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Ontogeny of Protein Phosphatases 1 and 2A in Developing Rat Lung

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ABSTRACT. Protein phosphatase (PP) 1 and 2A activities were measured in soluble and particulate fractions of rat lung homogenates, obtained from 18- to 22-day gestation fetal rats, from neonates, and from adults. PP 1 activity in the particulate fraction increased 7-fold from 15 ± 2 to 102 ± 12 (mean ± SD) U/g lung between 18 and 22 days gestation, increased a further 1.5-fold to 152 ± 18 U/g in newborns, with no further increase in adults. PP 2A activity in the particulate fraction increased 3-fold from 10 ± 2 to 32 ± 5 U/g between 21 and 22 days gestation and did not change postnatally. PP 1 activity in the soluble fraction increased 7.8-fold from 4 ± 2 to 31 ± 4 U/g lung between 18 and 22 days gestation, increased a further 1.8-fold in newborns to 54 ± 8 U/g lung and increased 1.5-fold to 81 ± 6 U/g lung in adults. PP 2A activity in the soluble fraction increased 2.2-fold from 12 ± 4 to 28 ± 8 U/g lung between 18 and 22 days gestation, increased a further 1.7-fold in newborns to 47 ± 9 U/g lung, and increased 1.6-fold to 75 ± 10 U/g lung in adults. PP 1 activity was 1.6- to 3-fold higher in the particulate than the soluble fraction. PP 2A activity was 2-fold higher in the soluble than the particulate fraction after 21 days gestation, in neonates and adults. We conclude that protein phosphatase activity is regulated developmentally during the critical perinatal period of fetal lung ontogeny and suggest that these enzymes play an important role in regulating phosphorylation reactions in the developing lung. (*Pediatr Res* 24: 25–27, 1988)

Abbreviation

PP, protein phosphatase

The phosphorylation and dephosphorylation of proteins is a ubiquitous mechanism for regulating cellular activity that involves two classes of enzyme, termed protein kinases and PP (1). Molecular forms of PP 1, 2A and 2C account for almost all the phosphatase activity toward enzymes that regulate the major biosynthetic and biodegradative pathways in liver and muscle, although in most cases, the contribution of protein phosphatase 2C is minor. The molecular structure and function of these enzymes have been studied extensively, but little information is available on their specific roles in the regulation of development.

Some information on phosphorylation processes in the developing fetal lung has recently become available. Activities of cyclic AMP-dependent protein kinase, protein kinase C, and a calcium-calmodulin-dependent protein kinase (phosphorylase kinase) have been measured in fetal lung (2–4). Cyclic AMP-dependent phosphorylation of several cytosolic proteins has been described (5), and may be developmentally regulated. However, dephosphorylation reactions in the developing lung have not been studied. Herein we describe the ontogeny of protein phosphatase activity in developing rat lung.

METHODS

Preparation of lung homogenates; particulate and soluble fractions. Time-dated pregnant rats were obtained by placing virgin females with a sire for 24 h and noting the presence of a plug. The sire was then removed. Gestational age was counted from the day of mating. Adult male rates and pregnant female rats were killed with intraperitoneal injections of pentobarbital. Fetal rats were obtained by rapid postmortem hysterotomy of time-dated pregnant rats. Fetal and neonatal rats were killed by decapitation.

Lungs were rapidly removed from the thorax of fetal, neonatal, and adult rats and placed in 0–4° C homogenization buffer (4 mM EDTA, 250 mM sucrose, 0.1% (vol/vol) mercaptoethanol, 1 mM benzamidine, 0.1 mM phenylmethyl sulfonyl fluoride) and homogenized immediately in 2.5 vol buffer with a Polytron homogenizer at setting 2, for two bursts of 10 s each.

Homogenates were centrifuged for 10 min at 1000 × *g*. The supernatants were collected and recentrifuged for 40 min at 100,000 × *g*. The supernatants thus obtained (termed the soluble fraction) were kept on ice while the pellets were resuspended in the original volume of the same buffer and recentrifuged as above. The washed pellets were resuspended by ten strokes of a Dounce glass homogenizer on ice in the same volume of 20 mM Tris/HCl, pH 7.0, containing 0.6 M NaCl. The suspension was then recentrifuged as above and the supernatant (termed the particulate fraction) was used for assay.

