Dihydrotestosterone Inhibits Fetal Rabbit

Pulmonary Surfactant Production

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A B S T R A C T Males have a higher morbidity and mortality for neonatal respiratory distress syndrome (RDS) than females, and respond less well to hormone therapy designed to prevent RDS by stimulating fetal pulmonary surfactant production. We have shown that male fetuses exhibit delayed production of pulmonary surfactant. We tested the hypothesis that the sex difference in fetal pulmonary surfactant production is under hormonal control. Pulmonary surfactant was measured as the saturated phosphatidylcholine/sphingomyelin ratio (SPC/S) in the lung lavage of fetal rabbits at 26 d gestation. There was an association between the sex of neighboring fetuses and the SPC/S ratio of the female fetuses, such that with one or two male neighbors, respectively, females had decreasing SPC/S ratios ($P < 0.05$). We injected dihydrotestosterone (DHT) into pregnant does from day 12 through day 26 of gestation in doses of 0.1, 1.0, 10, and 25 mg/d, and measured the SPC/S ratio in fetal lung lavage on day 26. In groups with the normal sex difference in fetal serum androgen levels (controls, 0.1 mg DHT/d) the normal sex difference in the SPC/S ratio was also present (females > males, $P = 0.03$). In the 1-mg/d group there was no sex difference in androgen levels and the sex difference in the SPC/S ratio was also eliminated as the female values were lowered to the male level. Higher doses of DHT (10, 25 mg/d) further reduced the SPC/S ratios. We injected the anti-androgen Flutamide (25 mg/d) from day 12 through day 26 of gestation. This treatment eliminated the normal sex difference in the lung lavage SPC/S ratio by increasing the male ratios to that of the females. We conclude that androgens inhibit fetal pulmonary surfactant production. An understanding of the mechanism of the sex difference in surfactant production

may allow development of therapy that is as effective in males as in females for preventing RDS.

INTRODUCTION

In the respiratory distress syndrome (RDS)[1] of the newborn a male disadvantage in morbidity and mortality has been widely recognized (1, 2). The cause of RDS is a lack of pulmonary surfactant, usually in relation to premature birth. For several years prenatal glucocorticoids have been administered to mothers about to deliver prematurely to reduce the risk of RDS in the newborn infant by stimulating surfactant production. Recent reports have shown that glucocorticoid therapy is more effective in reducing the incidence of RDS in female newborns than in male newborns (3–5).

Naeye described the morphology of male fetal lung as less developed than that of female lungs between 25 and 32 wk gestation (6). This finding has been supported by the report of Kotas and Avery (7) in which the lung pressure-volume characteristics (a physiologic index of surfactant function) of male fetal rabbits were less mature than those of female fetal rabbits at 27 d gestation. Glucocorticoid therapy significantly enhanced the pressure–volume characteristics of both sexes but the males remained significantly less mature than the females.

These findings may be due to a sex difference in fetal pulmonary surfactant production. We have previously reported that various indices of fetal lung maturation in human amniotic fluid [lecithin/sphingomyelin (L/S) ratio, saturated phosphatidylcholine (SPC) concentration, and cortisol concentration] are all significantly lower in amniotic fluid of male fetuses during the last 10 wk of gestation (8). Similar findings for the L/S ratio have also been reported by Pearson (9). We have subsequently shown that in the fetal rab-

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[1] *Abbreviations used in this paper*: DHT, dihydrotestosterone; RDS, respiratory distress syndrome; SPC, saturated phosphatidylcholine.

bit pulmonary surfactant, measured both as the L/S ratio and as the saturated phosphatidylcholine/sphingomyelin ratio (SPC/S), is higher in the lung lavage and amniotic fluid of female fetuses than of male fetuses at 26 and 28 d gestation (10).

In this study we tested the hypothesis that the male disadvantage in fetal pulmonary surfactant production is under hormonal regulation. We report evidence that both exogenous and endogenous androgens inhibit fetal pulmonary surfactant production.

