Expression of type II Na-P\textsubscript{i} cotransporter in alveolar type II cells

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Traebert, Martin, Olaf Hattenhauer, Heini Murer, Brigitte Kaissling, and Jürg Biber. Expression of type II Na-P\textsubscript{i} cotransporter in alveolar type II cells. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L868–L873, 1999.—Type II Na-P\textsubscript{i} cotransporters (type IIa and type IIb) represent apically located Na-P\textsubscript{i} cotransporters in epithelia of proximal tubules (type IIa) and small intestine (type IIb). Here we provide evidence that the type IIb (but not the type IIa) Na-P\textsubscript{i} cotransporter is also expressed in the lung. With the use of immunohistochemistry, location of the type IIb protein was found exclusively in the apical membrane of type II cells of the alveolar epithelium. Such a location of the type IIb cotransporter suggests an involvement in the reuptake of phosphate necessary for the synthesis of surfactant. A possible regulation of the abundance of the type IIb cotransporter in the lung was studied after adaptation of mice to a low-P\textsubscript{i} diet. After a chronic adaptation to a low-P\textsubscript{i} diet, no changes in the type IIb protein and the type IIb transcript were observed. These results exclude dietary intake of phosphate as a regulatory factor of the type IIb Na-P\textsubscript{i} cotransporter in alveolar type II cells.

sodium-inorganic phosphate cotransporter; immunohistochemistry

The composition of alveolar fluid is maintained by transepithelial transports of a variety of solutes through the alveolar epithelium. In alveolar type II (ATII) cells, several epithelial transport processes such as sodium-dependent transport of glucose (1, 4) as well as transport of amino acids (3, 4) and protons (15) and of sodium-dependent transport of glucose (1, 4) as well as trans

MATERIALS AND METHODS

Animals. Male mice (National Medical Research Institute; 8 wk old) were obtained from RCC (Füllinsdorf, Switzerland). All animals received tap water ad libitum and a commercial diet. For the adaptation studies, the animals were fed either a 1.1 (high-P\textsubscript{i}) or 0.09% (low-P\textsubscript{i}) P\textsubscript{i} diet (Kliba) for 5 days. Lung tissue used for the preparation of membranes or isolation of RNA was immediately frozen in liquid nitrogen.

Antisera. Polyclonal antibodies were custom-made (Eurogentec) by injection into rabbits of keyhole limpet hemocyanin-coupled synthetic peptides. Antigenic peptides corresponded to either the NH\textsubscript{2} or COOH terminus of the type IIb Na-P\textsubscript{i} cotransporter amino acid sequence (8). Results obtained with both antisera were identical.

Membrane preparations. A crude membrane fraction from the lungs was obtained as follows. Frozen lungs were thawed on ice, and all the following procedures were carried out at 4°C. The lung was homogenized with a Polytron emulsifier (setting 5 for 1 min) in 15 ml of buffer A (300 mM mannitol, 5 mM EGTA, and 12 mM Tris·HCl, pH 7.1), and the suspension was centrifuged at 1,000 \texttimes g for 30 min. The resulting pellet was resuspended in 20 ml of buffer A and centrifuged at 27,000 \texttimes g for 30 min. The resulting pellet was resuspended in 2–3 ml of buffer A by passage through a 26-gauge needle, and aliquots were immediately frozen in liquid nitrogen and stored at −70°C until used.

Western blot analyses. Membrane proteins (70 μg/lane) were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Before electrophoresis, the membranes were denaturated in the presence of 2% SDS by heating for 2 min at 95°C in the absence of a reducing agent. Incubation with primary antibodies (dilution 1:4,000) was performed as previously described (6, 8). As a secondary antibody, a goat anti-rabbit IgG conjugated to horseradish

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peroxidase (Amersham) was used, and immunodetection was carried out by enhanced chemiluminescence (Pierce). The type IIa Na–Pᵢ cotransporter of isolated renal BBMV was assessed as previously described (6).

