Strain-specific differences in sensitivity to ischemia-reperfusion lung injury in mice

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In recent years, significant progress has been made in understanding the mechanisms responsible for I/R lung injury (8). Animal models and in vitro investigations have suggested that circulating humoral and cellular mediators, including cytokines and neutrophils, are pivotal to the cascade leading to injury after I/R (13, 29). For example, utilizing sheep and pig lung models of I/R, our laboratory demonstrated a significant role for neutrophils (33) and NADPH oxidase (11, 12), the major enzymatic source of neutrophil-derived reactive oxygen species (ROS). The contribution of these mechanisms and the underlying sensitivity to I/R, however, appears to vary across different experimental species (1, 9, 11, 12, 20, 33, 36). Some of this variability may result from underlying genetic pressures that interact with environmental factors and confound experimental outcomes.

One approach to understanding the genetic determinants that predispose humans to specific disease characteristics is to establish a murine model and perform interstrain comparisons (26). We recently developed a highly sensitive, in vivo mouse preparation of unilateral I/R lung injury capable of detecting increased pulmonary vascular protein permeability after brief periods of ischemia and reperfusion in spontaneously breathing mice (10). In the present study, we hypothesized that genetic factors may predispose to I/R lung injury. To test this hypothesis, we measured pulmonary vascular protein permeability in eight inbred strains of mice after 30 min of left lung ischemia and 150 min of reperfusion.

METHODS

Animals. The protocols in this study were approved by The Johns Hopkins Institutions Animal Care and Use Committee. Male mice of the following inbred strains were purchased from Jackson Laboratories (Bar Harbor, ME): SWR/J, C3H/HeJ, A/J, 129/J, C57BL/6J, CBA/J, SI/LJ, BALB/c. Reciprocal F1 offspring of the BALB/c and SWR/J progenitor strains had intermediate phenotypes but a differing variance. A similar pattern of right lung Evans blue dye content suggested the presence of contralateral injury because baseline vascular permeability was not different. Lung I/R injury was attenuated by NADPH oxidase inhibition, indicating a role for NADPH oxidase-derived reactive oxygen species (ROS). There was no strain-dependent difference in lung NADPH oxidase expression. Strain-related differences in zymosan-stimulated neutrophil ROS production did not correlate with I/R lung injury in that neutrophil ROS production in SWR/J mice was greater than C57BL/6J but not different from BALB/c mice. These data indicate the presence of a genetic sensitivity to lung I/R injury that involves multiple genes including a maternal-related factor. Although neutrophil-derived ROS production is also modulated by genetic factors, the pattern did not explain the genetic sensitivity to lung I/R injury.

vascular permeability; edema; Evans blue dye; NADPH oxidase; p22(phox)

ISCHEMIA-REPERFUSION (I/R) injury occurs when the blood flow to an organ is transiently interrupted. I/R lung injury complicates cardiopulmonary bypass surgery (17) and the recovery from lung transplantation (24). Acutely, I/R injury leads to increased pulmonary endothelial permeability, edema, and hypoxemia (8). Acute lung injury contributes significantly to the morality associated with cardiopulmonary bypass (31). Moreover, lung transplant recipients surviving I/R injury are more likely to have a prolonged hospital stay (24) and may have an increased incidence of obliterative bronchiolitis (14), a manifestation of chronic rejection and a major obstacle to long-term graft survival (5).
expiratory pressure of 1 cmH₂O. The left pulmonary artery was isolated through a left thoracotomy and occluded with a slipknot (8-0 Prolene suture). One end of the slipknot was exteriorized for later isolation through a left thoracotomy and occluded with a slipknot (8-0 Prolene suture). One end of the slipknot was exteriorized for later verification of Prolene suture). The inspired gas tensions were measured utilizing Evans blue dye (EBD) as previously described (10). The EBD method was previously validated in this preparation by comparison with a double-radiolabeled albumin index of protein escape (10). We did not find that 30 min of ischemia and 150 min of reperfusion increased the albumin concentration in bronchoalveolar lavage protein concentration, suggesting that this injury did not increase alveolar epithelial permeability (10).

