Nickel is a common cause of pneumoconiosis. Here, we show that nickel inactivates aquaporin (AQP)-3, the water channel expressed apically in epithelial cells of human terminal airways. Human AQP3 was transiently transfected into human lung cells, and water permeability was measured in transfected and neighboring untransfected cells. Incubation with NiCl₂ rapidly, dose-dependently, and reversibly decreased water permeability in AQP3-expressing cells. Acidification of the extracellular medium also caused rapid, dose-dependent, and reversible inhibition of AQP3. Sensitivity of AQP3 to nickel was lower at alkaline pH than at neutral and acidic pH. Cells transfected with human AQP4 and AQP5, which are also expressed in airway epithelia, were insensitive to nickel and extracellular acidification. Zinc and cadmium, other common causes of pneumoconiosis, had no effect on the water permeability of AQP3. Three extracellular residues, Trp²⁸⁸, Ser³⁵², and His⁵⁴⁴, were responsible for the blocking effect of nickel on human AQP3. Ser³⁵² was identified as a common site for nickel and pH sensitivity. His⁵⁴⁴ was shown to be important for the water permeability of AQP3. Our results imply that nickel and extracellular pH may modulate lung water clearance and that defective water clearance may be an early component of nickel-induced lung disease.

Nonenzymatic regulation of ion channels by Ni²⁺ and other divalent cations or by pH is a well established phenomenon with many important physiological and pathophysiological implications. Less is known about nonenzymatic regulation of water channels, aquaporins (AQPs) (1). Mercury inhibits most mammalian water channels via binding to cysteine residues (2–4) and has been an important tool in studies of AQPs. Gold and silver were recently reported to inhibit a water channel from human erythrocytes, presumably AQP1, but a molecular basis for this inhibition has not been revealed (5). The question of whether Ni²⁺ and other divalent ions known to regulate the activity of ion channels modulate the activity of AQPs has, to our knowledge, not yet been addressed.

Nickel is widely used in modern industry (reviewed in Ref. 6). Inhalation is the primary route of occupational exposure to nickel and other heavy metals, and inhalation of nickel compounds is a common cause of pneumoconiosis (6, 7). AQP3, AQP4, and AQP5 are expressed in the airway epithelia (8–10). AQP3 is located at the apical membrane of human lung epithelium (10). Here, we have examined the effects of Ni²⁺ on the water permeability of human AQP3, AQP4, and AQP5 expressed in a human lung cell line. Since AQP3 has, when expressed in oocytes, been reported to be pH-sensitive (11), we also examined the effect of extracellular acidification. We show that Ni²⁺ and pH regulate the water permeability of human AQP3, but not of human AQP4 and AQP5. We also address the question of whether Ni²⁺ and pH may interact in the regulation of human AQP3.

Identification of the molecular sites responsible for the Ni²⁺ and pH sensitivity of AQP3 is important for future development of therapeutic agents. Histidine, with a pKₐ of ~6.6, is the most likely molecular target for regulation by pH and is also a preferential site for Ni²⁺ binding. By performing a series of mutations of extracellular histidines and amino acids considered to interact with histidine, we identified several molecular determinants of pH and Ni²⁺ sensitivity and at least one common determinant of Ni²⁺ and pH sensitivity.

MATERIALS AND METHODS

DNA Constructs—cDNA fragments encoding full-length AQP3 and the long form of AQP4 were obtained by amplification from the human lung QUICK-Clone cDNA library (Clontech). cDNA encoding human AQP5 was a generous gift from P. Agre (Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD). The cDNA fragments were used for creation of two types of cDNA constructs: constructs that expressed a water channel fused with green fluorescent protein (GFP) and constructs that expressed a water channel and GFP as separate proteins present in the same cell. For the first type of construct, cDNA fragments were subcloned in-frame into the pEGFP-N2 vector for AQP3 tagged with GFP at the COOH terminus and in-frame into the pEGFP-C2 vector for AQP3 tagged with GFP at the NH₂ terminus. For the second type of construct, cDNA fragments were subcloned into the pRES2-EGFP vector (Clontech).

