Control of the development of the pulmonary surfactant system in the saltwater crocodile, *Crocodylus porosus*

LUCY C. SULLIVAN, SANDRA ORGEIG, AND CHRISTOPHER B. DANIELS
Department of Environmental Biology, University of Adelaide, Adelaide, South Australia 5005
Received 9 January 2002; accepted in final form 15 July 2002

Sullivan, Lucy C., Sandra Orgeig, and Christopher B. Daniels. Control of the development of the pulmonary surfactant system in the saltwater crocodile, *Crocodylus porosus*. Am J Physiol Regul Integr Comp Physiol 283: R1164–R1176, 2002. —Pulmonary surfactant is a mixture of lipids and proteins that controls the surface tension of the fluid lining the inner lung. Its composition is conserved among the vertebrates. Here we hypothesize that the in ovo administration of glucocorticoids and thyroid hormones during late incubation will accelerate surfactant development in the saltwater crocodile, *Crocodylus porosus*. We also hypothesize that the increased maturation of the type II cells in response to hormone pretreatment will result in enhanced responsiveness of the cells to surfactant secretagogues. We sampled embryos at days 60, 68, and 75 of incubation and after hatching. We administered dexamethasone (Dex), 3,5,3'-triiodothyronine (T3), or a combination of both hormones (Dex + T3), 48 and 24 h before each prehatching time point. Lavage analysis indicated that the saturation of the phospholipids (PL) in the lungs of embryonic crocodiles occurs rapidly. Only T3 and Dex + T3 increased total PL in lavage at embryonic day 60, but Dex, T3, and Dex + T3 increased PL at day 75. The saturation of the PLs was increased by T3 and Dex + T3 at day 68. Swimming exercise did not increase the amount or alter the saturation of the surfactant PLs. Pretreatment of embryos with Dex, T3, or Dex + T3 changed the secretion profiles of the isolated type II cells. Dex + T3 increased the response of the cells to agonists at days 60 and 68. Therefore, glucocorticoids and thyroid hormones regulate surfactant maturation in the crocodile.

PULMONARY SURFACTANT (PS) is a mixture of lipids and proteins that reduces the surface tension at the air-liquid interface in the lung. PS reduces the work of breathing, promotes lung stability and prevents pulmonary edema. The phospholipid (PL) components of surfactant include phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, and phosphatidylserine (33). For all vertebrate surfactants so far examined, PC comprises at least 50% of total PL (12). Although all PLs demonstrate some surface activity, the predominant surface-active components of PS are disaturated PLs (DSP), particularly dipalmitoylphosphatidylcholine (DPPC) (60). All tetrapod species so far examined contain a significant proportion of DSP, although it is not certain whether this is always predominantly DPPC. However, the level of DSP increases with increasing preferred body temperature of different species. Species with lower body temperatures undergo a trade-off between a highly surface-active surfactant (high DSP), capable of very low surface tensions, and one with a lower phase transition temperature (low DSP), which is capable of maintaining homeoviscosity during changes in temperature (12). Nonmammals generally are thought not to require such low surface tensions, due to their relatively larger respiratory units. Nevertheless, DSP appears to be a crucial component of all surfactants and is tightly regulated both in adults and newborns/hatchlings (24). PS also consists of surfactant-associated proteins (SP), four of which have been described to date: SP-A, SP-B, SP-C, and SP-D. The surfactant proteins are important in lipid homeostasis and have a major role in lung immunity (8, 62). The components of PS are synthesized in specialized cells located in the lung epithelium, termed type II cells. Type II cells are characterized by a cuboidal shape, apical microvilli, and the presence of lamellar bodies, which are dense, multilayered structures that store the surfactant. Despite the vast phylogenetic separation and variety of lung structures among the vertebrates, the morphology of type II cells is highly conserved (9).

Archosaurian reptiles, which include crocodiles, alligators, caiman, and gharials, have a complex, multichambered lung, termed multichameral. In the archosaurian reptiles, the chambers of the lung connect independently with an unbranched intrapulmonary bronchus, which is reinforced with cartilage in the cranial portion of the lung (43). The cranial part of the lung consists of three rows of branching chambers. In the middle of the lung, the intrapulmonary bronchus narrows, loses the cartilage reinforcement, and a variable number of small and large chambers branch off (42). The inner surface of the chambers is lined with depressions in the parenchyma, called ediculae, which...
form the respiratory units (15). The ediculae are shallow structures that end in a network of trabeculae, the epithelium of which is analogous to mammalian bronchii (43). The type I and type II cells are located in the trabecular epithelium. Despite the different lung structures between crocodiles and eutherian mammals, the lipid composition of PS is remarkably similar (9–11). The only major difference in the lipid composition of surfactant isolated from the saltwater crocodile is the absence of PG. An SP-A-like protein has also been isolated from the lavage of the saltwater crocodile (56). However, the development of the surfactant system has not been investigated in the crocodilians.

