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Influence of Aminophylline and Cyclic AMP on Glycogen Metabolism in Fetal Rat Lung in Organ Culture

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Summary

The glycogen content of fetal rat lung declines coincident with increased pulmonary phospholipid synthesis. Aminophylline, a methylxanthine cyclic adenosine 3',5' monophosphate (AMP) phosphodiesterase inhibitor, and cyclic AMP augment fetal lung phospholipid synthesis. Because lung glycogen breakdown may contribute to pulmonary phospholipid synthesis, the effects of aminophylline and cyclic AMP on glycogen metabolism were studied in explants of 19 day fetal rat lung in organ culture. Treatment with aminophylline or dibutyryl cyclic AMP for 24 hr, resulted in a 25% ($P < 0.025$) and 75% ($P < 0.001$) decrease, respectively, in the glycogen conent of the explants. Glycogen synthase I activity was reduced by 32% in aminophylline treated cultures ($P < 0.025$) and 25% in cyclic AMP treated cultures ($P < 0.025$). The percent of total synthase in the active form was significantly reduced in all treated cultures. Neither aminophylline nor cyclic AMP treatment resulted in significant changes in glycogen phosphorylase a or total phosphorylase activity.

Speculation

The enhancement of fetal pulmonary surfactant by cyclic AMP and aminophylline may be due, in part, to the effects of these agents on lung carbohydrate metabolism.

Pulmonary surfactant is the phospholipid rich material which lines the alveoli of the lungs, preventing collapse due to surface tension at the air-alveolar interface (30). As the fetal lung develops late in gestation, there is increased synthesis of pulmonary surfactant while the epithelial cell glycogen content decreases (4, 14, 18, 31). The role of glycogen in fetal lung maturation is not clear. The temporal relationship between glycogen depletion and the acceleration of surfactant synthesis suggests that glucose derived from lung glycogen breakdown may contribute to pulmonary phospholipid synthesis. It is known that glucose is actively incorporated into surfactant phospholipids in both adult and fetal lung slices (7, 9, 19). Furthermore, corticosteroid administration to the fetus both augments fetal lung surfactant synthesis (22) and decreases glycogen content (7, 13).

Theophylline, a methylxanthine cyclic adenosine 3'5' monophosphate phosphodiesterase inhibitor, increases intracellular cAMP content (21) and also stimulates fetal lung surfactant synthesis (1, 12). Aminophylline is the ethylene diamine salt of theophylline. It has been previously demonstrated that aminophylline and cyclic AMP augment choline incorporation into phosphatidylcholine in explants of fetal rat lung in organ culture (8). Theophylline and cyclic AMP have also been shown to stimulate glycogen breakdown in fetal liver (25, 26) and adult muscle and liver (11, 21). Because glucose derived from lung glycogen breakdown may contribute to fetal lung surfactant synthesis, the effects of aminophylline and cyclic AMP on glycogen metabolism in explants of fetal rat lung have been examined.

MATERIALS AND METHODS

CULTURE CONDITIONS

Lung explants were prepared as described previously (8). Timed 19-day pregnant Sprague-Dawley rats (term is 22 days) were killed by a blow to the head. The fetuses were removed under sterile conditions and the lungs were removed from the fetuses, pooled, and chopped into 1 mm^3 blocks by a mechanical tissue chopper. Approximately 20 explants were placed on a sterile millipore filter supported on a stainless steel grid in a plastic tissue culture dish. Waymouth's MB 752/1 medium (5.5 ml) was added to each culture dish so as to just wet the filter. The undersurface of the explants were, thus, exposed to the medium and the upper surface to the environment. The explants were incubated for 24 hr at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Experimental cultures were incubated in medium to which aminophylline, 0.1–5.0 mM, or dibutyryl cyclic AMP, 0.2 mM, had been added. This concentration of dibutyryl cyclic AMP was chosen because it resulted in maximal stimulation of choline incorporation into phosphatidylcholine in previous lung explant experiments (8). After 24 hr in culture, the explants were rapidly frozen in test tubes in a dry ice and ethanol bath and stored at −70°C until analyzed.