METHODS

New Zealand White rabbits were mated between 0900 and 1200 h. All investigations were made at 26 d gestation (day 0 = mating; term = 31 d). Pregnant does were killed at 26 d between 0900 and 1200 h by intravenous injection of pentobarbital (150 mg). The uterus was exposed and the fetuses killed with intraperitoneal injections of pentobarbital (15 mg) through the uterine wall. The fetuses were removed from the uterus, weighed, then tracheostomized with PE90 tubing and the lungs lavaged with 5 X 0.5-ml aliquots of ice-cold 0.9% saline. 80–95% of the instilled saline was recovered from both the control and experimental animals. Fetal blood was obtained from the internal jugular vein, spun at 1,000 g for 10 min, and the serum collected. Fetal lungs and duodena were removed after lung lavage was completed for subsequent assay of alkaline phosphatase activity. DNA content of the lung was also measured (11). Fetal sex was identified by histologic examination of the gonads.

Neighbor effect. Fetuses at 26 d gestation from our previous study (10) were ranked according to their sex and the sex of their two adjacent neighbors. The fetus at the distal end of each horn was not ranked. The lung lavage SPC/S ratio was plotted as a function of rank. Analysis of variance using linear regression techniques was used to test for an effect of the neighbors' sexes on the SPC/S ratio.

Dihydrotestosterone injection. Pregnant does were injected subcutaneously daily with dihydrotestosterone (5α-androstan-17β ol-3-one, DHT; Steraloids Inc., Wilton, N. H.) beginning on day 12 of gestation through the day of death. Doses of 25, 10, 1, and 0.1 mg/d were used. DHT was suspended in corn oil at 4 mg/ml for the 25- and 10-mg/d doses, 1 mg/ml for the 1-mg/d dose, and 0.1 mg/ml for the 0.1-mg/d dose. One set of control animals received no injections.

Flutamide injection. Pregnant does were injected subcutaneously daily with the anti-androgen Flutamide (4'-nitro-3'-trifluoromethylisofutyranilide, 25 mg/0.1 ml dimethysulfoxide) which was a gift from Dr. A. S. Watnick, Schering Corp., Bloomfield, N. J. Control animals received 0.1 ml dimethylsulfoxide subcutaneously over the same treatment period.

Phospholipid analysis. Phospholipids were extracted from the lung lavage with chloroform/methanol (2:1 by vol); saturated phosphatidylcholine was isolated by a modification of the Mason method (12). The individual phospholipids were separated by thin-layer chromatography, visualized by bromthymol blue staining, and quantitated by densitometry (10, 12). The results were expressed as SPC/S. Sphingomyelin concentration is reported to remain relatively constant in fetal rabbit lung lavage (13). We have therefore used it as a denominator in order to control for differences in lung lavage efficiency and reduce the possibility of an apparent sex difference or treatment differences being found because

of a difference in lavage effectiveness or a difference in total lung liquid volume in one group of fetuses. This method of expression has been used previously by ourselves and others and is widely accepted (10).

Alkaline phosphatase assay. Lung and duodenum were prepared as 20% homogenates in Tris-sucrose buffer (ph 7.4). Alkaline phosphatase activity was assayed by the continuous spectrophotometric technique of Bowers and McComb, using para-nitrophenylphosphate as the substrate (14). 1 U = liberation of 1 μmol of para-nitrophenol/min under standard conditions. Proteins were measured by the Lowry method (15).

Androgen assay. Total androgens (testosterone and DHT) were assayed by radioimmunoassay in the serum of DHT treated animals and controls (16).

Statistical analyses. Student's t test and linear regression were used for data analysis (17). Where multiple t test comparisons were performed, the method of least significant differences was used to avoid Type II error (17).

RESULTS

Neighbor effect. According to our system of ranking, a male fetus with a male on each side was lowest on the scale and a female fetus with a female on each side was highest. Fig. 1 shows the relationship of neighbors' sex and SPC/S ratio. Although there is an apparent decrease in the SPC/S ratio of male fetuses with female neighbors the magnitude of this change is very small and is not statistically significant. From the low base-line level of the male fetuses there is a significant increase in the female SPC/S ratio from females with two male neighbors through females with no male neighbors ($P < 0.05$).