In eutherian mammals, PS maturation occurs late in gestation, marked by an increase in the amount of surfactant proteins and total PL within the air spaces and a relative increase in the proportion of PL that is disaturated (4, 29). The development of the surfactant PLs in three oviparous vertebrates, the chicken, bearded dragon, and sea turtle, follows the same general pattern of development. However, in the reptilian species so far examined, the surfactant system matures over a relatively shorter period of time. For example, although DSP appears in the lavage of the embryonic chicken at 85% of incubation, DSP is not detectable at 92% of incubation in the bearded dragon (26). Similarly, the amount of total PL is highest in the embryonic chick at 85% of incubation, whereas total PL is highest at the onset of air breathing in the bearded dragon and the green sea turtle (25, 26). Furthermore, the secretion of surfactant from isolated type II cells is highest in the embryonic chicken at 85% of incubation (57), whereas secretion is maximal after hatching in the bearded dragon (57a) and green sea turtle (58).

The development of the surfactant system is under multifactorial control. Extensive evidence from mammalian research suggests that glucocorticoids (2, 19, 46), thyroid hormones (1, 19, 54), autonomic neurotransmitters (20, 45), and pulmonary distension (28, 41) contribute to surfactant maturation. Glucocorticoids and thyroid hormones are thought to predominantly influence surfactant synthesis, thereby increasing the extracellular content of surfactant PLs (34). When administered together, glucocorticoids and thyroid hormones have a greater effect than either hormone administered alone (54). Autonomic neurotransmitters primarily influence surfactant secretion. Adrenergic agonists act directly on type II cells, through β-receptors, and activate a cAMP-dependent cascade of reactions (31). Cholinergic agonists are believed to act indirectly to stimulate surfactant secretion in eutherian mammals (6, 32). Recently, we discovered that glucocorticoids, thyroid hormones, and autonomic neurotransmitters also regulate PC secretion from type II cells isolated from the chicken (57), sea turtle (58), and the bearded dragon (57a). However, no one has determined whether changes in in vivo levels of glucocorticoids and thyroid hormones can affect surfactant development in nonmammals.

In newborn mammals, ventilation is one of the most important regulators of surfactant secretion (41, 48). This effect is partially mediated by the effect of circulating catecholamines (7); however, a single deep breath increases surfactant secretion in the rat lung (36). In rats, swimming also results in a stimulation of surfactant secretion within minutes (37). A direct mechanical stimulus is partially responsible for the increase in surfactant, as in vitro, physical stretch of isolated type II cells results in an increase in surfactant secretion equivalent to a combination of agonists (16, 66). In mammals, type II cells are preferentially located in corners and crevices in the lung, where they are exposed to the maximum amount of distortion (65). It is unknown if mechanical factors contribute to surfactant maturation in hatching reptiles.

As many aspects of the surfactant system are conserved among the vertebrates, here we hypothesize that the in ovo administration of glucocorticoids and thyroid hormones at various stages during late incubation will accelerate surfactant development in the saltwater crocodile, Crocodylus porosus. We also hypothesize that the increased maturation of the type II cells in response to hormone pretreatment will result in enhanced responsiveness of the cells to surfactant secretagogues. The current study is matched with that of Shepherdley et al. (this issue, 52), which provides a detailed analysis of the glucocorticoid, thyroid hormone, and deiodinase enzyme responses in developing crocodile embryos after hormone treatment. As the same individual animals were used in both studies and it is important to know how in ovo hormone treatment affected the hormonal environment of the lung and its cells, the levels of plasma hormones throughout development both before and after hormone treatment (from 52) are directly relevant to the present study. In control animals, plasma corticosterone increased between days 68 and 75 of incubation (from <1 to ~10 ng/ml blood) and decreased between day 75 and hatching (to ~4 ng/ml). Treatment of embryos with 3,5,5’-triiodothyronine (T3) increased corticosterone levels compared with control embryos on days 60 and 68, but not on day 75. Treatment of embryos with the synthetic glucocorticoid dexamethasone (Dex) suppressed plasma corticosterone on day 75 as did treatment with Dex + T3. In control embryos, plasma free T3 levels increased between day 75 and hatching (from ~0.5 to ~2.5 ng/ml). Treatment with T3 significantly increased free T3 levels in the plasma on days 68 and 75 of incubation, whereas treatment with Dex alone did not affect the concentration of free T3 in plasma on days 68 or 75. However, treatment with both Dex + T3 increased the concentration of plasma free T3 on day 75 of incubation (52). It is clear, therefore, that exogenous hormone treatment of the egg during the latter stages of development is capable of interacting with the hypothalamo-pituitary axis of the embryo to regulate endogenous hormone treatment. Furthermore, it is evident that there are complex interactions between the two hormone systems in the crocodile. In the present study we, therefore, examine both the appearance of surfactant PLs in the lungs and the secretion of PLs from isolated type II cells under basal and stimulated conditions.
conditions to understand the hormonal regulation of surfactant development in embryonic crocodiles. We also investigate the effect of a physical stressor after hatching (swimming) on the alveolar levels of PS.