GLYCOGEN AND PROTEIN ANALYSIS

Glycogen concentration was determined by the enzymatic fluorometric method of Lowry and Passonneau (16) in which NADPH production is measured after the tissue sonicate is treated with glycogen phosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase (GGPD). Approximately 30 explants were sonicated in 0.2 ml cold H_2O. An aliquot of the sonicate was combined with an equal volume of 0.04N HCl and heated to 100°C for 10 min. The glycogen content of this sample was determined in medium which contained 50 mM imidizole buffer (pH 6.9), 3.0 mM KH_2PO_4, 30 mM NaF, 0.2 mM EDTA, 0.5 mM adenosine-5'-phosphate, 0.9 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.02% bovine serum albumin, 60 uM NADP, 0.5 uM glucose-1, 6-diphosphate, 0.09 U/ml G6PD, 0.45 U/ml phosphoglucomutase, and 0.25 U/ml rabbit muscle glycogen phosphorylase a in a final volume of 2.2 ml. The reaction was initiated by the addition of 0.05 ml of diluted tissue sonicate. The amount of NADPH generated was determined in a Perkin-Elmer fluorimeter (Perkin-Elmer, Norwalk, CT) when the reaction was complete, generally after 20–25 min at 20°C. Sample values were compared to a standard curve derived from rabbit muscle glycogen.

Protein concentration was determined by the method of Lowry *et al.* (17) using bovine serum albumin as the standard.

ENZYME ANALYSIS

Glycogen synthase (EC 2.4.1.11) activity of the explants was assayed by measuring the rate of incorporation of UDP[U-^{14}C] glucose into glycogen as described by Eisen *et al.* (6) after optimal pH and substrate concentrations were established. The explants were sonicated in buffer containing 20 mM glycylglycine, pH 7.5, 100 mM NaF, 3.0 mM dithiothreitol, and 5.0 mM EDTA. The final assay medium for glycogen synthase I contained 10 mM UDP[U-^{14}C]glucose (8.0 Ci/mole), 5 mg/ml glycogen, and 100 mM Tris-HCl, pH 7.2, in a final volume of 50 μl. The reaction was initiated by the addition of the sonicate, which contained approximately 0.2 mg protein. For determination of total glycogen synthase (I + D) activity, the final assay medium also contained 10 mM glucose-6-phosphate, and the specific activity of the UDP[U-^{14}C]glucose was 2.0 Ci/mole. After incubation for 10 min at 30°C, aliquots of the reaction mixture were spotted on filter paper discs (Whatman #31) for isolation of glycogen by the method of Thomas *et al.* (29). The filter paper discs were washed four times in 66% ethanol, rinsed in acetone, and allowed to dry. Radioactivity of the glycogen precipitate was determined in 10 ml of Econofluor (New England Nuclear, Boston, MA) in a scintillation spectrometer. The reaction was linear with protein concentration up to at least 0.5 mg per assay and with time for at least 30 min. Activity of glycogen synthase D was calculated as the difference between glycogen synthase I and total glycogen synthase (I + D) activities.

Glycogen phosphorylase (EC 2.4.1.1) activity of the explants was assayed by measuring the rate of generation of NADPH in an assay mixture which contained an excess of glycogen, phosphoglucomutase, and G6PD, by a modification of the method of Schwartz (25). Approximately 30 explants were sonicated in 0.20 ml of ice cold 75 mM NaF, 5.0 mM EDTA, pH 6.7. Activity of glycogen phosphorylase a was determined in medium containing 50 mM imidazole buffer, pH 6.9, 3.0 mM KH_2PO_4, 30 mM NaF, 0.2 mM EDTA, 0.7 mg/ml glycogen, 0.5 uM glucose-1, 6-diphosphate, 0.9 mM $MgCl_2$, 60 uM NADP, 0.09 U/ml G6PD 0.45 U/ml phosphoglucomutase, 0.4 mM caffeine, and tissue sonicate containing approximately 100 ug protein in a final volume of 2.2 ml. The reaction was initiated by the addition of the tissue sonicate, and the rate of generation of NADPH was measured fluorometrically at 20°C. Total glycogen phosphorylase (a + b) activity was assayed in medium without caffeine, but containing 1.5 mM adenosine-5′-phosphate. The reactions were linear with protein concentration up to at least 180 ug per assay and with time for at least 30 min. Saturating amounts of all substrates were used.

