Modulation of aquaporin 4 and the amiloride-inhibitable sodium channel in perinatal rat lung epithelial cells

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Modulation of aquaporin 4 and the amiloride-inhibitable sodium channel in perinatal rat lung epithelial cells. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L1066–L1072, 1998.—During the perinatal period, a dramatic reversal of lung transepithelial ion and water transport occurs that involves the amiloride-inhibitable Na+ channel (ENaC). Aquaporin (AQP) water channel proteins facilitate cell membrane water transport. We now report that AQP-4, localized to basolateral membranes of airway epithelial cells, increases its mRNA expression in developing lung eightfold during the 2 days before birth to reach a peak on the first postnatal day in the lungs but not in brains or kidneys of neonatal rats. AQP-4 and the α-, β-, and γ-subunits of ENaC are both expressed by cultured rat fetal distal lung epithelial (FDLE) cells. AQP-4 and ENaC expression increase in FDLE cells cultured on uncoated permeant filters compared with matched control cells cultured on filters containing extracellular matrix derived from fetal lung epithelial cells. Similarly, AQP-4 expression increases in FDLE cells exposed to 21% O2 compared with cells exposed to 3% O2. These data demonstrate that AQP-4 expression is highest on the first day after birth in neonatal rat lungs. Exposure to ambient 21% O2 may contribute to increases in AQP-4 and ENaC expression to facilitate water transport across neonatal airway epithelia in the immediate postnatal period.

Aquaporin water channel; lung development; neonatal pulmonary function

IN FETAL LIFE, mammalian lungs are filled with a Cl-rich fluid secreted by the epithelium that is necessary for proper lung development (reviewed in Refs. 3, 23, 36). The switch from placental to pulmonary gas exchange occurring after parturition requires that fluid be absorbed from both the alveoli and airways to create an air-liquid interface for adequate respiratory function. To accomplish this transition, transepithelial Na+ absorption occurs across the pulmonary epithelium, accompanied by modulation in both the expression and activity of basolateral Na+-K+-ATPase (4, 27, 29) as well as of the apical amiloride-inhibitable epithelial Na+ channel (ENaC; see Refs. 5, 26, 25, 28). To determine whether membrane aquaporin (AQP) water channel proteins (6, 38) are modulated concomitant with Na+ reabsorption during this interval, we characterized the temporal relationship between the expression of two lung AQPs, AQP-4 and AQP-5, compared with ENaC subunits in both intact lungs and fetal distal lung epithelial (FDLE) cells from neonatal rats.

At present, a total of eight AQPs have been identified from various rat tissues (6, 10, 14, 16–18, 37). Although their transmembrane domains share a large degree of structural homology, both NH2- and COOH-termini of AQPs are divergent. The specific roles of these AQPs in both adult and perinatal lung function remain unclear despite considerable effort by multiple laboratories. In rat lung, AQP-1 is expressed by pulmonary endothelial cells and may facilitate fluid clearance from lungs during the perinatal period (22). Despite demonstration that its expression in lung increases fivefold at parturition and is potentiated by maternal corticosteroid administration (15), evidence that AQP-1 plays a critical role in lung function is lacking, since human subjects lacking AQP-1 are phenotypically normal (32). Although AQP-5 is expressed in type I pneumocytes during the perinatal period, its expression increases significantly only 2–4 days after birth (18). Thus timing of maximal AQP-5 expression in perinatal lung does not coincide with the interval of maximal transepithelial fluid reabsorption in lung.

AQP-4 (14; also previously referred to as AQP-0, also referred to as mercurial-insensitive water channel) is present in adult rat lung at modest levels and is significantly increased during the perinatal period (37). However, a recent demonstration that mice lacking AQP-4 protein display neither abnormal lung development nor significant lung disease at birth suggests that AQP-4 is not critical for lung fluid clearance during the perinatal period (19).