MATERIAL AND METHODS

Animals. Two clutches of saltwater crocodile eggs were purchased from commercial farmers (Crocodylus Park, Darwin, Australia). The eggs were maintained at the crocodile farm at 32°C on wire racks for 40 days, then transported to Adelaide by air. On arrival, the eggs were weighed and buried in vermiculite (Peats, Adelaide, Australia) and moistened with water (1:1, vermiculite:water) in plastic containers (9 × 26 × 20 cm). A maximum of 10 eggs was placed in each container and incubated under normoxic conditions at 32°C in a constant temperature incubator. This temperature yields ~90% males and is considered the optimal constant incubation temperature (63). The containers were rotated throughout the incubator every second day, and the water content was adjusted twice weekly. The embryos were sampled after 60, 68, and 75 days of incubation and also 1 day after hatching. These time points correspond to Ferguson stages 25, 26, 27, and 28, respectively (17). At all time points, lung weights (postlavage) and embryo weights were recorded. Five or six individuals were used for each sampling time point under each condition. Representatives from both clutches were used at each time point under each of the conditions.

Pretreatment of eggs and embryos. Forty-eight and twenty-four hours before the sampling day, the embryos were pretreated with water-soluble Dex (2 × 50 μg, Sigma, Sydney, Australia), T3 (10 μg, Sigma, Sydney, Australia) or a combination of hormones (Dex + T3). The doses were based on hormone pretreatment of snapping turtle (40) and chicken eggs (13, 68) and scaled up according to the differences in body weights. Dex was dissolved directly in isotonic saline, 68) and scaled up according to the differences in body weights. T3 was dissolved in a small volume of 0.1 M NaOH before diluting in isotonic saline. A small hole was buried in vermiculite (Peats, Adelaide, Australia) and moistened with water (1:1, vermiculite:water) in plastic containers (9 × 26 × 20 cm). A maximum of 10 eggs was placed in each container and incubated under normoxic conditions at 32°C in a constant temperature incubator. This temperature yields ~90% males and is considered the optimal constant incubation temperature (63). The containers were rotated throughout the incubator every second day, and the water content was adjusted twice weekly. The embryos were sampled after 60, 68, and 75 days of incubation and also 1 day after hatching. These time points correspond to Ferguson stages 25, 26, 27, and 28, respectively (17). At all time points, lung weights (postlavage) and embryo weights were recorded. Five or six individuals were used for each sampling time point under each condition. Representatives from both clutches were used at each time point under each of the conditions.

Pretreatment of eggs and embryos. Forty-eight and twenty-four hours before the sampling day, the embryos were pretreated with water-soluble Dex (2 × 50 μg, Sigma, Sydney, Australia), T3 (10 μg, Sigma, Sydney, Australia) or a combination of hormones (Dex + T3). The doses were based on hormone pretreatment of snapping turtle (40) and chicken eggs (13, 68) and scaled up according to the differences in body weights. Dex was dissolved directly in isotonic saline, whereas the T3 was dissolved in a small volume of 0.1 M NaOH before diluting in isotonic saline. A small hole was drilled in the top of the crocodile eggshell using a dental drill and 50 μl of isotonic (0.15 M) saline containing the agonists was injected. Control animals were injected with 50 μl of saline alone. At day 60 it was necessary to draw off ~100 μl of albumin before the experimental 50 μl was injected. At the later time points, the albumin had decreased in volume to such an extent that this procedure was unnecessary. After the injection, the hole in the top of the shell was sealed with a drop of candle wax, and the eggs were returned to the incubator.

Posthatch swimming. One day posthatch, four crocodile hatchlings were placed individually into water heated to 30°C in a recirculating flume, set at a speed of 0.5 m/s (courtesy of R.V. Baudinet, Department of Environmental Biology, University of Adelaide, for details see Ref. 18). The crocodiles were allowed to swim until showing obvious signs of fatigue (4–5 min). The animals were removed from the water and were then immediately killed with an overdose of pentobarbital sodium (Lethabarb, 150 mg/kg body wt, Arnolds of Reading, Sydney, Australia). A blood sample (~100 μl) was taken from the postoccipital venous sinus for lactate analysis both before and after the swim from each crocodile.

Analysis of PLs in lavage. The embryos were removed from the shells (days 60, 68, and 75) and killed using an overdose of pentobarbital sodium (Lethabarb, 150 mg/kg body wt, Arnolds of Reading). A sample for plasma hormone analysis was taken from the postoccipital venous sinus (for more details of hormone measurements, see Ref. 52). The animals were then cannulated with a tracheal cannula. The lungs were filled to maximal volume (1.5–3.5 ml) with 0.15 M ice-cold NaCl instilled and withdrawn three times. The lavage was centrifuged at 150 g for 5 min to remove any macrophages and other cellular debris. The lavage was lyophilized and the lipids were extracted in chloroform:methanol (1:2) (5). The amount of total inorganic phosphorus in the samples was measured (3) and converted to a measure of total PL, given that PLs comprise 4% phosphorus. Disrupted PLs (DSP) and neutral lipids were separated by adsorption column chromatography after reaction with osmium tetroxide (30). DSP content was also measured using the method of Bartlett (3). Because total PL in lavage was so low at day 60, an analysis of DSP was not possible. To obtain a measure of micrograms PL per gram dry lung weight for comparison with previous studies, we multiplied values of micrograms PL per gram wet lung weight by 5.5 (11). This was necessary, as the lung tissue after lavage was used to isolate lung cells.

