Acute renal failure leads to dysregulation of lung salt and water channels

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Background. Renal ischemia/reperfusion (I/R) injury and the acute respiratory distress syndrome (ARDS) frequently coexist in the intensive care setting, and this combination is associated with a high mortality. Recent experimental data demonstrate that renal I/R injury leads to an increase in pulmonary vascular permeability, similar to that observed in ARDS. However, the effects of renal I/R injury on alveolar fluid clearance—of potential importance in the setting of increased permeability—are unknown. We investigated the effects of renal I/R injury on pulmonary epithelial sodium channel (ENaC), Na,K-ATPase and aquaporin expression as a first step in addressing this question.

Methods. Sprague Dawley rats were subjected to four protocols: (1) surgery for bilateral I/R injury, (2) sham surgery, (3) surgery for unilateral I/R injury, or (4) bilateral nephrectomy. Lung tissue was examined for Na channel, Na,K-ATPase, aquaporin-1, and aquaporin-5 expression. Northern and Western blots were performed.

Results. Renal I/R injury and bilateral nephrectomy both led to marked down-regulation of pulmonary ENaC, Na,K-ATPase and aquaporin-5 but not aquaporin-1 compared to sham surgery. These changes were not influenced by the animals’ volume status. In contrast, unilateral I/R with an intact contralateral kidney did not lead to down-regulation of channel down-regulation.

Conclusions. Ischemic acute renal failure leads to down-regulation of pulmonary ENaC, Na,K-ATPase and aquaporin-5, but not aquaporin-1. Since bilateral nephrectomy but not single kidney I/R injury also leads to lung changes, these changes are likely mediated by systemic effects of acute renal failure (ARF), such as “uremic toxins,” rather than reperfusion products. These changes may modulate lung dysfunction, susceptibility to lung injury, or both.

Despite the development of dialysis and other novel therapies in the critically ill patient, the mortality associated with acute renal failure (ARF) remains unacceptably high, between 40% and 60% [1]. Leading causes of death in ARF in technologically developed countries include cardiorespiratory failure, sepsis, and withdrawal of dialysis [2]. ARF is an independent, additional risk factor for mortality in the multiorgan dysfunction syndrome [3]. A strong association has been repeatedly demonstrated with ARF and noncardiogenic acute respiratory distress syndrome (ARDS) [4]. However, the links between ARF and ARDS are poorly understood. Capillary leakage and accumulation of salt and water in areas of gas exchange are among the hallmarks of ARDS [5]. We have recently demonstrated that renal ischemia/reperfusion (I/R) injury in rats leads to increased pulmonary vascular permeability, which is partially macrophage mediated [6]. In addition, impaired clearance of salt and water from alveoli may further compromise gas exchange in ARDS [5]. The importance of the epithelial sodium channel (ENaC) and aquaporins in pulmonary salt and water clearance has recently been highlighted [7, 8]. We hypothesized that ARF could lead to down-regulation of ENaC, Na,K-ATPase and aquaporins, potentially contributing to deleterious salt and water accumulation in the lungs. We addressed this question using an established model of acute renal failure in the rat [6, 9] and examined effects on expression of pulmonary ENaC, Na,K-ATPase, and aquaporin-1 and aquaporin-5.

METHODS

Overview

Four groups of animals (with four or more animals in each group were studied: (1) those with bilateral I/R injury, (2) those who underwent sham surgery, (3) those who underwent bilateral nephrectomy, and (4) those with unilateral I/R injury. Animals were sacrificed at 24
or 48 hours following surgery with collection of blood and lung samples.

**Surgical procedures**

The model of renal I/R injury employed has been described in detail [6, 9]. Briefly, male 200 to 300 g Sprague-Dawley rats were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally) and placed on a heating pad to maintain constant temperature (monitored with a rectal thermometer). The kidneys were exposed via midline abdominal incisions, and the renal pedicles were dissected free. Renal ischemia was induced by nontraumatic vascular clamps (Roboz Surgical Instruments, Washington, DC, USA) over either one or both pedicles (as appropriate for group assignment) for 30 minutes. After the clamp(s) were released, the incision was closed in two layers with 2-0 sutures. Sham animals underwent anesthesia, laparotomy, and renal pedicle dissection only. A final group underwent bilateral nephrectomy using standard techniques. All animals received warm saline solution instilled in the peritoneal cavity during the surgical procedure and were then allowed to recover with ad libitum access to food and water. Animals were sacrificed by exsanguination under surgical anesthesia 24 or 48 hours after experimental intervention. Blood samples were collected for measurement of serum creatinine.