GLUCOSE OXIDATION

After the 24 hr culture period the rate of oxidation of [U-^{14}C] glucose to $^{14}CO_2$ was measured in the experimental and control explants. The culture medium was aspirated and replaced by Krebs-Ringers-phosphate solution (pH 7.4) containing 10 mM [U-^{14}C]glucose (specific activity 0.075 Ci/mole). The culture flask was placed in a sealed glass dish with side arm and a plastic well. The dish was flushed with 100% O_2 for 1 min and the side arm sealed. After a 2¼ hr incubation at 37°C, 0.3 ml hyamine hydroxide was injected into the plastic well and the incubation continued for another ¾ hr. $^{14}CO_2$ dissolved in the medium was released by acidification. The radioactivity of the hyamine hydroxide was determined in Econofluor.

All biochemicals were purchased from Sigma, St. Louis, MO and all radiochemicals were obtained from New England Nuclear, Boston, MA.

Table 1. *The influence of aminophylline and dibutyryl cyclic AMP on the glycogen content of fetal rat lung in organ culture*[1]

Experiment	Glycogen content (μg/mg protein)	Treated / Control	*P*
Aminophylline			
0	165.8 ± 7.7		
0.1 mM	169.7 ± 15.9	1.01 ± 0.08	ns
0.5 mM	156.0 ± 33.1	0.99 ± 0.25	ns
1.0 mM	126.0 ± 9.4	0.76 ± 0.04	<0.025
5.0 mM	251.0 ± 22.9	1.50 ± 0.14	<0.010
Dibutyryl cyclic AMP:			
0	173.1 ± 7.0		
0.2 mM	43.9 ± 2.6	0.25 ± 0.01	<0.001
Fresh 19-day lung:	236 ± 11.9		

[1] Explants from 19 day fetal lungs were incubated for 24 hr as described in the text. The values represent the mean ± SE of 3–4 experiments. Data from aminophylline and dibutyryl cyclic AMP treated explants were compared to those from untreated explants by *t* test for independent variables (ns = not statistically significant).

RESULTS

As is shown in Table 1, there was a significant decrease in the glycogen content of the explants cultured in the presence of 1.0 mM aminophylline as compared to controls. There was also a 75% decrease in the glycogen content of explants cultured in 0.2 mM dibutyryl cyclic AMP. At high doses of aminophylline (5.0 mM), the glycogen content was, however, greater than in the control cultures, but not different from fresh uncultured 19-day fetal lung. In organ culture, nutrients and pharmacologic agents reach the cells of the explant by diffusing through a block of tissue, which in this study, was 1 mm thick. It has been a common finding (8, 24) that relatively high concentrations of these substances are required for organ culture experiments and, for this reason, it is probably not appropriate to relate the drug levels used in this study to those that are used therapeutically.

Activity of glycogen synthase I, the active form of the enzyme, was significantly less in the explants cultured with 1.0 mM aminophylline (Table 2). The percent of total glycogen synthase activity which was in the I form was also significantly less in the treated cultures. The activities of glycogen synthase D and total glycogen synthase (I + D) however, were unchanged. Essentially the same results were obtained when the explants were cultured in the presence of 0.2 mM dibutyryl cyclic AMP (Table 3).

Table 2. *The influence of 1.0 mM aminophylline on glycogen synthase activity*[1]

Enzyme[2]	(*n*)	Control	Aminophylline	Aminophylline / Control	*P*
Synthase I	(5)	323 ± 26	218 ± 28	0.67 ± 0.05	<0.025
Synthase D[3]	(5)	2831 ± 219	2905 ± 268	1.02 ± 0.05	ns
Total Synthase	(5)	3154 ± 214	3128 ± 288	0.99 ± 0.05	ns
I: total (%)	(5)	10.5 ± 1.1	7.0 ± 0.6	0.68 ± 0.05	<0.025

[1] Culture conditions and statistical analysis were as described in Table 1.

[2] Enzyme activity is expressed as pmole/min/mg protein.

[3] Synthase D activity was calculated as the difference between total synthase and synthase I activity.

