Role of aquaporin water channels in pleural fluid dynamics

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In this study we investigated the role of aquaporin-type water channels in pleural fluid dynamics. Aquaporins are integral membrane proteins that function as bidirectional transporters of water and, in some cases, small solutes such as glycerol (reviewed in Refs. 10 and 28). Water movement across aquaporins can be driven by osmotic, oncotic, or hydrostatic forces. Recent studies in mice lacking specific aquaporins have indicated an important role for aquaporins in several organs (30). For example, mice lacking aquaporin-1 (AQP1) or AQP3 have nephrogenic diabetes insipidus with marked polyuria (13, 24), mice lacking AQP4 manifest reduced cerebral edema in response to brain injury (17), and mice lacking AQP5 have defective saliva secretion (14). Humans lacking AQP2, the vasopressin-regulated water channel, have a rare autosomal form of hereditary nephrogenic diabetes insipidus (6).

The rationale for investigation of the role of aquaporins in pleural fluid transport is the observation that AQP1 is present in most microvascular beds including those in lung (21) and that AQP1 deletion in mice produces marked alterations in the water permeability of microvasculature in lung (2), renal vasa recta (22), and the peritoneal barrier (36). Immunostaining revealed strong AQP1 protein expression in microvascular endothelia near the pleural surface and the peritoneal barrier (36). Immunostaining revealed strong AQP1 protein expression in microvascular endothelia near the pleural surface and movement across a mesothelial barrier lining the pleural space (4, 32). Fluid clearance from the pleural space is thought to occur primarily by lymphatic drainage (18). Under normal conditions Starling forces provide the driving force for fluid accumulation in the pleural space (8, 18).

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provided direct information about the role of AQP1 in pleural fluid transport.

METHODS

Transgenic mice. Transgenic knockout mice deficient in AQP1 or AQP3 were generated by targeted gene disruption as described previously (13, 16). Measurements were done in litter-matched mice (8–10 wk of age) produced by intercrossing of heterozygous mice in a CD1 genetic background. The investigators were blinded to genotype information for all physiological measurements. Protocols were approved by University of California, San Francisco, Committee on Animal Research.

Osmotic water permeability measurements. Mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and mechanically ventilated through a tracheostomy (Harvard Instruments, tidal volume 7–10 ml/kg, respiratory rate 70–90/min, room air). After 5 min, 0.25 ml of fluid [either saline containing 200 mM sucrose and 1% BSA (500 mosmol/kgH2O) or saline diluted with water containing 1% BSA (200 mosmol/kgH2O)] was infused into the pleural cavity bilaterally (0.15 ml right side, 0.1 ml left side) using a 1-ml syringe and 27-gauge needle. The chest wall was punctured laterally between the 5th and 6th ribs, with the tip of needle parallel to the lung surface to avoid lung puncture. If needed, small amounts of pentobarbital were given to maintain anesthesia. At specified times (0.5–20 min), the abdominal cavity was rapidly exposed and the descending aorta was transected. The chest was opened by a midline incision, and pleural fluid was withdrawn using a micropipette. After low-speed centrifugation (1,000 rpm, 5 min), supernatant osmolality was measured in duplicate using a freezing-point depression osmometer (Precision System, Natick, MA).

Equation 1 gives the time course of pleural fluid osmolality in response to infusion of an anisomolar solution at zero time. The equation assumes that surface area is constant, that no significant nonscopic fluid movement occurs during the brief measurement time, and that the osmotic properties of the pleural barrier are time independent. For computation of P1 from experimentally measured half-times (t1/2) for osmotic equilibration, Eq. 1 was evaluated at t = t1/2, in which C1(t) = [C0 + C1(0)]/2.

Isosmolar pleural fluid clearance. Mice were briefly anesthetized by halothane inhalation. Isosmolar fluid (0.25 ml) consisting of saline containing 1% albumin and added NaCl (320 mosmol/kgH2O) was infused into the right pleural cavity. After discontinuation of anesthesia, mice resumed normal activity without signs of distress. Mice were given free access to water. After 0.5, 30, 60, or 90 min, mice were reanesthetized, and pleural fluid was withdrawn as described above. Care was taken to withdraw as much fluid as possible. After centrifugation, the weight of the supernatant fluid was determined.

