Expression of Plasma Phospholipid Transfer Protein mRNA in Normal and Emphysematous Lungs and Regulation by Hypoxia*

Xian-cheng Jiang‡§, Jeanine D’Armiento‡, Rama K. Mallampalli‡, Jefferson Mar‡, Shi-Fang Yan‡, and Min Lin‡

From the ‡Division of Molecular Medicine, Department of Medicine, Columbia University, New York 10032 and the ¶Department of Internal Medicine and Department of Veterans Affairs Medical Center, The University of Iowa College of Medicine, Iowa City, Iowa 52242

The lung is the major site expressing plasma phospholipid transfer protein (PLTP) mRNA in humans and mice, suggesting that this protein might have an important role in maintaining normal function of this organ. In the lung of human collagenase transgenic mice, an emphysematous animal model, PLTP mRNA was 3-fold higher than in control mice. However, the mRNA in other tissues was not changed. To further assess the expression and function of PLTP, we measured PLTP mRNA level in lung tissue of two emphysematous patients and found that the mRNA was 4-fold higher than in control subjects. In situ hybridization on mouse lung suggested positive staining in alveolar type II epithelial cells. In addition, immortalized rat alveolar pre-type II epithelial cells and freshly isolated mature rat alveolar epithelial cells both highly expressed PLTP mRNA, and the former cells actively secreted PLTP activity into the medium. To examine the possible mechanisms leading to high levels of PLTP expression in vivo, we exposed the pre-type II cells to hypoxia and demonstrated induction of PLTP mRNA and a coordinate increase in secreted PLTP activity. Thus, the PLTP gene is highly expressed in alveolar type II epithelial cells and is induced during hypoxia and in emphysema. These observations suggest that a hypoxic stimulus occurring in emphysema may be a novel mechanism that contributes to enhanced expression of PLTP.

The plasma phospholipid transfer protein (PLTP)1 transports phospholipids among lipoprotein particles (1, 2) and from lipid bilayers to high density lipoproteins (HDL) (1). There is accumulating evidence indicating that PLTP plays an integral role in the remodeling of lipoproteins (3–5). PLTP transgenic mouse with moderate overexpression showed increased preβ-high density lipoprotein (HDL), apolipoprotein AI, and phospholipid in the apolipoprotein AI transgenic background (6). Overexpression of PLTP using adenovirus in mice leads to increased levels of newly generated preβ-HDL, but decreased plasma HDL and enhanced hepatic uptake of phospholipids and cholesteryl esters from HDL (7). However, aside from these processes, the physiological role of PLTP is almost unknown. In humans and mice, the lung is the dominant site expressing PLTP mRNA (8, 9). These observations suggest that PLTP might serve as an important regulator of phospholipid and lipoprotein metabolism in the lung.

The alveolar system of the lung exhibits an enormous surface area, which is in contact with the environment. To maintain the integrity of this air-surface interface, the alveolar lining is composed of two types of epithelial cells, pneumocytes I and II (type I and type II), with distinct functions. Alveolar type II epithelial cells are cuboidal cells that secrete surfactant, a complex heterogeneous material comprised predominantly of the phospholipid, phosphatidylycholine (PC), and several key hydrophobic proteins, which reduce alveolar surface tension. Alveolar type I epithelial cells are elongated cells that provide the majority of the alveolar surface area (10).

There is evidence that lipoproteins can influence surfactant phospholipid metabolism in type II cells. HDL and low density lipoprotein (LDL) can stimulate primary cultures of type II cells to secrete PC (11). Very low density lipoproteins (VLDL) stimulate surfactant lipids in immortalized or primary rat alveolar pre-type II epithelial cells (12). Because PLTP is highly expressed in the lung, and regulates lipoprotein metabolism, it is conceivable that this protein might play a role in surfactant metabolism.