The activities of glycogen phosphorylase a, the active form of the enzyme, total glycogen phosphorylase and the percentage of total activity in the active form were not affected by exposure to 1.0 mM aminophylline (Table 4). (Glycogen phosphorylase b activity was decreased by 24% in these explants.) Similar results were obtained when the explants were exposed to 0.2 mM cyclic AMP (Table 5).

The glycogen content of the control explants after 24 hr of culture was lower than the glycogen content of fresh uncultured 19 day lung (Table 1). This decrease in glycogen content was associated with a significant increase in the activities of glycogen phosphorylase a and total glycogen phosphorylase in the explants, while the activity of glycogen synthase I was unchanged. In order to investigate the observation that explants cultured in 5.0 mM aminophylline had a glycogen content similar to that of fresh 19-day lung, the activities of glycogen synthase and phosphorylase were examined after exposure to this dose of the drug. The explants had decreased activity of glycogen synthase I (174 pmole/min/mg protein) while total glycogen synthase activity (3139 pmole/min/mg protein) was not changed. There was also, however, decreased activity of both glycogen phosphorylase a and total glycogen phosphorylase. (2.08 nmole/min/mg protein and 3.79 nmole/min/mg protein, respectively.) The fraction of total phosphorylase in the active form in these explants was less than in control cultures (55%). Thus, the relative increase in glycogen content observed after exposure to this presumably toxic dose was probably due to the inhibition of glycogen phosphorylase activity.

Production of $^{14}CO_2$ from [U-^{14}C]glucose was measured to compare the overall viability of the aminophylline treated and control explants. As is shown in Table 6, exposure to 1.0 mM aminophylline resulted in twice the rate of CO_2 production from glucose.

Theophylline itself directly affects *in vitro* assays of glycogen phosphorylase (28) and glycogen synthase (5), probably by direct effects on the enzyme. To rule out the possibility that the changes in enzyme activities were due, in part, to direct *in vitro* effects of aminophylline which may have been transferred with the tissue into the assay medium, we performed a series of assays with varying amounts of added aminophylline. No direct effect of aminophylline on the activities of glycogen phosphorylase or synthase was noted.

DISCUSSION

It has been demonstrated that cyclic AMP and aminophylline, agents which enhance fetal lung surfactant production, also decrease the glycogen content of fetal rat lung explants. The decreased glycogen content correlated with decreased glycogen synthase I activity, but no significant stimulation of phosphorylase activity was found. In adult muscle and liver, glycogen breakdown is regulated by a complex series of reactions which result in activation of glycogen phosphorylase and inactivation of glycogen synthase (10, 27). This series of reactions may be initiated by activation of a cyclic AMP dependent protein kinase. In fetal liver explants, Sherline *et al.* (26) reported that acute treatment with cyclic AMP resulted in increased glycogen phosphorylase activity and decreased glycogen synthase activity, suggesting that regulation of glycogen metabolism in fetal and adult liver may be similar. Schwartz and Rall (25), however, found that whereas cyclic AMP treatment decreased the glycogen content of fetal rat liver explants, this effect was mediated only by decreased glycogen synthase I activity and not by increased phosphorylase activity. The authors' results with fetal lung explants are, thus, similar to those of Schwartz and Rall. The decreased glycogen content in

Table 3. *The influence of 0.2 mM dibutyryl cyclic AMP on glycogen synthase activity*[1]

				Cyclic AMP	
Enzyme[2]	(*n*)	Control	Cyclic AMP	Control	*P*
Synthase I	(4)	295 ± 24	217 ± 7	0.75 ± 0.05	<0.025
Synthase D[3]	(4)	2474 ± 212	2851 ± 177	1.16 ± 0.06	ns
Total synthase	(4)	2770 ± 204	3068 ± 178	1.11 ± 0.04	ns
I: Total (%)	(4)	10.9 ± 1.4	7.2 ± 0.4	0.68 ± 0.08	<0.05

[1] Culture conditions and statistical analysis were as described in Table 1.
[2] Enzyme activity is expressed as pmole/min/mg protein.
[3] Synthase D activity was calculated as the difference between total synthase and synthase I activity.