Hydrostatically driven pleural effusions. A fluid overload model of pleural effusion consisted of bilateral renal vessel ligation followed by intraperitoneal saline infusion. Renal vessel ligation was required because the polyuria in AQP1 null mice is not responsive to the V2 agonist 1-desamino-8-D-arginine vasopressin (DDAVP) (13). Mice were anesthetized with pentobarbital (50 mg/kg ip). The lateral abdominal skin was exposed, and a 2- to 3-cm incision was made in the back just lateral to the midline. The renal vessels were isolated and exposed by blunt dissection without damaging the peritoneum. The renal vessels were ligated bilaterally with 3–0 silk suture, and the skin was sutured closed. Isosmolar saline (40% body wt) was infused into the peritoneal cavity, and body weight was determined. After 3 h, body weight was measured to confirm anuria. After anesthesia with pentobarbital (150 mg/kg ip), pleural fluid was collected as described above, and lungs were harvested for determination of wet-to-dry weight ratio.

Thiourea-induced endothelial injury. Thiourea (10 mg/kg) in isosmolar saline (5 mg/ml concentration) was infused into the peritoneal cavity. At 2, 3, or 5 h, the mice were anesthetized with pentobarbital (150 mg/kg ip), pleural fluid was collected as described above, and lungs were harvested for determination of wet-to-dry weight ratio as described previously (26).

RT-PCR and immunocytochemistry. Pleural membranes were carefully microdissected from three wild-type and three AQP1 knockout mice and one AQP3 knockout mouse after euthanasia by pentobarbital overdose. Perfusion of the pulmonary artery with saline at high pressure facilitated detachment of the visceral pleura from lung parenchyma. Tissues were immediately homogenized in Trizol reagent (GIBCO BRL) for mRNA isolation using Oligotex mRNA mini kit. After reverse transcription, PCR was carried out using gene-specific primers designed to amplify portions of the coding sequences of each of the nine mouse aquaporins as described previously (37). Control PCR reactions were done in parallel using as template a cDNA mixture prepared from brain, lung, kidney, and liver. Immunofluorescence localization of AQP1 and AQP3 protein in cryostat sections of paraformaldehyde-fixed pleural membranes was done using specific rabbit polyclonal antibodies.

RESULTS

RT-PCR was carried out to identify transcripts encoding mammalian aquaporins in pleura. Reverse-transcribed cDNA from microdissected pleural membranes was PCR amplified using specific primers for the nine mammalian aquaporins cloned in mice. Figure 1A shows a strongly amplified AQP1 fragment and weakly amplified bands for AQP3s 3, 7, and 9. The weakly amplified transcripts encoding AQP3s 3, 7, and 9 may represent contamination of the pleural membranes by fat and blood/immune cells. In the control lanes in which the template consisted of cDNA derived from a tissue mixture containing all aquaporins, all nine aquaporins were amplified. Based on these results, immunolocalization was done for AQP1 and AQP3 (good antibodies not available for AQP7 and AQP9).
AQP1 protein was strongly expressed in microvessels near diaphragmatic (Fig. 1B-α), visceral (β), and parietal (c) pleura of wild-type mice. AQP1 was found at endothelial cell plasma membranes in small and medium-sized vessels. These results are consistent with previous data showing AQP1 expression in the microvasculature of many organs (21). AQP1 was also expressed in surface mesothelial cells of visceral pleura (Fig. 1B-b, arrowheads) and to a lesser extent in parietal and diaphragmatic pleura. No specific immunostaining was found in pleura from AQP1 null mice (Fig. 1B, bottom). No immunostaining of AQP3 was seen in wild-type or AQP1 null mice (not shown).

Osmotically driven water transport across the pleural surface was measured in anesthetized, mechanically ventilated mice in which 0.25 ml of hyperosmolar (500 mosmol/kgH₂O) or hyposmolar (200 mosmol/kgH₂O) fluid was instilled into the pleural space. Mice were killed at specified times, and pleural fluid was collected from the chest cavity for measurement of osmolality. After instillation of 500 mosmol/kgH₂O fluid into the pleural space, wild-type mice had consistently more fluid in the pleural space than AQP1 null mice killed at the same time, indicating more osmotic water entry and hence higher osmotic water permeability. As measured at a single time point of 5 min, AQP3 deletion did not reduce water permeability. Rates of osmotic equilibration were even faster in response to instillation of the hyposmolar fluid, with \( t_{1/2} \) values of 0.7 min (wild type) and 1.8 min (AQP1 null).

Apparent osmotic water permeability coefficients \( (P_o) \) were computed from \( t_{1/2} \) values as described in Methods, assuming a smooth pleural surface of area 10 cm². In response to infusion of hyperosmolar (500...
mosmol/kgH₂O) fluid, moved water into the pleural space with a $P_f$ of 0.024 cm/s in wild-type mice. $P_f$ was reduced to 0.006 cm/s in AQP1 null mice. In response to infusion of hyposmolar fluid (200 mosmol/kgH₂O), $P_f$ values were 0.086 cm/s (wild type) and 0.033 cm/s (AQP1 null). These values are subject to more uncertainty than those for the hyperosmolar study because of the rapid equilibration times. The interesting asymmetry in pleural fluid transport is discussed in DISCUSSION.