Chronic bronchitis, asthma, and emphysema are chronic obstructive lung diseases associated with disturbance of the surfactant system (13, 14). Cigarette smoking, the major contributor to chronic obstructive lung disease, reduces the amount of surfactant on the alveolar surface (15) but increases the numbers of alveolar type II epithelial cells (16). Surfactant deficiency leads to atelectasis and thus may contribute to hypoxia and hypercapnia observed in chronic lung disease (17, 18). Further, gas-exchange abnormalities such as hypoxia have been observed to alter surfactant metabolism in human lung tissue (19). These observations have led to the investigational use of surfactant replacement therapy in chronic obstructive lung disease. For example, high-dose bovine surfactant treatment in very premature infants greatly reduces mortality and incidence of pulmonary interstitial emphysema (20) and may be beneficial in asthma (21). Both emphysema and asthma are associated with elevated HDL levels (22, 23).

Human collagenase transgenic mice express the transgene in their lungs under the direction of the haptoglobin promoter. Histological analysis demonstrated disruption of the alveolar walls and coalescence of the alveolar spaces with no evidence of fibrosis or inflammation (24). This pathology is strikingly similar to the morphological changes observed in human emphysema (24) and therefore implicates interstitial collagenase as a

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‡ To whom correspondence should be addressed: Div. of Molecular Medicine, Dept. of Medicine, Columbia University, 630 W. 168th St., New York, NY 10032. Tel.: 212-305-7720; Fax: 212-305-5052; E-mail: xcj1@columbia.edu.

¶ From the §Division of Molecular Medicine, Department of Medicine, Columbia University, New York 10032 and the Department of Internal Medicine and Department of Veterans Affairs Medical Center, The University of Iowa College of Medicine, Iowa City, Iowa 52242

1 The abbreviations used are: PLTP, phospholipid transfer protein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very LDL; LBP, lipopolysaccharide binding protein; HIF-I, hypoxia-induced factor I.
possible etiological agent in the disease process. The close association between chronic obstructive lung disease and surfactant deficiency together with high levels of PLTP in the lung led us to investigate whether PLTP expression would be altered in emphysematous lung and if PLTP mRNA is regulated by hypoxia.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture media and reagents were obtained from Life Technologies, Inc. Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). Cholesterol and phospholipid measurement kits were obtained from Wake Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were from Sigma.

**Isolation of Total Lung RNA**—Human lung samples were obtained from two patients undergoing lung transplantation (IRB# X042 1) at Columbia Presbyterian Medical Center and from a normal nonsmoking human as a control. The major etiological factor for emphysema in the patients was cigarette smoking. Lung samples were also obtained from transgenic mice that were shown to develop emphysema secondary to increased collagenase expression within their lung (24). Total RNA was prepared from fresh tissue using the guanidinium thiocyanate-cesium chloride method as described previously. The total RNA was finally resuspended in diethyl pyrocarbonate-treated water, and the absorbance was read at A260 and A280. The quality of the RNA was checked on a 1.2% denaturing agarose gel to ensure the presence of the 28 S and 18 S ribosomal bands.

**Northern Blotting and RNase Protection Assay**—For PLTP mRNA Northern blot, total RNA (20 μg) from emphysematous patients and control was analyzed for PLTP mRNA as described previously (9); for hypoxia-induced factor I (HIF-I) mRNA Northern blot, total RNA (15 μg) from immortalized rat fetal alveolar pre-type II epithelial cells was analyzed for HIF-I mRNA as described previously (9) using a 900-base pair monkey HIF-I cDNA as a probe (a gift from Dr. David Stern); for mouse and rat PLTP mRNA assay, a fragment of the mouse PLTP cDNA (160 base pairs, codons 750–910) was cloned into Bluescript KS+ (Stratagene) and used to prepare radiolabeled cRNA probes for all of the RNase protection assays. The assay was performed as described previously (9). For mouse lipopolysaccharide binding protein (LBP) mRNA assay, a fragment of the mouse LBP cDNA (220 base pairs, 5′-untranslated region, 1581–1800) was cloned into Bluescript KS+ (Stratagene) and used to prepare radiolabeled cRNA probes for all of the RNase protection assays. The assay was performed as described previously (9).