^{32}P-labeled substrate (10^6 cpm/nmol). Phosphorylase *a* (glycogen phosphorylase EC 2.4.1.11; 1 mol phosphate/mol 97 Da subunit) was phosphorylated using phosphorylase kinase (EC 2.7.1.38) and [γ-^{32}P]ATP was removed as described previously (6).

PP assays. PP 1 and 2A were assayed at 30° C in an incubation mixture (0.03 ml) containing 50 mM Tris-Cl, 0.1 mM EGTA, 0.1% (vol/vol) 2-mercaptoethanol, 5 mM caffeine, bovine serum albumin (1.0 mg/ml), and phosphorylase *a* (1.0 mg/ml, 10.3 μM). Reactions were initiated with phosphorylase *a* after preincubating the other components for 5 min at 30° C. Assays were carried out for 10 min and terminated and analyzed as previously

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(6). Release of radioactivity was limited to <30% to ensure linear rates of dephosphorylation with respect to time. Assays were performed in duplicate, and control incubations were included in which PP was replaced by buffer. These control values were <5% of the total radioactivity and were subtracted from the values obtained in the presence of phosphatase. When inhibitor-2 or protamine were present in the assays, they were preincubated with the phosphatase for 10 min at 30° C before the addition of the substrate. PP 1 was defined as the activity inhibited in the presence of 30 nM inhibitor-2, the concentration at which authentic catalytic subunit of PP 1 was inhibited by about 90%. PP 2A was the activity stimulated by 15 μg/ml protamine in the presence of 30 nM inhibitor-2. One U of PP 1 or 2A activity (U) was defined as that amount of enzyme which catalyzed the dephosphorylation of 1.0 mol phosphorylase *a* per min. Assays of the soluble and the particulate fraction were carried out at a final dilution of 100-fold.

Other procedures. Inhibitor-2 (7) was purified from rabbit skeletal muscle. Protein was measured by the procedure of Bradford (8) using bovine serum albumin (A/1%/280 nM = 6.5) as a standard.

Statistical methods. The experiments were repeated five times. Results are expressed as mean ± SD. The developmental data were analyzed by analysis of variance. Statistical significance was accepted with $p < 0.05$.

RESULTS

PP activity in the particulate fraction. PP 1 and 2A are the only enzymes in mammalian tissue with significant phosphorylase phosphatase activity (9, 10), and experiments were therefore carried out using phosphorylase *a* substrate. PP activity in the particulate fraction of neonatal lungs increased with dilution to 156 U/g wet weight lung and a specific activity of 1.8 mU/mg protein at a final dilution of 100-fold. Activity was 4.2-fold lower at a dilution of 5-fold. Similar observations were made in the corresponding fractions for adult and fetal lung. Approximately 90% of the phosphorylase phosphatase activity could be extracted from the particulate fraction with 0.6 M NaCl, and this activity was inhibited by 80% by 30 nM inhibitor-2 purified from rabbit skeletal muscle.

The dephosphorylation of phosphorylase *a* by PP 1 is inhibited by low concentrations of protamine, whereas this polybasic protein is an activator of PP 2A at low concentrations and only an inhibitor at higher concentrations (11, 12). Protamine can therefore be used like inhibitor-2 to distinguish between PP 1 and 2A. In the particulate fraction, PP activity was not stimulated at low concentrations of protamine, but was inhibited at high concentrations of protamine in the absence of inhibitor-2. In the presence of inhibitor-2, PP activity was inhibited by >80% in the absence of protamine, and was inhibited by >60% in the presence of protamine, 30 μg/ml, confirming that PP 1 activity predominated in the particulate fractions (Fig. 1).

PP activity in the soluble fraction. Identical experiments to those described above were carried out using the soluble fractions (100,000 × *g* supernatant). The results were similar to those obtained with the particulate fractions, except that the proportion of PP 2A activity was higher. Only about 55% of the PP activity in the soluble fraction could be inhibited by 30 nM inhibitor-2, compared to 80% in the particulate fraction. PP activity was stimulated at low concentrations of protamine and inhibited at higher concentrations (Fig. 1). This suggested that inhibition of PP 1 was counterbalanced by activation of PP 2A at low concentrations of protamine.