The high pleural fluid osmolality at the 30-s time point in the AQP1 null mice infused with 500 mosmol/kgH₂O solution suggests that the pleural space contains little fluid at the time of infusion, which is consistent with reported volumes of 0.1–0.2 ml/kg in many species (27). Indeed, very little pleural fluid could be collected from control wild-type or AQP1 null mice the were killed without fluid infusion (26). To estimate the amount of pleural fluid directly, the pleural spaces of wild-type and AQP1 null mice were instilled with 0.25 ml of isosmolar fluid containing $^{125}$I-albumin as a volume marker. Mice were killed immediately for collection of pleural fluid and assay of $^{125}$I-labeled albumin concentration. The measured ratios of instilled-to-collected $^{125}$I-albumin concentrations [1.09 ± 0.02, wild type; 1.11 ± 0.01, AQP1 null (± SE); $n$ = 3] confirmed that initial pleural space fluid volume was under ~25 μl in the mice studied here.

To determine whether the decreased pleural osmotic water permeability results in impaired clearance (absorption) of pleural fluid, the pleural space was instilled with isosmolar fluid, and the pleural fluid volumes remaining at specified time points were measured. Figure 3 shows an approximately linear decrease in collected pleural fluid volume with averaged volume absorption rates of 3.8 (wild type) and 4.0 ml·kg⁻¹·h⁻¹ (AQP1 null). It is concluded that AQP1 deletion does not affect isosmolar pleural fluid clearance. Since measurements are comparative, the validity of this conclusion is unlikely to be affected by the imperfect recovery of pleural fluid by the micropipette collection method (0.23 ml recovered at time 0 of 0.25 ml instilled).

To determine whether AQP1 deletion affects pleural fluid accumulation in response to a hydrostatic driving force, a model of fluid overload with renal artery ligation was used. An excess of isosmolar fluid was infused into the peritoneal cavity, where it was absorbed to produce intravascular volume overload and pleural effusions. Although the classic model of acute volume expansion in rodents utilizes fluid overload (39), urinary losses were a concern here. Because DDAVP is ineffective in AQP1 null mice (13), bilateral renal artery occlusion was required to eliminate the kidney as a significant route of fluid clearance. The acute volume overload produced a marked accumulation of pleural fluid (Fig. 4A) but no significant elevation in lung water (Fig. 4B). AQP1 deletion did not affect the accumulation of pleural fluid.

Last, an established model of endothelial lung injury was used to create transient pleural effusions. In mice, intraperitoneal administration of thiourea (10 mg/kg) produces rapid accumulation of pleural fluid with peak volumes at 3 h in wild-type mice, followed by slow spontaneous clearance of the pleural effusion (23, 35). We tested whether the kinetics of thiourea-induced pleural fluid accumulation or clearance was affected by AQP1 deletion. Mice were killed at specified times after thiourea administration for assay of pleural fluid volume and lung water. AQP1 deletion did not significantly affect the kinetics of pleural fluid volume after 2, 3, and 5 h (Fig. 5A), nor did it affect lung water accumulation (Fig. 5B).
DISCUSSION

The principal finding of this study was that, whereas AQP1 provides a major pathway for osmotically driven water transport across the pleural barrier, AQP1 did not appear to play a role in physiologically and clinically important pleural fluid balance. Mouse models were developed for measurement of pleural fluid volume and lung water content. A: pleural fluid volume. B: lung wet-to-dry weight ratio. Data are means ± SE for 5 mice at each time point.

Osmotic water permeability across the pleural barrier was reduced by greater than fourfold by AQP1 deletion, while the anatomy of the pleural surface and microvasculature was not affected at the light microscopic level. Since AQP1 is expressed primarily in microvascular endothelial cells near the pleural surface, the reduced osmotic water permeability in AQP1 null mice suggests that the microvascular endothelium is a principal barrier for osmosis. Similar conclusions were reported for osmosis across the microvascular barriers in kidney (22) and lung (2). Indeed, 50% osmotic equilibration across the pleural surface in wild-type mice occurred in <1.7 min, much less than that of >10 min for osmotic equilibration in the peritoneal cavity (36). The apparent osmotic permeability coefficient, $P_o$, of the pleural barrier (∼0.02 cm/s) in wild-type mice was quite high, probably because of the excellent microvascular supply and the relatively leaky mesothelial barrier. The pleural surface thus provides an exceptionally efficient route for rapid water addition to or removal from the vascular compartment.