Cell culture and agonist stimulation. Type II cells and fibroblasts were isolated together and cultured at 30°C following previously published methods (58). Fibroblasts were necessary, as they are required for the glucocorticoid response (49). The tissue was dissociated in a solution of trypsin (0.1%, Commonwealth Serum Laboratories, Adelaide, Australia), EDTA (0.02%), and amphotericin B (10 μg/ml, Sigma) and DNase type I (1 mg/ml, Worthington Biochemicals, Freehold, NJ). The tissue was dispersed and filtered, and the resulting suspension was centrifuged. The cell pellet was resuspended in DMEM containing 24 mM NaHCO3 and 10 mM HEPES (Sigma). Immune cells and macrophages were removed from the mixture by differential adherence on rat IgG (Calbiochem, La Jolla, CA). The unattached cells were centrifuged, and the pellet was resuspended and incubated in DMEM containing 10% FBS, 24 mM NaHCO3, 25 U/ml penicillin, 25 μg/ml streptomycin, and 10 μg/ml amphotericin B (Sigma). At this time point, differential cell counts indicated that type II cells and fibroblasts were in a ratio of approximately 1:1. Therefore, this ratio is the same as used in other studies (38, 57, 58). Cells were diluted to 2.5 × 106 cells/ml (total number), plated at a density of 250,000 cells per well on 96-well culture plates, and cultured overnight in the presence of [methyl-3H]choline chloride (1 μCi/ml, Amersham Pharmacia, Sydney, Australia). This label is predominantly incorporated into PC (57). After ~18 h of incubation, an aliquot of cells from each animal was incubated in the presence of fresh DMEM alone (no FBS) for the determination of basal secretion levels for that animal. A number of other aliquots were incubated in the presence of agonists in the cell culture media. The agonists dissolved in DMEM were ATP (1 mM), epinephrine hydrochloride (100 μM), carbachol (100 μM), water soluble Dex (10 μM), T3 (100 nM), or a combination of Dex and T3 (10 μM Dex + 100 nM T3). The effect of ATP was not tested at day 60 as we were unable to isolate enough cells to perform all of the experimental manipulations. For the treatment groups, only the effect of Dex, T3, and epinephrine was tested (at the same concentrations) to determine if pretreatment of the embryos with Dex and T3 would change the cellular response to the hormones. Epinephrine was also tested as Dex and T3 have been reported to increase the number of adrenergic receptors. All drugs were obtained from Sigma. We previously used these agonists and doses to stimulate secretion in adult lizards and other non-eutherian vertebrates (39, 57, 58, 70, 71). After a 4-h incu-
Experimental Design

Materials and Methods

Materials

Animals

Results

Table 1. Body weight and wet lung weights of saltwater crocodile embryos and hatchlings (Crocodylus porosus)

<table>
<thead>
<tr>
<th>Incubation Day</th>
<th>Body wt (g)</th>
<th>Wet lung wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 60 (n = 6)</td>
<td>33.54 ± 1.12†</td>
<td>0.612 ± 0.04‡</td>
</tr>
<tr>
<td>Day 68 (n = 5)</td>
<td>55.71 ± 2.32‡</td>
<td>0.871 ± 0.06‡</td>
</tr>
<tr>
<td>Day 75 (n = 6)</td>
<td>84.22 ± 1.38†</td>
<td>0.815 ± 0.02†</td>
</tr>
<tr>
<td>Hatch (n = 9)</td>
<td>81.49 ± 1.07</td>
<td>0.791 ± 0.03†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE in g. Pairs of symbols indicate a significant difference between time points. †P < 0.001, ‡P < 0.001, §P < 0.05.

Table 2. Effect of in ovo Dex (2 x 50 µg), T3 (2 x 5 µg), or a combination of Dex + T3 (2 x 50 + 2 x 5 µg) on body weight and wet lung weights of saltwater crocodile embryos (Crocodylus porosus) at day 75

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Body wt (g)</th>
<th>Wet lung wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>84.22 ± 1.38†</td>
<td>0.815 ± 0.02‡</td>
</tr>
<tr>
<td>Dex (n = 6)</td>
<td>79.37 ± 1.33‡</td>
<td>0.897 ± 0.03‡</td>
</tr>
<tr>
<td>T3 (n = 6)</td>
<td>81.73 ± 0.82</td>
<td>0.851 ± 0.02†</td>
</tr>
<tr>
<td>Dex + T3 (n = 6)</td>
<td>78.67 ± 1.42†</td>
<td>0.826 ± 0.05†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE in g. Dex, dexamethasone; T3, 3,5,3′-triiodothyronine; Dex + T3, combination of Dex and T3. Pairs of symbols indicate a significant difference between time points. *P < 0.05, †P < 0.05, ‡P < 0.05.
ent hormone treatments is shown in Fig. 4, A-C. Cells demonstrate the same characteristics as control cells (Fig. 3), but a qualitative examination indicates that there is an increase in the number of lamellar bodies under all of the hormone pretreatments.

