Biochemical Assessment of Fetal Lung Maturity

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ABSTRACT

The relationship between the enzyme phosphatidate phosphohydrolase (PAPase) and lecithin/sphingomyelin (L/S) ratio in amniotic fluid was evaluated in normal human pregnancies and in several pregnancies complicated by Rh isoimmunization. An increase in PAPase activity in amniotic fluid appears to parallel the increase in L/S ratio after 33 weeks gestation in normal subjects. These data suggest that amniotic fluid PAPase may originate from the fetal lung and play a role in the regulation of the synthesis of lecithin. Its usefulness as an indicator of fetal lung development requires further correlation and confirmation.

Introduction

It is well established that a lipoprotein with surface active properties is required for lung alveolar stability. The major component of this lipoprotein is the surfactant dipalmitoyl lecithin (saturated phosphatidyl choline), the concentration of which increases with advancing gestation. Measurement of this phospholipid by the lecithin/sphingomyelin ratio (L/S), lecithin phosphorus determination, disaturated lecithin assay, measurement of the fatty acid side chains of phosphatidyl choline or quantitatively by the foam stability index test have proven valuable for the prenatal evaluation of fetal lung maturity. More recently, phosphatidyl glycerol has been suggested as an indicator of fetal lung maturation, since it may function as a potent activator of the phosphocholinecytidyltransferase enzyme.

The major pathway for the de novo synthesis of surfactant lecithin is the cytidine diphosphatidylcholine (CDP-choline) pathway (figure 1). This latter mechanism initiates the maturation of the phosphocholine transferase system at about 33 to 35 weeks of the gestation period in humans and animals. Key enzymes responsible for the regulation of phosphatidyl choline synthesis could serve as another index of fetal lung maturation, and the activity of the enzyme phosphatidate phosphohydrolase (PAPase EC 3.1 3.4)
may play an important role in the biosynthesis of glycerophospholipids.\textsuperscript{1,11} This enzyme catalyzes the hydrolysis of phosphatidic acids, a reaction that gives rise to diglycerides, a co-substrate in lecithin biosynthesis (figure 1). In measuring the activity of PAPase in highly purified lamellar bodies isolated from type II alveolar cells and human amniotic fluid, Jimenez et al\textsuperscript{12} suggested that PAPase is a functional component of the lamellar body. The specific activity of PAPase in human amniotic fluid throughout normal gestation has also been reported to increase proportionately with the rise in L/S ratios in amniotic fluid after 32 weeks gestation.\textsuperscript{10}

As described in this paper, currently available methodology has been used to study the relationship between the activity of PAPase in amniotic fluid and the development of fetal lung maturity.

Materials and Methods

AMNIOTIC FLUID SAMPLING

Amniotic fluid was collected by transabdominal amniocentesis. Samples most often were obtained for the management of Rh isoimmunization and determination of fetal maturity. Inevitably, a significant number of women on whom amniocentesis was done subsequently were shown to be normal. When amniocentesis was carried out on apparently normal patients the subjects gave prior informed consent. The variability observed in values of phospholipid quantification probably resulted from the method of selecting patients which was by gestational age only, rather than separated by medical problem. Amniotic fluid (37 samples) was collected from 23 women from 28 weeks gestation until time of induction of delivery or Cae-sarean section. Samples visibly contaminated with blood or meconium were excluded from the study.

On collection, each sample of amniotic fluid was divided into two portions. One portion, to be used for the determination of the L/S ratio, was centrifuged at low speed (500 × g) at 4°C for 10 minutes to remove cell debris as soon as possible after collection, then used immediately or stored frozen. The remaining portion was similarly centrifuged at 4°C and stored for no longer than four weeks at −20°C. The latter sample was assayed for phosphatidate phosphohydrolase (PAPase) by the modified method of Coleman and Hubscher.\textsuperscript{1,21}

**DETERMINATION OF L/S RATIO**

Three milliliters of the supernatant was extracted with one volume of methanol and two volumes of chloroform (vol/vol).\textsuperscript{*} The solution was mixed for 15 seconds and centrifuged at 500 × g for 10 minutes at 4°C. The lower phase was aspirated and evaporated under nitrogen. The dried extract was redissolved in 150 μl of chloroform, and aliquots of this solution were analyzed for lecithin and sphingomyelin according to a modification of the chromatographic procedure of Gluck et al\textsuperscript{6,8} without employing an acetone precipitation step. The developed plate was sprayed with a phosphoric acid-copper sulfate reagent, heated to 100°C on a hot-plate until charring occurred (about 15 minutes), and the spots quantitated by densitometry.\textsuperscript{6}

**DETERMINATION OF PAPASE ACTIVITY**

Phosphatidate phosphohydrolase in amniotic fluid appears to be stable for ap-
proximately three weeks if stored at —20°C in silicone coated tubes.