Ontogeny of PP activity. PP 1 activity in the particulate fraction increased 7-fold from 15 ± 2 (mean ± SD, $n = 5$) to 102 ± 12 U/g lung, $p < 0.001$ between 18 and 22 days gestation, and increased a further 1.5-fold in newborns, $p < 0.01$ with no further increase in adults (Fig. 2). PP 2A activity in the particulate fraction increased 3-fold from 10 ± 2 to 32 ± 5 U/g lung, $p <$

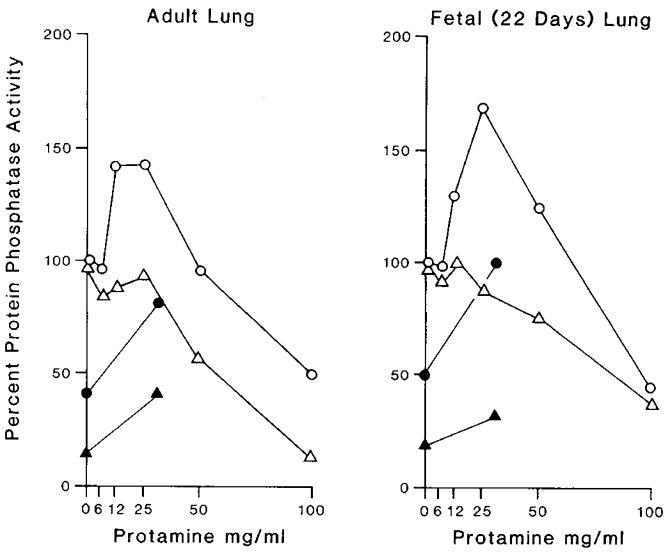

Fig. 1. Effect of protamine on phosphorylase phosphatase activity in adult and fetal (21-day gestation) rat lung in the presence and absence of inhibitor-2. Soluble and particulate fractions were obtained by centrifugation at 100,000 × *g* for 40 min. The fractions were diluted to 0.5 U/ml and aliquots (0.01 ml) were preincubated with protamine either in absence or in the presence of 30 nM inhibitor-2 before assaying for phosphorylase phosphatase activity as described in "Methods." The graphs show phosphorylase phosphatase activity in the soluble (○) and particulate (△) fractions in the absence of inhibitor-2 and phosphorylase phosphatase activity in the soluble (●) and particulate (▲) fractions in the presence of inhibitor-2.

0.05 between 21 and 22 days gestation and then did not change postnatally. PP 1 activity in the soluble fraction increased 7.8-fold from 4 ± 2 to 31 ± 4 U/g lung between 18 and 22 days gestation, $p < 0.001$, and increased a further 1.8-fold in newborns to 54 ± 8 U/g lung, $p < 0.01$. PP 1 activity was 1.6- to 3-fold higher in the particulate than in the soluble fraction, $p < 0.01$. In contrast, PP 2A activity was 2-fold higher in the soluble than the particulate fraction in neonates and adults, $p < 0.01$.

Inasmuch as the protein content of the lungs only increased 1.2-fold between 18 days gestation and adulthood, the ontogeny of PP specific activity (U/mg protein) did not differ significantly from the ontogeny of its activity (U/g lung).

DISCUSSION

The *in vivo* phosphorylation state of any protein not only depends on the activity of specific protein kinases, but also on the activities of relatively few protein phosphatases, reviewed in (1). PP 1, 2A, and 2C have been shown to account for nearly all phosphatase activity in liver and muscle toward 13 enzymes that regulate the major biosynthetic and biodegradative pathways of these tissues (9). PP 1 is enriched in glycogen-protein particles and is particularly active toward the enzymes of glycogen metabolism. It is also the major phosphatase found in hepatic microsomes and reticulocyte ribosomes, suggesting a role in processes such as cholesterol and protein synthesis. PP 2A and 2C are largely cytosolic. PP 2A is particularly active toward enzymes that regulate glycolysis/gluconeogenesis, fatty acid synthesis, and aromatic amino acid breakdown in liver, whereas PP 2 C displays it highest activity toward enzymes that regulate cholesterol biosynthesis. However, the phosphorylase phosphatase activity of PP 2C is very low (9) and in most cases, its contribution to total PP activity is also low (9). PP activity is also strongly dependent on dilution in liver extracts (10), in which dilution activates both PP 1 and 2A activities.