Table 4. *The influence of aminophylline on glycogen phosphorylase activity*[1]

				Aminophylline	
Enzyme[2]	(*n*)	Control	Aminophylline	Control	*P*
Phosphorylase a	(5)	3.81 ± 0.03	3.89 ± 0.19	1.04 ± 0.07	ns
Phosphorylase b[3]	(5)	1.97 ± 0.12	1.45 ± 0.12	0.76 ± 0.10	<0.025
Total Phosphorylase	(5)	5.79 ± 0.11	5.34 ± 0.23	0.92 ± 0.03	ns
a: Total (%)	(5)	65.6 ± 3.0	72.8 ± 1.9	1.12 ± 0.06	ns

[1] Culture conditions and statistical analysis were as described in Table 1.
[2] Enzyme activity is expressed as nmoles NADPH generated/min/mg protein.
[3] Phosphorylase b activity was calculated as the difference between total phosphorylase and phosphorylase a activity.

Table 5. *The influence of 0.2 mM dibutyryl cyclic AMP on glycogen phosphorylase activity*[1]

				Cyclic AMP	
Enzyme[2]	(*n*)	Control	Dibutyryl cyclic AMP	Control	*P*
Phosphorylase a	(4)	3.38 ± 0.10	3.84 ± 0.24	1.14 ± 0.10	ns
Phosphorylase b	(4)	1.96 ± 0.03	1.81 ± 0.12	0.93 ± 0.07	ns
Total phosphorylase	(4)	5.34 ± 0.08	5.65 ± 0.13	1.06 ± 0.04	ns
a: Total (%)	(4)	63.1 ± 1.11	67.9 ± 2.7	1.08 ± 0.06	ns

[1] Culture conditions and statistical analysis were as described in Table 1.
[2] Enzyme activity is expressed as nmoles NADPH generated/min/mg protein.

Table 6. *The influence of 1.0 mM aminophylline on the rate of glucose oxidation to CO_2*[1, 2]

			Aminophylline	
(*n*)	Control	Aminophylline	Control	P
(3)	9.8 ± 0.1	20.6 ± 0.3	2.10 ± 0.01	<0.001

[1] Culture conditions and statistical analysis were as described in Table 1.

[2] Data is expressed as nmoles glucose oxidized/hour/mg protein.

these explants is probably the result of inhibition of glycogen synthase activity. Because glycogen phosphorylase activity was greater in control explants than in fresh uncultured lung, further activation with aminophylline or cyclic AMP may not be possible. Hess *et al.* (11) have also noted failure to increase phosphorylase a activity with theophylline in the rat diaphragm.

The function of glycogen in fetal lung is obscure, but its developmental pattern in late gestation lung (4, 18) suggests that it may contribute to pulmonary surfactant synthesis. Glucose derived from prenatal lung glycogenolysis may provide energy, substrates, or cofactors necessary for pulmonary phospholipid synthesis. Fetal lung readily incorporates glucose into phospholipids, especially phosphatidylcholine (7, 9). Furthermore, *de novo* fatty acid synthesis requires NADPH, and glucose metabolized through the hexose monophosphate pathway may be a source of this cofactor. Other indirect evidence which suggests that glycogen may contribute to fetal lung surfactant synthesis includes the observation that glucocorticoids both accelerate pulmonary surfactant synthesis and decrease glycogen content (9, 13, 22) and the demonstration that there is increased lung glycogen and decreased pulmonary maturation in fetal rats decapitated *in utero* (2). These data, which show lung glycogen depletion by agents which augment surfactant synthesis, provide further indirect support of this hypothesis.

It is clear that theophylline itself has direct effects on *in vitro* assays for glycogen phosphorylase and synthase (5, 28). In this study, these effects were probably minimal because there was no difference in activity when these two enzymes were assayed with and without aminophylline in the reaction medium.

Cyclic AMP and theophylline have wide-ranging effects on pathways of carbohydrate metabolism other than that of glycogen. These include induction of fetal liver glucose-6-phosphatase activity (23), stimulation of hepatic phosphofructokinase (20), and pyruvate kinase (3) activity, and augmentation of glucose uptake by hamster embryo cells (15). The enhancement of fetal lung phospholipid synthesis by cyclic AMP could thus be mediated by a direct action on the enzymes of phospholipid synthesis or by its multiple effects on carbohydrate metabolism.

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