The activity of PAPase was assayed in duplicate on the amniotic fluids obtained from 23 patients at various gestational ages. Activity of PAPase was studied in two groups of patients; those in the third trimester to 32 weeks of gestation (28 to 32 weeks) and those of 33 weeks or more (33 to 40 weeks). The gestational age was established by the date of the last menstrual cycle. Seven patients were included in the first group and 16 patients in the second group. In most cases, the ratio between PAPase and L/S was carried out on the fluid collected from each subject. As a general rule, serial assays were not able to be obtained over the gestation period for most of the subjects studied. A few follow-up assays for PAPase and L/S ratio on an individual are included in figure 2 but are not identified since the data were uncommon.

**ASSAY OF PAPASE**

Each incubation vessel (10 × 60 mm capped tube) contains 0.1 ml of maleate buffer (0.6 mol per L, pH 6.0), 0.1 ml phosphatidic acid (1 µMOL), and 300 µL of amniotic fluid. The mixture incubates for 40 minutes at 37°C. The reaction is stopped by the addition of 0.1 mL of 50 percent trichloroacetic acid (TCA) and cooled to 0°C. Each reaction vessel receives 0.5 mL of 5 percent TCA containing 12.5 percent purified acid washed Norit A. An additional volume of 5 percent TCA (0.2 mL) is added to each tube. The mixture is then centrifuged for 10 minutes at 1500 × g, and a sample of the filtrate taken for determination of inorganic phosphate.

Total inorganic phosphate is determined by the method of Fiske and Subbarow. The total protein concentration is measured by the Lowry procedure.

The amount of inorganic phosphate released is corrected for the amount liberated from the enzyme mixture (in the absence of substrate) in which 0.1 mL of phosphatidic acid is replaced by 0.1 mL of distilled water. In the absence of enzyme, very little inorganic phosphate appears to be liberated from phosphatidic acid.

One unit of activity is defined as the amount of enzyme liberating 1 µMol of inorganic phosphate per hour under the experimental conditions described. Activity of PAPase can be also expressed in n moles of inorganic phosphate released per milligram of protein per hour:

\[
\frac{n \text{ mols P}_4}{\text{mg Protein} \times \text{h}}
\]

**COMMENTS ON PAPASE METHODOLOGY**

Preparation of the substrate, phosphatidic acid derived from egg phosphatidylcholine requires special techniques in order to ensure a highly stable suspension. Phosphatidic acid (60 µMol) is dissolved in 0.4 mL benzene and 6.0 mL of deionized water added. The concentration of the suspension is 1 µMol per 0.1 mL. The suspension is sonicated for 15 seconds while chilled. The process is carried out for six cycles. Sonication causes the benzene and lipid to be dispersed to form a milky emulsion, which is then dialyzed in a 6.35 mm cellophane tubing for 24 hours against three times 100 volumes of glass-distilled water in order to remove all traces of benzene. Dialysis causes most of the milky appearance of the emulsion to disappear. Complete removal of benzene from the resulting phosphatidate dispersion can be checked spectrophotometrically between 230 and 270 nm. This stabilized substrate retains its analytical usefulness in the emulsified state for two weeks if stored at 4°C in silicone-coated tubes.

Several properties of amniotic fluid PAPase have been established. Tween

* Sigma Chemical Co., St. Louis, MO 63178.
20, potassium fluoride, and certain divalent cations (Mg\(^{2+}\), Be\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\)) are inhibitors of this enzyme. The pH optimum of PAPase is at 6.0. The enzyme is unaffected by cyanide, glutathione, or cysteine at concentrations which would inhibit alkaline phosphatase. Inhibition of the enzyme may be caused by \(1 \times 10^{-3}\) mol per L of p-chloromercuribenzoate. This inhibition can be reversed by excess glutathione and suggests that thiol groups are essential for the activity of PAPase. There also appears to be no requirement for metal ion activators, since chelating agents have little effect on the activity. The enzyme can be completely inactivated if incubated at 56°C for 10 minutes.