The point mutations in the extracellular loops of human AQP3 were generated by PCR-based mutagenesis using wild-type cDNA as a template. The presence of each point mutation and absence of other modifications were confirmed by sequence analysis of the whole insert.

The transmembrane structure of human AQP3 was predicted using ClustalW Version 1.81 (13). The protein sequences were aligned with ClustalW Version 1.81 (13).
Distribution and Water Permeability of Human AQP3 Transfected into Lung Cells—Human AQP3 was transiently transfected into a bronchial epithelial cell line (BEAS-2b). GFP was used to identify cells expressing AQP3. In cells transfected with AQP3 tagged with GFP at the COOH terminus (AQP3-GFP), a distinct GFP signal was observed in the plasma membrane, whereas the signal was very weak or undetectable in the cytoplasm (Fig. 1a). The plasma membrane signal was evenly distributed along the apical and basal sides of the cells (Fig. 1b). In cells transfected with AQP3 and GFP as separate proteins (AQP3-GFP), the GFP signal was distributed as separate proteins. At pH 5.5, the AQP3-GFP fusion protein was a fully functional water channel.

Sensitivity of Human AQP3 to pH and Ni2+—Acidification of the extracellular solution dose-dependently decreased the P_f in cells expressing AQP3-GFP without affecting untransfected cells (Fig. 1e). At pH 5.5, the P_f in transfected cells was not different from that in untransfected cells. The pH effect was similar in cells expressing AQP3-GFP and in cells expressing AQP3-GFP. Inhibition of AQP3-mediated water permeability by acidic pH was rapid and reversible (Fig. 1, f and g). There was no detectable change in the distribution of human AQP3-GFP in cells exposed to low extracellular pH. The ratio of GFP signal in membranes to that in the cytosol was 2.52 ± 0.27 before and 3.00 ± 0.27 (n = 8) after 1 min in pH 5.5 solution, when the AQP3-mediated water permeability was completely inhibited. Notably, the intracellular pH was only little affected when the extracellular pH was reduced from 7.4 to 5.5 (7.34 ± 0.01 and 7.27 ± 0.01, respectively; n = 58).

Nickel also decreased the P_f in cells expressing human AQP3-GFP in a dose-dependent manner (Fig. 2a). The effect was rapid and reversible (Fig. 2b). The P_f in untransfected cells was not affected by Ni2+. There was no detectable change in the distribution of AQP3-GFP in cells exposed to Ni2+. The ratio of GFP signal in membranes to that in the cytosol was 3.89 ± 0.34 before and 4.05 ± 0.31 (n = 8) after 1 min in solution with 1 mM NiCl2, when the AQP3-mediated water permeability was dramatically down-regulated.

The effect of Ni2+ was pH-dependent (Fig. 2c). At neutral and acidic pH, the AQP3-mediated water permeability was completely inhibited by 1 mM NiCl2. At pH 7.4 and 8.0, the P_f in transfected cells was decreased by Ni2+, but remained significantly higher than that in untransfected cells.

Specificity of the pH and Ni2+ Effect on Human AQP3—AQP4 and AQP5 are, like AQP3, expressed in the epithelial cells of the lower airways of the human lung (10). BEAS-2b cells were transfected with either human AQP4 or AQP5 using cDNA constructs encoding GFP and the water channels as separate or fusion proteins. The water permeability was increased 6–8-fold in cells expressing AQP4 or AQP5 compared with untransfected cells. Lowering the extracellular pH to 5.5

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*Available at rsb.info.nih.gov/ji/index.html.*
or exposing these cells to Ni$^{2+}$ had no effect on the $P_f$ (Fig. 3, a and b).

Like nickel, zinc and cadmium are well documented causes of pneumoconiosis (6, 7). Cells transfected with AQP3-GFP were incubated with 1 mM ZnCl$_2$ or CdCl$_2$. The $P_f$ in cells expressing AQP3-GFP or in untransfected cells was not affected by either of these compounds (Fig. 3c).