Briefly, EBD (30 mg/kg, Sigma) was administered intravenously during the period of left pulmonary artery ischemia. After 150 min of reperfusion, the mice were reanesthetized, intubated, and briefly mechanically ventilated to allow removal of intravascular EBD by saline flush. The left lower lobe and right lungs were separately weighed and incubated for 24 h at 37°C in formamide (1 ml formamide/100 mg lung, Sigma Chemical, St. Louis, MO). After incubation for 24 h, 200-µl aliquots of the formamide supernatant were placed in 96-well plates for colorimetric evaluation using a spectrophotometer (620 nm). The ratio of left lung to right lung EBD content was utilized as an index of the increased pulmonary vascular permeability resulting from I/R (10).

Baseline pulmonary vascular permeability. BALB/c, SWR, and their F1 mice (25–30 g) were anesthetized with pentobarbital sodium (60 mg/kg) administered intraperitoneally. The trachea was cannulated and the mice were mechanically ventilated as described above. A median sternotomy was performed, and the pulmonary artery and left atrium were cannulated via the right and left ventricles, respectively by utilizing a water-jacketed (37°C) chamber designed for vascular permeability was measured utilizing Evans blue dye (EBD). The left atrium was cannulated and occluded with a slipknot (8-0 Prolene suture). The inspired gas tensions were measured utilizing Evans blue dye (EBD) as previously described (10). The EBD method was previously validated in this preparation by comparison with a double-radiolabeled albumin index of protein escape (10). We did not find that 30 min of ischemia and 150 min of reperfusion increased the albumin concentration in bronchoalveolar lavage protein concentration, suggesting that this injury did not increase alveolar epithelial permeability (10).

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Protocols. Mice from the eight inbred strains described above (n = 6–10 mice per group) were randomly studied to determine strain-specific sensitivity to I/R lung injury. Additional groups of BALB/c mice (n = 11) and SWR/J (n = 11) and their F1 offspring (BALB/c-F1 from BALB/c females and SWR/J males, n = 10 and SWR/J-F1 from SWR/J females and BALB/c males, n = 9) were evaluated randomly to determine the mode of inheritance responsible for strain differences in I/R lung injury. The lung harvesting and tissue processing for these experiments were performed by an investigator who was blinded to the identity of each mouse strain. To assess the effect of inbred strain on baseline pulmonary vascular permeability, Kᵢ was measured in uninjured control mice from both strains (SWR/J and C57BL/6) and side-scatter fluorescence-activated cell sorter profile in whole blood samples after red cell lysis by 0.168 M ammonium chloride (1:8 whole blood-ammonium chloride, 20 min). Isolated neutrophils (29,000–50,000 in 1 ml PBS with 10% FBS) were placed in a stirred, warmed (37°C) cuvette of a Chronolog 560-CA luminometer (Chronolog Instruments, Havertown, PA). Luminal (Sigma Chemical, St. Louis, MO) stock solution (62.5 mM in DMSO) was added to achieve a final concentration of 0.5 mM, and baseline chemiluminescence was recorded for 2 min. Serum-treated zymosan was added to achieve a final solution of 1.2 mg/ml and peak luminal-enhanced chemiluminescence (LEC) was recorded. Serum-treated zymosan was prepared by boiling zymosan A (Sigma Chemical) in HBSS for 20 min. It was then incubated twice (30 min each) in serum (porcine) and reconstituted in HBSS (10 mg/ml). Serum-treated zymosan was frozen and stored at −80°C until use. In some experiments, the effect of superoxide dismutase (2,000 U/ml; Sigma Chemical, St. Louis, MO) was examined to determine the contribution of superoxide anion to the LEC.

To determine the role of NADPH oxidase in I/R injury, C57BL/6 (n = 10) and SWR/J (n = 6) were pretreated with 500 µl of 60 mM apocynin (4-hydroxy-3-methoxy-acetophenone) (12) via intraperitoneal injection 45 before ischemia followed by an additional 200 µl 10 min before ischemia. Apocynin was dissolved in 0.9% normal saline. Apocynin-treated mice were compared with mice of the same strain injected with diluent (n = 10 C57BL/6; n = 9 SWR/J). In separate untreated control BALB/c (n = 11), C57BL/6 (n = 5), and SWR/J (n = 10) mice, neutrophils were isolated from cardiac blood to allow an interstrain comparison of NADPH oxidase activity as described above. In eight of these experiments (4 BALB/c and 4 SWR/J), neutrophil LEC was measured in the presence and absence of superoxide dismutase.