**RNA isolation**

Lung tissue was snap frozen in liquid nitrogen and then stored at −70°C until processed. Total cellular RNA was extracted from lung tissues by the method of Chomczynski and Sacchi [10]. In brief, 0.5 to 1 g tissue was homogenized at room temperature in 10 mL Tri reagent (Molecular Research Center, Cincinnati, OH, USA). RNA was quantitated by spectrophotometry and stored at −80°C.

**Northern hybridization**

Total RNA samples were fractionated on a 1.2% agarose-formaldehyde gel. The samples were transferred to a nylon membrane, cross linked by ultraviolet light, and baked for 1 hour. Hybridization was performed according to Church and Gilbert [11]. The cDNA probes were labeled with 32P-deoxynucleotide, using the Rad Prime DNA labeling kit (Invitrogen, Carlsbad, CA, USA). Following hybridization, the membranes were washed, blotted dry, and exposed to a Phosphorimagaser cassette at room temperature for 24 to 48 hours, and read by Phosphorimagaser (Amersham Bioscience Co., Piscataway, NJ, USA). The following rat polymerase chain reaction (PCR) product fragments were used as probes for Northern blot analyses: Na channel beta subunit, nucleotides 1012–1848; aquaporin-1, nucleotides 494–736; and aquaporin-5, nucleotides nucleotides 308–678.

**Electrophoresis and Western blotting**

Semi quantitative immunoblotting experiments were carried out according to standard protocols. Briefly, the solubilized membrane proteins from lungs of control or I/R injury rats were size fractionated on polyacrylamide minigels (Novex, San Diego, CA, USA) and were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% milk proteins, and then probed with either an aquaporin-5 or an αNa,K-ATPase-specific antibody. For aquaporin-5, an affinity-purified polyclonal antibody (raised against a 17 aa synthetic peptide from the C-terminal portion of mouse; aquaporin-5, Alpha Diagnostic, San Antonio, TX, USA) was used at an immunoglobulin (IgG) concentration of 0.8 μg/mL. The secondary antibody was donkey anti rabbit IgG conjugated to horseradish peroxidase (Pierce). For Na,K-ATPase, a monoclonal antibody against the α subunit of the pump (a generous gift from Dr. Jerry Lingrel, Department of Molecular Genetics, University of Cincinnati) was used at 1/1000 dilution. The secondary antibody was a goat antimouse IgG antibody. The sites of antigen-antibody complexation on the nitrocellulose membranes were visualized using chemiluminescence method (SuperSignal Substrate, Pierce Biotechnology Inc., Rockford, IL, USA) and captured on light-sensitive imaging film (Kodak, Rochester, NY, USA). Aquaporin-5 was identified as a 27 to 30 kD band. Na,K-ATPase was identified as a 95 to 98 kD band. The equity in protein loading in all blots was first verified by gel staining using the coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA, USA).

**Statistical methods**

Results are expressed as mean ± SEM. Statistical analysis was performed using ANOVA or unpaired Student t test, with a P value less than 0.05 considered significant.

**RESULTS**

**Survival and weight**

All animals survived until sacrifice. Sham I/R injury animals, despite undergoing operation, had little change in body weight (mean 0.7% increase over 48 hours). Animals with bilateral renal I/R injury tended to lose weight (6.3% loss in 48 hours), which has been well established and thought to be due to both cachexia and polyuria [12]. The group with bilateral nephrectomy and associated anuria tended to gain weight (14.8% increase over 48 hours). Animals with unilateral nephrectomy plus I/R injury lost weight (3.0% loss over 48 hours).

**Renal function**

Renal I/R injury led to a significant rise in serum creatinine compared to sham animals and unilateral ischemia
animals (Fig. 1). Bilateral nephrectomy led to much higher serum creatinine levels.