RNA isolation and Northern blot analyses. Total RNA and poly(A)⁺ RNA were isolated with TRIzol Reagent (GIBCO BRL) and polyATtract (Promega) according to the manufacturers’ protocols. After electrophoresis on a 1.2% agarose-formaldehyde gel, mRNAs were transferred onto nylon membranes (Biodyan). The blots were hybridized with a type IIb full-length probe (8) that was labeled by random priming in the presence of [α-³²P]dCTP. A probe for the ribosomal protein L28 served as a loading control. Hybridization was performed in 6× saline-sodium citrate (SSC), 5× Denhardt’s solution, 0.5% SDS, and 100 mg/ml of herring sperm DNA at 65°C. The blots were washed sequentially with 2× SSC-0.1% SDS (10 min at room temperature), 1× SSC-0.1% SDS (10 min at 40°C), and 0.5× SSC-0.1% SDS (20 min at 55°C).

Immunohistochemistry. A 100 mg/kg body wt of thiopental intraperitoneally, the mice were perfused with a hypodermic syringe via the right cardiac ventricle. The fixative consisted of 3% paraformaldehyde and 0.05% picric acid in 0.1 M cacodylate buffer (pH 7.4; adjusted to 300 mosM with NaCl). After anesthesia (100 mg/kg body wt of thiopental intraperitoneally), the mice were perfused with 3% paraformaldehyde and 0.05% picric acid in 0.1 M cacodylate buffer (pH 7.4; adjusted to 300 mosM with NaCl), and after rinsing again with 0.1% glutaraldehyde in PBS; and rinsed again with PBS, then with cacodylate buffer, and finally with distilled water. The sections were contrasted in 2% (wt/vol) methylcellulose containing 0.2% uranyl acetate for 10 min and examined on a Phillips CM 100 electron microscope.

RESULTS

Based on RT-PCR and Northern blotting, expression of the type IIb Na–Pᵢ cotransporter in the lung tissue of mice and Xenopus laevis, respectively, has been previously suggested (8, 9). With Northern blot analysis performed with poly(A)⁺ RNA isolated from mouse lungs, we now have confirmed the presence of type IIb mRNA (Fig. 1A). A type IIb-specific probe hybridized with lung poly(A)⁺ RNA at a position of ~4 kb, corresponding to the size of the type IIb transcript present in the small intestine (8). By using a probe specific for the renal type IIa Na–Pᵢ cotransporter, a weak reaction at 4 kb was detected (data not shown; see Ref. 12). This reaction, however, has to be regarded as a cross-hybridization with type IIb mRNA because it has been shown earlier with RT-PCR that in the lung the type IIa cotransporter gene is not expressed (8) and, moreover,
that the type IIa protein could not be detected on Western blots (see below).

Further evidence for the expression of the type IIb Na-Pi cotransporter in the lung was obtained by immunoblotting of a "crude" membrane fraction. With an anti-NH2 terminus antiserum, a specific immunoreaction with a band corresponding to a molecular mass of \( \sim 108 \) kDa was observed (Fig. 1B). Specificity of this immunoreaction was confirmed by inclusion of the antigenic peptide (B; consecutive section of A). A cross section through a bronchus is shown in E and demonstrates complete absence of type IIb cotransporter in bronchial epithelium. Inset: enlargement of top right corner in E documenting parallel staining of apical membrane of type II cells. Bars, 30 µm.

**Fig. 2.** Localization of type IIb cotransporter in mouse lung tissue by light microscopy. A–E: cryostat sections stained by immunofluorescence for type IIb Na-Pi cotransporter alone (A, B, and E) or costained for type IIb cotransporter (C) and \( \beta \)-actin (D). Specific type IIb-associated immunostaining is observed in apical regions of alveolar type II cells located in corner of alveolus. This appearance was completely blocked by inclusion of antigenic peptide (B; consecutive section of A). A cross section through a bronchus is shown in E and demonstrates complete absence of type IIb cotransporter in bronchial epithelium. Inset: enlargement of top right corner in E documenting parallel staining of apical membrane of type II cells. Bars, 30 µm.