Results

The activity of PAPase was determined in duplicate on the amniotic fluids obtained from a total of 23 patients at various gestational ages from 28 to 40 weeks. The comparison of PAPase activity in two groups of apparently normal patients studied, those in the third trimester from 28 to 32 weeks gestation and those of 33 weeks to term, are shown in figure 2. A lecithin/sphingomyelin ratio was determined in parallel with the PAPase activity. The L/S ratio was measured in duplicate and the mean value after densitometric quantification is plotted on figure 2. This group of women was subsequently shown to be normal (few higher risk problems including Rh incompatibility). Several patients were assayed at more than one interval during the gestation period. The figure does not specifically denote these patients. In one case, PAPase assayed at 36 weeks (see figure 2) demonstrated zero activity after multiple assays and was omitted from the figure. Four subjects were included in the first group (28 to 32 weeks) and seven patients in the second group (33 to 40 weeks). The mean value of PAPase in amniotic fluid for these patients with pregnancies at 28 to 32 weeks was 21 n mols of inorganic phosphate released per mg protein per hour. In contrast, those patients with pregnancies in excess of 32 weeks gestation, the mean value was 72 n mols. This represents a 3.4 fold increase in the activity of PAPase in the latter stages of gestation. The difference in the means of the two groups is highly statistically significant (\( p < 0.001\)). The L/S ratio was in the range of <1.0 to 1.3 in the first group and had a broad range of 1.1 to 6.8 in the second group.

In table I are illustrated a number of newborns with unequivocal respiratory problems who had PAPase levels under 42 n mols and L/S ratios between 0.9 to 1.2 at the time of induction of delivery by Caesarean section. This finding is not conclusive evidence of a causal relationship between PAPase activity and lecithin synthesis.

Discussion

It is generally agreed that the principal mechanism of surfactant synthesis in the lung is the cytidine diphosphocholine...
TABLE I

<table>
<thead>
<tr>
<th>Gestational Age at Delivery Weeks</th>
<th>Number of Subjects</th>
<th>PAPase (n Mols)</th>
<th>L/S Ratio</th>
<th>Hyaline Membrane Disease (HMD) or Other Respiratory Problem (RP)</th>
<th>Maternal Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>1</td>
<td>29</td>
<td>1.2</td>
<td>HMD</td>
<td>Rh incompatibility</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>51</td>
<td>2.7</td>
<td>Normal</td>
<td>Diabetes, pre-existing</td>
</tr>
<tr>
<td>38</td>
<td>1</td>
<td>79</td>
<td>6.2</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>36</td>
<td>1</td>
<td>63</td>
<td>2.9</td>
<td>Normal</td>
<td>Threatened premature labor (hemorrhage)</td>
</tr>
<tr>
<td>36</td>
<td>1</td>
<td>42</td>
<td>0.9</td>
<td>RP</td>
<td>Essential hypertension</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>81</td>
<td>2.8</td>
<td>Normal</td>
<td>Elective Caesarean</td>
</tr>
</tbody>
</table>

PAPase = Phosphatidate phosphohydrolase

| L/S | Lecithin/sphingomyelin |

pathway.12 Phosphatidate phosphohydrolase (PAPase) plays a central and perhaps regulatory role in the biochemical maturation of the fetal lung. It also catalyzes the hydrolysis of phosphatidic acids, a reaction that gives rise to 1, 2-diacylglycerols and orthophosphate. The diglycerides, in turn, can serve as acceptors of phosphocholine in the biosynthesis of lecithin. Also, PAPase has been shown to catalyze the hydrolysis of phosphatidylglycerophosphate forming phosphatidylglycerol.19 This latter compound has been reported by Gluck to improve the properties of surfactant in stabilizing the alveoli.20 Considering the important position of phosphatidic acid in the biosynthetic chain leading to phosphatidylcholine, further evaluation of the biochemical usefulness of PAPase as an amniotic fluid marker of fetal lung maturity is encouraged.

This study indicates that PAPase activity is present and measurable in amniotic fluid. Since the optimum pH of PAPase is 6.0, which is different than the pH optimum of either alkaline phosphatase or acid phosphatase, the enzyme appears to have a distinct phosphohydrolase specificity. The enzyme is also heat-inactivated at 56°C for 10 minutes which separates it from placental alkaline phosphatase.

The presence of this enzyme (PAPase) in amniotic fluid and the finding that it increases with gestational age emphasizes the role it may play in the regulation of lung phospholipid biosynthesis. The observation that patients with gestations of 33 weeks or more show a statistically significant increase in PAPase compared to gestation of 32 weeks or less is consistent with this concept. The changes in PAPase activity concomitant with an increase in L/S ratio imply that this enzyme marker and the surfactant lecithin may have associated biochemical pathways.

Summary

In this study, PAPase and L/S ratios were determined in the amniotic fluid of 23 patients from 28 to 40 weeks gestation. In most cases, the activity of PAPase increased during the latter stages of gestation and mirrored the surge of lung phosphatidylcholine biosynthesis. An increased activity of this enzyme in amniotic fluid may be used as an added indicator in the prediction of fetal lung maturity.

References