**Extracellular Determinants of the Ni$^{2+}$ and pH Sensitivity of Human AQP3**—To examine the molecular determinants of Ni$^{2+}$ and pH sensitivity, we performed a series of point muta-
The following amino acids were chosen for mutation: histidine, which can interact with Ni$^{2+}$ (15) and which is the only amino acid with a $pK_a$ in the range for AQP3 pH sensitivity; serine, aspartate, tryptophan, and tyrosine, since they may interact with histidine (16–18). The results from the mutation studies are summarized in Table I, and the most relevant results are presented in Figs. 5 and 6. All mutants except D219A and W231A (Fig. 5, a and b) were targeted to the plasma membranes of the cells. In two cases (S49A and H154A), mutant AQP3 was water-impermeable. In five cases (H53A, H53F, H53Y, Y124A, and H154Y), the maximal water permeability in cells expressing mutant AQP3 was significantly decreased compared with that in cells expressing wild-type AQP3.

Three residues, Trp$^{128}$ and Ser$^{152}$ in the second extracellular loop and His$^{541}$ in the third extracellular loop, were identified as determinants of AQP3 Ni$^{2+}$ sensitivity (Fig. 5, c and d). The $P_f$ in cells expressing AQP3(W128A), AQP3(S152A), or AQP3(H241A) was similar to that in cells expressing wild-type AQP3. Ni$^{2+}$ had no effect on the water permeability of any of these mutants.

None of AQP3 mutants completely abolished the pH dependence of the water channel. In cells expressing AQP3(H53A), AQP3(Y124A), or AQP3(H154F), the range of pH sensitivity was shifted to more alkaline values (Fig. 6, a–c). In cells expressing AQP3(S152A), the range of pH sensitivity was shifted to more acidic pH (Fig. 6d). The water permeability of wild-type AQP3 was completely inhibited at pH 5.5. At this pH, the $P_f$ in cells expressing AQP3(S152A) was 3.9-fold higher compared with that in the surrounding untransfected cells.

### DISCUSSION

The biological importance of membrane transporters is, to a large extent, dependent on their capacity to be regulated. Here, we show that the water permeability of human AQP3 expressed in human lung cells is regulated by changes in extracellular pH and by Ni$^{2+}$. Ser$^{152}$ in the second extracellular loop of AQP3 was identified as a common determinant of Ni$^{2+}$ and pH sensitivity. Our findings have several important potential implications for the understanding of lung physiology and pathophysiology, as well as for the physiology of other organs in which AQP3 is expressed, such as the kidney.

It is well documented that Ni$^{2+}$ can up- or down-regulate the activity of ion channels (19–24), but this is, to the best of our knowledge, the first demonstration of Ni$^{2+}$ regulation of the activity of a water channel. The effect of Ni$^{2+}$ was immediate, reversible, and dependent on at least three extracellular residues. In studies of the mechanisms by which Ni$^{2+}$ modulates the activity of cyclic nucleotide-gated channels and the epithelial sodium channel, extracellular histidines were found to be the molecular determinants of Ni$^{2+}$ sensitivity (19, 24). Nitrogen in the imidazole side chain of histidine is the preferred target for Ni$^{2+}$ binding (15). Human AQP3 has one histidine in the first extracellular loop, two in the second, and one in the
third. In this study, we found that one of these four extracellular histidine residues, His\(^{241}\), is crucial for the Ni\(^{2+}\) sensitivity of human AQP3. The side chain of tryptophan has a structure that is similar to that of the imidazole ring of histidine. Therefore, we reasoned that tryptophan may also be a candidate for Ni\(^{2+}\) binding. Indeed, we found that substitution of Trp\(^{128}\) with alanine completely abolished the Ni\(^{2+}\) sensitivity of AQP3. Negatively charged amino acids such as aspartate and glutamate can also bind Ni\(^{2+}\) (25). None of the examined extracellular aspartates proved to be important for the regulation of AQP3 by Ni\(^{2+}\). Glutamate residues are not present in the extracellular loops of human AQP3. Ser\(^{152}\) was the third residue in the extracellular loops of AQP3 that was also found to be crucial for Ni\(^{2+}\) sensitivity. This residue, as well as Tyr\(^{124}\), was studied because the effect of Ni\(^{2+}\) was pH-dependent. Mutation of His\(^{241}\), Trp\(^{128}\), or Ser\(^{152}\) led to complete elimination of Ni\(^{2+}\) sensitivity. This indicates that these three amino acids together are essential for Ni\(^{2+}\) binding to human AQP3.