**PLTP Activity Assay**—3 μl of plasma or 20 μl of the cell medium (10-fold concentrated from original) from immortalized rat fetal alveolar pre-type II epithelial cells were incubated for 1 h at 37 °C with radiolabeled liposomes (25 μl) and isolated human HDL3 (250 μg of protein) in a final volume of 400 μl. Following incubation, phospholipid liposomes were selectively precipitated and radioactivity in the supernatant was measured. PLTP activity was expressed as the rate of total radiolabeled phospholipids transferred from radiolabeled liposomes to the HDL3.

**Lipids and Lipoproteins Assays**—For small volumes of mouse plasma, HDL was separated from apoB-containing lipoproteins by using a HDL cholesterol reagent (Sigma). Using this method, an insignificant amount of mouse apoAI is precipitated (25). The total cholesterol and phospholipids in plasma and HDL were assayed by enzymatic methods (Wako).

**In Vivo Hybridization**—The cRNA probes were produced from a 666-base pair mouse PLTP cDNA subcloned into pBluescript. T7 and T3 promoters flanking the cDNA fragment were used to make the sense and antisense cRNA probe by in vitro transcription. Riboprobes were labeled with digoxigenin 11-UTP according to the protocol of Boehringer Mannheim. Nonisotopic in situ hybridization was performed using a modification of the manufacturer's protocol as described previously (26).

**Cell Culture and Hypoxia**—Immortalized rat fetal alveolar pre-type II epithelial cells were generated by infection with a retroviral construct expressing the adenoviral 12 S E1A gene product (27). In the current study, we used cells that have undergone about 110 passages. Cells were maintained in Waymouth's 572/1 medium with 10% fetal calf serum at 37 °C in atmosphere containing 5% CO2. After reaching confluence, the cells were harvested using 0.25% trypsin with 0.1% EDTA and plated onto 100-mm tissue culture dishes (2 × 106 cells/dish). After incubation overnight (95–100% confluence), the cells were divided into two groups; one group was incubated continuously for 6, 12, and 24 h, the other group was incubated under hypoxia for 6, 12, and 24 h. The cells were subjected to hypoxia by placing them in an environmental chamber (Coy Laboratory Products, Ann Arbor, MI), which provided a controlled temperature (37 °C) and atmosphere with the indicated amount of O2 and CO2 (5%) and the balance made up of N2 (28). Use of this chamber for cell culture has been described previously (28). The pH of the medium remained unchanged throughout the experiments; pO2 in the medium was ~12–15 torr (oxygen was leached continuously from the tissue culture plasticware during the course of the experiments). At different time points, cell media were collected for PLTP activity assays, and the cells were collected for total RNA extraction.

**Isolation of Mature Alveolar Type II Epithelial Cells from Rat**—The mature rat alveolar type II cells were isolated as described previously (29). The yield of cells was approximately 40 × 106 cells and viability was >95% as determined by trypan blue exclusion.

**Statistical Analysis**—Results are expressed as mean ± S.D. The statistical significance of the differences between the groups was estimated by the Student's t test. A p value less than 0.05 was considered significant.

**RESULTS**

PLTP mRNA, as determined by the RNase protection assay, in the lung of human collagenase transgenic mice (an emphysematous animal model) (24) was found to be significantly higher than that of control (303 ± 16% versus 100 ± 30%, p < 0.0001), whereas other tissues studied did not show any changes (Fig. 1). As a control we also measured the mRNA level of LBP, which belongs to the same gene family as PLTP, in the lung and liver of the transgenic mice, and we did not find changes in these two tissues (Fig. 2). Northern blot analysis demonstrated that PLTP mRNA level in the lung of two emphysematous patients was much higher than that of a non-smoking control (Fig. 3). The results suggest an effect of emphysema on PLTP gene expression in the lung.

Despite significant differences in PLTP mRNA expression between normal and emphysematous lung, plasma PLTP activity and total plasma cholesterol, phospholipids, HDL cholesterol, and HDL phospholipids did not demonstrate significant differences between emphysematous and control mice (Table I), suggesting the possibility that the effects of elevated PLTP expression were restricted to the lung.