In this report, we quantify the AQP-4 mRNA expression in the lungs, kidneys, and brain of fetal, neonatal, and adult rats to determine if lung AQP-4 expression is modulated in a manner similar to that exhibited by the α-, β-, or γ-subunits of ENaC. Furthermore, we quantified alterations in AQP-4 expression in rat FDLE cells cultured under conditions in which either extracellular matrix (ECM) or ambient fraction of inspired O2 was varied. Together, these data demonstrate that AQP-4 expression is modulated in a manner similar to that displayed by ENaC. The eightfold increase in AQP-4 within a 24- to 72-h interval from embryonic (E) day 20 (E20) to postnatal day 1 suggests that AQP-4 may play some noncritical role in lung airway transepithelial water reabsorption, perhaps in conjunction with ENaC during the early postnatal period.
MATERIALS AND METHODS

Isolation of AQP-4 and AQP-5 probes. RT-PCR was utilized to amplify cDNAs using primers corresponding to nucleotides (nt) 4–84 (5′-GCTGATCATGTTGCCTTCACAAGGCGTCTG-3′ with an engineered Bsl I site), nt 934–951 (5′-CCCGCATGACTCGTGTCCTGCTCGTACGTTGTTGCTCG-3′ with an engineered Sph I site) for AQP-4 (14), and nt 104–122 (5′-GGCACCATGAAAAGGAGGTG-3′) and nt 906–924 (5′-CATGGTGTTGCTGAGTCTG-3′) for AQP-5 (33). After 10 ng of cDNA from total lung RNA of Sprague-Dawley rats (Charles River Laboratories, Billerica, MA) were prepared using the Superscript preamplification system (GIBCO-BRL, Gaithersburg, MD), PCR amplifications were achieved by 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min plus 72°C, 7 min) using 50 pmol of each primer per reaction. The resulting 879-bp AQP-4 fragment and the 820-bp AQP-5 fragment were subcloned into PCR II (Invitrogen, San Diego, CA), and both strands were sequenced by the Department of Genetics DNA Sequencing Facility (Children's Hospital, Boston, MA) using the dye terminator technique (9).

RNA isolation and Northern analyses. After RNA was isolated from embryonic days E18–E21 (E18–E21), postnatal (postnatal days 1, 3, 7, and 14), and adult rat tissues and cultured cells using a STAT 60 Kit (Teltest B, Friendswood, TX), it was size fractionated by electrophoresis in formaldehyde–agarose gels, transferred to nylon membranes (Duralon-UV; Stratagene, La Jolla, CA), and ultraviolet cross-linked as described previously (13). After prehybridization, blots were hybridized with either 32P-labeled AQP-4, AQP-5, or full-length (3.7 kb)-, or 3.2 kb)-ENA C probes (5′ 8GGCACCATGAAAAAGGAGGTG-3′; see Ref. 5), washed with 0.1× SSC + 0.1% SDS at 55°C for 30 min (AQP-4 and AQP-5) or 0.2× SSC + 0.1% SDS at 42°C (α-, β-, and γ- ENAs), and then subjected to autoradiography at −70°C (Lightening Plus intensifier screens and XAR-5 film; Eastman Kodak, Rochester, NY). Individual lanes of autoradiograms were quantified using either photoanalytic image processing with National Institutes of Health (Bethesda, MD) Image or laser densitometry as described previously (34), and the signal for each band was normalized by probing the same blots with a similarly labeled cDNA probe for glyceraldehyde-3-phosphate dehydrogenase and comparing signal intensity. Results are expressed as means ± SE. Significant differences between samples were determined using ANOVA. Statistical significance was considered at P values of <0.05.

Preparation of affinity-purified anti-AQP-4 antiserum. A 16-mer peptide corresponding to amino acids 277–292 of AQP-4 (HVIDIRGDGKEKKDGDC) was synthesized and purified by HPLC (QC8, Hopkinson, MA), conjugated to keyhole limpet hemocyanin, and used to immunize rabbits. Subsequent immune sera were affinity purified using SulfoLink Coupling gel (Pierce, Rockford, IL) as described previously (21).