Cell culture. Trypan blue was excluded from >95% of the cells after the isolation period in all experiments. There was no detectable LDH release from either control or agonist-treated cells after the experimental period. After 15–18 h of incubation, differential cell counts indicated that fibroblasts and type II cells were

Table 3. Lavage analysis of total PL and DSP from hatching saltwater crocodiles, Crocodylus porosus, under control and swimming conditions

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Control (n = 5)</th>
<th>Swimming (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PL, g PL/mg wet lung wt</td>
<td>1.01 ± 0.16</td>
<td>1.07 ± 0.21</td>
</tr>
<tr>
<td>Total DSP, g DSP/mg wet lung wt</td>
<td>0.246 ± 0.03</td>
<td>0.251 ± 0.07</td>
</tr>
<tr>
<td>% DSP/PL</td>
<td>24.98 ± 1.33</td>
<td>22.35 ± 2.94</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. PL, phospholipid; DSP, disaturated PL.
cultured in a ratio of ~2:1. Therefore, this indicates that the fibroblasts were slowly dividing in culture. This proportion was maintained in all of the age groups. The secretion of PC was used as a general indicator of surfactant secretion. Basal PC secretion did not change between the different time points (Table 4, ANOVA \( P = 0.21 \)). For easy comparison, basal secretion for each time point was converted to 100; therefore any increase in secretion with addition of the agonists/hormones is represented by values over 100.

At day 60 in control animals, only carbachol significantly increased PC secretion over that of the wells containing media alone (basal secretion) (\( P < 0.01 \), Fig. 5A). At day 68, only epinephrine (\( P < 0.05 \)) and carbachol (\( P < 0.01 \), Fig. 5B) increased PC secretion over basal levels. However, ATP (\( P < 0.01 \)), epinephrine (\( P < 0.01 \)), carbachol (\( P < 0.01 \)), Dex (\( P < 0.05 \)), \( T_3 \) (\( P < 0.05 \)), and a combination of Dex and \( T_3 \) (\( P < 0.001 \)) all increased PC secretion over basal levels at day 75 (Fig. 5C). After hatching, only ATP (\( P < 0.05 \)), epinephrine (\( P < 0.001 \)), and Dex + \( T_3 \) (\( P < 0.05 \)) increased PC secretion over basal levels (Fig. 5D). The effect of carbachol on isolated cells was significantly greater at day 60 than after hatching (\( P < 0.01 \)). The effect of Dex at day 75 was also significantly higher than that after hatching (\( P < 0.05 \)). In addition, the effect of Dex + \( T_3 \) was significantly higher at day 75 than at day 68 (\( P < 0.05 \)).

**Effect of hormone treatment on PC secretion.** At day 60, only cells isolated from animals pretreated with Dex + \( T_3 \) increased PC secretion over basal levels in response to epinephrine, Dex, and \( T_3 \) (\( P < 0.05 \)) (Fig. 6A). Furthermore, when comparing between treatment groups, the response of the cells to epinephrine was significantly greater in the Dex + \( T_3 \) pretreatment group compared with the response of cells from both control animals and those pretreated with Dex only. There were no other significant between-group comparisons at day 60. At day 68, cells isolated from all treatment groups increased PC secretion in response to epinephrine. Cells isolated from animals pretreated with Dex also increased PC secretion in response to \( T_3 \) (\( P < 0.05 \)). Also at day 68, cells isolated from animals pretreated with Dex + \( T_3 \) increased PC secretion in response to both Dex and \( T_3 \) (\( P < 0.05 \)) (Fig. 6B). At day 75, cells isolated from control, Dex, and Dex + \( T_3 \) increased PC secretion in response to all the agonists (\( P < 0.05 \)). Cells isolated from the \( T_3 \) pretreatment group increased PC secretion in response to epinephrine and \( T_3 \) (\( P < 0.05 \)), but not to Dex (Fig. 6C). However, both at day 68 and at day 75, there were no significant between-group differences.