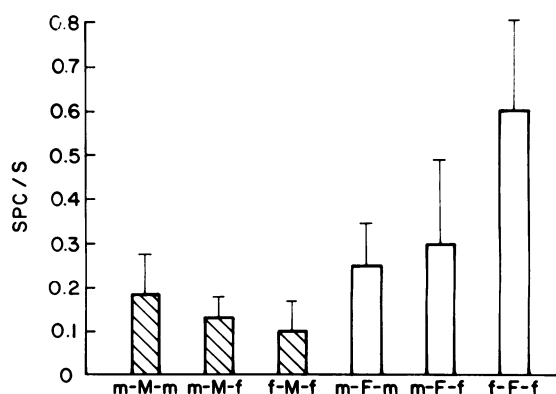*DHT effect.* Treatment with DHT at doses of 25 and 10 mg/d resulted in significantly increased male

FIGURE 1 Neighbor effect on fetal rabbit pulmonary surfactant production. M or F, male or female being studied, m or f, male or female neighbor. Bars represent mean±SEM of 3–10 fetuses from six does. Regression techniques show an inverse correlation in the female between number of male neighbors and SPC/S ratio, $P < 0.05$. There is no significant relationship in the male fetus between the presence of female neighbors and the SPC/S ratio.

and female androgens (Table I). The 1-mg/d dose produced female androgen levels that were of the same order of magnitude as those reported for normal males (18) and of our control male fetuses. Treatment with 0.1 mg/d did not significantly alter the normal male–female difference in androgen levels as compared with controls. In the DHT-treated animals there was a trend for fetal body weights to be lower; however the differences in body weight were not significant after correcting for multiple comparisons (Table I). Placental wet weight was significantly elevated in fetuses of both sexes in the 1-, 10-, and 25-mg/d groups. Lung wet weights were not utilizable since the lungs had been lavaged.

Fig. 2 shows the dose-response for the effect of DHT treatment on the lung lavage SPC/S ratios of males and females. Similar to our previous report, we found a significant sex difference (mean female SPC/S ratio > mean male SPC/S ratio, $P < 0.05$) in both the un-injected controls and the 0.1 mg DHT/d treatment group. Treatment with 1, 10, and 25 mg DHT/d eliminated the sex difference by lowering the mean female SPC/S ratio to the level of the males. A dose-response effect in the female was apparent as the mean SPC/S ratio in the 25-mg/d group was significantly lower than in the 1-mg/d group ($P < 0.01$). A suggestion of
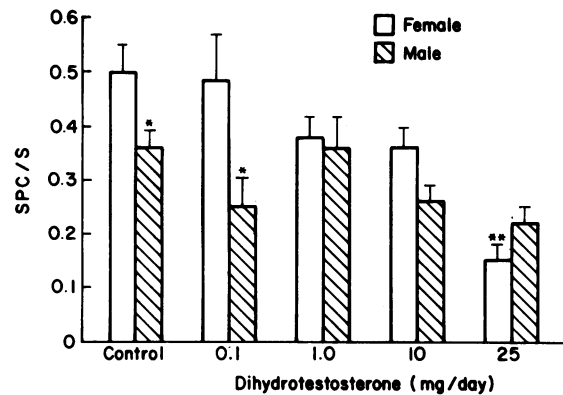

FIGURE 2 Effect of DHT treatment on the lung lavage SPC/S ratio in males and females. Bars represent mean±SEM. The number in each category is the same as in Table I under body weight. The control and 0.1 mg/d groups show a sex difference, all other groups show no sex difference. A dose-response effect of DHT on the SPC/S ratio is apparent. *Male SPC/S ratio is less than female SPC/S ratio, $P = 0.03$. **Female SPC/S ratio in 25-mg/d group is less than female SPC/S ratio in 1-mg/d group, $P < 0.01$. Multiple comparisons were not used.

a dose–response relationship was apparent in the males, but was not statistically significant.

The alkaline phosphatase activity for duodenum and

TABLE I
Body Weight, Placenta Weight, and Serum Androgen Levels of Control and DHT-treated Fetuses