Immunohistochemistry. Tracheas of anesthetized rats were intubated, and lungs were perfused with optimum cutting temperature (OCT) compound (Miles, Elkhart, IN). Lung tissue samples were then embedded in OCT compound and snap-frozen in liquid nitrogen, and 6-µm-thick frozen sections were mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were incubated at 37°C for 15 min, fixed with acetone for 5 min, exposed to 0.3% Triton X-100 in phosphate-buffered saline for 5 min, and blocked with PBS containing 2% bovine serum albumin (BSA) and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Chester, PA) for 1 h at 25°C. After a 1.5-h exposure to affinity-purified anti-AQP-4 peptide antibody (1:50 dilution in PBS with 2% BSA), slides were incubated (1 h) with alkaline phosphatase-conjugated affinity-purified donkey anti-rabbit IgG (1:75; Jackson ImmunoResearch Laboratories), and then washed slides were developed using fast red chromagen (Sigma Chemicals, St. Louis, MO) and counterstained with methyl green.

Isolation of FDLE cells and fetal matrix. FDLE cells were isolated from E20 Wistar rats as described previously and seeded at confluence (5 × 105 cells/cm2) on either lung cell-derived matrix (mixed-lung cell [MLC]) derived from embryonic day 17 (E17) fetal rats or uncoated filters (31). Previous work (31) has demonstrated that FDLE cells are derived primarily from prealveolar epithelium. FDLE cell filters were then cultured in Dulbecco's modified Eagle's medium with 10% FBS in humidified 21% O2-5% CO2-74% N2 or 3% O2-5% CO2-92% N2 atmospheres for 24 h (30). After replacement of the medium in 24 h, adherent cells were harvested for RNA at 24 h (uncoated vs. MLC experiments) or 72 h (3 vs. 21% O2 concentration experiments).

RESULTS

Maximal AQP-4 expression occurs in rat lungs during the perinatal period. RT-PCR amplifications of adult rat lung cDNA with primers specific for AQP-4 and AQP-5 yielded probes possessing nucleotide sequences identical to those reported previously (14, 33). As shown in Fig. 1A, Northern analyses of brain, lung, and kidney total RNA from adult rats confirmed (14) high levels of AQP-4 expression in adult rat brain (100%, n = 4) that were significantly different (P < 0.001) compared with either lungs (36 ± 10.8%) or kidneys (17 ± 6%) from the same animals. AQP-4 is detectable on E20 (Fig. 1C, lane 1), and its maximal expression occurs within the immediate perinatal period corresponding from birth (D1; lane 3) to postnatal day 3 (D3; lane 4) as shown in Fig. 1, A and C. Lung AQP-4 expression within 24 h of birth is 830 ± 20% (P < 0.005, n = 3) higher compared with that in E20 rats or 200 ± 10% (P < 0.001, n = 3) higher than levels present in adult lung (Fig. 1C, lane 7). In contrast, AQP-5 expression in developing lung is distinct from that exhibited by AQP-4 (Fig. 1D). AQP-5 is detectable in total lung RNA at E21 (lane 2) and only increases gradually over the first weeks of life such that adult lung AQP-5 expression (Fig. 1D, lane 6) is 299 ± 54% (P < 0.005, n = 4) greater compared with that present in the lungs of D1 rats.

AQP-4 expression in the developing lung is tissue specific, since identical analyses of both brain (Fig. 2, A and B) and kidney (Fig. 2C) reveal different age-specific patterns. For example, expression of AQP-4 in brain reaches adult levels that are 210 ± 32% (n = 3, P < 0.001) of those of D1 rats only after day 14 (Fig. 2A).

AQP-4 is localized to the basolateral surfaces of airway epithelial cells in both the adult and neonatal lung. Affinity-purified antibody raised to a 16-mer peptide containing a sequence present in the COOH-terminal domain of AQP-4 revealed distinct staining of the basolateral membranes of epithelial cells lining both cartilaginous large airways and bronchi as well as of medium-sized bronchi and bronchioles in lungs of newborn and adult rats (Fig. 3, A and C). Staining is not present in other lung structures, including pulmonary vessels and alveolar spaces. In all cases, AQP-4
staining is specific as demonstrated by its ablation after addition of excess (50 µg/ml) corresponding peptide (Fig. 3B). No specific staining is observed in the lungs of E20 or E21 fetal rats (data not shown). As reported previously by others (8, 19, 37), anti-AQP-4 antiserum also identifies AQP-4 protein in the basolateral membranes of epithelial cells lining the tubules in the kidney medulla (Fig. 3D).