The location of the type IIb cotransporter protein in mouse lung tissue was assessed by immunofluorescence (Fig. 2) and immunogold electron microscopy (Fig. 3). Throughout the lung, tissue-specific type IIb

**Fig. 3.** Ultrathin section labeled for type IIb Na-Pi cotransporter by immunogold technique (A). Gold particles were present in high density in microvilli (B; enlargement of box in A) and were barely detectable in cytoplasm. Arrows, region of tight junctions. Bars, 1.5 µm.
Na-P\(_i\) cotransporter-mediated immunostaining was detected only in the respiratory tissue and was limited to large, roughly cuboidal cells where the alveolar walls unite and form angles. No specific immunostaining was detected in other cells of the alveolar epithelium or in the epithelium of the bronchi (Fig. 2E). Co-staining for \(\beta\)-actin revealed that the type IIb protein is located at sites of high abundance of \(\beta\)-actin, representing the apical pole of type II alveolar cells (Fig. 2, C and D).

With immunogold electron microscopy, the type IIb protein was detected along the short microvilli present at the apical pole of large cuboidal cells (Fig. 3). The vaculated subapical area and the tight junctions in the margins clearly identify these cells as ATII cells (21). Besides, in microvilli, type IIb protein-associated gold particles were also rarely observed in vacuoles present in the subapical cytoplasm but were absent in the so-called multilamellar bodies that are critically involved in surfactant storage and secretion (16).

Under conditions of reduced dietary intake of phosphate (low-P\(_i\) diet), Na-P\(_i\) cotransport in the small intestine and proximal tubules is upregulated (5, 11). In both tissues, increased transepithelial \(P_i\) transport is due to an increase in the amount of type II Na-P\(_i\) cotransporters in the apical membrane (5, 11; Hatten-hauer, Traebert, Murer, and Biber, unpublished data). To answer the question of whether the dietary content of \(P_i\) also affects the amount of type IIb cotransporter in the lung, mice were fed a low-P\(_i\) diet and, as a control, a high-P\(_i\) diet (chronic adaptation) for 5 days. As indicated in Fig. 4, no evidence for an upregulation of the type IIb protein with a low-P\(_i\) diet was obtained. As a control for the physiological response of the diets given, the amount of renal type IIa Na-P\(_i\) cotransporter was determined by Western blots in isolated renal proximal tubular BBMVs (Fig. 4A). As indicated, a low-P\(_i\), diet resulted in a large increase in the amount of type IIa protein compared with that in BBMVs isolated from control (high-P\(_i\), diet) animals and was similar in size to that reported in another study (11). Furthermore, as documented by Northern blotting, a low-P\(_i\), diet also did not result in a change in the amount of the type IIb transcript (Fig. 4B).

**DISCUSSION**

In agreement with previously reported results obtained by Northern blot analysis (9) and RT-PCR (8), the data presented indicate that the type IIb Na-P\(_i\) cotransporter is expressed in the lung tissue of mice. By Northern and Western blots, mRNA and protein species of the same sizes as observed in the small intestine (8) were observed. Furthermore, by immunocytochemistry, the type IIb Na-P\(_i\) cotransporter was identified as an apical protein of ATII cells. This cell type of the alveolar epithelium is characterized by formation of tight junctions, a high concentration of actin in the microvilli, and multilamellar bodies involved in surfactant synthesis (16, 19, 21). In all cases, type IIb-associated immunoreactions were found only in such cells, suggesting that the type IIb Na-P\(_i\), cotransporter is a specific marker for ATII cells and, moreover, for the apical membrane of these cells. No evidence for a location different (e.g., the bronchial epithelium) from type II cells was obtained.