To determine whether NADPH oxidase expression differed as a function of inbred strain after I/R injury, p22phox expression was measured in snap-frozen lungs of additional BALB/c (n = 6) and SWR/J (n = 5) mice subjected to I/R and compared with the lungs from uninjured control mice from both strains (n = 4 BALB/c, n = 4 SWR/J).

Statistical analysis. The homogeneity of variance (Hartley’s F-max) was determined across strains before utilization of parametric (single-factor ANOVA and Fischer least significant difference) or nonparametric (Kruskal-Wallis with post hoc Dunn’s multiple comparison) tests as appropriate. Values presented in the text and figures are means ± SE. Differences were considered significant when P < 0.05.

RESULTS

The order of sensitivity to I/R lung injury among the eight strains tested was SWR/J > C3H/HeJ > A/J > 129J > C57BL/6J > CBA/J > SJL/J > BALB/c (Fig. 1). More specifically, I/R lung injury did not differ between the SWR/J and C3H/HeJ groups but both were significantly more injured than the BALB/c, SJL/J, CBA/J, C57BL/6/J, and 129J groups (P < 0.05). The SWR/J group was also significantly more
injured than the A/J group \((P < 0.05)\). Moreover, there were no overlapping values between the SWR/J mice and either BALB/c or SJL/J mice. There was no difference in the right lung EBD values in this data set \((P/H11005=0.12)\) with the right lung EBD in SWR/J mice averaging 0.14 ± 0.01 compared with BALB/c which averaged 0.10 ± 0.01 relative absorbance units (RAU) (data not shown).

Figure 2 shows the comparison between additional groups of BALB/c and SWR/J progenitors and both combinations of their reciprocal F1 offspring. Similar to the results in Fig. 1, there was no overlap in susceptibility to left lung I/R injury between the BALB/c and SWR/J progenitors. Furthermore, both reciprocal F1 progeny from the BALB/c and SWR/J strains had comparable sensitivity to I/R injury (left-to-right lung ratio of EBD content 2.03 ± 0.15 and 2.12 ± 0.32 RAU, respectively). These two nonsegregant progeny did differ, however, in their within-strain variance of left lung I/R injury in that the variance of the BALB/c-F1 was smaller than that of the SWR/J-F1. We therefore chose to use the F1 progeny from BALB/c mothers for statistical comparison with the parental strains. In that regard, left lung I/R injury of the F1 progeny from BALB/c mothers was significantly greater \((P < 0.05)\) than that of the BALB/c progenitor but not different from the SWR/J progenitor.

Figure 3 shows EBD content of the right lung in the two progenitors and BALB/c-F1 filial mouse strains after left lung I/R. In a pattern similar to that of I/R lung injury to the left lung, the EBD content in the right lung of BALB/c mice was significantly less \((P < 0.05)\) than the EBD content in the right lung of SWR/J mice. Furthermore, the EBD content of the right lung of the F1 progeny of BALB/c mothers after left lung I/R was significantly \((P < 0.05)\) greater than that of the SWR/J progenitor. As shown in Fig. 4, there were no significant differences in baseline Kf or WW/DW (measured after increasing pulmonary vascular pressure) between these groups.

The NADPH oxidase inhibitor apocynin significantly attenuated I/R injury in both C57BL/6 and SWR/J strains as evidenced by the ~50% decrease in left lung EBD content (Fig. 5). Apocynin pretreatment had no effect on the right lung EBD content. Of note, the increase in left lung permeability was somewhat greater in the diluent-treated C57BL/6 and SWR/J mice compared with the original groups shown in Fig. 1. We have found that the permeability injury does vary over time within any given strain. As a result, all relevant comparison groups in this study were performed contemporaneously to control for any unexpected differences that may occur as a function of season or mouse condition.
The major finding of this study was a significant variation among eight inbred mouse strains with respect to sensitivity to unilateral I/R lung injury. These data suggest that genetic factors were important determinants of the observed differences in sensitivity to lung I/R injury. The two most phenotypically distinct strains, namely SWR/J and BALB/c, demonstrated consistent and discreet sensitivity to this injury. Specifically, the SWR/J mice were most sensitive to I/R lung injury whereas the BALB/c mice were most resistant as determined by EBD-labeled albumin extravasation.

