable data indicates that the major factor governing steroid responsiveness is the cytosolic GR (DuBois et al., 1995) and hence expression of this cellular marker was followed. b) Surfactant proteins are of two main types (hydrophobic and hydrophilic) and we chose the hydrophobic protein B as our second marker. Surfactant protein B represents the most critical component of lung surfactant since it imparts spreading capability to glycerophospholipids that produce a surface active film on the alveolar surface. Furthermore, normal surfactant biosynthesis, storage, and secretion depend on surfactant protein B (Weaver and Conkright, 2001). c) The hydrophilic protein of choice was surfactant protein A since it is the most abundant of all the surfactant proteins. This marker has the distinct quality that its ontogeny reflects the development of glycerophospholipids that primarily make up lung surfactant (Alcorn et al., 2004). It also plays the crucial role of acting as the first line of defense against inhaled microbes and pathogens and therefore serves as indicator of lung immune function (McCormack and Whitsett, 2002). Surfactant protein A also has the advantage that it is exquisitely sensitive to corticosteroid exposure such that excessive exposure destabilizes

its mRNA (Boggaram et al., 1989). In designing optimal regimens this marker therefore helps in deciding the upper safety threshold for steroid exposure.

We chose to follow the message levels of all of these markers. The advantage of studying message levels is that advancement of technologies such as TaqMan® based quantitative reverse transcription-polymerase chain reaction (RT-PCR) allow measurement of extremely low copy numbers for markers of interest. This becomes important when evaluating fetal maturation where expression levels can be extremely low due to the immature state of the developing organ. Furthermore, the biggest advantage of the chosen markers is that the protein levels of these markers as a function of gestation in control fetal rat lung are available in the literature (Ballard et al., 1984; Schellhase et al., 1989; Shimizu et al., 1991). By establishing a mathematical relationship between the measured message and protein data from the literature in the control group, the behavior of the marker proteins in the DEX treated animals can be predicted. PK/PD modeling will be used in designing an optimal steroid regimen based on the in-depth biomarker profiles for glucocorticoid induced fetal lung maturation reported here.

Materials and Methods

mRNA Measurement

RNA Preparation. Fetal lung was obtained from 54 control and DEX treated pregnant rats described in the companion paper. Fetal lungs from fetuses belonging to each litter were pooled. Fetal lung was ground into powder using liquid nitrogen chilled pestles and mortars. Extraction of total RNA was carried out using Trizol (Invitrogen Corp, Carlsbad, CA) according to the manufacturer instructions. An external standard psuedomessage (GRG-1 cRNA) was added to each sample prior to homogenization of tissue in Trizol to allow correction for variable extraction yield (DuBois et al., 1993). GRG-1 was chosen as the external standard because it was originally cloned from Neurospora and did not share homology with any mammalian gene (McNally and Free, 1988). The extracted total RNA was resuspended in nuclease free water (Ambion, Austin, TX) and stored at -80°C. To assess the purity and integrity of total RNA, the ratio of 260/280 nm absorbance was computed and electrophoresis on formaldehyde agarose gels was performed. Finally, total RNA concentrations were determined using absorbance readings at 260 nm.

Preparation of cRNA Standards. The construction of GR and GRG-1 cRNAs has been described previously (DuBois et al., 1993; DuBois et al., 1995). Rat surfactant protein A and B cDNA clones were a generous gift from Dr. JH Fisher and have been described previously (Sano et al., 1987; Emrie et al., 1989). Surfactant protein A cDNA was subcloned into the EcoR1 site of the pGEM-3Z (Promega, Madison, WI) vector, linearized with HindIII restriction enzyme, and transcribed in vitro with T7 polymerase (MEGAScript^{TM} T7 Polymerase kit, Ambion Inc.) to synthesize cRNA standards. The concentration of the cRNA stock was determined by absorbance at 260 nm. Purity and

integrity of the RNA was confirmed by 260/280 nm absorbance and electrophoresis in 5% acrylamide/8M urea gels. Working dilutions were prepared from the cRNA stock. Surfactant protein B cDNA provided by Dr. Fisher was extremely dilute and was therefore first amplified using PCR. The PCR product was cloned using TOPO™ TA cloning (Invitrogen Corp, Carlsbad, CA) according to manufacturer instructions. Thereafter, the procedure for cRNA standard generation was identical to that for surfactant protein A.

Absolute Quantification of mRNA using Real Time Quantitative RT-PCR. Assays for all messages of interest (GR, surfactant protein A and B, and GRG-1) in extracted lung samples were developed. These assays utilized the in vitro synthesized cRNA as standards. The kinetic based RT-PCR methodology made use of the Stratagene MX4000 fluorescence based thermal cycler and TaqMan® probe technology. TaqMan® probes and primers were designed using PrimerExpress software (Applied Biosystems, Foster City, CA) under the condition that sequences sharing homology with other genes were excluded. Primers/probes were custom synthesized by Biosearch Technologies, Inc. (Novato, CA). The probes were synthesized with the fluorescent reporter (FAM or HEX) attached to the 5′-end and the quencher BHQ attached to the 3′-end. Forward and reverse primer design allowed positioning of the two oligonucleotides as close to one another without overlapping the probe. The amplicons that were generated were between 76 and 107 base-pairs. The assays employed a one tube/two enzyme Brilliant® 1-Step Quantitative RT-PCR Core Reagent Kit (Stratagene, La Jolla, CA) that was used according to manufacturer instructions. The concentrations of the primers/probes and magnesium chloride were optimized and the reaction conditions along with the

oligonucleotide sequences are reported in Table 1. The cRNA standards were run in duplicate concurrently on the same plate with unknown samples which were run in triplicate. The RT minus controls to test for possible genomic DNA contamination in extracted RNA were run for each sample and in all cases gave no measurable amplification signal. The construction of the standard curves allowed estimation of moles of mRNA rather than relative message expression. Fractional yields were calculated based on the recovered GRG-1 cRNA relative to the quantity added prior to tissue homogenization. This allowed molar quantification of mRNA per gm of fetal lung tissue. As a time-saving measure the RT-PCR multiplex assay for GR and GRG-1 message was run in a single tube without reduction in sensitivity. The intra- and inter-assay coefficients of variation for all transcripts of interest were under 18%.

Additional Data Sources

Total GR concentrations in fetal rat lung were obtained from Ballard et al. (1984). GR data were reported as fmol/mg DNA, which were converted to fmol/mg protein using the protein/DNA ratio of 7.2 reported by the same authors. Ontogeny of surfactant protein A in male and female fetal rat lung has been published by Schellhase et al. (1989). Finally, Shimizu et al. (1991) have reported developmental patterns of surfactant protein A and B during normal ontogenic stages of rat lungs. The three datasets for surfactant protein A are in excellent agreement and therefore all the information was used during the modeling exercise. Data were captured by computer digitalization (Sigma Scan, Jandel Scientific, Corte Madera, CA).

PK/PD Model

Pharmacokinetics. The equations and parameters describing unbound DEX and corticosterone plasma concentrations in control and DEX-treated fetuses were from the companion report.

Mechanistic Basis for Pharmacodynamics. Free glucocorticoids in fetal plasma can rapidly equilibrate with intracellular steroid concentrations because their lipophilicity allows ready passage across cellular membranes. Steroids interact with the cytosolic GR based on their relative receptor affinity. Binding of the steroid to its receptor produces an activated complex, which can transcriptionally induce expression of surfactant components and related enzymes (Ballard and Ballard, 1995). For surfactant proteins A and B, induction of mRNA synthesis occurs via a mechanism that is consistent with a receptor-mediated process (Liley et al., 1988; Liley et al., 1989). In addition, higher concentrations of corticosteroids in vitro reduce surfactant protein A mRNA stability leading to increased degradation (Boggaram et al., 1989). This paradoxical effect also occurs via a receptor-mediated process (Boggaram et al., 1991), producing an inhibitory effect on surfactant protein A during steroid over-exposure in cell culture systems. Finally, the altered message expression translates into corresponding changes in protein concentration, which prepares the fetal lung for life outside the womb. Thus the steroid-induced lung maturation primarily occurs via transcriptional regulation at the message level (Liley et al., 1988; Ballard et al., 1996). Most interestingly, GR in the fetus (unlike the adult situation) is not under feedback transcriptional regulation by its own ligand (Kalinyak et al., 1989; Ghosh et al., 2000). Thus heightened exposure to glucocorticoids

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is not associated with down-regulation of its own receptors, which serves as a mechanism of amplifying glucocorticoid effects during fetal development.

GR Dynamics. The cellular mechanisms of glucocorticoid effects in the fetal lung are portrayed in Fig. 1 and 2 for mRNA and surfactant proteins A and B. GR binding component is identical for both markers, since the steroid bound receptor complex drives the PD effects. It will be shown later that the message levels for GR were identical in control and treatment groups, which agrees well with the general notion that GR is not under transcriptional feedback regulation. Furthermore, GR exhibited a peculiar temporal pattern where the message in the fetal lung behaved like a short infusion. This influx of GR message was followed by appearance of GR after a modest translational delay. The delay in the appearance of the receptor can be recognized visually by comparing the receptor message data from the current study vs. the GR profile reported by Ballard et al. (1984). Thus GR information in treatment and control groups were described by:

$$\frac{dmRNA_R}{dt} = k_{s,\,Rm(0-T)} - k_{d,\,Rm} \bullet mRNA_R, \qquad mRNA_R(0) = mRNA_{R0} \qquad (1)$$

$$\frac{dR_{Total}}{dt} = \frac{1}{\tau_r} \bullet (mRNA_R{}^{\gamma r} - R_{Total}), \qquad R_{Total}(0) = 0 \qquad (2)$$

Symbols include receptor message ($mRNA_R$) and total protein (R_{Total}) as a function of time (t), $k_{s,Rm}$ represents the zero-order input of receptor message defined as $k_{s,Rm} = k_{s,Rm}$ when $0<t\leq T$; else $k_{s,Rm} = 0$, $k_{d,Rm}$ is the first-order rate constant for receptor mRNA degradation, and $mRNA_{R0}$ is the average receptor message observed on gestational day 17 defined as time zero. The production and loss of total receptor was described as being dependant upon a first-order rate constant that is equivalent to the reciprocal of the transit time τ_r and γr is the amplification factor indicating that, on average, a single mRNA

transcript can be used to translate multiple copies of GR. Finally, the baseline value of receptor ($R0_{Total}$) was incorporated by setting the measured total receptor equal to $R0_{Total} + R_{Total}$ (Mager and Jusko, 2001).

