Synergistic effect of cortisol and thyrotrophin releasing hormone on lung maturation in the fetal sheep entails connective tissue maturation

GERMAN A. CAMPOS, FRANCISCO A. GUERRA, ELLEN BRÜMMER, MARIA L. MUÑOZ and INES A. VEGA.

Departaments of Physiology and Obstetrics & Gynecology, Faculty of Medicine, Universidad Austral de Chile, Valdivia, Chile.

Pressure-volume relationships and collagen and elastin contents were measured in the lungs of fetal sheep infused either with saline (n = 4), thyrotrophin-releasing hormone (TRH; n = 6), cortisol (n = 9) or TRH plus cortisol (n = 10) at 128 days of gestation (term = 149 days) for 7 days. Lung distensibility (V_{40} = 1.8 ± 0.1 ml/g wet wt; mean ± SD) and stability (V_5 = 0.6 ± 0.1) increased along with collagen (C) (10.1 ± 2.7 μg/mg) and elastin (E) contents (128 ± 35 ng/mg) in the animals infused with TRH plus cortisol and were significantly higher ($p < 0.05$) than those observed in TRH (V_{40} 0.62 ± 0.07; V_5 0.32 ± 0.04; C 3.53 ± 1.3; E 38.2 ± 8.3), cortisol (V_{40} 0.66 ± 0.6; V_5 0.27 ± 0.03; C 4.27 ± 0.8; E 41.02 ± 12.7) or saline infused fetuses (V_{40} 0.40 ± 0.1; V_5 0.20 ± 0.06; C 3.28 ± 0.9; E 31.5 ± 9.2). Plasma concentrations of prolactin (PRL), triiodothyronine (T3) and cortisol (F) were also higher in the group of fetuses infused with both hormones in comparison with the other groups. In fetuses treated with TRH plus cortisol, PRL (32 ± 8.3 ng/ml) and T3 (308.3 ± 36 μg/dl) were significantly higher than in those infused with cortisol alone (PRL 3.7 ± 2.3; T3 128 ± 30) or with saline (PRL 4.2 ± 1.6; T3 < 5 μg/dl). In the group treated with TRH alone, PRL also increased significantly (37 ± 6.4), but T3 increased only slightly (18 ± 3.4).

We conclude that there is a synergism between TRH and cortisol to induce lung maturation in the fetal sheep and that its mechanism entails connective tissue maturation.

INTRODUCTION

Lung maturation in the fetus is induced by hormones such as glucocorticoids, triidothyronine (T3), prolactine (PRL), adrenocorticotrophin, etc., given alone or in combinations (Liggins *et al.*, 1981; Liggins & Schellenberg, 1986). Most of the hormones studied have been shown to exert their effects on the surfactant system and on lung structure (Liggins *et al.*, 1981; Mitzner *et al.*, 1979; Crone *et al.*, 1983). Kitterman *et al.* (1981) showed that in the fetal lamb endogenous cortisol correlates well with the prepartum maturation of the lung. However, it is difficult to separate the effect of cortisol with that of the other hormones which also increase before birth (Challis & Olson, 1988). Furthermore, glucocorticoids given to pregnant women induce lung maturity in their fetuses (Liggins & Howie, 1972; Collaborative study, 1981); however, they are not equally effective at earlier gestational ages, suggesting that other factors are also involved in the physiological process of fetal lung maturation.

Some authors (Schellenberg *et al.*, 1988; Liggins *et al.*, 1988) have shown a synergistic effect between cortisol an thyrotrophin releasing hormone (TRH) on lung maturation in the fetal lamb. They found a significant

Correspondence to: Germán A. Campos, M.D., Department of Obstetrics & Gynecology, Faculty of Medicine, Universidad Austral de Chile, P.O. Box 567, Valdivia, Chile. Fax: (56-63) 212953 or 214475.

increase in the surfactant system, in the alveolization of the lung and in alveolar distensibility and stability in animals infused with TRH plus cortisol in comparison with those infused either with saline, cortisol or TRH alone. Furthermore, they also demonstrated that the collagen and elastin contents were higher in the fetuses with distensible lungs in comparison with the group of fetuses with non-distensible lungs (Schellenberg *et al.*, 1987). Kendall *et al.* (1990) showed that cortisol infused to near term lamb fetuses accelerates pulmonary alveolization studied through morphometric analysis.