The protection from I/R injury characteristic of the inbred BALB/c strain was lost in the F1 offspring of BALB/c females mated with SWR/J males (Fig. 2). Of note, there was also a trend toward protection from injury when SWR/J females were mated with BALB/c males. Susceptibility in these offspring, however, was not significantly different from that in the SWR/J males and was much more variable than that in the first filial progeny of BALB/c females crossed with SWR/J males. The complete separation of the response to I/R injury in the progenitor strains combined with the intermediate sensitivity to I/R injury of their progeny strongly suggests that multiple genes (rather than simple Mendelian genetics) determine sensitivity to I/R injury in this model (25). Moreover, the presence of a maternal-related influence on the first filial phenotype indicates that at least one of the modifying determinants is either maternally inherited (through nuclear or mitochondrial DNA) or related to differences in maternal uterine or postnatal nesting environments between inbred strains (35).

The pattern of strain sensitivity to I/R lung injury was paralleled by the accumulation of EBD in the contralateral lung. Specifically, right lung EBD content in BALB/c mice after unilateral I/R lung injury was significantly less than that of SWR/J mice, whereas the BALB/c-F1 filial offspring demonstrated an intermediate phenotype that was not statistically different from either parental strain (Fig. 3). This difference in right lung EBD was less robust than the strain-related differences in I/R injury because it was not statistically detectable when the initial eight strains were compared in the same analysis. We previously found a small increase in right lung EBD content (compared with sham surgical controls) after left lung I/R that correlated with left lung ischemic time in C57BL/6J mice (10). This EBD increase, however, was not associated with a corresponding increase in right lung radiolabeled albumin extravasation, suggesting that the increase in right lung EBD was not due to a contralateral lung injury (4) but rather was secondary to a transfer of EBD from albumin to an unknown lung tissue protein (7). Moreover, this transfer only occurred when the right lung received the entire cardiac output, suggesting that the increased flow and pressure facilitated endothelial binding. In the present study, each mouse strain was exposed to the same ischemic and reperfusion times, indicating a different explanation for the right lung EBD differences. Thus this current difference in right lung EBD content could represent a strain-related difference in baseline pulmonary vascular permeability vs. a true contralateral I/R lung injury in SWR/J mice. To examine the first possibility, we
measured the $K_i$ in normal mice from the two extreme strains and their F1 progeny as rapidly as possible after death. This was accomplished by leaving the lungs in situ and increasing static vascular pressure to avoid a reperfusion injury, which our laboratory previously showed can occur after a 5-min ischemic time in this preparation (10). We did not find any difference in baseline $K_i$ or WW/DD (measured after increasing vascular pressure) between the three groups (Fig. 4), suggesting that strain-related differences in baseline endothelial barrier function were a less likely explanation for the results shown in Fig. 3. Whether the differences in right lung EBD content represent a true contralateral I/R injury will require further study.

In considering the genes that explain the variable sensitivity to I/R lung injury, reasonable possibilities would include proteins involved in either the production or destruction of ROS given the known involvement of these toxic products in I/R lung injury (12, 13, 16, 21). Interestingly, the pattern of sensitivity among wild-type strains observed in our lung I/R injury model differs from the strain sensitivity to hyperoxic exposure (22) or ozone inhalation (27), two other examples of ROS-mediated lung injury. Specifically, hyperoxia resulted in lung injury that was greatest in BALB/c mice followed in order of severity by C57BL/6 and C3H/HeJ mice (22). SWR/J mice were not evaluated. A similar pattern of sensitivity was observed across eight mouse strains after ozone inhalation with BALB/c being the most and C3H/HeJ being the second to least sensitive (27). Although the genes responsible for the differential sensitivity to hyperoxia or ozone across these inbred strains remain unknown, previous studies have identified candidate genes involved in both antioxidant defense systems (6) and proinflammatory pathways (28).

The marked difference in strain sensitivity pattern between lung I/R injury and ROS-mediated inhalational injury is not necessarily surprising given the potential mechanistic differences in ROS production as well as differences in lung tissue compartments where ROS may be generated and scavenged. We reasoned that the genes behind the interstrain differences in lung I/R injury could affect ROS concentrations in the vascular compartment or the response of the endothelium to ROS. Thus genes that control 1) differences in neutrophil ROS production, 2) neutrophil-endothelial interactions, 3) ROS-induced endothelial signaling, or 4) endothelial antioxidant defenses are possible candidates. Consistent with the first possibility, inbred strain-related differences in cardiac NADPH oxidase expression and activity were reported in MF1, 129sv, and C57BL/6J mice (3). Moreover, SWR/J mice have been shown to respond to Heligmosomoides polygyrus adult worm infection with a greater intravascular oxygen radical production compared with BALB/c mice (2), suggesting a possible genetic difference in neutrophil ROS production that would correlate with the pattern of sensitivity to lung I/R injury in the present study. Antioxidant enzyme activity has also been shown to vary as a function of inbred mouse strain (30, 32, 34), but a recent study (23) found that lung activities of glutathione peroxidase, glutathione $S$-transferase, and superoxide dismutase were greater in C57BL/6 compared with BALB/c mice, a finding that would not be consistent with our data (Fig. 1).