Mutation of His\(^{53}\) to alanine attenuated and of His\(^{154}\) to alanine abolished the water permeability of AQP3. We hypothesized that the imidazole rings of His\(^{53}\) and/or His\(^{154}\) may participate in stacking interactions, important for the proper conformation of the water path. To address this question, we replaced histidine with the aromatic amino acid phenylalanine. We found that AQP3(H154F), but not AQP3(H53F), had the same maximal water permeability as wild-type AQP3.

Human AQP3 was pH-sensitive in the range from pH 5.5 to 7.0. The imidazole group of histidine is the only amino acid side chain affected within this pH range. At pH 5.5, the group is protonated and positively charged, whereas at pH 7.0, it is electrically neutral. His\(^{53}\) from the first extracellular loop and His\(^{154}\) from the second extracellular loop were found to participate in the regulation of the water permeability by pH. Mutation of Ser\(^{152}\) was also associated with a modification of AQP3 pH sensitivity. Serine is a common partner of histidine in a number of enzymes that have an active-site motif known as the catalytic triad (reviewed in Ref. 16). We suggest that, in AQP3, Ser\(^{152}\) may play a role similar to that in the catalytic triad of enzymes. Tyrosine has been shown to closely interact with histidine in the photosystem of cyanobacteria (17). Cells expressing AQP3(Y124A) had significantly lower maximal water permeability than cells expressing wild-type AQP3. It is possible that the aromatic side chain of Tyr\(^{124}\) may participate in stacking interactions with histidine or some other aromatic residue of AQP3.

The finding that AQP3 water permeability is pH-dependent is in line with previous observations by Zeuthen and Klaerke (11). Two other mammalian AQPs have been found to be pH-sensitive; AQP0, which is expressed in the lens (26), and AQP6, which is expressed in intercalated cells of kidney collecting ducts (27). In contrast to AQP3, the maximal water permeability for AQP0 and AQP6 is low at pH 7.5 and significantly increases at acidic pH. A histidine residue in the first extracellular loop was identified as a determinant of the pH sensitivity of AQP0. Amino acid residues controlling the pH sensitivity of AQP6 were not identified. No attempt has been previously made to identify the molecular determinants of AQP3 pH sensitivity.

Alignment of the protein structures showed that all amino acid residues involved in the regulation of AQP3 by Ni\(^{2+}\) or pH are absent in AQP4 and AQP5. Neither AQP4 nor AQP5 was Ni\(^{2+}\)- or pH-sensitive. Nickel and acidic pH can therefore be used as tools to discriminate between the activities of AQP3 on one hand and AQP4 and AQP5 on the other.

This study was performed on a bronchial epithelial cell line derived from the human lung. Nickel is widely used in modern industry, and inhalation is the primary route of occupational nickel exposure. High doses of nickel have been suggested to predispose to asthma, lung fibrosis, and lung cancer (reviewed in Ref. 6). Could interaction of nickel with AQP3 contribute to one or more of these conditions? AQP3 is, like AQP4 and AQP5, abundantly expressed in the airway epithelium. AQP3-null mice have few signs of lung dysfunction except for a small but significant reduction of airway humidification (28, 29). Mice lacking AQP4 and AQP5 also have few signs of lung dysfunction. This has raised the question of whether lung AQPs are important for airway fluid transport. However, there appears to be important species differences with regard to the lung expression of AQPs and particularly with regard to AQP3 expression. AQP3 is present in the apical membranes of bronchial epithelial cells in the human lung, but not in the rodent lung (8–10). Hence, the extracellular loops of AQP3 will, in the human lung, face the airway lumen. Here, we found that Ni\(^{2+}\) binds to the extracellular loops of human AQP3 and that AQP3 is sensitive to extracellular pH. Other investigators have shown that the epithelial sodium channel, which is essential for apical sodium entry into the airway epithelium, is also Ni\(^{2+}\)-sensitive (24). Taken together, these observations are compatible with the concept that the initial stages in human lung disease caused by nickel are related to the effect of Ni\(^{2+}\) on apical water and ion transporters. The interaction between nickel and pH becomes interesting in this context. The effect of Ni\(^{2+}\) was more pronounced at acidic and neutral pH than at alkaline pH. Thus, it may be important to ensure that the airway liquid is alkaline in the situation of nickel exposure.