AQP-4 is expressed in cultured FDLE cells where its expression is modulated by alterations in fetal ECM and ambient Po2 in a manner similar to that exhibited by the α-, β-, and γ-subunits of ENaC. Previous work in one of our laboratories has utilized cultured FDLE cells to demonstrate that amiloride-inhibitable transepithelial Na+ transport is modulated by multiple factors, including ECM components synthesized by MLC isolated from fetal rats (30, 31, 36). To determine if the expression of ENaC subunits and AQP-4 is modulated in FDLE cells in a similar manner, we incubated FDLE cells on uncoated filters or filters coated with fetal ECM. As shown in Fig. 4A–C, the expression of the α-, β-, and γ-ENaC subunits as well as of AQP-4 were reduced on coated filters compared with paired FDLE cells cultured on uncoated filters. In contrast to FDLE cells from uncoated filters (Fig. 4, lanes 1 and 3), all ENaC subunits of FDLE cells cultured on fetal ECM filters are reduced significantly (P < 0.05, n = 4), including the α-subunit (96 ± 3.5% reduction; Fig. 4C,

Fig. 1. Aquaporin (AQP)-4 expression in the lung peaks in the perinatal period. A: expression of AQP-4 in the adult rat. Twenty micrograms of total RNA prepared from whole brain (lane 1), lung (lane 2), and kidney (lane 3) were subjected to Northern blotting analyses using a 32P-labeled AQP-4 cDNA probe. Resulting single autoradiogram (exposed for 18 h) displayed here shows a single 5.5-kb transcript present in all lanes and is representative of 4 separate experiments. Bottom of A shows data from the same blot after reprobing with a 32P-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to correct for differences in RNA loading. Filled circles denote locations of the 28S and 18S ribosomal RNA bands.

B: expression of AQP-4 in developing rat lung. Total RNA was prepared from embryonic day 20 (E20; lane 1), embryonic day 21 (E21; lane 2), neonatal day 1 (D1; lane 3), neonatal day 3 (D3; lane 4), neonatal day 7 (D7; lane 5), neonatal day 14 (D14; lane 6), and adult lungs (lane 7) using both AQP-4 and GAPDH probes. Relative expression of AQP-4 represented by the autoradiographic densities of AQP-4/GAPDH normalized for the mean AQP-4 expression in adult lung RNA is shown. C: individual autoradiogram exposed for 18 h displaying prominent expression of AQP-4 in the lungs of neonatal rats. Lane designations correspond with bars in B. D: expression of AQP-5 in developing lung. This single autoradiogram is representative of a total of 4 Northern analyses performed as described in B. Total lung RNA from E20 (lane 1), E21 (lane 2), D1 (lane 3), D3 (lane 4), D7 (lane 5), and adult lung (lane 6) was probed with both AQP-4 and GAPDH. Note that AQP-5 expression is not maximal in the immediate postnatal period.