Connective tissue is an important component of the lung and its composition is related to its mechanical properties, but information about changes at this level in fetuses receiving hormonal infusions to induce lung maturation is still scattered (Schellenberg, 1986). Schellenberg and Liggins (1987) showed that collagen and elastin increased during alveolar formation in the fetal sheep lung. Furthermore, the same authors demonstrated similar changes in connective tissue with the infusion to fetal sheep of hormones like cortisol, T3 and PRL, given alone or in combinations (Schellenberg *et al.*, 1987). The aim of this study was to investigate further and to confirm previous findings (Liggins *et al.*, 1988) about the synergism between cortisol and TRH, on connective tissue composition and mechanical properties of the fetal lung.

MATERIAL AND METHODS

Twenty nine Romney Marsh ewes were used for these experiments. Estrous cycle was synchronized with vaginal sponges containing 60 mg of medroxyprogesterone acetate (SIGMA Co., St. Louis) and injecting prostaglandin $PGF_{2\alpha}$ 5 mg i.m. at the moment of removal of the sponge (14 days). Pregnancy was confirmed with sonographic examination at about day 70 of gestation using an Aloka Ultrasound machine with a 7.0 MHz transrectal transducer. Pregnant ewes were operated under epidural anaesthesia at 117 days (term 149 days) and plastic catheters were placed in the fetal carotid artery, jugular vein and in the amniotic cavity using a technique previously described (Campos *et al.*, 1985). At 121 days of gestation, a group of 9 fetuses was continuously infused i.v. at a rate of 1.0 ml/h with a solution containing NaCl 0.9%, ampicillin (1 g/l) and heparin (100 IU/l) during 3.5 days. After that, cortisol was added to the solution at a concentration of 1 mg/h during 3.5 days. At a similar gestational age (121 days) another group of 14 fetuses was infused intra-arterially with the same solution containing TRH (ELEA Laboratories, Buenos Aires). The solution containing TRH was infused in 1 ml in 1 minute every hour, each pulse containing 25 μg of TRH. At 3.5 days cortisol (1 mg/h) was added i.v. to 10 TRH infused fetuses until the end of the infusion. The remaining 4 fetuses received only the TRH solution. A group of 6 fetuses was infused with saline solution during 7 days and was used as a control group.

An arterial blood sample (4 ml) was obtained daily for pH, blood gases analysis and plasma hormone determinations by established radioimmunoassays. To prevent premature delivery of the fetus during the infusions, 150 mg of Medroxyprogesterone acetate (Upjohn Co., Kalamazoo, USA) was injected i.m. to the ewe at the beginning of the experiment.

At the end of the experiment, the fetuses were killed with an overdose of sodium pentobarbitone i.v. and then delivered by Cesarean section under epidural anaesthesia. The fetuses were weighed and the trachea, lungs and heart were removed as a block. The lungs were dissected free from the heart and carefully separated one from the other at the level of the tracheal bifurcation. The left lung was used for quasi-static pressure-volume curves. We performed inflation and deflation pressure-volume studies using pressure increments of 5 cm H_2O to a maximum of 40 cm H_2O. For volume equilibration, we allowed 2 min at each pressure, except at 40 cm H_2O and at atmospheric pressure on deflation, where we allowed 5 min. As a check for leaks during pressure-volume studies, we measured the displacement volume in 0.9% NaCl for each lung after the pressure-volume study. Lung distensibility was expresed as the amount of air (ml/g of wet tissue) necessary to reach and maintain a pressure of 40 cm

H_2O (V_{40}). Lung stability (V_5; ml/g of wet tissue) was estimated by the amount of air remaining in the lung after deflation at atmospheric pressure.