The equilibration of steroids across cellular barriers and their binding to cytosolic GR was assumed to be an instantaneous process. The concentration of steroid-receptor complex (SR) in control (Con) and DEX treated groups is given by:

$$SR_{Con} = \frac{R_{Total} \bullet C_{free,Con}}{K_{d,Cort} + C_{free,Con}} \quad (3)$$

$$SR_{Dex} = \frac{\frac{R_{Total} \bullet C_{free,DEX}}{K_{d,Cort}}}{1 + \frac{C_{free,DEX}}{K_{d,Cort}} + \frac{D_{f,free}}{K_{d,Dex}}} + \frac{\frac{R_{Total} \bullet D_{f,free}}{K_{d,Dex}}}{1 + \frac{C_{free,DEX}}{K_{d,Cort}} + \frac{D_{f,free}}{K_{d,Dex}}} \quad (4)$$

where $C_{free,Con}$, $C_{free,DEX}$, and $D_{f,free}$ are the concentrations of unbound corticosterone (C) and DEX (D) in fetal plasma from the companion report. Equation 4 is the well-known Gaddum equation (Gaddum, 1937) that describes two ligands competing for binding to their receptor. The first component of Eq. 4 computes the portion of the SR containing corticosterone as the ligand and the second component expresses the quantity of SR containing DEX. The $K_{d,Dex}$ and $K_{d,Cort}$ represent the equilibrium dissociation constants for the two steroids. The $K_{d,Dex}$ for fetal rat lung GR has been published and $K_{d,Cort}$ can be calculated from its relative receptor affinity (Ballard, 1986). Thus, the values for $K_{d,Dex}$ and $K_{d,Cort}$ were fixed to 4.7 and 22.1 nM during the modeling procedure. The equations for SR will be used to drive PD under the assumptions that post receptor events are steroid independent and differences between corticosteroids can be explained by their relative receptor affinity and plasma temporal patterns. We have recently shown that these assumptions are suitable by applying quantitative structure-property relationship

theory to corticosteroid genomic effects (Mager et al., 2003). Once the parameter estimates were obtained for GR dynamics, they were fixed in the following analysis.

Biphasic Effect on Surfactant Protein A mRNA. Corticosteroids can affect surfactant protein A mRNA by dual mechanisms. The following equations were jointly fitted to describe the simultaneous action of SR on mRNA synthesis and degradation in control and treatment groups:

$$\frac{dmRNA_{A,Con}}{dt} = k_{s,A} \bullet (1 + \frac{Smax_{As} \bullet SR_{Con}^{\gamma As}}{SC_{50,As}^{\gamma As} + SR_{Con}^{\gamma As}}) - k_{d,A} \bullet (1 + \frac{Smax_{Ad} \bullet SR_{Con}^{\gamma Ad}}{SC_{50,Ad}^{\gamma Ad} + SR_{Con}^{\gamma Ad}}) \bullet mRNA_{A,Con} \quad (5)$$

$$\frac{dmRNA_{A,Dex}}{dt} = k_{s,A} \bullet (1 + \frac{Smax_{As} \bullet SR_{Dex}^{\gamma As}}{SC_{50,As}^{\gamma As} + SR_{Dex}^{\gamma As}}) - k_{d,A} \bullet (1 + \frac{Smax_{Ad} \bullet SR_{Dex}^{\gamma Ad}}{SC_{50,Ad}^{\gamma Ad} + SR_{Dex}^{\gamma Ad}}) \bullet mRNA_{A,Dex} \quad (6)$$

Surfactant protein A message ($mRNA_A$) is synthesized in a zero-order process ($k_{s,A}$) and degraded by a first-order process ($k_{d,A}$). $Smax_{As}$ and $SC_{50,As}$ represent the maximum possible stimulation of $k_{s,A}$ and the concentration of SR required for half-maximal stimulation. $Smax_{Ad}$ and $SC_{50,Ad}$ are analogous parameters affecting $k_{d,A}$. The Hill coefficients γAs and γAd adjust for the degree of sigmoidicity in the PD profiles. Stationary baseline was assumed at time zero and the following rate constant was derived from Eq. 5:

$$k_{d,A} = \frac{k_{s,A} \bullet (1 + \frac{Smax_{As} \bullet SR_0^{\gamma As}}{SC_{50,As}^{\gamma As} + SR_0^{\gamma As}})}{mRNA_A^0 \bullet (1 + \frac{Smax_{Ad} \bullet SR_0^{\gamma Ad}}{SC_{50,Ad}^{\gamma Ad} + SR_0^{\gamma Ad}})} \quad (7)$$

The baseline initial condition values of surfactant protein A message ($mRNA_A^0$) for Eq. 5 and 6 were fixed as the mean value from control animals on gestational day 17 at which time the steroid receptor complex (SR_0) contained only corticosterone as the binding

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ligand. Eq. 7 reduces one parameter during estimation. Once the parameter estimates were obtained for the message dynamics, they were fixed in the following analysis.

Surfactant Protein A Dynamics. The protein was translated from its mRNA and its production and loss was described as being dependant upon a first-order rate constant that is equivalent to the reciprocal of the transit time τ_A. The amplification factor γAp indicates that, on average, a single mRNA transcript can be used to translate multiple copies of the protein. The equations used to describe protein synthesis and degradation in control and DEX groups were:

$$\frac{dSP\text{-}A_{Con}}{dt} = \frac{1}{\tau_A} \bullet (mRNA_{A,Con}{}^{\gamma Ap} - SP\text{-}A_{Con}), \qquad SP\text{-}A_{Con}(0) = 0 \qquad (8)$$

$$\frac{dSP\text{-}A_{Dex}}{dt} = \frac{1}{\tau_A} \bullet (mRNA_{A,Dex}{}^{\gamma Ap} - SP\text{-}A_{Dex}), \qquad SP\text{-}A_{Dex}(0) = 0 \qquad (9)$$

On gestational day 17 surfactant protein A was not detectable and hence the initial conditions were set at 0.

Induction of Surfactant Protein B mRNA. The following equations were jointly fitted to describe the inductive effect of SR on mRNA synthesis in control and DEX groups:

$$\frac{dmRNA_{B,Con}}{dt} = k_{s,B} \bullet (1 + \frac{Smax_B \bullet SR_{Con}{}^{\gamma B}}{SC_{50,B}{}^{\gamma B} + SR_{Con}{}^{\gamma B}}) - k_{d,B} \bullet mRNA_{B,Con} \qquad (10)$$

$$\frac{dmRNA_{B,Dex}}{dt} = k_{s,B} \bullet (1 + \frac{Smax_B \bullet SR_{Dex}{}^{\gamma B}}{SC_{50,B}{}^{\gamma B} + SR_{Dex}{}^{\gamma B}}) - k_{d,B} \bullet mRNA_{B,Dex} \qquad (11)$$

Surfactant protein B message ($mRNA_B$) is synthesized through a zero-order process ($k_{s,B}$) and degraded by a first-order process ($k_{d,B}$). $Smax_B$ and $SC_{50,B}$ represent the

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maximum possible stimulation of $k_{s,B}$ and the concentration of SR required for half-maximal stimulation. The Hill coefficient γB adjusts for the degree of sigmoidicity in the PD profiles. Stationary baseline was assumed at time zero and the following rate constant was derived from Eq. 10, which reduces one parameter during the estimation procedure:

$$k_{d,B} = \frac{k_{s,B} \bullet (1 + \frac{Smax_B \bullet SR_0^{\gamma B}}{SC_{50,B}^{\gamma B} + SR_0^{\gamma B}})}{mRNA_B^0} \qquad (12)$$

The baseline initial conditions ($mRNA_B^0$) for Eq. 10 and 11 were fixed as the mean value from control animals on gestational day 17. Once the parameter estimates were obtained for the message dynamics, they were fixed in the following analysis.

Surfactant Protein B Dynamics. The protein was translated from its mRNA and its production and loss was described as being dependant upon a first-order rate constant that is equivalent to the reciprocal of the transit time τ_B. The amplification factor γBp indicates that, on average, a single mRNA transcript can be used to translate multiple copies of the protein. The equations used to describe protein synthesis and degradation in control and DEX groups were:

$$\frac{dSP\text{-}B_{Con}}{dt} = \frac{1}{\tau_B} \bullet (mRNA_{B,Con}{}^{\gamma Bp} - SP\text{-}B_{Con}), \qquad SP\text{-}B_{Con}(0) = 0 \qquad (13)$$

$$\frac{dSP\text{-}B_{Dex}}{dt} = \frac{1}{\tau_B} \bullet (mRNA_{B,Dex}{}^{\gamma Bp} - SP\text{-}B_{Dex}), \qquad SP\text{-}B_{Dex}(0) = 0 \qquad (14)$$

On gestational day 17 surfactant protein B was not detected and hence the initial conditions were set at 0.