---

**Fig. 3.** Appearance of representative type II cells isolated from embryos of the saltwater crocodile, *Crocodylus porosus* (control animals), after 60 (A), 68 (B), and 75 (C) days of incubation and after hatching (D). All cells are cuboidal in shape, have a large nucleus, and demonstrate characteristic lamellar bodies and microvilli. A qualitative examination indicates an increase in lamellar body number with advancing incubation (scale bars = 2 μm).
DISCUSSION

Development of surfactant PLs in crocodiles. At equivalent time points, the amount of total PL and DSP in lavage (expressed as a ratio of dry lung weight) were similar to the levels found in the bearded dragon (26), but much lower than those found in embryonic green sea turtles (25). However, the pattern of development of the PLs is similar in all three species, in that the amounts of both total PL and DSP in lavage increase dramatically after the onset of air breathing. This increase in both total PL and DSP after the onset of hatching correlates with a dramatic increase in plasma T3 levels (52). It is possible that the elevated T3 levels stimulated the secretion of lipids into the lungs, although this is unlikely, as T3 did not stimulate secretion from type II cells isolated from hatchlings (Fig. 5D). Alternatively, T3 stimulates the maturation and/or synthesis of β-receptors (14, 64), which are then stimulated by increased catecholamines at hatching to increase surfactant secretion into the lung.

In the saltwater crocodile, DSP/PL increased rapidly to maturity, between days 68 and 75. This increase correlates exactly with an increase in circulating corticosterone levels in the crocodile embryos (52) and appears to indicate that corticosterone is responsible for increasing surfactant synthesis (i.e., increased PL saturation). This is also supported by the observation that at day 68, Dex pretreatment resulted in an increase in the number of lamellar bodies.

Effect of hormone treatment on surfactant PLs. This is the first study to demonstrate that the in ovo administration of hormones can influence the amount of alveolar surfactant in nonmammals. Treating chick embryos with thyroxine (67) and Dex (68) increases the

Table 4. Percent basal secretion of [%3H]PC from cultured type II cells isolated from embryonic and hatchling saltwater crocodiles, Crocodylus porosus

<table>
<thead>
<tr>
<th>Incubation Day</th>
<th>Day 60 (n = 5)</th>
<th>Day 68 (n = 5)</th>
<th>Day 75 (n = 6)</th>
<th>Hatch (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% [%3H]PC secretion</td>
<td>2.259 ± 0.357</td>
<td>2.404 ± 1.085</td>
<td>0.914 ± 0.190</td>
<td>1.958 ± 0.234</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. [%3H]phosphatidylcholine (%3H]PC) secretion from the type II cells was calculated on the basis of the amount of [%3H]PC in the media, expressed as a percentage of total [%3H]PC (i.e., the amount of [%3H]PC in the media plus the cellular fraction).
synthesis of PC in whole lung homogenates. However, whether the hormones increased surfactant pools was not determined in these studies. Administration of both Dex and T₃ increased the total PL content in the lavage of the saltwater crocodile, but this effect is dependent on gestational age. At days 60 and 75, both T₃ and Dex + T₃ increased total PL, whereas at day 75, Dex alone also increased total PL. It is possible that the T₃ effect is mediated by corticosterone, because the major effect of T₃ administration at days 60 and 68 was the increase in circulating corticosterone (52). However, at day 75, when endogenous corticosterone levels are already extremely elevated, T₃ did not elevate corticosterone any further. Hence, at this time point, T₃ does appear to have a specific effect. At day 75, the administration of Dex caused endogenous corticosterone production to be completely abolished (52). Hence, the effect of increased PL content in lavage at this time point is directly mediated by Dex. Glucocorticoids probably primarily act to increase lipid synthesis, thereby increasing the concentration of both tissue and alveolar surfactant. This effect may be achieved primarily by stimulation of enzymes regulating PL synthesis (34). Thyroid hormones have also been reported to increase the activities of the PL synthesizing enzymes (21).

It is clear that the administered hormones have variable responses at the different stages of incubation, as is also the case in the fetal sheep (22) and rat (47). We speculate that this effect may be the result of the natural variation in maturation rates of different receptors and changes in the rates of PL production throughout incubation. As the amount of total PL in the lungs was extremely low at day 60, any effects of the agonists would be easier to detect. By day 75, the number of receptors on the type II cells may have greatly increased, therefore amplifying the effect of the hormones. Control animals have similar amounts of total PL in their lungs at both days 68 and 75. However, the hormone pretreatments were only able to increase the amounts of total PL in the lavage of the crocodile at day 75. It is possible that, although the amounts of PL are stable between days 68 and 75, there is a rapid maturation of the receptors on type II cells between these time points, thereby making the system more responsive to the administered hormones at day 75.

Whereas the hormone pretreatments did not influence total PL at day 68, they had a profound effect on the relative saturation of the PLs. Both T₃ and Dex + T₃ significantly increased the saturation of the lipids at this time. It has been reported that glucocorticoids and
thyroid hormones increase the percentage of saturated PC in the lung (34). Although the hormones increased PL saturation at day 68, there was no further increase in day 75 animals. Furthermore, from the evidence presented, we hypothesize that the hormones have different roles at different stages in the developmental period. Hence, we suggest that day 68 is primarily a stage of surfactant synthesis and day 75 is a stage of surfactant secretion. Therefore, at day 68, the hormones increased surfactant lipid saturation, whereas at day 75 they increased the secretion of surfactant into the air space.