After completion of the pressure-volume studies, the left lung was dissected free from the pleura, major bronchia and vessels. The tissue obtained was lyophilized and stored at –20° C until analyzed for hydroxyproline and desmosine.

Hydroxyproline and desmosine analyses

The lyophilized lung tissue (200 mg) was subjected to acid hydrolysis with HCl 6 N, 1% Phenol in vacuum-sealed ampoules at 110° C for 12 hours. Recovery of hydroxyproline and desmosine was determined by subjecting vials with known amounts of the aminoacids to the same process of hydrolysis. The sample was then analyzed for hydroxyproline (collagen) using an Aminoacid Analyzer according to a method previously described (Powell & Whitney, 1980). For desmosine (elastin) analysis, the hydrolysate was directly applied to a chromatography column fitted in a Gas Chromatograph Perkin-Elmer model Sigma 300, equipped with a flame ionization detector. The data were integrated and registered using an integrator Perkin-Elmer model LCI-100. The standards for desmosine and isodesmosine were a gift from Prof. G.C. Liggins, Auckland, New Zealand. Briefly, the assay's conditions included: silanized glass column of 8 feet long, 2 mm internal diameter and 0.25 inch external diameter. Stationary phase: GP 3% SP-2310 / 2% SP-2300 over 100/120 mesh Chromosorb W AW. The elution program was as follows: initial temperature 80° C for an initial time equal 0; speed 30° C/min, final temperature 240° C in 8 minutes. Under the assay's conditions, desmosine elutes as a clear peak at 5.625 min from the beginning of the procedure (Figs. 1 and 2). Results were corrected according to the recovery efficiency of the assays' methods, which in the case of hydroxyproline was 85 ± 2.3% (mean ± SD) and for desmosine was 81 ± 1.8%.

The data were statistically analyzed using Duncan's Multiple Range Test. A p value of less than 0.05 was considered significant.

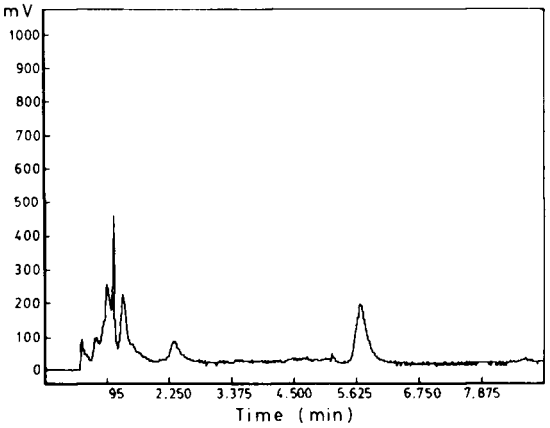

Fig. 1: Gas chromatogram of an hydrolysate containing 6 nm of desmosine. Assay's conditions described in text.

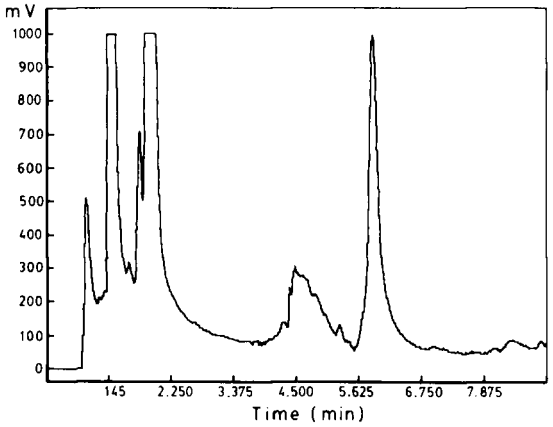

Fig. 2: Gas chromatogram showing a clear peak at 5.625 min, corresponding to the desmosine content of fetal lung hydrolysate. Assay's conditions described in text.