To determine which cell types were expressing PLTP mRNA, mouse lung tissue was analyzed by in situ hybridization, using a mouse cRNA probe that was labeled with digoxigenin 11-UTP. The positive signal was visualized as dark blue. The sections were also counterstained with hematoxylin, and nuclei were stained green. As shown in Fig. 4A, scattered cuboidal cells lining alveoli (a) stained intensely with a dark blue color (long arrows), whereas in the sense cRNA control, dark blue staining of cells was not detected (Fig. 4B). These data are highly suggestive that type II cells express PLTP and might regulate PLTP production. Without suitable markers, however, we cannot exclude the possibility that PLTP is also expressed...
in cells other than type II cells. In this regard, positive staining was also occasionally observed in cells with flat morphology surrounding the terminal airways (short arrows), suggestive of expression of the protein in reserve cells (Fig. 4A).

To investigate whether alveolar type II epithelial cells are a site for PLTP gene expression, we measured the PLTP mRNA in mature rat type II alveolar epithelial cells as well as lung, liver, heart, kidney, and small intestine (Fig. 5). Similar to findings in the human and mouse (8, 9), rat lung is also a major site for PLTP mRNA expression. In addition, PLTP mRNA was detected at high levels in rat liver. As shown in Fig. 5, PLTP was expressed in type II cells, and the amount of the mRNA in mature type II cells was comparable with that of liver.

To further confirm that type II cells are responsible for PLTP gene expression, we measured PLTP mRNA in rat immortal-

TABLE I

| Mice         | Total cholesterol | Phospholipid | HDL cholesterol | HDL phospholipid |
|--------------|-------------------|--------------|-----------------|-----------------|
| Transgenic   | 77 ± 6            | 166 ± 21     | 61 ± 6          | 139 ± 19        |
| Control      | 82 ± 6            | 151 ± 31     | 66 ± 9          | 150 ± 22        |

FIG. 2. RNase protection analysis for LBP mRNA in lung and liver from human collagenase transgenic and control mice. 30 μg of total RNA was used for the RNase protection assay. See “Experimental Procedures” for details. n = 6.

FIG. 3. Northern blot analysis of total RNA from the lung of emphysematous patients and control. Total RNA (20 μg) from emphysematous patient and control was used for Northern blots probed with PLTP cDNA as described under “Experimental Procedures.” We exposed the x-ray film for three days to obtain the PLTP mRNA signal, then the signal was scripted away from the membrane and reprobed with actin cDNA probe. The actin mRNA signal was obtained after 12 h of exposure. This result is a representative of two similar experiments.

FIG. 4. In situ detection of PLTP in mouse lung. A, section labeled by antisense PLTP digoxigenin-cRNA and visualized by alkaline phosphatase immunostaining, × 40. B, sense PLTP cRNA control, ×40. Countersained nuclei can be seen. The arrows point to type II pneumocytes. The triangles point to mesenchymal cells. The letter “B” refers to an airway (bronchiole), whereas the letter “A” refers to an alveolus.

FIG. 5. RNase protection analysis for PLTP mRNA in mouse and rat different tissues and cells. 30 μg of total RNA was used for the RNase protection asssay. See “Experimental Procedures” for details.
FIG. 6. Effect of hypoxia on the PLTP activity in immortalized rat alveolar pre-type II epithelial cells medium. The immortalized pre-type II cells (2 × 10⁶ cells/dish) were exposed to hypoxia at various time-points, see “Experimental Procedures” for details. The cells and the media were collected at each time point for activity measurements. This result is a combination of three experiments.

FIG. 7. Effect of hypoxia on the PLTP mRNA in immortalized rat alveolar pre-type II epithelial cells. The immortalized pre-type II cells (2 × 10⁶ cells/dish) were exposed to hypoxia at various time-points, see “Experimental Procedures” for details. The cells were collected at each time point for activity measurements. This result is a combination of three experiments.

FIG. 8. Effect of hypoxia on the HIF-I mRNA in immortalized rat alveolar pre-type II epithelial cells. The immortalized pre-type II cells (2 × 10⁶ cells/dish) were exposed to hypoxia at various time-points, see “Experimental Procedures” for details. The cells were collected at each time point for activity measurements. This result is a combination of three experiments.