Data Analysis. Data from multiple animals were pooled and the ADAPT II program (D'Argenio and Schumitzky, 1997) with the maximum likelihood method was applied for all fittings. The following variance model was used:

$$\text{Variance} = \text{Coefficient}^2 \bullet Y(t)^{\text{Power}} \quad (15)$$

where Coefficient and Power are variance parameters, and Y(t) represents the model output function. The goodness-of-fit was assessed using correlation coefficients, examination of residuals, visual inspection of the fitted curves, estimator criterion value, sum of squared residuals, and coefficients of variation of the estimated parameters.

Simulation Study

The PK/PD equations described in this and the companion report were used to design an infusion regimen for DEX that would cause stimulation of surfactant proteins A and B without affecting the stability of surfactant protein A mRNA. The study aims to find an optimal dosing regimen which is defined as the least possible DEX dose that would reproduce the endogenous prenatal steroid exposure and up-regulation of fetal lung maturational markers precociously.

Results

Temporal Patterns of Free Steroids in Plasma. Free concentrations of steroids that have the ability to gain access to the intracellular targets are depicted in Fig. 3. The injection of DEX caused suppression of corticosterone secretion via a negative feedback mechanism. In the treatment group the endogenous and exogenous steroids initiate the PD processes while free corticosterone is the active species in the control group. The degree of inter-individual variability was relatively low and hence the average fitted free steroid profiles were allowed to drive the lung maturational effects.

GR Dynamics. The temporal pattern of GR mRNA in control and treatment groups is shown in Fig. 4. The message levels in control fetal lung start to rise in concert with the rising free corticosterone in fetal plasma. The increasing message concentrations are striking because in adult animals GR exists under negative transcriptional regulation (Sun et al., 1998). Thus, rising steroid concentrations in adult animals causes suppression of its own receptor (Sun et al., 1998). In contrast, the fetus does not exhibit suppression of GR message in a high corticosteroid milieu. The injection of an even more potent steroid DEX does not inhibit the rising GR message, which further confirms the notion of a lack of auto-regulatory feedback in the fetus. However, at 72 hours there appeared to be a clustering of mRNA values below the controls for DEX treated animals. The mean (± standard deviation) concentrations of GR mRNA in the control and treatment groups were 7.8±0.5 and 5.7±0.7 fmol/gm. It would be very difficult to explain mechanistically (and to capture mathematically) why the message data would overlay over one another in the control and treatment groups except at this one time point. It is therefore likely that this difference in GR mRNA at 72 hours between DEX treated and control animals is a

reflection of inter-litter variability rather than a true difference. Since majority of the GR mRNA data did not exhibit a difference between control and treatment animals, both profiles were captured using identical equations representing a brief influx of GR message. The temporal pattern of the message was followed by a similar temporal pattern for GR, albeit with a moderate translational delay (Fig. 5). The delayed GR profile was assumed to be similar between control and treatment groups because the message profiles did not display any difference between the two groups. The delay in the GR profile was captured using a transit compartment transduction approach (Mager and Jusko, 2001). Thus, although the total concentration of receptor sites in the fetal lungs of treated animals was not measured, these concentrations could be easily computed based on GR message data. Parameters describing GR dynamics are presented in Table 2.

Surfactant Protein A. Message levels for this protein increased ~ 100 fold during the last days of gestation in control fetal rat lung, which was followed by increased protein synthesis (Fig. 6). In the treatment group the message levels began to rise earlier than the control group. However, the initial increase was followed by an inhibitory phase (Fig. 6). The concentrations of surfactant protein A in the DEX group therefore did not reach the same high plateau as was seen in the control group. Thus, the chosen regimen of six 1 umol/kg doses of DEX cannot be considered as an optimal regimen. The parameters describing surfactant protein A dynamics are reported in Table 3.

Surfactant Protein B. Message levels of this protein increased ~ 400 fold during the final days of gestation in control fetal rat lung (Fig. 7). This increase was followed by an increase in protein levels for this critical lung maturational marker. Fetal lungs from DEX treated animals exhibited an even higher plateauing of message levels (Fig. 7). The

message not only reached a higher plateau, but also began to rise earlier as compared to the control group. Thus, surfactant protein B exhibited the optimal properties desired from an antenatal steroid regimen. This marker exhibited a monophasic stimulatory pattern such that higher steroid exposure produced a higher response without any inhibitory effects. The control group data indicated that increased message levels translate into correspondingly high protein levels. Thus the treatment group would be expected to exhibit heightened protein levels because of higher message expression observed in this group (Fig. 7). The parameters estimated for surfactant protein B dynamics are shown in Table 4.

The Driving Force Behind PD Effects. To understand the basis behind the varied effects of corticosteroids on the two surfactant proteins, the driving force for these effects was simulated using Eq. 3 and 4. Fig. 8 shows the profiles for the steroid receptor complex in the two groups and compares them to the various SC_{50} values obtained from curve fitting of the surfactant mRNA data. In the control group the concentrations for the complex exceed the two lower SC_{50} values needed for inducing mRNA synthesis. Maintenance of the free plasma corticosterone concentrations by the endogenous system at approximately twice the $K_{d,Cort}$ value of 22 nM (Fig. 3) allowed occupation of 2/3rd of the available receptors in the fetal lung. The $SR_{Control}$ concentrations never approached the higher degradation enhancing SC_{50} value, thus allowing the endogenous biology to selectively induce both surfactant proteins without any inhibitory effects. Thus controlled and sustained exposure to the endogenous steroid was the key factor that produced an inductive effect on both surfactant proteins. In contrast to the control group, SR was predominantly occupied by the exogenous steroid in the treatment group (Fig. 8). The

concentrations of SR exceeded the two lower stimulatory SC_{50} values after each intramuscular injection of DEX. However, the degradation enhancing SC_{50} was also exceeded during the third to sixth DEX doses. This excessive exposure to corticosteroids therefore produced a predominant inhibitory effect on surfactant protein A in the treatment group.

Simulation Study. The predominant inhibitory effect observed in the treatment group for surfactant protein A demonstrated that six 1 μmol/kg doses were not optimal for inducing fetal lung maturation. The gathered information was therefore applied to a simulation study to ascertain an optimal dosing regimen. It was soon recognized that given the narrow window available for SR (Fig. 8) and the relatively short DEX half-life of 3 hr, intramuscular injection may not serve as the most optimal dosing route. Steroid administration would be required too frequently (every 4-6 hr) for three days to achieve the targeted window displayed in Fig. 8. From a study design point of view, such a dosing regimen would be highly impractical. We therefore performed a series of simulations that involved zero-order infusion regimens, which can be readily implemented in future studies using osmotic pumps or slow release pellets. The PK parameters obtained from the intramuscular regimen are directly applicable to an infusion regimen because intramuscular input produces complete bioavailability for DEX (Samtani and Jusko, 2005). The infusion regimen that met the requirements of an optimal DEX regimen was 31 nmol/kg/hr (12 ug/kg/hr) on gestational days 18-21. The temporal patterns of the steroids in the fetal circulation arising from this steroid regimen are presented in Fig. 9. Interestingly, the DEX steady-state concentration achieved during this simulation (9 nM) is roughly twice the $K_{d,Dex}$ value of 4.7 nM. Thus the system

responds optimally when 2/3rd of the GR sites are occupied regardless of the steroid in circulation. This reflects the assumption that post receptor events are independent of the steroid. Although the concentration of free DEX reached only 9 nM, the simulations indicate that this concentration is still sufficient to cause inhibition of corticosterone secretion. This is not unanticipated because the concentration of DEX required for 50% inhibition of corticosterone secretion is less than 1 nM, indicating that any steroid regimen that targets the $K_{d,Dex}$ value of 4.7 nM will invariably cause adrenosuppression. Thus the hypothalamus-pituitary-adrenal axis is exquisitely sensitive to exogenous steroid exposure leading to negative feedback inhibition even during moderate glucocorticoid exposure. The simulated temporal pattern for the driving force SR is also portrayed in Fig. 9. The SR would primarily contain DEX as the bound ligand because of its higher receptor affinity and low circulating corticosterone. The temporal pattern for SR was designed with the goal of preventing its concentrations from reaching the degradative SC_{50} of 400 nM. This requirement was also met by the 31 nmol/kg/hr dosing regimen.

The simulated PD profiles for the infusion regimen are presented in Fig. 10. For comparison purposes, the ontogeny of the two surfactant proteins in control animals is also provided. The simulated regimen not only produced higher protein concentrations but also hastened the appearance of the two markers. The proposed infusion protocol may therefore reflect a dose sparing regimen that produces precocious lung maturation.

In designing regimens it is also important to consider the cumulative dose of the drug being administered. Two dosing regimens have been described in this report. The administered six doses of 1 umol/kg DEX doses translate into 2250 nmol, while the

simulated three day 31 nmol/kg/hr infusion is equivalent to 826 nmol DEX. This estimate assumes a body weight of 375 gm for pregnant rats, which was the average weight of our 54 animals. The lower cumulative DEX associated with the infusion regimen may therefore allow beneficial effects on lung maturation and permit dose reduction. Thus administering a smaller quantity of the steroid with sustained controlled exposure might be the optimal strategy for dosing corticosteroids antenatally.

Discussion

In vivo effects of glucocorticoids on surfactant proteins in fetal lung have only been looked at in a limited manner. The two main studies (Schellhase and Shannon, 1991; Phelps and Floros, 1991) report single time point data for various lung maturational markers after single or multiple doses of DEX. Most markers were measured using either qualitative techniques or relative values in treated animals compared to controls. The noteworthy feature of this report is that all markers quantified or adapted from the literature represent absolute quantification. The term absolute is not meant to describe current state of knowledge in this area. The term absolute is a description of the methodological procedures adopted as part of this research. Such measurements are amenable to PK/PD modeling and offer the advantage that the knowledge gained can be extended to new situations using simulations.