RESULTS

Data on body and lung weights are shown in Table I. Values for arterial pH and blood gas tensions were not significantly different between the groups, either before or during the infusion, or at the end of each experiment (data not shown).

During the infusions, the plasma concentration of cortisol increased, reaching a level of 42.4 ± 7.8 µg/dl in the fetuses receiving TRH plus cortisol and 45.2 ± 7.5 µg/dl in the group receiving only cortisol, whereas it remained not detectable (< 1 µg/dl) in the fetuses treated with TRH or saline ($p > 0.05$). The plasma levels of T3 increased progressively during the experiment in all the groups, except in the fetuses infused with saline. At the end of the infusion, plasma T3

TABLE I

Effects of the infusions of TRH and/or Cortisol on plasma levels of various hormones

	TRH + Cortisol	Cortisol	TRH	Saline
n	10	9	4	6
Age (days)	128.9 ± 1.6	129.7 ± 1.2	128.3 ± 1.4	127.9 ± 1.8
Body wt (g)	2720 ± 252	2860 ± 302	2650 ± 328	2890 ± 293
Lung wet wt (g)	70.7 ± 6.5	77.2 ± 8.1	70.2 ± 8.7	69.3 ± 7.0
Prolactin (ng/ml)	32 ± 8.3[a]	3.7 ± 2.3[b]	37 ± 6.4[a]	4.2 ± 1.6[b]
Cortisol (μg/dl)	42.4 ± 7.8[a]	45.2 ± 7.5[a]	< 1.0	< 1.0
T3 (μg/dl)	308.3 ± 36[a]	128.0 ± 30[b]	18.0 ± 3.4[c]	ND

Values, means ± SD at end of infusions. ND, not detectable. Values with different superscripts show statistically significant differences ($p < 0.05$).

concentration in the fetuses infused with TRH and cortisol was about 17 times higher ($p < 0.001$) than in those treated with TRH alone. Prolactin increased more than 40 times in the fetuses infused with TRH or with TRH and cortisol, compared with those fetuses infused only with cortisol or saline ($p < 0.01$) (Table I).

Pressure-volume studies showed estimates of lung distensibility (V_{40}) and stability (V_5) significantly higher ($p < 0.05$) in the fetuses infused with TRH plus cortisol than in those animals receiving either TRH or cortisol alone or saline solution (Table II).

Both desmosine and hydroxyproline increased significantly in the group of fetuses treated with TRH and cortisol compared to those receiving only TRH, cortisol or saline. In the group of fetuses infused with both hormones, the rise in the concentration of desmosine was proportionately higher than the increase in hydroxyproline, determining a significant fall in the ratio of hydroxyproline to desmosine ($p < 0.01$) (Table II).

DISCUSSION

Lung maturation in fetal sheep infused with TRH and/or cortisol has been studied analyzing its mechanical properties and surfactant content in both the alveolar fluid and in the lung tissue (Schellenberg *et al.*, 1988; Liggins *et al.*, 1988). In these studies, a synergistic effect of both hormones on lung maturation has been clearly demostrated. Both lung distensibility and stability increased in these experiments along with a rise in the concentration of saturated phosphatidylcholine in the alveolar lavage fluid and in lung tissue. They concluded that lung maturation before birth is dependent on the synergism between several hormones. At least three of such hormones - cortisol, prolactin and T3 - can play an important role in that process.

Our results, using the same experimental approach that of Liggins *et al.* (1988), showed that the plasma concentrations of prolactin and T3 in the fetuses infused with TRH and cortisol rose to levels normally found at term in the fetal sheep (Challis *et al.*, 1988). These values were in the range of those reported by Liggins *et al.* (1988). Both lung distensibility (V_{40}) and stability (V_5) were significantly higher in the fetuses infused with both TRH and cortisol, compared with values of fetuses infused either with each hormone by separate or with saline. Present results agree with those previously reported, confirming the synergistic effect of TRH and cortisol on lung maturation in the sheep fetus (see Liggins *et al.*, 1988; Schellenberg *et al.*, 1988).