We found a possible pathogenic role for NADPH oxidase because apocynin significantly decreased the left-to-right lung EBD ratio in both C57BL/6 and SWR/J mice (Fig. 5). We previously found that apocynin 1) blocked whole blood LEC (12, 2) completely prevented the increased pulmonary vascular permeability and edema caused by I/R in a blood-reperfused sheep lung preparation (12), and 3) attenuated hypoxemia in an intact pig model of cardiopulmonary bypass lung injury (11), suggesting a major role for NADPH oxidase-derived ROS in lung I/R. The specific cellular sources of the injurious NADPH oxidase were not determined in the present study, but neutrophils may have played an important role based on our previous data in sheep lung I/R (33). Others have implicated the non-phagocytic NADPH oxidase present in endothelial cells as a significant source of ROS during lung I/R injury (15), and our laboratory previously showed that apocynin effectively blocks pulmonary endothelial NADPH oxidase activity (11).

On the basis of these data and those of Ben Smith et al. (2), we looked for strain-related differences in NADPH oxidase expression as assessed by p22$^\text{phox}$ expression. We did not find any differences between strains in lung p22$^\text{phox}$ protein, suggesting that SWR/J mice were not more susceptible because of increased NADPH oxidase complex expression. Interestingly, the appearance of p22$^\text{phox}$ expression in the experimental groups did not differ in the reperfused left lung compared with the right lung. The explanation for this result is unknown, but there are several potential explanations, including a nonspecific effect of the anesthesia or surgical manipulation involved in this preparation as well as bilateral lung injury from left lung I/R (4).

Regardless of the mechanism, it did not appear that differences in lung NADPH oxidase protein expression could explain the strain-dependent differences in I/R injury. We therefore selected the three strains that spanned the entire spectrum of I/R lung injury (BALB/c, C57BL/6, and SWR/J) and compared the ability of their isolated blood neutrophils to generate ROS after serum-treated zymosan stimulation in vitro. We chose serum-treated zymosan as a method for activating neutrophils because, similar to lung I/R (13), it stimulates complement receptors and triggers both NADPH oxidase activation and neutrophil degranulation (19). Interestingly, we did find that in vitro neutrophil NADPH oxidase activity differed as a function of inbred strain. Specifically, C57BL/6 neutrophils produced 71% less ROS compared with neutrophils from the most I/R-sensitive SWR/J strain (Fig. 6). There was no difference, however, between the neutrophils from the extreme ends of the injury spectrum (BALB/c vs. SWR/J), suggesting that genetic differences in neutrophil NADPH oxidase were not the likely explanation for the variable sensitivity to lung I/R. Similar to our results, Gardi et al. (18) reported that formyl-Met-Leu-Phe-stimulated peritoneal neutrophils from C57BL/6 and BALB/c mice generated similar amounts of superoxide anion as measured by cytochrome $c$ reduction, but they did not study SWR/J mice.

In conclusion, our results demonstrate significant inbred strain differences in the pulmonary endothelial barrier dysfunction caused by lung I/R injury in the intact mouse, suggesting a strong genetic influence on an as yet unknown component of the injury process. An NADPH oxidase inhibitor attenuated injury, but lung NADPH oxidase expression was similar across strains and a strain-dependent pattern of neutrophil-derived in vitro ROS production did not correlate with I/R injury, suggesting that genetic differences in neutrophil NADPH oxidase activity were not involved. Additional studies comparing endothelial NADPH oxidase activity from these strains are...
planned. The pattern of inheritance suggests the likely involvement of multiple genes, including a maternal-related factor. The presence of many candidate genes, makes this type of approach to this problem difficult. Experiments are therefore underway to generate the appropriate F2 and backcross mice necessary for a genome-wide scan to identify quantitative trait loci (26) responsible for the strain-dependent sensitivity to lung I/R.

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