Literature data suggest that glucocorticoid effects on surfactant protein B are stimulatory and induction occurs at all doses and durations of steroid exposure. Such observations agree with our results. Literature results for in vivo surfactant protein A induction appear to be relatively low, variable, and dependant on the gestational age and duration of exposure. These results have been attributed to the possible biphasic effects of glucocorticoids observed in vitro where low concentrations (10^{-9} nM) produce stimulation while higher concentrations (10^{-7} nM) cause inhibitory effects. Our data confirm that the biphasic effect is also true in vivo and selective induction of surfactant protein A can be achieved under controlled and sustained exposure to glucocorticoids.

Another in vitro phenomenon investigated is the reversibility of surfactant protein and enzyme induction upon removal of corticosteroids from the medium (Ballard and Ballard,

1995; Vidaeff et al., 2004). Our pilot experiments (data not shown) support this reversible induction theory. Use of fewer than six doses of DEX in rats produced transient effects that would start returning to baseline after stopping steroid administration. All of the above findings have important physiologic consequences when considering the two most popular antenatal corticosteroid regimens. The commonly used single course of glucocorticoids (NIH Consensus Panel, 1995) will probably lose its efficacy after a few days due to reversibility of glucocorticoid action. The seminal clinical trial by Liggins and Howie (1972) supports this hypothesis because the study reported a high incidence of respiratory distress in infants born 7-20 days after a single course of glucocorticoids. To prevent this problem, clinicians have adopted another antenatal regimen where women at risk of preterm labor are administered multiple courses of corticosteroids for weeks to months (Newnham, 2001). It is possible that fetuses exposed to repeated doses of corticosteroids may become deficient in surfactant protein A and exhibit adult onset metabolic disorders (Iannuzzi et al., 1993, Newnham, 2001). These possible adverse effects therefore warrant reassessment of optimal antenatal dosing strategies.

Our data from control animals shows that the endogenous biology circumvents the reversibility of inductive effects and selectively induces both surfactant proteins. The innate biology does this by maintaining the concentrations of the free endogenous corticosteroid above a critical threshold which is the K_d value for the steroid/receptor interaction. Excessive endogenous steroid exposure is prevented by maintaining the concentration at ~ twice the threshold which leads to $2/3^{rd}$ occupation of available receptors. Thus, by sustaining the steroid concentrations at a steady plateau, over-exposure to glucocorticoids is prevented, paradoxical inhibitory effects are not permitted,

fetal programming that causes adult onset metabolic disorders is avoided, and most importantly critical lung maturation is achieved in control animals. Based on the simulation results, it is therefore proposed that conservative use of steroids involving small divided doses producing sustained exposure may be more beneficial rather than weekly repeated courses over prolonged periods of time. Rational and conservative use of steroids becomes even more important in light of the finding that fetal GR is not under negative feedback regulation by its own ligand (Fig. 4). Lack of this protective mechanism in the fetus makes the possibility of adverse corticosteroid effects even more likely. This should therefore serve as additional encouragement for investigators to intensity the search for rational and safe antenatal doses of corticosteroids.

The knowledge gained from the pregnant rodent model in understanding glucocorticoid effects on fetal lung during the past few decades has been immense (Brown and Seckl, 2005). However, there are limitations with this animal model. Unlike in humans, rat gestation is extremely short. Glucocorticoid exposure and GR concentrations rise in tandem very late in gestation, which also represents the time frame where rodent fetuses are large enough for experimental manipulation. Precocious lung maturation is therefore difficult to induce in this animal model, which is obvious from Fig. 10, where the extent of early lung maturation is small. GR peaks only after gestational day 20 in the fetal rat lung. Thus, apart from the transcriptional and translational delays, the main factor regulating the slow and late appearance of fetal lung maturation is the availability of GR. Although this animal model is not ideal for producing precocious lung maturation, the study of DEX effects in fetal rat lung almost reflects the clinical situation. This is because DEX administration severely inhibits endogenous corticosterone secretion. Thus the

treatment effects are primarily driven by DEX in a low corticosterone environment. This corticosteroid milieu reflects the clinical situation of steroid administration in preterm labor where lung maturation is primarily driven by the exogenous steroid because the endogenous steroid surge hasn't begun yet.

Finally, it is important to reflect on the multitude of mechanisms that the endogenous biology draws together to ensure efficient lung maturation during the late gestational corticosteroid surge. These represent a combination of changes in the disposition of the endogenous steroid and alterations in the system pharmacology. These mechanisms are supported by the data presented in our two reports and are as follows: a) Increased plasma free fraction for corticosterone is manifested by decreasing corticosteroid binding globulin in plasma, thus providing a bigger pool of steroid for driving PD effects. b) There is increased maternal to fetal corticosterone transfer which probably occurs due to shutting down of placental corticosteroid metabolizing enzymes. This equilibration of steroid concentrations across the placenta leads to increased fetal glucocorticoid exposure. c) The concentration of GR in the fetal lung rises markedly in concert with the plateauing of corticosterone to its critical steady-state value of two times $K_{d,Cort}$. The heightened concentrations of the ligand and GR lead to production of a greater driving force (i.e. SR) which is necessary for mediating fetal lung maturation. d) Unlike the adult situation, GR is not under negative regulatory feedback. This allows GR levels to increase in a high glucocorticoid environment. The mechanisms related to GR, although essential for normal functioning of the endogenous biology, also predispose the fetus to adverse effects in the event of over-exposure to exogenous glucocorticoids. It is therefore essential to use these highly potent steroids in a rational, safe, effective and

conservative fashion to ensure improvement of maternal/fetal health without causing foreseeable adverse effects.

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Footnotes

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Figure legends

Fig. 1: Model for receptor-mediated transcriptional effects of corticosteroids on surfactant protein A. Effects are driven by corticosterone in control animals (top) and by DEX and corticosterone in treated animals (bottom). Dotted arrows and rectangles indicate stimulation of processes via indirect mechanisms. Dashed arrows arising from the mRNAs do not affect their concentrations since protein synthesis from its message is not a degradation route. Processes occurring intracellularly are enclosed within a box.

Fig. 2: Receptor-gene mediated model for surfactant protein B driven by corticosteroids. The notation for different processes is the same as in Fig. 1.

Fig. 3: Model fittings from the companion article depicting fetal free plasma corticosteroid concentrations that are capable of gaining intracellular access and driving PD. The solid line is fetal free corticosterone in control animals. Dashed and dotted lines represent fetal free DEX and corticosterone in treated animals.

Fig. 4: GR mRNA in fetal lung from control (●) and DEX treated (○) animals. Solid line represents model fitting.

Fig. 5: Total GR concentrations in fetal rat lung from Ballard et al. (1984). Solid line is the result of the model fitting.

Fig. 6: Surfactant protein A mRNA and protein concentrations in control (top) and DEX treated (bottom) animals. Dashed lines are the results of the simultaneous fitting of the control and treatment mRNA data. Solid lines are model predictions for protein concentrations.

Fig. 7: Surfactant protein B mRNA and protein concentrations in control (top) and DEX treated (bottom) animals. Dashed lines are the results of the simultaneous fitting of the

control and treatment mRNA data. Solid lines are model predictions for the protein concentrations.

Fig. 8: Profiles for the steroid receptor complex in control and DEX treated animals. Lines are defined in the graph. The lower horizontal lines represent the SC_{50} for stimulating mRNA production of the two surfactant proteins. The upper horizontal line represents the SC_{50} for stimulating the mRNA degradation for surfactant protein A.

Fig. 9: Simulated curves for fetal free corticosterone (dashed line) and DEX (dotted line) during a 31 nmol/kg/hr maternal DEX infusion on gestational days 18 to 21. The solid line represents the simulated profile for the steroid receptor complex in fetal rat lung which will serve as the driving force for PD effects.

Fig. 10: Simulated PD profiles (dashed lines) for surfactant protein A (thick line) and surfactant protein B (thin line) during a 31 nmol/kg/hr maternal DEX infusion on gestational days 18 to 21. For comparison purposes the normal ontogeny for these proteins in control fetal rat lung is also presented (solid lines).

Table 1. Primer/probe sequences and optimized reaction conditions for quantitative RT-PCR

	Oligonucleotide	nM	Sequence 5′-3′	$MgCl_2$ (mM)	dNTP (mM)
Surfactant protein A	Forward primer	250	CCTTTAGAGCAGGAGGCAACA	3	0.8
	Reverse primer	250	AATCATGCCCAAGTAGACATAGTTGT		
	FAM labeled probe	100	CTTCGCAATACTTGCAATGGCCTCGT		
Surfactant protein B	Forward primer	250	GATGACCAAGGAAGACGCTTTC	3	0.8
	Reverse primer	250	CAAGCAGCTTCAAGGGTAGGAT		
	FAM labeled probe	200	TCACATTCTTGTTCCAGGAACTTCCGGA		
Multiplex	Receptor forward primer	300	AACATGTTAGGTGGGCGTCAA	5	1.0
	Receptor reverse primer	300	GGTGTAAGTTTCTCAAGCCTAGTATCG		
	FAM labeled receptor probe	100	TGATTGCAGCAGTGAAATGGGCAAAG		
	GRG-1 forward primer	300	CGGTTCTGGTGTAATGCTAAAGCT		
	GRG-1 reverse primer	300	AGTTCGCCAAGGGCTTCTC		
	HEX labeled GRG-1 probe	100	CCCTTCGAAATTCCAAGCCAAGTATGTCAT		