Treatment	Control	DHT			
		0.1 mg/d	1.0 mg/d	10 mg/d	25 mg/d
Female					
Body wt, g	24.7±0.72	24.3±0.71	21.9±0.94	23.0±0.81	22.5±0.35
	($n = 26$)	($n = 13$)	($n = 12$)	($n = 28$)	($n = 16$)
Placenta wt, g	3.21±0.20	3.90±0.25	4.32±0.36*	5.20±0.26‡	4.96±0.44§
	($n = 15$)	($n = 13$)	($n = 12$)	($n = 27$)	($n = 17$)
Serum androgen, ng/ml	0.22±0.08	0.32±0.13	0.90±0.14	3.31±1.06	9.65±0.98
	($n = 4$)	($n = 2$)	($n = 6$)	($n = 5$)	($n = 2$)
Male					
Body wt, g	25.8±0.77	25.7±0.87	22.9±0.97	22.1±0.98	23.2±1.00
	($n = 24$)	($n = 9$)	($n = 12$)	($n = 23$)	($n = 10$)
Placenta wt, g	3.44±0.14	4.01±0.26	4.23±0.27*	5.21±0.41§	5.31±0.41‡
	($n = 15$)	($n = 9$)	($n = 12$)	($n = 23$)	($n = 10$)
Serum androgen, ng/ml	0.31±0.08	0.52±0.06	0.88±0.09	3.10±0.64	9.92±2.38
	($n = 5$)	($n = 4$)	($n = 6$)	($n = 8$)	($n = 2$)

All numbers represent mean±standard error (number of observations) from four to six does. Each n for serum androgens represents a pool of serum from three to six fetuses. Control values of body and placenta weights were compared with treated values by t test: a $P < 0.01$ was the cut-off for statistical significance.
* Difference from control, $P = 0.01$
‡ Difference from control, $P < 0.0001$.
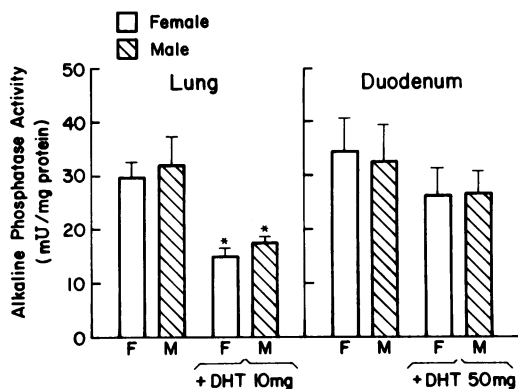§ Difference from control, $P = 0.0002$

FIGURE 3 Alkaline phosphatase activity in male and female fetuses of controls and a selected treatment group in lung, and duodenum. Each bar represents the mean±SEM. No sex differences were observed. DHT treatment lowered activity in lung but not in duodenum. *Treatment group lower than sex-matched control group, $P < 0.01$.

for lung is shown in Fig. 3. There was no sex difference in control animals in alkaline phosphatase activity of either duodenum or lung. DHT treatment at 10 mg/d reduced lung alkaline phosphatase activity to half of the control activity. There was no effect of DHT on duodenal alkaline phosphatase activity at doses of up to 50 mg DHT/d.

No sex difference was found in lung DNA/protein ratio in either control or treated animals. There was no effect of DHT treatment on the lung DNA/protein ratio (data not shown).

The anti-androgen Flutamide (Table II) had no apparent effect on either fetal body weight or serum androgen levels. There was, however, a significant effect of this drug on placental weight ($P < 0.01$). Flutamide eliminated the sex difference in the lung lavage SPC/S ratio by increasing the male ratios up to that of the females (0.38±0.05 vs. 0.39±0.07, respectively).

DISCUSSION

Fetal sexual development and differentiation are hormonally regulated events. Jost et al. (19) has formulated the hypothesis for mammals that fetal sexual development proceeds along female pathways unless androgens are present, thus leading to male development. Experiments in polytocous mammals have shown that fetal development can be affected by hormonal events in fetal neighbors. In rats, androgen levels of fetal females are successively higher if the fetus has one or two male neighbors, respectively (20). In both rats and mice external sex characteristics (ano-genital distance) and the adult sexual behavior of the female have been shown to be more masculine for females who have male neighbors in utero (20, 21). In this context, our observation that the presence of male neighbors is associated with lower SPC/S ratios in the female fetus strongly suggests a hormonal origin to the observed male delay in fetal lung surfactant production.