**Lung epithelial sodium channel expression**

Bilateral renal I/R injury significantly decreased lung sodium channel mRNA expression at 48 hours postischemia compared to sham-operated animals (51% ± 5% vs. 100% in sham, $P < 0.01$, $N = 4$; Fig. 2). Lung sodium channel mRNA expression was mildly decreased, but not significantly, at 24 hours (data not shown).

**Lung aquaporin-5 expression**

Bilateral renal I/R injury markedly decreased lung aquaporin-5 mRNA expression at 48 hours postischemia (decreased by 74% ± 5% 48 hours compared to sham-operated animals, $P < 0.01$, $N = 4$; Fig. 3).

**Lung aquaporin-1 expression**

Renal I/R injury did not alter aquaporin-1 expression at either 24 or 48 hours (Fig. 4).
Effect of azotemia on aquaporin-5 expression

To determine whether the reduction in the expression of aquaporin-5 was due to I/R products or renal failure itself, the experiments were repeated in animals subjected to either unilateral ischemia (I/R injury but no renal failure) or bilateral nephrectomy (renal failure but no I/R injury). As indicated in Figure 5, the aquaporin-5 mRNA expression significantly decreased in bilateral nephrectomy or bilateral I/R injury, but not with unilateral ischemia. The results of studies on four separate animals indicated that, at 48 hours, the expression of aquaporin-5 decreased by 78% ± 5% in bilateral ischemia, and by 71% ± 6% in bilateral nephrectomy (P < 0.01 vs. sham in both groups). The expression of aquaporin-5 in unilateral ischemia did not change (P > 0.05 vs. sham, N = 4).

Western blotting

In order to evaluate if the change in lung aquaporin-5 mRNA level was consistent with changes at the protein level, we used an antibody to aquaporin-5. Immunoblot of aquaporin-5 protein demonstrated a significant decrease during bilateral I/R injury compared to sham-I/R injury animals, consistent with results at the mRNA level (Fig. 6). Given the potential importance of Na,K-ATPase in pulmonary fluid clearance, immunoblotting was performed to detect this protein. There was a marked decrease in lung Na,K-ATPase protein content during bilateral I/R injury compared to sham-I/R injury animals (Fig. 7).

DISCUSSION

Our results demonstrate that ARF resulting from either bilateral I/R injury or bilateral nephrectomy can down-regulate pulmonary ENaC, Na,K-ATPase and aquaporin-5 expression. The failure of unilateral renal I/R injury to induce similar down-regulation indicates that these changes are likely mediated by systemic effects of ARF rather than reperfusion products alone. Furthermore, the observation that bilateral renal I/R injury with weight loss yielded the same observations as bilateral nephrectomy with weight gain makes it unlikely that changes in fluid volume played an important role in these changes. We further demonstrated that in the case of aquaporin-5, changes at the protein level were consistent with those at the mRNA level. Our findings suggest that ARF may exert deleterious effects on lung physiology, effects that may be of particular importance in the settings of antecedent or concurrent lung injury or mechanical ventilation.

The down-regulation of ENaC we observed is temporally consistent with the pulmonary inflammation, increased albumin permeability, and interstitial edema seen in the same rat model after ARF [6]. The amiloride-sensitive epithelial sodium channel, ENaC, is a heterodimeric protein composed of three homologous subunits [7]. ENaC promotes sodium absorption in epithelial cells of the distal renal tubule, distal colon, and lung. ENaC is critical in airway fluid clearance, as demonstrated by the rapid death of ENaC-deficient mice after birth due to pulmonary edema and respiratory failure [7]. Further, inhibition of the amiloride-sensitive epithelial sodium channel augments edema formation in lungs subjected to pulmonary I/R injury [13]. Accordingly, our findings of depressed ENaC expression in rat lungs during ARF suggest that ARF may contribute to impaired pulmonary fluid clearance. Decreased edema clearance may be of particular importance in the setting of increased pulmonary permeability, a hallmark of ARDS.