Table 2. Estimated and fixed parameters for receptor dynamics

Parameter (units)	Definition	Estimate	CV%
$k_{s,Rm}$ (fmol/g lung/h)	Zero-order input of receptor message	0.13	12
Tinf (h)	Duration of receptor message input	40.3	12
$k_{d,Rm}$ (h^{-1})	First-order rate of receptor mRNA degradation	0.0067	26
τ_R (h)	Receptor transit time	71.8	33
γr	Amplification factor for the translation of receptor message to protein	2.98	3
$K_{d,Cort}$ (nM)	Equilibrium dissociation constant for corticosterone	22.1	Fixed
$K_{d,Dex}$ (nM)	Equilibrium dissociation constant for dexamethasone	4.70	Fixed
$mRNA_{R0}$ (fmol/g lung)	Average receptor message observed at time zero (Gestational day 17)	4.13	Fixed
$R0_{Total}$ (fmol/mg protein)	Receptor baseline value	326	Fixed

Table 3. Dynamic parameters for effects of corticosteroids on surfactant protein A regulation

Parameter (units)	Definition	Estimates	CV %
Surfactant protein A mRNA dynamics			
γAs	Hill coefficient for stimulation of $k_{s,A}$	8.96	23
$k_{s,A}$ (fmol/g lung/h)	Zero-order rate constant for mRNA synthesis	0.32	35
$Smax_{As}$	Maximum possible stimulation of $k_{s,A}$	99.3	43
$SC_{50,As}$ (fmol/mg protein)	Concentration required for half-maximal stimulation of $k_{s,A}$	296	9
$Smax_{Ad}$	Maximum possible stimulation of $k_{d,A}$	5.65	54
γAd	Hill coefficient for stimulation of $k_{d,A}$	35.2	176
$SC_{50,Ad}$ (fmol/mg protein)	Concentration required for half-maximal stimulation of $k_{d,A}$	400	5
$k_{d,A}$ (h^{-1})	First-order rate constant for mRNA degradation	0.035	34[a]
$mRNA_A^0$ (fmol/g lung)	Baseline value for surfactant protein A mRNA	9.10	Fixed
Surfactant protein A dynamics			
τ_A (h)	Surfactant protein A transit time	21.1	26
γAp	Amplification factor for the translation of message to protein	0.97	2
SP-A (0)	Baseline value for surfactant protein A	0	Fixed

[a] Secondary parameter

Table 4. Dynamic parameters for effects of corticosteroids on surfactant protein B regulation

Parameter (units)	Definition	Estimates	CV %
Surfactant protein B mRNA dynamics			
γB	Hill coefficient for stimulation of $k_{s,B}$	13.9	11
$k_{s,B}$ (fmol/g lung/h)	Zero-order rate constant for mRNA synthesis	0.30	34
$Smax_B$	Maximum possible stimulation of $k_{s,B}$	448	18
$SC_{50,B}$ (fmol/mg protein)	Concentration required for half-maximal stimulation of $k_{s,B}$	305	3
$k_{d,B}$ (h^{-1})	First-order rate constant for mRNA degradation	0.0287	34[a]
$mRNA_B^0$ (fmol/g lung)	Baseline value for surfactant protein B mRNA	10.3	Fixed
Surfactant protein B dynamics			
τ_B (h)	Surfactant protein B transit time	16.5	94
γBp	Amplification factor for the translation of message to protein	1.53	3
SP-B (0)	Baseline value for surfactant protein B	0	Fixed