Fig. 2. Expression of AQP-4 in developing rat brain and kidney is distinct compared with lung. A: quantitation of AQP-4 expression in developing rat brain. Quantitative Northern analyses were performed as described in Fig. 1 using 20 µg of total RNA prepared from E20 (lane 1), E21 (lane 2), D1 (lane 3), D3 (lane 4), D7 (lane 5), D14 (lane 6), and adult (lane 7) rats. Relative expression of AQP-4 vs. GAPDH is expressed as mean ± SE for a total of 3 separate individuals. Significant differences (P < 0.001) are observed between adult vs. fetal, D1, D3, and D7 but not D14 lanes. B: individual autoradiogram exposed for 14 h displaying prominent expression of AQP-4 in rat brain after D7. Lane designations correspond to rat ages in A. C: expression of AQP-4 in developing rat kidney. Expression of AQP-4 in total RNA (20 µg/lane) prepared from either embryonic day 18 (E18; lane 1), E20 (lane 2), D1 (lane 3), or adult (lane 4) whole kidney is shown (n = 3) and was determined as described in Fig. 1. Filled circles denote locations of the 28S and 18S ribosomal RNA bands.
lanes 2 and 4) as well as the β- and γ-subunits (67 ± 9 and 90 ± 3% reductions, respectively, Fig. 4B, lanes 2 and 4). In a similar manner, AQP-4 mRNA expression in FDLE cells cultured on fetal ECM is also significantly reduced by 48 ± 8% (P < 0.005 n = 4). No AQP-5 expression was detectable in these FDLE cells despite prolonged autoradiography (data not shown). In a similar manner, FDLE cells cultured in 21% O2 express significantly (50.2 ± 5.3%, P < 0.01, n = 4) more AQP-4 compared with FDLE cells cultured in 3% O2 (Fig. 5).

**DISCUSSION**

It is now generally accepted that ENaC plays an important role in net fluid absorption from airways and alveoli at the time of birth (11, 24, 25, 28). Targeted deletion of α-ENaC gene function results in mice that exhibit a lack of amiloride-sensitive Na⁺ transport, fail to clear their lung liquid, and die shortly after birth (11). However, the transepithelial pathways for water reabsorption that accompany the establishment of ionic gradients in newborn lungs are not known.

AQP proteins have been demonstrated to increase the water permeability of both apical and basolateral membranes where net water reabsorption is driven by the establishment of small osmotic gradients (1, 16). In contrast, a combination of AQP-2 (apical membrane) and AQP-3 as well as AQP-4 (basolateral membrane) facilitates selective water reabsorption in response to large transepithelial osmotic gradients in kidney collecting duct (1, 12, 16, 21, 38). However, the exact physiological roles of various AQPs in transepithelial water transport in mammalian tissues are currently under active investigation. Whereas humans lacking AQP-1 expression do not exhibit major alterations in either kidney or pulmonary physiology (32), mutations in AQP-2 cause non-X-linked nephrogenic diabetes insipidus (7). At present, no natural human mutations of AQP-3, -4, or -5 have been reported. However, recent targeted deletion of AQP-4 gene function in mice has established that a knockout of AQP-4 gene function does not alter normal morphology of developing lung or cause significant changes in the expression of AQP-5 in lung that could possibly compensate for lack of AQP-4 lung function (19).

To investigate if the expression of AQP-4 and AQP-5 is similar to that exhibited by ENaC, we studied...
selected aspects of both the temporal expression and location of these AQP water channels in the lungs of fetal and postnatal rats as well as in cultured FDLE cells isolated from lung tissue.

Northern analyses (Fig. 1) show that AQP-4 expression begins by E20 in fetal rats and increases eightfold over a 48- to 72-h interval to achieve maximal levels during the first postnatal day. Thereafter, lung AQP-4 mRNA levels decline over the first week of postnatal life to adult levels that remain approximately one-half of those present during the brief interval of maximal AQP-4 expression. This pattern of lung AQP-4 expression is tissue specific (Fig. 2). These data confirm and extend those published recently by Umenishi et al. (37). Our immunohistochemistry of AQP-4 protein in adult and developing rat lung is similar to previous reports (8, 10, 37) and reveals that AQP-4 is present on the basolateral surfaces of epithelial cells lining large and small airways but not in alveolar epithelial cells (Fig. 3). The lack of detectable AQP-4 protein in airways of fetal rats is consistent with Northern analyses described above. Localization of AQP-4 to airway epithelial cell basolateral membranes suggests that it may augment transepithelial water in a manner similar to that proposed for AQP-4 in the basolateral membrane of kidney inner medullary collecting duct (8, 16, 19). However, at the present time, no apical membrane AQP (corresponding to AQP-2 in kidney) has been identified for these airway epithelial cells.