The asymmetry in pleural osmotic water permeability was an unanticipated observation. In general, water permeability is symmetric for simple membrane and epithelial barriers. However, osmotic water transport across the complex pleural barrier may be asymmetric for a number of reasons that cannot be distinguished from the data here, including changes in microvascular blood flow in response to hypo- vs. hyperosmolar pleural instillates, changes in pleural morphology in response to water exit vs. entry, and changes in effective pleural surface area. The computation of a single apparent osmotic water permeability coefficient for the complex pleural barrier may thus not be valid. In any case, the results here indicate that pleural osmotic water permeability is high and significantly reduced by AQP1 deletion.

Whereas osmotically induced water transport across the pleural surface was reduced 10-fold by AQP1 deletion, hydrostatically driven accumulation of pleural and lung fluid was not affected. To compare results from wild-type and AQP1 null mice, it was necessary to ligate the renal artery because of the polyuria and nephrogenic diabetes insipidus in AQP1 null mice. The insensitivity of pleural fluid accumulation to AQP1 deletion indicates that AQP1-independent pathways such as paracellular transport provide the major route for fluid movement. Also, because AQP1 is a waterspecific transporter that does not transport salts, pleural fluid accumulation would require a route for salt movement, which probably involves transient breakdown of the pleural barrier (34). Any accumulation of solute-free water in the pleural space would promptly be reabsorbed by osmotic forces. Our results thus provide evidence against a role for AQP1 in pleural fluid accumulation. In a perfused lung model, we reported previously that AQP1 deletion caused a small decrease in lung water in response to large increases in pulmonary artery pressures (25). Although this observation is unlikely to be explained by accumulation of solute-free water in the lung interstitial compartment, we believe that AQP1 deletion is associated with differences in the integrity of the pulmonary microvasculature. Another difference between the pleural and lung interstitial compartments is that the pleural space is open and expandable, whereas the lung interstitium is filled with an extracellular matrix that mechanically restricts volume changes.

The spontaneous clearance of isosmolar fluid instilled into the pleural space was ∼4 ml·kg⁻¹·h⁻¹ in the mice studied here. This value is substantially greater than measured clearance rates of 0.017 ml·kg⁻¹·h⁻¹ in rabbits (5) and 0.32 ml·kg⁻¹·h⁻¹ in sheep (34). The reduced thickness of the pleural barrier in rodents compared with larger animals (27) may account for the more rapid fluid clearance, as well as the high capacity of the mouse lymphatic system to

![Diagram](https://example.com/diagram.png)
clear fluid. AQP1 deletion did not affect clearance of isosmolar fluid instilled into the pleural space. Because Starling forces favor fluid accumulation, it is likely that the majority of fluid exiting the pleural space passes through lymphatics located throughout the parietal pleura (3, 9, 18). Thus the expression of AQP1 in pleural microvasculature does not facilitate pleural fluid clearance.

Thiourea toxicity was studied as an experimental model of endothelial lung injury associated with transient pleural effusions (12, 23). After injection of thiourea, lung fluid accumulation and pleural effusions were maximal at 3 h (12). AQP1 deletion did not alter the time course or magnitude of pleural effusions formed in the first 3 h after thiourea administration. After 5 h, both pleural fluid volume and wet-dry weight ratio of lung were decreased, but there was no significant difference. We conclude that AQP1 does not affect pleural fluid dynamics in response to endothelial injury.

In summary, although AQP1 provides a major pathway for osmotically induced water transport across the pleural barrier, it does not play an important role in pleural fluid dynamics in normal physiology and disease. The question arises as to why AQP1 is expressed in a number of microvascular beds where its deletion is not associated with physiological defects, including lung (2, 25), salivary gland (14), and lacrimal gland (19). Although we think that it is unlikely, AQP1 may be important in pleural fluid dynamics in stresses not tested here. AQP1 may play a non-water-transporting role in the microvasculature, possibly in endothelial growth and proliferation based on its identification as an early response gene (11) and its expression in tumor microvasculature (7). AQP1 has been proposed to play a role in carbon dioxide transport in oocyte studies (20), but does not appear to be important in erythrocytes and lung in vivo (38). As found recently for AQP4 expression in skeletal muscle (37) and gastric parietal cells (31), the data here support the conclusion that the tissue-specific expression of an aquaporin does not ensure a physiological function.

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