[a] Secondary parameter

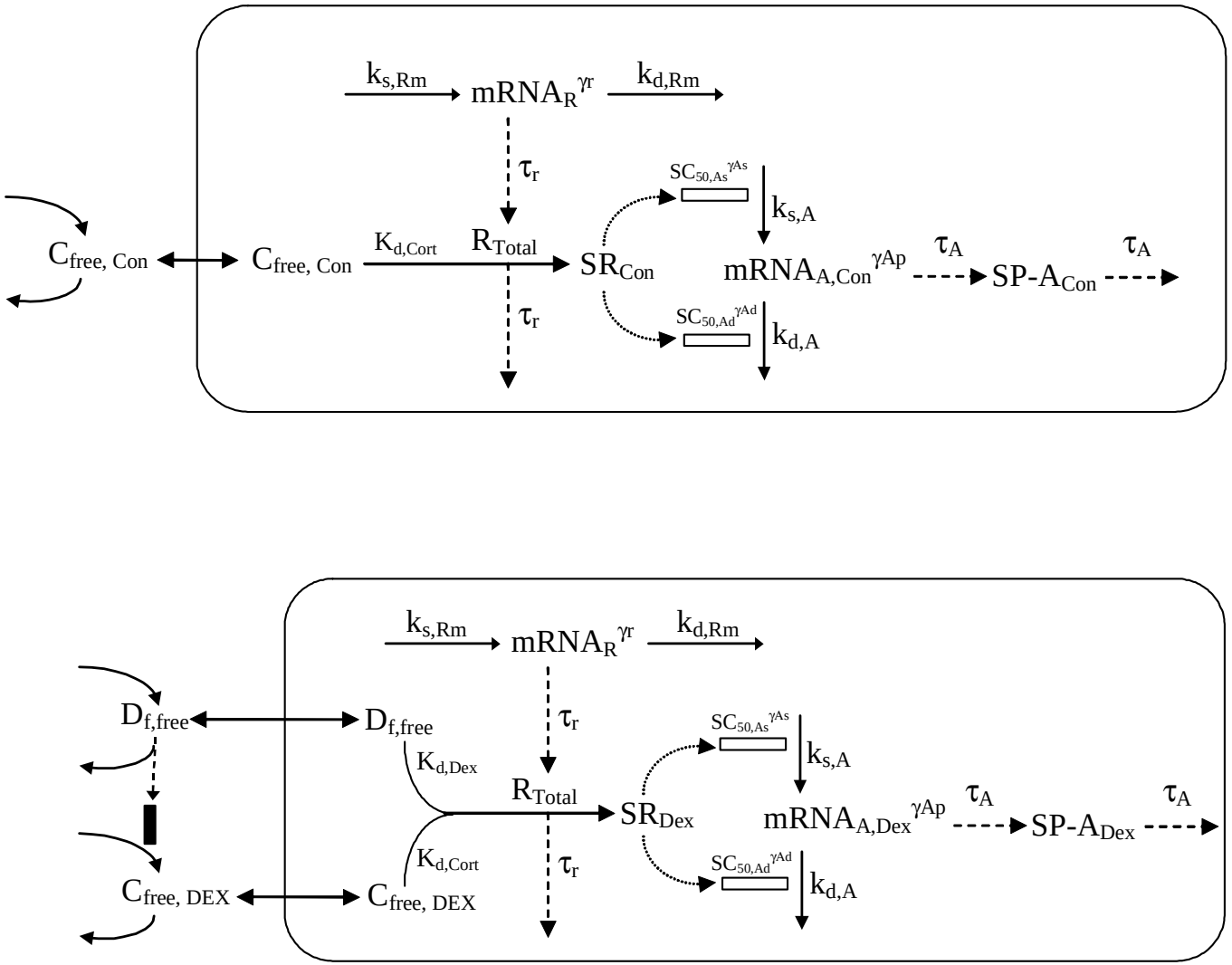

Fig. 1

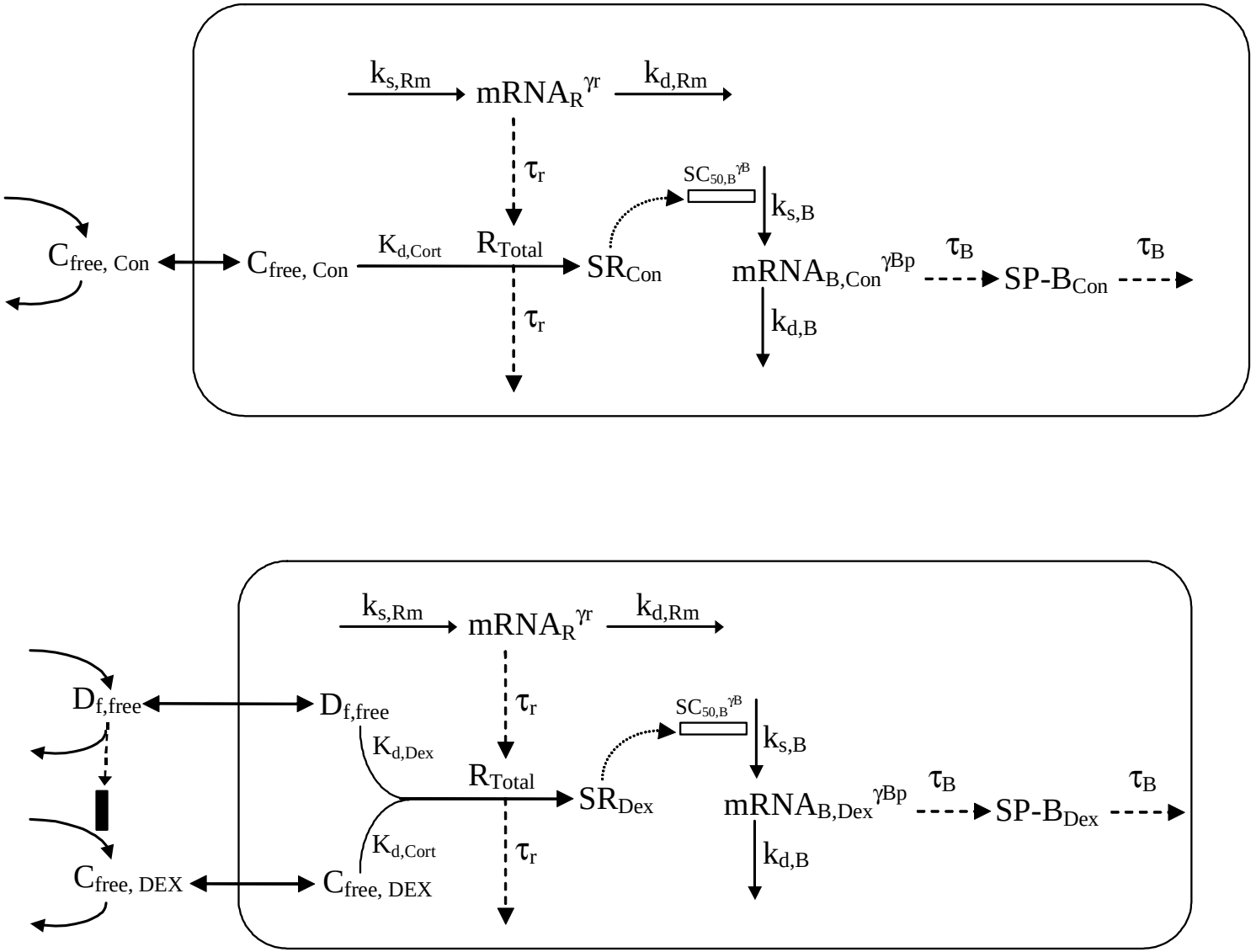

Fig. 2

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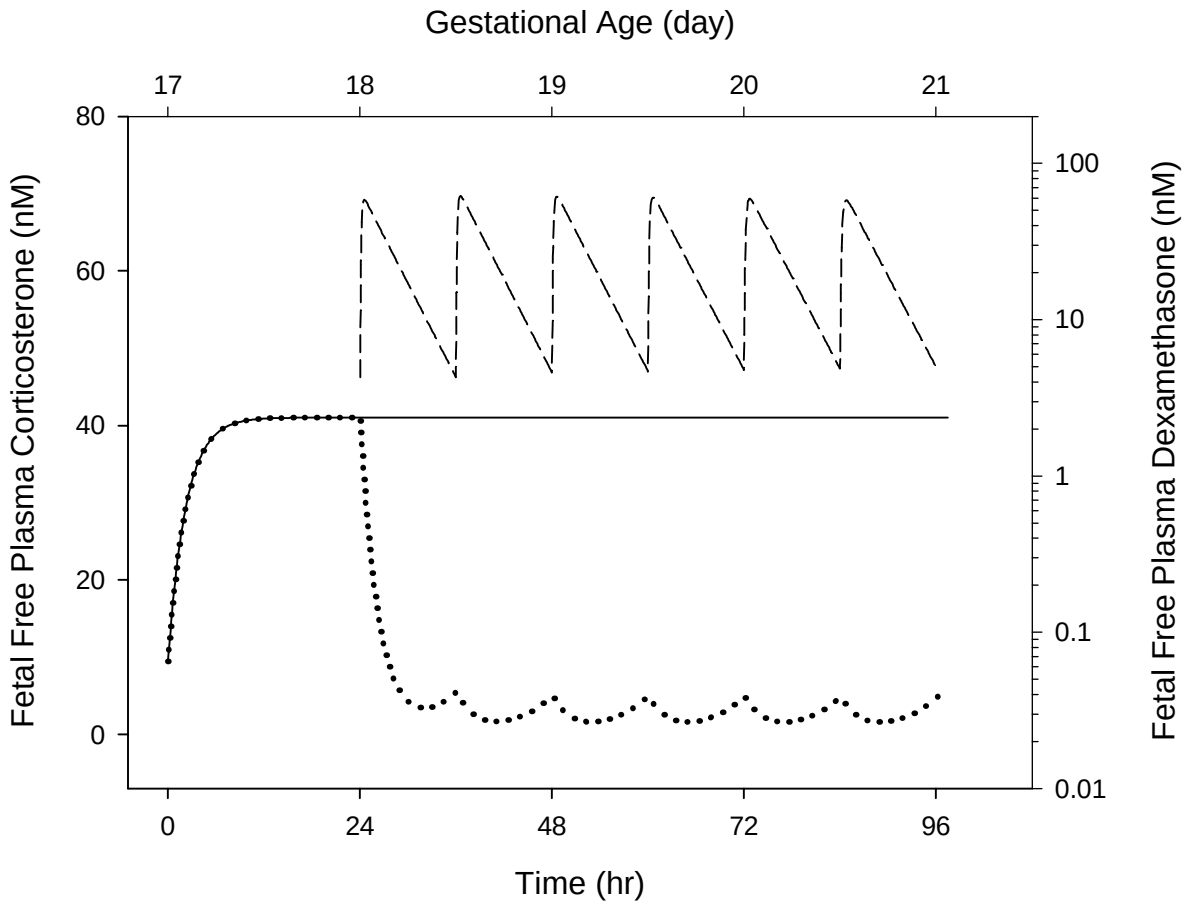

Fig. 3

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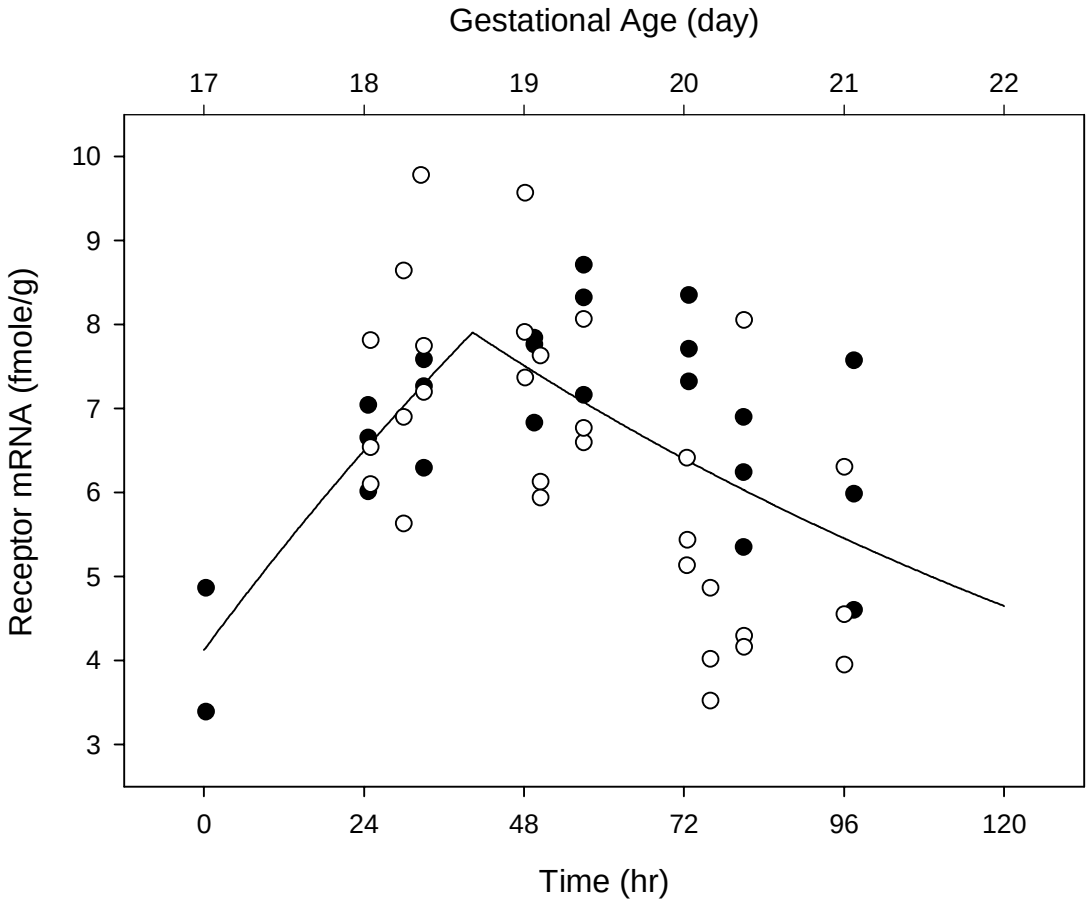

Fig. 4

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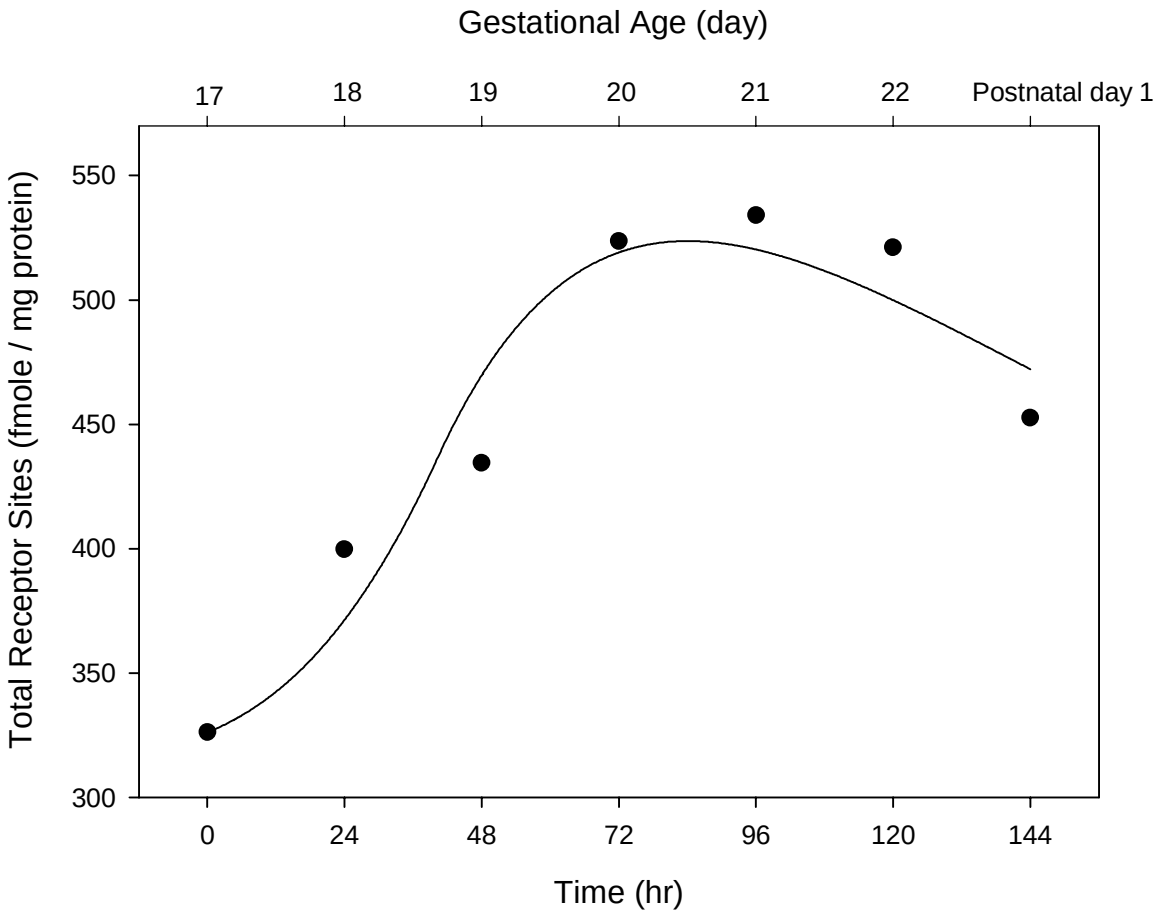