Our results showed that the intrafetal infusion of TRH plus cortisol caused a significant increase in desmosine (elastin) and hydroxyproline (collagen) in lung tissue, being the rise in elastin higher than that of collagen. Therefore, the ratio collagen/elastin changed from 0.1 in saline or cortisol-infused animals to 0.07 in the fetuses infused with both

TABLE II

Effects of the infusions of TRH and/or Cortisol on distensibility (V_{40}), stability (V_5), and hydroxyproline (HPL) and desmosine (DES) contents of fetal sheep lungs.

	TRH + Cortisol	Cortisol	TRH	Saline
n	10	9	4	6
HPL (μg/mg)	10.1 ± 2.7^a	4.27 ± 0.80^b	3.53 ± 1.3^b	3.28 ± 0.9^b
DES (ng/mg)	128.2 ± 35.2^a	41.0 ± 12.7^b	38.2 ± 8.3^b	31.5 ± 9.2^b
Ratio HPL/DES	0.076 ± 0.004^a	0.105 ± 0.08^b	0.094 ± 0.10^b	0.103 ± 0.08^b
V_{40} (ml/g)	1.8 ± 0.11^a	0.66 ± 0.06^b	0.62 ± 0.07^b	0.40 ± 0.1^c
V_5 (ml/g)	0.60 ± 0.11^a	0.27 ± 0.03^c	0.32 ± 0.04^b	0.20 ± 0.06^c

Values, means ± SD at end of infusions. Values with different superscripts show statistically significant differences ($p < 0.05$).

hormones ($p < 0.01$). The cause of the increase in collagen and elastin in the animals treated with TRH plus cortisol is not clear. It is known that corticosteroids given to pregnant animals stimulated collagen and elastin deposition in the developing lung of the fetal monkey, but causing an increase rather than a decrease in the collagen/elastin ratio (Beck *et al.*, 1981). In our experiments, cortisol given alone caused a small and non-significant increase in both collagen and elastin in the lung when compared with the saline-infused animals. However, the combination of TRH plus cortisol –probably through an interaction between prolactin, T3 and cortisol– was able to increase the collagen and elastin contents of the fetal lung.

The increased lung distensibility and stability observed in the fetuses treated with TRH plus cortisol correlate well with the rise in collagen and elastin. Several reports have suggested that changes in the connective tissue may be more important than an increase in surfactant in determining the mechanical properties of the hormone-induced maturation of the fetal lung (Brumley *et al.*, 1977). The contents of collagen and elastin in the fetal lung are significantly higher in animals with distensible lungs in comparison with those with non-distensible lungs (Schellenberg, 1987), a finding that agrees well with the results of the present experiments.

The changes in connective tissue found in the present experiments are similar to those observed during normal lung maturation in the fetal sheep. Rapid elastin accumulation and a mild increase in the collagen content (Schellenberg, 1986; Schellenberg *et al.*, 1987) appear to occur before alveolization of the lung in the fetal sheep. Our preliminary observations (Campos *et al.*, 1990) show that fetal sheep infused with TRH and cortisol had lungs with more air spaces and thinner alveolar walls than the lungs of fetuses infused only with cortisol. These structural changes occurred along with increases in lung distensibility and stability, suggesting that connective tissue changes and alveolar formation (structural maturation) could be closely related.

From the above, we may conclude that the synergism between TRH and cortisol to induce lung maturation in the fetal sheep is expressed in different ways, including structural changes such as alveolar formation and thinning of the alveolar wall, stimulation of surfactant synthesis and –at a biochemical level– stimulation of collagen and elastin accumulation in the fetal lung.

ACKNOWLEDGMENTS

We thanks the financial support from Grant FONDECYT 932/88.