Herein we have shown that the activities of PP 1 and 2A are regulated during the critical perinatal period of fetal lung ontog-

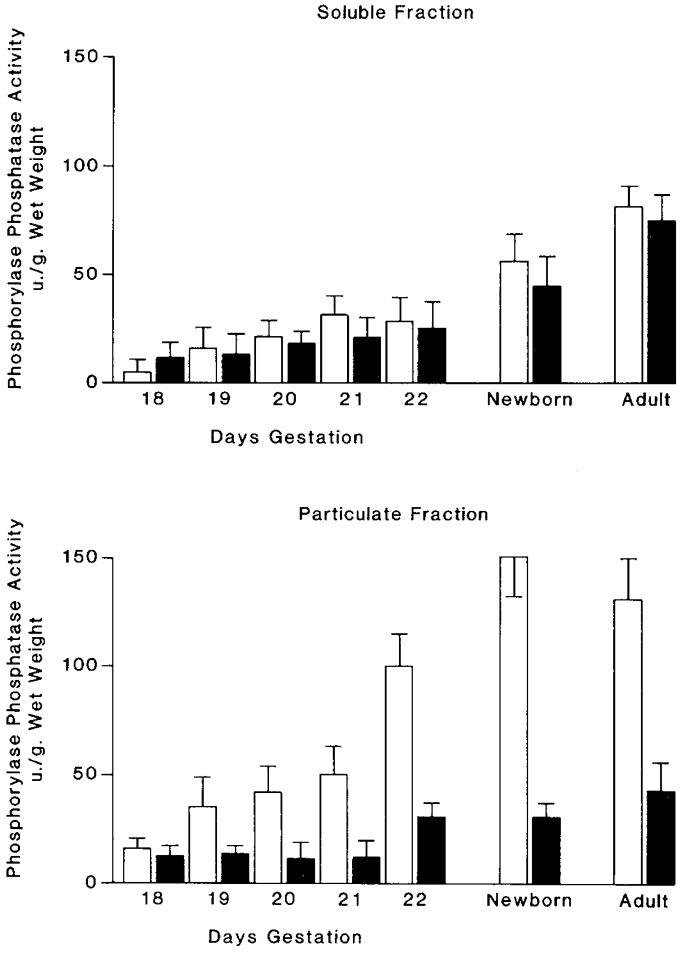

Fig. 2. PP 1 (*open bars*) and 2A (*hatched bars*) activities, mean + SD ($n = 5$), in soluble and particulate fractions of fetal, neonatal, and adult rat lungs. PP 1 assays were carried out as described in "*Methods*" in the presence of 30 nM inhibitor-2. PP 2A assays were carried out in the presence of 30 nM inhibitor-2 and 15 μg/ml protamine. The assays were performed in duplicate.

eny, suggesting that these enzymes play an important role in regulating phosphorylation reactions in developing lung. As has been found in other mammalian tissues, PP 1 is the major activity associated with the particulate fractions, whereas PP 2A is largely cytosolic.

Phosphorylation mechanisms are known to regulate pulmonary surfactant secretion. Dobbs and Mason (13) showed that adult rat type II pneumocytes secrete surfactant in response to β-agonists. Rice *et al.* (14) have reported increased amounts of cyclic AMP and cyclic AMP-dependent protein kinase activity in response to terbutaline, forskolin, or both agents, and correlated these effects with the release of surfactant. Sano *et al.* (3) have also recently reported protein kinase C activity in type II pneumocytes and have proposed a major role for protein kinase C in the regulation of surfactant secretion. Whitsett *et al.* (5) have reported that cyclic AMP-dependent phosphorylation of actin is developmentally regulated in the fetal rat lung, increasing at the same time as fetal lung surfactant near term. Phosphorylation and dephosphorylation of cytoskeletal elements such as actin may regulate surfactant exocytosis.

The rate-limiting enzymes of fatty acid and phospholipid biosynthesis in liver, namely acetyl-CoA carboxylase and phosphatidylcholine cytidylyltransferase are also rate-limiting in the pathway for surfactant phospholipid biosynthesis in the lung. The hepatic enzymes are inactivated by phosphorylation (15–17), and this is also likely to be true in lung, inasmuch as Maniscalco *et al.* (18) have shown that acetyl-CoA carboxylase in fetal rat lung is inhibited under phosphorylating conditions and reactivated under dephosphorylating conditions. Pulmonary glycogenolysis also occurs during the last few days of fetal rat gestation (19) and is regulated in this and other species by three enzymes likely to undergo phosphorylation-dephosphorylation, namely glycogen phosphorylase, phosphorylase kinase, and glycogen synthase.

The striking changes in PP 1 and 2A activities in the critical perinatal period of fetal rat ontogeny strongly suggest that these enzymes play a role in regulating processes that prepare the fetal lung for air breathing, such as the biosynthesis and secretion of pulmonary surfactant.

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