The pH dependence of human lung AQP3 may be of great clinical importance. The pH of airway surface liquid is ~6.8, and it responds rapidly to changes in systemic acid-base status.
In a recent study in which human airway surface epithelial cells were used, it was found that the addition of mast cells causes substantial increases in proton secretion (32). In line with this, it was found that the pH of exhaled airway vapor condensate is significantly lower in patients with acute asthma than in healthy controls (33). In our study, AQP3 water permeability decreased sharply when the pH fell below 7.0. Interestingly, there is evidence that airway surface liquid pH may be dynamically regulated and that activation of the adenylate cyclase-cAMP pathway results in alkalinization, whereas treatment with histamine and ATP results in acidification of airway surface liquid (32, 34, 35).

AQP3 is expressed in several other organs, including the kidney, salivary and lacrimal glands, and skin (9, 36–38). The most prominent phenotype in AQP3 knockout mice is a decrease in urinary concentrating capacity, resulting in severe nephrogenic diabetes insipidus (39). Hence, it will be important to explore the relationship between renal medullopapillary pH and urinary concentrating capacity. Nickel is one of the most common causes of contact allergic dermatitis (6, 40). Nickel inhibition of AQP3 may be a contributor to this condition. AQP3 and another member of the aquaporin family, AQP7, have recently been found in dendritic cells, which play an important role in the innate immune system (41, 42). Mercury, which is well known blocker of AQPs (2–4) and a cause of contact dermatitis (6, 43), inhibits the regulatory cell volume decrease after macropinocytosis in dendritic cells (42). Given the finding that nickel blocks the water permeability of AQP3, it is tempting to speculate that perturbation of volume regulation of dendritic cells is an important contributor to nickel dermatitis and to the inflammatory component of nickel pneumoconiosis.

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FIG. 5. Mutations affecting the Ni$^{2+}$ sensitivity of human AQP3. Data are presented as differences between the $P_f$ in transfected cells and the corresponding untransfected cells from the same coverslips ($n = 13–25$ cells). a, the $P_f$ in cells expressing AQP3(D219A) was retained in the endoplasmic reticulum of the cells. AQP3(D219A) had a similar distribution. c and d, in contrast to cells expressing wild-type AQP3 (WT), the $P_f$ in cells expressing AQP3(W231A), AQP3(H241A), or AQP3(S152A) was not decreased by 1 mM NiCl$_2$.

FIG. 6. Mutations affecting the pH sensitivity of human AQP3. Data are presented as differences between the $P_f$ in transfected cells and the corresponding untransfected cells from the same coverslips ($n = 12–74$ cells). a, the $P_f$ in cells expressing AQP3(H53A) or AQP3(H53F) was significantly lower than that in cells expressing wild-type AQP3 (WT). The range of pH sensitivity was shifted to higher pH values in cells expressing AQP3(W231A), b, the mutation H154A rendered human AQP3 water-impermeable. The maximal $P_f$ in cells expressing AQP3(H154F) was similar to that in cells expressing wild-type AQP3, but was achieved at higher pH values. c, the mutation Y124A decreased the maximal water permeability of AQP3 and shifted the pH sensitivity curve to higher pH values. d, the water permeability in cells expressing AQP3(S152A) was less responsive to acidic pH. At pH 5.5, the $P_f$ in transfected cells was 3.9-fold higher than that in untransfected cells, whereas in cells expressing wild-type AQP3, the water channel-mediated $P_f$ was completely inhibited.
Nickel and Extracellular Acidification Inhibit the Water Permeability of Human Aquaporin-3 in Lung Epithelial Cells
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