Fig. 5

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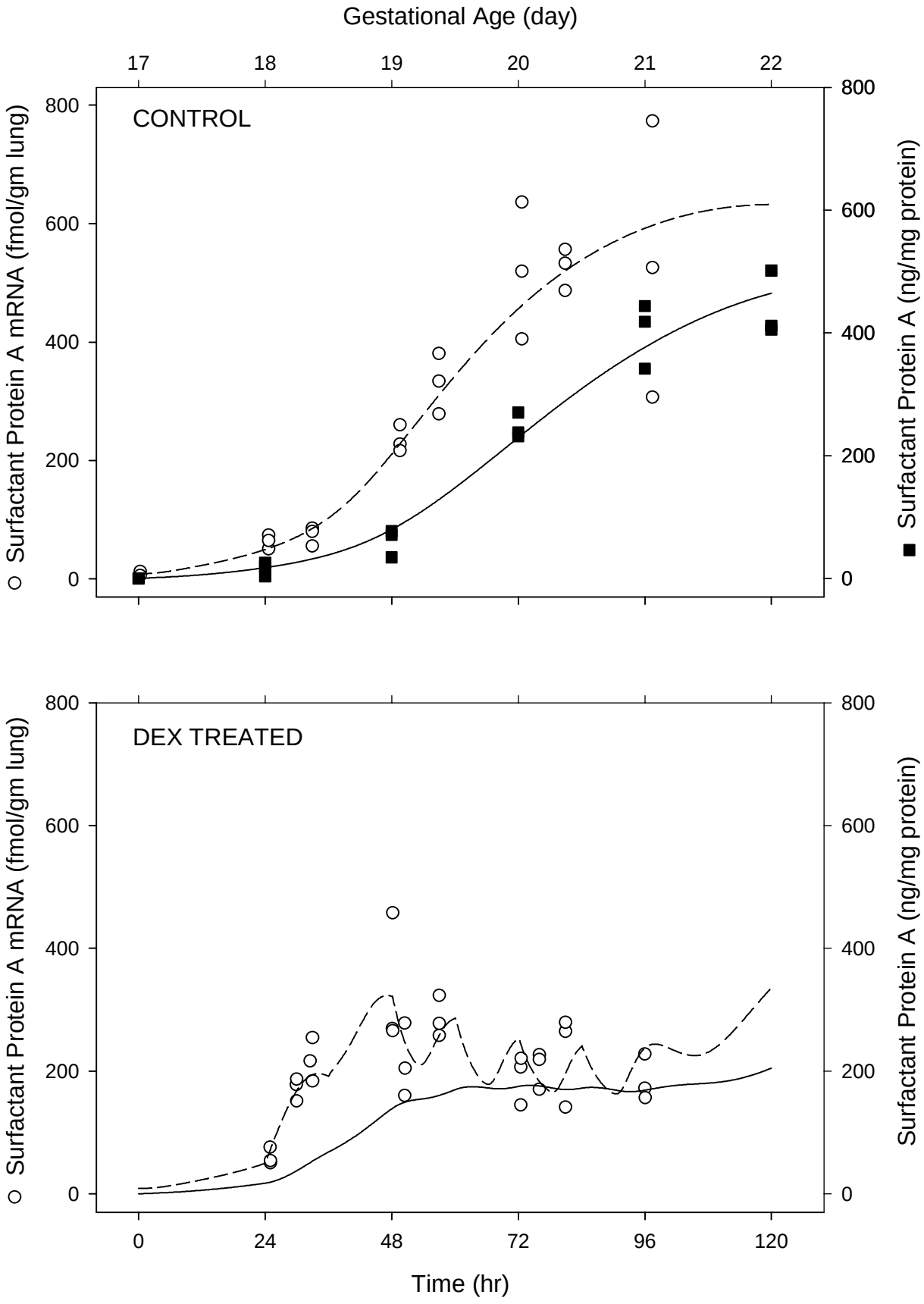

Fig. 6

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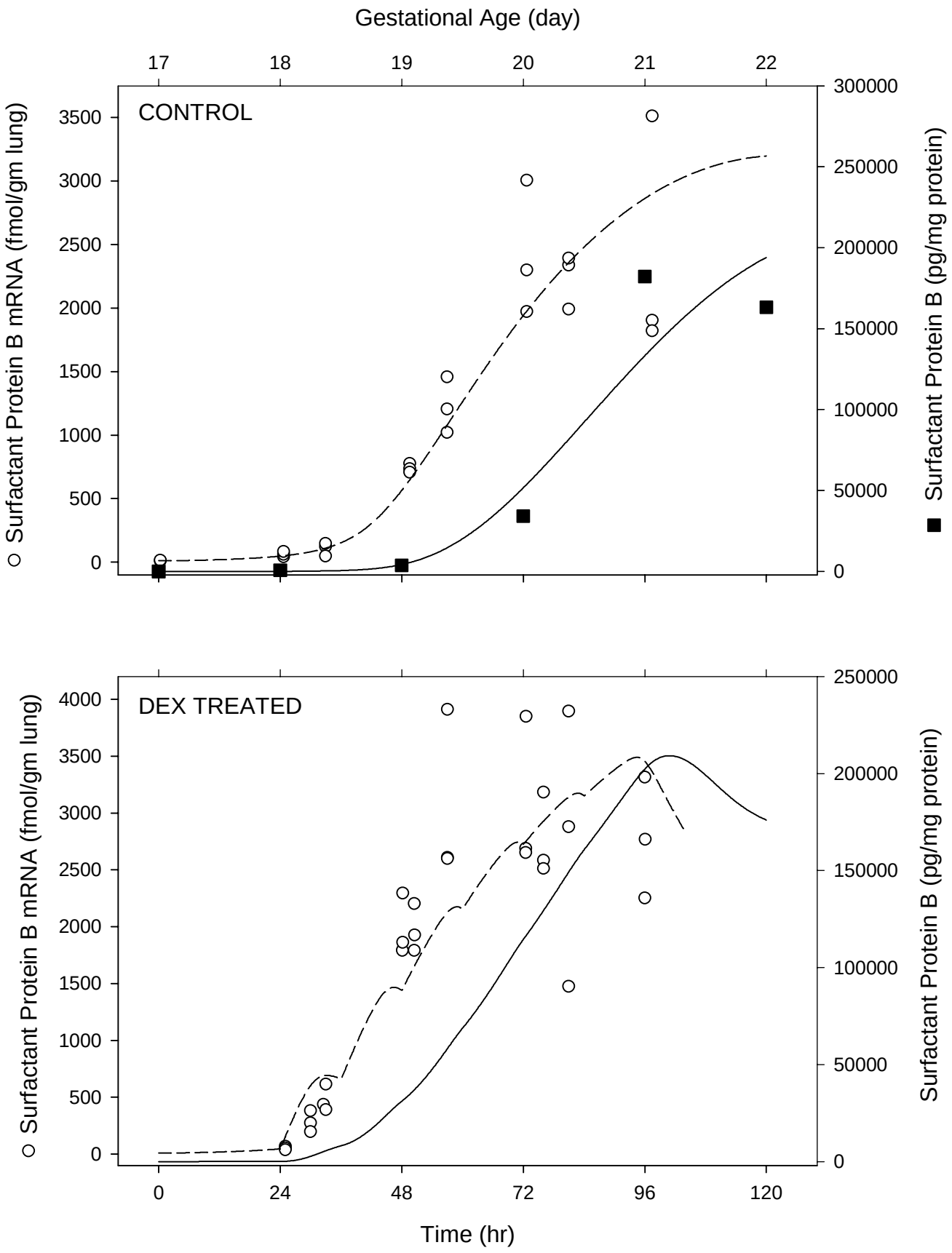