**Effect of swimming exercise on surfactant PLs.** Post-hatching swimming did not alter the amount or saturation of the surfactant PLs. This is despite the finding that swimming increases surfactant secretion in rats (37), presumably by increases in ventilation. However, swimming is not a normal activity performed by rats, whereas hatching crocodiles can swim within minutes of hatching. Therefore, the large amount of surfactant PL present in the lungs of hatching crocodiles may already prepare the animal for vigorous activity. It is also possible that hatching crocodiles have an already greatly increased surfactant pool and that further increases in response to exercise are not necessary at this age. It is possible therefore that adult crocodiles may respond to swimming exercise by increasing their surfactant pool. However, in mammals, lungs are sensitive to distortion at all ages, as surfactant secretion is stimulated by ventilation in both adult (37) and newborn mammals (28, 41). In contrast to mammals, changes in breathing pattern do not appear to mediate surfactant release in lizards (69). Given that the saltwater crocodile can experience long and variable nonventilatory periods (53), breathing parameters may not be an appropriate regulator of surfactant secretion in this species.

**Cell culture.** The exclusion of trypsin blue and the lack of LDH release indicate that neither the culture period nor the pharmacological agents affected cell viability. In addition, the cell morphology was maintained after the culture period. Therefore, any increase in PC secretion during the culture period was directly due to the effect of the agonists in the cell culture medium.

The culture of type II cells was performed in the presence of fibroblasts. As glucocorticoids have a minimal effect on isolated type II cells (44, 53, 59), it is proposed that they act through interstitial fibroblasts. Post et al. (44) isolated a factor produced from fibroblasts, which they termed fibroblast-pneumocyte factor (FPF), that increases PC production in fetal type II cells. The release of FPF from interstitial fibroblasts is believed to be mediated by glucocorticoids. The factor is proposed to act ultimately at the level of the type II cell by stimulating the activity of choline-phosphate cytidylyltransferase (44). Therefore, fibroblasts were cultured with type II cells in this study, as they are believed to be required for the glucocorticoid response. We used the natural ratios of these two cell populations that were yielded on isolation as the minimal ratio of fibroblasts to type II cells required to elicit the glucocorticoid response. We used the natural ratios of these two cell populations that were yielded on isolation as the minimal ratio of fibroblasts to type II cells required to elicit the glucocorticoid response. This ratio would likely also be species dependent. Furthermore, the amount of surfactant produced from type II cells is enhanced in the presence of fibroblasts (51, 57). This may be due to the release of factors from fibroblasts that stimulate surfactant synthesis or because type II cells tend to aggregate into alveolar-like structures when in the presence of fibroblasts (51). However, fibroblasts are known to divide in culture and may
therefore overgrow the type II cells. However, our results indicate only a single round of division in the 15–18 h attachment period. This result is confirmed in another study, which reported only the slow growth of fibroblasts in a similar coculture system over a 5-day period (51). However, as type II cells are the surfactant-producing component of this cell culture system, the discussion will focus on the release of surfactant from type II cells.

In control animals, basal secretion did not change significantly between the different time points. Similarly, Griese et al. (20) found that the level of basal PC secretion does not change in type II cells isolated from fetal, newborn, and adult rats. This implies that the dramatic increase in extracellular surfactant pools seen after hatching in the crocodile (and also birthing in the rat) is due to factors other than the constitutive secretion of surfactant PLs from the type II cells. Given the potent effect of the agonists in the culture medium on PC secretion and that all three hormone pretreatments increased total PL at day 75 of incubation, it is likely that an increase in circulating levels of hormone (predominantly T3 in the crocodile as this was elevated at hatching (52)) or neurotransmitter provide the stimulus for surfactant secretion before hatching. A change in the response of type II cells to surfactant secretagogues is also seen in the rat (20). In the rat, the developmental change in secretory response is agonist specific, which implies that the different pathways mature at different rates. It also appears that the adrenergic pathway matures slightly before other signaling pathways in the saltwater crocodile. Likewise, the full response to adrenergic agonists is reached 7 days after birth in the rat, whereas the response to ATP does not reach that of adult cells until 14 days after birth (20). However, in the saltwater crocodile, the response peaked at day 75 for all the other agonists. This may be explained by the rapid maturation of the surfactant system in the crocodile between day 75 (94% of total incubation) and hatching, requiring synchronization of signal transduction pathways. It is likely that this rapid maturation and high cellular responsiveness at day 75 is mediated by corticosterone, because circulating levels of this hormone are highest at day 75 (52).

The analysis of lavage PLs indicated that pretreatment with T3 and Dex + T3 increased the amount of PLs in lavage at day 60, yet treating the cells with the agonists in the cell culture media did not increase surfactant secretion. This discrepancy may be due to the 4-h time period over which the agonists were in the culture media. The pretreatment of the eggs occurred 48 h before lavage and, therefore, would allow a much greater period of time for both synthesis and secretion of surfactant. Alternatively, the in ovo administration of the hormones may have other effects on the embryo and may, therefore, be indirectly influencing the secretion of surfactant. For example, the administration of the hormones may lead to the release of epinephrine or norepinephrine from the adrenal medulla, which then act to increase surfactant secretion.