DISCUSSION

In this study, we demonstrate that the PLTP mRNA is up-regulated in emphysematous lungs but not in other tissues studied, and PLTP is highly expressed in primary and immortalized rat alveolar type II epithelial cells. The induction of mRNA level in emphysematous lung is specific for PLTP, because the mRNA level of LBP, another member of the gene family, did not show changes. Under conditions of hypoxia, immortalized rat alveolar type II epithelial cells exhibit induction of the PLTP mRNA with an increase in PLTP activity in the cell medium. Collectively, these observations suggest that hypoxia, resulting from alveolar-capillary destruction in emphysematous lungs, serves as an important stimulus for PLTP expression in the lung.

The widespread distribution of PLTP mRNA in peripheral tissue suggests that PLTP may play a key role in regulating extracellular phospholipid and lipoprotein metabolism (8, 9). In this study, PLTP gene expression appears to be markedly increased in murine emphysematous lung without a change in plasma lipoprotein levels. In cultured alveolar type II cells, however, PLTP mRNA was induced by hypoxia, leading to an increase in the secretion of PLTP activity in medium. These results suggest that a hypoxic driving force increases PLTP in emphysema, but effects on lipid metabolism may be restricted to the lung. Although we did not directly test the hypothesis, it is tempting to speculate that this increase in local PLTP activity could play a role in regulating alveolar surfactant metabolism. Alternatively, induction of PLTP expression might simply be a consequence of surfactant deficiency which leads to microatelectasis and attendant hypoxia in chronic obstructive lung disease.

The emphysematous patients used in this study were smoking-related. Cigarette smoking reduces the amount of surfactant on the alveolar surface (15) but has been shown to increase the number of alveolar type II epithelial cells (16). We speculate that type II cell hyperplasia could also stimulate PLTP production to transport more surfactant to the alveolar membrane to rescue the pathological damage characteristic of emphysema.

How might the PLTP work locally? On the one hand, it has been reported that injecting partially purified PLTP increases the turnover rate of [³H]phosphatidylcholine ether-labeled HDL by 50%, suggesting that the PLTP could facilitate transfer of radiolabeled phospholipids from HDL to tissues or facilitate the uptake of HDL particles by tissues (31). Interestingly, one of the main sites of uptake of [³H]phosphatidylcholine ether-labeled HDL is the lung (31). On the other hand, PLTP is able to transfer phospholipids from artificial vesicles to HDL (1). PLTP could conceivably facilitate uptake of surfactant lipids that are secreted by type II cells or released by HDL into the lung interstitial fluid compartment and ultimately targeted for deposition into the alveolar membrane. Thus, PLTP might play an integral role in surfactant lipid trafficking and reutilization in type II alveolar epithelial cells.
PLTP Gene Expression in Lung

The induction of PLTP mRNA in murine and human emphysematous lung and in alveolar type II cells suggests that hypoxia may be an important stimulus for induction of PLTP gene expression. In human emphysematous lung, however, there may also be a strong inflammatory component, suggesting a possible role of local cytokines. In the mouse model the inflammatory component is minimal. Moreover, after endotoxin injection, PLTP mRNA was decreased in mice (9). Thus, although we cannot rule out a role for other stimuli, our data suggests that hypoxia is likely to be a major stimulus for PLTP gene induction in the emphysematous lung.

A number of genes are known to be induced by hypoxia (30), and the mechanism may be either increased transcription or mRNA stability. It is known that hypoxic effects are involved in a variety of cellular and global responses including anaerobic metabolism, erythropoiesis, angiogenesis, and breathing (32), which allow the cell to survive at low oxygen tension and help restore the cell to normal levels. We demonstrated in this study that gene(s), like PLTP, which are involved in lipid metabolism can also be regulated by hypoxia. Although there are no hypoxia consensus response elements, HIF-1 (30), existing either in the promoter or 3′-end flanking regions of the PLTP gene,2 the mechanism responsible for the induction of PLTP mRNA deserves further study.

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2 X.-C. Jiang and A. Tall unpublished observation.