Fig. 7

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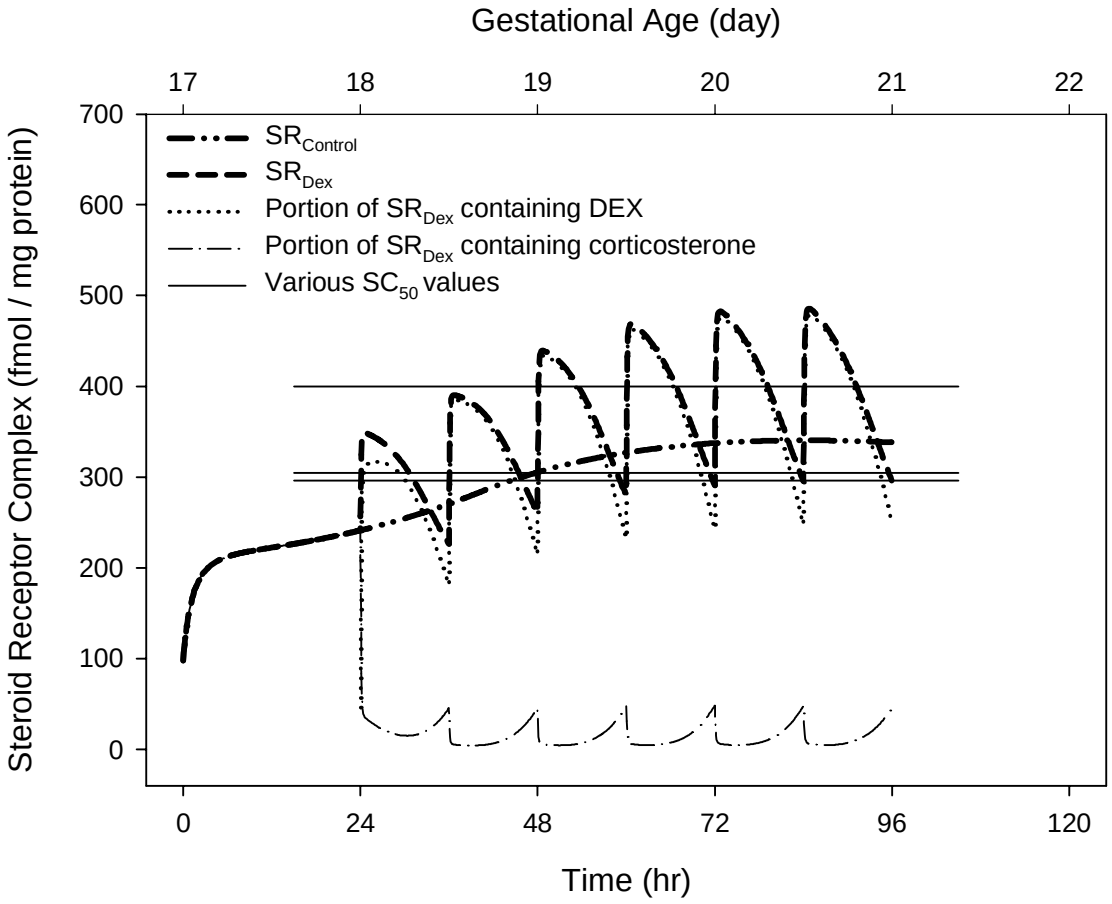

Fig. 8

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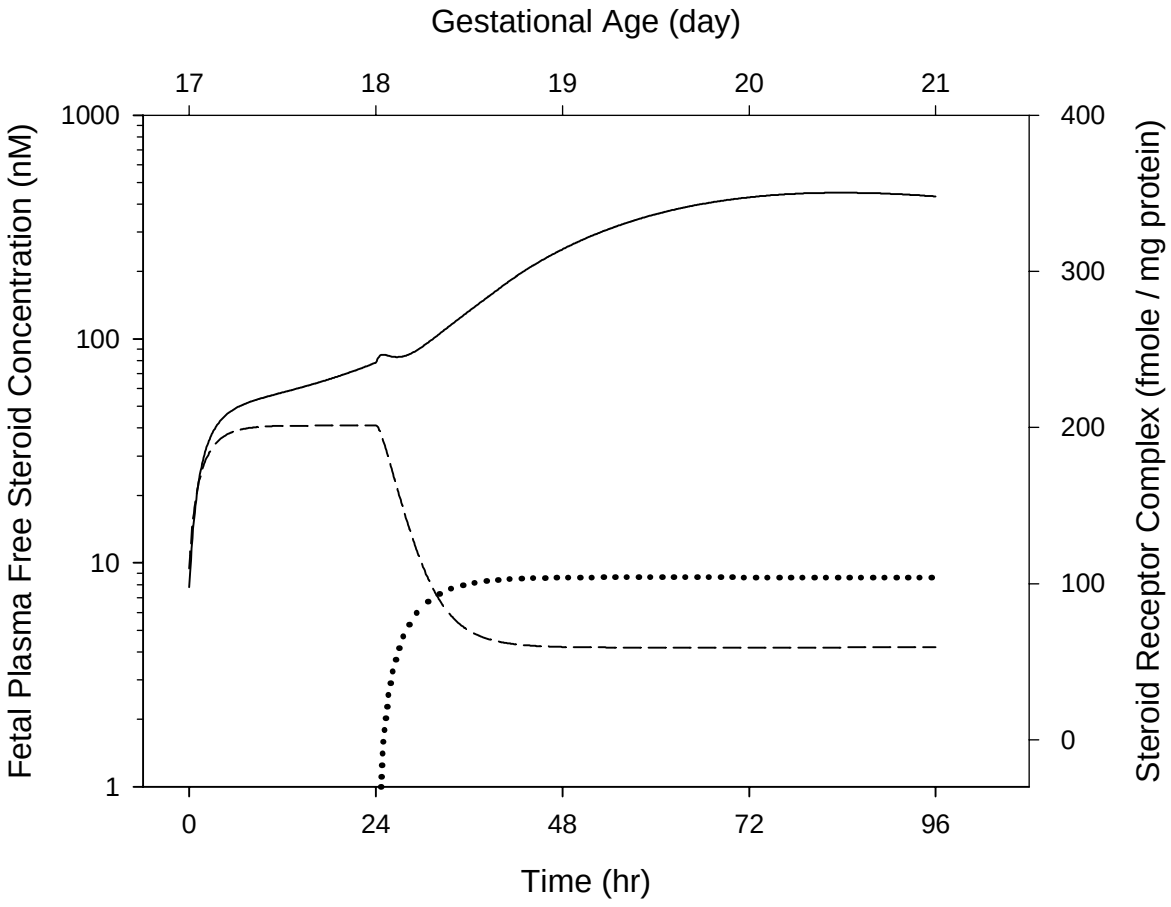

Fig. 9

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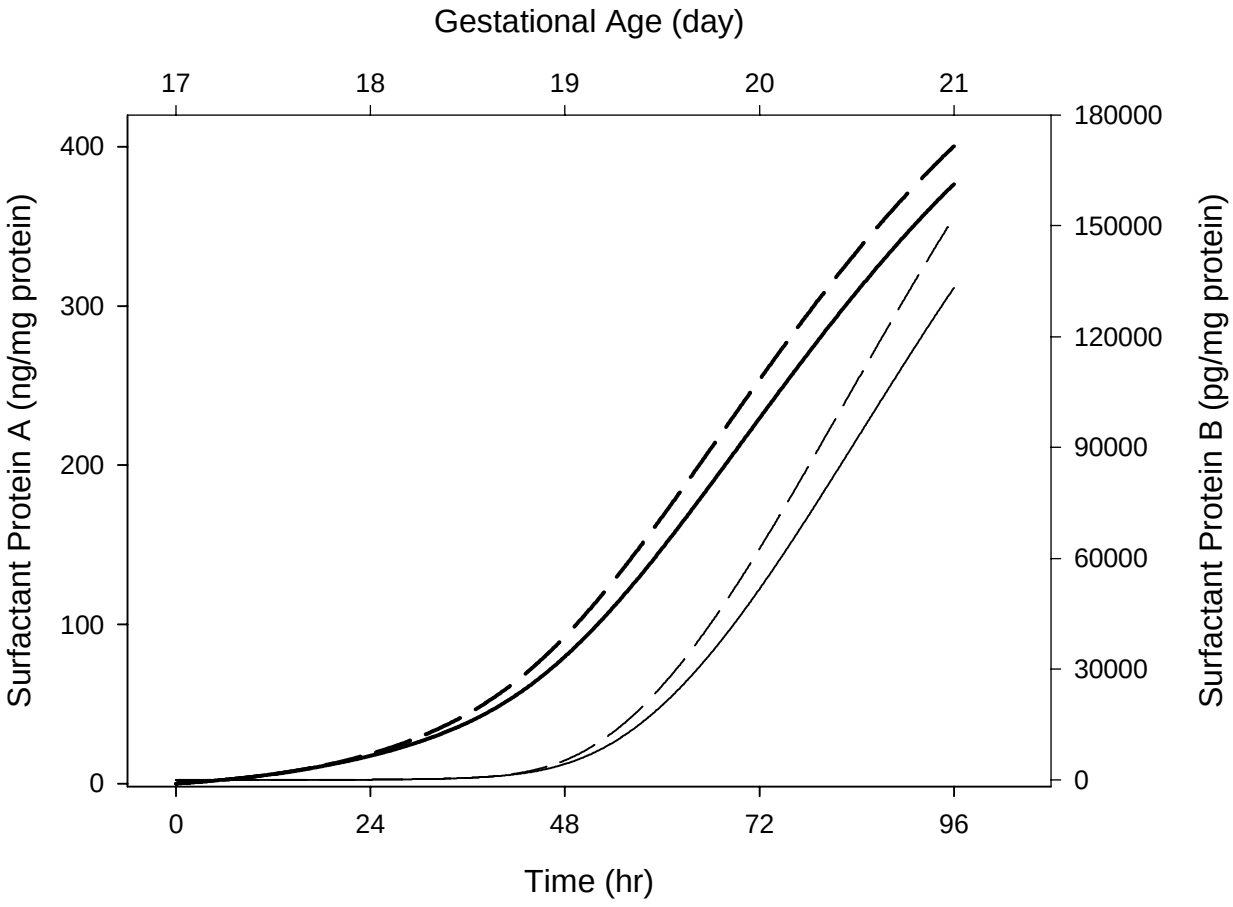

Fig. 10