REFERENCES

BECK JC, JOHNSON JWC, MITZNER W, LEE PA, LONDON WT, SLY DL (1981) Glucocorticoids, hyperinsulinemia and fetal lung maturation. Am J Obstet Gynecol 139: 465-470

BRUMLEY GW, KENLSON JH, SHOMBERG DW, CRENSHAW C (1977) Whole and disaturated lung

phosphatidylcholine in cortisol-treated, intrauterine growth-retarded and twin control lambs at different gestational ages. Biol Neonate 31: 155-166

CAMPOS GA, GUERRA FA, ISRAEL EJ (1986) Effects of cholic acid infusion in fetal lambs. Acta Obstet Gynecol Scand 65: 581-583

CAMPOS GA, CROVETTO E, GUERRA FA (1990) Effects of cortisol and thyrotropin releasing hormone (TRH) on morphology and connective tissue composition of the fetal lung. Arch Biol Med Exp 23: R257 (Abstract)

CHALLIS JRG, OLSON DM (1988) Parturition. In: KNOBIL ED, NEILL J (eds) The Physiology of Reproduction: Ist ed. New York: Raven Press. pp. 2117-2216

COLLABORATIVE GROUP ON ANTENATAL STEROID THERAPY (1981). Effect of antenatal dexamethasone administration on the prevention of respiratory distress syndrome. Am J Obstet Gynecol 141: 276-286

CRONE RK, DAVIES P, LIGGINS GC, REID L (1983) The effect of hypophysectomy, thyroidectomy and replacement therapy of cortisol and ACTH on ovine fetal lung structure. J Dev Physiol 5: 281-288

KENDALL JZ, LAKRITZ J, PLOPPER CG, RICHARDS GE, RANDALL GCB, NAGAMANI M, WEIR AJ (1990) The effects of hydrocortisone on lung structure in fetal lambs. J Dev Physiol 13: 165-172

KITTERMAN JA, LIGGINS GS, CAMPOS GA, CLEMENTS JA, FORSTER CS, LEE CH, CREASY RK (1981) Prepartum maturation of the lung in fetal sheep: Relation to cortisol. J Appl Physiol 51: 384-390

LIGGINS GC, HOWIE RN (1972) A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics 50: 515-525

LIGGINS GC, KITTERMAN JA, CAMPOS GA, CLEMENTS JA, FORSTER CS, LEE CH, CREASY RK (1981) Pulmonary maturation in the hypophysectomized ovine fetus. Differential responses to adrenocorticotrophin and cortisol. J Dev Physiol 3: 1-14

LIGGINS GC, SCHELLENBERG J-C (1986) Hormones and Lung Maturation. In: JOHNSTON BM, GLUCKMAN PD (eds) Respiratory control and lung development in the fetus and newborn. 1st ed. New York: Perinatology Press. pp 108-127

LIGGINS GC, SCHELLENBERG J-C, MANZAI M, KITTERMAN JA, LEE CH (1988) Sinergism of cortisol and thyrotropin-releasing hormone in lung maturation in fetal sheep. J Appl Physiol 65: 1880-1884

POWELL JT, WHITNEY PL (1980) Postnatal development of rat lung. Biochem J 188: 1-8

SCHELLENBERG J-C (1986) The development of connective tissue and its role in pulmonary mechanics. In: JOHNSTON BM, GLUCKMAN PD (eds) Respiratory control and lung development in the fetus and newborn. 1st ed. New York: Perinatology Press. p 45

SCHELLENBERG J-C, LIGGINS GC (1987) Elastin and collagen in the fetal sheep lung. I. Ontogenesis. Pediatr Res 22: 335-338

SCHELLENBERG J-C, LIGGINS GC, KITTERMAN JA, LEE CH (1987) Elastin and collagen in the fetal sheep lung. II. Relationship to mechanical properties of the lung. Pediatr Res 22: 339-343

SCHELLENBERG J-C, LIGGINS GC, MANZAI M, KITTERMAN JA, LEE CH (1988) Synergistic hormonal effects on lung maturation in fetal sheep. J Appl Physiol 65: 94-100