Thus far, three different mammalian Na-P\(_i\), cotransporters have been identified and were grouped into three families: type I, type II, and type III cotransporters (22). Expression of type I cotransporters in the lung seems unlikely because no evidence for type I gene expression was obtained in earlier studies (22 and references therein). Also, in preliminary studies, we could not detect specific immunostaining with an anti-type I antibody (data not shown). With respect to type II Na-P\(_i\), cotransporters, two subfamilies were distinguished: type IIa and type IIb. In mammals, expression of the type IIa isoform is largely restricted to the proximal tubules (22). Expression of the type IIb isoform has been demonstrated in the small intestine and (based on RT-PCR) was suggested to occur in other tissues as well (8). In this study, expression of the type IIb cotransporter in the lung is described. Expression of the type IIa cotransporter was not evident as shown by the lack of immunoreaction with a type IIa-specific antibody. This is in agreement with an earlier study (8) that indicated, by RT-PCR, that the type IIa gene is not expressed in the lung. On the other hand, expression of the type III Na-P\(_i\), cotransporter in lung tissue seems to be very likely. Expression of this Na-P\(_i\), cotransporter/
retrovirus receptor family was shown to be ubiquitous, including in the lung (10, 22). Due to the lack of suitable antibodies, the exact cellular location of the type III Na-Pi cotransporter in different tissues and/or cells is not yet known. The broad expression pattern, however, suggests that the location of the type III protein is not cell specific.

The precise role of the type IIb Na-Pi cotransporter located in the apical membrane of ATII cells is not known. The alveolar surface fluid consists of an aqueous and a lipid phase acting as a surfactant. The latter contains high amounts of phosphatidylcholine, phosphoglycerol, and cholesterol (17). Thus because phosphate is a major constituent of surfactant and, therefore, is also needed in high amounts for surfactant synthesis, one may assume that P, liberated in the alveolar fluid compartment is efficiently taken up (recycled) by ATII cells via the type IIb Na-Pi cotransporter.

Extracellular concentration of P, is largely controlled by the rate of renal proximal tubular P, reabsorption and also by the capacity of the small intestine to reabsorb P,. It has been demonstrated that in both epithelia the rate of transepithelial P, transport is determined by the abundance of type II Na-Pi cotransporters residing in the apical membranes. Among other factors, limited intake of phosphate via the diet (low-P, diet) results in an increased amount of type II Na-Pi cotransporters in brush borders of proximal tubules (11) and enterocytes (5; Hattenhauer, Traebert, Murer, and Biber, unpublished data). Although, in the kidney, upregulation occurs, to a large part, independent of other factors such as parathyroid hormone (11), upregulation of the type IIb cotransporter in the small intestine is mediated via 1,25-dihydroxyvitamin D3 (5). To investigate whether restriction of dietary P, may also lead to an upregulation of the amount of type IIb cotransporters in the lung, the relative abundance of the type IIb protein and transcript was analyzed after a chronic low-P, diet. Although, by this condition, upregulation of the renal type IIa cotransporter was clearly detected, no evidence for upregulation of the amount of the type IIb Na-Pi cotransporter in the lung was obtained. This suggests that the type IIb Na-Pi cotransporter in ATII cells is not regulated by the dietary content of phosphate directly or indirectly via 1,25-dihydroxyvitamin D3. It remains to be determined whether the amount of type IIb Na-Pi cotransporters in ATII cells is regulated at all or if this Na-Pi cotransporter is constitutively expressed in these cells.

In summary, we have shown that the type IIb Na-Pi cotransporter is specifically expressed in the apical membranes of ATII cells. Furthermore, evidence was obtained that this transporter is not regulated by changes in the dietary content of P,. Although the exact role of Na-Pi cotransport through the apical membrane of the ATII cells remains to be determined, a role in surfactant synthesis is suggested.

The first two authors contributed equally to this work.

This work was supported by Swiss National Fund Grants 31-052853.97 (to J. Biber) and 31-47742-96 (to B. Kaissling).

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Received 9 March 1999; accepted in final form 19 July 1999.

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