To explore the relationships between factors that modulate expression of both AQP-4 and the ENaC subunits, we utilized primary cultures of FDLE cells exposed to differences in either ECM and O2 concentrations (Figs. 4 and 5). Compared with control FDLE cells cultured on uncoated filters, FDLE cells cultured on fetal lung ECM exhibit a combination of significant reductions in amiloride-inhibitable Na+ transport (31) during the first week of postnatal life, β- and γ-ENaC expression begin 24–48 h before birth and reach maximal levels of expression during the first week of life on postnatal day 1 (36). In contrast, similar Northern analyses of lung AQP-5 expression (Fig. 1D) reveal that, although it is detectable in E21 fetal rats, maximal adult AQP-5 mRNA levels are not achieved until after the first week of postnatal life (15, 37).

Fig. 4. Expression of AQP-4 and amiloride-inhibitable Na+ channel (ENaC) subunits α, β, and γ in cultured fetal distal lung epithelial (FDLE) cells or E20 rat lung. Northern analyses were performed on total RNA (15 µg/lane) prepared from either FDLE cells cultured for 48 h after their isolation from the lungs of E20 fetal rats (lanes 1–4) or whole lungs of E20 rats (lane 5). Paired samples of FDLE cells were cultured on either uncoated Transwell filters (lanes 1 and 3) or filters coated with extracellular matrix produced by mixed-lung cells from embryonic day 17 fetal rat lungs (lanes 2 and 4). A: comparison of expression of AQP-4 and corresponding GAPDH transcript. B: identical blot showing expression of γ (3.1 kb)- and β (2.4 kb)-ENaC subunits. C: identical blot showing expression of α (3.8 kb)-ENaC. Locations of the 28S and 18S ribosomal RNA bands are indicated by filled circles.

Interestingly, the pattern of AQP-4 expression in developing rat lung is similar to that described previously for ENaC (25, 36), resulting in coincident maximal coexpression of α-, β-, and γ-subunits and AQP-4 on day 1 of life. Although α-ENaC expression peaks immediately before birth and then declines transiently dur-
as well as significant decreases in $\alpha$, $\beta$, and $\gamma$-ENaC subunits as well as in AQP-4 (Fig. 4).

Recently, Pitkanen et al. (30) reported that switching cultured FDLE cells from fetal 3% to postnatal 21% O$_2$ concentrations significantly increases both the ENaC and ENaC subunit expression. Data shown in Fig. 5 demonstrate that exposure of FDLE cells to 21% O$_2$ vs. 3% O$_2$ increases AQP-4 expression by ~50%. These findings may suggest the existence of an O$_2$-responsive mechanism that may participate in modulation of both AQP-4 and ENaC subunits at the level of transcription. In contrast, FDLE cells do not display detectable levels of AQP-5 mRNA when cultured under any of these conditions described above.

In summary, the data reported here provide evidence that the expression of ENaC subunits and AQP-4 is highest on the first day after birth in neonatal rats. In this regard, exposure to ambient 21% O$_2$ may contribute to the increases in both AQP-4 and ENaC expression. Because the majority of lung liquid is absorbed during labor and the 6-h interval immediately after birth (when AQP-4 protein levels in lung are low; see Ref. 26), AQP-4 protein is not likely a rate-limiting step in transepithelial water flux. These data may provide a partial explanation for the absence of phenotypic abnormalities of AQP-4 in knockout animals (19). We speculate that AQP-4 may become more important in the overall process of transepithelial water flux in airways after the immediate postnatal period when endogenous hormonal levels such as epinephrine have declined (24). Although these data suggest that coordinate regulation of ENaC and AQP-4 expression may occur and imply that AQP-4 may participate in increasing the water permeability of the basolateral membranes of airway epithelial cells to facilitate Na$^+$-mediated reabsorption of fetal lung liquid, both the role of AQP-4 protein in airway water reabsorption and its relationship to transepithelial Na$^+$ flux mediated by ENaC require further study.

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