Therefore, we have applied Jost's concept of fetal sexual differentiation to the developing lung, hypothe-

TABLE II

Body Weight, Placenta Weight, Serum Androgen Levels, and Lung Lavage SPC/S Ratios of Control and Flutamide-treated Fetuses

	Body wt	Placenta wt	Serum androgens	SPC/S
	g		*ng/ml*	
Female				
Control	24.90±0.80	3.32±0.20	0.23±0.09	0.36±0.13
	(11)	(11)	(3)	(11)
Flutamide, 25 mg/d	24.46±0.75	4.76±0.22*	0.22±0.09	0.39±0.07
	(10)	(10)	(3)	(9)
Male				
Control	25.88±0.79	3.45±0.15	0.33±0.08	0.14±0.03
	(10)	(10)	(3)	(10)
Flutamide, 25 mg/d	25.41±1.50	4.58±0.17*	0.32±0.07	0.38±0.05*
	(13)	(13)	(4)	(12)

All numbers represent the mean±SE (number of observations). Each n for serum androgens represents a pool of three fetuses. Control values of body and placenta weights were compared with treated values by t test.
* Difference from control, $P < 0.02$.

sizing that fetal surfactant will be produced according to a female timetable unless androgen is present. There is good evidence to indicate that the fetal lung may be a target organ for sex steroids. Estrogen (22) and luteotrophic hormone (23) have been shown to augment SPC surfactant production, and lung receptors for estrogen have been demonstrated (24). Specific androgen receptors have also been identified in both adult rat lung (25) and human fetal lung fibroblasts (26). In the human fetal lung the males have more androgen receptor activity than the females, reflecting the fact that androgens stimulate their own receptors (26). Testosterone is activated locally to DHT in target cells by the enzyme 5α-reductase. This enzyme has also been found in both adult rabbit lung (27) and in human fetal lung fibroblasts (25). The human fetal lung 5α-reductase activity is higher in males (25).

The effect of DHT was relatively specific to the lung. Body weights were not significantly affected; therefore the inhibitory effect on the SPC/S ratio was not due to a generalized toxic effect on fetal growth. The lack of effect on duodenal alkaline phosphatase activity together with an effect on lung alkaline phosphatase activity also supports the concept that the observed effect on the SPC/S ratio occurs because the lung is a target organ for DHT, as the fetal human duodenum lacks DHT receptor activity (26).

We have previously quantified a sex difference in pulmonary surfactant production by expressing the data as a ratio of SPC to sphingomyelin (10). Similar results were obtained in the present study for control animals; the effects of DHT and Flutamide on pulmonary surfactant production were monitored using this same end-point. The treatment regimens had no effect on the efficiency in washing the lungs or on the amount of sphingomyelin recovered. We, therefore, conclude that the observed changes in the ratio of SPC to sphingomyelin reflects changes in the amount of SPC.

The interrelationship between changes in plasma androgen levels and the inhibition of SPC/S ratios in the male and female fetuses is noteworthy. The lowest dose of DHT at which a relative decrease in the SPC/S ratio could be discerned (1 mg) resulted in a fourfold increase in the level of plasma androgens in the females (i.e. from 0.22 to 0.90 ng/ml). To significantly reduce the absolute SPC/S ratio in the female necessitated a 40-fold increase in plasma androgens (from 0.22 to 9.65 ng/ml). It is not surprising, therefore, that the 0.1 mg dose did not affect either the relative or absolute SPC/S ratios for either sex, despite the fact that the mean female plasma androgen level was comparable to the male control at this dose (0.32 vs. 0.31 ng/ml). The relative insensitivity of the females to DHT may be due to differences in the number and

binding affinity of androgen receptors in the lung (25). The lack of any measurable effect on the male SPC/S ratios may be because the males are already maximally inhibited by endogenous androgens.

The marked effect of the anti-androgen Flutamide only on the male SPC/S ratios again reflects the relative insensitivity of the female fetuses to circulating androgens since there was no change in the SPC/S ratios of the Flutamide-treated female fetuses (which are also exposed to comparable androgen levels). Furthermore, elimination of the naturally occurring sex difference in lung lavage SPC/S ratios by Flutamide suggests that endogenous androgens inhibit pulmonary surfactant production.

The mechanism by which DHT interacts with regulation of fetal pulmonary surfactant is unknown. We propose (a) that the observed male disadvantage in clinical responsiveness to glucocorticoid, as well as in the morbidity and mortality from RDS, is due at least in part to a delay in the male fetus of pulmonary surfactant production, and (b) that androgens inhibit fetal surfactant production. An understanding of the mechanisms for this sex difference may allow development of therapy that is as effective in male as in female fetuses.

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