Carbachol stimulated PC secretion from cells at all prehatch stages, most significantly at day 60, but not after hatching. The role of cholinergic agonists is proposed to increase surfactant production in heterothermic animals at low body temperatures (71). This acts to maintain lung and surfactant function without an increase in metabolic rate. The saltwater crocodile experiences significant fluctuations in daily body temperatures, particularly in smaller individuals (50). In fact, the saltwater crocodile has a lower and more variable body temperature than most Crocodilia (even lower than the American alligator, A. mississippiensis (27)).

It is not known why carbachol did not increase PC secretion after hatching. However, it is possible that the response to carbachol is temperature dependent as it is in adult bearded dragons (70). Carbachol increases the amount of PL secreted from type II cells isolated from the bearded dragon at 18°C, but not at 37°C (70). Therefore, the cells from hatching crocodiles may not have increased PC secretion in response to carbachol as the culture temperature used in this experiment was relatively warm (30°C). Alternatively, the mechanism of “thermal switching” may not develop until after hatching, which may explain why a response to carbachol was observed at all prehatching time points and was most pronounced at the earliest age.

Effect of hormone treatment on PC secretion. Pretreatment of saltwater crocodiles with Dex, T3, or Dex + T3 changed the secretion profiles of the type II cells. We found that only the cells isolated from the Dex + T3 pretreatment group demonstrated a response to epinephrine at day 60. An epinephrine response at this stage of development was absent in control animals. Furthermore, a significant between-group pretreatment effect of Dex + T3 compared with control and Dex alone suggests that pretreatment of the embryo with the combination of hormones enhanced the responsiveness of the isolated cells to epinephrine stimulation. Glucocorticoids and thyroid hormones increased β-receptor density on type II cells isolated from fetal lambs (61). It is possible, therefore, that the combination of Dex and T3 increased the number of adrenergic receptors on the type II cells of the saltwater crocodile, thereby accelerating the maturation of the β-adrenergic pathway and yielding a secretory response to epinephrine at day 60. Pretreatment of animals at days 60 and 68 with Dex + T3 also caused an increase in PC secretion in response to Dex and T3 added to the cell culture medium when compared with the within-group basal secretion levels. However, the between-group comparisons failed to indicate a significant pretreatment effect on the responsiveness of the cells to the hormones. This lack of response may be an artifact of small sample size and/or high variation between individuals, such that elevated secretions can occur in individual groups compared with their own controls, but this difference is not evident in the between-group comparisons. However, despite these constraints, it is possible that the pretreatments cause different responses to agonists at different times. In particular, it appears that pretreatment of the embryo...
has little effect at later stages of development, but the altered cellular responses to epinephrine (Fig. 6A) suggest that pretreatment with hormones may uncouple developmental processes before day 60. This style of experiment is worthwhile pursuing with larger sample sizes and earlier time points. A further example of the apparently enhanced pretreatment effect early during development is evident when comparing the response of the cells to Dex and T3 in culture between the different age groups. As Dex and T3 in the culture medium increased PC secretion only at day 75 in control animals, it appears that the hormone pretreatment at days 60 and 68 enhanced the development of the hormonal signaling pathways. Dex increases the response of type II cells to the surfactant secretagogues terbutaline, ATP, UTP, and N-ethylcarboxymidoadenosine (23). As Dex increased the response to a number of secretagogues, it is likely that Dex affects signaling events downstream of the receptor level (23).

In conclusion, this is the first study to demonstrate that in vivo glucocorticoid and thyroid hormone levels can influence the pattern of surfactant development in reptiles. Furthermore, the exogenous administration of hormones early during development (before 75–80%) is capable of enhancing the maturation rate of cellular components and processes. Large-scale changes in surfactant pools were detected by lavage analysis and more subtle effects were detected by a change in PC secretion from type II cells. Although further research is required to elucidate the mechanisms involved in the control of surfactant development in reptiles, the use of such animals may provide a novel approach to surfactant research, as many aspects of the surfactant system, including composition, function, and control are highly conserved among the vertebrates. Further advantages of these animals for models in surfactant research include the fact that the egg and embryo provide a closed system that is more easily controlled and manipulated than a mammalian mother and fetus.

The authors gratefully acknowledge C. Shepherdly, N. Miller, J. Griffiths, S. Munns, and P. Wood for technical assistance. Crocodile eggs were exported (#9652), imported (#4243), and kept (Q20168) under permits from National Parks and Wildlife. Animal associations with the Conservation Commission of the Northern Territory, edited by Webb GJW, Manolis SC, and Whitehead PJ. Chipping Norton: Surrey Beatty and Sons Pty Ltd in association with the Conservation Commission of the Northern Territory, 1987.

REFERENCES


57a. Sullivan LC, Orgeig S, and Daniels CB. Regulation of pulmonary surfactant secretion in the developing lizard, Pogona vitticeps. Comp Biochem Physiol A In press.