Aquaporins are water-transporting proteins that are expressed in various tissues, including lung, kidney, brain, and salivary tissue [14]. To date, ten mammalian members of the aquaporin family have been identified [15]. Aquaporin-1 is expressed in pulmonary microvascular
endothelia and is important for osmotically driven water transport across alveolar capillaries [16]. Aquaporin-5 is expressed in type 1 alveolar epithelial cells and is important in airspace-capillary osmotic water permeability [8]. Aquaporin-1 and aquaporin-5 are down-regulated after murine adenovirus infection, and thus may be involved in disease-related pulmonary edema [17]. However, recent studies in the aquaporin-5–deficient mice did not reveal any defect in active fluid absorption under normal conditions [8] or acute lung injury [18]. Nonetheless, it is plausible that aquaporin-5 may serve other functions such as generation and maintenance of an aqueous surface layer, mediating carbon dioxide exchange, or maintaining volume and structure of the type I pneumocyte [19]. Recent findings suggest that aquaporin inhibition may increase susceptibility to ventilator-induced lung injury, consistent with this hypothesis [20].

Impaired pulmonary fluid clearance arising from depressed ENaC expression, and perhaps derangements arising from decreased aquaporin-5 expression, may have significant deleterious effects in the context of increased pulmonary permeability, such as that observed in ARDS. To the extent that augmented edema retention predisposes to gross alveolar flooding (particularly in gravitationally dependent regions of the lung), apparent pulmonary compliance will be decreased and shunt fraction will increase. Less overt increases in alveolar fluid may inactivate surfactant, thereby decreasing lung compliance, increasing alveolar wall tension, and promoting further fluid filtration into the alveolar spaces. Increased
alveolar surface tension may enhance stress-induced fracturing of the alveolar blood-air barrier [21]. Surfactant inactivation will also promote focal microatelectasis, increasing shear stresses during lung inflation and augmenting transmission of alveolar tension to interstitial structures (such as fragile pre- and post-alveolar capillary vessels) [22–24]. Damage to interstitial vessels appears of importance in several models of lung injury [24, 25]. These effects would be expected to increase susceptibility to ventilator-induced lung injury, an entity which is of increasing importance in the intensive care setting [26–29]. In addition, to the extent that the injured lung, acting via cytokine release, can “drive” the systemic inflammatory response, a “vicious cycle” may be initiated or perpetuated [30].

The role of the Na,K-ATPase in clearance of lung salt and water has been the subject of considerable recent investigation in both normal and injured lungs and is the topic of recent reviews [31, 32]. In brief, sodium enters the alveolar type II cell primarily through the luminal sodium channel, with a smaller contribution from other transporters, and is subsequently extruded via the Na,K-ATPase [33, 34]. Water is thought to follow passively (isomotically). β-adrenergic stimulation of the type 2 cell promotes incorporation of the Na,K-ATPase in the basolateral membrane, up-regulating the extrusion of sodium and augmenting sodium clearance; an effect which can be blocked by disruption of cytoskeletal microtubular elements [34]. This active transport mechanism continues to be operative in several models of lung injury, augmenting clearance of edema fluid [35, 36]. The down-regulation of apical sodium-positive channel and basolateral Na,K-ATPase that we observed suggests that both the sodium entry across the apical membrane and the sodium exit across the basolateral membrane are impaired in the lungs during renal I/R injury. Whether the down-regulation of Na,K-ATPase is secondary to the down-regulation of sodium channel or is independent of that remains speculative. In addition, specific dopaminergic stimulation (DA-1) also increases edema clearance, albeit through a mechanism that appears distinct from that induced by β-adrenergic stimulation [37].

Our findings indicate that ARF down-regulates pulmonary expression of ENaC, Na,K-ATPase and aquaporin-5. As noted, these molecular derangements may have detrimental effects on lung fluid balance, especially in the settings of antecedent or concurrent structural injury of the lung. Impaired pulmonary fluid handling may impair lung function and increase the susceptibility of the lung to injury, particularly during mechanical ventilation. It is important to note that the functional importance of the changes in the lung channels was not addressed in our study. Furthermore, observations in rats have to be cautiously interpreted for relevance in humans. The potential for “communication” between the injured kidney and the lung suggests that further study of kidney-lung interaction may prove both fruitful in the effort to reduce the high mortality associated with ARF.

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